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THE ANTINOCICEPTIVE EFFECTS OF HN AND RELATED PEPTIDES IN THE MOUSE WRITHING AND TAILFLICK TESTS: THE POSSIBLE ROLE OF CENTRAL D2 RECEPTOR

By

© **Hong Ruan**

Dissertation submitted to
the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Master of Science in Pharmacology

Under the Supervision of
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August, 1999

ACKNOWLEDGEMENTS

I would like to thank all of those people who have made my last three years at the University of Ottawa so enjoyable. However, without the help of a special few, this work may not have been possible.

A greatest appreciation will be delivered to my supervisor Dr. Simon Lemaire. It is his theoretical direction and encouragement that guide me through both the exciting and frustrating periods, in successfully accomplishing the courses and thesis objectives. His good nature, patience and generosity, provided an excellent environment for learning.

In the laboratory, I was fortunate to meet Dr. Michel Dumont, Dr. Thanh Lee and Yanmei Wu, who provided excellent support and technical instruction whenever I need it. Their stimulating conversation, pleasant smile make the years passed more easily. I would like to thank for their good friendship and enjoyable laboratory moments. I am also grateful to the effort from Dr. Prasad in preparation all the synthesized peptides for my study.

I would like to offer my thanks to the examiners who took the precious time to review my thesis and present their valuable feedback and stimulating questions.

To the friends and family, I thank my parents and fiancée who keep my perspective on life balanced and enjoyable.

Finally, I would like to thank the financial support from the Medical Research Council which allowed me to complete this thesis work over the courses.

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LIST OF ABBREVIATIONS:

AC	= adenylate cyclase
AD_{50}	= the concentration to produce 50% analgesia
AMPA	= α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
B_{max}	= maximal number of binding sites
BSA	= bovine serum albumin
CNS	= central nervous system
COX	= cyclo-oxygenase
DA	= dopamine
δ	=delta
DSLET	= [D-Ser^2, Leu^5]enkephalinyl-Thr
D1	= dopamine receptor subtype 1
D2	= dopamine receptor subtype 2
EAA	= excitatory amino acid
HN	= histogranin
HPLC	= high performance liquid chromatography
i.c.v	= intracerebroventricular injection
IC_{50}	= 50% inhibitory concentration
i.p.	= intraperitonial
i.t.	= intrathecal injection
K_d	= equilibrium dissociation constant
κ	= kappa
K_H	= inhibitory constant for the high affinity sites
K_L	= inhibitory constant for the low affinity sites
Ki	= inhibitory constant
MPE	= maximal possible effect
μ	= mu

NAS = nucleus accumbens septi

n_H = hill coefficient

NMDA = N-methyl-D-aspartate

NSAID = non-steriod anti-inflammatory drugs

OGP = osteogenic growth peptide

PAG = periaqueductal gray

PCP = phencyclidine

RVM = rostral ventral medulla

SAR = structure activity relationship

VTA = midbrain ventral tegmental area

ABSTRACT

Histogranin (HN) is an adrenal medullary peptide that was shown to display N-methyl-D-aspartate (NMDA) receptor antagonist activities. Recent data indicated that [Ser^1]HN, a stable synthetic analogue of HN, administrated intrathecally (i.t.) can produce significant analgesic effects in rat models of tonic and chronic pain. Such effects were attributed to the ability of the peptide to modulate the activity of the spinal NMDA receptor. The aims of the present study were to determine what type(s) of pain HN and related peptides are able to alleviate and which receptor type(s) is(are) involved in such activity. HN and related peptides (intracerebroventricularly: *i.c.v.*) displayed significant analgesic activity in both the the mouse writhing (visceral type of pain) and tailflick (acute reflex type of pain) assays. In the writhing test, they showed dose and structure dependency with the following order of potency: HN-(7-15) > histone H4-(86-100) > HN (AD_{50}=24.2 nmol/mouse) ≈ [Ser^1]HN ≈ HN-(7-10) > OGP ≈ HN-(1-10). The minimal active core resided in the middle portion of HN, HN-(7-10) displaying significant analgesia at 50 nmol/mouse (*i.c.v.*) while HN-(6-9) and HN-(8-10) were inactive. On the other hand, the lengths of action of the peptides strongly depended on the integrity of the N-and C-terminal portions of the peptides, since the analgesic effects of HN-(7-15), HN-(1-10), and HN-(7-10) were short lasting (5 min) as compared with those of HN and [Ser^1]HN (15-30 min). The analgesic activity of [Ser^1]HN (50 nmol/mouse) was not affected by opioid (naloxone, 1 nmol/mouse), NMDA (CPP, 0.3 nmol/mouse) and D1 (SCH-23390, 0.5 nmol/mouse) receptor antagonists, but markedly diminished by the D2 receptor antagonist raclopride

(0.5 nmol/mouse). The interaction of the peptides with central D2 receptor was also demonstrated in *in vitro* binding assays using [^{3}H]raclopride (D2 antagonist) with rat brain membranes. [^{3}H]raclopride bound to a high affinity (K_d of 4.87± 0.54 nM) saturable (B_{max} of 47.2±3.01 fmol/mg protein). HN dose-dependently inhibited the binding of [^{3}H]raclopride (2.5 nM), the competition curve presenting the biphasical profile of D2 agonists with a K_H of 2.05±0.04 nM and a K_L of 2.88±0.71 μM and being affected by the presence of NaCl in the same way as dopamine. Thus, the presence of NaCl (10 mM) induced the conversion of the high affinity site into the low affinity site, resulting with an overall K_i of 6.54 ± 1.2 μM. Finally, the relative potencies of HN and related peptides in inhibiting [^{3}H]raclopride binding correlated well with their potencies in inducing analgesic effects in the mouse writhing test (r = 0.86). Our results indicate that HN and related peptides can produce analgesia in thermal and chemical pain assays by a mechanism that involves the participation of the dopamine D2 receptor.

SECTION 1

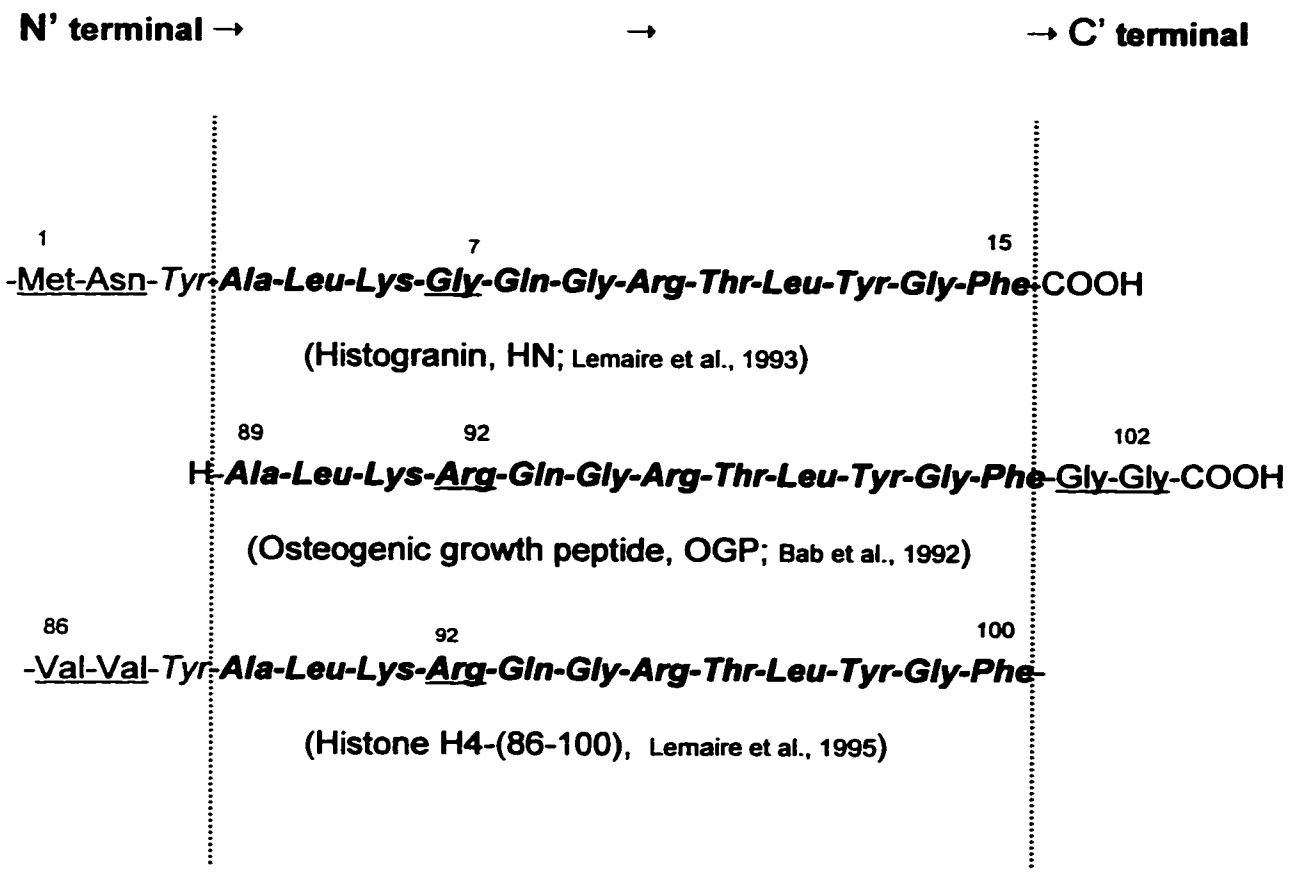
GENERAL INTRODUCTION

1. 1 THE PHARMACOLOGY PROFILE OF HISTOGRANIN (HN)

1.1.1 HN and Related Peptides

Histogranin (HN), a pentadecapeptide (H-Met-Asn-Tyr-Ala-Leu-Lys-Gly-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-COOH), was isolated as a co-fraction of neuromedin C during the extraction of bombesin-like peptides from bovine adrenal medulla (Lemaire et al., 1993). The name "histogranin" was derived from the facts that the peptide possesses 80% structural homology with fragment-(86-100) of histone H4 (Histo) (Fig. 1) and is concentrated in adrenomedullary chromaffin granules (granin). Histones are highly conserved proteins whose synthesis are characteristically linked to DNA replication and whose locations are typically within the chromatin structure of the nucleus (Wu and Bonner, 1981; Weisbrod, 1982). The production of histone mRNA variants, contrary to that of histone $mRNA_s$, is generally associated with the expression of cell cycle-independent histones. The possibility that HN is derived from some variant of histone H4 is supported by the recent discovery of an inducible poly A-containing histone H4 mRNA variant (H4-v.1) in bovine adrenal medulla (Gendron et al., 1997). In addition, the findings of immunoreactive histone H4 on cell surface of lymphocytes (Watsom et al.,1995) and osteogenic growth peptide (OGP), or histone H4-(89-102) (Fig. 1) in blood plasma are consistent with the assumption that variants of histone H4 may be expressed and processed into small peptides to play extranuclear function(s).

Figure 1 Comparison of the structures of HN and Histone H4 derivative (Histone H4-(86-100)) and OGP (Histone H4-(89-102)). The area within the dashed line indicates the highly reserved amino acid fragments among these three peptides.


N' terminal →
→
→ C' terminal
1
7
15
-Met-Asn-Tyr-Ala-Leu-Lys-Gly-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-COOH
(Histogranin, HN; Lemaire et al., 1993)
89
92
102
H-Ala-Leu-Lys-Arg-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-Gly-Gly-COOH
(Osteogenic growth peptide, OGP; Bab et al., 1992)
86
92
100
-Val-Val-Tyr-Ala-Leu-Lys-Arg-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-
(Histone H4-(86-100), Lemaire et al., 1995)

Immunoreactive-HN (ir-HN) is mainly observed in the pituitary, adrenal medulla and lymphoid tissues (spleen, lung), but significant levels of the peptide can also be detected in the brain (Lemaire et al.,1993). The distribution of ir-HN in the subcellular fractions of bovine adrenal medulla indicated that the peptide is concentrated in the chromaffin granules. A large increase in the release of HN can be induced by the cholinergic receptor agonist carbamylcholine in perfused adrenal glands (Lemaire et al., 1993). These results suggest a neuroendocrine function for HN.

1.1.2 Anti-N-Methyl-D-aspartate (NMDA) Receptor Activity

The modulating effect of HN on NMDA receptor activity was first demonstrated in a *in vivo* mouse model of anti-convulsion test. Intracerebroventricular (*i.c.v*) injection of HN and its synthetic stable analog, [Ser^1]HN dose-dependently (5-100nmol/mouse) inhibited the convulsions induced by NMDA, but not by the non-NMDA glutamate receptor agonists α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), kainic acid, bicuculline and pentylentetrazol (Lemaire et al., 1993; Shukla et al.,1995). The selectivity of HN against NMDA receptor stimulation suggested that this peptide may act as an endogenous modulator of central NMDA receptor activity. The blockade of NMDA-induced convulsions by HN and related peptides requires the integrity of the molecule of HN (Prasad et al.,1995), as most HN fragments show no or non-significant anticonvulsant activity. HN-(2-15) is the only tested fragment that showed significant protection against NMDA induced convulsions.

In agreement with the anti-NMDA receptor activity of HN, [Ser^1]HN (*i.c.v.)* in rats

also produced dose-related stereotypy, ataxia and locomotion, a behavior profile typical to that of PCP , a non-competitive NMDA antagonist (Shukla et al., 1995). In addition, central administration of HN was shown to impair passive avoidance learning in mice (Maurice et al.,1995). Accumulating evidence indicates that cognitive and learning processes involve the NMDA receptor transmission (Morris et al., 1986; DeNoble et al.,1990; Venable and Kelly, 1990). Both competitive (AP5) and non-competitive (MK-801, PCP) NMDA receptor antagonists blocked the working memory in radial maze and impaired the acquisition of the passive avoidance response in rats.

The possible interaction of HN with the NMDA receptor was also examined in *in vitro* binding studies. [Ser^1]HN (10^{-9} -10^{-5} M) dose-dependently inhibited the specific binding of [^{3}H]CGP-39653, a competitive NMDA antagonist, with maximal inhibitory effect reaching about 34% of specific binding. The saturation binding performed in the presence of [Ser^1]HN (2uM) revealed that the peptide significantly decreases the maximal binding capacity (B_{max}), without significantly affecting the dissociation constant (K_d), indicating a non-competitive nature of the interaction between HN and the NMDA receptor. It was therefore suggested that the blockade of NMDA receptor-mediated activity could be due to an allosteric modulation in the conformation of the NMDA binding site. This conclusion was further supported by the *in vitro* binding assays (Dumont et al., 1994) using [^{3}H]dextromethorphan ([^{3}H]DM, a non-competitive NMDA antagonist), wherein [Ser^1]HN enhanced [^{3}H]DM binding to rat brain membranes. This latter effect appeared to involve exclusively the NMDA receptor complex since it was reversed by NMDA itself, but not by TCP or (+)-pentazocine (PCP and sigma receptor ligands, respectively).

1.1.3 HN Binding Sites in the Rat Brain Membrane

In vitro binding studies with the stable synthetic analogue [^{125}I][Ser^{1}]HN revealed that the peptide possesses specific binding sites in membrane preparations of rat brain (Rogers and Lemaire,1993) and human peripheral blood lymphocytes (Lemaire et al., 1993b). The specific binding of [^{125}I][Ser^{1}]HN (5 nM) to membrane preparations from rat brain displayed several characteristics typical to membrane-bound receptors (Bennett and Yamamura, 1985), namely high affinity (K_d = 25.0 nM), saturability, selectivity, reversibility and sensitivity to trypsin and heat treatments. The Hill coefficients (n_H) calculated from both the saturation and competition binding experiments were close to unity, indicating that the peptide bound to a single population of sites. The regional distribution of [^{125}I][Ser^{1}]HN binding in rat brain membranes revealed that the hippocampus and cerebral cortex contain higher density of sites than the cerebellum and striatum, a distribution binding profile similar to that observed with the non-competitive NMDA antagonist [^{3}H]MK-801 (Rogers and Lemaire, 1993). [^{125}I][Ser^{1}]HN binding was selective for HN and closely related structures and was unaffected by the presence of unrelated peptides, such as substance P, β-endorphin, neuropeptide Y, enkephalins, dynorphin A-(1-13) and neuromedin C. The abilities of HN related peptides to compete with the high affinity site for [^{125}I][Ser^{1}]HN required the intact 15 amino-acid structure of HN since removal of a single amino acid from either N- or C-terminals provided HN fragments with significantly reduced displacing potency (Rogers and Lemaire, 1993; Prasad et al.,1995). Removal of additional amino acids from either C- or N-terminals of HN led to further decreases in the binding potency. This large reduction in the binding potency of HN

fragments correlated well with their lack of effect antagonizing NMDA-induced convulsions (Prasad et al., 1995). Therefore, the modulation of NMDA receptor activities by HN and related peptides was most likely to be mediated through an interaction of the peptides with the central high affinity site for [Ser^1]HN.

[^{125}I][Ser^1]HN binding to rat brain membranes was specific to HN and related peptides, not being displaced by various modulators of the NMDA receptor complex including CPP, AP5, MK-801, TCP, Gly and Glu (Rogers et al., 1993). On the other hand, polyamines (spermidine, spermine, DA10, DET) known to play a role in the control of NMDA receptor (among others) functions (Williams et al.,1991; Brackyley et al., 1990; Sprosen and Woodruff, 1990; Ransom and Stec,1988), inhibited the binding of [^{125}I][Ser^1]HN with an IC_{50} similar to that for inhibition of [^{3}H]spermidine binding (London et al., 1991). However, the fact that HN was unable to displace the binding of [^{3}H]spermidine (20 nM) to rat brain membranes and the inhibitory effect of DET on [^{125}I][Ser^1]HN binding was non-competitive, indicating that the high affinity site for HN is distinct from the polyamine binding domain on the NMDA receptor complex and that the interactions between these two sites (HN and polyamine) is allosteric.

1.1.4 The Antinociceptive Effects of HN

The investigation of the possible analgesic action of HN was initially triggered by the finding that transplantation of adrenal chromaffin cells into the subarachnoid space of the rat spinal cord alleviates the nociceptive response in several chronic type of pain models associated with the overactivation of the spinal NMDA receptor, such as

hyperalgesia or allodynia induced by sciatic nerve injury (Hama and Sagen, 1993), administrations of complete Freund's adjuvant (Sagen et al., 1990) and formalin (Siegen and Sagen,1993) into rat hind paws. Adrenal chromaffin cells are known to release various neuroactive substances that can alter nociception. These include the opioid peptides Met- and Leu-enkephalins and catecholamines (Carmichael and Winkler, 1985). However, in the formalin assay, the reduction of the second phase of noxious response by the chromaffin cell transplantation showed resistance to the opioid antagonist, naloxone, and the alpha (α) adrenergic antagonist, phentolamine administered alone or in combination (Siegen and Sagen,1993). The insensitivity of these effects to opioid and adrenergic antagonists suggested that other pain reducing agents may be released from the adrenal medulla. In addition to opioid peptides and catecholamines, chromaffin cells are known to produce a large variety of neuroactive substances including neuropeptides and neurotrophic factors (Lemaire et al., 1993; Usicker, 1993). One of the neuroactive peptides secreted from the adrenal medulla that could modulate NMDA receptor activity (or overactivity) is HN (Lemaire et al.,1993).

[Ser^1]HN administrated intrathecally (*i.t.*) was recently shown to fully mimic the effects of adrenomedullary transplants by suppressing the second phase (tonic) of pain in the formalin test (Siegan and Sagen, 1997) and hyperalgesia and allodynia consecutive to sciatic nerve injury (Siegan et al., 1997). The first phase (acute) of pain induced by formalin, an NMDA-independent phase of pain, was not affected by [Ser^1]HN (Siegan and Sagen, 1997). In both pain assays, the intermediate dose of the peptide was found to be most effective, resulting in bell-shaped dose-response curves. Current theories

regarding the mechanisms of chronic pain suggest that excessive activation of the NMDA receptor in the spinal cord evokes a series of neuroplastic changes that lead to persistent hyperexcitability (Coderre et al.,1993; Dickenson, 1990; Dubner and Ruda, 1992). In support of this, NMDA antagonists are effective in suppressing hyperalgesia resulting from neuropathy or inflammation (Davar et al., 1990; Mao et al.,1993; Ren et al., 1992). The rat pain assays of peripheral (sciatic) nerve injury and formalin (tonic phase) are typical tests involving NMDA receptor mechanisms (Coderre et al., 1993). The effectiveness of [Ser^1]HN in these two pain assays suggested that the adrenal medullary peptide HN could contribute to the analgesic activity of adrenal medullary implants. Further more, the analgesic effects of [Ser^1]HN were proposed to result from the NMDA receptor antagonist activity of the peptide. On the other hand, the reduced effectiveness of higher doses of the peptide suggested that HN could also possess some NMDA agonist activity (Siegan et al., 1997; Siegan and Sagen, 1997).

More recently, the same research team found that [Ser^1]HN (*i.t.*) dose-dependently blocks mechanical and thermal hyperalgesia and allodynia induced by *i.t* administration of NMDA (Hama et al., 1999). The distinct profile and robust analgesic activity of [Ser^1]HN in this latter test suggest that the administration of HN may be an efficient novel approach for the management of pain resulting from abnormal excitatory neurotransmission.

1.2 PAIN: PATHOPHYSIOLOGY AND PHARMACOLOGICAL TREATMENT

1.2.1 Transmission and Central Modulation of Nociceptive Stimuli

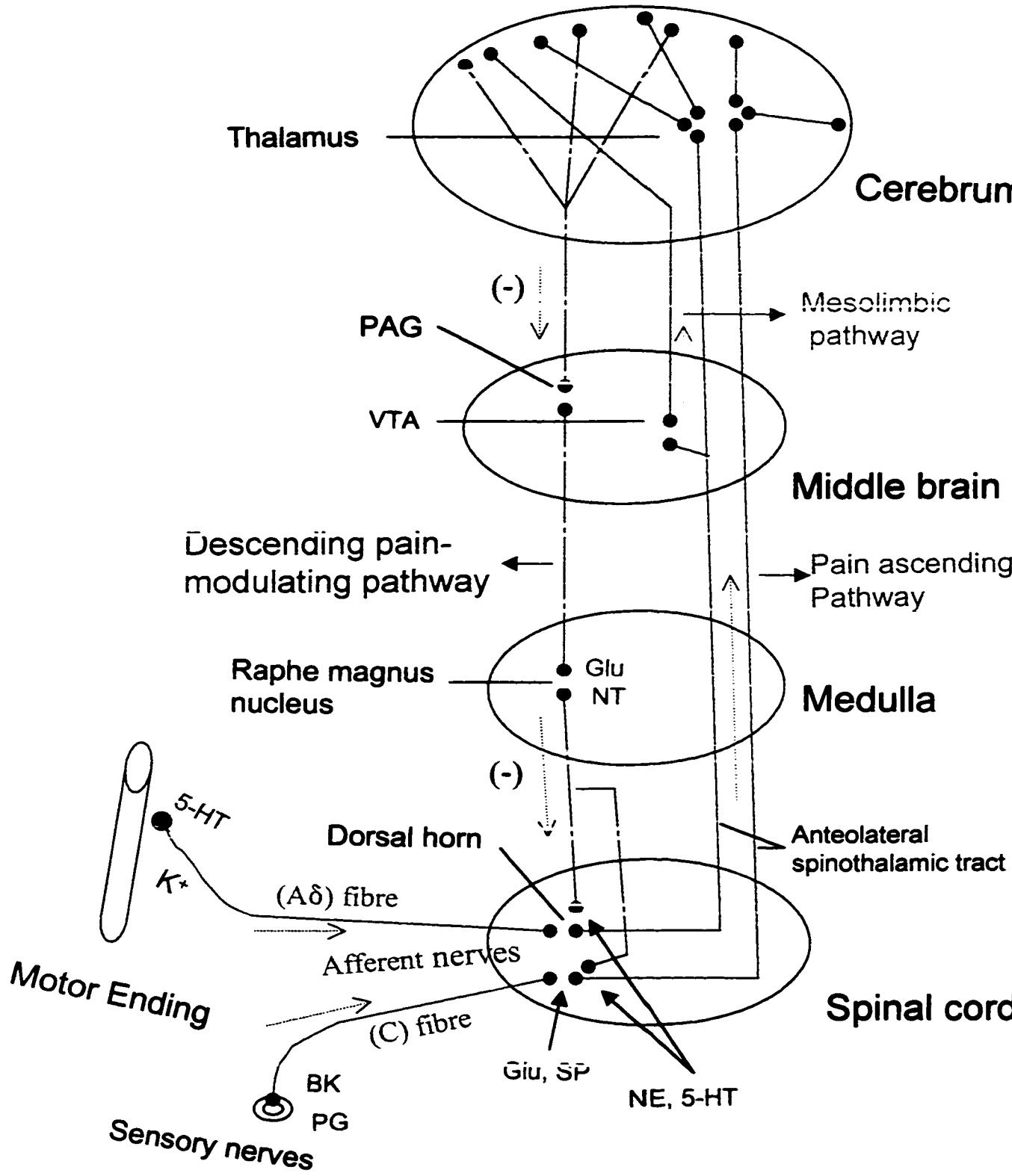
Pain signals are initiated by the stimulation of nociceptors located on primary afferent nerves and aimed at receiving and transmitting painful stimuli. Nociceptive neurons are depolarized by various pain-producing chemicals, such as K^+, bradykinin, 5-HT, prostaglandins, released in an area of tissue injury or inflammation. In addition, prostaglandins increase the sensitivity of the pain receptor to bradykinin, the most potent pain producing agent (Lombardo and Wilson, 1997). The afferent A-δ and C nerves fibers transmitting respectively fast and slow pain impulses enter the spinal cord at the dorsal roots and synapse in the substantia gelatinosa (laminae II and III), across the spinal cord, and ascend to the brain in either the neospinothalamic tract or paleospinothalamic tract (Lombardo and Wilson, 1997). The neuropeptide substance P (Kuraishi et al., 1985; Wiesenfeld-Hallin, 1986) and the excitatory amino acid, L-glutamate (Ransom et al., 1977; Watkins and Evans, 1981; Salt and Hill, 1983) have been identified as two major candidates mediating the pain transmission at the primary afferent synapses in the spinal cord (Fig. 2).

The mammalian central nervous system possesses well-defined networks that modulate nociceptive transmission via a feedback mechanism. The most extensively studied is the descending pathway that is constituted of three linked areas (Fig. 2) (Fields et al., 1991). The first area is the midbrain periaqueductal gray (PAG). The neurons of PAG send impulses to the raphe magnus nucleus located in the rostral ventral medulla (RVM). Impulses are further transmitted from the raphe nucleus down to a pain inhibitory

complex located in the dorsal horns of the spinal cord. In animal studies, electrical stimulation of the PAG area or raphe nucleus area can completely suppress strong pain signals via the dorsal spinal roots. The input to the RVM from the PAG is predominantly excitatory (Fields and Anderson, 1978; Vanegas et al., 1984). The evidence that PAG-induced excitation of RVM neurons are mediated at least in part by EAAs has been provided via several approaches (Clements et al., 1987; Wiklund et al., 1988; Aimone and Gebhart, 1986). Non-selective EAA agonists injected in the PAG and RVM are, in general, found to suppress nociceptive responses in the spinal dorsal horn (Aimone and Gebhart, 1986; Carstens et al., 1988; Jensen and Yaksh, 1992; Zhuo and Gehart, 1992).

Another transmitter that mediates PAG-induced excitation of RVM nuclei is neurotensin. Both behavioral and anatomical evidence suggest that neurotensin is involved in the nociceptive modulatory influence of the PAG on the RVM (Quirion et al., 1982; Fang et al., 1987). Microinjection of neurotensin into the RVM produces a dose-dependent analgesic effect. The down-regulation by RVM of dorsal horn nociceptive transmission is known to involve both the serotonergic and noradrenergic mechanisms. RVM contains a high percentage of serotonergic and noradrenergic neurons that project to the spinal dorsal horn (Ruda et al., 1981;1982). Both 5-HT1 and 5-HT3 receptors localized on the primary efferent terminals are involved (El-Yassir and Fleetwood-Walker, 1990; Glaum et al., 1988). Norepinephrine also has a well established antinociceptive effect when applied *i.t* (Proudfit and Hammond, 1981). The inhibition of nociceptive transmission by norepinephrine has been demonstrated to involve mainly α_2 receptors (Fleetwood-Walker et al., 1985; Millar and Williams, 1989; Zhao and Duggan, 1988).

Figure 2 Schematic representation of the major components of the pain system and modulatory relations among them. Afferent input (red line) generated at peripheral sensory endings converges in the spinal dorsal horn nuclei. Ascending pathways convey the sensory signals to numerous locations in the brainstem, cortical, and subcortical divisions of the limbic system, and via thalamic relays to somatosensory areas of the cerebral cortex. The responsiveness to the nociceptive input is modulated by descending control pathways (green line) that originate directly or indirectly from midbrain, pons, medulla and cerebral cortex.
5-HT, serotonin; BK, bradykinin; Glu, glutamate;
NE, norepinephrine; NT, neurotensin; SP, substance P;
PG, prostaglandin; PAG, periaqueductal gray;
VTA, ventral tegmental area.


Thalamus
Cerebrum
(-)
PAG
Mesolimbic pathway
VTA
Middle brain
Descending pain-modulating pathway
Pain ascending Pathway
Raphe magnus nucleus
Glu
NT
Medulla
(-)
5-HT
K+
Dorsal horn
Anteolateral spinothalamic tract
(Aδ) fibre
Afferent nerves
Motor Ending
Spinal cord
(C) fibre
Glu, SP
NE, 5-HT
BK
PG
Sensory nerves

1.2.2 Pharmacological Approaches to Manage Pain

Pain has been divided into acute transient pain and tonic chronic pain based on its time lasting, severity of tissue injury and the physiological function it serves (Table 1) (Perkins and Dray, 1996). Acute pain is associated with negligible tissue damage, and serves as a physiological warning of potential tissue damage. Typical animal models to measure this type of pain include the tail-flick (d' Amour and Smith, 1941) and hot plate (Eddey et al., 1953) tests. Chronic pain can be further divided into two states : the mild to medium state of pain induced by mild tissue injury or inflammation; and the hyperalgesic or allodynic state. While the former state is still a protective response and resolves rapidly once the injury is healed, the later state (hyperalgesia or allodynia) of chronic pain outlasts its biological purpose which arises from chronic pathological lesions or degenerative processes. Various animal models have been developed to mimic these two states of chronic pain, eg. abdominal writhing test (mild to medium type of pain) (Sigmund et al., 1957); formalin assay, neuropathic pain model, arthritic pain model (hyperalgesia and allodynia).

The present treatments for most pain states heavily rely on the use of non-steroid anti-inflammatory drugs (NSAID) and opioid analgesics. NSAIDs are commonly used for treatment of mild to moderate chronic pain or inflammation-induced hyperalgesia. They act peripherally at the site of tissue injury and block the synthesis of prostaglandins. Almost all the NSAIDs are potent inhibitors of cyclo-oxygenase (COX) which convert arachnoid acid precursors into prostaglandins. Two types of cyclo-oxygenase have been found (Xie et al., 1991). COX-1 is virtually present in all tissues and is important for the

maintenance of normal cell function while COX-2 is absent in normal tissues but is induced following tissue damage or inflammation (for review see :Dubois et al., 1998). Therefore, it has been proposed that the inhibition of COX-2 produces an anti-inflammatory and analgesic effect whereas the inhibition of COX-1 may induce toxic side effects. All currently used NSAIDs inhibit the two enzyme isoforms. However, most of them, such as indomethacin, aspirin or derivatives, are more potent inhibitors of COX1 than COX2 (Mitchell et al., 1993). Chronic use of these compounds is associated with toxic side-effects, such as gastrointestinal upset, increased bleeding time and reduced renal function. Recently, selective COX-2 inhibitors, such as NS389 and BF-389, have been developed. These compounds were shown to possess anti-inflammatory and analgesic properties in animal models of chronic pain, with no evidence of gastric toxicity (Seibert et al., 1994; Masferrer et al., 1994).

Opioids are currently the most potent analgesics available in treating various pain states, including acute pain and most types of hyperalgesia. In contrast to NSAIDs, the opioids exert their analgesic effect centrally. Three subtypes of opioid receptors have been characterized: mu(μ), kappa (κ) and delta (δ) receptors (Chang and Cuatrecasas, 1979; Kosterlitz and Paterson, 1980). Concomitant with the identification of opioid receptor subtypes, several endogenous opioid peptides, such as Leu- and Met-enkephalin, β-endorphin and dynorphin, were discovered in the limbic system, thalamus, PAG, substantia gelatinosa of the dorsal horn, and gut (Hunt and Lovick, 1982; Bugnon et al., 1979; Glazer and Basbaum, 1981; Cruz and Basbaum, 1985). Receptor binding studies indicate that morphine and enkephalin bind preferentially to μ and δ receptors

(Gillan et al., 1980), respectively, while μ- and κ-receptors show high affinity and marked selectivity for β-endorphin and dynorphin, respectively (Schoffelmeer et al., 1991; Huidoro-Toro et al., 1981). Both exogenous and endogenous opioids exert their analgesic effects by activating the opioid receptors concentrated in supraspinal and spinal sites (Lombardo and Wilson, 1997). For instance, by binding to the μ receptor in the RVM nucleus, morphine and opioid peptides inhibit the GABA-containing interneurons which tonically inhibit the descending pathway (Fields et al., 1991). In addition, at the spinal dorsal horn, opioids acting at δ receptors on the terminals of the primary afferent mediate the inhibition of the release of nociceptive transmitters such as substance P (Collin et al.,1991). Being very potent for the treatment of severe and chronic pain states, the opioid drugs are often associated with some serious side effects, including respiration depression, gastrointestinal disturbance, sedation, tolerance and dependence, which limit their use for long term treatment. In addition, the pain associated with inflammation or neuropathy has been shown to be largely resistant to opioid analgesics (Dray et al., 1994).

Recently, some advances have been reviewed and are leading a conceptual shift in pain pathophysiology and management (for review see: Perkins and Dray, 1996). The novel proposed approaches are the following: A. Peripherally acting: inhibitors of kinin receptors. B. Centrally acting: (1) inhibitors of nitric oxide synthase; (2) antagonists of tachykinin receptors; (3) antagonists of excitatory amino acid receptors.

While the blockade of bradykinin and kallidin receptors has substantial support for the treatment of inflammation induced pain (Hargreaves et al., 1988; Shibata et al., 1986), the inhibitors of nitric oxide synthesis (Handy et al., 1995; Sakurada et al., 1996) and the

antagonists of neurokinin (Birch et al., 1992; Urban et al., 1994) or NMDA receptors (Ren et al., 1992; Davar et al., 1991) have been shown to be effective in attenuating neuropathic pain and inflammation-induced hyperalgesia. Thus, the development of these novel approaches is susceptible to offer ways to supplement or replace the traditional use of NSAID and opioids in the treatment of pain.

Table 1. (Intra) Classification of pain

	Acute transient pain	Chronic tonic pain	
		[a] Mild to medium	[b] Hyperalgesia, allodynia
Cause	Negligible tissue injury	Mild tissue injury or inflammation	Serious tissue injury; inflammation; neuropathology
Function	Physiological warning	Protective response	Pain sensation outlasts biological usefulness
Animal model	Tail-flick test; Hot plate test; Paw pressure test	Abdominal writhing test	Formalin test (tonic phase); Neuropathic pain models; Arthritic pain models
Treatment	Opioids; [c] Kinin antagonists	NSAID; Opioids	Opioids; [c] NMDA antagonists [c] Neurokinin antagonists

[a] mild to medium state of chronic pain, [b] hyperalgesic or allodynic state of chronic pain, [c] in preclinical trial

1.3 CENTRAL DOPAMINE D2 RECEPTOR AND ITS RELATED ANALGESIC EFFECTS

1.3.1 Classification of Dopamine Receptors

Dopamine (DA) receptors were first proposed to exist in two discrete populations on the basis of pharmacological and biochemical evidence. One population was positively coupled to adenylate cyclase (AC) and the other one was independent of the adenosine 3',5'-cyclic monophosphate (cAMP)-generating system (Spano et al., 1978). Kebabian and Calne (1979) summarized these observations and suggested the following nomenclature: D1 for the receptor that stimulated AC and D2 for the one that was not coupled to this effector. However, in the early 1980s, it was shown that the stimulation of the D2 receptor can inhibit AC in the pituitary (De Camilli et al., 1979; Enjalbert and Bockaert, 1983; McDonald et al., 1984) and the CNS (Onali et al., 1985). The concept of the duality of DA receptors was considerably strengthened by the development of selective agonists and antagonists of the D1 and D2 receptors. The benzazepine derivative SCH 23390 is a selective D1 receptor antagonist while SKF 38393 and CY208-243 are preferential D1 receptor agonists. Raclopride is a selective D2 antagonist while quinpirole is a selective D2 agonist (Ögren et al., 1986). Using the radiolabelled forms of these compounds, it has been possible to study the distribution of D1 and D2 receptors in the brain by autoradiography.

Molecular cloning studies have revealed the existence of five (5) distinct DA receptor subtypes termed D1, D2, D3, D4 and D5 (for review see Sibley and Monsma, 1992). All these DA receptors contain the same primary structure that allow them to be classified in the seven transmembrane (TM) domain G-protein-coupled receptor family.

On the basis of structure and pharmacological criteria, the five DA receptors have been categorized as either D1- or D2- like receptors. The D1 and D5 receptors share a very high homology in their transmembrane domains. Similarly, the transmembrane sequences are highly conserved among D2, D3 and D4 receptors (for review see Civelli et al., 1993; Gingrich and Caron, 1993). Pharmacologically, although the profile of D1 and D2 receptors are substantially different, the D5 receptor exhibits the classical ligand-binding characteristics of D1 receptors, and the D3 and D4 receptors bind the hallmark D2-selective ligands with relatively high affinity (for review see Civelli et al., 1993; Gingrich and Caron, 1993). In addition, the initial distinction between D1 and D2 receptors in term of signaling events (positive or negative coupling to AC), appears to apply to the novel members of the DA receptor family, the D5 receptor being coupled to stimulation of AC (Dearry et al., 1990) and the D3 (Chio et al., 1994) and D4 (Chio et al., 1994; Cohen et al.,1992) receptors to inhibition of cAMP formation.

In addition to their link to AC activity, the DA receptors have also been reported to couple to other signaling transduction pathways, including the modulation of Ca^{2+} and K^+ channel activities and phospholipase and Na^+-K^+-ATPase activities (for review see Missale et al., 1998). Based on current data (Rashed et al.,1996; Morra et al., 1991; review see Missale et al., 1998), almost all these intracellular events have been linked to some of the G protein-coupled subtypes of DA receptors.

1.3.2 D2 Receptor Mediated Analgesia

Pharmacological data have been gathered suggesting an important role of the

dopaminergic transmission on the elicitation of behaviors that are used to measure analgesia. Various investigations have demonstrated that enhancement of DA activity can produce antinociception on different behavior situations (Paalzow and Paalzow, 1983; Barasi and Duggal, 1985; Drago et al., 1984, Morgan and Franklin, 1991). The evidence that demonstrated that the analgesia produced by the indirect and direct DA agonists lacks opioidergic link and predominantly involves the central D2 receptor system was provided by the following experiments. First, in the formalin pain assay, the analgesic effects induced by indirect dopaminergic stimulants, such as cocaine and amphetamine, were significantly blocked by D2 antagonists but not naloxone, an opioid antagonist (Lin et al., 1989; Morgan and Franklin, 1991; Bittencourt and Takahashi, 1997; Altier and Stewart, 1998). Even though in some situations, these analgesic effects were somewhat attenuated by the D1 antagonist, SCH-23390 (Morgan and Franklin, 1991; Altier and Stewart,1998), the selective D1 agonist, SKF 38393, did not display any significant analgesic activity (Morgan and Franklin, 1991). It was suggested that the D1 receptor may play an "enabling" role in D2 receptor-mediated analgesia in the formalin test. Second, apomorphine, a mixed D1/D2 agonist and the D2 selective agonists bromocriptine and RU 24926, have been found to produce dose-dependent analgesic effects in mouse writhing (Frussa-Filho et al., 1996) and hot plate tests (Michael-Titus et al., 1990), whereas SKF38393, a D1 receptor agonist, had no influence on pain sensitivity. The inhibitions of the visceral pain by apomophine and bromocriptine were reversed by haloperidol, a D2 antagonist, and not modified by naloxone, SCH-23390, or domperidone (a D2 antagonist acting only peripherally) (Frussa-Filho et al., 1996).

While the above nociceptive tests revealed an apparent and important role of the enhancement of D2 receptor transmission in analgesia (Morgan and Franklin, 1991; Gonzales-Rios et al., 1986; Frussa-Filho et al., 1996), the results concerning the effects of D2 agonists in modulating the pain in the tailflick test, a typical acute reflex type of pain, are discordant (Barasi and Duggal, 1985; Verma and Kulkarni, 1991; Jensen and Yaksh, 1984; Gonzales-Rios et al., 1986). For instance, Barasi and Dugal (1985) reported that i.t or systemic administrations of small doses of apomorphine and the D2 agonist, LY171555, increase the tailflick latency in lightly anaesthetised rats. Conversely, Jensen and Yaksh (1984) found no increase in the tailflick latency in the rats with intact neuraxis after intrathecal administration of apomorphine. A common view is that the tailflick pain assay is more sensitive to opioid analgesics than non-narcotic drugs. Thus, it is not surprising that the activation of the D2 receptor may be less effective than morphine and congeneers in producing analgesia in this test.

The site(s) of pain transmission pathway that is(are) involved or modulated by the D2 receptor activation is(are) still not well defined. It has been proposed that midbrain ascending D2 neurons arising from the cell bodies of the VTA and projecting to various forebrain structures such as the prefrontal cortex, NAS and amygdala play an important role in mediating the suppression of tonic pain (Franklin et al., 1989; Morgan and Franklin 1990; Manning et al.,1994; Anderson and Rompré, 1996). In agreement with this hypothesis, the most recent study of Altier and Stewart (1998) demonstrated that the analgesic effects of intra-ventral tegmental infusion (VTA) of substance P or morphine, or intra-nucleus accumbens septi (NAS) infusion of amphetamine in the rat formalin assay

can be attenuated efficiently by intra-NAS infusion of the D2 antagonist, raclopride. These data further indicate the importance of the mesolimbic system and more particularly the D2 receptors located in this system for the mediation of the analgesic activity of morphine and amphetamine.

In addition, D2 receptor mRNAs were shown to be expressed in discrete subpopulations of neurons of rat trigeminal ganglion (Peterfreund et al., 1995). Therefore, the function of subsets of sensory neurons that display the D2 receptors may be influenced by dopamine or D2 receptor agonists. D2 receptors present in the trigeminal ganglion may be involved in the modulation the clinical pain syndromes of headache and trigeminal neuralgia.

1.3.3 Distribution of D2 Receptor Subtypes and Other Related Biological Functions

In situ hybridization has been extensively used to study the distribution of DA receptor mRNA in the brain. Expression of D2 receptors is highest in striatum, nucleus accumbens, olfactory tubercle and substantia nigra (Meador-Woodruff et al., 1989; Mengod et al., 1989; Najlerahim et al., 1989; Mansour et al., 1990). The distribution patterns of D3 and D4 receptors are more restricted to the mesolimbic system. The highest concentrations of D3 receptor mRNA are found in the nucleus accumbens, whereas D4 receptor mRNA is more abundant in frontal cortex, amygdala, thalamus, hypothalamus and pituitary and somewhat less abundant in the hippocampus (Joyce and Meador-Woodruff, 1997). Overall, it appears that the D2 receptors are more dominantly expressed in areas associated with motor function, while D3 and D4 receptors are more

exclusively located in areas where the dopamine system is thought to serve a role in modulating emotion and cognition.

Although the underlying causes of schizophrenia remain unknown, much attention has been focused on the brain DA system. Since dopaminergic agents could induce a schizophrenia-like psychosis (Randrup and Munkvad, 1965; Snyder, 1973; Lieberman et al., 1987), and these effects could be inhibited by D2 receptor antagonists (Seeman and Lee, 1975), it was proposed that the positive symptoms of schizophrenia were related to enhanced dopaminergic neurotransmission. Additionally, the clinic potencies of different classes of antipsychotics correlated well with their affinities for the D2 receptor (Seeman and Lee, 1975; Creese et al., 1976; Seeman, 1992), suggesting that this receptor was a common target for clinical action. While blockade of limbic D2 receptor has been proposed as a critical component in mediating the positive antipsychotic effects, excessive blockade of D2 receptors in striatum is believed to be associated with the frequently reported extrapyramidal side effects (for review see Deutch et al., 1991). Blockade of dopamine D2 receptors in the pituitary is associated with hyperprolactinaemia (Meltzer et al., 1989). Activation of the D3 receptor has been reported to show some antidepressant activity, which would be useful for the treatment of Parkinson's disease when depression occurs concomitantly with motor dysfunction (for review see Cummings, 1992).

1.3.4 Dopaminergic pathways

Using histofluorescence techniques for visualizing catecholamines and tyrosine hydroxylase, the rate-limiting enzyme (Shiman et al., 1971) in catecholamine biosynthesis,

it has been shown that DA is the principal neurotransmitter in four major neural systems in the brain (Dahlström et al., 1964; Fallon et al., 1978; Graybiel et al., 1983; Lindvall and Björklund., 1983). The largest of these neuronal systems is the nigrostriatal pathway, which originates in the midbrain substantia nigra complex and projects to the dorsal striatum (caudoputamen). Degeneration of this pathway leads to Parkinson's disease (Hornykiewicz, 1966). The mesolimbic system, relatively spared in Parkinson's disease, arises in the midbrain ventral tegmental area and innervates the ventral striatum (nucleus accumbens and olfactory tubercle) and parts of the limbic system. This system is thought to influence motivated behaviors, including motor activity related to reward (Koob and Bloom, 1988; Koob, 1992). The ventral tegmental area also gives rise to the smaller mesocortical pathway, which projects to the frontal cortex, and may be involved in certain aspects of learning and memory (Le Moal and Simon, 1991). The fourth DA-containing pathway is the hypothalamic tuberoinfundibular system, which innervates the pituitary stalk and regulates prolactin secretion (Fuxe et al., 1969).

1.4 *In vitro* BINDING PROPERTIES OF CENTRAL D2 RECEPTOR

1.4.1 The Marker for Central D2 receptors

Some earlier studies have used either [^{3}H]haloperidol (Burt et al.,1976), [^{3}H]spiperone (Creese and Snyder, 1978) or [^{3}H]sulpiride (Theodorou et al., 1979) as ligands of choice to label the D2 receptor; however, these ligands are less than ideal. For example, [^{3}H]haloperidol has been characterized and shown to have a high affinity for the σ receptor while [^{3}H]spiperone appears to bind to the 5-HT receptor (Creese and Snyder, 1978) as well as to the α-adrenoceptor (Morgan et al.,1984). Furthermore, saturation binding studies using [^{3}H]spiperone have documented a higher number of D2 receptors in the CPU than found with either [^{3}H]sulpiride or with [^{3}H]raclopride (Hall and Wedel, 1986). Such greater density of striatal [^{3}H]spiperone sites most likely reflects the binding of this ligand to other types of receptors (besides the D2 receptor). In contrast to [^{3}H]spiperone, [^{3}H]sulpiride displays a high degree of selectivity for the D2 site (Zahniser and Dubocovich, 1983; Stefanini et al., 1987). However, the affinity of sulpiride for this site is low (Dewar et al., 1989; Köhler et al., 1985)

More recently, a novel substituted benzamide, raclopride, has been introduced as a pharmacological probe for studies of the central D2 receptor *in vivo* and *in vitro* (Köhler et al., 1985). Similar to other substituted benzamides, raclopride has a high affinity for the binding site labelled by the dopamine D2-antagonist [^{3}H]spiperone and [^{3}H]domperidone (Leysen et al., 1978; Seeman, 1980), but much lower affinity for binding sites labelled by the D1 antagonist [^{3}H]flupenthixol (Seeman, 1980) and little or no affinity for adrenergic, serotoninergic, histaminergic and muscarinic receptors (Köhler et al., 1985). Besides,

[^{3}H]raclopride binds to to the D2 site with higher affinity and lower nonspecific background as compared with [^{3}H]sulpiride (Köhler et al.,1985; Dewar et al.,1989).

1.4.2 The Binding Profiles of D2 Agonists and Antagonists

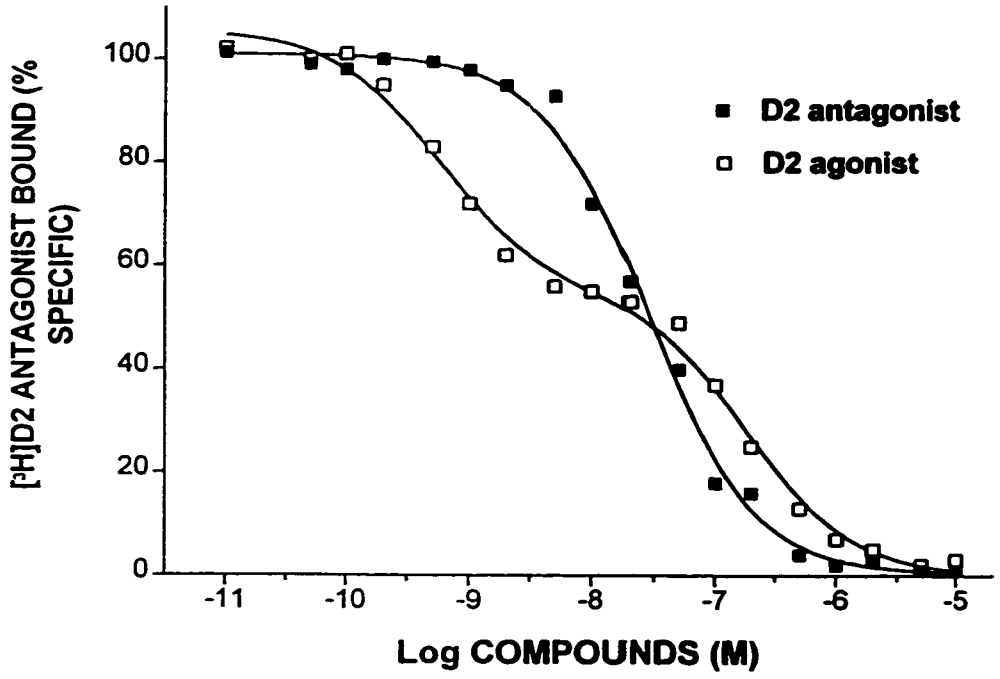
Activation of the D2 class of receptors is known to inhibit adenylate cyclase, via intermediary proteins, Gi proteins (Onali P et al., 1984), the coupling of which can be inhibited by pertussis toxin (Fujita et al., 1985). According to a ternary complex model, these Gi-proteins are constituted of 3 subunits, the α, β, and γ subunits, the α subunit constituting the binding site of GDP. The agonist by binding to the D2 receptor triggers the transformation of GDP to GTP, which, in turn, leads to a dissociation of the α-subunit from the receptor -$\beta\gamma$ subunits (Hall et al., 1992). D2 receptors that are coupled to G proteins exhibit high affinity (D2 high), while those receptors in the low-affinity state (D2 low) are believed to be dissociated from the nucleotide-binding protein (Lean et al., 1980; Sibley et al., 1982).

Consistent with this G-protein regulated acting model, accumulating binding data have provided evidence to suggest the existence of two affinity states for the D2 receptor with approximately equal proportion (Seeman et al., 1986; Seeman and Grigoriadis, 1987). [^{3}H]agonists are not capable of differentiating these two affinity states of D2 receptors and thus show monophasic (n_H=1) agonist/[^{3}H]agonist and antagonist/[^{3}H]agonist competition curves (Sibley et al.,1982). However, it has been experimentally found that the competition binding models between the agonist/[^{3}H]antagonist and the antagonist/ [^{3}H]antagonist are notably distinct (Fig 2.) (Sibley et al., 1982; Dewar et al.,1989; Reader

et al., 1990; Hall et al., 1991). The affinity of antagonist for the D2 receptor is not dependent on the formation of the receptor-G protein complex. The competition curves with D2 antagonists have steep slopes with a hill coefficient close to the unit and are better fitted to one rather than to a two-site model (Dewar et al., 1989). In contrast, DA and other D2 receptor agonists inhibit [^{3}H]antagonist binding in a heterogenous fashion displaying much shallower competition curves (nH<<1), thus fitting a two-site competition model (Reader et al., 1990; Hall et al., 1991). K_H and K_L are binding constants for the high and the low affinity agonist binding sites, respectively. Interpreted by the ternary complex model, agonists bind with high affinity to D2 receptor when the G($\alpha\beta\gamma$) protein are associated, with low affinity when the α-subunit is dissociated from the receptor-$\beta\gamma$ subunit.

Although there is some indirect evidence which suggests that the low affinity binding state is the functional state of the postsynaptic D2 receptor (Scatton, 1981; Starke et al., 1983), a reasonable conclusion is that the high affinity D2 binding state is an active receptor conformation coupled to G protein whereas the low affinity binding state may possibly represent a desensitized state, similar to the situation for the beta-adrenoreceptor (Sibley and Lefkowitz, 1985; Toews et al., 1983).

Figure 3 Schematic representation of D2 agonist or D2 antagonist in competition with the [^{3}H]D2 antagonist binding. It is important to note that the competition curve of D2 antagonist fit best to a single site binding model, with the slope factor close to 1, whereas the curve for D2 agonist is fitted best by assuming a two-site model, with the slope factor is far below 1.


[³H]D2 ANTAGONIST BOUND (% SPECIFIC)
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
Log COMPOUNDS (M)
D2 antagonist
D2 agonist

1.4.3 Regulation of D2 agonist Binding by Na^+ and Guanine Nucleotides

The previous studies have demonstrated that the binding of agonists to the D2 receptor was sensitive to the addition of Na^+ or guanine nucleotides to the incubation medium (Grigoriadis and Seeman, 1985; Reader et al., 1990) while the binding of [^{3}H] D2 antagonists was not influenced by the addition of Na^+ (Reader et al., 1990). A possible explanation is that Na^+ and guanine nucleotides, by favoring the dissociation of the Gi protein from D2 receptor, decrease the affinity of agonist binding to the D_2 receptor, thus inducing a transition from the D_2 high state to a D_2 low state (Reader et al., 1990). However, the literature data concerning the fashion in which Na^+ regulates the inhibition of [^{3}H]antagonist binding by D2 agonists is discordant. In the binding of [^{3}H]spiperone, [^{3}H]domperidone and [^{3}H]YM-09151-2 (Grigoriadis and Seeman, 1985; 1986; Niznik et al., 1985), the alteration by Na^+ of the affinity of DA for the D2 receptor appeared to be related to the partial conversion of the high to the low affinity state of the receptor. In contrast, Reader et al (1990) reported that Na^+ decreases the affinity of DA for both the high and low states of the D2 receptor in [^{3}H]raclopride binding without significantly altering the relative proportion (%R_H and %R_L) of these sites. In the above referred studies, the regulatory effects of Na^+ were investigated using membrane prepared from rat or rabbit neostriatum. It has been shown that the regulation pattern of Na^+ on the D2 receptor in the pituitary glands differs largely from that in the striatum (George et al., 1983), the presence of Na^+ (100 mM) alone is sufficient to fully convert the high-affinity state of the D2 agonist binding into the low-affinity state. Thus, the regulation pattern of Na^+ in the competition binding of D2 agonist/ [^{3}H]antatgonist could vary depending on the structure

of the [^{3}H]D2 antagonist used or the membrane preparation in which the D2 receptor is being characterized.

Compared with Na^+, the regulatory effects of guanine nucleotides on [^{3}H] D2 agonist binding are of less variance. Guanine nucleotides enable the complete conversion of $D2^{high}$ into $D2^{low}$ in the presence of a physiological concentration of Na^+, while having little influence on the proportion of the two states in the absence Na^+ (Grigoriadis and Seeman, 1985; Hall et al., 1992; Reader et al., 1990; Sibley et al., 1982), suggesting that Na^+ is required before this conversion can occur (Grigoriadis and Seeman, 1985; Usdin et al., 1980; Watanabe et al., 1985).

1.5 MODULATION OF NMDA RECEPOR FUNCTIONING THROUGH AN INTERACTION WITH D2 RECEPTOR

Substantial *in vivo* evidence has suggested the complex modulatory action of DA on NMDA receptor functions. The direction of this modulation varies with the specific subtype of central DA receptor activated. For instance, the increase in locomotor activity induced by microinjection of NMDA into the NAS is significantly reduced by D2 agonists, but not D1 agonists (Wu et al., 1993), indicating that NMDA-induced hyperlocomotion is modulated by DA released from terminals in the nucleus accumbens through the stimulation of mesolimbic D2 receptors. Besides, there are studies which show the participation of the D2 receptor in the three types of behavior induced by PCP: ataxia, stereotypy (Pipoli et al., 1990) and hyperlocomotion (Orgen and Goldstein, 1994), although the D1 receptor might play an important role in the expression of ataxia and stereotypy.

The functional interaction between DA, the D2 receptor and the NMDA receptor was also investigated in various *in vitro* studies. In the mammalian neostriatum, Cepeda et al. (1993) demonstrated that the neuron response evoked by iontophoretic application of NMDA and non-NMDA receptor agonists were attenuated by bath application of the D2 agonist, quinpirole. In contrast, SKF38393, a D1 receptor agonist, potentiated the NMDA-evoked excitation. The later effect was also mimicked by the administration of DA. This negative modulating effect of the D2 agonist on the NMDA receptor system was in agreement with the results of *in vivo* studies. In the CA1 neurons of rat hippocampal slices (Miyakawa, 1996), both dopaminergic and NMDA receptor systems have been

shown to be involved in the modulation of the long-term depression (LTD). The induction of LTD was blocked by the NMDA receptor antagonists or D2 agonists, while the activation of the D1 receptor produced significantly larger LTD than the control LTD. The recent finding (Leslie et al., 1998) that the non-competitive NMDA receptor antagonists, PCP or MK-801, have synergistic effects on the suppression of immediate gene zif 268 expression by D2 agonists in striatopallidal neurons, provided further evidence of the interaction between the NMDA receptor channel system and the D2-DA system at the molecular level.

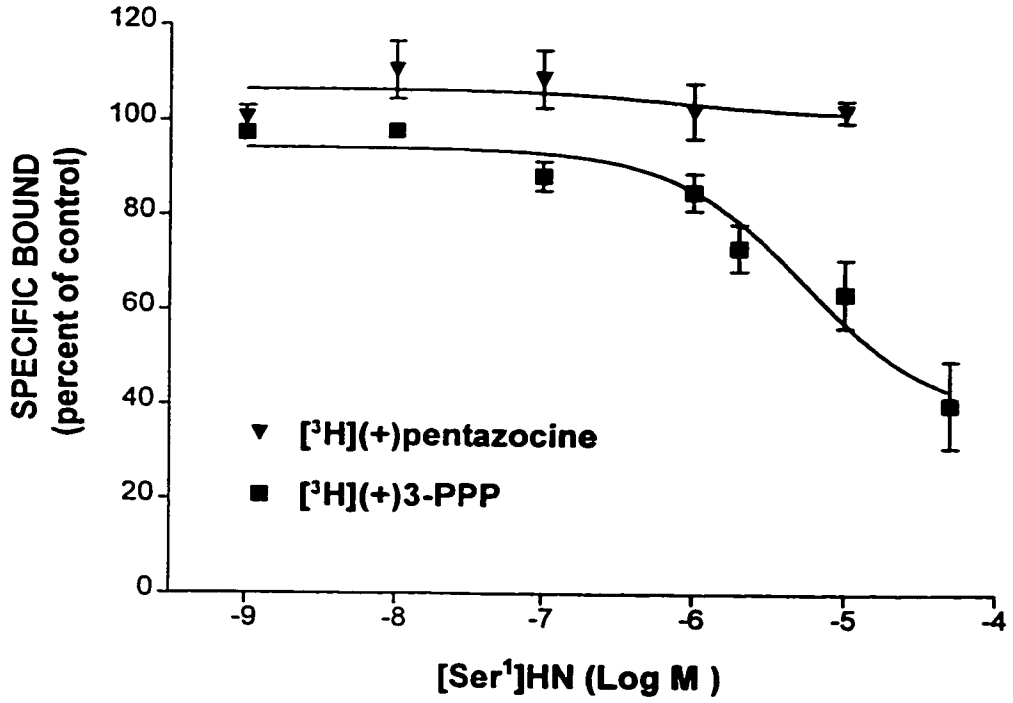
Consistent with its modulating effect on the NMDA receptor function, the D2 receptor is able to regulate the release of glutamate in the striatum (Yamamoto and Davy, 1992). D2 but not D1 agonists can block the K^+-induced release of glutamate in a concentration dependent manner. This result implicates that part of the modulatory effect of the D2 receptor may be due to its action on the presynaptic release of glutamate.

1.6 APPENDIX:

An early result from our lab has demonstrated that [Ser^1]HN is able to inhibit the binding of [^{3}H](+)3-PPP to rat brain membranes (unpublished data). The inhibition of [^{3}H](+)3-PPP binding by [Ser^1]HN was further confirmed by some preliminary experiments in the present study (Fig 3). [Ser^1]HN dose-dependently displaced the binding of [^{3}H](+)3-PPP. The competition curve analysis revealed that the presence of [Ser^1]HN inhibited maximally 60% of [^{3}H](+)3-PPP binding sites with an IC_{50} of 5.32 μM.

[^{3}H](+)3-PPP has been known to bind with high affinity to the σ1 receptor (Quirion et al., 1992) and low affinity to the D2 receptor (Wikstrom et al., 1987). Therefore, the inhibitory effect of [Ser^1]HN on [^{3}H](+)3-PPP binding could be due to its interaction with either of these two binding sites. However, the possibility that [Ser^1]HN acts as a σ receptor ligand is weak, since the binding of [^{3}H](+)pentazocine (σ1 receptor ligand, Steinfels et al., 1988) was not affected by [Ser^1]HN (Lemaire et al., 1993; Shukla et al., 1995). These results may thus implicate the D2 receptor as a potential target to modulate the inhibitory effect of [Ser^1]HN in [^{3}H](+)3-PPP binding. To better identify the interaction between HN and the D2 receptor, *in vitro* binding assays using a more selective D2 receptor radioligand are required.

Figure 4 Effects of [Ser^{1}]HN on the [^{3}H](+)3-PPP and [^{3}H](+)pentazocine binding. Binding of [^{3}H](+)3-PPP (5nM) and [^{3}H](+)pentazocine (5nM) to rat brain membrane (0.8 mg) was conducted for 30 min at room temperature. the specific binding in both assays were determined by difference counts in the absence and presence of 10 μM of haloperidol. The curve represents the mean ± S.E. of three replicate experiments carried out in duplicate.


120
100
80
60
40
20
0
SPECIFIC BOUND
(percent of control)
[3H](+)pentazocine
[3H](+)3-PPP
-9
-8
-7
-6
-5
-4
[Ser1]HN (Log M)

SECTION 2

THESIS AIMS AND OBJECTIVES

HN is an adrenal medullary peptide that has been shown to display NMDA receptor antagonist activities including the blockade of NMDA-induced convulsions and the induction of PCP-like behaviors (stereotypy, ataxia, locomotion, and blockade of passive avoidance learning) (Shukla et al., 1995; Maurice et al., 1995). The anti-NMDA receptor activity of HN has been suggested to be mediated through an allosteric modulation of the NMDA receptor complex by the high affinity HN binding site (Rogers and Lemaire, 1993). Recent findings from another laboratory have revealed the ability of [Ser^1]HN (*i.t*) to block the hyperalgesic states in animal models of chronic pain, namely the tonic phase response in the formalin test and hyperalgesia and allodynia induced by sciatic nerve injury and *i.t* injection of NMDA (Siegan et al., 1997; Siegan and Sagen, 1997; Hama et al., 1999). These beneficial effects of [Ser^1]HN were associated with the ability of the peptide to negatively modulate NMDA receptor activity. Such results triggered the present investigation on the possible analgesic effects of HN and its related peptides (*i.c.v.*) in other experimental models of pain. The hypothesis was extended to examine the receptor interactions involved in such activity of the peptides using *in vivo* and *in vitro* assays. Therefore, the aims of the present study were:

(1) To verify the analgesic effects of HN and related peptides in the mouse writhing (visceral pain) and tailflick (acute reflex pain) assays after *i.c.v* administration of the peptides in mice. In these studies, the dose and time response curves were established

as well as the structure-dependence of the effects. Meanwhile, the validity of the analgesic tests was assessed by the demonstration of the lack of motor effect of the peptides in the rotarod assay.

(2) To examine the receptor(s) involved in the analgesic effects of HN using specific antagonists for various receptors (opioid, NMDA, D1 and D2). The analgesic activity of HN in the two pain assays was specifically blocked by the D2 antagonist raclopride.

(3) To characterize the interaction of HN and related peptides with the D2 receptor using the [^{3}H]raclopride *in vitro* binding test with rat brain membranes. These studies established the structure-potency relationships, the selectivity of action and the nature of interaction between the peptides and the D2 receptor. In addition, a correlation was made between the abilities of the peptides to compete with [^{3}H]raclopride and cause analgesia in the mouse writhing assay.

SECTION 3

INTRODUCTION TO ARTICLE 1

The following paper demonstrating the significant analgesic effects of (i.c.v.) HN and its related peptides and fragments in two different animal models of pain: mouse writhing and tail-flick tests. The mechanism (the receptor(s) involved) underlying these analgesic effects was investigated by coadministration of [Ser^1]Hn with selective antagonists of opioid, NMDA, D2 or D1 receptor. HN and its related peptides were synthesized by Dr. Prasad (the second author) in our lab. I, the first author was responsible for conducting all the in Vivo antinociceptive and motorcoordination tests, and analyzing the experiment data.

Running title: Analgesic effect of Histogranin

Non-opioid antinociceptive effects of supraspinal histogranin and related peptides: possible involvement of the dopamine D2 receptor

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RUAN, H., J.A. PRASAD AND S. LEMAIRE. *Non-opioid antinociceptive effects of supraspinal histogranin and related peptides: possible involvement of the dopamine D2 receptor. PHARMACOL BIOCHEM BEHAV.* The antinociceptive effects of intracerebroventricular (i.c.v.) administration of histogranin (HN) and related peptides were assessed in the mouse writhing and tail-flick assays. In the writhing test, the peptides displayed dose-dependent analgesic effects with an AD_{50} of 23.9 nmol/mouse for HN and the following order of potency: HN-(7-15) > histone H4-(86-100) = HN = HN-(7-10) > [Ser^1]HN > OGP ≈ HN-(1-10). HN-(6-9) and HN-(8-10) did not show any significant analgesic activity at 50 nmol/mouse. The importance of the C- and N-terminal amino acids in the analgesic activity of the peptides was demonstrated by the prolonged effects of HN and [Ser^1]HN (≈ 30 min) as compared with those of HN fragments (HN-(7-15), HN-(1-10) and HN-(7-10): 5-10 min). The analgesic activity of [Ser^1]HN (50 nmol/mouse) was not affected by the coadministration of opioid (naloxone, 1 nmol/mouse), NMDA (CPP, 0.3 nmol/mouse) and D1 (SCH-23390, 0.5 nmol/mouse) receptor antagonists, but it was significantly antagonized by the coinjection of the D2 receptor antagonist raclopride (0.5 nmol/mouse). In the mouse tail-flick assay, HN and related peptides (50 nmol/mouse) also showed significant analgesic activity (15%-35% MPE). The analgesic effect of [Ser^1]HN was dose-dependent and, at 75 nmol/mouse, lasted for up to 45 min and was blocked by the coadministration of raclopride (1 nmol/mouse), but not naloxone (2 nmol/mouse). In the mouse rotarod assay, relative high doses (75 ~ 100 nmol/mouse) of HN and related peptides did not significantly affect motor coordination. These results indicate that supraspinal administration of HN and related peptides induce significant non-

opioid analgesic effects devoid of motor activity by a mechanism that involves the participation of dopamine D2 receptors.

Histogranin Analgesia Dopamine receptor Mouse writhing Tail-flick

HISTOGRANIN (HN ; H-Met-Asn-Tyr-Ala-Leu-Lys-Gly-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-COOH), a pentadecapeptide whose structure presents 80% homology with that of fragment-(86-100) of histone H4, was first isolated in our laboratory from extracts of bovine adrenal medulla (22). In the adrenal medulla, the peptide was concentrated in chromaffin granules and it was released from perfused adrenal glands upon cholinergic receptor stimulation (22). The immunoreactive peptide was also detected in various rat tissues, including the pituitary, adrenal glands, heart, spleen, lungs and brain (23). A possible physiological role for the peptide was suggested by its antagonism of N-methyl-D-aspartate (NMDA)-induced convulsions in mice (22, 23, 38). Recently, [Ser^1]HN, a chemically stable analog of HN (38), was shown to attenuate hyperalgesia and allodynia caused by sciatic nerve injury (39) and intrathecal (i.t.) administration of NMDA (17). In addition, the suppression by [Ser^1]HN of the late phase but not the early phase of pain in the formalin assay raised the possibility that HN could be an endogenous blocker of tonic or persistent pain (41). Since NMDA receptors in the dorsal horn of the spinal cord are predominantly involved in the mediation of prolonged nociceptive information (11,29,34,46), the antinociceptive effects of [Ser^1]HN at this level were then proposed to be due to its ability to modulate the activity of the NMDA receptor.

In *in vitro* binding studies with rat brain membranes, [^{125}I][Ser^1]HN displayed a high affinity site with characteristics (saturability, reversibility, sensitivity to trypsin and heat treatments) of a membrane-bound receptor (35). However, the binding of [^{125}I][Ser^1]HN was unaffected by NMDA and competitive (CPP) and non-competitive (AP5, MK-801) NMDA antagonists. Polyamines, including spermine and spermidine, were the only

NMDA regulatory agents which affected the binding of [^{125}I][Ser^{1}]HN, although [Ser^{1}]HN itself was unable to displace the binding of [^{3}H]spermidine (35). Therefore, it was suggested that HN may bind to its own site in the brain and affect NMDA receptor-mediated activity through an allosteric interaction with the NMDA receptor (33,35). On the other hand, we have recently observed that [Ser^{1}]HN was a potent inhibitor of the binding of the σ_1/D2 receptor ligand [^{3}H](+)3-PPP (45) to rat brain membranes (unpublished observation). The insensitivity of the binding of the specific σ_1 ligand [^{3}H](+)pentazocine to [Ser^{1}]HN (22) led us to hypothesize that HN could specifically interact with the central dopamine D2 receptor.

The dopamine D2 receptor is a G_i protein-bound receptor consisting of three subtypes: D2, D3 and D4 receptors (28). Previous studies have indicated that drugs such as bromocriptine, quinpirole, apomorphine, cocaine and amphetamine can alleviate pain in various animal models by a mechanism that involves the participation of the dopamine D2 receptor (8,14,21,24,30). Their analgesic activity was mainly observed in the writhing (14,16), formalin (2,30) and hot plate (21,24) assays, the tail-flick assay being somewhat less sensitive to the analgesic action of the compounds (16,27). Herein, we investigated the effects of HN and related peptides in the mouse writhing and tail-flick assays. The results suggest that HN and related peptides can produce, first in the writhing but also in the tail-flick assays, dose-, structure- and time dependent non-opioid analgesic effects that are blocked by the D2 receptor antagonist raclopride with no significant effect on motor function.

MATERIALS AND METHODS

Animals

Mice (male 20-25g, swiss webster) obtained from Charles River (Canadian Breeding Farm, St. Constant, Quebec) were housed five per cage in a room with controlled temperature (22 ± 2°C), humidity and artificial light (6.30 a.m.-19 p.m.). The animals had free access to food and water and were used after a minimum of 4 days of acclimation to housing conditions. Experiment were carried out between 10:00 a.m. and 4:00 p.m. in an air-regulated and soundproof laboratory (23± 1°C , 40 % humidity). Mice were habituated at least 30 min before experiment. The experiments were authorized by the animal care committee of the University of Ottawa in accordance with the guidelines of the Canadian Council on Animal Care.

Drugs and Peptides

(±)3-(2 carboxypiperazine-4-yl)-propyl-propionic acid (CPP), raclopride and SCH-23390 were purchased from Research Biochemical Incorporated (Natick, MA). Naloxone was the product of ENDO laboratory Inc (Garden City, New York). HN and related analogues and fragments were synthesized in our laboratory by the solid-phase procedure as described previously (32). The purity of the synthetic peptides was assessed by analytical HPLC on μ-Bondapack C18 (Waters) and by thin-layer chromatography on silica gel plates (60 F 254; BDH chemical, Darmstadt, Germany) in the following solvent system (v/v): 1-butanol /acetic acid/ water/pyridine (15/3/10/12). Their composition and molecular weight were determined by amino acid analysis of acid (HCl) hydrolysates and fast atom

bombardment mass spectrophotometry (FAB MS). Peptides were dissolved in double-distilled sterile water (vehicle) and 10 µl peptide solutions and vehicle were delivered gradually through Intracerebroventricular (i.c.v.) injection within 3 s. Free hand *i.c.v* injections into the lateral ventricles of the conscious mouse were performed as described in previous study (38). The injections were made using a No. 27 gauge, 0.25-in needle attached to 500-µl Hamilton syringe and an automatic dispenser (PB 600; Hamilton Col, Reno, NV). The needle was filled with polyethylene tubing, leaving 3 mm of the needle tip exposed. The site of injection confirmed by injecting Indian ink in preliminary experiments was on an imaginary line drawn through the anterior lobe of the ears and from an imaginary midsagital line. The whole injection procedure was completed within 10 -15 s, so that the animals should suffer minimal discomfort and pain.

Mouse Writhing Test

Antinociceptive activity of HN and its related peptides was evaluated using the acetic acid-induced writhing test according to a modification (37) of the method of Hayashi and Takemori (18). Male swiss webster mice were injected intraperitoneally (i.p.) with 1.0% acetic acid (10 ml/kg) 5 min (or the indicated time) after *i.c.v* injection of 0 (distilled water), or three to five effective doses (between 0.5 and 100 nmol) of HN or related peptides. The number of writhes displayed by each mouse was counted for a period of 10 min after the injection of the acetic acid solution. An abdominal stretch is characterized by the contraction of the abdominal muscles, the arching of the back ventrally such as the abdomen touches the bedding surface and the extension of one or both hind limbs. Mice

were used only once and killed immediately. Groups of 10 mice were used per dose and the average number of writhes per mouse was compared with that of the control group. Antinociceptive activity was expressed as % analgesia as calculated by the formula: [(mean number of writhes in control group - mean number of writhes for the test group) / (mean number of writhes in control group) × 100]. The percent analgesia for various effective doses was then used to calculate the AD_{50} and potency ratio by the method of Litchfield and Wilcoxon (26) using the procedure 47 of the computer program of Taliarida and Murray (43). The times of action of HN and related peptides were determined by injection of 1% acetic acid at various times after the administration of the peptides. The results represent the mean percent analgesia ± S.E.M. in groups of 10 mice and statistical analysis of the analgesic effect of the peptides were carried out by the student t-test. For antagonism experiment, selective antagonists of opioid , NMDA, D2 or D1 receptors were administrated i.c.v. in groups of 20 mice in an aliquot of 10 ul, alone or in combination with [Ser^1]HN as indicated. The results are expressed as the mean number of writhes per mouse ± S.E.M. One way analysis of variance was used to compare the effect of drug combination, followed by the Newman-Keuls multiple comparison test. The criterion for statistical significance in all tests was $P < 0.05$.

Mouse Tail-flick Assay

The nociceptive thresholds of all mice treated with HN and related peptides were also assessed using a noxious reflexive pain model by the heat tail-flick test (10). Experiments were performed between 10:00 and 15:00 hr. Mice were lightly restrained

under paper wadding and their tails were placed gently on a beam radiation window. Noxious stimulation was provided by a beam of high density light focused on the tail. The light intensity was set at 40 to give a control reading of about 3 s. The response time latency was measured automatically and was defined as the interval between the onset of the thermal stimulus and the abrupt flick of the tail. Each determination was performed in at least 10 animals. The mean score was taken as the response latency. A cut-off latency of 12 s was employed to prevent the possibility of tissue damage. The antinociceptive effects in this assay were expressed as the percentage of the maximum possible effect (% MPE), which was calculated by the formula:

$$\% \text{ MPE} = \frac{(\text{postinjection latency} - \text{baseline latency}) \times 100}{(\text{cutoff latency} - \text{baseline latency})}$$

The use of % MPE takes into account differences in baseline nociceptive latencies so that these difference will not bias the quantification of antinociception. The significance of the possible analgesic effects of HN and related peptides (i.c.v.) alone were determined using student t-test by comparing the % MPE ± S.E.M. in peptide-treated mice with that of vehicle-treated mice. One way analysis of variance was employed to access the possible effect of drug combination, followed by Newman Keuls multiple comparison test.

Effects on Motor Movements

The ability of HN and its related peptides to interfere with the coordinated motor movements was evaluated using a rotarod assay. Impairment of coordinated motor

movements was defined as inability of the mice to remain on the rota-rod for a 2-min test period (12). The rotarod apparatus (Ugo-Basile, Varese, Italy) consisted of rotating bar above a series of plates connected to an automatic counter. Mice were placed upon the bar and the time to fall onto the plate was immediately registered. Two hours prior to evaluation of drug action, mice underwent a condition selection. Rotation velocity was set at 20 rpm. After two trials, mice which remained on the bar for over 120 s (cut off time) were used for evaluating the effects of peptides. Rotarod assays were conducted 5 and 10 min after the *i.c.v* administration of vehicle (distilled water) or peptides at a dose that provided maximal analgesic response in the writhing test. Rotarod performance (up to 120 s) which represented the mean time of mice remaining on the rotating bar was compared between the vehicle-treated group and the peptide-treated groups. Each group consisted of 15-20 animals. Statistical significance were determined using the Student t- test .

RESULTS

Dose- and Structure-Dependency of HN and Related Peptides in Mouse Writhing Test

I.c.v. administration of HN and related peptides in mice induced dose- and structure-dependent analgesic activities as assessed by their ability to inhibit writhing in response to i.p. administration of acetic acid (Fig. 1, Table 1). The chemically stable HN analogue, [Ser^1]HN, displayed an analgesic potency similar to that of HN (AD_{50} of 31.7 nmol/mouse as compared with 23.9 nmol/mouse for HN). The C-terminal fragment of HN, HN-(7-15), was 2.79 times as potent as HN itself with an AD_{50} of 8.5 nmol/mouse, whereas the N-terminal fragment, HN-(1-10), was less potent than HN (AD_{50}: 54.8 nmol/mouse). The minimal core for producing the analgesic effect appeared to reside in sequence 7-10 of HN since HN-(7-10) had a potency comparable to that of HN itself and the shorter peptides HN-(6-9) and HN-(8-10) did not produce significant analgesic activity at a dose of 50 nmol/mouse (Table 1). The analgesic potencies of histone H4-(86-100) (23) and osteogenic growth peptide (OGP) (6), two unmodified C-terminal fragments of histone H4, were comparable to those of HN and HN-(1-10), respectively.

Time of Action

The time responses of these analgesic effects induced by HN, [Ser^1]HN, and selective HN fragments are illustrated in Fig. 2. HN (50 nmol/mouse) produced an analgesic effect that lasted for about 30 min, the maximal effect (67 ± 12% analgesia) being observed 10 min after the administration of the peptide. The full length HN analogue, [Ser^1]HN, had a time response profile that was comparable to that of HN. The lack of either

C-terminal or N-terminal portions of HN resulted in HN fragments with reduced times of action. At five min, HN-(7-15) (50 nmol/mouse) produced a stronger analgesic effect than HN itself (70% analgesia as compared with 54% analgesia for HN); but its analgesic effect rapidly decreased with only 5.6% analgesia remaining 10 min after the administration of the peptide. Likewise, the HN fragments, HN-(1-10) and HN-(7-10), displayed rapid losses in their analgesic activities.

Non-involvement of Opiate and NMDA Receptors

The possible involvement of opiate and NMDA receptors in the analgesic effect of HN in the mouse writhing test was verified by coadministration of [Ser^1]HN (50 nmol/mouse) with specific opiate (naloxone, 1 nmol/mouse) or NMDA (CPP, 0.3 nmol/mouse) receptor antagonists (Fig. 3). Analysis of the mean number of writhes in animals treated with [Ser^1]HN alone (3.3 ± 0.5) as compared with that of the control group (7.4 ± 0.6) revealed a significant effect corresponding to 54.5± 7.4 % analgesia. The mean numbers of writhes in mice treated with naloxone (7.8 ± 1.3) and CPP (6.5 ± 1.0) alone did not differ significantly from that of vehicle treated mice. Coadministration of naloxone with [Ser^1]HN did not modify the antinociceptive activity of the peptide (4.2 ± 0.5 writhes as compared with 3.3 ± 0.5 for [Ser^1]HN alone; $P > 0.05$). Similarly, the coadministration of the NMDA antagonist CPP with [Ser^1]HN did not affect the analgesic activity of the peptide (4.5 ± 0.9 writhes; $P > 0.05$).

Specific Blockade by the D2 receptor Antagonist Raclopride

Figure 4 shows the effects of D2 (raclopride) and D1 (SCH-23390) receptor antagonists on the antinociceptive activity of [Ser^1]HN (50 nmol/mouse) in the mouse writhing test. Raclopride (Fig. 4A) and SCH-23390 (Fig. 4B) administered alone (0.5 nmol/mouse, i.c.v.) did not affect the pain response. Coadministration of raclopride with [Ser^1]HN significantly and almost completely reversed the antinociceptive activity of the peptide, increasing the mean number of writhes from 3.2 ± 0.9 (mice receiving [Ser^1]HN alone) to 6.9 ± 1.3 ($P < 0.05$) (Fig. 4A). In contrast, coadministration of SCH-23390 with [Ser^1]HN failed to significantly affect the analgesic activity of the peptide (3.0 ± 1.05 writhes as compared with 2.4 ± 0.9 in [Ser^1]HN-treated group, $P > 0.05$) (Fig. 4B).

Antinociceptive Activity of HN and Related Peptides in Mouse Tail-flick Assay

HN and some related peptides, which were previously shown to be effective in the mouse writhing test, were also tested in the mouse tail-flick assay, an animal model of acute reflex type of pain. All tested peptides (50 nmol/mouse, i.c.v.) induced small but significant increases (13.3-34.7 % MPE) in the TF latency as compared with the vehicle-treated group (3.4% MPE), the most efficient peptides being HN and OGP (Fig. 5). [Ser^1]HN was chosen to assess the dose- and time-dependence of this increase in pain reflex latency (Fig. 6A). Significant analgesia was observed up to 45 min after i.c.v. administration of 75 nmol/mouse of the peptide. At a lower dose (50 nmol/mouse), the peptide produced a smaller analgesic effect that remained significant up to 30 min after the administration. Finally, the analgesic effect of [Ser^1]HN (75 nmol/mose) was partially but significantly reversed by the coadministration of raclopride (1 nmol/mouse), but not by

naloxone (2 nmol/mouse) (Fig. 6B).

Effects of HN and Related peptides in the Mouse Rotarod Assay

The possibility that HN and related peptides affect motor coordination was evaluated by the mouse rotarod assay (Table 2). At the highest doses tested in the mouse writhing test, none of the HN peptides or fragments significantly affected the abilities of mice to stay on the rotarod as assessed by the percent of mice falling and the mean performance in each group of animals ($P > 0.05$ as compared with vehicle treated animals).

DISCUSSION

HN was first isolated from the bovine adrenal medulla (22), a tissue recognized to contain various pain reducing substances, including the endogenous opioid peptides Met- and Leu-enkephalins and catecholamines (6, 25). Recently, Sagen and colleagues have developed a technique of transplantation of adrenal medullary tissue or chromaffin cells into the spinal subarachnoid space for the alleviation pain in various rat models of tonic and chronic pain (36,40,42). While most of the analgesic effects of adrenal transplants were attributed to opioid peptides and catecholamines released from adrenomedullary chromaffin cells, the alleviation of the noxious response in the second phase of the formalin assay was not completely antagonized by naloxone and phentolamine, alone or in combination (42), suggesting that other factor(s) were involved in the mediation of the analgesic activity of the adrenal transplants. Recently, the same group of investigators found that i.t. administration of [Ser^1]HN, a stable synthetic analogue of the adrenal medullary peptide HN (38), can mimic the beneficial effects of the adrenal transplants (17,39,41). The analgesic activity of [Ser^1]HN was then ascribed to its property to inhibit the NMDA receptor (23,33,38) on the basis that the spinal NMDA receptor plays an important role in the mediation of prolonged nociceptive transmission (11,29,34,46). However, no attempt was made to define the nature of the receptor involved in the actions of the HN peptide at this level.

The present data show that supraspinal administration of HN and related peptides induce marked antinociceptive activity in the mouse writhing and tail-flick assays. The non-reversal of the analgesic effects of [Ser^1]HN by naloxone in both tests confirms their non-

opioid nature. While a large body of evidence supports the potential utility of NMDA antagonists (i.t.) for the treatment of chronic pain (9,11,34,46), the supraspinal administration (i.c.v.) of competitive (CPP, AP5) and non-competive (MK-801) NMDA antagonists has been shown not to affect nociception (31). These data are consistent with our results which show the non-effectiveness of the competitive NMDA antagonist CPP (i.c.v.) in the mouse writhing test (Fig. 3). Therefore, the analgesic activities of HN and related peptides in the current assays cannot be explained by the NMDA receptor antagonist properties of the peptides (24,33,38).

On the other hand, there exists a descending pain control pathway (13), the major components being the midbrain periaqueductal gray (PAG), several nuclei of the rostral ventral medulla (RVM) and the spinal dorsal horn. The excitation of the RVM by the PAG projection is mediated partly by excitatory amino acids (EAA) (1,13). EAA agonists injected into the PAG and some of the nuclei of RVM suppressed nociceptive responses in the spinal dorsal horn (1,20,48). Therefore, the analgesic effects of supraspinal HN and related peptides could be due to the stimulation of excitatory amino acid receptors. Such a possibility was also suggested by the observation that high doses of [Ser^1]HN (i.t.) produce NMDA agonist-like behavior (39, 41). However, the inability of the NMDA antagonist CPP to block the analgesic activity of [Ser^1]HN in the mouse writhing test (Fig. 3) indicated that such effect of the peptide does not involve the stimulation of the NMDA receptor.

The observation that the D2 selective antagonist raclopride markedly antagonizes the analgesic effects of [Ser^1]HN in the mouse writhing and tail-flick assays suggests that

HN and related peptides produce their effects by an interaction with some central dopamine D2-like receptor(s) either directly or through a modulation of dopaminergic transmission. Several studies have indicated that both D2 agonists and indirect dopamine receptor stimulants produce analgesia in different animal models of pain (14,16,21,30). Their analgesic effects were suggested to be mediated through enhanced dopamine D2 receptor transmission in terminals of the mesolimbic pathway. In support of this concept, Altier and Stewart (2) have recently reported that the analgesic effects of intra-NAS amphetamine are prevented by raclopride. While some D2 receptor-mediated behaviors such as locomotion and stereotypy are expressed only when the D1 receptor system is costimulated (3,7,19), the analgesic effects of direct D2 receptor agonists or indirect dopamine receptor stimulants generally do not involve the activation of the D1 receptor (5,8,14,32). The non-involvement of the D1 receptor in the analgesic response to [Ser^1]HN was evidenced by its insensitivity to the D1 receptor antagonist SCH-23390. Thus, the analgesic activities HN and related peptides, like those of other analgesics acting through the D2 receptor, did not involve the participation of the D1 receptor.

Another approach to elucidate the site of action of HN and related peptides was provided by the structure-activity relationship (SAR) study of selected HN analogues and fragments. Analysis of SAR revealed several interesting characteristics for the analgesic effects of HN and related peptides. First, in accordance with previous findings (33, 35), [Ser^1]HN, a chemically stable analog of HN, displayed the same range of potency as HN in the mouse writhing test, suggesting that the synthetic analogue possesses the structural requirements of the natural peptide for biological activity. However, in contrast with

previous findings that the removal of a single amino acid from either the N- or C-terminal portions of HN provides HN fragments with dramatic losses in [^{125}I][Ser^{1}]HN binding potencies (35) and anticonvulsant (33) activities, the analgesic potencies of the N-terminal (HN-(1-10)) and C-terminal (HN-(7-15)) fragments were largely retained and even improved as compared with that of HN (Fig. 1, Table 1 and Fig. 5). These data indicate that the mechanism underlying the analgesic activities of supraspinal HN and related peptides in the mouse writhing and tail-flick assays is distinct from that involved in their binding to the high affinity HN site in the brain (35) and inhibition of NMDA-induced convulsions (33). The possibility that HN binds to a lower affinity site in the brain has not yet been examined, but HN was recently shown to compete with the binding of [^{3}H]raclopride, a D2 selective ligand (unpublished data). HN may thus act as a modulator of the central D2 receptor, a Gi protein-coupled receptor (28), and cause D2 agonist-like antinociceptive activity either by a direct interaction with D2 sites or in an allosteric manner after acting on its own receptor.

The minimal active core for inhibition of mouse writhing in response to visceral pain appears to reside in the middle portion (amino acid sequence 7-10) of HN, since HN-(7-10) displayed an analgesic potency comparable to that of HN while HN-(8-10) and HN-(6-9) were inactive at the dose of 50 nmol/mouse. Even though both C- and N-terminal portions of HN were not essential for production of the analgesic activity, their presence markedly prolonged the action of the peptides (Fig. 2). Thus, HN-(1-10), HN-(7-15) and HN-(7-10), possibly due to their reduced resistance to the action of peptidases, displayed short times of action as compared with the full length peptides, HN and [Ser^{1}]HN.

Fragment-(86-100) of histone H4, a pentadecapeptide in histone H4 that shows 80% structural homology with HN (22), displayed an analgesic potency comparable to that of HN (Table 1). Osteogenic growth peptide (OGP) (4), another C-terminal fragment of histone H4, was 2.2 times less potent than HN (Table 1). The possible processing of histone H4 into its C-terminal fragment, H4-(86-100), has not yet been demonstrated, but the recent finding of the presence of histone immunoreactivity on the cell surface of lymphocytes (44), the existence of C-terminal histone H4 related peptides in blood plasma (OGP) (4) and histogranin in the adrenal medulla (22) are compatible with the hypothesis that histone H4 or some variant of histone H4 such as H4-v.1 (17) may generate C-terminal histone H4 fragments with extranuclear and/or extracellular function(s).

I.c.v. administration of HN and related peptides in mice significantly but less effectively attenuated the pain response in the tail-flick assay (a thermal acute pain assessed by a reflex response) than the writhing test. The tail-flick assay was recognized to be less sensitive to the analgesic effects of non-narcotic analgesics (9). Although the analgesic effects of drugs acting through the D2 receptor were well pronounced in animal models of abdominal pain (14), hyperalgesia associated with peripheral nerve injury (30) and the late phase of pain due to formalin (24), the antinociceptive effects of D2 agonists in the tail-flick assay were less prominent (16, 27). Therefore, the observation that HN and related peptides are less potent and efficient analgesics in the tail-flick than writhing assays correlates with the previous findings which indicated that the tail-flick assay is less sensitive than other pain assays to D2 agonists. On the other hand, the possibility that the

analgesic effects of HN and related peptides in both writing and tail-flick assays resulted from an impairment of motor functions was weakened by the observation that the peptides (i.c.v.) at doses which produced maximal analgesic activities, did not impair motor function as assessed by the mouse rotarod assay (Table 2).

In conclusion, these results, along with previous findings, indicate that HN and related peptides are potent and effective analgesics in various animal models of pain. While the analgesic properties of the HN and related peptides in the mouse writhing and tail-flick assays most likely involve the participation of dopamine D2-like receptor(s), the possible involvement of this (these) receptor(s) in other pain-relieving activities (tonic pain induced by formalin (41), hyperalgesia induced by sciatic nerve injury (39) and i.t. administration of NMDA (17)) remains to be established.

ACKNOWLEDGEMENTS

This work was supported by the Medical Research Council of Canada (MT-11170).

REFERENCES

1. Aimone, L.D.; Gebhart, G.F.: Stimulation-produced spinal inhibition from the midbrain in the rat is mediated by an excitatory amino acid neurotransmitter in the medial medulla. J.Pharmacol. Exp. Ther. 6 : 1803-1813; 1986.

2. Altier, N; Stewart, J.: Dopamine receptor antagonists in the nucleus accumbens attenuate analgesia induced by ventral tegmental area substance P or morphine and by nucleus accumbens amphetamine. J. Pharmacol. Exp. Ther. 285: 208-215;1997

3. Arnt, J.; Bogeso, K.P.; Hyttel, J.; Meier, E.: Relative dopamine D1 and D2 receptor affinity and efficacy determine whether dopamine agonist induced hyperactivity or oral stereotype in rats. Pharmacol. Toxicol. 62:121-130; 1988.

4. Bab, I.; Gazit, D.; M.; Chorev, M.; Muhlrad, A.; Shteyer, Z.; Greenberg, Z.; Namdar, M.;.Kahn, A.: Histone H4- related osteogenic growth peptide (OGP): a novel circulating stimulator of osteoblastic actiity. EMBO J. 11:1867-1873; 1992.

5. Bittencourt, A.L.; Takahashi, R.N.: Mazindol and lidocaine are antinociceptives in the mouse formalin models: involvement of dopamine receptor. Eur. J. Pharmacol. 330: 1803-1813; 1997.

6. Boarder, M. R.; Lockfeld, A. J.; Barchas, J. D.: Met-enkephalin [Arg6, Phe7] immunoreactivity in bovine caudate and bovine adrenal medulla. J. Neurochem. 39:149-154; 1982.

7. Braun, A. R.; Chase, T. N.: Obligatory D1/D2 receptor interaction in the generation of dopamine agonist related behaviors. Eur. J. Pharmacol. 131: 301-306; 1986.

8. Carr, K.D.: Dopaminergic mechanisms in the supraspinal modulation of pain. Pain(Supple) 2: S223; 1984.

9. Coderre T. J.; Van Empel, I.: The utility of excitatory amino acid (EAA) antagonists as analgesic agents. I. Comparision of the antinociceptive activity of various classes of EAA antagonists in mechanical, thermal and chemical nociceptive tests. Pain 59: 345-352; 1994.

10. D'Amour, F.E.; Smith, D.L.: A method for determining loss of pain sensation. J. Pharmacol. Exp. Ther. 72: 74-77; 1941

11. Dickenson, A.H.: A cure for wind-up: NMDA receptor antagonist as a potential analgesics. Trends Pharmacol. 11: 307-309; 1990.

12. Dunham, N.W.; Miya, T.S.: A note on a simple apparatus for detecting neurological deficit in rats and mice. J. Am. Pharm. Assoc. 46: 208-209;1957.

13. Field, H.L.; Heinricher, M.M.; Mason, P.: Neurotransmitters in nociceptive modulatory circuits. Annu. Rev. Neurosci. 14:219-245; 1991.

14. Frussa-Filho, R.; Rocha, J.B.T.; Conceicao, I.M.; Mello, C.F.; Pereira, M.E.: Effects of dopaminergic agents on visceral pain measured by the mouse writhing test. Arch. Int. Pharmacodyn. 331: 74-93; 1996.

15. Gendron, N.; Dumont, M.; Gagne, M.F.; Lemaire, S.: Poly A-containing histone H4 mRNA variant (H4-V.1): Isolation and sequence determination from bovine adrenal medulla. Biochim. Biophys. Acta. 1396:32-38; 1997.

16. Gonzales-Rios, F.; Vlaiculescu, A.; Ben Natan, L.; Protais, P.; Costentin, J.: Dissociated effects of apomorphine on various nociceptive responses in mice. J.

Neural Transmission. 67:87-103; 1986.

17. Hama, A.T.; Siegan, J.B.; Herzberg, U.; Sagen, J.: NMDA-induced spinal hypersensitivity is reduced by naturally derived peptide analog [Ser^1]Histogranin Pharmacol. Biochem. Behav. 62: 67-74; 1999.

18. Hayashi, G.; Takemori, A.E.: The type of analgesic receptor interaction involved in certain analgesic assays. Eur. J. Pharmocol. 16: 63-66; 1971.

19. Jackson, D. M.; Hashizume, M.: Bromocriptine induces marked locomotor stimulation in dopamine-depleted mice when D1 dopamine receptor are stimulated with SK&F38393. Psychopharmacol. 90: 147-149; 1986.

20. Jensen, T.S.; Yaksh, T.L.: The antinociceptive activity of excitatory amino acids in the rat brainstem: an anatomical and pharmacological analysis. Brain Res. 569: 255-267; 1992.

21. Kostrzewa, R.M.; Brus, R.; Kalbfleisch, J.: Ontogenetic homologous sensitization to the antinociceptive action of quinpirole in rats. Eur. J. Pharmacol. 209: 157-161; 1991.

22. Lemaire, S.; Shukla, V.K.; Rogers, C.; Ibrahim, I.H.; Lapierre, C.; Dumont, M.: Isolation and characterization of histogranin, a natural peptide with N-methyl-D-aspartate antagonist activity. Eur. J. Pharmacol. 245: 247-256; 1993.

23. Lemaire S.; Rogers, C.; Dumont, M.; Shukla, V.K.; Lapierre, C.; Prasad, J.; Lemaire; I.: Histogranin, a modified histone H4 fragment endowed with N-methyl-D-aspartate antagonist and immunostimulatory activities. Life Sciences 56: 1233-1241; 1995

24. Lin, Y.; Morrow, T.J.; Kiritsy-Roy, J.A.; Terry, L.C.; Casey, K.L.: Cocaine: evidence for supraspinal, dopamine-mediated, non-opiate analgesia. Brain Res. 479: 306-312; 1989.

25. Liston, D.; Datey, G.; Rossier, J.; Verbanck, J.-J.; Vanderhaeghem J.-J.: Processing of proenkephalin is tissue specific. Science 225: 734-737; 1984.

26. Litchfield, J.T.; Wilcoxon, F.: A simplified method of evaluating dose-effect experiments. J. Pharmacol. Exp. Ther. 96: 99-113;1949.

27. Liu, Q.S.; Qiao, J.T.; Dafny, N.: D2 dopamine receptor involvement in spinal dopamine-produced antinociception. Life Sciences. 51: 1485-92, 1992.

28. Missale, C.; Nash, S.R.; Robinson, S.W.; Jaber, M.; Caron, M.G.: Dopamine receptors: from structure to function. Physiol. Rev. 78:189-225, 1998

29. Moore, R.D.; Mokler, D.J.; Winterson, B.J.: Intrathecally administered N-methy-D-aspartate increases persistent hindlimb flexion in rat. Neurosci. Let. 146: 223-226; 1990.

30. Morgan, M.J.; Franlin, K.B.J.: Dopamine receptor subtypes and formalin test analgesia, Pharmacol. Biochem. Behav. 40: 317-322; 1991.

31. Nasstrom, J.; Karlsson, U.; Berge, O.G.: Systemic or intracerebroventricular injection of NMDA receptor antagonist attenuates the antinociceptive activity of intrathecally administered NMDA receptor antagonists. Brain Res. 623: 47-55; 1993.

32. Ohkubo, Y.; Momura, K.; Yamaguchi, I.: Involvement of dopamine in the mechanism of action of FR64822, a novel non-opioid antinociceptive compound. Eur. J.

Pharmacol. 204: 121-125; 1991.

33. Prasad, J.A.; Shukla, V.K.; Lemaire, S.: Synthesis and biological activity of histogranin and related peptides. Can. J. Physiolo. Pharmacol. 73:209-214; 1995.

34. Ren, K.; Hylden, J.L.K.; Williams, G.M.; Ruda, M. A.; Dubner, R.: The effects of a non-competitive NMDA receptor antagonist, MK-801, on behavior hyperalgesia and dosal horn neuronal activity in rats with unilateral inflammation. Pain 50: 331-344;1992.

35. Rogers, C.; Lemaire, S.: Charactrization of [^{125}I][Ser^{1}]Histograin binding sites in rat brain. J. Pharmacol. Exp. Ther. 267: 350-356; 1993.

36. Sagen, J.; Wang, H.; Pappas, G.G.: Adrenal medullary implants in the rat spinal cord reduce nociception in a chronic model of pain. Pain 42: 69-79;1990.

37. Shukla, V.K.; Bansinath, M.; Dumont, M.; Lemaire, S.: Selective involvement of kappa opioid and phencyclidine receptors in the analgesic and motor effects of dynorphine-A-(1-13)-Tyr-Leu-Phe-Asn-Gly-Pro. Brain Res. 591: 176-180; 1992.

38. Shukla, V.K.; Lemaire, S.; Dumont, M.; Merali, Z.: N-methyl-D-Asparate receptor antagonist activity and phencyclidine-like behavioral effects of the pentadecapeptide, [Ser^{1}]Histogranin. Pharmcol. Biochem. Behav. 50: 49-54; 1995.

39. Siegan, J. B.; Hama, A.T.; Sagen, J.: Suppression of neuropathic pain by a naturally-derived peptide with NMDA antagonist activity. Brain res. 755: 331-334; 1997.

40. Siegan, J.B.; Sagen, J.: Attenuation of NMDA-induced spinal hypersensitivity by adrenal medullary transplants. Brain Res. 680: 88-98; 1995.

41. Siegan, J. B.; Sagen, J.: A natural peptide with NMDA inhibitory activity reduces tonic pain in the formalin model. Neuroreport 8: 1379-1381; 1997.

42. Siegan, J.B.; Sagen, J.: Attenuation of formalin pain responses in the rat by adrenal medullary transplants in the spinal subarachnoid space. Pain 70: 279-285;1997.

43. Tallarida, R.J.; Murray, R.B.: Manual of pharmacological calculations with computer programs. 2nd ed., springer, New York; 1987.

44. Watson, K.; Edwards, R.J.; Shaunak, S.; Parmelee, D.C.; Sarraf, C.; Gooderham, N.J.; Davies, D.S.: Extra-nuclear location of histone in activated human peripheral blood lymphocytes and cultured T-cells. Biochem. Pharmacol. 50: 299-309; 1995.

45. Wikstrom, H.; Andersson, B.; Elebring, T.; Svensson, K.; Carllson, A.; Argent, B. N-substituted 1,2,3,4,4a,5,6,10b-Octahydrobenzo[f]quinolines and 3-Phenylpiperidines: Effects on central dopamine and σ receptors. J. Med. Chem. 30: 2169-2174; 1987.

46. Woolf, C.J.; Thompson, S.W.N.: The induction and maintenance of central sensitization is dependent on N-methy-D-aspartic acid receptor activation; implications for the treatment of post-injury pain hypersensitivity states. Pain 44: 293-299; 1991.

47. Yamamoto, T. ; Yaksh, T.L.: Spinal pharmacology of thermal hyperalgesia induced by constriction injury of sciatic nerve. Excitatory amino acid antagonists. Pain 49: 121-128;1992.

48. Zhuo, M.; Gebhart, G.F.: Characterization of descending facilitation and inhibition of spinal nociceptive transmission from the nuclei reticularis gigantocellularis and

gigantocellularis pars alpha in the rats. J. Neurophys. 67: 1599-1614; 1992.

Table 1

Relative potencies of HN (i.c.v.) and related peptides in producing analgesia in the mouse writhing pain assay.

Peptides	AD_{50} (nmol/mouse) (95% CL) [a]	Potency ratio [b] (95% CL) [a]
HN	23.9 (12.5-45.5)	1.0
[Ser^1]HN	31.7 (19.2-52.3)	0.75 (0.33 - 1.70)
HN-(7-15)	8.5 (1.9 - 15.4)	2.79 (1.36 -13.9) *
HN-(1-10)	54.8 (34.8 - 80.2)	0.45 (0.21 - 0.97) *
HN-(7-10)	25.8 (5.9 - 112)	0.92 (0.18 - 4.6)
HN-(6-9)	NA[c]	-
HN-(8-10)	NA[c]	-
H4-(86-100)	23.5 (14.0 - 39.4)	1.01 (0.44 - 2.30)
H4-(89-102) (OGP)	52.6 (35.7 -77.7)	0.45 (0.21-0.96) *

[a] 95% Confidence limit. [b] As compared with HN. OGP: Osteogenic growth peptide.

[c] NA: Not active at 50 nmol/mouse, the mean number of writhes was not significantly different from that in the control group (6.5 writhes/mouse for HN-(6-9) and 6.1 writhes/mouse for HN-(8-10) as compared with 7.2 writhes/mouse in the control group). * $P \leq 0.05$ as compared with HN.

Table 2

The effects of HN and related peptides in the mouse rotarod assay.

Treatment	Dose (nmol/mouse)	% of mice falling [a]		Rotarod performance [b] (seconds)	
		5 min [c]	10 min [c]	5 min [c]	10 min [c]
Vehicle	~	8.8	11.1	112.1±4.5	110.7±6.6
Histogranin (HN)	75	10	20	114.8±5.2	102.9±11.4
[Ser^1]HN	75	0	0	120.0±0.0	120.0±0.0
HN-(1-10)	75	13.3	6.7	110.7±6.3	115.1± 4.9
HN-(7-15)	75	11.1	0	109.4±10.6	120.0±0.0
H4-(86-100)	75	26.7	20	96.8±10.1	99.9±10.8
H4-(89-102) (OGP)	100	20	20	105.8±7.8	103.5±10.5

[a] Percent of mice falling during the 2 min assay. [b] Mean times ± S.E.M. for the mice to remain on the rotarod. [c] Time interval after i.c.v administration of the peptides or vehicle.

Legend to Figures

Fig 1. Dose-dependent analgesic effects of HN and its fragments in the mouse writhing pain assay. Peptides were administered i.c.v. at the indicated doses 5 min prior to i.p. administration of 1 % acetic acid and their analgesic effects were monitored as indicated in the Method section. Results are expressed as the mean percent analgesia (n = 10) and the AD_{50s} were calculated as indicated in the Method section.

Fig 2. Time-dependent analgesic effects of HN, [Ser^1]HN, HN-(7-15), HN-(1-10) and HN-(7-10) in the mouse writhing test. Peptides (50 nmol/mouse, i.c.v.) were administered at various times prior to the administration of 1 % acetic acid and analgesia was monitored as described in the Method section. Results are expressed as the mean percent analgesia ± S.E.M. (n=10). * $P < 0.05$ as compared with the control group (Student-t test) .

Fig 3. Effects of opioid (naloxone) and NMDA (CPP) receptor antagonists on the analgesic effect of [Ser^1]HN in the mouse writhing test. [Ser^1]HN (50nmol/mouse, i.c.v.) was administered alone or in combination with naloxone (1 nmol/mouse) or CPP (0.3 nmol/mouse) 5 min prior to i.p. administration of 1 % acetic acid as indicated in the method section. Results are expressed as the mean number of writhes ± S.E.M. (n=20). * $P < 0.05$ is considered significant as compared with the control group (One way ANOVA followed by the Keuman Keuls multiple comparison

test).

Fig 4. Effects of D2 and D1 receptor antagonists on the analgesic effect of [Ser^1]HN in the mouse writhing test. [Ser^1]HN (50nmol/mouse, i.c.v.) was administered alone or in combination with raclopride (0.5 nmol/mouse) (A) or SCH-23390 (0.5 nmol/mouse) (B) 5 min prior to i.p. administration of 1 % acetic acid. Results are expressed as the mean number of writhes ± S.E.M. (n=20). * $P < 0.05$ is considered significant as compared with the control group; $^+$ $P < 0.05$ is considered significant as compared with the group of mice treated with [Ser^1]HN alone (One way ANOVA followed by the Keuman Keuls multiple comparison test).

Fig. 5 Analgesic effects of HN and related peptides in the tail-flick assay. Peptides were administered i.c.v. at 50 nmol/mouse 10 min prior to the tail-flick assay as described in the method section. Results are expressed as the mean percentage of maximal possible effect (% MPE) ± S.E.M. (n=10). * $P<0.05$ is considered significant as compared with that of vehicle-treated group (Student t-test).

Fig 6. (A) Time- and dose-dependent analgesic effects of [Ser^1]HN in the mouse tail-flick assay. Mice were administered with [Ser^1]HN (50 or 75 nmol i.c.v.) or vehicle at the indicated times prior to the measurement of analgesia. Results are expressed as the mean percentage of maximal possible effect (% MPE) ± S.E.M. (n=10). * $P<0.05$ is considered significant as compared with vehicle treated group; (B) Effect

of D2 (raclopride, 1 nmol/mouse) and opioid (naloxone, 2 nmol/mouse) receptor antagonists on [Ser^1]HN (75 nmol/mouse) induced-analgesia in the mouse tail-flick assay. * $P<0.05$ is considered significant as compared with the [Ser^1]HN treated group (One way ANOVA followed by the Keuman Keuls multiple comparison test).

Figure 1

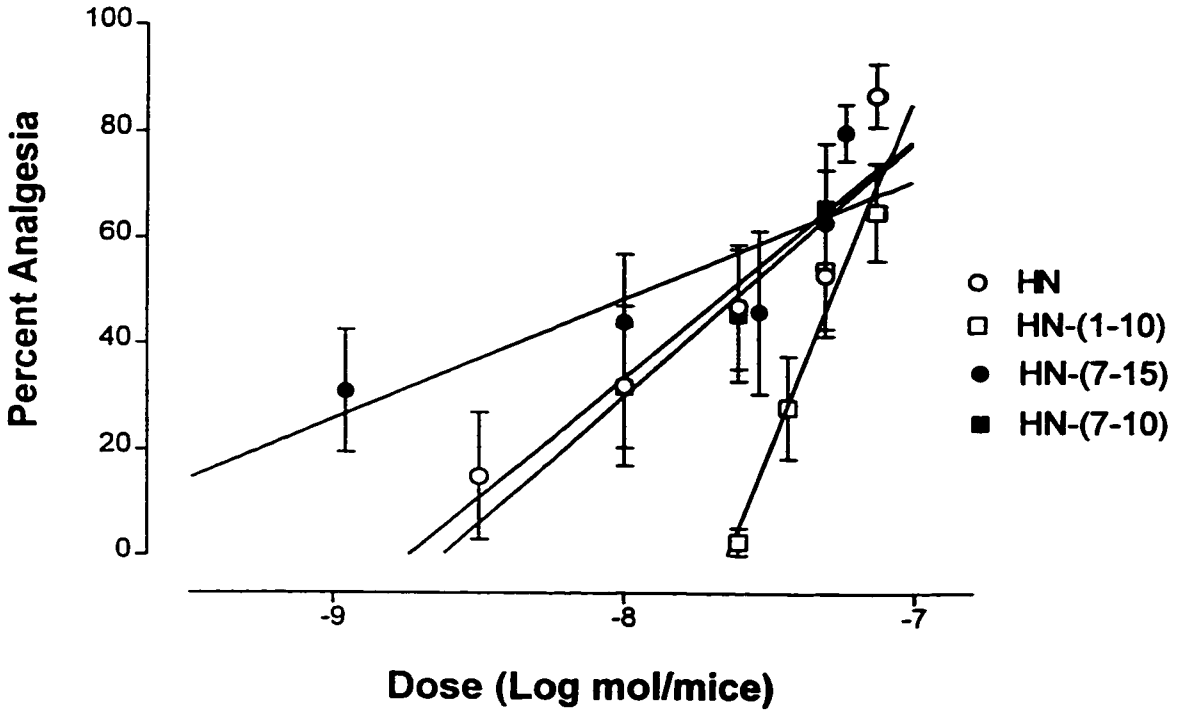

100
80
60
40
20
0
Percent Analgesia
-9
-8
-7
Dose (Log mol/mice)
HN
HN-(1-10)
HN-(7-15)
HN-(7-10)

Figure 2

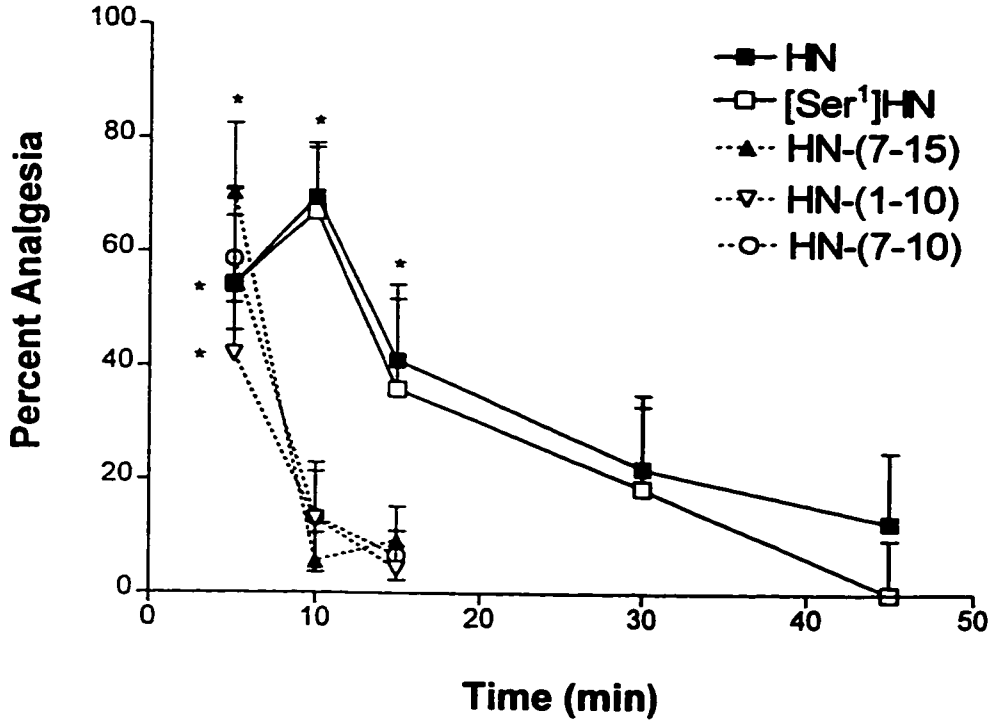

Percent Analgesia
100
80
60
40
20
0
0
10
20
30
40
50
Time (min)
HN
[Ser1]HN
HN-(7-15)
HN-(1-10)
HN-(7-10)

Figure 3

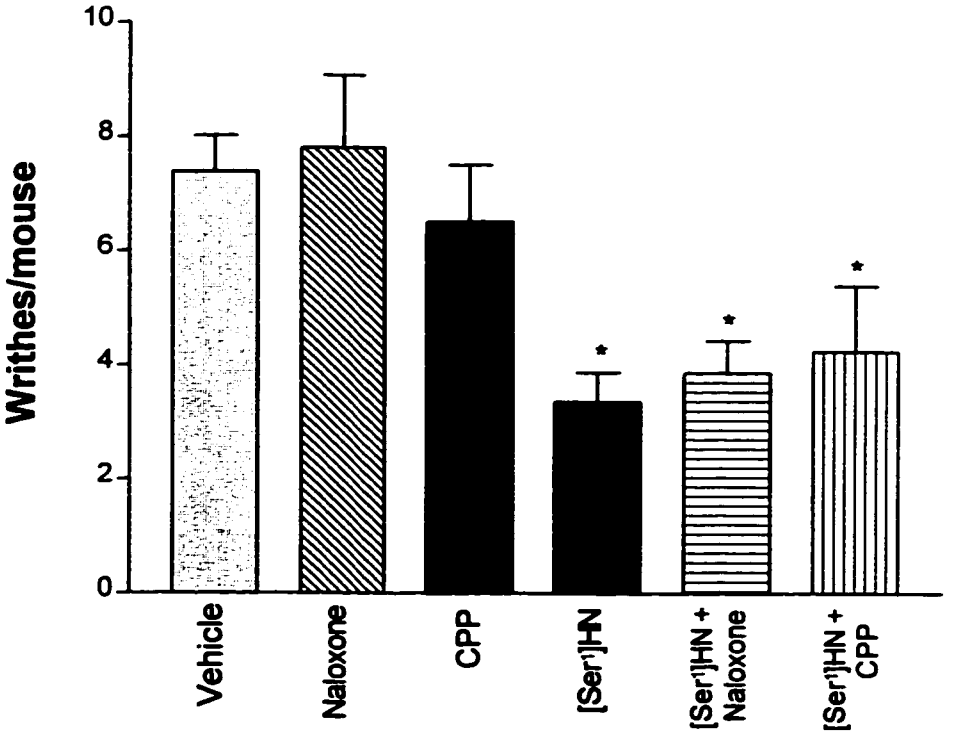

Writes/mouse
10
8
6
4
2
0
Vehicle
Naloxone
CPP
[Ser¹]HN
[Ser¹]HN + Naloxone
[Ser¹]HN + CPP
*
*
*

Figure 4

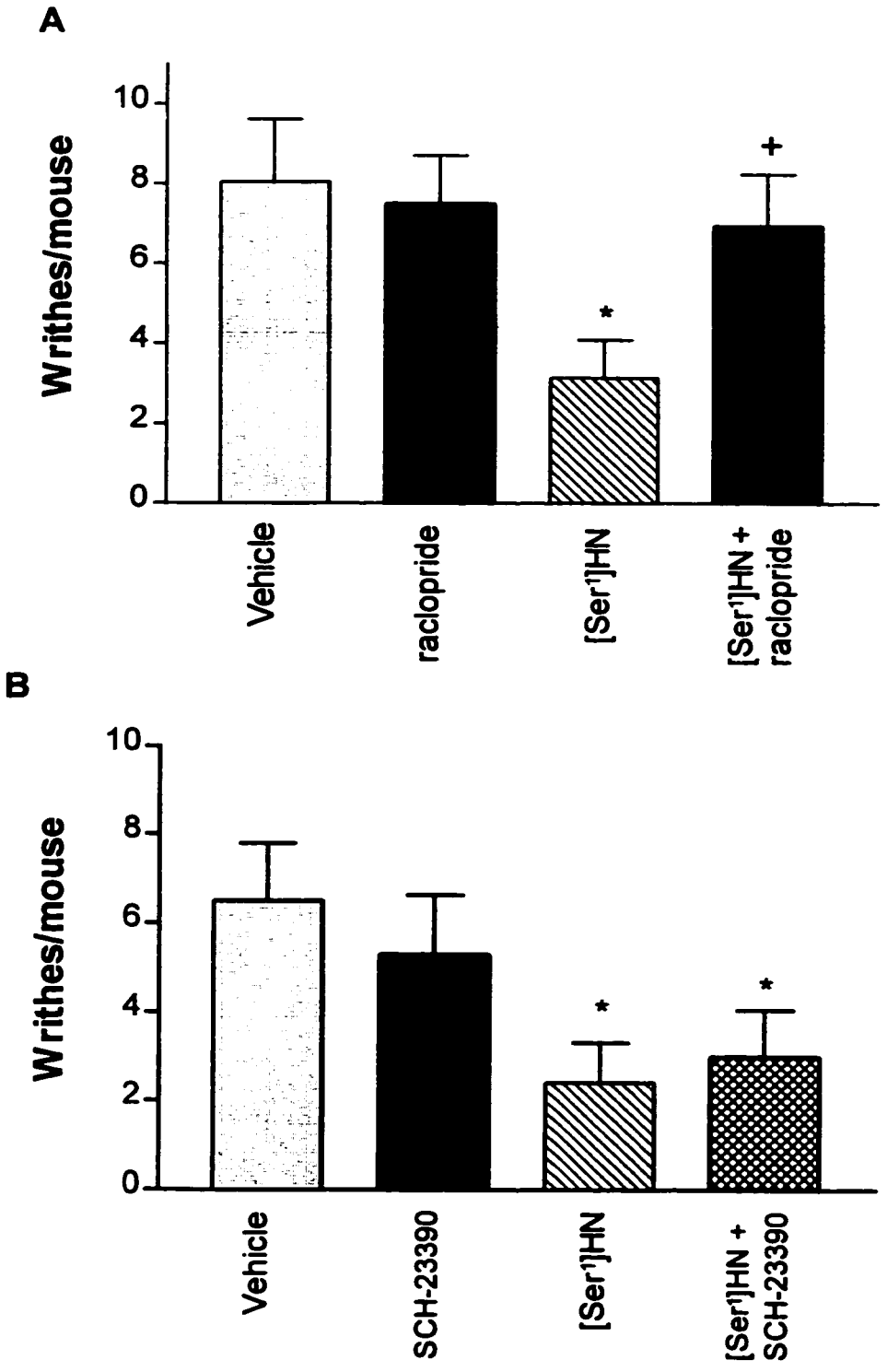

A
Writhes/mouse
10
8
6
4
2
0
*
+
Vehicle
raclopride
[Ser1]HN
[Ser1]HN + raclopride
B
Writhes/mouse
10
8
6
4
2
0
*
*
Vehicle
SCH-23390
[Ser1]HN
[Ser1]HN + SCH-23390

Figure 5

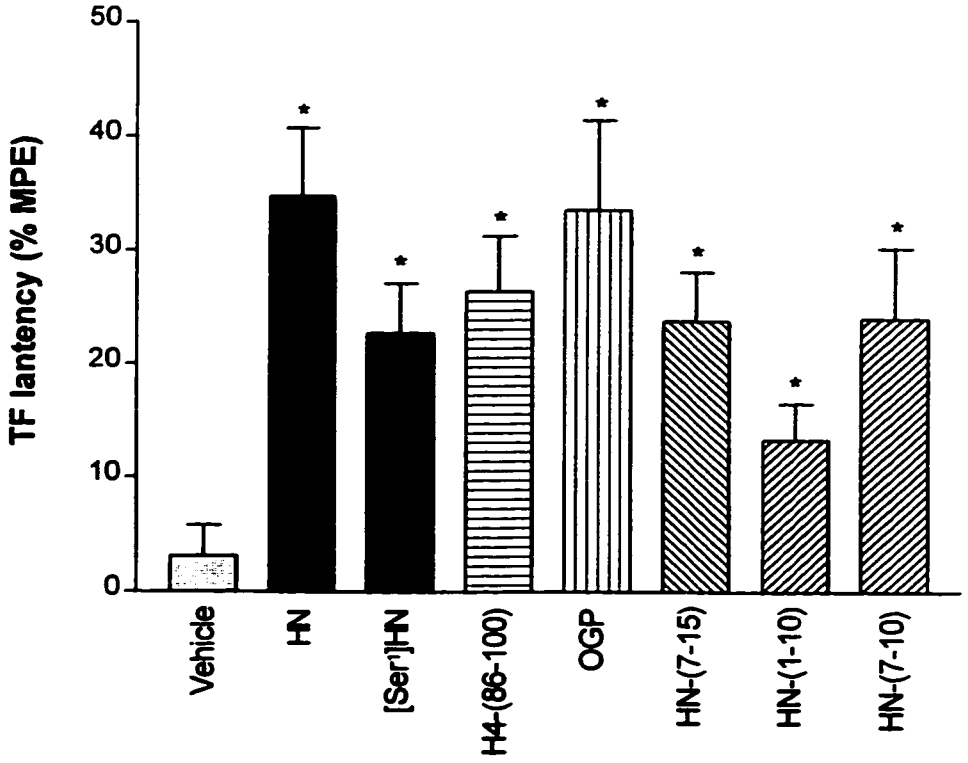
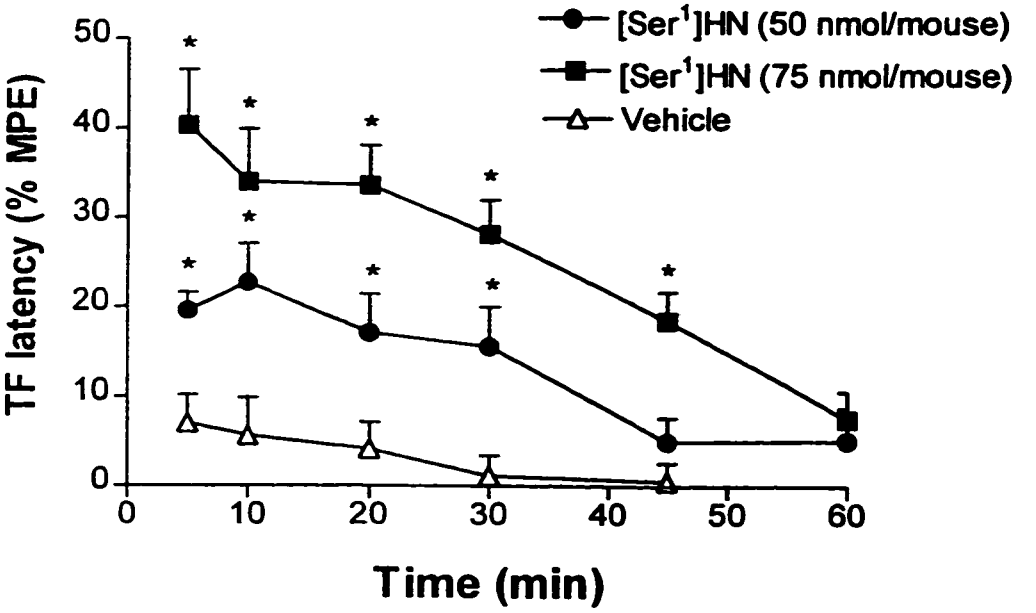
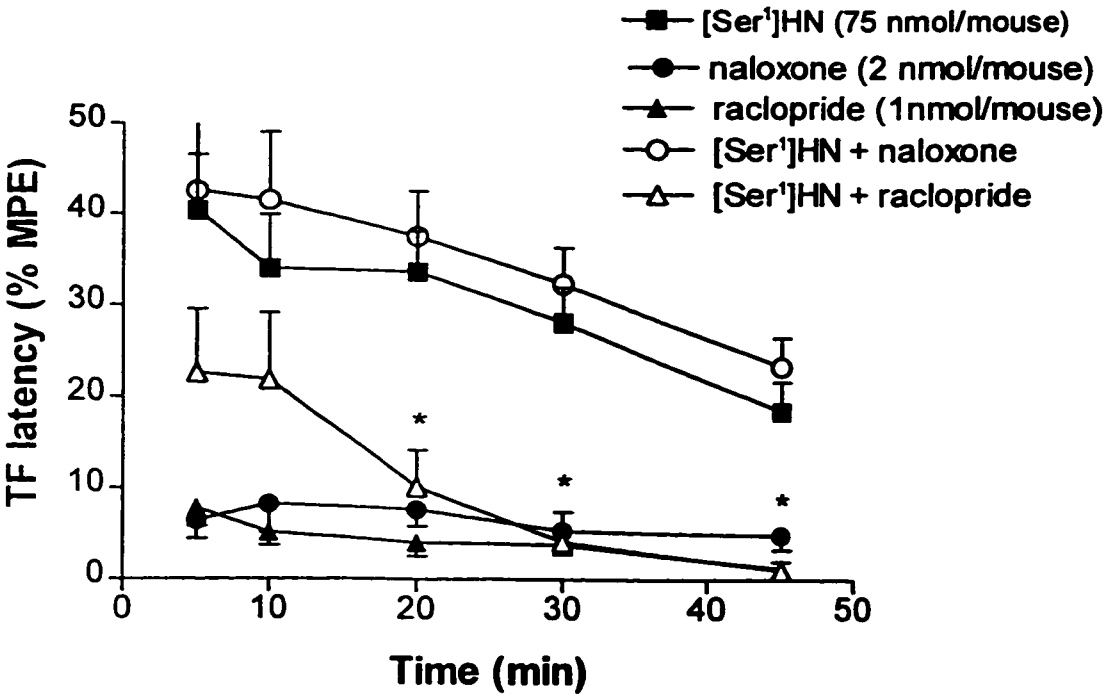

TF lantency (% MPE)
50
40
30
20
10
0
Vehicle
HN
[Ser1]HN
H4-(86-100)
OGP
HN-(7-15)
HN-(1-10)
HN-(7-10)

Figure 6

A


[Ser¹]HN (50 nmol/mouse)
[Ser¹]HN (75 nmol/mouse)
Vehicle
TF latency (% MPE)
Time (min)
0
10
20
30
40
50
60

B


[Ser¹]HN (75 nmol/mouse)
naloxone (2 nmol/mouse)
raclopride (1nmol/mouse)
[Ser¹]HN + naloxone
[Ser¹]HN + raclopride
TF latency (% MPE)
Time (min)
0
10
20
30
40
50

SECTION 4

INTRODUCTION TO ARTICLE 2

As the first paper suggested a specific involvement of D2 receptor in mediation of the analgesia induced by i.c.v. administration of HN and related peptides, the following paper was aimed to characterize the interaction of HN and related peptides with the D2 receptor using the in vitro ([^{3}H]raclopride) binding assay to rat brain membranes. The binding potencies of HN and related peptides was compared with their abilities to produce analgesia, and the correlation was made. All the peptides were prepared by Dr. Prasad (see acknowledgements). The displacement of [^{3}H]raclopride binding by dopamine in the absence and presence of Na^+ (Fig 4B) was performed by Yanmei Wu (the second author). I (the first author) was responsible for carrying out the rest of the assays as well as the data analysis.

Interactions of Histogranin and Related Peptides with Dopamine D2 Receptor in Rat Brain Membranes

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This study was supported by the grant from Medical Research Council of Canada (MT-11170).

Running title: **Histogranin and D2 Receptor Interaction**

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The number of text pages: 16

The number of tables: 1

The number of figures: 5

The number of references: 33

The number of words in the Abstract : 240

The number of words in the Introduction: 637

The number of words in the Discussion: 1437

List of abbreviation:

AMPA, α-amino-3-hydroxy-5-methyl-isoxazole-4-propionate; B_{max}, maximal binding capacity; DSLET, [D-Ser^2]Leu-enkephalin-Thr^6; HN, histogranin; K_d, equilibrium dissociation constant; K_H, inhibitory constant for the high affinity state; K_L, inhibitory constant for the low affinity state; NAS, nucleus accumbens septi; n_H, hill coefficient; NMDA, N-methyl-D-aspartate; OGP, osteogenic growth peptide; (+)3-PPP, 3-(3-hydroxyphenyl-)-N-(1-propyl)piperidine; R_H, receptor in high affinity state; VTA, ventral tegmental area

ABSTRACT

HN and related peptides were assessed for their ability to modulate the specific binding of the D2 receptor ligand [^{3}H]raclopride to rat brain membranes. A high affinity saturable site for [^{3}H]raclopride was found with a K_d of 4.87 ± 0.54 nM and a B_{max} of 47.2 ± 3.01 fmol/mg protein. [Ser^1]HN (1 μM), a chemically stable analogue of HN, produced a competitive inhibition of [^{3}H]raclopride binding, resulting in a two fold increase in the K_d (9.35 ± 1.46 nM) but no significant change in the B_{max} (44.9 ± 5.67 fmol/mg protein). In competition binding studies with 2.5 nM [^{3}H]raclopride, [Ser^1]HN evoked a biphasic competition curve with a K_i for high affinity state of 32.1 ± 10.8 nM and a K_i for low affinity state of 4.43 ± 2.35 μM. Such competition model was comparable to those of the dopamine agonists, dopamine and (+)3-PPP, but distinct from those of the dopamine antagonists, (-)sulpiride and (+)sulpiride (monophasic competition curves). The competition binding profile of [Ser^1]HN also resembled to those of HN or related peptides but differed from that of neurotensin and DSLET. The presence of NaCl (10 mM) affected the competition curve of [Ser^1]HN in a similar manner as that of dopamine, inducing the conversion of the high affinity state into the low affinity state with an overall K_i value of 6.54 ± 1.2 μM. The relative potencies HN and related peptides in inhibiting [^{3}H]raclopride binding are compared with their abilities to induce analgesia in the mouse writhing test (Lemaire et al., 1997; and submitted data). The results indicate that HN and related peptides compete with the binding of [^{3}H]raclopride in a D2 agonist-like manner, such action of the peptides correlating well with their analgesic activity.

HN is a bovine adrenomedullary peptide (Lemaire et al., 1993) whose structure possesses 80% and 78% homology with to those of histone H4-(86-100) and osteogenic growth peptide (OGP: histone H4-(89-102)) (Bab et al., 1992), respectively. The peptide was found to be present in various rat tissues including the lungs, heart, and brain (Lemaire et al., 1995). HN and its chemically stable analogue, [Ser^1]HN, dose-dependently blocked convulsions in mice induced by intracerebroventricular (i.c.v) administration of NMDA, but not by the non-NMDA excitatory amino acids AMPA and kainic acid, suggesting that the peptide is a specific endogenous modulator of NMDA receptor function (Lemaire et al., 1993; Shukla et al., 1995). High affinity saturable binding sites for [^{125}I][Ser^1]HN were found in rat brain membranes (Roger and Lemaire, 1993). The relative potencies of HN and related peptides in competing with [^{125}I][Ser^1]HN binding correlated well with their abilities to inhibit NMDA-induced convulsions (Prasad et al., 1995).

[Ser^1]HN administrated intrathecally (i.t.) was recently shown to cause analgesia in rat models of chronic pain associated with allodynia and hyperalgesia. For instance, [Ser^1]HN was effective in blocking hyperalgesia and allodynia induced by sciatic nerve injury (Siegan et al., 1997) or i.t. administration of NMDA (Hama et al., 1999). In addition, [Ser^1]HN blocked the tonic phase of pain induced by intraplantar administration of formalin (Siegan and Sagen, 1997). More recently, we found that i.c.v. administrations of HN and related peptides are also potent pain blocking agents in the mouse writhing and tail-flick assays (Lemaire et al., 1997; submitted data). Such effects were blocked by the

dopamine D2 receptor antagonist, raclopride, but not the D1 (SCH-23390), opioid (naloxone) or NMDA (CPP) receptor antagonists. The latter findings indicated that at least some of the analgesic effects of HN and related peptides involve the participation of the D2 receptor.

Dopamine receptors have been classified into two distinct categories by virtue of their ability to interact with adenylate cyclase (Kebabian et al., 1979). The D1 receptor, comprising the D1 and D5 receptor subtypes, is associated to a stimulatory Gs protein which, when stimulated, induces an increase in the activity of adenylate cyclase, whereas the D2 receptor, comprising the D2, D3 and D4 receptor subtypes, is linked to an inhibitory Gi protein which may or may not induce the inhibition of adenylate cyclase. It has been established that the D2 receptor exists in high and low affinity states (Seeman and Grigoriadis, 1987). Agonists bind with high affinity to the D2 receptor when it is associated with the Gi protein, but with low affinity when the receptor is dissociated from the Gi protein (De Lean et al., 1980; Sibley et al., 1982). On the other hand, the affinity of the antagonist for the D2 receptor is not dependent upon the formation of the receptor-G protein complex (Dewar et al., 1989). This can be experimentally observed in competition binding studies with radiolabelled antagonists wherein agonists and antagonists display biphasic and monophasic displacement curves, respectively (Dewar et al., 1989). In addition, both Na^+ and guanine nucleotides selectively decrease the affinity of agonist binding to the D2 receptor through a dissociation of the Gi protein from the receptor (George et al., 1983; Grigoriadis and Seeman, 1985; Reader et al., 1990; Hall et al., 1991).

The aim of the present investigation was to characterize the possible interaction of HN and related peptides with the D2 receptor in rat brain membranes using the D2 selective antagonist [^{3}H]raclopride as the radiolabelled ligand. The type of interaction between [Ser^1]HN and the D2 receptor was defined in saturation and competition binding studies by comparison with prototypic dopamine D2 receptor agonists and antagonists under different affinity states of the receptor. Finally, the potencies of HN and related peptides to compete with [^{3}H]raclopride binding were compared with their abilities to induce analgesia in the mouse writhing test.

Materials and methods

Materials

[^{3}H]raclopride (78.4 Ci/mmol) was obtained from New England Nuclear (Boston,MA). (+)3-PPP, dopamine HCl, (-)sulpiride, (+) sulpiride, (+)butaclamol and neurotensin were purchased from the Research Biochemicals Inc. (Natick, MA). DSLET was purchased from the Peninsula Laboratories Inc. (Belmont, CA). Bacitracin, bestatin, captopril, thiorphan, and bovine serum albumin were obtained from the Sigma Chemical Co. (St louis, MO). HN and related peptides were synthesized in our laboratory as described by Prasad et al. (1995).

Preparation of rat brain membranes

After decapitation, fresh forebrains from six male Wistar rats (200-250 g, St Constant, Québec) were rapidly removed and homogenized in 25 volumes of ice-cold Tris-HCl (5 mM, pH 7.4; buffer A) with a glass teflon homogenizer. The homogenate was centrifuged at 27, 000 g for 30 min at 4°C. The pellet was resuspended in buffer A and centrifuged. The resulting pellet was homogenized and incubated on ice in 1 L of buffer A containing 0.3 M KCl for 60 min (Lee et al., 1982). The suspension was centrifuged at 27,000 g for 30 min at 4°C and the pellet was resuspended in buffer A. The later washing procedure was repeated three times and the final membrane pellet was resuspended in 5 volumes of buffer A and kept frozen at -80°C until the binding assay. Protein concentration was measured in an unfrozen aliquot by the method of Lowry et al. (1951)

using bovine serum albumin as the standard.

[^{3}H]Raclopride binding assay

The binding assays with [^{3}H]raclopride (2.5 nM) to rat brain membranes, unless indicated, were carried out at room temperature (22°C) for 60 min in an aliquot of 2 ml of buffer A containing the peptidase inhibitors bacitracin (25 µM), bestatin (30 µM), captopril (10 µM) and thiorphan (0.3 µM). The tubes were then placed in an ice water bath (4° C) for 10 min and binding was terminated by rapid filtration under reduced pressure over 934-AH Whatman filters which were presoaked for at least 1 hr in buffer A. The filters were washed with 4 aliquots of 3ml ice-cold buffer A, placed in 10 ml Ecolume (ICN Biochemicals Inc., Mississauga, Ontario) and counted a Wallac Scintillation counter (WinSpectral 1414). Specific binding of [^{3}H]raclopride was defined as the difference between the total radiolabel bound and that bound in the presence of 10 µM (+)butaclamol. Saturation experiments with increasing concentrations of [^{3}H]raclopride (0.2-20 nM) were performed in the absence or presence of 1 µM [Ser^1]HN. Competition experiments were performed with 2.5 nM [^{3}H]raclopride in the presence of 10 to12 increasing concentrations (10^{-10} - 10^{-4} M) of the peptides or competing ligands. To avoid oxidation, dopamine was dissolved in oxygen-free boiled water containing 0.1% sodium metabisulfite.

Data analysis

All values, Scatchard-type (B_{max} , K_d) or competition-type (IC_{50}), were generated by

the iterative nonlinear least square curve-fitting procedure using the computer program "PRISM" (Graphpad Software Inc, San Diego, CA). Curves shown are representative of three to four experiments. The competition curves can be fitted to single- or multi-site model using the "extra sum of squares" principle. This analysis, as an extra index, allowed user to determine the statistical competition model that best fit the experiment data by appropriate F test. The inhibition constant (K_i) was calculated according to the equation: $K_i = IC_{50}/(1+[L]/K_d)$, wherein [L] is the concentration of [^{3}H]raclopride and K_d its equilibrium dissociation constant (Cheng and Prusoff, 1973). All data represent the mean ± S.E. of at least 3 experiments conducted in duplicate. The criteria of $P < 0.05$ was considered statistically significant.

Results

Protein, time and temperature dependencies of [^{3}H]raclopride binding.

The specific binding of [^{3}H]raclopride (2.5 nM) to rat brain membranes increased linearly with respect to protein concentration from 0.08-0.8 mg within 2ml incubation medium (Fig. 1). All following binding experiments were performed with 0.8 mg membrane protein. At room temperature (22°C), the association of [^{3}H]raclopride (2.5 nM) was rapid and reached a steady state within 10 min (Fig. 2). The maximum binding was observed 45 min after the beginning of incubation and remained stable for up to 90 min, indicating that neither binding sites nor radioligands degraded during the incubation time. A reduction in the incubation temperature from 22° C to 4°C significantly decreased the specific binding of [^{3}H]raclopride (* $P < 0.05$, Paired t-test), the maximum binding obtained after 30 min at 4°C representing only 46.4% of that obtained at room temperature. Thus, the subsequent binding experiments were carried out at room temperature with an incubation time of 60 min.

Saturation binding of [^{3}H]raclopride.

The specific binding of [^{3}H]raclopride displayed concentration-dependency between 0.25 and 20 nM and was saturable at higher concentration (Fig. 3). Scatchard plot analysis indicated that [^{3}H]raclopride binds to one single site ($r = 0.97$) with a K_d of 4.87 ± 0.54 nM and a B_{max} of 47.2 ± 3.01 fmol/mg protein. The presence of [Ser^1]HN (1 µM) in the incubation medium increased the K_d value by almost double (from 4.87 ± 0.54 nM to

9.35±1.46 nM), but failed to significantly affect the B_{max} (44.9 ± 5.67 fmol/mg as compared with 47.2 ± 3.01 fmol/mg protein in the absence of the peptide).

Competition binding studies with D2 agonists and antagonists

Figure 4A illustrates the effects of (+)-sulpiride and (-)-sulpiride on [^{3}H]raclopride binding. Both isomeric compounds displayed dose-dependent inhibition of the binding of [^{3}H]raclopride, the D2 specific stereoactive isomer (-)-sulpiride was 10 times more potent than less active isomer (+)-sulpiride with a K_i of 0.68 ± 0.36 µM (Table 1). The competition curves obtained with the two compounds were monophasic with n_H close to unity. On the other hand, D2 agonists, such as dopamine and (+)3-PPP inhibited the binding of [^{3}H]raclopride in a biphasic manner (n_H<<1, Fig. 4B, Table 1). Fine analysis of the competition curve with dopamine provided a high affinity site representing 33.7 % of binding activity with a K_i for this high affinity site (K_H) of 2.05 ± 0.04 nM (Table 1). The low affinity site represented the rest of binding activity with a K_i for the low affinity (K_L) of 2.88 ± 0.71 µM. The addition of NaCl (10 mM) to the incubation medium induced a marked conversion of the high affinity site for dopamine into a low affinity site, with only 14.2 ± 2.64 % receptor remaining in the high affinity state. Analysis of the dopamine competition curve in the presence of NaCl in the monophasic mode provided a K_i of 1.47 ± 0.18 µM and a n_H of 0.8 (Fig. 4B).

Effect of [Ser^1]HN on [^{3}H]raclopride binding.

[Ser^1]HN dose-dependently inhibited the binding or [^{3}H]raclopride with a K_i of 0.67

± 0.25 μM and a n_H of 0.21 (Fig. 5; Table 1). Fine analysis of the competition binding data with [Ser^1]HN revealed the presence of two affinity sites: the high affinity site represented 40.3 % of binding activity and had a K_H of 32.1 ± 10.8 nM; the low affinity site represented the rest of binding activity and had a K_L of 4.43 ± 2.35 μM. Addition of NaCl (10 mM) to the incubation medium resulted in a complete conversion of the high affinity state into the low affinity state with a K_i of 6.54 ± 1.2 μM and a n_H close to unity (0.91).

Structure-activity relationship of HN and related peptides.

HN analogues and fragments were compared for their ability to displace the binding of [^{3}H]raclopride to rat brain membranes (Table 1). HN displaced [^{3}H]raclopride binding with a potency that was comparable to that of [Ser^1]HN (K_i of 0.42±0.04 μM as compared with 0.67±0.25 μM for [Ser^1]HN). Both C- and N-terminal fragments of HN that is (HN-(7-15) and (HN-(1-10), respectively, were potent inhibitors of [^{3}H]raclopride binding. The order of potency of HN and related peptides in competing with [^{3}H]raclopride binding was the following: HN-(7-15) > HN > HN-(7-10) ≈ [Ser^1]HN ≈ histone H4-(86-100) > HN-(1-10) > OGP. Both HN-(8-10) and HN-(7-9) were inactive up to a concentration of 50 uM (Table 1). HN related peptides all displayed a low Hill coefficient, indicating that they affected more than one affinity site. The analysis of displacement curves of all the peptides revealed existence of two sites competition mode, providing K_H in the subnanomolar range and representing 27-44% of specific binding activity.

Effects of HN unrelated peptides on [^{3}H]raclopride binding

Two neuropeptides, neurotensin and DSLET were also examined in the current competition study. The inhibition of [^{3}H]raclopride binding by neurotensin was dose dependent, starting at high concentrations (>10 μM). The competition curve of neurotensin fits best for a single site model (n_H = 0.84) with a K_i of 17.3 ± 0.3 μM. DSLET, a delta opioid ligand, was inactive in affecting the binding of [^{3}H]raclopride up to a concentration of 50 μM.

Discussion

[^{3}H]raclopride was previously found to be a selective marker of the D2 receptor (Köhler et al., 1985; Dewar et al., 1989). In the current study, the binding of [^{3}H]raclopride to a membrane preparation of rat brain displays characteristics typical to a membrane-bound receptor, such as saturability, reversibility, structure selectivity and stereospecificity. The temperature (22°C) dependent association curve of [^{3}H]raclopride to rat brain membranes is consistent with the previous results obtained with the membranes prepared from the rat brain striatum (Köhler et al., 1985). Therefore, the binding of [^{3}H]raclopride to rat brain membranes can be employed as a useful tool to investigate the central D2 receptor binding property as well as the modulation its activities by other agents.

Scatchard plot analysis of the saturation binding data with [^{3}H]raclopride revealed the presence of a single high affinity site with a K_d of 4.87 nM. This result is in agreement with the binding characteristics of D2 antagonists which are known to bind with equal affinity to the high and low affinity states of the D2 receptor (Dewar et al., 1989; Grigoriadis and Seeman, 1985). In the presence of [Ser^1]HN, the affinity of [^{3}H]raclopride was markedly decreased as evidenced by the higher K_d value. However, the B_{max} remained unchanged, implying a competitive nature for the interaction between [Ser^1]HN and the central D2 receptor. This type of modulating effect of [Ser^1]HN on [^{3}H]raclopride binding is similar to that observed with neurotensin on the binding of the D2 receptor

agonist [^{3}H]NPA (von Euler, 1991). In that study, neurotensin at low concentration (1-10nM) was shown to increase the K_d without significantly influencing the B_{max}. However, the modulating effect of neurotensin on D2 agonist binding was shown to result from an allosteric interaction between the D2 receptor and the neurotensin receptor (Kitabgi et al., 1977), and not to a direct effect of the peptide on the D2 receptor (Fuxe et al., 1984; Fuxe et al., 1992; von Euler, 1991).

In competition binding studies with tritiated D2 antagonists, it has been found that D2 agonists exhibit displacement curves distinct from those of D2 antagonists. For instance, agonists inhibit [^{3}H]raclopride binding in a heterogenous manner displaying biphasic competition curves, while the competition curves of antagonists are monophasic (Sibley et al., 1982; Hamblin et al., 1984; Dewar et al., 1989; Reader et al., 1990). Thus, while the competition curves with the dopamine agonists, dopamine and (+)3-PPP, best fitted the biphasic competition model (n_H<<1), the D2 antagonists, (-)sulpiride and (+)sulpiride, were steep (n_H close to 1) and best fitted the one site model. [Ser^1]HN, HN and related peptides, like D2 agonists, displaced the binding of [^{3}H]raclopride in a biphasic manner (n_H<<1). These results provide the first evidence which indicate that HN and related peptides behave as D2 agonists at the receptor level.

The Na^+ ion, which was shown to regulate the binding of agonist/[^{3}H]antagonist to D2 receptors (Grigoriadis and Seeman, 1985; Reader et al., 1990; Hall et al., 1991), was compared for its modulating effects in the [Ser^1]HN and dopamine competition binding

experiments. The inclusion of Na^+ (10 mM) in the incubation medium induced in both instances marked conversion of the high affinity state (R_H) into the low affinity state (R_L), as the $\%R_H$ were significantly diminished with dopamine or completely abolished with [Ser^1]HN. The resulting K_i values for overall displacement curves with [Ser^1]HN (6.54 ± 1.2 μM) and dopamine (1.47 ± 0.18 μM) in the presence of Na^+ were close to the K_L values in absence of Na^+ (4.43 ± 2.35 μM and 2.88 ± 0.71 μM, respectively). These data are discordant from the previous finding of Reader et al. (1990) which indicated that Na^+ decreases the binding affinity of dopamine for the displacement of [^{3}H]raclopride binding to both high and low affinity states without affecting the relative proportions ($\%R_H$ and $\%R_L$) of the sites. This discrepancy may result from the difference in the membrane preparation between our current study and the above mentioned study, in which membranes were prepared from the rabbit neostriatum. The regulation patterns of Na^+ in agonist/[^{3}H]antagonist binding can vary largely depending on the different distribution of the D2 receptor. For example, the presence of 100 mM Na^+ alone in rat anterior pituitary is sufficient to fully convert the high-affinity state to the low affinity state (George et al., 1983). In the present study, the similarity of the effect of Na^+ in its regulation [^{3}H]raclopride binding by [Ser^1]HN and dopamine supports the hypothesis that HN related peptides may possess an agonist-like binding activity on the D2 receptor.

In competition binding studies with [^{3}H]raclopride, neurotensin was also shown to be effective only at high concentrations (>10 μM) with a K_i of 17.3 μM as compared with 0.42 μM for HN (Table 1). Besides, the competition curve of neurotensin fitted best the

one site model with an n_H of 0.84, whereas that of HN fitted best the two site model with an n_H of 0.33. The non-effectiveness of neurotensin in displacing [^{3}H]raclopride binding at low concentrations is consistent with the observations of Li et al. (1993).

Previous *in vitro* binding studies (Rogers et al., 1993) indicated the presence of a high affinity site for [^{125}I][Ser^1]HN in rat brain membranes. Both binding and anti-NMDA properties of HN required the full length of the peptide, since the removal of a single amino acid from either C- or N-terminals greatly reduced the ability of the peptide to compete with [^{125}I][Ser^1]HN binding (Rogers et al., 1993) or block NMDA-induced convulsions in mice (Prasad et al., 1995). In contrast with these previous findings, the abilities of HN fragments to compete with the binding of [^{3}H]raclopride was not significantly reduced by the absence of either N- or C-terminal fragments (Table 1). Hence, the structure-dependence of this latter effect was demonstrated by the distinct potencies of the various HN fragments (HN-(7-15) > HN > HN-(7-10) > HN-(1-10)). The inability of HN-(6-9) and HN-(7-10) to inhibit [^{3}H]raclopride binding suggested that the minimal active core is HN-(7-10). Therefore, the modulation of [^{3}H]raclopride binding by HN and related peptides is most likely not mediated through the previously characterized high affinity HN binding site in the brain (Rogers and Lemaire, 1993).

[Ser^1]HN has already been shown to be a potent blocker of pain in rats induced by formalin (Siegan and Sagen, 1997), sciatic nerve injury (Siegan et al., 1997) and intrathecal administration of NMDA (Hama et al., 1999). Recently, HN and related

peptides were also shown to display antinociceptive effects in the mouse writhing and tail-flick assays (Lemaire et al., 1997; submitted data). Interestingly, the abilities of the peptides to compete with the binding of [^{3}H]raclopride correlated well ($r = 0.86$; Fig. 6) with their potencies in producing analgesia in the mouse writhing test . In this latter assay, [Ser^1]HN and H4-(86-100) displayed analgesic potencies comparable to that of HN, whereas HN-(1-10) and HN-(7-15) showed significant decrease and increase in activity as compared with HN, respectively, the minimal active core being HN-(7-10) (Lemaire et al., 1997; submitted data). Accumulating evidence suggests an important role of central D2 receptor in the modulation of pain. Both D2 agonists and indirect D2 receptor stimulants administrated peripherally or centrally alleviate pain in animal models that include the mouse writhing (Frussa-Filho et al., 1996), the rat hot plate (Michael-Titus et al., 1990) and the rat formalin (Mogan and Franklin, 1991; Altier and Stewart, 1998) assays. The activation of the D2 receptor in the mesolimbic pathway has been proposed to be a major component in mediating this analgesic activity. Such a possibility was supported by the recent finding that the analgesic effect of intra-VTA infusion of amphetamine in the rat formalin assay can be attenuated by intra-NAS infusion of the D2 receptor antagonist, raclopride (Altier and Stewart, 1998). Interestingly, the analgesic effects of [Ser^1]HN in both writhing and tail-flick assays were also shown to be blocked by raclopride (submitted data).

In conclusion, the present results indicate that HN and related peptides inhibit the binding of the D2 receptor iigand [^{3}H]raclopride to rat brain membranes in dose- and

structure-dependent manners. The interaction of the peptides with the D2 site is competitive and resembles to that of D2 agonists. The D2 agonist-like binding potencies of HN and related peptides correlate well with their analgesic potencies in the mouse writhing test but differ from their abilities to bind to the high affinity [Ser^1]HN site in rat brain membranes (Rogers and Lemaire, 1993) or inhibit NMDA-induced convulsions in mice (Prasad et al., 1995). Thus, the interaction of HN and related peptides with the D2 receptor is more likely to be responsible for their analgesic activity, being either direct or indirect by an allosteric modulation of the D2 receptor (Agnati et al., 1993) via the stimulation of a still uncharacterized low affinity HN binding site.

Acknowledgements

We are grateful to Dr. Michel Dumont for his valuable comments and technical assistance. We also thank Yanmei Wu for her participation in the competition of [^{3}H]raclopride binding by dopamine.

References

Agnati LF, Fuxe K, Benfenati F, von Euler G, and Fredholm B (1993) Intramembrane receptor-receptor interactions: integration of signal transduction pathways in the nerves system. *Neurochem Int* **22:** 213-222.

Altier N and Stewart J (1998) Dopamine receptor antagonists in the nucleus accumbens attenuate analgesia induced by ventral tegmental area substance P or morphine and by nucleus accumbens amphetamine. *J Pharmacol Exp Ther* **285:** 208-215.

Bab I, Gazit D, Chorev M, Muhlrad A, Shteyer A, Greenberg Z, Namdar M and Kahn A (1992) Histone H4-related osteogenic growth peptide (OGP): a novel circulating stimulator of osteoblastic activity. *EMBO J* **11:** 1867-1873.

Cheung YC and Prusoff WH (1973) Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50% inhibition (IC50) of an enzymatic reaction. *Biochem Pharmacol* **22:** 3099-3102.

De Lean A, Stadel JM and Lefkowitz RJ (1980) A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase coupled β-adrenergic receptor. *J Biol Chem* **255:** 7108-7117.

Dewar KM, Montreuil B, Grondin L and Reader TA (1989) Dopamine D2 receptors labeled with [^{3}H]raclopride in rat and rabbit brains. Equilibrium binding, kinetics, distribution and selectivity. *J Pharmacol Exp Ther* **250:** 696-706.

von Euler G (1991) Biochemical characterization of the intramembrane interaction between neurotensin and dopamine D2 receptors in the rat brain. *Brain Res* **561:** 93-98.

Frussa-Filho R, Rocha JB, Conceicao IM, Mello CF and Pereira ME (1996) Effects of dopaminergic agents on visceral pain measured by the mouse writhing test. *Arch Int Pharmacodyn Ther* **331:** 74-93.

Fuxe K, Agnati LF, Andersson K, Eneroth P, Härfstrand A, Goldstein M and Zoli M (1984) Studies on neurotensin-catecholamine interactions in the hypothalamus and in the forebrain of the male rat. *Neurochem Int* **6:** 735-750.

Fuxe K, O'Connor WT, Antonelli T, Osborne PG, Tanganelli S, Agnati LF and Ungerstedt U (1992) Evidence for substrate of neuronal plasticity based on pre- and postsynaptic neurotensin-dopamine receptor interactions in the neostriatum. *Proc Natl Acad Sci USA* **89:** 5591-5595.

George SR, Binkley K and Seeman P (1983) Dopamine receptors sites and states in human brain. *J Neurol Transm* **18 (supple):** 149-156.

Grigoriadis D and Seeman P (1985) Complete conversion of brain D2 receptors from the high- to the low-affinity state for dopamine agonist, using sodium ions and guanine nucletide. *J Neurochem* **44:** 1925-1935.

Hall H, Halldin C and Sedvall G (1991) Gpp(NH)p stimulates [^{3}H]raclopride binding to homogenates from human putamen and accumbens. *Neurosci Lett* **136:** 79-82.

Hama AT, Siegan JB, Herzberg U and Sagen J (1999) NMDA-induced spinal hypersensitivity is reduced by naturally derived peptide analog [Ser^1]histogranin. *Pharmacol Biochem & behav* **62:** 67-74.

Hamblin MW, Leff SE and Creese I (1984) Interaction of agonists with D-2 dopamine receptors: evidence for a single receptor population existing in multiple agonist

affinity-states in rat striatal membranes. *Biochem Pharmacol* **33:** 877-887.

Kebabian JW and Calne DW (1979) Multiple receptors for dopamine. *Nature* **277:** 93-96.

Kitabgi P, Carraway R, van Rietschoten F, Morgat JL, Menez A, Leeman SE and Freychet P (1977) Neurotensin: specific binding to synaptic membranes from the rat brain. *Proc. Natl. Acad. Sci. USA* **74:** 1846-1850.

Köhler C, Hall H, Ogren SO and Gawell L (1985) Specific in vitro and in vivo binding of ^{3}H-Raclopride: a potent substituted benzamide drug with high affinity for dopamine D-2 receptors in the rat brain. *Biochem Pharmacol* **34:** 2251-2259.

Lee CM, Javitsch JA and Snyder SH (1982) Characterization of [^{3}H]desipramine binding associated with norepinephrine uptake sites in rat brain membranes. *J neurosci* **10:**1515-1525.

Lemaire S, Shukla VK, Rogers C, Ibrahim IH, Lapierre C and Dumont M (1993) Isolation and characterization of histogranin, a natural peptide with N-methyl-D-aspartate antagonist activity. *Euro J Pharmacol* **245:** 247-256.

Lemaire S, Rogers C, Dumont M, Shukla VK, Lapierre C, Prasad J and Lemaire I (1995) Histogranin, a modified histone H4 fragment endowed with N-methyl-D-aspartate antagonist and immunostimulatory activities. *Life Sciences* **56:** 1233-1241.

Lemaire S, Ruan H. and Prasad J.A. (1997) Central and peripheral non-opioid analgesic activity of histogranin and related peptides. Soc. Neurosci. **23:** 674.

Li X.M, von Euler G, Hedlund PB, Finnman UB and Fuxe K (1993) The C-terminal neurotensin-(8-13) fragment potently modulates rat neostriatal dopamine D_2

recptors. *Eur J Pharmacol* **234:** 125-128.

Lowry OH, Roserough NJ, Farr AL and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265-270.

Michael-Titus A, Bousselmame R and Costentin J (1990) Stimulation of dopamine D2 receptors induces an analgesia involving an opioidergic but non enkephalinergic link. *Eur J Pharmacol* **187:** 201-207.

Morgan MJ and Franklin KBJ (1991) Dopamine receptor subtypes and formalin test analgesia. *Pharmacol Biochem Behav* **40:** 317-322.

Prasad JA, Shukla VK and Lemaire S (1995) Synthesis and biological activity of histogranin and related peptides. *Can J Physiol Pharmacol* **73:** 209-214.

Reader TA, Boulianne S, Molina-Holgado E and Dewar K (1990) Effects of monovalent cations on neostriatal dopamine D2 receptors labeled with [^{3}H]raclopride. *Biochem Pharmacol* **40:**1739-1746.

Rogers C, Lemaire S (1993) Characterization of [^{125}I][Ser^{1}]histogranin binding sites in rat brain. *J Pharmacol Exp Ther* **267:** 350-356.

Seeman P and Grigoriadis D (1987) Dopamine receptors in brain and periphery. *Neurochem Int* **10:** 1-25.

Shukla VK, Lemaire S, Dumont M and Merali Z (1995) N-methyl-D-Asparate receptor antagonist activity and phencyclidine-like behavioral effects of the pentadecapeptide, [Ser^{1}]Histogranin. *Pharmcol Biochem Behav* **50:** 49-54.

Sibley DR, De Lean A and Creese I (1982) Anterior pituitory dopamine receptors. Demonstration of interconvertable high and low affinity states of D2 dopamine

receptor. *J Biol Chem* **257:** 6351-6361.

Siegan JB, Hama AT and Segan J (1997) Suppression of neuropathic pain by a naturally-derived peptide with NMDA antagonist activity. *Brain res* **755:** 331-334.

Siegan JB and Sagen J (1997) A natural peptide with NMDA inhibitory activity reduces tonic pain in the formalin model. *Neuroreport* **8:** 1379-1381.

Table 1. Inhibition of the [^{3}H]raclopride binding to rat brain membrane by HN, HN-related peptides, HN-unrelated peptides and D2 receptor ligands.

Compounds	K_i [a] (μM)	$n_{(H)}$ [b]	K_H (nM)	K_L (μM)	R_H (%)
1. HN related peptides or fragments					
HN	0.42±0.04	0.33	27.5±9.31	4.29±0.51	33.2±4.01
[Ser^1]HN	0.67±0.25	0.21	32.1±10.8	4.43±2.35	40.3±5.64
HN-(7-15)	0.36±0.13	0.44	19.0±7.40	3.32±0.63	38.1±2.36
HN-(1-10)	4.45±0.99	0.13	38.2±3.59	7.09±0.97	44.2±1.83
HN-(7-10)	0.61±0.07	0.58	33.1±15.5	5.51±1.01	25.3±5.03
H4-(86-100)	0.70±0.20	0.15	35.2±10.2	5.42±1.12	42.4±4.39
OGP	5.38±1.43	0.25	38.1±11.3	7.61±0.85	26.6±2.25
HN-(6-9)	NA[c]				
HN-(8-10)	NA[c]				
2. Other peptides					
Neurotensin	17.3±0.32	0.84			
DSLET	NA[c]				
3. D2 agonists					
Dopamine	0.25±0.05	0.12	2.05±0.04	2.88±0.71	34.1±1.65
(+)3-PPP	0.73±0.32	0.26	37.5±11.8	4.88±0.92	45.3±4.08

4. D2 antagonist		
(-)Sulpiride	0.68±0.36	0.79
(+)Sulpiride	6.85±0.39	1.22

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[a] The inhibitory constant (K_i) were calculated by the method of Cheung and Prusoff (1973).

[b] The Hill coefficient (n_H) was determined by the computer program "PRISM" using the linear analysis of the slope log [$B/(B_{max} - B)$] vs. the log ligand concentration.

[c] NA: not active in displacing the binding of [^{3}H]raclopride up to concentration of 50 μM.

Legend to Figures

Fig. 1 Effect of increasing protein concentration on [^{3}H]raclopride binding to rat brain membranes. Binding of [^{3}H]raclopride (2.5 nM) to rat brain membrane (0.08-0.8 mg) was conducted for 60 min as described under "Material and Method". Data represent the mean ± S.E. of three experiments performed in duplicate.

Fig. 2 Time and temperature dependence of [^{3}H]raclopride binding. Rat brain membranes (0.8 mg) were incubated with [^{3}H]raclopride (2.5 nM) at 4°C (closed triangle) or room temperature (22°C, closed square) and the incubations were terminated at various time intervals. Data represent the mean ± S.E. of three experiments carried out in duplicate. * $P < 0.05$ indicates significant difference as compared the [^{3}H]raclopride bound at room temperature with that at 4 °C (Paired t-test).

Fig. 3 Saturation curves showing the effect of [Ser^1]HN on [^{3}H]raclopride binding to rat brain membranes. Specific binding was measured with 7 increasing concentrations of [^{3}H]raclopride (0.25-20 nM) in the presence or absence of 10 μM (+) Butaclamol. The K_d and B_{max} values were 4.87 ± 0.54 nM and 47.2 ± 3.81 fmol/mg protein for control (open square), and 9.35 ± 1.46 nM and 44.9 ± 5.67 fmol/mg protein in the presence of [Ser^1]HN (closed

square). The data represent the mean± S.E. of four independent experiments carried out in duplicate. The inset shows corresponding Scatchard plots of the saturation binding data.

Fig. 4 Competition of [^{3}H]raclopride binding by the D2 agonist (dopamine) and D2 antagonists ((+)sulpiride and (-)sulpiride). (A) Inhibition of specific [^{3}H]raclopride binding (2.5 nM) to rat brain membrane by the D2 antagonists, (-)sulpiride and less active isomer (+)sulpiride. The binding experiments were performed at 22° C for 60 min in the presence of 10 concentrations of the compounds. (B) The effect of Na^+ on the displacement of the [^{3}H]raclopride binding by dopamine. The competition curves of dopamine were determined in the presence of 12 concentrations of dopamine in the incubation mediums containing 0 or 10 mM NaCl. Data points are the mean ± S.E. of three separate experiments, each conducted in duplicate.

Fig. 5 The effect of Na^+ on the displacement of the [^{3}H]raclopride binding by [Ser^1]HN. Using the condition identical to Fig. 4, the competition curves of [Ser^1]HN were determined in the presence of 10 concentrations (5 nM-50 μM) of peptide in the incubation mediums containing 0 or 10 mM NaCl. Data points are the mean± S.E. of three separate experiments, each conducted in duplicate.

Index term

Histogranin peptides, D2 receptor, [^{3}H]raclopride binding, rat brain membrane, analgesia.

Figure 1

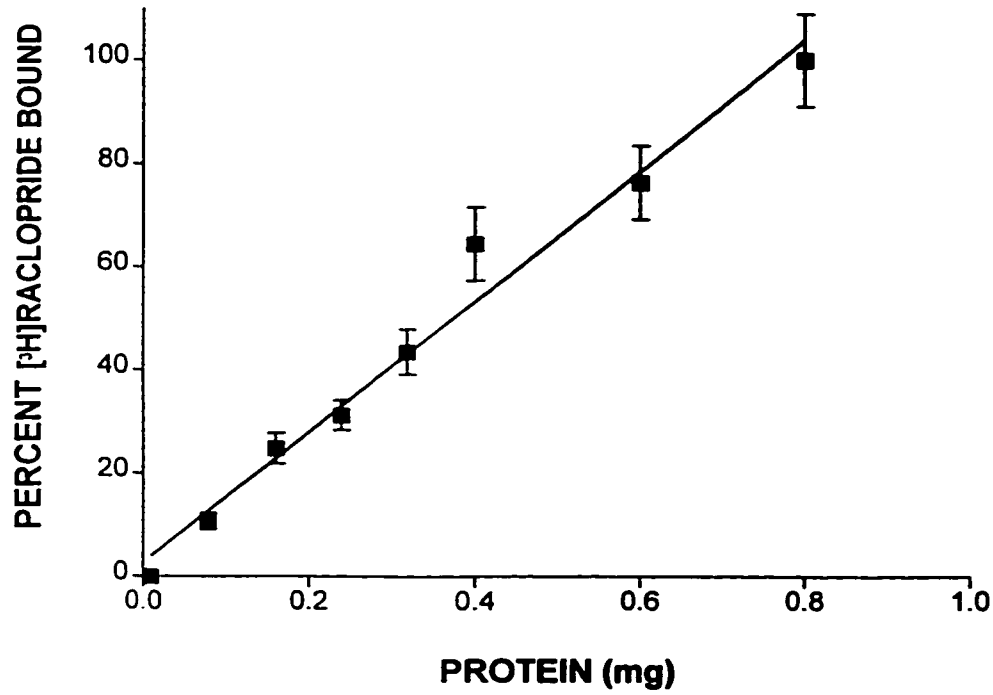

PERCENT [³H]RACLOPRIDE BOUND
100
80
60
40
20
0
0.0
0.2
0.4
0.6
0.8
1.0
PROTEIN (mg)

Figure 2

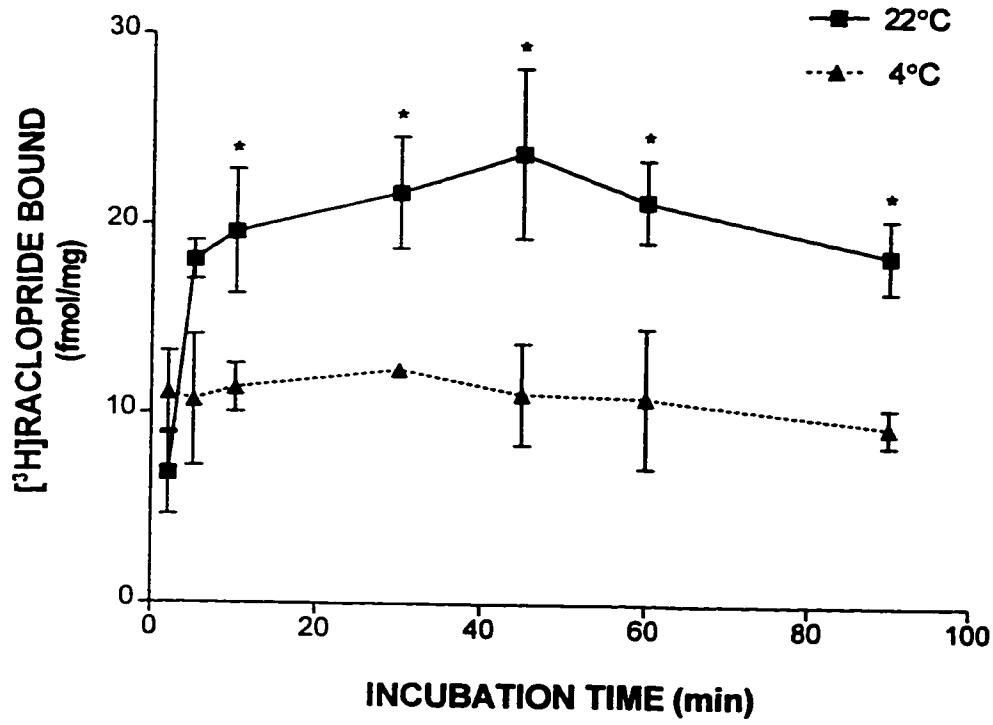

22°C
4°C
[³H]RACLOPRIDE BOUND (fmol/mg)
30
20
10
0
0
20
40
60
80
100
INCUBATION TIME (min)

Figure 3

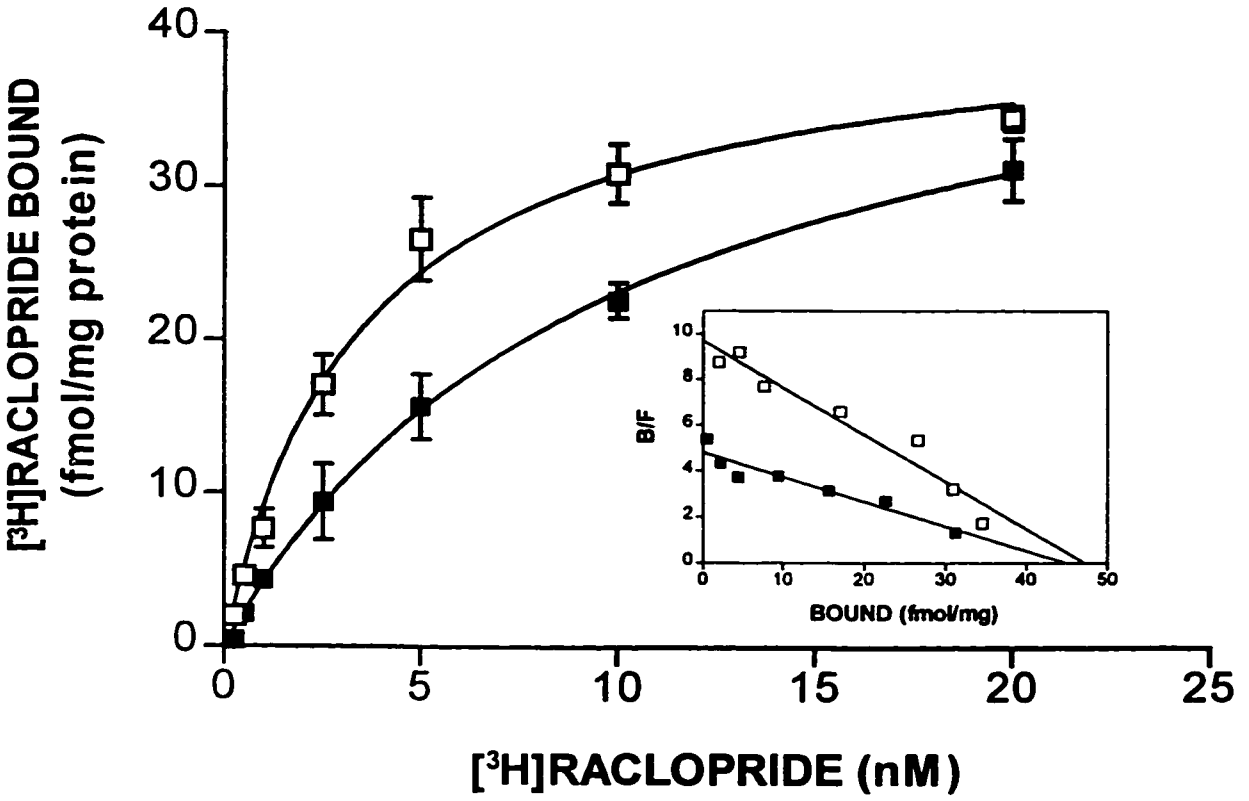
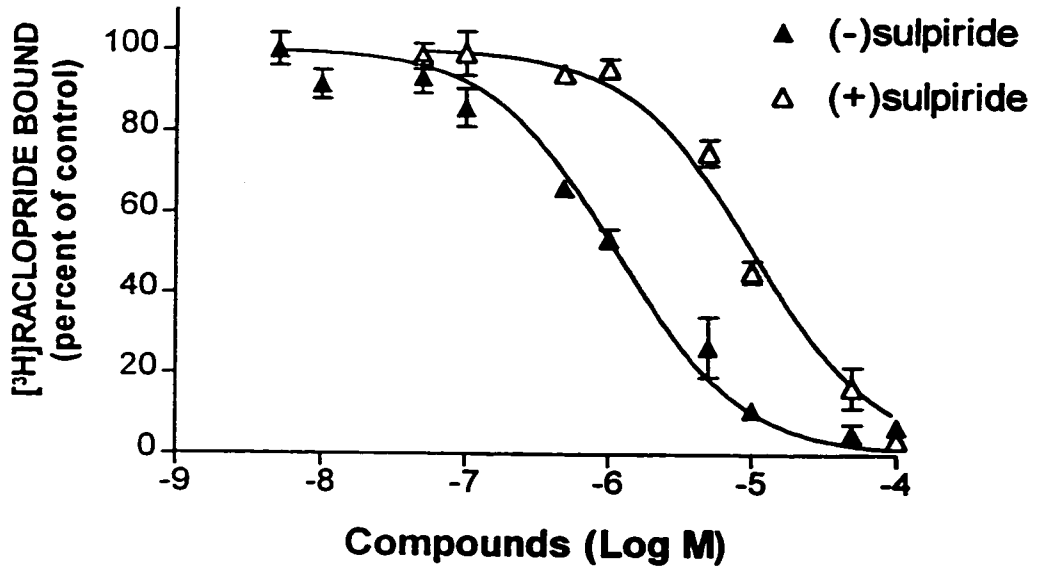

[3H]RACLOPRIDE BOUND
(fmol/mg protein)
40
30
20
10
0
0
5
10
15
20
25
[3H]RACLOPRIDE (nM)
B/F
10
8
6
4
2
0
0
10
20
30
40
50
BOUND (fmol/mg)

Figure 4

A


[³H]RACLOPRIDE BOUND
(percent of control)
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
Compounds (Log M)
(-)sulpiride
(+)sulpiride

B.

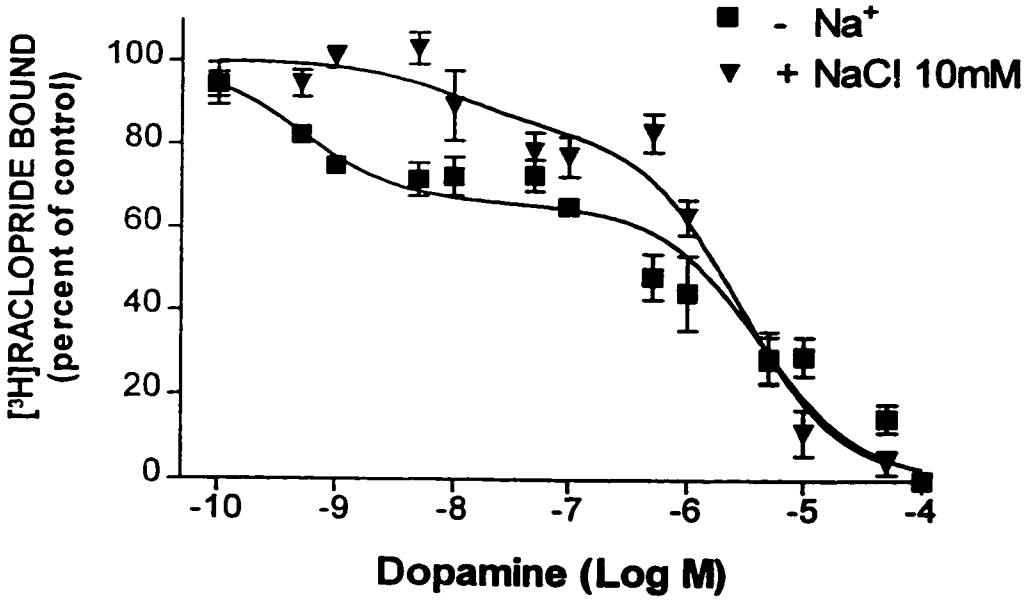

[³H]RACLOPRIDE BOUND
(percent of control)
100
80
60
40
20
0
-10
-9
-8
-7
-6
-5
-4
Dopamine (Log M)
- Na+
+ NaCl 10mM

Figure 5

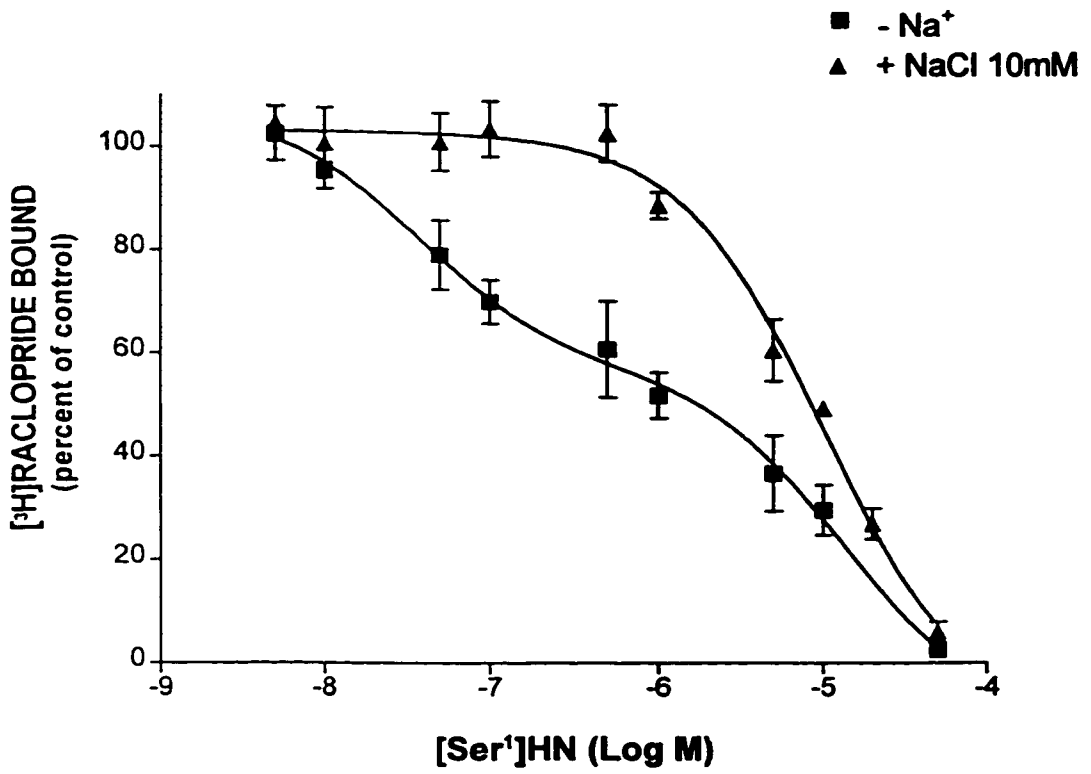
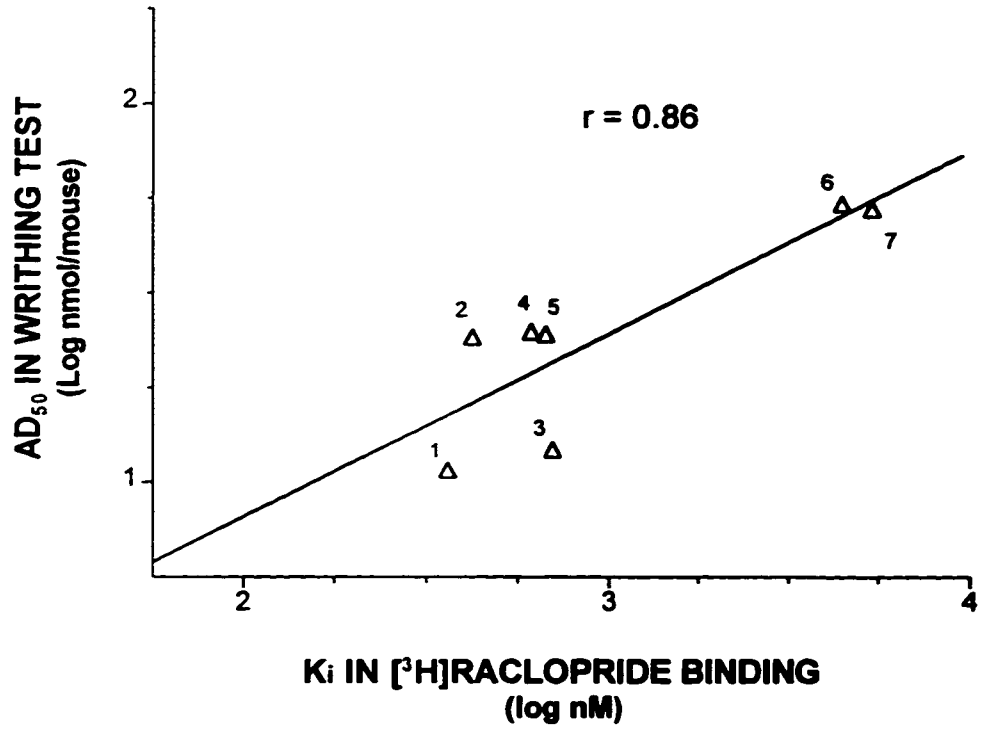

■ - Na+
▲ + NaCl 10mM
[3H]RACLOPRIDE BOUND
(percent of control)
100
80
60
40
20
0
-9
-8
-7
-6
-5
-4
[Ser1]HN (Log M)

Figure 6

1. HN-(7-15)
2. HN
3. H4-(86-100)
4. [Ser^1]HN
5. HN-(7-10)
6. HN-(1-10)
7. OGP



SECTION 5

GENERAL DISCUSSION

5.0 General Overview

HN, a novel peptide isolated from the bovine adrenal medulla was found to be present at high concentration in various rat tissues, including the pituitary, adrenal glands, lungs and brain (Lemaire et al., 1993). The peptide was first proposed to be an endogenous modulator of the central NMDA receptor on the basis of its NMDA antagonist activities as evidenced by its selective blockade of NMDA-induced convulsions in mice and exhibition of PCP-like behaviors in rats and mice (Lemaire et al., 1993; Shukla et al., 1995; Maurice et al., 1995). Recently, [Ser^1]HN, a stable synthetic analogue of HN, was shown to significantly attenuate hyperalgesia and allodynia in various animal models of chronic pain (Siegan et al., 1997; Siegan and Sagen, 1997; Hama et al., 1999). The antinociceptive activities of [Ser^1]HN in these tests were attributed to its negative modulating effect on spinal NMDA receptor(s), even though no attempt was made to define the nature of the receptor(s) involved.

Based on these former results, the current study was undertaken with the aims of verifying if HN and related peptides could produce analgesia in other animal models of pain and establishing a possible mechanism for the analgesic effects of the peptides.

5.1 Structure-dependency.

Supraspinal (i.c.v) administration of HN and related peptides induced marked

antinociceptive activity in both the mouse writhing and tail-flick tests, the pharmacology of which was compared with the ability of the peptides to compete with the binding of the D2 receptor antagonist, [^{3}H]raclopride. The structure-activity relationship study in both *in vivo* (mouse writhing) and *in vitro* ([^{3}H]raclopride binding) assays revealed several interesting features. For instance, the abilities of HN and related peptides to block writhing and displace [^{3}H]raclopride binding greatly diverged from the previously reported potencies of these peptides in blocking NMDA-induced convulsions (Prasad et al., 1995) and competing with the binding of [^{125}I][Ser^1]HN to rat brain membranes (Rogers and Lemaire , 1993). While previous findings indicated the requirement of full length peptides (15 amino acids) for biological activities, the present results indicate that the interaction of HN and related peptides with the dopamine D2 site in rat brain membranes as well as their analgesic effects require only the portion 7 to 10 of the molecule of HN (Fig 4 & Table 2; Table 4), HN-(7-15) displaying the highest potency among the tested peptides in both binding and behavioral tests. Thus, the mechanism underlying the analgesic activities of supraspinal HN and related peptides is distinct from that involved in the inhibition of NMDA-induced convulsions and displacement of the binding of [^{125}I][Ser^1]HN to its high affinity low capacity site in rat brain membranes (Prasad et al., 1995; Rogers and Lemaire , 1993). Such results speak for the possible presence of a second site for HN, presumably a lower affinity site present on or close to a dopamine D2 receptor and responsible for the supraspinal analgesic activities. Interestingly, the abilities of HN and related peptides to cause analgesia corresponded well with their abilities to compete with the binding of the D2 antagonist [^{3}H]raclopride to rat brain membranes (r=0.86) (Fig 15). These latter results provided some evidence that the analgesic activities of HN and related peptides could be

due to their direct or indirect interaction with the central D2 receptor.

Even though the presence of either the N-terminal or C-terminal portions in HN was not necessary for its analgesic and binding activities, it greatly enhanced the length of action of the peptide in the pain assays (Fig 5), suggesting that the action of peptidases may be an important factor to be considered for the design of long-acting HN related peptides and evaluation of the analgesic activity of HN. Indeed, the amino acids in the terminal regions of HN and related peptides play an important role in prolonging their time of action, indicating that they must confer to the peptides some resistance to the actions of physiological degrading enzymes (aminopeptidases, carboxypeptidases or endopeptidases).

Fragment-(86-100) in histone H4, a pentadecapeptide that shows 80% structural homology with HN (Lemaire et al., 1993), displayed an analgesic potency comparable to that of HN (Table 2). OGP (Bab et al., 1992), another C-terminal fragment in histone H4 (fragment 89 to 102), was two times less potent than HN (Table 2). The production of histone H4 -(86-100) has not yet been demonstrated, but Bab et al. (1999) have recently indicated that histone H4-(85-103) can be biosynthesized via an alternative translational initiation codon present in histone H4 mRNA. Thereafter, H4-(86-100) could be produced through the processing of H4-(85-103) by the action of a dipeptidyl peptidase at both N- and C-terminals. The recent finding of histone immunoreactivity on the cell surface of lymphocytes (Watson et al., 1995), the existence of C-terminal histone H4 related peptides in blood plasma (OGP) (Bab et al., 1992) and histogranin in the adrenal medulla (Lemaire et al., 1993) are compatible with the hypothesis that the histone H4 gene or some variant of histone H4 such as H4-v.1 (Gendron et al., 1997) may generate C-terminal

fragments of histone H4 with extranuclear and/or extracellular function(s).

5.2 Receptor(s) Involved in the Analgesic Activities.

Coadministration of [Ser^1]HN with naloxone (i.c.v.), an opioid receptor antagonist, failed to affect the analgesic activity of the peptide in both writhing and tailflick assays (Fig 6; Fig 9B). Naloxone at the dose of 1 nmol/mouse (i.c.v.) has been shown not to be analgesic *per se* but to block the action of opioids when coadministered with the tested compounds (data not shown). The insensitivity of [Ser^1]HN-induced analgesia to naloxone in the mouse writhing test suggests a non-opioid nature for this action. This result is in accordance with previous findings which indicated that the analgesic effects of [Ser^1]HN (i.t.) in rat chronic pain assays are insensitive to naloxone (Siegan et al., 1997).

The CPP insensitivity of the analgesic effect of [Ser^1]HN in the mouse writhing test (Fig 5) suggests that the NMDA receptor is also not involved in the action of the peptide. While a large body of evidence supports the potential utility of spinal NMDA antagonists for the treatment of chronic pain (Coderre and von Empel, 1994; Dicksnson, 1990; Ren et al., 1992; Woolf and Thompson, 1991), the supraspinal (i.c.v.) administration of competitive and non-competive NMDA antagonists (CGS, MK-801) has been shown to have no effect on nociception (Nasstrom et al., 1993). The marked effectiveness of HN and related peptides in the tail-flick test (Fig 8) also speaks for a differentiation between the analgesic activities of HN and related peptides and their ability to modulate NMDA receptor functions (Shukla et al., 1995) since many investigators have indicated that NMDA receptor antagonists are ineffective as analgesics in reflexive or brief nociceptive tests,

such as the tail-flick assay (Aanonsen and Wilcox, 1987; Millan and Seguin, 1994; Coderre and Van Empel, 1994).

The analgesic effects of [Ser^1]HN in the mouse writhing (Fig 7A) and tail-flick (Fig 9B) tests were either completely blocked or strongly attenuated by the D2 receptor antagonist raclopride, revealing a novel mode of action for HN and related peptides. There is increasing evidence which indicates that the enhancement of central dopaminergic transmission produces antinociception (Paalzow and Paalzow, 1983; Barasi and Duggal, 1985; Drago et al., 1984). The mechanism behind the analgesic activity of D2 agonists is not clearly defined. However, midbrain ascending DA neurons in the mesolimbic pathway have been implicated in the inhibition of tonic or persistent pain (Morgan and Franklin, 1990; Altier and Stewart, 1993; Altier and Stewart, 1998).

As demonstrated by some early studies, some D2 receptor mediated behaviors, such as locomotion and stereotype behaviors are expressed only when the D1 receptor is costimulated (Braun and Chase, 1986; Waddington, 1986; Arnt et al., 1988). However, substantial evidence supports the concept of a lack of participation of the D1 receptor in the analgesic activity of D2 agonists (Carr, 1984; Frussa-Filho et al., 1996; Ohkubo et al., 1991). The non-involvement of the D1 receptor in the analgesic response of [Ser^1]HN (i.c.v.) was evidenced by its insensitivity to the coadministration with the D1 receptor antagonist SCH-23390 (Fig 7B). Thus, the analgesic activities HN and related peptides, like those of prototypic D2 agonists, does not involve the participation of the D1 receptor.

A recent report that the analgesic activity of the bombesin-like peptide ranatensin (i.c.v.) is blocked by the D2 antagonist sulpiride , but not by the D1 antagonist SCH-23390,

nor the opiate antagonist naloxone (Zhu et al., 1991), indicates that some neuropeptides, other than HN, can also produce analgesia by a mechanism that involves the participation of the D2 receptor. The distinctions and/or similarities between the analgesic effects of HN and these other peptides remain to be established.

5.3 Lack of Motor Effect.

The analgesic activity of a substance may be difficult to evaluate if such a compound also causes an impairment of motor functions. For example, the analgesic activity of dynorphin A-(1-13) (Dyn A-(1-13) (i.t.) in the tail-flick assay has always been difficult to evaluate due to its marked motor effects, causing hindlimb paralysis (Stevens and Yaksh, 1986), loss of tail-flick reflex (Caudle and Isaac, 1987) and ataxia (Walker et al., 1982) at analgesic doses. However, in the pain assays described herein, none of the tested peptides caused any significant change in motor function as assessed by the performance of the animals in the rotarod assay (Table 3). Besides, HN and related peptides did not cause either any motor-stimulant activity (gross observation, data not shown) within the analgesic dose range. Therefore, the decrease in the acetic acid-induced writhing frequency as well as the increase in the tail-flick latency observed after the administration of the peptides is not interfered with the disruption of motor -function, and can be interpreted as analgesia.

5.4 D2 Agonist-like Competition Binding Activity against [^{3}H]Raclopride.

Since the pharmacology of the antinociceptive tests revealed the involvement of the D2 dopamine receptor in the analgesic activities of HN and related peptides, it became important to verify their mode of action at the receptor level using rat brain membranes with the specific D2 antagonist [^{3}H]raclopride as the radiolabelled ligand (Kohler er al., 1985). Saturation binding studies provided linear Scatchard plots, revealing the presence of a single high affinity site (K_d = 4.87 nM). The addition of [Ser^1]HN in the [^{3}H]raclopride saturation binding assays significantly decreased the affinity of [^{3}H]raclopride for the D2 receptor, as evidenced by a doubling of the K_d value. The B_{max} remained unchanged, implying a competitive nature of interaction of [Ser^1]HN with the central D2 receptor (Fig 12).

It has been widely accepted that the inhibition of [^{3}H]antagonist binding by unlabelled D2 agonists exhibits characteristics that are different from those of D2 antagonists. For instance, agonists inhibit [^{3}H]antagonist binding in a heterogenous manner displaying biphasical competition curves, while antagonists present monophasic competition curves (Sibley et al., 1982; Hamblin et al., 1984; Dewar et al., 1989; Reader et al., 1990). Thus, while dopamine and the D2 agonist (+)3-PPP exhibited biphasic competition curves with low Hill coefficients (nH$\leq$1) (two sites model), (-)sulpiride and its less active isomer (+)sulpiride exhibited monophasic competition curves with Hill coefficients close to unity ($n_H \approx 1$), implying a one site model (Fig 13). [Ser^1]HN and some HN related peptides displaced the binding of [^{3}H]raclopride biphasically ($n_H << 1$), similar to that observed with the D2 agonists dopamine and (+)3-PPP but not the D2 antagonists

(+)/(-)-sulpiride (Table 4; Fig 14). These results therefore provide the first *in vitro* binding characteristics which indicate that HN and related peptides in [^{3}H]raclopride competition binding experiments behave as D2 agonists.

Na^+ ions which are known to regulate the competition binding profiles of D2 agonists by a conversion of the high affinity site into a low affinity site (Grigoriadis and Seeman, 1985; Reader et al., 1990; Hall et al., 1991) were herein shown as a modulator of the binding of [Ser^1]HN in competition binding experiments. The inclusion of Na^+ (10 mM) into the incubation medium induced a complete conversion of the high affinity state (R_H) into the low affinity state (R_L), with a resulting n_H close to an unit (Fig 14). Similar observation were extended to dopamine with the percent of the binding sites in a high affinity state (R_H %) significantly diminished (Fig 13B), indicating that the effects of Na^+ ions on the inhibition profile of [Ser^1]HN were comparable to those of the dopamine agonist dopamine itself.

Neurotensin (NT) was also shown to compete with the binding of [^{3}H]raclopride (Table 4), but such interaction is different from that observed with HN in that NT inhibited the binding of [^{3}H]raclopride only at high concentrations (>10uM, data not shown), thus resulting in a lower potency (K_i, 17.3 uM) as compared with HN and related peptides. Besides, the competition curve of NT can be best described as monophasical model (n_H = 0.84), which is typical for the D2 antagonist and parallel with its antipsychotic activity.

5.5 Summary and Conclusions:

HN and related peptides administrated i.c.v. produce significant analgesic effects in both mouse writhing (visceral pain) and tail-flick (acute reflex pain) assays. The analgesic effects in the writhing test are dose dependent and structure-related. The minimal structure necessary for producing the analgesic effect resides in the middle portion of HN (amino acid sequence 7-10). The mechanism underlying the analgesia-induced by HN and related peptides involves the interaction of the peptide with D2 but not opioid, NMDA or D1 receptors. The analgesic effects of HN and related peptides were not due to their interference with motor activity. The involvement of the D2 receptor in the analgesic effects of HN and related peptides is also substantiated by the result from *in vitro* binding of [^{3}H]raclorpide to rat brain membranes. The saturation binding study suggests a competitive interaction between [Ser^1]HN and the D2 receptor. [Ser^1]HN displays a biphasic competition curve (high and low affinity state) in the [^{3}H]raclopride binding assay, resembling that of D2 agonists but not D2 antagonists. The displacement of [^{3}H]raclopride binding with [Ser^1]HN is regulated by Na^+ mainly by a conversion of the high affinity state into the low affinity state, a regulation pattern similar to that observed with dopamine. Finally, the potencies (K_i) of HN and related peptides in displacing the binding of [^{3}H]raclopride to rat brain membranes correlate well with that their abilities to cause analgesia in the mouse writhing test, further supporting the concept that the analgesic activities of the peptides are mediated through their modulation of G-protein linked D2 binding sites.

SECTION 6

FUTURE WORK

An extension of this study can be pursued in the four aspects:

1) Other animal models of chronic pain can be developed to examine the analgesic effect of HN and related peptides. Since the enhancement of central DA D2 receptor transmission has been frequently reported to be effective in relieving the noxious response in the tonic phase of the formalin test, HN related peptides or derivatives are expected to be effective pain relievers in the tonic phase of pain in the formalin test.

2) HN and related peptides could be examined for their possible abilities to cause tolerance or dependence.

3) The *in vitro* [^{3}H]raclopride binding study suggested that HN may act as a D2 receptor agonist. However, the question wether presynaptic or postsynaptic D2 receptors are affected by HN has not been resolved. It has been reported that the D2 receptor is predominantly presynaptic, and that the activation of such a receptor is able to regulate the release of various neurotransmitters, such as DA, glutamate in the CNS and noradrenaline in the heart. A recent finding from our lab demonstrated that HN can attenuate the release of [^{3}H]noradrenaline from cardiac synaptosomes exposed to ischemic conditions, the inhibory effect of HN being reversed by raclopride. This result implies that HN may have a protecting function in some neurological diseases such as stroke and cerebral ischemia

which are accompanied by an excessive release of neurotransmitters in certain brain areas. The possible modulatory effects of HN and related peptides on the release of central neurotransmitters, such as Glu or DA, remain to be established.

4) The binding properties of HN on the D2 receptor could be examined in different brain sections. The D2 receptors expressed in the striatum, limbic structures and pituitary are known to be involved in different biological activities. Therefore, the specific displacement [^{3}H]raclopride binding by HN in some of these brain regions may be helpful to determine the physiological role of the peptide.

SECTION 7

REFERENCES

Aanonsen, L.M. and Wilcox, G.L. (1987) Nociceptive action of excitatory amino acids in the mouse: effects of spinally administered opioids, phencyclidine and sigma agonists. J. Pharmacol. Exp. Ther. 243: 9-19.

Aimone, L.D. and Gebhart, G.F. (1986) Stimulation-produced spinal inhibition from the midbrain in the rat is mediated by an excitatory amino acid neurotransmitter in the medial medulla. J. Neurosci. 6: 1803-1813.

Altier, N. and Stewart, J. (1993) Intra-VTA infusions of the substance P analogue, DiMe-C7, and intra-accumbens infusions of amphetamine induce analgesia in the formalin test for tonic pain. Brain Res. 1993 628: 279-285.

Altier, N. and Stewart, J. (1998) Dopamine receptor antagonists in the nucleus accumbens attenuate analgesia induced by ventral tegmental area substance P or morphine and by nucleus accumbens amphetamine. J. Pharmacol. Exp. Ther. 285: 208-215.

D'Amour, F.E. and Smith, D.L. (1941) A method for determining loss of pain sensation. J. Pharmacol. Exp. Ther. 72: 74-77.

Anderson, R. M. and Rompré, P.P. (1996) Rewarding electical stimulation of the ventral tegmental area attenuates pain during the formalin test. Soc. Neurosci. (Abstract) 22: 686.

Arnt, J., Bogeso, K.P., Hyttel, J. and Meier, E. (1988) Relative dopamine D1 and D2 receptor affinity and efficacy determine whether dopamine agonist induced hyperactivity or oral stereotype in rats. Pharmacol. Toxicol. 62:121-130.

Bab, I., Gazit, D., Massarawa, A. and Shteyer, A. (1988) Regenerating bone marrow produces a potent growth-promoting activity to osteogenic cells. Endocrinology 123: 345-352.

Bab, I., Gazit, D., Muhlarad, A., Shteyer, A., Greenberg, Z., Namdar, M. and Kahn, A. (1992) Histone H4-related osteogenci growth peptide (OGP): a novel circulating stimulator of osteoblastic activity. EMBO. 11: 1867-1873.

Bab, I., Smith, E., Gavish, H., Attar-Namdar, M., Chorev, M., Chen, Y.C., Muhlrad, A., Birnbaum, M.J., Stein, G. and Frenkel, B. (1999) Biosynthesis of osteogenic growth peptide via alternative translational initiation at AUG85 of histone H4 mRNA. J. Biol. Chem. 274: 14474-14481.

Barasi, S. and Duggal, K.N. (1985) The effect of local and systemic application of dopaminergic agents on tail flick latency in the rat. 117: 287-294.

Bennett, J.P. and Yamamura, H. I. (1985) Neurotransmitter, hormone or drug receptor binding methods. In Neurotransmitter receptor binding, 2 nd Ed, ed. by Yamamura, H. I., Raven Press, New York, 1985.

Birch, P.J., Harrison, S.M., Hayes, A.G., Rogers, H. and Tyers, M.B. (1992) The non-peptide NK1 receptor antagonist, (+/-)-CP-96,345, produces antinociceptive and anti-oedema effects in the rat. Br. J. Pharmacol. 105: 508-510.

Bittencout, A.L. and Takahashi, R.N. (1997)Mazindol and lidocaine are antinociceptives in mouse formalin model: involvement of dopamine receptor. Eur. J. Pharmacol. 330: 109-113.

Brackley, P., Goodnow, R. Jr., Nakanishi, K., Sudan, H.L. and Usherwood, P.N. (1990) Spermine and philanthotoxin potentiate excitatory amino acid responses of Xenopus oocytes injected with rat and chick brain RNA. Neurosci. Let. 114: 51-56

Braun, A.R. and Chase, T.N. (1986) Obligatory D1/D2 receptor interaction in the generation of dopamine agonist related behaviors. Eur. J. Pharmacol. 131: 301-306.

Burris, T.P., Stringer, L.C. and Freeman, M.E. (1991) Pharmacologic evidence that a D2 receptor subtype mediates dopaminergic stimulation of prolactin secretion from the anterior pituitary gland. Neuroendocrinology 54:175-183.

Burt, D.R., Creese, I. and Snyder, S.H. (1976) Properties of [^{3}H]haloperidol and [^{3}H]dopamine binding associated with dopamine receptors in calf brain membranes. Mol. Pharmacol. 12: 800-812.

Carmichael, S.W. and Winkler, H. (1985) The adrenal chromaffin cells. Sci. Ame. 253:40-49.

Carr, K.D. (1984) Dopaminergic mechanisms in the supraspinal modulation of pain. Pain(Supple) 2: S223.

Carstens, E., Stelzer, B. and Zimmermann, M. (1988) Microinjections of glutamate or morphine at coincident midbrain sites have different effects on nociceptive dorsal horn neurons in the rat. Neurosci. Let. 95:185-191.

Caudle, R.M. and Isaac, L. (1987) Intrathecal dynorphin(1-13) results in an irreversible loss of the tail-flick reflex in rats. Brain Res. 435: 1-6.

Cepeda, C., Buchwald, N.A. and Levine, M.S. (1993) Neuromodulatory actions of doapmine in the neostriatum are dependent upon the excitatory amino acid receptor subtypes activated. Proc. Nat. Acad. Sci. (USA) 90: 9576-9580.

Chang, K.J. and Cuatrecasas, P. (1979) Multiple opiate receptors. Enkephalins and morphine bind to receptors of different specificity. J. Biol. Chem. 254: 2610-2618.

Chio, C.L. , Lajiness, M.E. and Huff, R.M. (1994) Activation of heterologously expressed D3 dopamine receptors: comparison with D2 dopamine receptors. Mol. Pharmacol. 45: 51-60.

Civelli, O., Bunzow, J.R. and Grandy, D.K. (1993) Molecular diversity of the dopamine receptors. Annu. Rev. Pharmacol. Toxicol. 32: 281-307.

Clement, J.R., Madl, J.E., Johnson, R.L, Larson, A.A. and Beitz, A.J. (1987) Localization of glutamate, glutaminase, aspartate, and asparatate aminotransferase in the rat midbrain periaqueductal gray. Exp. Brain Res. 67: 594-602.

Coderre, T.J., Katz, J., Vaccarino, A.L. and Melzack, R. (1993) Contribution of central neuroplasticity to pathological pain: review of clinical and experimental evidence. Pain 52: 259-285.

Coderre, T.J. and Van Empel, I. (1994) The utility of excitatory amino acid (EAA) antagonists as analgesic agents. I. Comparison of the antinociceptive activity of various classes of EAAantagonists in mechanical, thermal and chemical nociceptive tests. Pain 59: 345-352.

Cohen, A. I., Todd, R.D., Harmon, S. and O'Malley, K.L. (1992) Photoreceptors of mouse retinas possess D4 receptors coupled to adenylate cyclase. Proc. Natl. Acad. Sci. (USA) 89: 12093-13097.

Collin, E., Mauborgne, A., Bourgoin, S., Chantrel, D., Hamon, M. and Cesselin, F. (1991) In vivo tonic inhibition of spinal substance P (-like material) release by endogenous opioid(s) acting at delta receptors. Neuroscience 44: 725-731.

Creese, I., Burt, D.R. and Snyder, S.H. (1976) Dopamine receptor binding predicts clinical and pharmacological potencies of antischizophrenic drugs. Science 192: 481-483.

Creese, I. and Snyder, S.H. (1978) 3H-Spiroperidol labels serotonin receptors in rat cerebral cortex and hippocampus. Eur. J. Pharmacol. 49: 201-202.

Cruz, L. and Basbaum, A. (1985) Multiple opioid peptides and the modulation of pain:immunohistochemical analysis of dynorphin and enkephalin in the trigeminal nucleus caudalis and spinal cord of the cat. J. Comp. Neurol. 240: 331-348.

Cummings, J.L. (1992) Depression and Parkinson's disease: a review. Am. J. Psychiatry 149: 443-454.

Dahlstrom, A. and Fuxe, K. (1964) Localization of monoamines in the lower brain stem. Experientia 20: 398-399.

Davar, G., Hama, A., Deykin, A., Vos, B. and Maciewicz, R. (1991) MK-801 blocks the development of thermal hyperalgesia in a rat model of experimental painful neuropathy. Brain Res. 553: 327-330.

Davar, G., Hama, A., Deykin, A., Vos, B. and Maciewicz, R. (1991) MK-801 blocks the development of thermal hyperalgesia in a rat model of experimental painful neuropathy. Brain Res. 553: 327-330.

Dearry, A., Gingrich, J.A., Falardeau, P., Fremeau, R.T., Bates, M.D. and Caron, M.G.(1990) Molecular cloning and expression of the gene for a human D1 dopamine receptor. Nature 347: 72-76.

De Camilli, P., Macconi, D. and Spada, A. (1979) Dopamine inhibits adenylate cyclase in human prolactin-secreting pituitary adenomas. Nature 278: 252-254.

De lean, A. and Stadel, J.M. (1980) Lefkowitz, R.J. A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase coupled β-adrenergic receptor. J. Biol. Chem. 255: 7108-7117.

DeNoble,V.J., Jones, K.W., Schaeffer, C.L. and Bauerle,L.M. (1990) 3-((+-)-2-carboxypiperazin-4-yl) propyl-1-phosphonic acid (CPP) and phencyclidine produce a deficit of passive avoidance retention in rats. Eur. J. Pharmacol. 175: 197-202.

Deutch, A.Y., Moghaddam, B., Innis, R.B., Krystal, J.H., Aghajaian, G.K., Bunney, B.S. and Charney, D.S. (1991) Mechanisms of action of atypical antipsychotic drugs. Implications for novel therapeutic strategies for schizophrenia. Schizophr. Res. 4: 121-156.

Dewar, K.M., Montreuil, B., Grondin, L. and Reader, T.A. (1989) Dopamine D2 receptors labeled with [^{3}H]raclopride in rat and rabbit brains. Equilibrium binding, kinetics, distribution and selectivity. J. Pharmacol. Exp. Ther. 250: 696-706.

Dickenson, A.H. (1990) A cure for wind up: NMDA receptor antagonists as potential analgesics. Treads Pharmacol. Sci. 11: 307-309.

Drago, F.G., Caccamo, G., Continella, G. and Scapagnini, U. (1984) Amphetamine-induced analgesia does not involve brain opioids. Eur. J. Pharmacol. 101: 267-269.

Dray, A., Urban, L. and Dickenson, A. (1994) Pharmacology of chronic pain. Trends Pharmacol. Sci. 15: 190-197.

Dubner, R., and Ruda, M.A. (1992) Activity-dependent neuronal plasticity following tissue injury and inflammation. Trends Neurol. Sci. 15: 96-103.

Dubois, R.N., Abramson, S.B., Crofford, L., Gupta, R.A., Simon, L.S., Van De Putte, L.B.

and Lipsky, P.E. (1998) Cyclooxygenase in biology and disease. FASEB J. 12: 1063-1073.

Dumont, M., Prasad, J. and lemaire, S. (1994) Interaction of histogranin and related peptides with [^{3}H]dextromethorphan binding sites in rat brain. Neurosci. Let. 173: 135-138.

Eddey, W.B. and Leimbach, D. (1953) Synthetic analgesics (II): dithenylbutenyl and dithenylbutylamines. J. Pharmacol. Exp. Ther. 107: 385-389.

Enjalbert, A. and Bockaert, J. (1983) Pharmacological characterization of the D2 dopamine receptor negatively coupled with adenylate cyclase in rat anterior pituitary. Mol. Pharmacol. 53: 576-584.

Fallon, J.H. and Moore, R.Y. (1978) Catecholamine innervation of the basal forebrain. III. Olfactory bulb, anterior olfactory nuclei, olfactory tubercle and piriform cortex. J. Comp. Neurol. 180: 533-544.

Fang, F.G., Moreau, J.L. and Fields, H.L. (1987) Dose-dependent antinociceptive action of neurotensin microinjected into the rostroventromedial medulla of the rat. Brain Res. 420: 171-174.

Fields, H.L. and Anderson, S.D. (1978) Evidence that raphe-spinal neurons mediate opiate and midbrain stimulation-produced analgesia. Pain 5: 333-349.

Fields, H.L., Heinricher, M.M. and Mason, P. (1991) Neurotransmitters in nociceptive modulatory circuits. Annu. Rev. Neurosci. 14: 219-245.

Fleetwood-Walker, S.M., Mitchell, R., Hope, P.J., Molony, V. and Iggo, A. (1985) An alpha 2 receptor mediates the selective inhibition by noradrenaline of nociceptive responses of identified dorsal horn neurones. Brain Res. 334: 243-254.

Franklin, K.B.J. (1989) Analgesia and neural substrate of reward. Neurosci. Biochem. Behav. Rev. 13: 149-154.

Frussa-Filho, R., Rocha, J.B., Conceicao, I.M., Mello, C.F. and Pereira, M.E. (1996) Effects of dopaminergic agents on visceral pain measured by the mouse writhing test. Arch. Int. Pharmacodyn. Ther. 331: 74-93.

Fujita, N., Nakahiro, M., Fukuchi, I., Saito, K. and Yoshida, H. (1985) Effects of pertussis toxin on D2-dopamine receptor in rat striatum: evidence for coupling of Gi regulatory protein with D2-receptor. Brain Res. 333: 231-236.

Fuxe, K., Hökfelt, T. and Nilsson, O. (1969) Factors involved in the control of the activity of the tubero-infundibualr dopamine neurons during pregancy and lactation. Neuroendocrinology. 5: 257-270.

Gendron, N., Dumont, M., Gagne, M.-F. and Lemaire, S. (1997) Poly A-containing histone H4 Mrna variant (H4-V.1): Isolation and sequence determination from bovine adrenal medulla. Biochim. Biophys. Acta. 1396: 32-38.

George, S. R., Binkley, K. and Seeman, P. (1983) Dopamine receptor sites and states in human brain. J. Neural. Transm. 18(supple): 149-156.

Gillan, M.G., Kosterlitz, H.W. and Paterson, S.J. (1980) Comparison of the binding characteristics of tritiated opiates and opioid peptides. Br. J. Pharmacol. 70: 481-490.

Gingrich, J.A. and Caron, M.G. (1993) Recent advances in the molecular biology of dopamine receptors. Annu. Rev. Neurosci. 16: 299-321.

Glaum, S.R., Proudfit, H.K. and Anderson, E.G. (1988) Reversal of the antinociceptive effects of intrathecally administered serotonin in the rat by a selective 5-HT3 receptor antagonist. Neurosci. Let. 95: 313-317.

Glazer, E.J. and Basbaum, A.I. (1981) Immunohistochemical localization of leucine-enkephalin in the spinal cord of the cat: enkephalin-containing marginal neurons and pain modulation. J. Comp. Neurol. 196: 377-389.

Gonzales-Rios, F., Vlaiculescu, A., Ben Natan, L., Protais, P. and Costentin, J. (1986) Dissociated effects of apomorphine on various nociceptive responses in mice. J. Neural. Transm. 67: 87-103.

Graybiel, A.M. and Elde, R.P. (1982) Somatostatin-like immunoreactivity characterizes neurons of the nucleus reticularis thalami in the cat and monkey. J. Neurosci. 3:1308-1321.

Grigoriadis, D. and Seeman, P. (1985) Complete conversion of brain D2 dopamine receptors from the high- to the low-affinity state for dopamine agonists, using sodium ions and guanine nucleotide. J. Neurochem. 44: 1925-1935.

Grigoriadis, D. and Seeman, P. (1986) [^{3}H]-domperidone labels only a single population of receptors which convert from high to low affinity for dopamine in rat brain. Naunyn Schmiedebergs Arch Pharmacol. 332: 21-25.

Hall, H., Halldin, C. and Sedvall, G. (1992) Gpp(NH)p stimulates [^{3}H]raclopride binding to homogenates from human putamen and accumbens. Neurosci. Let. 136: 79-82.

Hama, A.T. and Sagen, J. (1993) Reduced pain-related behavior by adrenal medullary transplants in rats with experimental painful peripheral neuropathy. Pain 52: 223-231.

Hama, A.T., Siegan, J.B., Herzberg, U. and Sagen, J. (1999) NMDA-induced spinal hypersensitivity is reduced by naturally derived peptide analog [Ser^1]histogranin. Pharmacol. Biochem. Behav. 62: 67-74.

Hamblin, M.W., Leff, S.E. and Creese, I. (1984) Interactions of agonists with D-2 dopamine receptors: evidence for a single receptor population existing in multiple agonist affinity-states in rat striatal membranes. Biochem. Pharmacol. 33: 877-887.

Handy, R.L., Wallace, P., Gaffen, Z.A., Whitehead, K.J. and Moore, P.K. (1995) The antinociceptive effect of 1-(2-trifluoromethylphenyl) imidazole (TRIM), a potent inhibitor of neuronal nitric oxide synthase in vitro, in the mouse. Br. J. Pharmacol. 116: 2349-2350.

Hargreaves, K.M. Troullos, E.S., Dionne, R.A., Schmidt, E.A., Schafer, S.C. and Joris, J.L. (1988) Bradykinin is increased during acute and chronic inflammation: therapeutic implications. Clin. Pharmacol. Ther. 44: 613-621.

Hornykiewicz, O. (1966) Dopamine and brain function. Pharmacol. Rev. 18: 925-964.

Huidobro-Toro, J.P., Yoshimura, K., Lee, N.M., Loh, H.H. and Way, E.L. (1981) Dynorphin interaction at the kappa-opiate site. Eur. J. Pharmacol. 72: 265-266.

Hunt, S.P. and Lovick, T.A. (1982) The distribution of serotonin, met-enkephalin and beta-lipotropin-like immunoreactivity in neuronal perikarya of the cat brainstem. Neurosci. Let. 30: 139-145.

Jensen, T.S. and Yaksh, T.L. (1992) The antinociceptive activity of excitatory amino acids in the rat brainstem: an anatomical and pharmacological analysis. Brain Res. 569: 255-267.

Jensen, T.S. and Yaksh, T.L. (1984) Effects of an intrathecal dopamine agonist, apomorphine, on thermal and chemical evoked noxious responses in rats. Brain Res. 296: 285-293.

Joyce. J.N. and Meador-Woodruff, J.H. (1997) Linking the family of D2 receptors to neuronal circuits in human brain: insights into schizophrenia. Neuropsychopharmacol. 16: 375-384.

Kebabian, J.W. and Calne, D.B. (1979) Multiple receptors for dopamine. Nature 277:93-96.

Kohler, C., Hall, H., Ogren, S.O. and Gawell, L. (1985) Specific in vitro and in vivo binding of ^{3}H-raclopride. A potent substituted benzamide drug with high affinity for dopamine D-2 receptors in the rat brain. Biochem. Pharmacol. 34:2251-2259.

Koob, G.F. (1992) Drugs of abuse: anatomy, pharmacology and function of reward pathways. Trends Pharmacol. Sci. 13: 177-184.

Koob, G.F. and Bloom, F.E. (1988) Cellular and molecular mechanisms of drug dependence. Science 242: 715-723.

Kosterlitz, H.W. and Paterson, S.J. (1980) Characterization of opioid receptors in nervous tissue. Proc. R. Soc. Lond. B. Biol. Sci. 210:113-122.

Kuraishi, Y., Hirota, N., Sato, Y., Kaneko, S., Satoh, M. and Takagi, H. (1985) Noradrenergic inhibition of the release of substance P from the primary afferents in the rabbit spinal dorsal horn. Brain Res. 359: 177-182.

Lemaire, S., Shukla, V.K., Rogers, C., Ibrahim, I.H., Lapierre, C., Parent, P. and Dumont, M. (1993) Isolation and characterization of histogranin, a natural petpide with NMDA receptor antagonist activity. Eur. J. Pharmacol. 245: 247-256.

Lemaire, S., Rogers, C., Dumont, M., Shukla, V.K., Lapierre, C., Prasad, J. and Lemaire, I. (1995) Histogranin, a modified histone H4 fragment endowed with N-methyl-D-aspartate antagonist and immunostimulatory activities. Life Sci. 56: 1233-1241.

Le Moal, M. and Simon, H. (1991) Mesocorticolimbic dopaminergic network: Functional and regulatory roles. Physiol. Rev. 71: 155-234.

Leslie, C.A., Jung, A. and Bennett, J.P. Jr. (1998) Potentiation of D2-dopamine receptor-mediated suppression of zif268 by non-competitive NMDA receptor antagonists in reserpinized rats. Brain Res. 59: 40-49.

Leysen, J.E., Gommeren, W. and Laduron, P.M. (1978) Spiperone: a ligand of choice for neuroleptic receptors. 1. Kinetics and characteristics of in vitro binding. Biochem. Pharmacol. 27: 307-316.

Lieberman, J.A., Kane, J.M. and Alvir, J. (1987) Provocative tests with psychostimulant drugs in schizophrenia. Psychpharmacology (Berlin) 91: 415-433.

Lin, Y., Morrow, T. J., Kiritsky-Roy, J.A., Terry, L.C., and Casey, K.L. (1989) Cocaine: evidence for supraspinal, dopamine-mediated, non-opioid analgesia. Brain Res. 479: 306-312.

Lindvall, O. and Björlund, A. (1983) Dopamine and norepinephrine-containing neuron systems: their anatomy in the rat brain. Chemical Neuroanatomy, P.C. Emson, ed. New York: Raven press, 299-255.

Lombardo, M.C. and Willson, L.M. (1997) Pain in Pathophysiology: Clinic concept of disease process (Price, S.A.; Wilson, L.M., Eds. 5), St. Louis, pp. 818-848.

London, E.E., Dawson, V.L. and Mantione, C.R. (1991) Specific binding sites for polyamines in mammalian brain. In NMDA receptor related agents, ed. by Kameyama, T., Nabeshima, T. and Domino, E.F., pp 71-78, NPP Books, Ann Arbor, MI.

Mansour, A., Meng, F., Meador-Woodruff, J.H., Taylor, L.P., Civelle, O. and Akil, H. Site-

directed mutagenesis of the human dopamine D2 receptor. Eur. J. Pharmacol. 227: 205-214.

Manning, B.H., Morgan, M.J. and Franklin, K.B. (1994) Morphine analgesia in the formalin test: evidence for forebrain and midbrain sites of action. Neuroscience 63: 289-294.

Mao, J., Price, D.D., Hayes, R.L., Lu, J., Mayer, D.J. and Frenk, H. (1993) Intrathecal treatment with dextrorphan or ketamine potently reduces pain-related behaviors in a rat model of peripheral mononeuropathy. Brain Res. 605: 164-168.

Masferrer, J.L., Zweifel, B.S., Manning, P.T., Hauser, S.D., Leahy, K.M., Smith, W.G. and Isakson, P.C. (1994) Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. Proc. Natl. Acad. Sci. (USA) 91: 3228-3232.

Maurice, T., Privat, A. and Lemaire, S. (1995) The pentadecapeptide [Ser^1]histogranin impairs passive avoidance learning in mice. Eur. J. Pharmacol. 283: 251-254.

McDonald, W.M., Sibley, D.R., Kilpatrick, B.F. and Caron, M.G. (1984) Dopaminergic inhibition of adenylate cyclase correlates with high affinity agonist binding to anterior pituitary D2 dopamine receptors. Mol. Cel. Endocrinol. 36: 201-209.

Meador-Woodruff, J.H., Mansour, A., Bunzow, J.R., Vantol, H.H.M., Watson, S.J. and Civelli, O. (1989) Distribution of D2 dopamine receptor mRNA in rat brain. Proc. Natl. Acad. Sci. (USA) 86: 7625-7628.

Mengod, G., Martinez-Mir, M.I., Vilaro, M.T. and Palacios, J.N. (1989) Localization of the mRNA for the dopamine D2 receptor in brain by in situ hybridization histochemistry, Proc. Natl. Acad. Sci. (USA) 86:8560-8564.

Michael-Titus, A., Bousselmame, R. and Costentin, J. (1990) Stimulation of dopamine D2 receptors induces an analgesia involving an opioidergic but non enkephalinergic link. Eur. J. Pharmacol. 187: 201-207.

Millan, M.J. and Seguin, L (1994) Chemically-diverse ligands at the glycine B site coupled toN-methyl-D-aspartate (NMDA) receptors selectively block the late phase of formalin-induced pain in mice. Neurosci. Let. 178: 139-143.

Millar, J. and Williams, G.V. (1989) Effects of iontophoresis of noradrenaline and stimulation of the periaqueductal gray on single-unit activity in the rat superficial dorsal horn. J. Comp. Neurol. 287: 119-133.

Missale, C., Nash, S.R., Robinson, S.W., Jaber, M. and Caron, M.G. (1998) Dopamine receptors: from structure to function. Phsiol. Rev. 78: 189-225.

Mitchell, J.A., Akarasereenont, P., Thiemermann, C., Flower, R.J. and Van, J.R. (1993)

Selectivity of nonsteriodal antiinflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. Proc. Natl. Acad. Sci. (USA) 90:11693-11697.

Morgan, D.G., Marcusson, J.O. and Finch, C.E. (1984) Contamination of serotonin-2 binding sites by an alpha-1 adrenergic component in assays with [^{3}H]spiperone. Life Sci. 34: 2507-2514.

Morgan, M.J. and Franklin, K.B. (1990) 6-Hydroxydopamine lesions of the ventral tegmentum abolish D-amphetamine and morphine analgesia in the formalin test but not in the tail flick test. Brain Res. 519: 144-149.

Morgan, M.J. and Franklin, K.B.J. (1991) Dopamine receptor subtypes and formalin test analgesia. Pharmacol. Biochem. Behav. 40: 317-322.

Morra, M., Leboulenger, F., Desrues, L., Tonon, M.C. and Vaudry, H. (1991) Dopamine inhibits inositol phosphate production, arachidonic acid formation, and corticosteroid release by frog adrenal gland through a pertussis toxin-sensitive G-protein. Endocrinology 128: 2625-2632.

Morris, R.G.M., Anderson, E., Lynch, G.M. and Baudry, M. (1986) Selective impairment of learning and blockade of long-term potentiation by a N-methyl-D-aspartate receptor antagonist, AP5. Nature 319: 774-776.

Najlerahim, A., Barton, A.J., Harrison, P.J., Heffernan, J. and Pearson, R.C. (1989) Messenger RNA encoding the D2 dopaminergic receptor detected by in situ hybridization histochemistry in rat brain. FEBS Let. 255: 335-339.

Niznik, H.B., Grigoriadis, D.E., Pri-Bar, I., Buchman, O. and Seeman, P. (1985) Dopamine D2 receptors selectively labeled by a benzamide neuroleptic: [^{3}H]-YM-09151-2. Naunyn Schmiedebergs Arch Pharmacol. 329: 333-343.

Ögren, S.O. and Goldstein, M. (1994) Phencyclidine- and dizocilpine-induced hyperlocomotion are differentially mediated. Neuropsychopharmacol. 11: 167-177.

Ögren, S.O., Hall, H., Kohler, C., Magnusson, O. and Sjostrand, S.E. (1986) The selective dopamine D2 receptor antagonist raclopride discriminates between dopamine-mediated motor functions. Psychopharmacol. (Berl) 90: 287-294.

Ohkubo, Y., Nomura, K. and Yamaguchi, I. (1991) Involvement of dopamine in the mechanism of action of FR64822, a novel non-opioid antinociceptive compound. Eur. J. Pharmacol. 204:121-125.

Onali, P., Olianas, M.C. and Gessa, G.L. (1984) Selective blockade of dopamine D-1 receptors by SCH 23390 disclose striatal dopamine D-2 receptors mediating the inhibition of adenylate cyclase in rats. Eur. J. Pharmacol. 99:127-128.

Onali, P.L., Olianas, M.C. and Gessa, G.L. (1985) Characterization of dopamine receptors mediating inhibition of adenylate cyclase activity in rat striatum. Mol. Pharmacol. 28: 138-145.

Paalzow, G.H. and Paalzow, L.K. (1983) Opposing effects of apormophine on pain in rats. Evaluation of the dose-response curve. Eur. J. Pharmacol. 5: 93-99.

Perkins, M. and Dray, A. (1996) Novel pharmacological strategies for analgesia. Ann. Rheum. Dis. 55: 715-722.

Peterfreund, R.A., Kosofsky, B.E. and Fink, J.S. (1995) Cellular localization of dopamine D2 receptor messenger RNA in the rat trigeminal ganglion. Anesth. Analg. 81: 1181-1185.

Popoli, P., Caporali, M.G. and Scotti de, C.A. (1990) Evidence of the involvement of D1 dopamine receptors in PCP- induced stereotypy and ataxia in rabbits. Pharmacol. Res. 22: 197-205.

Prasad, J.A., Shukla, V.K. and Lemaire, S. (1995) Synthesis and biological activity of histogranin and related peptides. Can. J. Physiolo. Pharmacol. 73: 209-214.

Proudfit, H.K. and Hammond, D.L. (1981) Alterations in nociceptive threshold and morphine-induced analgesia produced by intrathecally administered amine antagonists. Brain Res. 218: 393-399.

Quirion, R., Gaudreau, P., St-Pierre, S., Rioux, F. and Pert, C.B. (1982) Autoradiographic distribution of [^{3}H]neurotensin receptors in rat brain:visualization by tritium-sensitive film. Peptides 3: 757-763.

Quirion, R., Bowen, W.D., Itzhak, Y., Junien, J.L., Musacchio, J.M., Rothman, R.B., Su, T.B., Tam, S.W. and Taylor, D.P. (1992) A proposal for the classification of sigma binding sites. Trens Pharmacol. Sci. 13, 85-86.

Randrup, A. and Munkvad, I. (1965) Special antagonism of amphetamine-induced abnormal behaviour. Inhibition of stereotyped activity with increase of some normal activities. Psychopharmacologia 7: 416-422.

Ransom, R.W. and Stec, N.L. (1988) Cooperative modulation of [^{3}H]MK-801 binding to the N-methyl-D-aspartate receptor-ion channel complex by L-glutamate, glycine, and polyamines. J. Neurochem. 51: 830-836.

Rashed, S.M. and Songu-Mize, E. (1996) Regulation of Na^+, K^+-ATPase activity by dopamine in cultured rat aortic smooth muscle cells. Eur. J. Pharmacol. 305: 223-230.

Ren, K., Hylden, J.L., Williams, G.M., Ruda, M.A., and Dubner, R. (1992) The effects of a non-competitive NMDA receptor antagonist, MK-801, on behavioral hyperalgesia and dorsal

horn neuronal activity in rats with unilateral inflammation. Pain 50: 331-344.

Rogers, C. and Lemaire, S. (1993) Characterization of [^{125}I][Ser^{1}]histogranin sites in rat brain. J. Pharmacol. Exp. Ther. 267: 350-356.

Ruda, M.A. (1982) Opiates and pain pathway: demonstration of enkephalin synapses on dorsal horn projection neurons. Science 215: 1523-1525.

Ruda, M.A., Allen, B. and Gobel, S. (1981) Ultrastructural analysis of medial brain stem afferents to the superficial dorsal horn. Brain Res. 205: 175-180.

Sagen, J., Wang, H. and Pappas, G.D. (1990) Adrenal medullary implants in the rat spinal cord reduce nociception in a chronic pain model. Pain 42: 69-79.

Sakurada, T., Sugiyama, A., Sakurada, C., Tan-No, K., Yonezawa, A., Sakurada, S. and Kisara, K. (1996) Effect of spinal nitric oxide inhibition on capsaicin-induced nociceptive response. Life Sci. 59: 921-930.

Salt, T.E. and Hill, R.G. (1981) Neurotransmitter candidates of somatosensory primary afferent fibres. Neuroscience 10: 1083-1103.

Scatton, B. (1982) Further evidence for the involvement of D2, but not D1 dopamine receptors in dopaminergic control of striatal cholinergic transmission. Life Sci. 31: 2883-2890.

Schoffelmeer, A.N., Warden, G., Hogenboom, F. and Mulder, A.H. (1991) Beta-endorphin: a highly selective endogenous opioid agonist for presynaptic mu opioid receptors. J. Pharmacol. Exp. Ther. 258: 237-242.

Seeman, P. and Lee, T. (1975) Antipsychotic drugs: direct correlation between clinical potency and presynaptic action on dopamine neurons. Science 188: 1217-1219.

Seeman, P. (1980) Brain dopamine receptors. Pharmacol. Rev. 32: 229-313.

Seeman, P., Grigoriadis, D., George, S.R., Watanae, M. and Ulpian, C. Function states of dopamine receptors. In: Dopaminergic systems and their regulation (Eds. Woodruff GN, poat JA and Roberts PJ), pp 97-109. Macmillan, London, 1986.

Seeman, P. and Grigoriadis, D. (1987) Dopamine receptors in brain and periphery. Neurochem. Int. 10: 1-25.

Seibert, K. (1994) Selective inhibition of inducible cyclooxygenase 2 in vivo is antiinflammatory and nonulcerogenic. Proc. Natl. Acad. Sci. (USA) 91: 3228-3232.

Shiman, R., Akino, M. and Kaufman, S. (1971) Solubilization and partial purification of

tyrosine hydroxylase from bovine adrenal medulla. J. Biol. Chem. 246: 1340-1350.

Sibley, D.R., De Lean, A. and Creese, I. (1982) Anterior pituitary dopamine receptors. Demonstration of interconvertible high and low affinity states of the D-2 dopamine receptor. J. Biol. Chem. 257: 6351-6361.

Sibley, D.R. and Lefkowitz, R.J. (1985) Molecular mechanisms of receptor desensitization using the beta-adrenergic receptor-coupled adenylate cyclase system as a model. Nature 317: 124-129.

Sibley, D.R. and Monsma, F.J. Jr. (1992) Molecular biology of dopamine receptors. Trends Pharmacol. Sci. 13: 61-69.

Siegan, J.B., Hama, A.T. and Sagen, J. (1997) Suppression of neuropathic pain by a naturally-derived peptide with NMDA antagonist activity. 755: 331-334.

Siegan, J.B. and Sagen, J. (1997a) Attenuation of formalin pain responses in the rat by adrenal medullary transplants in the spinal subarachnoid space. Pain 70: 279-285.

Siegan, J.B. and Sagen, J. (1997b) A natural peptide with NMDA inhibitory activity reduces tonic pain in the formalin model. Neuro. Rep. 8: 1397-1381.

Sigmund, F., Cadmus, R. and Lu, G. (1957) Method for evaluating both non-narcotic and narcotic analgesics. Proc. Soc. Exp. Biol. Med. 95: 729-735.

Shukla, V.K., Lemaire, S., Dumont, M. and Merali, Z. (1995) N-methyl-D-Asparate receptor antagonist activity and phencyclidine-like behavioral effects of the pentadecapeptide, [Ser^1]Histogranin. Pharmcol. Biochem. and Behav., 50 : 49-54.

Snyder, S.H. (1973) Amphetamine psychosis: a model schizophrenia mediated by catecholamine. Am. J. Psychiatry 130: 61-67.

Spano, P.F., Govoni, S. and Trabucchi, M. (1978) Studies on the pharmacological properties of dopamine recetors in various areas of the central nervous system. Adv. Biochem. Psychopharmacol. 19: 155-165.

Sprosen, T.S. and Woodruff, G.N. (1990) Polyamines potentiate NMDA induced whole-cell currents in cultured striatal neurons. Eur. J. Pharmacol. 179: 477-478.

Starke, K., Spath, L., Lang, J.D. and Adelung, C. (1983) Further functional in vitro comparison of pre- and postsynaptic dopamine receptors in the rabbit caudate nucleus. Naunyn Schmiedebergs Arch Pharmacol. 323: 298-306.

Stefanini, E., Ortu, A.M., Vernaleone, F. and Gessa, G.L. (1987) [^{3}H] (-)sulpiride binding in rat striatum, cortex and anterior pituitary: an improved assay. Pharmacol. Res.

Commun.19: 777-791.

Steinfels, G.F., Alberici, G.P., Tam, S.W. and Cook, L. (1988) Biochemical, behavioral, and electrophysiologic actions of the selective sigma receptor ligand (+)-pentazocine. Neuropsychopharmacology 1: 321-327.

Stevens, C.W. and Yaksh, T.L. (1986) Dynorphin A and related peptides administered intrathecally in the rat: a search for putative kappa opiate receptor activity. J. Pharmacol. Exp. Ther. 238: 833-838.

Theodorou, A., Crockett, M., Jenner, P. and Marsden, C.D. (1979) Specific binding of [^{3}H]sulpiride to rat striatal preparations. J. Pharm. Pharmacol. 31: 424-426.

Unsicker, K. and Stogbauer, F. (1992) Screening of adrenal medullary neuropeptides for putative neurotrophic effects. Int. J. Dev. Neurosci. 10: 171-179.

Urban, L., Thompson, S.W. and Dray, A. (1994) Modulation of spinal excitability: co-operation between neurokinin and excitatory amino acid neurotransmitters. Trends Neurosci. 17: 432-438.

Usdin, T.B., Creese, I. and Snyder, S.H. (1980) Regulation by cations of [^{3}H]spiroperidol binding associated with dopamine receptors of rat brain. J. Neurochem. 34: 669-676.

Vanegas, H., Barbaro, N.M. and Fields, H.L. (1984) Midbrain stimulation inhibits tail-flick only at currents sufficient to excite rostral medullary neurons. Brain Res. 321: 127-133.

Venable, N. and Kelly, P.H. (1990) Effects of NMDA receptor antagonists on passive avoidance learning and retrieval in rats and mice. Psychopharmacology 100: 215-221.

Verma, A. and Kulkarni, S.K. (1991) Alpha 2-adrenoceptor- and D2-dopamine receptor-mediated analgesic response of B-HT 920. J. Pharm. Pharmacol. 43: 131-133.

Waddington, J.L. (1986) Behavioural correlates of the action of selective D-1 dopamine receptorantagonists. Impact of SCH 23390 and SKF 83566, and functionallyinteractive D-1:D-2 receptor systems. Biochem. Pharmacol. 35: 3661-3667.

Walker, J.M., Moises, H.C., Coy, D.H., Baldrighi, G. and Akil, H. (1982) Nonopiate effects of dynorphin and des-Tyr-dynorphin. Science 218: 1136-1138.

Watanabe, M., George, S.R. and Seeman, P. (1985) Dependence of dopamine receptor conversion from agonist high- to low-affinity state on temperature and sodium ions. Biochem. Pharmacol. 34: 2459-2463.

Watkins, J.C. and Evans, R.H. (1981) Excitatory amino acid transmitters. Annu. Rev. Pharmacol. Toxicol. 21: 165-204.

Watsom, K., Edwards, R.J., Shaunak, S., Parmelee, D.C., Sarraf, C., Gooderham, N.J. and Davies, D.S. (1995) Extra-nuclear location of histones in activated human peripheral blood lymphocytes and cultured T-cells. Biochem. Pharmacol. 50: 299-309.

Weisbrod, S. (1982) Active chromatin. Nature 297: 289-95.

Wiesenfeld-Hallin, Z. (1986) Substance P and somatostatin modulate spinal cord excitability via physiologically different sensory pathways. Brain Res. 372: 172-175.

Wiklund, L., Behzadi, G., Kalen, P., Headley, P.M., Nicolopoulos, L.S., Parsons, C.G. and West, D.C. (1988) Autoradiographic and electrophysiological evidence for excitatory aminoacid transmission in the periaqueductal gray projection to nucleus raphemagnus in the rat. Neurosci. Let. 93: 158-163.

Wikstrom, H., Andersson, B., Elebring, T., Svensson, K., Carlsson, A. and Largent, B. (1987) N-substituted 1,2,3,4,4a,5,6,10b-Octahydrobenzo[f]quionlines and 3-phenylpiperidines: Effects on central dopamine and σ receptors. J. Med. Chem. 30: 2169-2174.

Williams, K., Romano, C., Dichter, M.A. and Molinoff, P.B. (1991) Modulation of the NMDA receptor by polyamines. Life Sci. 48: 469-498.

Wu, M., Brudzynski, S.M. and Mogenson, G.J. (1993) Functional interaction of dopamine and glutamate in the nucleus accumbens in the nucleus accumbens in the regulation of locomotion. Can. J. Physiol. Pharmacol. 71: 407-413.

Wu, R.S. and Bonner, W.M. (1981) Separation of basal histone synthesis from S-phase histone synthesis in dividing cells. Cell 27: 321-330.

Xie, W.L., Chipman, J.G., Robertson, D.L., Erikson, R.L. and Simmons, D.L. (1991) Expression of a mitogen-responsive gene encoding prostaglandin synthase is regulated by mRNA splicing. Proc. Natl. Acad. Sci. (USA) 88: 2692-2696.

Yamamoto, B.K. and Davy, S. (1992) Dopaminergic modulation of glutamate release in striatum as measured by microdialysis. J. Neurochem. 58: 1736-1742.

el-Yassir, N. and Fleetwood-Walker, S.M.A. (1990) 5-HT_1-type receptor mediates the antinociceptive effect of nucleus raphe magnus stimulation in the rat. Brain Res. 523: 92-99.

Zahniser, N.R. and Dubocovich, M.L. (1983) Comparison of dopamine receptor sites labeled by [^{3}H]-S-sulpiride and [^{3}H]-spiperone in striatum. J. Pharmacol. Exp. Ther. 227: 592-599.

Zhao, Z.Q. and Duggan, A.W. (1988) Idazoxan blocks the action of noradrenaline but not spinal inhibition from electrical stimulation of the locus coeruleus and nucleus Kolliker-Fuse

of the cat. Neuroscience 25: 997-1005.

Zhu, X.Z., Ji, X.Q., Wu, S.X. and Zou, G. (1991) Sulpiride attenuates ranatensin-M-induced antinociception. Chung Kuo Yao Li Hsueh Pao 12: 291-293.

Zhuo, M. and Gehart, G.F. (1992) Characterization of descending facilitation and inhibition of spinal nociceptive transmission from the nuclei reticularis gigantocellularis and gigantocellularis pars alpha in the rat. J. Neurophysiol. 67: 1599-1614.