THE DIRECT AND MODULATORY ANTINOCICEPTIVE ACTIONS OF ENDOGENOUS AND EXOGENOUS OPIOID DELTA AGONISTS

by

TODD WILLIAM VANDERAH

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Todd W. Vanderah

entitled The Direct and Modulatory Antinociceptive Actions of Endogenous and Exogenous Opioid Delta Agonists.

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Henry Yamamura, Ph.D.	March 23, 95 Date
Frank Porreca, Ph.D.	March 23, 1995 Date
William Roeske, M.D.	March 23, 1995 Date
Hugh Laird, Ph.D.	April 11, 1995 Date
Josephine Lai, Ph.D.	March 24, 1995 Date

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Frank Porreca, Ph.D. / Henry Yamamura Ph.D.	4/17/95 / April 17, 95 Date

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Table

1. Morphine A_{50} (and 95% C.L.) following pretreatment with L365,260 or naltrindole.

ABSTRACT

Opioids are responsible for a number of effects but are employed primarily as analgesics. The discovery of endogenous opioids and multiple receptors have led to a better understanding of how analgesics function, and how both opioid receptors and endogenous ligands are regulated. The **hypothesis** of this dissertation states that levels of endogenous enkephalins are modulated by stress, inflammation and the endogenous peptide cholecystokinin (CCK) to alter the antinociceptive efficacy of μ and, possibly, δ opioids. Endogenous enkephalin release results in either direct antinociception or synergistically enhances the antinociceptive effects of the μ agonist morphine *via* δ receptors. This thesis will first detail how the administration of exogenous δ compounds can enhance the antinociceptive effects of a μ agonist.

Exposure of mice to a cold-water swim-stress (CWSS) paradigm resulted in direct antinociception that was attenuated by opioid antagonists. Exposure to CWSS for a limited amount of time did not produce significant antinociception, but produced a marked enhancement of morphine antinociception. This enhancement was blocked by the administration of δ antagonists and [Leu^5]enkephalin antisera.

An antisense oligodeoxynucleotide was designed from the δ opioid receptor and administered to animals. Animals treated with the δ antisense and

exposed to the CWSS paradigm showed a significant decrease in antinociception.

Inflamation produced in the hind-paw of the mouse significantly enhanced the antinociceptive effects of morphine that was inhibited by δ antagonists and [Leu^5]enkephalin antisera. The administration of a selective CCK_B antagonist, L365,260 or CCK_B antisense, enhances the antinociceptive potency of morphine. This enhancement is attenuated by δ antagonists, [Leu^5]enkephalin antisera, as well as exhibits a two-way cross tolerance with δ agonists. L365,260 and an "enkephalinase" inhibitor, when coadministered, produced significant antinociception that was blocked by a δ antagonist and [Leu^5]enkephalin antisera.

Using polymerase chain reaction in search of a δ opioid receptor subtype, an orphan receptor was cloned and characterized as a member of the G protein-coupled receptor family.

These data suggest that stress results in the release of [Leu^5]enkephalin that enhances the antinociceptive effects of a μ-selective agonist morphine. Release of endogenous enkephalins may be regulated by the interactions of cholecystokinin at the CCK_B receptor. Such information may lead to appropriate use of opiates in conjunction with CCK antagonists for the management of appropriate pain states.

INTRODUCTION

Many illnesses are in some way associated with the sensation of pain. Pain is primarily a protective mechanism meant to bring to conscious awareness the fact that tissue damage is occurring or is about to occur. The sensation of pain, also called nociception (Perl, 1980), is complex as there is a sensory input, as well as an emotional entity. Pain is often associated with motivated behavioral responses such as withdrawal or defense. Unlike other sensations, the subjective perception of pain can be modulated by other past or present experiences. For example, the heightened pain response accompanying fear of the dentist may intensify one's sensation of pain or an injured athlete during a competitive event may perceive less pain. Pain is also classified as either chronic or acute. Chronic pain is usually associated with untreatable conditions that often have long term tissue destruction, whereas acute pain arises from short term tissue damage (Bonica 1953). In general, pain sensations serve as warning signals of damage and play a protective role in man.

This dissertation will primarily deal with acute pain mechanisms with the exception of an inflammation study using a mycobacterium referred to as Freund's Complete Adjuvant. The introduction will serve as a review of the pain pathways, opiates both endogenous and exogenous, opioid receptors, and how opioid receptors interact in pain relief.

The undesirable feeling of pain has driven man to seek physicians and modern medicines for relief. The early pain-relieving medicines such as alcohol and barbiturates were nonspecific in function with many side effects. Today, although we have efficacious opioid analgesics, they are not without significant side effects. Continual studies about pain pathways and receptors, inflammation and chemical mediators and both intra- and extracellular functions after analgesic exposure are broadening the foundations for the development of new and better pain-relieving compounds.

Nociception and Pain Pathways

The nervous system in the periphery consists of pain receptors termed nociceptors that respond to either mechanical, thermal or chemical stimuli (Burgess and Perl, 1973; Georgopoulos, 1977; Beitel and Dubner, 1976). These nociceptors do not have specialized receptor structures and are noted as simply "naked" nerve endings. They are located in a number of tissues such as cutaneous, subcutaneous, visceral, blood vessels, muscle and joints. Nociceptors, unlike other sensory receptors, do not adapt to sustained or repetitive stimuli presumably to decrease the possibility of severe tissue damage and to assure survival. Once tissue is damaged, a cascade of events occurs which results in enhanced pain to natural stimuli, termed hyperalgesia. The corresponding increase in the responsiveness of nociceptors is termed

peripheral sensitization. These receptors can be sensitized by a number of chemical mediators, including substances such as prostaglandins. These lipid-derived compounds are released during inflammation and greatly enhance the nociceptor's response to a noxious stimuli, hence, explaining, in part, the analgesic effects of aspirin and other inhibitors of prostaglandin synthesis. Our knowledge on sensitization is limited and, is currently, an active field of research.

These different nociceptors convert noxious stimuli into electrical signals and conduct information to the central nervous system by two different but specialized sensory fibers in the periphery (Collins et al., 1960; Meyer and Campbell, 1981). Signals that arise from either thermal or mechanical nociceptors are transmitted over large myelinated Aδ-fibers at a rate of 2-20 meters/sec (Campbell et al., 1979). Impulses that arise from chemical, thermal and mechanical polymodal nociceptors are delivered by small unmyelinated C-fibers at a slower rate of 0.5-2 meters/sec (Darian-Smith et al., 1979). The Aδ-fibers have typical thermal thresholds above temperatures of 50°C, whereas the C-fiber thresholds are between 38-50°C (Meyer et al., 1994). It has been shown that only half of the cutaneous Aδ-fiber nociceptors and approximately 30% of the C-fibers are responsive to mechanical stimuli (Meyer et al., 1991; Handwerker et al., 1991).

The primary afferent cell bodies are located in the dorsal root ganglia along the spinal cord and synapse with second-order interneurons in the dorsal horn of the spinal cord. Anatomically, the gray matter of the spinal cord is separated into ten laminae (Rexed, 1954), and highly organized for functional purposes. The afferents for nociceptive transmission synapse with interneurons and ascending neurons located in laminae I-VI (Dubner and Bennett, 1983; Willis, 1985a; Woolf, 1994). Although interneurons play a large role in conducting and modulating information, including both excitatory and inhibitory type properties, and are located both pre- and postsynaptically, their local circuitry is unknown (Woolf, 1994). The electrical impulses from afferent neurons are converted to chemical impulses and include a unique neurotransmitter to pain fibers called substance P (Woolf, 1994). Synaptic transfer of information is governed by the nature and amount of neurotransmitter released, by the number and type of postsynaptic receptors, the coupling of the receptors to second messenger systems and ion channels, the kinetics involved in both receptor and channel-mediated events, autofeedback of the transmitter on the presynaptic neuron as well as factors responsible for either uptake or breakdown of the transmitter.

The axons of cells in laminae I (and the outer part of II) and IV-VI give rise to the main projection pathways from the dorsal horn to the thalamus. Although many projection neurons can be activated by afferent activity in

nociceptors, these spinothalamic tract neurons may have a primary role in initiating the sensation of pain (Mayer et al., 1975; Dubner et al., 1989; Simmone et al., 1991), although this may not be exclusive. Schaible et al. (1991) suggest that one role of projection neurons appears to be the activation of descending control systems which in turn modulate the activity of the dorsal horn neurons. Nociceptive transmission to the brain mainly involves two spinothalamic pathways. First, the neospinothalamic tract projects directly to the thalamus from lamina I without relay to other sites (Albe-Ressard et al., 1974). These neurons primarily project to the somatosensory cortex from the thalamus (Price and Dubner, 1977). The other tract, termed paleospinothalamic, projects from lamina V and terminates in a number of brain regions including the medulla, pons and midbrain, with a minimal amount of direct projections to the thalamus (Brodal, 1981). The neospinothalamic tract is responsible for processing rapid information and is involved in transmitting the information regarding the location, intensity and duration of a noxious stimuli (Melzack and Dennis, 1978). The paleospinothalamic tract includes slower conduction and processing with information eventually projecting to the limbic system and to numerous parts of the brain and may be involved in the emotional portion of pain (Melzack and Dennis, 1978). Several other ascending tracts have also been mentioned in pain-processing such as the

spino(trigemino)pontoamygdalian tract and to a lesser extent the spinohypothalamic tract.

Central processing of various components of pain include the thalamus, midbrain, forebrain and cortex (primary and secondary somatosensory areas). According to Melzack and Casey (1968), there are three components that the central nervous system must deal with: (1) a sensory-discriminitive component, (2) a motivational component and (3) a cognitive and evaluative component. It is the sensory-discriminitive and cognitive components of pain where incoming information from the periphery regarding tissue damage is compiled and associated with memory and discrimination in order to characterize the meaning of pain. The supraspinal processing of pain includes a number of nuclei. The medullary nucleus gigantocellularis has major nociceptive inputs and may be an important area in pain behavior (Casey, 1971b, 1980). The ventrobasal complex is important in associating thermal and tingling sensations (Cerevo and Morrison, 1986). Other nuclei involved in nociception include ventrocaudal parvocellular, intralamina thalamic and posterior nuclei (Tasker et al., 1983; Albe-Ressard, 1985; Willis, 1985b). Although it seems the mechanisms of peripheral and spinal nociception are investigated and understood to some extent, the mechanisms of supraspinal nociception is still a "black box" and only recently have there been studies on the supraspinal aspects of pain.

Opiates

Plants are the first, and probably best, medicinal chemists known to mankind. The plant *Papaver somniferum* produces an extract that is referred to as opium which is derived from the Greek word for "juice". The medicinal use of poppy seeds has been recorded as early as 2100 BC by the Sumerians and possibly as early as 8000 BC in Greece. Opiates include the compounds morphine, codeine and other congeners that are extracted from opium. The isolation of crude morphine was done by Louis C. Derosne in 1803-1804, but he did not realize that it was the active substance of opium. Friedrich W.A. Serturner isolated crystalline morphine in 1806 and showed it caused sleep in dogs. He gave the active compound the name morphine after the Ovid's God of Dreams, Morpheus. The pure alkaloid preparations were then preferred over the crude extracts and have served as the most effective analgesics. The American Civil War helped introduce the hypodermic needle which has also led to the unfortunate abuse and misuse of opiates. A number of morphine analogs, including meperidine and methadone, were synthesized with the hopes of their being less addictive, yet analgesic. This led to the production of opioids with mixed agonist and antagonist properties such as nalorphine. Although opiates are a major and important part in today's pharmacopeia, their liability for abuse potential, including severe physical dependence, their inhibition of gastrointestinal activity, their ability to depress respiration and to

bring about tolerance to the desired effects requires continual research and understanding of how opiates interact with mammalian physiology.

Pain and the Medicinal Use of Opiates

The management of pain is something every human has faced since the beginning of time. A number of treatments have been used over time that have not radically changed over the years. Common treatments included the application of cool water or heat, plant extracts, herbs (Tainter and Ferris, 1969), as well as more novel pharmaceutical compounds such as nonsteroidal antiinflammatory drugs (NSAIDs) and narcotics (Sunshine and Olson, 1994). The non-narcotic analgesics such as the NSAIDs have been thought to have their main pharmacologic action in the periphery where the pain originates. More recently, however, it has become clear that cyclooxygenase inhibitors can produce a pronounced antihyperalgesic action at the level of the spinal cord and this effect may be of great significance in persistent pain (Malmberg and Yaksh, 1992). The NSAIDs are distinct from the narcotic analgesics in that they do not bind to narcotic receptors. The NSAIDs have a ceiling effect in that increasing the dose beyond a certain level does not produce additional analgesic effects, yet the benefit of using these compounds is that tolerance and physical dependence has not been reported with use (Sunshine and Olson, 1994).

Although NSAIDs are in wide use today, the management of severe pain, as in the case of certain cancers, includes a wide use of narcotic analgesics.

Poppy was known and used over 5000 years ago by the Sumerians of the Middle East in the treatment of pain (Kramer, 1954). Opiates were used in the treatment of conditions such as headaches in Egypt around 1500 B.C., and arthritic pain, chest pain, cough, as well as for the induction of sedation in Europe during the Greek and Roman periods (Benedetti and Premuda, 1990). The establishment of experimental science in the seventeenth century led to the therapeutic study of opiates in the management of pain. In 1805 the German chemist, Friedrich Serturner, extracted an active alkaloid from the capsule of the *Papaver somniferum* that he termed morphium, after the Greek God of dreams, Morpheus. Eventually, morphium was abbreviated to "morphia" and "morphine" (Jaffe and Martin, 1990; Fulop-Miller, 1938). Over 25 different alkaloids have been extracted from the poppy with morphine and codeine being the most widely used. In the twentieth century, many semi-synthetic and synthetic opioid compounds have been produced in the hope of reducing side effects of the extracted alkaloids. The synthesis of opioid antagonists such as N-allylnormorphine otherwise known as naloxone, (Unna, 1943; Foldes et al., 1963; Lasagna, 1965; Blumberg et al., 1966; Jasinski et al., 1967; McClane and Martin, 1967) has provided unique therapeutic advantages in drug overdose, as well as instrumental "tools" for important opiate research.

Opioids have been described as "the mainstay of cancer pain management" (World Health Organization 1986); in many countries morphine and codeine are not available for this purpose (Stjernsward, 1991). Although many specialists agree that opioids should be used in the management of pain, there are a number of drawbacks with the use of the modern opioid analgesics such as: lack of responsiveness of the neuropathic pain to opioids, respiratory depression, gastrointestinal distress, tolerance, addiction and even route of administration (Portenoy and Coyle, 1990). Even though opiate pharmacology was introduced over 5000 years ago, our knowledge and understanding is not yet comprehensive. In the 1970's, a large step forward in the pharmacology of opiates was brought about by the discovery of opioid receptors and endogenous opioid peptides. Only recently have the central opiate receptors been cloned and identified (Evans et al., 1992; Kieffer et al., 1992) and will, no doubt, lead to further pharmacological understanding of opiate pain relief. As the goal of treatment is pain relief with a minimum of adverse side effects, a rationale is the understanding of opiate pharmacology and its clinical application.

Opioid Receptors and Endogenous Opioids

A variety of approaches have been developed in order to identify whether receptors are involved in *in vivo* physiological responses to drugs. Some of the

approaches employed are: the establishment of significant structure-activity relationships, correlation of potency for a particular effect with activity in a presumably understood bioassay, the estimation of the affinity of an antagonist for the receptor acted upon by two or more agonists (i.e., pA_2 analysis), the establishment of tolerance and the subsequent cross-tolerance between substances with known activity, and finally, the use of differential antagonism by antagonists with selectivity for a particular receptor (Porreca and Burks, 1993). Receptors for the opiates were assumed based on pharmacological evidence. Such evidence includes a structure-activity relationship of a number of compounds including antagonists (Harris, 1974). For example, countless variations of morphine have been synthesized, yet all possess the basic center and an aromatic moiety. Substitutions on the N-methyl moiety of morphine with different lengths of carbon chains lead to products of varying agonist and antagonist properties (Casey, 1971a). The concept that opioids act at receptors was supported by evidence suggesting that stereoisomers of opiate compounds may have different pharmacological activities such as levorphanol and dextrorphan (Portoghese, 1970). Naturally occurring morphine is of the levo (-) enantiomer and acts as an analgesic, yet the dextro (+) enantiomer was virtually useless at relieving pain. Additionally, detecting and analyzing compounds that differ in efficacy (partial agonists) have elucidated opiate activity via receptors as well. The partial agonists, nalorphine and nalbuphine,

produce antinociception but are not as efficacious as morphine, yet they will produce a withdrawal syndrome in morphine-dependent rhesus monkeys (Young et al., 1984). Receptor mediated activity of opiates was also suspected when selective antagonists such as naloxone were used to antagonize the antinociceptive effects of morphine (McClane and Martin, 1967).

In the localization of opiate receptors, Goldstein et al., (1971) used the stereoisomers of (-) levorphanol and (+) dextrorphan to determine binding sites in mouse brain homogenates. His group used radioactive levorphanol binding in mouse brain tissue in the presence of nonradioactive dextrorphan or in the presence of nonradioactive levorphanol. They hypothesized that the nonradioactive levorphanol would displace the radioactive levorphanol and that the inert isomer, dextrorphan, would not displace the radioactive levorphanol. Goldstein's work was unsuccessful to the extent that he found only 2% of the binding to be stereospecific, yet his work lead to the establishment of the now well-known radioligand binding technique (Goldstein et al., 1971).

Soon after the invention of radioligand binding techniques, three different groups found high specific binding of opioid receptors in neural tissue of the rat. Pert and Snyder (1973) demonstrated specific and saturable binding using [^{3}H]naloxone in rat brain homogenates. Simon et al. (1973), using the potent opioid agonist, [^{3}H]etorphine, also demonstrated high affinity binding in rat brain homogenates. Using a fraction of rat synaptosomal membranes, Terenius

(1973) is also credited for finding high affinity binding using radioactive dihydromorphine. The evidence supported involvement of pharmacological receptors for opioid binding in the central nervous system, and were correlated with a role in antinociception (Quirion et al., 1983). More importantly, was the theory that the central nervous system may also have its own endogenous opioids that interact with these receptors (Akil et al., 1976).

In the mid 1970's, opioid research was at a new peak with the discovery of endogenous opioids, as well as the discovery of multiple receptor types. In 1976, Martin and colleagues (Martin et al., 1976) used a dog model to provide evidence that three different receptor types existed. They introduced an initial classification of opiate receptors postulated on the basis of the spectrum of activity produced by several prototype drugs (Martin et al., 1976; Gilbert and Martin, 1976) and reported that morphine, ketocyclazocine, and SKF-10047 each produced different effects. Although morphine and ketocyclazocine had some similar effects, the pharmacology of SKF-10047 was quite different. It was shown, however, that effects of all the compounds were antagonized by the opioid antagonist naltrexone and that tolerance developed to the with all three compounds. Martin and colleagues proposed the existence of μ-receptors which were activated by morphine, κ-receptors acted upon by ketocyclazocine and σ-receptors activated by SKF-10047 (Martin et al., 1976). It is generally accepted that both μ and κ receptors are important in opiate pharmacology, yet

the role of σ receptors appears to be different in opiate activity. During the same period Martin and colleagues were initially classifying opiate receptors, a novel opiate receptor was suggested based on differences in rank order potency of the recently identified endogenous peptides [Met^5]- and [Leu^5]enkephalin (Hughes, 1975) in bioassay tissues (Henderson et al., 1972). The differences in potency of these peptides, when compared with opiate alkaloids in the guinea pig ileum (GPI) and mouse vas deferens (MVD) assays, lead to the suggestion of a δ-receptor. It was termed δ since it exhibited a more potent response in the vas deferens (50-fold for [Leu^5]enkephalin and 9-fold for [Met^5]enkephalin) than in the guinea-pig ileum (Lord et al., 1976,1977). In contrast, morphine, and the benzomorphan MR2034, were more potent in the ileum (7- and 21-fold, respectively). The pharmacology of the μ, κ and δ receptors has since been extensively studied by a number of groups. (e.g., Kosterlitz et al., 1981; Dray and Nunan, 1984; Pasternak and Wood, 1986; Miller et al., 1986; Heyman et al., 1986a,b,1987; Millan, 1989,1990; Mattia et al., 1991a,b).

During this same time period, several groups wondered why nature would create a plant alkaloid that binds stereospecifically to receptors on neuronal membranes and display such dramatic effects on the mammalian nervous system. There were also curious reports showing that electrical stimulation of the periaqueductal brain region induces analgesia in both humans

and animals (Reynolds, 1969; Mayer et al., 1971), and could be attenuated by the opioid antagonist, naloxone (Akil et al., 1976; Hosobuchi et al., 1977). These questions and studies led to the search for an endogenous opioid. The first reported data on the existence of endogenous opioids was by Terenius and Wahlstrom (1974) using brain and spinal extracts. In 1975, Kosterlitz and colleagues (Hughes et al., 1975) reported the first isolation and purification of two pentapeptides from porcine brain, and demonstrated opioid agonist activity in *in vitro* tissue assays, including pharmacological antagonism by naloxone. The two peptides were named "enkephalins" translated from the Greek meaning "in the head". They were identified as either [Met^5]enkephalin (Tyr-Gly-Gly-Phe-Met) or as [Leu^5]enkephalin (Tyr-Gly-Gly-Phe-Leu). Further studies have shown that the enkephalins are found spinally (Khachaturian et al., 1982), as well as systemically (Yang et al., 1980). Enkephalin activity remained following the modification of the C-terminus via amidation or addition of amino acids. The N-terminal tyrosine residue, however, appeared to be an absolute requirement, in that removal of the N-terminal positive charge either by acetylation or other means completely obliterated opioid activity (Weber et al., 1981; Liebisch et al., 1986; Seizinger et al., 1985).

Subsequently, it was reported that the C-fragment of β-lipotrophin (β-LPH) incorporated the [Met]enkephalin sequence and that an extended peptide, recedes-91 of β-LPH, had opioid activity (Li and Chung, 1976). This peptide

was isolated from the camel pituitary and was named β-endorphin from "endogenous morphine" (Li and Chung, 1976). β-LPH contained other sequences with opioid activity, β-LPH_{61-76} and β-LPH_{61-77}, which were named α- and γ-endorphin, respectively (Ling et al., 1976). Additional extended [Met^5]enkephalin peptides have also been identified in the pituitary, including [Met^5]enkephalin-Arg^6 and [Met^5]enkephalin-Arg^6-Phe^7 (Huang et al., 1979; Stern et al., 1979), as well as in the brain and adrenal medulla (Lewis et al., 1980; Kangawa et al., 1980; Kilpatrick et al., 1981). Goldstein and coworkers, in 1979, discovered a group of extended [Leu^5]enkephalin peptides from pituitary tissue. One of the peptides discovered was termed dynorphin (1-13) from the Greek word dynamis meaning "powerful", and 1-13 representing the amino acid chain length (Goldstein et al., 1979). For the next several years, several extended [Leu^5]enkephalins were discovered including α-neo-endorphin (Kangawa and Matsuo, 1979), dynorphin 1-8 (Minamino et al., 1980), and β-neo-endorphin (Minamino et al., 1981). A larger, 32 amino acid dynorphin peptide containing dynorphin 1-17 in the amino terminal was renamed dynorphin A (Fischli et al., 1982). The carboxy terminal tridecapeptide was called dynorphin B. The two peptides were cleaved at the processing signal Lys-Arg.

The three categories of endogenous peptides, enkephalins, endorphins and dynorphins, were not synthesized directly but derived from larger, inactive,

precursor polypeptides. Large 3-5KDa peptides were found in the adrenal medulla which were probable precursors for [Leu^5] and [Met^5]enkephalin (Kimura et al., 1980). Noda et al. (1982) first sequenced the pre-cursor to [Met^5]- and [Leu^5]enkephalin, termed pre-proenkephalin, from bovine adrenal medulla. Within the pre-proenkephalin peptide were the shorter peptides [Met^5] and [Leu^5]enkephalin, [Met^5]enkephalin-Arg^6-Gly^7-Leu^8 and [Met^5]enkephalin-Arg^6-Phe^7. The pre-proenkephalin peptide contained six copies of [Met^5]enkephalin, yet only one copy of [Leu^5]enkephalin, confirming the observations by Hughes et al. (1975) that 3 to 4 times greater quantity of [Met^5]enkephalin was isolated versus [Leu^5]enkephalin. A large 31KDa precursor peptide was isolated from the pituitary gland and named proopiomelanocortin (POMC)(Mains et al., 1977) since it contained corticotropin (ACTH), β-endorphin, β-LPH and melanocyte stimulating peptides (Rubenstein et al., 1978; Chretien et al., 1979). Finally, the discovery of the dynorphin precursor was reported in 1982 by Kakidani and coworkers using a cloning technique from porcine brain cDNA. The dynorphin precursor also contains three copies for [Leu^5]enkephalin, α- and β-neoendorphin, but does not contain a copy of [Met^5]enkephalin and was named pre-proenkephalin B (Kakidani et al., 1982). However, Rossier (1982) redefined the precursor peptide of dynorphin as pre-prodynorphin. It should also be noted that a copy of [Met^5]enkephalin was later identified in the POMC precursor peptide and it

was found that further processing of β-endorphin may lead to increased levels of [Met^5]enkephalin. On the other hand, Rossier et al. (1977) reported that the processing of β-endorphin to [Met^5]enkephalin was not the major source of [Met^5]enkephalin since the concentration of β-endorphin in the brain is only 5-10% of the concentrations of enkephalins. Watson et al. (1978) reported that the distribution of enkephalin immunoreactive material was distinctly different from that of β-endorphin. Several groups have done *in situ* experiments describing the anatomical distribution of the three major classes of endogenous opioids (i.e., endorphins, enkephalins and dynorphins), and their probable involvement with antinociceptive activity (Finley et al., 1981; Watson et al., 1982; Cruz and Basbaum, 1985).

Endogenous Opioids and Stress

Endogenous opioid systems are widely represented in regions which are involved in the stress response, e.g., hypothalamus, pituitary and adrenals. Similarly, autonomic nervous system centers have been shown to be innervated by central and peripheral opioidergic neurons. Although the endogenous opioids are not thought to be tonically active, they are activated by stressful stimuli, thus allowing them to influence certain physiological effects of such stimuli (Przewlocki, 1993). The potential involvement of endogenous opioids in stress-induced antinociception has been widely reported and is a complex

phenomenon whose characteristics are highly dependent on species (e.g., mouse or rat) and strain of animal (Moskowitz et al., 1985; Marek et al., 1987). In particular, the nature of the stress stimulus (Terman et al., 1986) is critical to the type of antinociceptive response that is observed (see Watkins and Mayer, 1982 and Terman et al., 1984 for reviews). Many studies have demonstrated both opioid and non-opioid forms of antinociception (e.g., Lewis et al., 1980; Terman et al., 1983; Tierney et al., 1991). Of the many forms of stress-induced antinociception, swim-stress in mice and rats has been consistently shown to produce opioid-induced effects (e.g., Cooper and Carmody, 1982; Carmody and Cooper, 1987; Willow et al., 1980). these effects appear to be mediated via opioid δ receptors (Jackson et al., 1989; Kitchen and Pinker, 1990) since antinociception is antagonized by the δ selective antagonist naltrindole. Footshock stress (McGivern et al., 1983) and social isolation (Raab et al., 1985) have been shown to decrease the hypothalamic content of the proenkephlin peptides. This decrease, presumably, results from an increase in peptide release. Stressful conditions have also been shown to enhance proenkephalin gene expression in cells localized in the parvocellular part of the hypothalamus (Harbuz and Lightman, 1989; Lightman and Young, 1989). This work was complemented by the discovery of an increase in the biosynthetic activity of the hypothalamic proenkephalin system in stressed rats (Hong et al., 1985). Various manipulations which affect the

function of the adrenals, e.g., adrenalectomy, demedullation, or medullary denervation, diminish stress-induced antinociception (Lewis et al., 1980a), demonstrating the role of peripheral enkephalins in stress. Both a forced swim (Christie et al., 1981) and conditioned fear-induced stress (Chance et al., 1978; Sumova and Jakoubek, 1989) resulted in a decrease in [^{3}H]leu-enkephalin binding in rat brain membranes. Swim-stress has been shown to significantly decrease [^{3}H]diprenorphine binding in the hypothalamus and other brain structures, as measured by autoradiography (Seeger et al., 1984). Thus, acute stress appears to decrease opioid receptor binding theoretically due to an enhanced release of endogenous opioids. Chronic, recurrent stress has been shown to cause down-regulation of both δ- and μ-receptors in some structures of the rat brain (Nakata et al., 1985).

Cloning and Molecular Mechanisms of Opioid Receptors

Although extensive pharmacologic studies have been done to characterize both the endogenous peptides and opioid receptors, our knowledge of the structural and molecular entities of the opioid receptors has been extremely limited. This, in turn, has arrested the development of new agonists and antagonists. The recent cloning of the δ (1992), μ (1993) and κ (1993) opioid receptors will facilitate molecular studies, agonist/antagonist interactions and lead to a better understanding of their functional properties.

Cloning of the δ opioid receptor from a NG108-15 mouse neuroblastoma cell line cDNA was reported by two different groups (Evans et al., 1992; Kieffer et al., 1992). Both groups used similar expression cloning techniques and a cell line that expresses a high density of δ opioid receptors. The cDNA from the cells was transfected into monkey kidney (COS) cells, with subsequent screening of the cells with either [^{125}I]-labeled [D-Ala^2, D-Leu^5]enkephalin (DADLE) (Evans et al., 1992) or ^{3}H-labeled Tyr-D-Thr-Gly-Phe-Leu-Thr (DTLET) (Kieffer et al.,1992), both agonists for the δ opioid receptor. The cloned mouse δ opioid receptor was found to be a 372 amino acid protein whose sequence was most closely related to that of the somatostatin receptor, among members of the G-protein coupled family of receptors. Soon thereafter, Yasuda et al., (1993) reported the isolation of a mouse δ opioid receptor clone from a brain cDNA library using the polymerase chain reaction (PCR) method and degenerate primers. Fukuda et al., (1993) also reported the cloning of a rat δ opioid receptor from brain cDNA using PCR. There was 97% similarity between the protein sequences of the rat and mouse receptors. In 1994, Yamamura and coworkers published the cloning of a human δ opioid receptor from two different cDNA libraries (Knapp et al., 1994). Portions of the human clone were found in either a human striatal cDNA (3'end) or a human temporal cotex cDNA (5' end) using a hybridization screening method (Knapp et al., 1994). The two pieces of cDNA contained a large identical overlap area where the two

were ligated together and expressed in COS-7 cells. The human clone amino acid sequence was 93% homologous to the cloned mouse and rat receptors (Knapp et al., 1994). Simonin et al. (1994) also reported the cloning of a human δ opioid receptor using PCR from a human neuroblastoma cell line and showed that the gene for the human δ opioid receptor contains two introns located after transmembrane domains 1 and 4. The human clone was 94% homologous to the mouse clone. Seven transmembrane segments can be predicted in all of the δ receptors cloned thus far, a characteristic structural feature of the G-protein coupled receptor family.

The cloned δ receptors in all cases, fulfill the properties described of those endogenous opioid receptors in the nervous system. They have higher affinity for [Met^5]- and [Leu^5]enkephalin than for the dynorphins (Yasuda et al., 1993). Many delta-selective ligands were used such as [D-Pen^2, D-Pen^5]enkephalin (DPDPE), [D-Ala^2, Glu^4]dletorphin, [D-Ser^2, Leu^5, Thr^6]enkephalin (DSLET), naltrindole, naltriben (NTB) and BNTX bound to the different δ cloned receptors with high affinity whereas κ and μ selective agonists did not (Evans et al., 1992; Kieffer et al., 1992, 1994; Yasuda et al., 1993; Fukuda et al., 1993; Knapp et al., 1994). Knapp et al., (1994) have also suggested that the cloned δ receptor is a δ_2 subtype since the more δ_2 selective ligand, NTB bound the human clone with an 8-fold higher affinity than the more δ_1 selective ligand BNTX. Further evidence to suggest that δ

subtypes exist were reported by Reisine et al. (1993). He showed that the pharmacological studies of the expressed δ receptor cDNA correlate unevenly with parallel studies of the δ receptor expressed in brain membranes. These results differ from the good correlations observed in studies comparing the cloned and endogenous μ and κ receptors.

The cloned δ opioid receptor has been reported as being coupled to pertussis toxin-sensitive G-proteins and to mediate agonist inhibition of cAMP formation, indicating that the receptor is functionally coupled to adenylyl cyclase. Forskolin stimulation of cAMP was inhibited in δ opioid transfected cells when δ selective agonists were presented to the cells. The effects were reversed by naloxone (Evans et al., 1992; Yasuda et al., 1993; Tamir and Kushner, 1993; Simonin et al., 1994; Yamamura personal communication, 1994). Delta receptor mRNAs of 8.5-9 kb (Kieffer et al., 1992; Evans et al., 1992) and 12 kb (Evans et al., 1992) manifest high expression in the mouse striatum. Kieffer and colleagues (Simonin et al. 1994) demonstrated transcript for the receptor in cortical areas, including the olfactory bulb, hippocampus, and amygdala, as well as in the basal ganglia and hypothalamus by using a Southern hybridization technique with the human δ opioid receptor as a probe. The mouse δ opioid receptor has been mapped to the distal region of chromosome 4 (Bzdega et al., 1993). Further work with the cloned δ opioid receptor has shown that the gene for this receptor is located on chromosome

1p34 (Uhl et al., 1994). Anti-δ receptor antisera localized δ receptors in the superficial dorsal horn synaptic terminals of the dorsal root ganglion cells (Dado et al., 1993). Elde and coworkers noted that terminals containing the δ opioid receptor appeared to be closely apposed to fibers and terminals containing enkephalin peptide (Dado et al., 1993).

Molecular characterization of the receptors has been done in order to determine what part of the receptor is important in binding agonists and antagonists for the δ opioid receptor. The construction of chimeric open reading frames (ORF) was done with the rat δ and μ clones in order to determine which portion of the receptor is important in ligand binding (Onogi et al., 1995). Replacement of the region around the first extracellular loop of δ receptor with the corresponding region of the μ receptor gave the resultant chimeric receptor a similar affinity to the μ agonist DAMGO as compared with the native μ receptor (Onogi et al., 1995). They also demonstrated that the reciprocal chimera deprived the mainly μ receptor of DAMGO binding. Interestingly, similar results were seen with other peptidic compounds, but not with non-peptidic ligands such as morphine and naloxone (Onogi et al., 1995). Site-directed mutagenesis studies have identified that the aspartate at residue 128 is necessary for the binding of many selective and non-selective agonists for the δ opioid receptor, but not for antagonist binding (Livingston et al., 1994). The decrease in agonist binding in the mutant receptor was not due to

an uncoupling of the receptor from G proteins since the mutant efficiently mediated the inhibition of forskolin-stimulated cAMP accumulation induced by opioid agonists (Livingston et al., 1994). Antisera for the N-terminal portion (1-16 aa) of the δ opioid receptor have been raised and administered, *i.c.v.* to mice (Garzon et al., 1994). Animals administered the antisera had an impaired antinociceptive response to several δ selective opioid agonisst but not to μ agonists, implying that the N-terminal portion of the receptor is important in relaying the antinociceptive effects of opiates. They also reported a decrease in DPDPE binding in antisera treated animals, but no change in DAMGO binding (Garzon et al., 1994).

A fairly recent technique being employed in many laboratories today (Mirabelli et al., 1991; Wahlestedt et al., 1993a,b; Zhou et al., 1994), termed gene "knockdown", has been used in determining the physiologic function of the cloned δ opioid receptor. A 20 base pair antisense oligodeoxynucleotide (oligo) designed from the N-terminal of the mouse cloned δ opioid receptor when centrally administered to mice twice a day for three days significantly inhibited the antinociceptive effects of a δ_2 agonist [D-Ala^2, Glu^4]deltorphin, but not the δ_1 agonist [D-Pen^2, D-Pen^5]enkephalin, the μ agonist DAMGO, or the κ agonist U69,593 in the tail-flick assay (Bilsky et al., 1994; Lai et al., 1994a). A 20 base pair mismatch oligo administered to mice in the same fashion as the antisense had no effect on the antinociception of δ, μ, or κ agonists. Notably,

when the δ antisense was administered spinally, the antinociception of both δ agonists [D-Ala^2, Glu^4]deltorphin and [D-Pen^2, D-Pen^5]enkephalin were significantly reduced, yet there was no change with DAMGO, U69,593 (Bilsky et al., 1994) or U50,488 (Tseng et al., 1994). Delta antisense central administration twice a day from three to six days significantly reduced [^{3}H]naltrindole, but not [^{3}H]DAMGO or [^{3}H]U69,593 binding in mouse whole brain (Bilsky, personal communication, 1994). Delta receptor binding, as well as analgesia, gradually returned three to five days after the last treatment of the antisense, indicating no irreversible damage or toxicity (Bilsky et al., 1994; Standifer et al., 1994). Delta mismatch central administration had no effect on radioligand binding of the three different ligands. Pasternak and colleagues (1994) have also reported a 40%-50% decrease in δ receptor binding in δ antisense treated NG108-15 cells, but no change in δ mismatch treated cells. The cloning of the δ receptor led to the discovery of other structurally similar receptors which did not bind opioids or demonstrate any characteristic function leading to their classification as structurally related orphan receptors.

Yasuda et al. (1993) reported the cloning and functional characterization of a mouse brain κ opioid receptor cDNA clone. The cloned receptor is a 380 amino acid protein with 61% homology to the mouse δ opioid receptor. The majority of the homology was found in the transmembrane spanning regions with the amino- and carboxyl-termini showing substantial differences in both

sequence and length (Yasuda et al., 1993). A rat brain κ opioid receptor from the striatum was cloned using a low-stringency screening technique (Meng et al., 1993). The cloned κ receptors have high affinity binding to selective κ agonists such as U69,593, U50,488, dynorphin and ethylketocyclazocine, yet exhibited low affinity binding to δ agonists such as DPDPE, DADL and the enkephalins. The κ receptor selective antagonist nor-BNI, as well as the general antagonist (-)-naloxone, but not (+)-naloxone, bound potently to the cloned κ receptor (Yasuda et al., 1993; Meng et al., 1993). The cloned κ receptors associate with pertussis toxin-sensitive G-proteins. Selective κ agonists were shown to inhibit forskolin-stimulated cAMP accumulation in transfected COS-1 cells with the effects completely reversed by naloxone (Yasuda et al., 1993; Meng et al., 1993). Some unpublished findings by Reisine and colleagues have demonstrated that repeated exposure of the κ opioid receptor to U50,488 (κ agonist) resulted in the uncoupling of the receptor from G-proteins and adenylyl cyclase. This desensitization involved the enzyme β-adrenergic receptor kinase (BARK) based on some coexpression of the κ receptor and a BARK dominant-negative mutant.

The cloning of the rat and the guinea pig κ opioid receptors were identified with approximately 90% homology to the mouse clone with similar pharmacologic techniques as employed above, (Chen et al., 1993b; Nishi et al., 1993; Minami et al., 1993; Xie et al., 1994). The human κ opioid receptor was

reported by two different groups with similar pharmacological findings as mentioned with the mouse and rat clones with two introns (Mansson et al., 1994; Zhu et al., 1995). The deduced amino acid sequences of the human clones were approximately 93.9% and 93.2% identical to mouse and rat κ receptors, respectively (Zhu et al., 1995). Also reported was that the human κ receptor was approximately 60% homologous to the human δ and μ receptors (Zhu et al., 1995). In all cases, the κ cloned receptors exhibited a κ_1 receptor binding profile. Lai et al. (1994b) have reported that the cloned mouse κ receptor demonstrated high affinity binding with bremazocine and α-neoendorphin, compounds for the κ_{1b} subtype, and low affinity for fedotozine and oxymorphindole, compounds for the κ_{1a} subtype. CI-977 and U69,593 bind equally well at both sites (Lai et al., 1994b) leading to the conclusion that the cloned κ receptor may be of the κ_{1b} subtype.

A Northern blot identified only one mRNA transcript of 6.0 kb for the human κ receptor (Zhu et al., 1995). High levels of mRNA for the κ receptor were found in the amygdala, caudate nucleus, hypothalamus and subthalamic nucleus (Zhu et al., 1995). Chimeric studies in which the second extracellular loop of the human μ receptor was replaced with the human κ receptor resulted in high affinity binding of several κ selective ligands such as dynorphin (1-17) and (1-13) (Wang et al., 1994c). The chimera retained its high affinity binding for μ receptor ligands such as morphine and DAMGO (Wang et al., 1994c).

Reisine and colleagues (1994) have also done some extensive chimeric studies using the mouse cloned δ and κ receptors. They have reported that the amino-terminus of the κ receptor is important in receptor antagonist binding, yet the amino-terminus of the κ receptor was not important in κ agonist binding (Kong et al., 1994). Xue et al. (1994) have constructed six chimeric receptors using the cloned rat κ and μ receptors and expressed them in COS cells. All of the six chimeras bound the nonselective opioid antagonist diprenorphine with high affinity, hence retaining their opioid activity. The second extracellular loop and the adjoining C-terminal portion of the fourth transmembrane (TM) helix were essential for high affinity binding peptidic (dynorphin A, dynorphin B and α-neo-endorphin) ligands of the κ receptor (Xue et al., 1994). The third extracellular loop and the sixth and seventh TM helices played an important role in determining the selectivity of the nonpeptidic κ antagonist nor-BNI. U50,488H and U69,593, non peptidic κ agonists, require the entire κ receptor except for the second extracellular loop (Xue et al., 1994). Therefore, these studies have shown that the the cloned κ receptor is of the κ_{1b} subtype and that the second extracellular loop is important in κ agonist binding with the N-terminal important in antagonist binding.

The rat μ opioid receptor was cloned from a rat brain cDNA library. Using the polymerase chain reaction technique, a probe from the rat δ opioid receptor was generated and used in a low stringency hybridization screening

method in order to find the rat μ receptor (Chen et al., 1993a). This clone contains an open reading frame of 1194 base pairs , with a deduced polypeptide sequence of 398 amino acid residues. The rat μ amino acid sequence showed 64% and 60% identity with the sequences of the mouse δ and κ receptors, respectively (Chen et al., 1993a). All three opioid receptor types exhibit a seven transmembrane domain structure based on the hyrophobic/hydrophilic characteristics of the amino acids. Comparison of the amino acid sequences of the three cloned rat opioid receptors shows that the sequences of membrane spanning segments 2, 3 and 7 are highly conserved, as well as the sequences of the intracellular loops, especially the second intracellular loop (Reisine and Bell, 1993). The high degree of sequence similarity in the intracellular loops may suggest that they interact with similar G-proteins since these intracellular regions have been identified in other G-protein coupled receptors as the site of coupling interaction (Reisine and Bell, 1993). The amino and carboxyl terminal differ among all three receptors , as well as the second and third intracellular loops. These areas may constitute the site of ligand-binding for the opioid receptors. Also noted by Reisine and Bell (1993) is that all three receptor types contain consensus sequences for asparagine (N)-linked glycosylation on the extracellular domains. The δ and the κ receptors have two sites for glycosylation whereas the μ receptor has four potential glycosylation sites.

Cloning of the μ opioid receptor from rat brain cDNA has also been reported by two other groups with the same sequence as reported by Chen et al. 1993a; Wang et al., 1993; Fukuda et al., 1993. These three groups have shown high affinity binding in μ receptor transfected COS cells (Wang et al., 1993; Chen et al., 1993a), as well as in Chinese hamster ovary cells (Fukuda et al., 1993) with selective μ agonists such as DAMGO, morphine and β-FNA. Selective δ compounds such as DPDPE and DSLET, as well as κ selective compounds U50,488 and bremazocine, were found to bind with low affinity. They also demonstrated that general opioid compounds like dipernorphine and (-)-naloxone also bound with high affinity to the cloned μ receptor. Chen et al. (1993a) also reported that forskolin-stimulated accumulation of cAMP in μ transfected COS cells was inhibited selectively by the μ agonist DAMGO and that this effect was reversed by naloxonazine. These results suggest that the μ opioid receptor, like the other opioid receptors, are functionally coupled to the inhibition of adenylyl cyclase. A human μ opiate receptor has been identified from a cerebral cortical cDNA library using partial sequences from the rat μ opiate receptor (Wang et al., 1994b). The partial μ sequences were used as a probe and hybridization screening was performed on the human cDNA library. The clone identified by Wang et al. (1994b) displayed an overall nuclotide identity of 87% with the rat cDNA coding regions. The open reading frame of this clone displayed 95% homology identity to amino acids encoded by the rat

μ opioid receptor, 59% amino acid and 62% amino acid identity with the κ and δ receptors, respectively (Wang et al., 1994b). Two introns were reported in this clone with one located in the second extracellular loop and another located in the C-terminus of the protein (Wang et al., 1994b). The human clone maintained the same binding characteristics as the rat clones with high affinity binding of DAMGO, morphine and another selective μ ligand CTOP. Damgo and morphine also inhibited forskolin-stimulated cAMP accumulation in human μ opioid receptor transfected COS cells that was reversed by naloxone. The human μ opioid receptor has been localized to the 6q24-25 human chromosome (Wang et al., 1994b). Further studies with the rat μ opioid receptor have identified several brain regions that may contain μ receptors.

Zastawny et al. (1994) using *in situ* hybridization have demonstrated that μ receptor mRNA was expressed in the cerebral cortex, caudate-putamen, nucleus accumbens, olfactory tubercle, septal nuclei, thalamus, hippocampus and medial habenular nucleus. Co-localization of μ opioid receptor mRNA and μ receptor protein was done by Mansour et al. (1994). They demonstrated that both the mRNA and the protein for μ receptors were found in the nucleus of the accessory olfactory bulb, anterior olfactory bulb, striatal patches of the nucleus accumbens and caudate-putamen, endopiriform nucleus, claustrum, diagonal band of Broca, globus pallidus, ventral pallidum, bed nucleus of stria terminalis, most thalamic nucleo medial and posteriocortical medial amygdala, lateral,

dorsomedial, posterior and mammillary nuclei of the hypothalamus, parabrachial nucleus, locus coeruleus, central gray, nucleus ambiguus and several other nuclei of the mid-brain region (Mansour et al., 1994). The high expression of mRNA for the μ receptor in the thalamic structures is found where μ agonists are thought to modulate pain transmission in the brain.

In order to study the sites of the μ receptor that are important in ligand binding and receptor coupling to proteins, several mutagenesis studies have been performed. Deletion of 64 N-terminal amino acids of the μ receptor produced very little effect on receptor-agonist interaction or receptor-G-protein coupling (Wang et al., 1993). Deletion of 33 C-terminal amino acids yielded a receptor at which morphine, but not DAMGO, inhibited adenylate cyclase (Surratt et al., 1994). Alanine substitution for each charged amino acid residue in the transmembrane (TM) regions of the N-terminally deleted receptor reduced affinities for DAMGO, morphine and the opiate antagonist naloxone. Replacement of the TM Asp^{114} with asparagine or glutamic acid increased the affinity for the antagonist naloxone. TMII and TMIII glutamic acid substitutions for Asp^{114} and Asp^{147} reduced agonist binding affinities but allowed for full inhibition of adenylate cyclase activity at high agonist concentrations (Surratt et al., 1994). TM VI histidine substitution with alanine yielded a receptor that produced almost twice the cyclase inhibition displaced by the wild type receptor in parallel transient expression assays (Surratt et al., 1994). These

studies have emphasized the importance of charged residues in TM domains and C- and N-terminals in the function of agonist and second messenger coupling of the μ opioid receptor.

Using an antisense strategy, Rossi and coworkers (1994) have designed and centrally administered an antisense or mismatch for the 5'-untranslated region of the mouse cloned μ opioid receptor. Animals receiving the μ antisense on days 1, 3, and 5 were administered morphine and tested in the tail-flick assay 24 hours after the last injection. They demonstrated that morphine antinociception was completely blocked in antisense treated animals, but mismatch or vehicle treated animals were not significantly affected (Rossi et al., 1994).

Continuous cloning studies have also identified a receptor that, in the rat, is 367 amino acids in length and has approximately 50-60% identity to the cloned rat κ, μ and δ receptors. This clone, to date, does not actively bind any known ligand with high affinity, earning its name "orphan" (Wang et al., 1994a; Fukuda et al., 1994; Chen et al., 1994). An orphan receptor was cloned from mouse brain cDNA and shown by *in situ* hybridization to express mRNA in the central nervous system of the mouse with high expression in the limbic system (Saeki et al., 1993). Mollereau et al. (1994) has cloned the human correlation of the orphan receptor showing 49-50% homology to the cloned opiate receptors. The mouse and human orphan receptor clones are

approximately 80% homologous in the TM domain regions with the N- and C-terminals varying in sequence and length with the other cloned opioid receptors (Vanderah and Yamamura, unpublished observations, 1993). Hence, the cloning of this orphan receptor in several species and its expression in the central nervous system displays the need to identify the endogenous agonist(s) for the receptor.

Delta Opioid Receptors and Their Relationship to Pain

Several pharmacological studies on δ opioid receptors *in vivo* have been reported using the endogenous enkephalins (Hughes et al., 1975; Cowan et al., 1976). The endogenous peptidic opioids were difficult to work with since they were broken down rapidly and lacked good antinociceptive efficacy and selectivity for the δ opioid receptor (Hughes et al., 1975; Cowan et al., 1976; Lord et al., 1977). Several of these problems were reduced or eliminated by the synthesis of new enkephalin peptides that contained D-amino acids, including [D-Ala^2]-met-enkephalin (DAME) (Pert et al., 1976) and [D-Ala^2, Leu^5]enkephalin (DADLE)(Miller et al., 1977). Although these D-amino acid containing peptides were more stable than the enkephalins and more selective for the δ receptors, they still exhibited less than 10-fold selectivity for the δ receptor over the μ receptor (Miller et al., 1977).

The δ selectivity of compounds was based on different tissues containing pre-dominantly one type of opioid receptor or radiolabeled ligands that were found to be selective for a particular receptor. The bioassays for receptor specificity have classically been the mouse vas deferens for δ receptors, and the guinea-pig ileum for μ receptors. The first examples of δ opioid receptor compounds that were both stable and highly selective for the δ opioid receptor were the constrained cyclic penicillamine containing enkephalin analogues [D-Pen^2, D-Pen^5]enkephalin (DPDPE), [D-Pen^2, L-Pen^5]enkephalin (DPLPE) and [D-Pen^2, L-Cys^5]enkephalin (DPLCE) (Mosberg et al., 1983). These compounds were shown to have up to 300-fold selectivity for the δ opioid receptor. Increasing the ring size of these cyclic compounds decreased the δ-affinity while the μ-affinity remains the same. This finding led to the synthesis of more μ selective compounds (Mosberg et al., 1988), suggesting that the compact structure of DPDPE does play a role in δ selectivity. Although DPDPE is stable and selective for the δ opioid receptor, it has a low affinity for the receptor (Mosberg et al., 1988). Investigation into a more potent compound led to the discovery of a para-chloro group at the Phe^4 postion, (pCl-DPDPE), an amino acid shown to be very important in δ receptor selectivity (Toth et al., 1990), is approximately 7-times more potent in the mouse vas deferens when compared to nonchlorinated DPDPE. In isolated tissue studies it shows 5400-fold selectivity and 574-fold selectivity in binding assays for the δ receptor

(Toth et al., 1990). Modifications of Tyr^1 of the DPDPE ligand lead to a decrease in δ receptor affinity (Toth et al., 1992), and addition of the unnatural amino acid 2, 6-dimethyltyrosine (DMT) resulted in a 10-fold increase in affinity at the δ-site accompanied by a 35-fold increase in potency at the μ-site (Hansen et al., 1992). Two [Leu^5]enkephalin analogues, [D-Ser^2, Leu^5]enkephalyl-Thr^6 (DSLET) (Gacel et al., 1980) and [D-Thr^2, Leu^5]enkephalyl-Thr^6 (DTLET) (Zajac et al., 1983), demonstrated a 3000-fold selectivity in the isolated tissue studies and 179-fold in the binding assays for the δ receptor. These two compounds were not very promising since their breakdown by peptidases occured rapidly.

The first δ selective antagonist, ICI 154,129, is a peptide based on the sequence of [Leu^5]enkephalin in which structural changes were introduced to improve the metabolic stability of the peptide (Shaw et al., 1982). The ICI antagonist was 30-fold more selective for the δ sites than for μ sites in isolated tissue studies, with a relatively poor affinity overall (Shaw et al., 1982). A second peptide antagonist, ICI 174,864, was found to have good affinity with the δ receptor (K_e 30 nM) and coupled with a greater than 150-fold selectivity for the δ over the μ opioid receptor (Cotton et al., 1984). A selective nonpeptidic δ antagonist, naltrindole (NTI), was synthesized by Portoghese et al. in 1988. NTI is the indole analogue of the nonselective opioid antagonist naltrexone. NTI demonstrates high affinity for the δ receptor and over 100-fold

selectivity for the δ receptor in the isolated tissue and binding assays (Portoghese et al., 1988; Rogers et al., 1990).

In 1989 two groups simultaneously published evidence for a new highly selective δ peptide isolated from the skin of *Phyllomedusa sauvagei* (a South African frog) and termed dermenkephalin (Mor et al., 1989) or the more widely accepted name, deltorphin (Kreil et al., 1989). Deltorphin, with a structure of Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2, was the most potent δ agonist thus far reported with an IC_{50} in the mouse vas deferens of less than 1nM, and a μ/δ ratio of close to 3000 (Kreil et al., 1989). Subsequently, two more deltorphin peptides were isolated from frog skin which contained a D-Ala residue at position 2 (Erspamer et al., 1989). The sequence of this peptide was Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2 and referred to as deltorphin I. The second deltorphin peptide isolated by Erspamer et al. (1989) only differed by a Glu in position 4, and is referred to as deltorphin II. Isolation of the cDNA's encoding for deltorphin I and deltorphin II indicated that the D-Ala residue was coded for by a normal GCG codon for L-Ala (Richter et al., 1990), suggesting that the insertion of the D-Ala in position 2 may be a post-translational event. The D-amino acid in the deltorphin peptides render them partially resistant to degradation by peptidases in the plasma and brain membranes of rats (Marastoni et al., 1991). The deltorphin II peptide was shown to have high affinity for the δ opioid receptor (K_i = 2.4 nM), but low affinities for the μ (K_i

= 1600 nM) and κ (K_i = >25,000 nM) opioid receptors (Kreil et al., 1989). Deltorphin II and DPDPE have been shown to elicit antinociception in rats and mice at both central sites (Porreca et al., 1984, 1987a,b; Galligan et al., 1984, Jensen and Yaksh, 1986; Heyman et al., 1987, 1988; Takemori and Portoghese, 1987; Jiang et al., 1990b,c, 1991; Mattia et al., 1991a,b; Vanderah et al., 1992, 1994), and at spinal sites (Tung and Yaksh, 1982; Porreca et al., 1984, 1987a,b; Heyman et al., 1987; Drower et al., 1991; Mattia et al., 1992).

Heyman et al. (1987) demonstrated that δ opioid receptors, both centrally and spinally, were responsible for antinociception and not μ receptors. They showed that the δ selective antagonist, ICI 174,864, blocked the antinociceptive effects of DPDPE, but not the antinociceptive effects of the μ selective agonists [D-Ala^2, N-Me-Phe^4, Gly-ol]enkephalin (DAMGO)(Handa et al., 1981) and morphine. Yet, pretreatment with the selective μ antagonist, β-funaltrexamine (β-FNA)(Ward et al., 1982; Ward and Takemori, 1982), blocked the antinociceptive effects of DAMGO and morphine, but not that of DPDPE. Studies with deltorphin II have shown that this peptide produces antinociception when administered centrally in mice for over 40 min, and is selectively antagonized by ICI 174,864, but not by μ or κ selective antagonists (Heyman et al., 1987; Jiang et al., 1990b). Further evidence for δ receptor involvement in antinociception was shown by microinjection of δ agonists into

the medullary reticular formation producing significant analgesia, whereas μ agonists were inactive (Jensen and Yaksh, 1986; Ossipov et al., 1995). These studies have demonstrated the importance of δ receptor antinociception, and, with further development of δ selective compounds, may lead to clinically administered δ opioids.

Subsequently, a peptidic antagonist was developed that exhibited selectivity for the δ opioid receptor. The peptide [D-Ala^2, Leu^5, Cys^6]enkephalin (DALCE) has been reported to covalently bind to δ receptors by a thiol-disulfide exchange mechanism (Bowen et al., 1987). DALCE posses a pharmacological profile *in vivo* that is consistent with a non-equilibrium δ receptor antagonist after short term agonist effects (Calcagnetti et al., 1989). Jiang et al. (1990a) demonstrated an entire dose response antinociceptive antagonism of DPDPE in animals pretreated with central administration of DALCE. The first non-peptidic, non-equilibrium δ antagonist which showed selectivity in *in vivo* studies was the isothiocyanate derivative of NTI (5'NTII) (Portoghese et al., 1990). The peak of effect in *in vivo* testing was 24 hours after central administration, theoretically after some type of covalent binding occurs. The non-peptidic antagonist 5'NTII has been shown to selectively antagonize the antinociceptive effects of the δ agonist [D-Ala^2, Glu^4]deltorphin, but not those of DPDPE (Vanderah et al., 1993), or μ and κ agonists (Vanderah and Porreca, unpublished observations). Recently, new δ selective antagonists have been

developed, including analogues of NTI with greater potency and selectivity (Takemori et al., 1990; Larson et al., 1990). These include the benzofuran analogue of NTI, naltriben (NTB) and the analogue of naltrexone, 7-benzylidenenaltrexone. NTB demonatrates good affinity for the δ receptor (K_e = 0.27nM) and shows 100-fold selectivity for δ- over μ-sites, and 178-fold selectivity over κ-sites in isolated tissue assays (Portoghese et al., 1991; Sofuoglu et al., 1991b). BNTX was also selective for the δ receptor and potent in antagonizing the antinociceptive effects of DPDPE, but not those of DSLET, morphine and U69,593 (Potoghese et al., 1992; Sofuoglu et al., 1993).

Major advances in δ selective opioid receptor analgesics was brought about by the synthesis of a nonpeptidic δ agonist, BW 373U86. This compound is more stable than the typical peptides and demonstrated high selectivity for the δ receptor in both the isolated tissue and binding assays (Lee et al., 1993; Wild et al., 1993). Multiple derivatives of the BW compound have been synthesized and are characteristically selective for the δ opioid receptor. One particular compound, SNC 80, has been extensively characterized and found to be highly selective for the δ opioid receptor in both isolated tissue and binding assays, as well as antinociceptive at the δ opioid receptor (Calderon et al., 1994; Bilsky and Porreca, 1995).

Evidence for Delta Opioid Receptor Subtypes

Significant pharmacological evidence has shown that subtypes of opioid δ receptors exist (Porreca et al., 1992; Jiang et al., 1991; Mattia et al., 1991a; Rothman et al., 1988; Vaught et al., 1982). Erspamer et al. (1989) demonstrated that [D-Ala^2, Glu^4]deltorphin, had approximately 13-fold greater affinity for the δ opioid receptor than the other selective δ agonist [D-Pen^2, D-Pen^5]enkephalin (DPDPE). [D-Ala^2, Glu^4]deltorphin exhibits higher selectivity in the δ receptor concentrated tissue, the mouse vas deferens, and very low selectivity in the μ site concentrated tissue, the guinea-pig ileum (Gyang and Kosterlitz, 1966; Henderson et al., 1972). [D-Ala^2, Glu^4]deltorphin, given by the intracerebroventricular (*i.c.v.*) route produced analgesia in less than 5 min after administration which persisted for approximately 35 to 40 min (Jiang et al., 1990b). Both [D-Ala^2, Glu^4]deltorphin's and DPDPE's analgesic effects are antagonized by the general opioid antagonist naloxone (Jiang and Vanderah, unpublished results 1991). The δ antagonist, ICI 174,864, blocks the antinociceptive effects of both δ opioid ligands, [D-Ala^2, Glu^4]deltorphin and DPDPE (Heyman et al., 1987; Jiang et al., 1990b). The μ antagonist, β-funaltrexamine (β-FNA), does not block the antinociceptive effects of either [D-Ala^2, Glu^4]deltorphin or DPDPE (Ward and Takemori, 1982; Heyman et al., 1987; Jiang et al., 1990b). The κ selective antagonist, nor-binaltorphimine (nor-BNI), does not block the analgesic activity of the δ agonists (Horan et al.,

1991a; Horan et al., 1991b). These results suggest that the antinociceptive properties of both [D-Ala^2, Glu^4]deltorphin and DPDPE are acting via an opioid δ receptor that lies in the central nervous system of the mouse.

The development of novel antagonists has allowed for identification of the δ subtypes. Bowen et al., 1987, have demonstrated that the peptide, [D-Ala^2, Leu^5, Cys^6]enkephalin (DALCE), produces a covalent bond with the δ opioid receptors in rat membranes via a thiol-disulfide exchange. The cysteine in postion 6 is suggested as being responsible for irreversible antagonism, since its similar cysteine lacking analogue, [D-Ala^2, Leu^5, Ser^6]enkephalin, has no antagonistic properties (Mattia et al., 1991b). Pretreatment with DALCE produces a dose- and time-related antagonism of the antinociceptive effects of the agonist DPDPE, but not those of the other δ agonist, [D-Ala^2, Glu^4]deltorphin, or the μ agonist, morphine (Jiang et al., 1990b; Calcagnetti et al., 1989). Portoghese et al., 1990, synthesized and characterized a new δ opioid antagonist called naltrindole-5'-isothiocyanate (5'-NTII). This compound antagonizes the analgesic effects of the agonist, [D-Ala^2, Glu^4]deltorphin, but not the analgesic effects of DPDPE in the mouse warm-water tail-flick test (Jiang et al.,1991; Porreca et al., 1992). Non-equilibrium antagonist actions have also been shown with 5'-NTII against [D-Ser^2, Leu^5, Thr^6]enkephalin (DSLET), but not against morphine or the κ agonist, U50,488H (Portoghese et al., 1990). The opioid agonists, [D-Ala^2, Glu^4]deltorphin and [D-Pen^2, D-

Pen^5]enkephalin, were shown to produce dose-response antinociception in the mouse warm-water tail-flick test at ICI 174,864-sensitive δ opioid receptors (Jiang et al., 1990a,b). However, [D-Ala^2, Glu^4]deltorphin antinociception was attenuated by 5'NTII and not DALCE, yet [D-Pen^2, D-Pen^5]enkephalin was attenuated by DALCE but not 5'NTII indicating two-way differential antagonism and the existence of δ receptor subtypes (Jiang et al., 1991). Therefore, subtypes of δ-opioid receptors have been identified pharmacologically, and termed δ_1 and δ_2 opioid receptors (Sofuoglu et al., 1991a; Jiang et al., 1991; Mattia et al., 1991a). On this basis, DPDPE has been suggested to produce its antinociceptive effects via a δ_1-opioid (i.e., DALCE-sensitive) receptor subtype, while [D-Ala^2, Glu^4]deltorphin appears to be selective for an δ_2-opioid (5'-NTII) receptor subtype.

In addition to the direct antinociceptive properties of δ-opioid receptor agonists, these compounds have been repeatedly demonstrated to produce a modulatory (i.e., increase or decrease in potency and efficacy) action on the effects of μ-opioid receptor agonists such as morphine (Vaught and Takemori, 1979; Lee et al., 1980; Heyman et al., 1986b,1989a,b; Horan et al., 1992a) in a variety of endpoints including antinociception in mice and rats. The interaction between δ-opioid receptor agonists and μ-opioid receptor agonists, such as morphine, is mediated via δ-opioid receptors. The modulation, but not the direct antinociception of morphine, is antagonized by δ-opioid receptor

selective antagonists such as ICI 174,864 (Heyman et al., 1989a,b). In addition, the selective δ-opioid receptor agonists, DPDPE and [D-Ala^2, Glu^4]deltorphin (given at subeffective doses), have been shown to positively modulate morphine antinociception, and further more, to interact with morphine in a synergistic fashion (Horan et al., 1992a). Based on data such as these, as well as on the basis of substantial evidence using radioligand binding approaches *in vitro*, one hypothesis explaining the modulatory actions of δ opioid receptor agonists on μ opioid receptor-mediated effects is that these interactions occur via an μ-δ opioid receptor complex (see Rothman et al., 1988, for review), though other interpretations of such data are possible.

The observation that both DPDPE and [D-Ala^2, Glu^4]deltorphin can be demonstrated to produce an ICI 174,864-sensitive modulatory effect on morphine antinociception (Heyman et al., 1989a,b) might be taken to suggest that both the δ_1 and δ_2 subtypes of the δ opioid receptors are involved in the observed modulatory actions. However, recent studies using subtype selective antagonists have demonstrated that the modulatory actions of both DPDPE and [D-Ala^2, Glu^4]deltorphin are sensitive to antagonism by 5'-NTII, but not by DALCE (Porreca et al., 1992), leading to the conclusion that only the δ_2 opioid receptor is involved in the modulatory effects of δ opioid receptor agonists on μ opioid receptor effects.

The observation that both the δ_1 opioid receptor agonist, DPDPE, as well as the δ_2 opioid receptor agonist, [D-Ala^2, Glu^4]deltorphin, both produce modulatory actions on morphine antinociception apparently via a δ_2 opioid receptor does not appear consistent with the observed selectivity of these agonists for the δ opioid receptor subtypes as identified using studies of direct antinociception (Jiang et al., 1991; Sofuoglu et al., 1991a; Mattia et al., 1991a).

Other competitive antagonists have been used to distinguish whether δ receptor subtypes exist. The synthesis of naltrindole (NTI), naltriben (NTB) and 7-benzylidenenaltrexone (BNTX) has demonstrated δ receptor heterogeneity. NTB antagonized the antinociceptive effects of DSLET better than those of DPDPE, whereas NTI antagonized the antinociceptive effects of DPDPE better than those of DSLET (Sofuoglu et al., 1991b). BNTX completely blocked the antinociceptive effects of DPDPE but not those of DSLET (Portoghese et al., 1992; Sofuoglu et al., 1993).

Further studies by Vanderah et al., 1992, characterized the antinociceptive response to cold water swim-stress (CWSS) in mice using opioid selective antagonists. The activation of endogenous opioid systems by CWSS was antagonized by ICI 174,864 and 5'-NTII but not by the other δ antagonist DALCE. Neither the κ antagonist nor-BNI nor the μ antagonist, β-FNA, had any effect on the antinociception induced by CWSS.

An additional approach to distinguish whether subtypes of the δ receptors exist, is to produce pharmacological tolerance using different δ agonists. Theoretically, if these agonists act at different receptors, tolerance to DPDPE would not create cross-tolerance to [D-Ala^2, Glu^4]deltorphin and *vice versa*. Mattia et al.(1991a) demonstrated that antinociceptive tolerance to the actions of these agonists, as well as a μ agonist, [Tyr-D-Ala-Gly-NMe-Phe-Gly-ol] (DAMGO), developed after three days of twice daily *i.c.v.* injections. No cross-tolerance between several doses of [D-Ala^2, Glu^4]deltorphin and DPDPE was observed, supporting the existence of the δ receptor subtypes. There was also no antinociceptive cross-tolerance between δ agonists and the μ agonist, DAMGO. Sofuoglu et al. (1991a) have also demonstrated a lack of antinociception cross-tolerance between the two δ agonists, DPDPE and DSLET, in mice. These studies have provided further evidence that two different δ opioid receptors exist in the mouse brain.

Negri et al. (1991) has demonstrated subtypes of the δ opioid receptors in rat brain using radiolabeled [^{3}H]deltorphin I . They have shown, in their binding assay, a biphasic displacement of [^{3}H]deltorphin I by DPDPE. A monophasic displacement of [^{3}H]DPDPE by deltorphin I was observed, demonstrating the existence of δ opioid receptor heterogeneity. Vaughn et al. (1990) also investigated the ability of [D-Pen^2, pCl-Phe^4, D-Pen^5]enkephalin (p-Cl-Phe^4-DPDPE) and [D-Ala^2, (2R, 3S)-cyclopropyl^{E}Phe^4, Leu^5]enkephalin methyl

ester (Cp-OMe) (Shimohigashi et al., 1987) to inhibit [^{3}H]p-Cl-Phe^4-DPDPE binding in both rat brain and in the mouse vas deferens (MVD) and showed that p-Cl-Phe^4-DPDPE recognized brain and MVD receptors with equal affinity, while CP-OMe showed 33-fold lower affinity in MVD compared to brain. These data are consistent with the existence of different δ opioid receptors in the brain and MVD. Xu et al.(1992) has demonstrated that naltrindole and oxymorphindole were selective for δ_1 (20-fold), whereas DPDPE and deltorphin I were 80-fold and 38-fold, respectively, for δ_2. Additionally, Rothman and coworkers have used site-directed acylating agents to deplete rat brain membranes of μ opioid receptors, and conducted binding competition studies using [^{3}H][D-Ala^2, D-Leu^5]enkephalin (DADLE) and several unlabeled δ opioid selective ligands (Xu et al., 1991, 1992). Furthermore, *in vitro* binding studies in rat brain membranes have shown that DALCE reduces the number of δ_1 sites without affecting δ_2 sites, suggesting that the DALCE-sensitive δ_1 receptor may be synonymous with the δ_{ncx} site (Rothman et al., 1992). The δ_2 receptor has also been referred to as the $\delta_{complex}$ receptor (Rothman et al., 1988; Vaught et al., 1982) and may exist as a complex with a μ receptor (Schoffelmeer et al., 1988). Delta receptor heterogeneity has also been shown using two different δ ligands, [^{3}H][D-Ser^2, Leu^5, Thr^6]enkephalin and [^{3}H]DPDPE (Sofuoglu et al., 1992).

In conclusion, based on the pharmacological tools that are available, there exist at least two subtypes of δ opioid receptors. The δ_1 receptor is responsive to the δ agonist DPDPE and the antagonist DALCE. The δ_2 receptor is responsive to the agonists [D-Ala^2, Glu^4]deltorphin, DSLET and to the antagonist 5'-NTII. Consequently, the δ_2 receptor is the receptor responsible for modulating or potentiating the effects of morphine (Porreca et al., 1992).

Modulatory Effects on Mu Agonists

Although many studies have shown that the different types of receptors, when presented with an agonist, result in antinociception, several studies have investigated the interactions between different opioid receptors and their antinociceptive function. Recent reports have suggested that δ opioid receptors may play an indirect role in antinociception produced at μ opioid receptors (Heyman et al., 1986b). Vaught and Takemori (1979) demonstrated that [Leu^5]enkephalin given *i.c.v.* or *i.p.*, at a dose that produces no significant antinociception in the warm-water tail-flick test, enhanced the antinociceptive effects of morphine by approximately 3-fold. This work was followed by studies that demonstrated the enhancement of morphine antinociception by [Leu^5]enkephalin was a synergistic effect, and not an additive effect (Porreca et al., 1990). Although these previous studies demonstrated that δ agonists could enhance the potency of morphine, Jiang et al. (1990c) reported that the

efficacy of morphine was also enhanced by δ agonists. Unlike the synergistic effect of [Leu^5]enkephalin, [Met^5]enkephalin shifted the morphine dose response curve to the left (Vaught and Takemori, 1979; Lee et al., 1980; Vaught et al., 1982). Enhancement of morphine antinociception has also been shown with subeffective doses of [D-Ala^2, Glu^4]deltorphin (Vaught and Takemori, 1979), that were antagonized by ICI 174,864 and 5'NTII, but not DALCE (Porreca et al., 1992). The *i.c.v.* administration of subeffective doses of DPDPE, DSLET and DADLE enhanced the antinociceptive effects of the μ agonist morphine, while doses of *i.c.v.* [D-Ala^2, Met^5]enkephalinamide (DAMA), antagonized the antinociceptive effects of morphine. The effects by both DPDPE and DAMA were antagonized by *i.c.v.* administration of the δ antagonist, ICI 174,864 (Vaught et al., 1982; Barrett and Vaught, 1982; Porreca et al., 1987a; Heyman et al., 1986a, 1989a,b). Jiang et al. (1990d) have reported that the co-administration of equi-antinociceptive actions of DPDPE or [Leu^5]enkephalin plus morphine, for three days, resulted in the development of less tolerance to morphine antinociceptive actions.

Although δ opioid agonists can enhance both the potency and efficacy of morphine, they are unable to enhance other μ agonists such as DAMGO, PL017, or β-endorphin (Heyman et al., 1989b). Experiments utilizing receptor-selective irreversible antagonists have helped to clarify which δ receptor mediates the modulation of morphine. Pretreatment with the μ opioid

antagonists, β-FNA and naloxonazine, did not alter the direct antinociceptive effects of DPDPE or DAMA (Heyman et al., 1987). However, β-FNA, but not naloxonazine, abolished both the change in potency derived from DPDPE and attenuation from DAMA on morphine antinociception (Heyman et al., 1989a,b). The pretreatment of mice with the δ_1 antagonist, DALCE (Bowen et al., 1987), had no effect on the ability of the subeffective doses of *i.c.v.* DPDPE or [Met^5]enkephalin to potentiate or attenuate morphine antinociception (Porreca et al., 1992). In contrast, the irreversible δ antagonist, 5'NTII, completely blocked the enhanced effect of the subeffective δ agonists on morphine, yet had no effect on morphine alone (Porreca et al., 1992).

Ligand binding studies have demonstrated apparent noncompetitive interactions between δ and μ binding sites (Rothman and Westfall, 1980,1982a,b), and led to the hypothesis that the modulatory effects of the δ agonists on μ-mediated antinociception occur through a δ binding site of an opioid receptor complex made up of distinct, yet interacting, μ and δ binding sites (Rothman and Westfall, 1983; Rothman et al., 1987a,b, 1988; Rothman et al., 1993). Ligand-binding data have demonstrated that β-FNA selectively alkylates the "μ-δ" opioid receptor complex resulting in δ receptors that can exist either seperately or in a physically-associated state with μ receptors (Rothman et al., 1988). The seperate δ receptors were termed $\delta_{\text{non-complexed}}$

(δ_{ncx}), or the receptors associated with the μ receptors termed $\delta_{complexed}$ (δ_{cx}) (Rothman et al., 1988).

Modulation of morphine antinociception has also been reported with compounds acting at CCK receptors. Administration of CCK has been shown to antagonize the antinociceptive effects of morphine (Itoh et al., 1982; Wiesenfeld-Hallin and Duranti, 1987), while antagonists of the CCK receptors have been consistently shown to enhance morphine antinociceptive potency (Hill and Woodruff, 1990). The latter observation to actions of antagonists preferentially at the CCK_B receptor subtype in rodents (Dourish et al., 1990; Hughes et al., 1990; Hill and Woodruff, 1990). Some investigators have suggested that stress may be a required component in order for the CCK antagonists to produce the commonly observed enhancement of the morphine effect (Lavigne et al., 1992), while others have observed the effect in apparently non-stressed rats. In this regard, Stanfa and Dickenson (1993) have studied halothane-anesthetized rats to demonstrate an enhancement of the morphine inhibition of C-fiber evoked responses in dorsal horn neurons by L365,260, a CCK_B antagonist, an experimental condition in which stress would presumably not be a factor.

The cloning of opioid receptors and the synthesis of more selective compounds will, hopefully, identify how the opioid receptors interact to produce some of the effects reported here. Although many studies have been

done since the discovery of the active alkaloid, morphine, our understanding of how opioids produce antinociception, interact with the different opiate receptors, activate second messenger systems, produce both tolerance and dependence, as well as whether all endogenous opioids and receptors have been discovered at present is far from forming a complete picture, preserving a desire both to learn and to work.

Hypothesis of the Dissertation

The use of opiates was one of the first documented pharmacological application of a plant extract (Kramer 1954). The use and abuse of opioids, as well as their clinical importance in the management of human pain, augments the need for continual research in the opiate field. The multiplicity of the endogenous opiate system, including multiple receptors and endogenous ligands, complicate our understanding of how the entire system functions. Critically, however, the analgesics most often used clincally today act via μ opioid receptors to produce analgesia and are responsible for a number of undesirable side effects including tolerance, physical (Cowan et al., 1988) and psychic dependence, respiratory depression (Jaffe and Matin, 1990), constipation (Kromer and Woinoff, 1980; Jaffe and Martin, 1990; Sheldon et al., 1990) and perhaps a lack of efficacy in neuropathic pain (Portenoy and

Coyle 1990). Recent work in the opiate field has focused on the synthesis of selective opioids for other receptor types including δ sites. Studies have shown that agonists at δ opioid receptors stimulate, rather than depress, respiratory function (Cheng et al.,1993; Kramer T, unpublished observation, 1990). Use of a selective agonist for the δ receptor may be less likely to produce both tolerance (Jiang et al., 1990d) and dependence (Horan P, personnal communication, 1991). Delta opioid receptors produce antinociception supraspinally, spinally and peripherially after opioid administration in rodents, and several studies have demonstrated that stress activates opidergic systems, proenkephalin synthesis and antinociception via δ opioid receptors.

Given the recent pharmacologic support for the existence of subtypes of δ opioid receptors in the mouse, we suggest that stress-induced activation of endogenous opioid systems may result in the production of antinociception, and further, that such an effect may involve subtypes of opioid δ receptors. In order to test this possibility, we have focused on antinociception associated with swim-stress, an effect thought to involve δ opioid receptors (Jackson et al., 1989; Kitchen and Pinker, 1990) and additionally, we sought to employ novel and selective opioid receptor antagonists to potentially elucidate the subtypes of opioid δ receptors which might be involved.

It has also been shown that δ opioid receptors play an important role in modulating the antinociceptive effects of the μ agonist morphine. Based on

the current literature and our understanding of opioids; it is the **hypothesis** of this dissertation that (1) the δ opioid receptor plays an important role in enhancing the potency of morphine, (2) that cold-water swim stress and inflammation of the mouse paw results in antinociception and enhancement of morphine antinociception via an endogenous enkephalin δ opioid receptor interaction, and (3) that cholecystokinin (CCK), via CCK_B receptors, tonically inhibits the antinociceptive interactions of endogenous enkephalins at δ opioid receptors. These studies are important in the development of techniques which may enable novel therapeutic approaches involving opioid δ agonists, and should play a role in the synthesis of new analgesics that act as agonists at δ opioid receptors and antagonists at CCK_B sites to enhance the clinically relevant effects of morphine. In addition, it is hypothesized that more than one δ opioid receptor exists in the mouse brain and that the δ opioid receptor subtypes can be cloned using a polymerase chain reaction (PCR) technique. By designing degenerate primers from published, homologous, G protein-coupled δ opioid, somatostatin and angiotensin receptors, amplification of a subtype of δ opioid receptor using PCR and the primers can be done. Therefore, also proposed in this dissertation is the cloning of an orphan opioid receptor subtype, the clones' sequence homology comparison to the cloned δ opioid receptor (Evans *et al.*, 1992; Kieffer *et al.*, 1992) and the expression of the subtype clone in a

transiently transfected COS-7 cell line. Subtype identification will be evaluated using selective opioid ligands in a radiolabeled ligand binding assay.

Overall, the work presented in this dissertation will aid in the design of new non-μ opioid agonists for the effective relief of pain, with promises of decreased tolerance, dependence and respiratory depression, as well as compounds which may have significant efficacy in neuropathic pain.

METHODS

Animals

Male, ICR mice (20-30 g, Harlan, Indianapolis, IN) were used for all experiments. Animals were kept in groups of four in a temperature controlled room with a 12 hr light-dark cycle (lights on 7:00 A.M. to 7:00 P.M.). Food and water were available *ad liberatum* until the time of the experiment. All procedures were approved by the Animal Care and Use Committee of the University of Arizona.

Chemicals

Morphine sulfate was purchased from Mallinckrodt Chemical Co., (St. Louis, MO). Cyclic Tyr-D-Pen-Gly-Phe-D-Pen (where Pen is D-penicillamine, DPDPE), [D-Ala^2, Leu^5, Cys^6]enkephalin (DALCE) and naltrindole-5'-isothiocyanate (5'-NTII) were synthesized as previously described (Bowen *et al.*, 1987; Portoghese *et al.*, 1990; Mosberg et al., 1983). [D-Ala^2, Glu^4]deltorphin was prepared by solid phase peptide synthesis methods similar to those previously described (Mosberg et al., 1983) and dissolved in 10% dimethylsulfoxide (DMSO). ICI 174,864 (N,N-diallyl-Tyr-*Aib*-*Aib*-Phe-Leu-OH, where *Aib* is α-aminoisobutyric acid) was purchased from Cambridge Research Biochemicals (Atlantic Beach, NY). Beta (β)-funaltrexamine (β-FNA) and nor-binaltorphimine (nor-BNI) were purchased from Research Biochemicals Inc.

(Natick, MA). L365,260 was a gift of Merck, Sharp and Dohme Research Laboratories (Rahway, N.J.) and was dissolved in 20% DMSO/propylene glycol. Thiorphan, naloxone, methysergide, yohimbine, cocaine, [Met^5]enkephalin and [Leu^5]enkephalin were purchased from Sigma Chemical Co. (St. Louis, MO). CCK-8 sulfate was purchased from Peptide International (Louisville, KY) and naltrindole HCl was a generous gift of Burroughs-Wellcome Co. (Research Triangle Park, N.C.). The Freund's Complete and Incomplete Adjuvant were purchased from Calbiochem (La Jolla, CA). All compounds were dissolved in distilled water unless noted otherwise, prior to use.

Antisera

Antisera against [Met^5]enkephalin and [Leu^5]enkephalin were produced by repeated injection of rabbits with peptides coupled to bovine thyroglobulin and were a generous gift of Dr. Leon Tseng. The specificities of the antibodies have been characterized by radioimmunoassay. Anti-[Met^5]enkephalin was obtained as crude rabbit serum and was shown to lack significant cross-reactivity (i.e., less than 2%) with dynorphin A(1-17), Gly-Gly-Phe-Met, Try-Gly-Gly-Phe, [Met^5]enkephalin-Leu, [Met^5]enkephalin-Arg-Gly-Leu, β-endorphin, cholecystokin-8 (sulfated), substance P, somatostatin, morphine sulfate, naloxone or neurotensin. Anti-[Met^5]enkephalin serum also showed less than 2% cross-reactivity to [Met^5]enkephalin-Arg-Phe. The anti-[Leu^5]enkephalin serum did not cross-react (i.e., less than 2%) with β-endorphin, dynorphin A-(1-

13) or dynorphin A-(1-17); this serum showed approximately 14% cross-reactivity with [Met^5]enkephalin (Dr. Leon Tseng, personal communication). Antisera were dissolved in distilled water just prior to use.

Injection Techniques

Intracerebroventricular Injections

Injections were by the intracerebroventricular (*i.c.v.*) route directly into the lateral ventricle according to the modified method of Haley and McCormick (1957) as previously described (Porreca *et al.*, 1984). Mice were lightly anesthetized with ether, an incision was made in the scalp, and the injection was made 2 mm lateral and 2 mm caudal to bregma at a depth of 3 mm using a 10 μl Hamilton (Reno, NV) microliter syringe with a 26-gauge needle.

Spinal Injections

Intrathecal (*i.t.*) injections were given into the spinal subarachnoid space at the L5-L7 level using the method of Hylden and Wilcox (1980) as modified by Porreca and Burks (1983). Lumbar punctures in unanesthetized mice were made using a 10-μl Hamilton (Reno, NV) syringe fitted with a 27-gauge needle. Both *I.c.v.* and *i.t.* injections were made manually in a volume of 5 μl over a period of 15 sec.

Subcutaneous and Intraperitoneal Injections

Subcutaneous (*s.c.*) administrations were made by injection into the subcutaneous space of the central abdominal location in a volume of 1 ml/kg.

Intraperitoneal (*i.p.*) administrations were directly injected into the peritoneal cavity from the left lower part of the abdomen. Injections were made in unanesthetized mice in a volume of 1ml/kg. Injections were given with a 1 ml tuberculin syringe fitted with a 26-gauge needle and all procedures were approved by the Animal Care and Use Committee if the University of Arizona.

Intraplantar Injections

Freund's Complete (FCA) or Incomplete Adjuvant (FIA) were delivered into the hind paw (5 μl) subcutaneously. The mice were covered with a blue pad and simply restrained by hand. No anesthetic was used.

Tail-flick Assay

The thermal nociceptive stimulus was 55°C warm water with the latency to tail-flick or withdrawal taken as the endpoint. Animals were tested by gently holding them by hand in a vertical position and recording the baseline latency. Control baseline latencies (i.e., prior to injection of test substances) ranged between 2-4 s. The latency to the first sign of a rapid tail-flick was taken as the endpoint according to the method of Jansen et al. (1963). Mice not responding within 5 s (<10%) during baseline testing were discarded from the experimental groups. After the determination of baseline latencies, the mice received graded doses of agonist in control mice or in mice pretreated with either antagonists, chronic agonists, antisera and enzyme inhibitors. Tail-flick latencies after agonist administration were determined during their peak time

of effect, a time either previously shown to result in a maximal antinociceptive response or shown by the studies done for this dissertation. In antagonist studies employing irreversible agents, DALCE (4.5 nmol), 5′-NTII (17.5 nmol), [Cys^4]deltorphin (3.0 nmol) and β-funaltrexamine (18.8 nmol) were given as a single pretreatment dose, 24 hr prior to testing. These doses and times have been previously established to be the times of peak agonist and antagonist actions (Heyman et al., 1989a; Jiang et al., 1990a,b,c; 1991; Horan et al., 1992; Porreca et al., 1992).

For the drug trials, a cut-off time of 15 sec was employed; if the mouse failed to respond within this time, the tail was removed from the water and that animal was assigned a maximum score. Mice not responding within 5 sec in the initial control trial were eliminated from the experiment. Antinociception at each time point was calculated according to the following formula: % antinociception = 100 x (test latency - control latency)/(15 - control latency). All testing was done in unanesthetized mice.

Evaluation of Hyperalgesia

In order to assess the possible development of a hyperalgesic response following FCA or FIA, mice were tested using the 50°C warm-water tail-flick test with 90 sec and 60 sec as the cut-off latencies, respectively. Hyperalgesia was calculated as (test latency - control latency)/(cut-off - control latency) where test latency refers to the response time following injection of FCA or FIA

into the mouse hind paw. To determine whether blockade of the hyperalgesic response following FCA injection would also result in blockade of the modulatory effect, mice were pretreated with cocaine (10 μl of 5% solution in the hind paw, concurrent with FCA) and (a) the potency of i.c.v. morphine was evaluated after 1 hr, and (b) were tested for hyperalgesia after 1 hr.

Cold-Water Swim-Stress (CWSS)

CWSS consisted of immersion of mice in water at either 20, 15 or 5° C using a cylindrical container, 17 cm in diameter, and 23 cm tall with water filled to a depth of 11 cm. Mice were placed in the water for a 3 min period and upon extraction, patted dry with paper towels. At various times after the swim, the mice were tested for their antinociceptive response as described below. With the exception of the antinociceptive time-course data, all additional experimentation employed the 5°C water temperature and all testing was done 10 min after the CWSS, the time of peak antinociception.

Cold-Water Swim-Stress (CWSS) Modulation

CWSS consisted of exposing mice to 5°C water for a 30 sec period and upon extraction, patting dry with paper towels.

Experimental Procedures

Agonist Studies

Direct antinociception was measured in the tail-flick assay 10 min after *i.c.v.* administration with DPDPE, [D-Ala^2,Glu^4]deltorphin, DAMGO and morphine in all studies.

Antinociception after *s.c.* or *i.p.* administration of morphine was measured either 30 or 20 min after injection, respectively. [Met^5] and [Leu^5]enkephalin were given by the *i.c.v.* route 10 min prior to antinociceptive testing or co-administered with morphine. The enkephalinase inhibitor, thiorphan, was either centrally or spinally administered at 100 μg 20 min prior to testing. Direct antinociception from CWSS in 5°C for 3 min was maximal at ten min after removal of mice from the cold water. For evaluation of morphine enhanced antinociception resulting from CWSS, mice were tested in the tail-flick assay 10 min after a 30 sec CWSS episode. In experiments involving the *i.c.v.* administration of morphine, test groups (i.e., CWSS exposed) received graded *i.c.v.* doses of drug 2 min prior to CWSS exposure (i.e., 12 min prior to testing); control groups were tested 12 min after *i.c.v.* morphine without exposure to CWSS.

Antagonist Studies

The general opioid antagonist, naloxone, was administered *i.p.* 20 min prior to tail-flick testing in the presence of two opioid agonists, DPDPE or [D-

Ala^2,Glu^4]deltorphin, resulting in a dose response (A_{50} of 0.38 mg/kg). In order to determine if antinociceptive effects of CWSS were the result of activity at opioid receptors, studies were conducted in the absence and presence of naloxone; this antagonist was given *s.c.* at 1 mg/kg 7 min prior to the 3 min CWSS and the animals tested after a further 10 min (i.e., 20 min after naloxone). Naloxone was also administered *i.th.* at a dose of 0.5 μg 1 min prior to CWSS exposure (i.e., 14 min prior to testing). In order to determine whether enhanced morphine antinociception by CCK_B receptor blockade was mediated via an opioid receptor, naloxone was administered 20 min prior to testing *s.c.* at 0.3 mg/kg.

The δ-selective opioid antagonist, ICI 174,864 (*N,N-diallyl*-Tyr-*Aib-Aib*-Phe-Leu-OH, where *Aib* is α-aminoisobutyric acid)(Cotton et al., 1984), was administered *i.c.v.* 10 min prior to tail-flick testing in the presence of the opioid agonists DPDPE or [D-Ala^2,Glu^4]deltorphin resulting in a dose response (A_{50} of approximately 1.3 nmol).

In order to determine whether the opioid δ receptor was involved in the CWSS-induced or the FCA-induced antinociceptive effect, the selective δ antagonist ICI 174,864 was given by *i.c.v.* (as well as *i.th.* in the CWSS case) (4.4 nmol) injection 2 min prior to the 3 min CWSS and antinociception determined after an additional 10 min (i.e., 15 min after the antagonist). In order to understand whether enhanced morphine antinociception by CCK_B

receptor blockade was δ-receptor mediated, ICI 174,864 was co-administered with morphine *i.c.v.* at 4.4 nmol.

Opioid κ-receptor involvement was evaluated by *i.c.v.* injection of the selective κ-antagonist, nor-BNI (Takemori et al., 1988), given at a dose (1 nmol) which selectively antagonizes the antinociceptive effects of U69,593 (Lahti et al., 1985), but not those of DAMGO, morphine, DPDPE or [D-Ala^2, Glu^4]deltorphin (Horan et al., 1991; 1992; Horan and Porreca, unpublished observations). Nor-BNI was given *i.c.v.* 7 min prior to the 3 min CWSS and antinociception determined after an additional 10 min (i.e., 20 min after the antagonist). Additionally, mice were pretreated with β-FNA (18.8 nmol, at -24 hr) in order to prevent activity at the μ receptor; this dose- and time-relationship for β-FNA has been previously established (Heyman et al., 1987, 1989a,b; Jiang et al., 1990c) to selectively block the antinociceptive effects of morphine and DAMGO, but not that of DPDPE or [D-Ala^2, Glu^4]deltorphin.

Pretreatment of mice with 5'-NTII or [Cys^4]deltorphin, previously shown to antagonize [D-Ala^2, Glu^4]deltorphin, but not DPDPE or morphine, was made 24 hr prior to testing as previously described (Jiang et al., 1991; Horan and Porreca, unpublished data 1991). Previous studies have shown maximal antagonist properties of DALCE following 24 hr pretreatment (Jiang et al., 1990a; Porreca et al., 1992); at this time, DALCE pretreatment blocked the

antinociceptive actions of DPDPE, but not those of morphine or DAMGO. Thus, this time was chosen for studies with DALCE.

Treatments with these antagonists at these doses and times did not produce any measurable antinociceptive or behavioral effects and were the time of maximal antagonist activity as shown in previous studies (Heyman et al., 1986a,1987; 1989a,b; Jiang et al., 1990b,c; 1991; Porreca et al., 1987b,1992). These previous studies (Jiang et al., 1990a,b; 1991; Porreca et al., 1992) also demonstrated that administration of vehicle for DALCE, 5'-NTII, [Cys^4]deltorphin, nor-BNI, ICI 174,864 or β-FNA did not alter the dose response line for *i.c.v.* morphine and for this reason, a vehicle control for the 24 hr pretreatments was not repeated in the present studies.

In experiments involving the use of antisera, these were given to the mice by the *i.c.v.* route 20 min prior to antinociceptive testing (i.e., 8 min prior to morphine and 10 min prior to CWSS or FCA); mice were tested 12 min after *i.c.v.* morphine administration. Antisera were administered *i.c.v.*, 20 min before testing (i.e., 10 min prior to morphine administration). Antisera were administered 2 min prior to L365,260 administration 22 min prior to testing. Antisera to [Leu^5]enkephalin and [Met^5]enkephalin were characterized by administering them to mice in the presence of both [Leu^5]enkephalin and [Met^5]enkephalin peptides. They had no direct antinociceptive effects by themselves, as well as no effect on morphine antinociception.

Enhanced antinociception of morphine by CCK_B receptor blockade was tested 20 min after L365,260 (1 μg, *i.c.v.* and *i.th.*) and 10 min after graded doses of morphine. For the *s.c.* experiments, L365,260 was given at a dose of 0.2 mg/kg, at 40 min prior to testing, while graded doses of morphine were given 20 min prior to testing. Naltrindole was given *s.c.* in all cases at a dose of 10 mg/kg, 35 min prior to testing. These doses and times were determined in pilot experiments to represent the times of peak agonist and antagonist actions and were used in previous studies in the rat (Ossipov et al., 1994). In order to confirm selectivity of this dose of naltrindole for δ receptors, *s.c.* naltrindole was administered at the same dose 35 min prior to administration of an A_{90} dose of *i.c.v.* DPDPE, [D-Ala^2, Glu^4]deltorphin or morphine; antinociceptive testing took place 10 min after the agonist. As a control, where necessary, the appropriate vehicle was injected at the designated times. Thiorphan was given either by the *i.c.v.* or the *i.th.* route at a dose of 100 μg 15 min prior to testing.

Administration of Oligodeoxynucleotides

Antisense and mismatch oligodeoxynucleotides (oligos) were reconstituted in sterile deionized water at a final concentration of 2.5 μg/μl. These oligos were injected directly into the lateral cerebral ventricle as described above (12.5 μg per injection) on a twice-daily (09.00 and 18.00 hr) schedule for three days. Three groups of 10 mice were employed (i.e. vehicle

injected, antisense oligo and mismatch oligo treated). Antinociceptive testing took place 12 hr after the last oligo administration (i.e. injection 6). Baseline latencies were determined just prior to agonist administration. Oligos were purchased from Midland Certified Reagent Co. (Midland, TX). Two 20 base oligos were derived from the 5′ coding sequence of the cloned mouse CCK_B receptor (Figure 62)(Nakata et al., 1992) (nucleotides 13 to 32) with the following sequences: 5′-TGC ACG CTG CTG TTC AGC TT-3′ (antisense) and 5′-CGC ATA CTG CCA TTC AAT TT-3′ (mismatch). These sequences were verified by a search for sequence homology (Genepro, Riverside, WA) to the gene sequences of the CCK_A, δ, μ and κ receptors to ensure no cross-hybridization.

CWSS antinociception was tested in animals treated with oligos designed from the δ opioid receptor in the exact same manner as stated above. Antisense and sense oligos derived from the 5′ end of the coding sequence of the cloned δ receptor (nucleotides 7 to 26) were designed and purchased from Midland. These two 20 base oligos were of the following sequence: 5′-GCA CGG GCA GAG GGC ACC AG-3′ (antisense) and 5′-CTG GTG CCC TCT GCC CGT GC-3′ (sense). Once again these sequences were verified by a homology search.

Tolerance and Cross-Tolerance Studies

Antinociceptive tolerance to selective μ and δ agonists was induced using the procedure described by Mattia et al. (1991a). Mice were treated twice daily with *i.c.v.* DPDPE, [D-Ala^2, Glu^4]deltorphin or DAMGO at 0800 and 1800 hr for three days. Tolerance to the enhanced antinociceptive effect by L365,260 or [Leu^5]enkephalin on morphine antinociception was achieved after mice were treated at 0800 and 1800 hr for 2 days. The doses chosen for repeated treatment were approximately equivalent to the A_{90} value for each compound in naive mice and were as follows: DPDPE, 46.5 nmol; [D-Ala^2, Glu^4]deltorphin, 12.8 nmol; and DAMGO, 0.2 nmol. The doses chosen for the CCK_B antagonist, L365,260, and [Leu^5]enkephalin for chronic treatment were the doses that effectively enhanced morphine antinociception after one injection. These doses and the injection schedule used to produce tolerance for DPDPE, DAMGO and [D-Ala^2,Glu^4]deltorphin, as assessed by a significant rightward displacement in the antinociceptive dose-response lines for each agonist, have been described previously (Mattia et al., 1991a). Testing with CWSS-induced antinociception took place on the morning of the fourth day after repeated treatment with DPDPE, DAMGO and [D-Ala^2,Glu^4]deltorphin. In order to verify the development of tolerance to each test agent, and possible cross-tolerance to the antinociceptive effects of CWSS, groups of control or agonist pretreated mice received an *i.c.v.* injection of an approximately A_{90} dose

of DPDPE, [D-Ala^2, Glu^4]deltorphin or DAMGO in the respective pretreatment group. Additionally, groups of control or agonist pretreated mice were subjected to CWSS and antinociception determined after 10 min as described above. On the morning of the 4th day in the case of chronic [D-Ala^2, Glu^4]deltorphin or on the evening, 20 min after four injections of L365,260 or [Leu^5]enkephalin injection, dose-response curves were determined for morphine enhancement by L365,260 and [Leu^5]enkephalin or for [D-Ala^2, Glu^4]deltorphin in seperate groups of mice.

Tolerance was also produced to the antinociceptive effect resulting from CWSS. In this procedure, different groups of mice were subjected to repeated CWSS beginning with the first exposure at 0800 hr and followed by a second (1800 hr, day 1), third (0800 hr, day 2), fourth (1800 hr, day 2), fifth (0800 hr, day 3) and sixth (1800 hr, day 3) exposure. That is, each group received a different number of exposures prior to testing for an antinociceptive response. Each group was tested for antinociceptive response only once after the appropriate number of CWSS exposures and antinociception recorded after 10 min. Tolerance to the antinociceptive response was seen beginning with the third swim exposure, but in order to ensure a significant degree of tolerance had developed, further testing took place with mice subjected to six CWSS exposures over a period of 3 days. On the morning of the fourth day, the mice received graded *i.c.v.* doses of the μ selective agonist, DAMGO, or the δ

selective agonists, DPDPE or [D-Ala^2, Glu^4]deltorphin and antinociception was recorded after 10 min.

Body Temperature

In order to assess the possible involvement of decreases in body temperature following CWSS-exposure in the observed antinociceptive effect, the rectal temperature of separate groups of mice was recorded using a YSI thermistor probe just before CWSS, and 10 min after the CWSS-exposure (i.e., the time of antinociceptive testing). Additionally, separate groups of mice were pretreated with *s.c.* vehicle, or naloxone (1 mg/kg, 7 min prior to CWSS) and body temperature recorded as described above. Further, body temperature was recorded prior to, and after each CWSS exposure in the tolerance procedure as described above involving six CWSS exposures over 3 days. Finally, body temperature was monitored prior to, and 24 hr after *i.c.v.* injection of mice with β-FNA, DALCE and 5'-NTII at the dose described above. The recording of body temperature was achieved using a YSI Tele-Thermometer, Model 44TA (Yellow Springs Instrument Co., Yellow Springs, OH) and a series 400 probe. The probe was inserted 2.5 cm into the rectum of the mouse and maintained for 3 min for the establishment of a steady temperature. During this period, the mice were gently restrained under a laboratory towel.

Statistics

Animals were randomly assigned to test groups and the experiments were conducted using a between-subjects design with at least 10 animals used per data point. Regression lines, A_{50} values (i.e., the dose producing a 50% antinociceptive response) and 95% confidence limits (C.L.) were determined using all individual data points with the computer program described by Tallarida and Murray (1986)(procedure 8). For the fitting of regression lines and calculation of the A_{50} values, only the linear portion of the dose-effect curve was used. Relative potencies were calculated by comparison of the regression line A_{50} values and significant changes in potency were defined as cases in which the 95% confidence limits of the A_{50} values did not overlap. The data were analyzed by ANOVA and by the Student's t test for grouped data. All data points shown are the mean values for at least 10 mice from different groups and error bars represent the standard error.

Cloning of an Orphan Receptor

In order to clone a novel subtype of opioid delta receptor, both sense and antisense degenerate primers were designed and used in the polymerase chain reaction (PCR). Degenerate primers were designed from three different receptors that have been cloned: the delta opioid (Evans et al., 1992), somatostatin (Yamada et al., 1992) and the angiotensin receptors (Sasaki et al., 1991). The primers were designed from areas of greatest homology

between all three receptors. The primers are 30 nucleotides in length and were synthesized on the Milligen Millipore Oligonucleotide Synthesizer. PCR was done using mouse brain cDNA library as the template and Taq polymerase as the enzyme to amplify the DNA. Forty five cycles of PCR were performed with the conditions as follows: denature 94°C for 1min, anneal 55°C for 2 min and 72°C for 3 min. The PCR products were then exposed to electrophoresis in order to determine their length. PCR products that were much smaller or much larger than the approximate size predicted, based on comparison to the published δ opioid receptor, were excluded. An appropriate size PCR product was extracted from the agarose gel and purified using glass milk (Qiagen).

The PCR product was subcloned into the pCRII vector from Invitrogen Corporation. This vector has T and A nucleotides flanking either ends in the cloning region, and anneals with the PCR T and A flanking product. Sequencing was done by the Molecular and Cellular Biology Department at the University of Arizona and by Stanford University. All sequencing was done by the standard Sanger Dideoxy Nucleotide Method using universal T7 and SP6 primers designed from the host vector. Sequence comparison using Genebank was used to identify whether the PCR product was unique as well as its degree of homology to the published δ opioid receptor. The unique PCR product was used as a probe to screen the mouse brain cDNA library (Stratagen) in order to locate a full length clone. The PCR product was labeled with [^{32}P] by the

random priming method from Boehringer Mannheim. A portion of the cDNA library was transferred to high-bond nylon filters (Amersham) in order for plaque hybidization to occur. The library was exposed to the labeled PCR product for 12 hours at 45°C for hybridization to occur. Screening conditions were as follows: 2XSSC (1XSSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0) and 0.1% SDS for 30 min at 65°C and 0.1XSSC and 0.1% SDS for 30 min at 65°C. The filters were exposed to x-ray film for approximately two days, and postive clones were identified and screened two more times, following the protocol above, in order to assure that all the colonies' DNA would hybridize with the probe. The postive clones were then excised from the lambda phage using a helper phage, exassist/solar system from Stratagene. The clones were exposed to electrophoresis in order to determine their approximate length. A positive clone was sequenced in order to assure the probe sequence was present, to identify the full length open reading frame and to compare its percent homology to the cloned mouse opioid δ receptor.

Northern blot hybridization was done using a Clontech Multiple Northern Tissue Blot containing approximately 10 μg of mRNA from several different tissues including mouse whole brain, skeletal muscle, heart, liver, spleen, kidney and testicles. The 700 base pair PCR product was labeled at with [^{32}p]CTP by the random priming method from Boehringer Mannheim. The mRNA blot was prehybridized in buffer containing 5 X SSPE, 10 X Denhardt's,

0.6 M naCl, 10 mM Tris-HCL, pH 7.5, 1 mM EDTA, 0.05% salmon sperm DNA, 10 mM dithiothreitol (DTT) and 20 mM β-mercaptoethanol for 4 hours at 65°C. Subsequent hybridization was done in prehybridization buffer plus labeled probe at 65°C for 18 hours. After hybridization, the blot was washed for 40 min at room temperature in 2 X SCC with 0.05% SDS and twice for 40 min at 50°C in 0.1 X SCC with 0.1% SDS. The blot was exposed to x-ray film (Kodak XAR) for 5 nights at -80°C and developed.

Expression and Characterization of the Orphan Receptor

The clone was inserted into the expression vector psv-SPORT from GIBCO, and transiently transfected into COS-7 cells. The cells were cultured in base media of 1:1Dulbecco's Modified Eagle's Medium (DMEM):HAMS F12 supplemented with 10% iron supplemented bovine calf serum, penicillin (100 units/ml) and streptomycin (100 mg/ml). All cells are grown under a humidified atmosphere of 5% CO_2:95% air, at a temperature of 37°C. Transfection was done using the standard calcium phosphate method with the Profection Kit from Promega. Standard controls with a reporter gene and a sham transfection was done alongside with the clone transfection. COS-7 cells have already been characterized for opioid binding and, therefore, were an appropriate cell line to use. Transfected cells were incubated for 48 hours before being detached with 5.0 mM EDTA in phosphate-buffered saline. The harvested cells were washed once with 20 ml Tris-Mg buffer (50 mM Tris-HCL, 5.0 mM magnesium chloride,

pH 7.4), homogenized using a Teflon-glass tissue grinder in 10 ml fresh Trig-Mg buffer, washed once again with 20 ml Tris-Mg buffer and then resuspended in fresh Tris-Mg buffer to between 50 and 100 μg/ml protein. Saturation binding experiments were performed using [^{3}H]naltrindole, [^{3}H]CTOP, [^{3}H]U69,593, [^{3}H][4'-CL-Phe^4]DPDPE, [^{3}H]SKF-10,047, [5-^{3}H]DTG and [^{125}I]-somatostatin analogues (SS14 and SMS) on crude membrane preparations.

Using the amino acid sequence and the computer program Seqaid, a hydropathy plot was done to determine whether the clone is homologous to other G-protein coupled receptors, whether it has seven transmembrane domains and if the protein contains a common DRY amino acid sequence after the third transmembrane domain .

RESULTS OF DELTA SUBTYPE STUDIES

Pretreatment of mice with DALCE (4.5 nmol, *i.c.v.*) or 5′-NTII (17.5 nmol, *i.c.v.*) 24 hr prior to testing produced no changes in basal tail-flick latency. DALCE pretreatment produced a significant blockade of the antinociceptive effects of a submaximal dose of *i.c.v.* DPDPE (46.5 nmol), but had no effect on the antinociceptive effects of [D-Ala^2,Glu^4] deltorphin (Figure 1). In contrast, pretreatment with 5′-NTII significantly antagonized the antinociceptive effects of a submaximal dose of [D-Ala^2, Glu^4]deltorphin (12.6 nmol) but had no effect on antinociception produced by DPDPE (Figure 2). The dose-response lines for *i.c.v.* [D-Ala^2, Glu^4]deltorphin in control, or in DALCE pretreated mice are shown in Figure 3. Coadministration of DPDPE (46.5 nmol) with [D-Ala^2, Glu^4]deltorphin in DALCE pretreated mice resulted in a marked rightward displacement of the [D-Ala^2, Glu^4]deltorphin dose-response line (Figure 3). The A_{50} values (and 95% confidence limits) for [D-Ala^2, Glu^4]deltorphin in control, DALCE-pretreated, and DALCE-pretreated plus DPDPE mice were 3.9 (3.36 - 4.53), 3.9 (3.28 - 4.73) and 31.7 (26.25 - 38.28) nmol, respectively. These data show that under δ_1-opioid receptor blocked conditions, DPDPE produced an approximately 8-fold rightward displacement of the [D-Ala^2, Glu^4]deltorphin dose-response curve. The dose-response line for [D-Ala^2, Glu^4]deltorphin in DALCE-pretreated mice was not different from that in controls. The dose-response lines for DPDPE in control, or in 5′-NTII-

pretreated mice are shown in Figure 4. Coadministration of [D-Ala^2, Glu^4]deltorphin (12.6 nmol) with DPDPE in 5′-NTII-pretreated mice had no significant effect on the DPDPE dose-response line (Figure 4). The A_{50} values (and 95% confidence limits) for DPDPE in control, 5′-NTII-pretreated, and 5′-NTII-pretreated plus [D-Ala^2, Glu^4]deltorphin mice were 19.3 (14.55 - 25.58), 25.38 (18.72 - 34.41) and 29.03 (18.48 - 45.61) nmol, respectively.

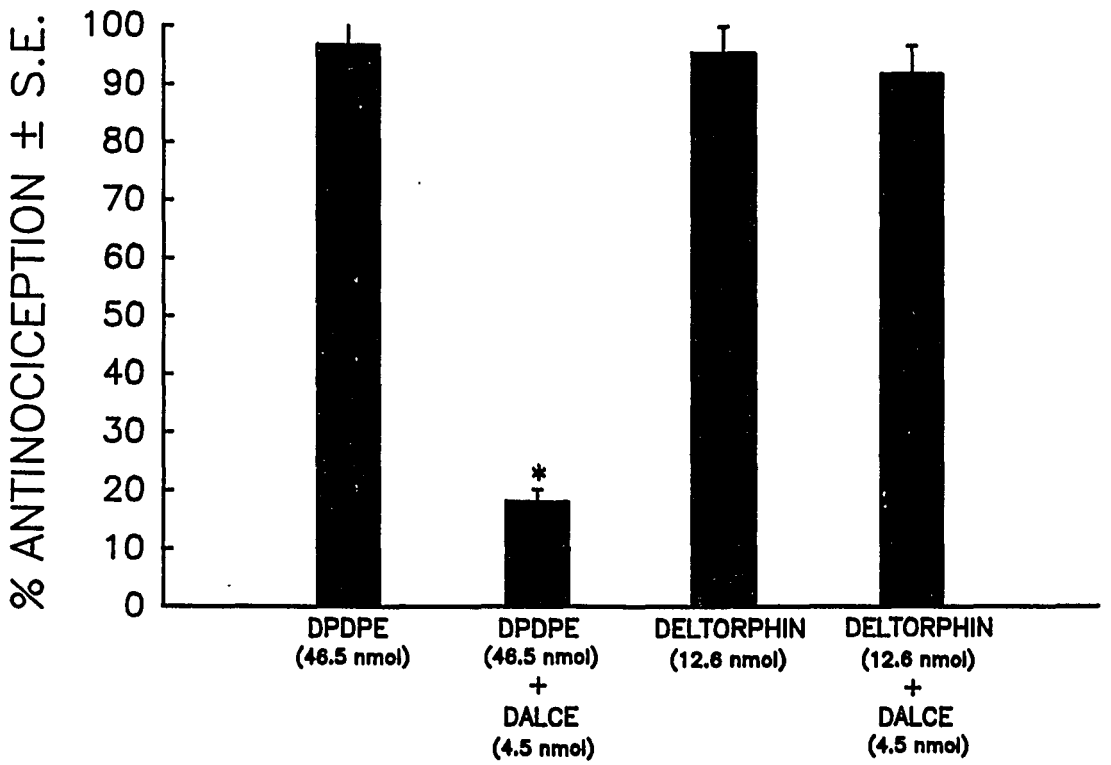

Figure 1. Antinociceptive effect of DPDPE or [D-Ala^2, Glu^4]deltorphin in control mice, or following pretreatment with DALCE; DALCE had no direct antinociceptive effect.

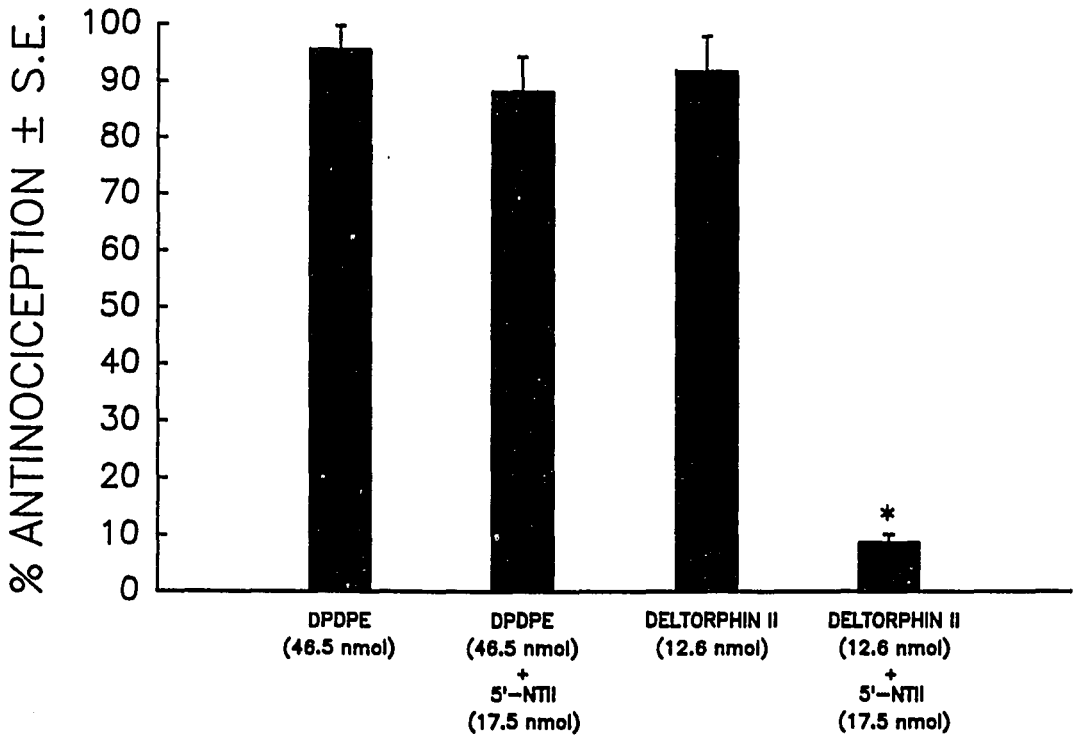

Figure 2. Antinociceptive effect of DPDPE or [D-Ala^2, Glu^4]deltorphin in control mice, or following pretreatment with 5′-NTII; 5′-NTII had no direct antinociceptive effect.

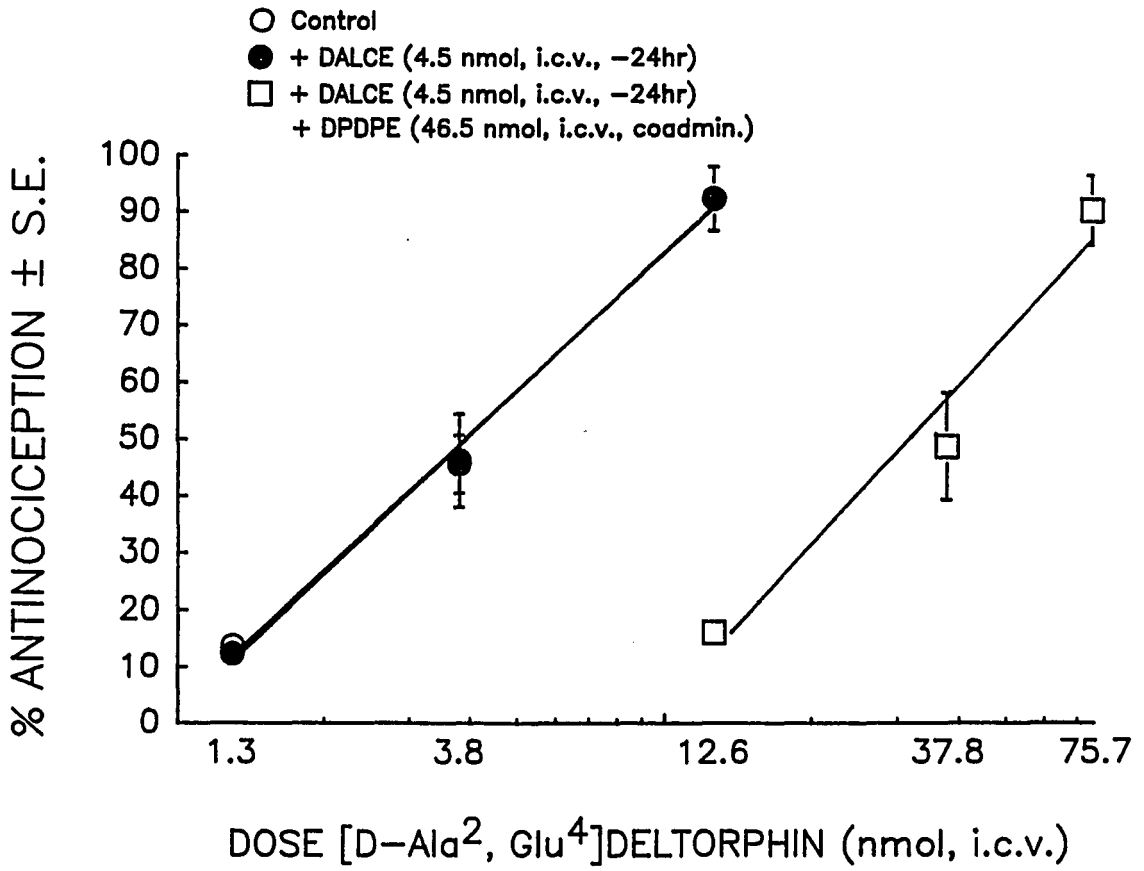

Figure 3. Antinociceptive dose-response curves of graded *i.c.v.* doses of [D-Ala^2, Glu^4]deltorphin in control mice, DALCE-pretreated mice, or in DALCE-pretreated mice in the presence of a sub-maximal dose of DPDPE; DPDPE produced virtually no effect in DALCE-pretreated mice.

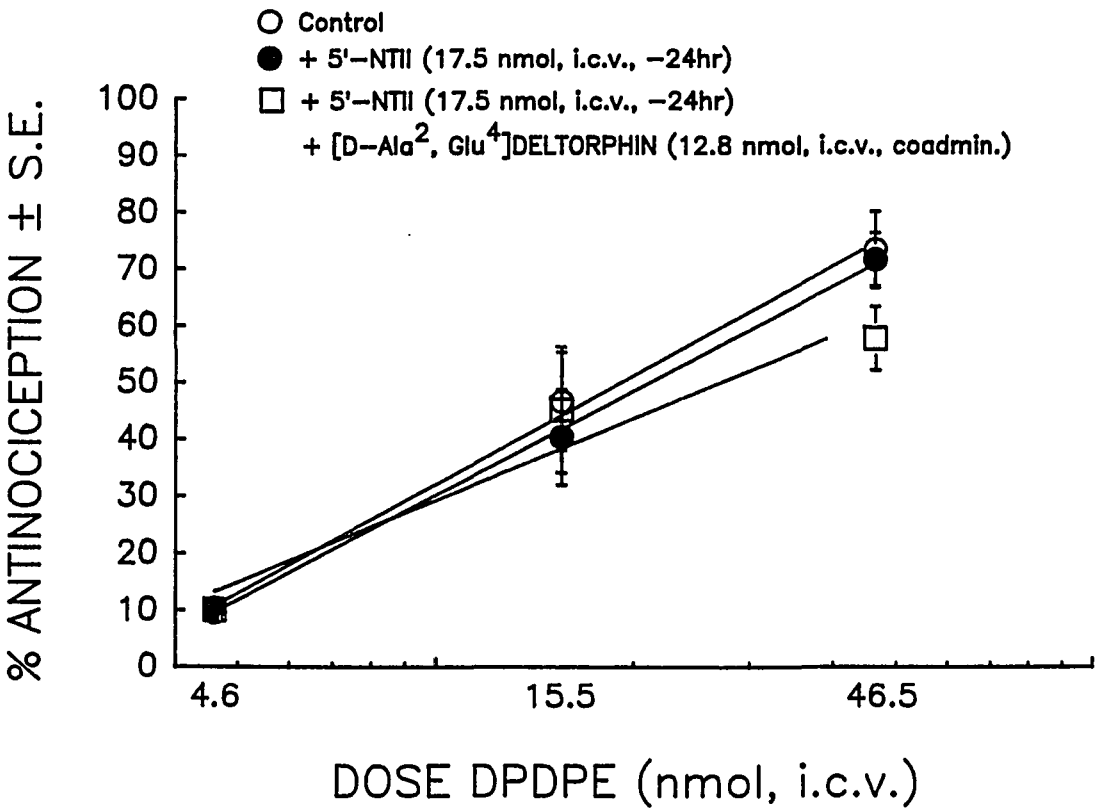

Figure 4. Antinociceptive dose-response curves of graded *i.c.v.* doses of DPDPE in control mice, 5′-NTII-pretreated mice, or in 5′-NTII-pretreated mice in the presence of a sub-maximal dose of [D-Ala^2, Glu^4]deltorphin; [D-Ala^2, Glu^4]deltorphin produced virtually no effect in 5′-NTII-pretreated mice.

SUMMARY OF DELTA OPIOID RECEPTOR SUBTYPE STUDIES

Recent studies of the direct antinociceptive properties of δ opioid receptor agonists have provided strong pharmacological evidence for the existence of subtypes of δ opioid receptors. Through the use of novel and selective δ opioid receptor antagonists, *i.c.v.* DPDPE and [D-Ala^2, Glu^4]deltorphin have been suggested to produce their antinociceptive effects in mice via subtypes of δ opioid receptors, termed δ_1 and δ_2, respectively (Sofuoglu et al., 1991; Jiang et al., 1990a,b;1991). This finding is supported by the observation of a two-way lack of cross-tolerance between DPDPE and [D-Ala^2, Glu^4]deltorphin, as well as to the μ opioid receptor agonist [D-Ala^2, NMPhe^4, Gly-ol]enkephalin (DAMGO)(Mattia et al., 1991a; Sofuoglu et al., 1991). It should also be noted that the antinociceptive effects of DPDPE and [D-Ala^2, Glu^4]deltorphin are not sensitive to μ or κ opioid receptor antagonists in this model, and the δ opioid receptor selective antagonists, DALCE and 5′-NTII, do not antagonize the antinociceptive actions of μ opioid receptor agonists such as morphine or DAMGO (Jiang et al., 1990a,b;1991).

In addition to a direct role for δ opioid receptors in the mediation of antinociception, it has long been recognized that agonists at δ opioid receptors can produce either a positive (i.e., increase in potency and efficacy) or negative (i.e., decrease in potency and efficacy) of the effects of μ opioid receptor

agonists. Interestingly, two endogenous ligands of the δ opioid receptor produce opposite modulatory actions; [Leu^5]enkephalin produces a positive, while [Met^5]enkephalin produces a negative, effect on μ opioid receptor mediated antinociception (Vaught et al., 1979; Lee et al., 1980). Both the positive and the negative modulatory actions of [Leu^5]enkephalin and [Met^5]enkephalin (but not the direct μ opioid receptor mediated antinociception) are sensitive to antagonism by the δ opioid receptor antagonist, ICI 174,864, leading to the conclusion that the modulatory effect is mediated via δ opioid receptors; such receptors have been hypothesized to be a part of a functional or physical μ-δ opioid receptor complex (see Rothman et al., 1988 for review).

Using the same approach as that taken for direct antinociceptive studies, recent data have demonstrated that the δ opioid receptor modulatory effect is sensitive only to δ_2-opioid receptor antagonists, suggesting the sole participation of this receptor subtype in μ opioid receptor modulation (Porreca et al., 1992). Additionally, induction of tolerance at the δ_2, but not the δ_1 opioid, receptor prevents the modulatory action (Vanderah and Porreca, unpublished observations).

The finding that both the positive and negative modulatory effects of δ opioid receptor agonists (Heyman et al., 1989a,b) could be antagonized by ICI 174,864 together with the identified subtypes of δ opioid receptors, initially suggested that these positive and negative modulatory effects might be

mediated via different δ opioid receptor subtypes. This concept was reinforced by the observation that DPDPE and [D-Ala^2, Glu^4]deltorphin produced their direct antinociceptive effects via subtypes of δ opioid receptors. However, both DPDPE and [D-Ala^2, Glu^4]deltorphin produced a positive modulatory effect, and further, this action was selectively sensitive to antagonism by δ_2 opioid receptor antagonists (Porreca et al., 1992). Such observations appeared inconsistent with the concept that these selective δ-opioid receptor agonists acted at subtypes of δ opioid receptors. This paradox was investigated in the present study by blocking the preferred receptor at which DPDPE and [D-Ala^2, Glu^4]deltorphin are hypothesized to act using irreversible δ-opioid receptor subtype-selective antagonists, DALCE and 5′-NTII. Possible interactions of these ligands with the non-preferred receptor might be revealed under such δ_1 and δ_2 opioid receptor blocked conditions.

Following blockade of the δ_1 opioid receptor using DALCE, the direct antinociceptive effects of DPDPE were almost completely blocked while those of [D-Ala^2, Glu^4]deltorphin were unaltered. Construction of the [D-Ala^2, Glu^4]deltorphin dose-effect curve in the presence of a dose of DPDPE (which produced a sub-maximal effect under control conditions) resulted in a marked rightward displacement of the [D-Ala^2, Glu^4]deltorphin dose-response curve. This observation suggests that DPDPE can bind to the δ_2-opioid receptor, but apparently, does not produce antinociception directly at this site. In contrast,

the converse experiment under δ_2 opioid receptor blocked conditions suggested that [D-Ala^2, Glu^4]deltorphin does not interact with the δ_1 opioid receptor site. No antagonism of the DPDPE dose-effect curve was observed in the presence of a sub-maximal dose of [D-Ala^2, Glu^4]deltorphin under conditions in which the effects of [D-Ala^2, Glu^4]deltorphin were almost completely blocked by 5′-NTII. The possibility that supramaximal doses of [D-Ala^2, Glu^4]deltorphin might eventually interact with the δ_1 opioid receptor cannot be excluded and was not investigated in the present study.

These observations suggest that DPDPE has actions at both δ-opioid receptor subtypes. Apparently, DPDPE mediates its direct antinociceptive actions via the δ_1 opioid receptor while its modulatory effects are mediated via the δ_2 opioid receptor (i.e., is a partial agonist at this site). In contrast, [D-Ala^2, Glu^4]deltorphin produces both actions via the δ_2 opioid receptor subtype. It is also of particular interest that the direct antinociceptive actions of these substances appear to be mediated predominately, if not exclusively, through its own receptor subtype, even though both compounds compete for sites labelled by δ-opioid receptor selective ligands. A possible explanation of this observation may relate to the efficacy of each compound at each receptor subtype. Though DPDPE may effectively bind to the δ_2 opioid receptor site, it may have only limited efficacy and be unable to transduce direct antinociception at this site. Such a view would be supported by

demonstrations of effective modulatory actions of [Leu^5]enkephalin (Vaught and Takemori, 1979) and [Met^5]enkephalin (Lee et al., 1980) on morphine antinociception, even though it is virtually impossible to demonstrate direct antinociceptive effects of these compounds in this test (Horan et al., 1992b). On this basis, it would be reasonable to suggest that the production of a modulatory action on μ opioid receptor mediated antinociception would occur with greater efficiency than that required for direct production of antinociception, and would appear to account for the actions of DPDPE at both subtypes of δ opioid receptors, as well as the significant differences in the direct antinociceptive pharmacology of DPDPE and [D-Ala^2, Glu^4]deltorphin.

RESULTS OF CWSS ANTINOCICEPTION

CWSS-induced antinociceptive time-course. Although an antinociceptive response was produced following exposure of mice to water at 20, 15 or 5°C, the response seen at the coldest temperature was more pronounced, achieving approximately a 90% effect 10 min after the CWSS (Figure 5). The antinociceptive response seen following exposure at all water temperatures appeared to be maximal at approximately 10 min after CWSS exposure and persisted for up to 20 min.

CWSS-induced changes in body temperature. Basal temperature in control mice, determined immediately prior to CWSS exposure, was 36.4 ± 0.31°C (n = 20), while the basal temperature in mice pretreated with naloxone (1 mg/kg, *s.c.*) 10 min prior to measurement (i.e., the time of exposure to CWSS) was 36.9 ± 0.35°C (n = 20) (Figure 6). These values did not differ significantly (Student's t test for grouped data). When the body temperature of these groups was redetermined 10 min after the CWSS exposure (i.e., the time of antinociceptive testing) the respective core temperatures of the control and naloxone treated animals were decreased to 25.5 ± 0.43 and 26.2 ± 0.51°C, respectively (Figure 6); these values did not differ significantly, but in each case were significantly decreased when compared to the respective group prior to CWSS exposure. While there was no significant difference in body temperature

of animals with or without naloxone pretreatment following CWSS exposure, evaluation of the antinociceptive response in the two groups showed a marked difference; the control antinociceptive response was 93.4 ± 4.1 percent while the naloxone-pretreated group had an antinociceptive response of 13.7 ± 2.1 percent, values which were shown to be significantly different (Student's t test for grouped data).

In separate groups of animals, mice were subjected to repeated exposure to CWSS, twice daily for 3 days and body temperature was monitored both immediately prior to each CWSS exposure and after 10 min. The body temperatures as determined immediately prior to CWSS exposures 1-6 were 36.1 ± 0.40, 35.8 ± 0.56, 35.1 ± 0.66, 35.8 ± 0.41, 34.4 ± 0.37 and 34.5 ± 0.22°C, respectively, while the corresponding values determined 10 min after the CWSS exposure were 26.0 ± 0.94, 25.2 ± 0.72, 26.0 ± 0.89, 26.8 ± 0.75, 26.1 ± 0.95 and 26.4 ± 0.66°C. Evaluation of the data by ANOVA revealed no significant differences in either the pre-CWSS group or in the post-CWSS group (Figure 7).

Finally, assessment of body temperature prior to, and 24 hr after injection with β-FNA, DALCE and 5'-NTII, revealed no changes between the groups (data not shown).

Characterization of the CWSS-induced antinociceptive response: studies with antagonists. The selective δ opioid agonist DPDPE at 30 nmol, *i.c.v.*, an A_{90} dose in the mouse tail-flick test (55°C)(Mattia et al., 1991a), is antagonized in a dose response fashion to the general opioid antagonist naloxone given *s.c.* (Figure 8). The calculated A_{50} for naloxone is 0.370 with (95% confidence intervals) of (0.215-0.636) mg/kg. Similar results were found using an A_{90} dose of the selective δ agonist [D-Ala^2,Glu^4]deltorphin (12.8 nmol, *i.c.v.*)(Mattia et al., 1991a)(Figure 9). The A_{50} for naloxone in the presence of [D-Ala^2,Glu^4]deltorphin is 0.388 (0.140-1.076) mg/kg.

Similar experiments were done using the same dose of DPDPE in the presence of the selective δ opioid receptor antagonist ICI 174,864. The A_{50} for ICI 174,864 in the presence of DPDPE was 1.37 (0.555-3.360) nmol (Figure 10) and 1.242 (0.461-3.343) nmol in the presence of [D-Ala^2,Glu^4]deltorphin (Figure 11).

Due to the robust antinociceptive effect produced by CWSS-exposure at the 5°C water temperature, all further characterization was done using CWSS at this temperature. Exposure of mice to CWSS resulted in a strong antinociceptive response when determined after 10 min (Figure 12). Pretreatment with naloxone (0.3, 1 or 3 mg/kg, *s.c.*, 7 min prior to the 3 min CWSS or 20 min prior to antinociceptive testing) produced a significant inhibition of the CWSS-induced antinociception; the naloxone AD_{50} (and 95%

C.L.) against CWSS-induced antinociception was calculated to be 0.19 (0.072 - 0.53) mg/kg. Similarly, pretreatment with graded *i.c.v.* doses of the δ selective antagonist, ICI 174,864 (2 min prior to CWSS or 15 min prior to antinociceptive testing) produced a significant antagonism of the antinociceptive response; the ICI 174,864 AD_{50} (and 95% C.L.) against CWSS-induced antinociception was 2.19 (0.43 - 11.1) nmol. Pretreatment of the mice with a dose of the κ selective antagonist, nor-BNI (1 nmol) which selectively blocks κ-mediated antinociception (Horan et al., 1991; 1992), failed to antagonize the CWSS-induced antinociceptive response.

Pretreatment 24 hr prior to testing with the μ-selective antagonist, β-FNA (18.8 nmol, *i.c.v.*), or with the δ_1 antagonist, DALCE (4.5 nmol, *i.c.v.*), did not alter the CWSS-induced antinociceptive response (Figure 12). In contrast, pretreatment with the δ_2-selective antagonist, 5'-NTII (17.5 nmol, *i.c.v.*, at -24 hr), effectively antagonized the CWSS-induced antinociceptive response (Figure 12).

Pretreatment with naloxone, (0.5 μg, *i.th.*, 2 min prior to the 3 min CWSS or 15 min prior to antinociceptive testing) produced a significant inhibition of the CWSS-induced antinociception (Figure 13). Similarly, pretreatment with an *i.th.* dose of the δ selective antagonist, ICI 174,864 (2 min prior to CWSS or 15 min prior to antinociceptive testing), produced a significant antagonism of the antinociceptive response (Figure 13).

Pretreatment with the α_2-adrenergic antagonist, yohimbine (1 μg, *i.th.*), or with the serotonin antagonist, methysergide (1 μg, *i.th.*), did not alter the CWSS-induced antinociceptive response (Figure 13).

CWSS-induced antinociception in mice tolerant to the antinociceptive effects of DAMGO, DPDPE and [D-Ala^2, Glu^4]deltorphin. Pretreatment of mice with the selective opioid agonists, DAMGO, DPDPE and [D-Ala^2, Glu^4]deltorphin, has previously been shown to produce antinociceptive tolerance to each agonist, but no cross-tolerance between these agents (Mattia et al., 1991a). Using the doses and injection schedule from these previous studies, the effect of induction of antinociceptive tolerance to each of these selective agonists on the CWSS-induced response was evaluated. CWSS-exposure produced an effective antinociceptive response in the control group, while administration of an A_{90} dose of DAMGO, DPDPE or [D-Ala^2, Glu^4]deltorphin produced a similar response in mice pretreated for several days with saline (Figure 14). Pretreatment of mice with DAMGO, DPDPE or [D-Ala^2, Glu^4]deltorphin, produced tolerance to each of these agents, as shown by the decreased response to each of the agonists in pretreated mice, respectively (Figure 14). While CWSS-exposure produced an effective antinociceptive response in control mice, or in mice tolerant to DAMGO or DPDPE, CWSS-exposure was

minimally active in mice tolerant to the antinociceptive actions of [D-Ala^2, Glu^4]deltorphin (Figure 14).

Evaluation of selective agonist-induced antinociception in mice tolerant to CWSS-induced antinociception. Repeated exposure of different groups of mice to CWSS resulted in a progressive diminution to the antinociceptive response observed after the first exposure (Figure 15). Significant decreases in CWSS-induced antinociception were observed by the third CWSS-exposure (i.e., morning of day 2), and the antinociceptive response continued to decrease so that both exposures on day 3 resulted in virtually no antinociceptive response. Antinociceptive dose-response lines to DAMGO, DPDPE and [D-Ala^2, Glu^4]deltorphin were generated in control animals, or in mice subjected to 6 CWSS-exposures (i.e., on the morning of day 4). While the dose effect lines for DAMGO (Figure 16) and DPDPE (Figure 17) were unaltered by prior exposure to 6 episodes of CWSS, the dose response line to [D-Ala^2, Glu^4]deltorphin was significantly displaced to the right by 3.4-fold in CWSS-tolerant mice (Figure 18). The calculated A_{50} values (and 95% C.L.) in control and in CWSS-tolerant mice were 0.06 (0.05-0.08) and 0.06 (0.05 - 0.08) for DAMGO, 15.9 (12.0 - 19.9) and 16.5 (13.2 - 19.9) for DPDPE and 4.2 (3.5 - 4.8) and 14.2 (10.0 - 18.4) for [D-Ala^2, Glu^4]deltorphin.

CWSS antinociception in the presence of either [Leu^5]- or [Met^5]enkephalin antisera. Exogenous *i.c.v.* administration of [Met^5]enkephalin at a dose of 17.4 nmol has no antinociceptive nor hyperalgesic effect by itself, but when coadministered with morphine will shift the dose response curve of morphine to the right 2-fold. Mice pretreated 10 min prior to morphine/[Met^5]enkephalin with antisera to [Met^5]enkephalin (200 μg/5 μl) but not antisera raised against [Leu^5]enkephalin (200 μg/5 μl) blocked the rightward shift (Figure 19).

The *i.c.v.* administration of [Leu^5]enkephalin at a dose of 4.5 nmol has no antinociceptive nor hyperalgesic effect by itself, but when coadministered with morphine will shift the dose response curve of morphine to the left over 3-fold. Mice pretreated 10 min prior to morphine/[Leu^5]enkephalin with antisera to [Leu^5]enkephalin (200 μg/5 μl) but not antisera raised against [Met^5]enkephalin (200 μg/5 μl) blocked the leftward shift (Figure 20).

Pretreatment of mice with graded doses of *i.c.v.* antibody to either [Leu^5]enkephalin or [Met^5]enkephalin had no direct antinociceptive, hyperalgesic or observable behavioral effects (data not shown) and did not alter morphine antinociceptive potency (Figure 21).

The direct antinociceptive effects of CWSS were antagonized in a dose-response fashion in animals pretreated with [Leu^5]enkephalin antisera. There was no change from control in CWSS exposed animals that were pretreated with antisera raised against [Met^5]enkepalin at three different doses (Figure 22).

CWSS antinociception in the presence of δ-opioid antisense or sense oligodeoxynucleotide

Animals treated twice a day for three days with either a δ-opioid antisense or sense oligo were exposed to the CWSS on day four. The antinocicpetive effect of CWSS was significantly attenuated in animals pretreated with δ antisense, yet the animals treated with the δ sense oligo were no different than control (Figure 23).

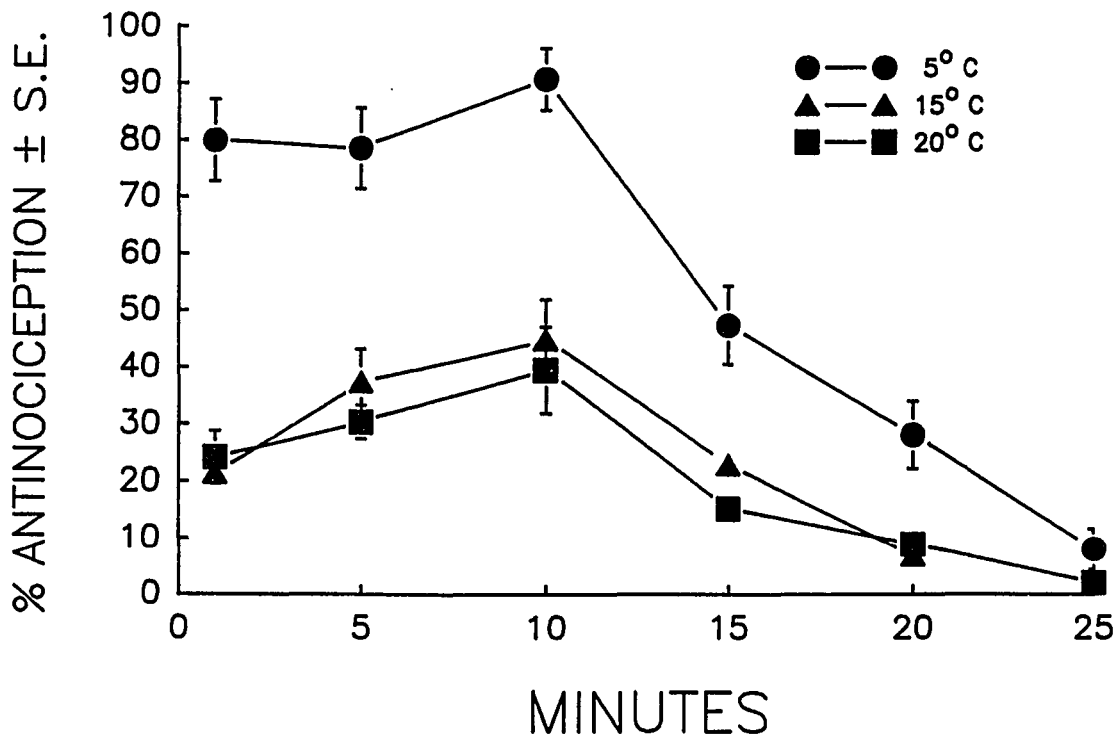

Figure 5. Time course of antinociception following exposure of mice for 3 min to water at 20, 15 and 5°C. Each data point represents the mean of data from 10 animals.

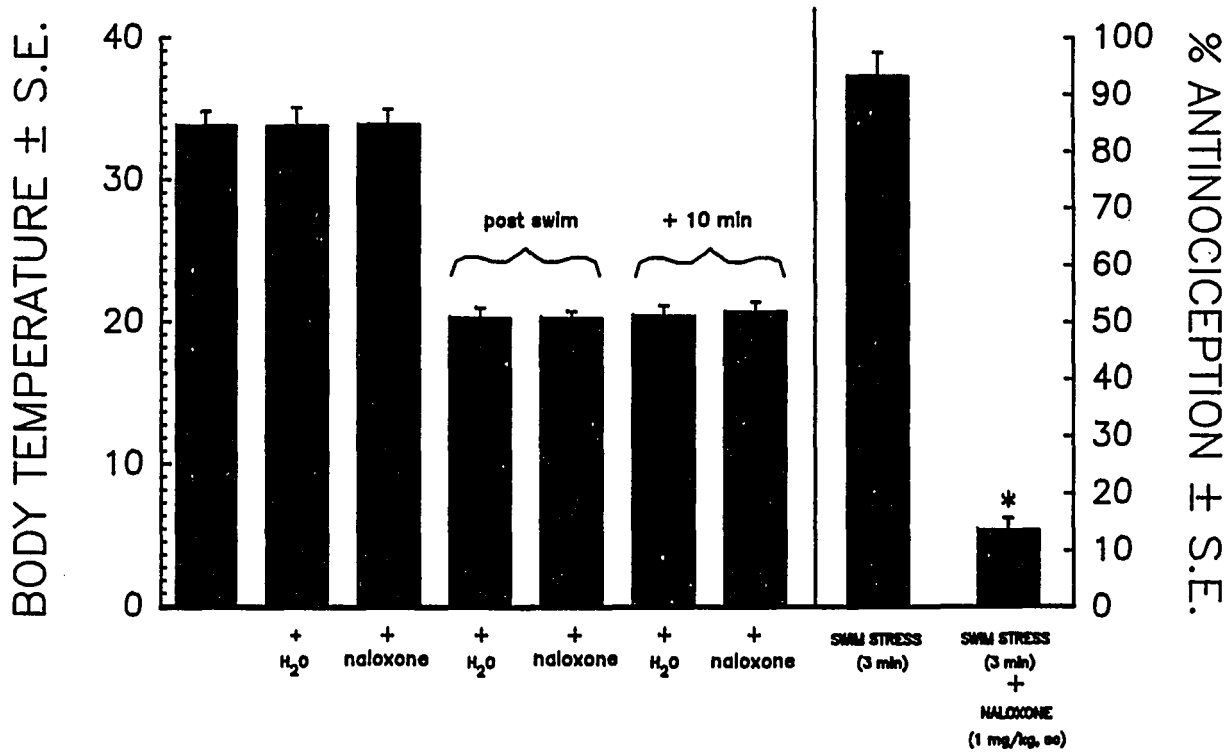

Figure 6. Core body temperature measurements in mice subjected to the following conditions: no CWSS, no CWSS/vehicle, no CWSS/naloxone, CWSS/vehicle (temperature immediately after CWSS), CWSS/naloxone (temperature immediately after CWSS), CWSS/vehicle (temperature at + 10 min after CWSS) and CWSS/naloxone (temperature at +10 min after CWSS). Naloxone or vehicle were always given *s.c.* 7 min prior to CWSS (i.e., corresponding to 20 min prior to antinociceptive testing). In the right panel, the antinociceptive effect of CWSS is shown in mice with and without naloxone given *s.c.* 20 min prior to antinociceptive testing.

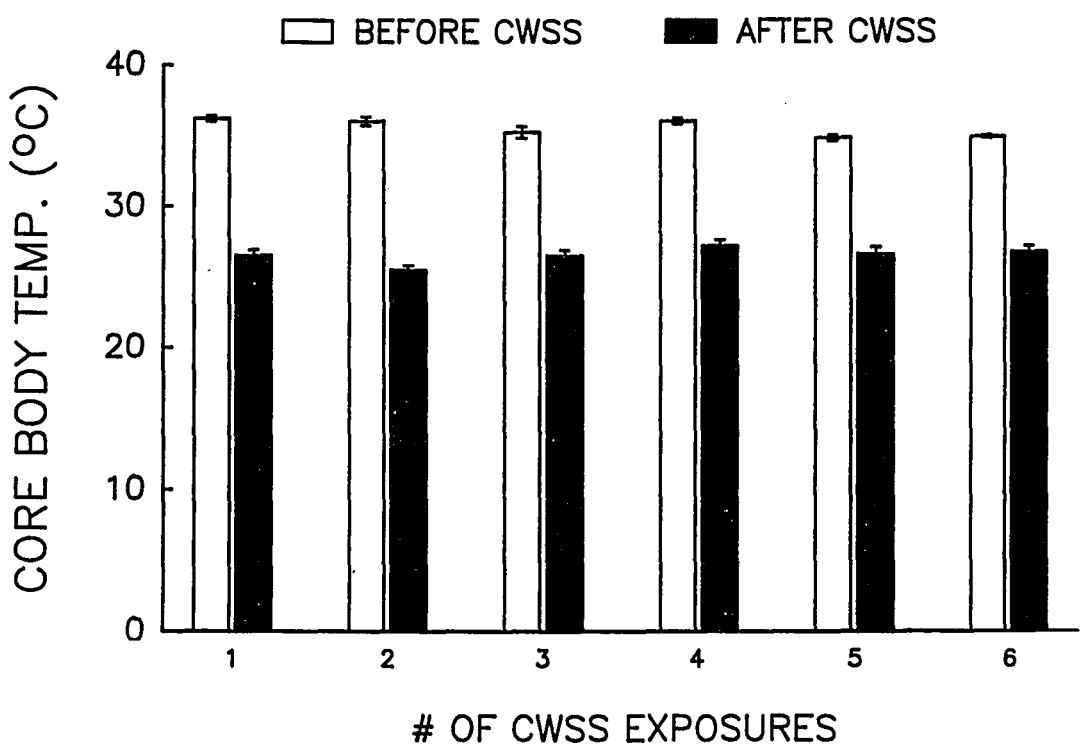

Figure 7. Core body temperature before (closed bars) and 10 min after repeated exposure to CWSS (3 min at 5° C). Body temperature was significantly reduced following each exposure.

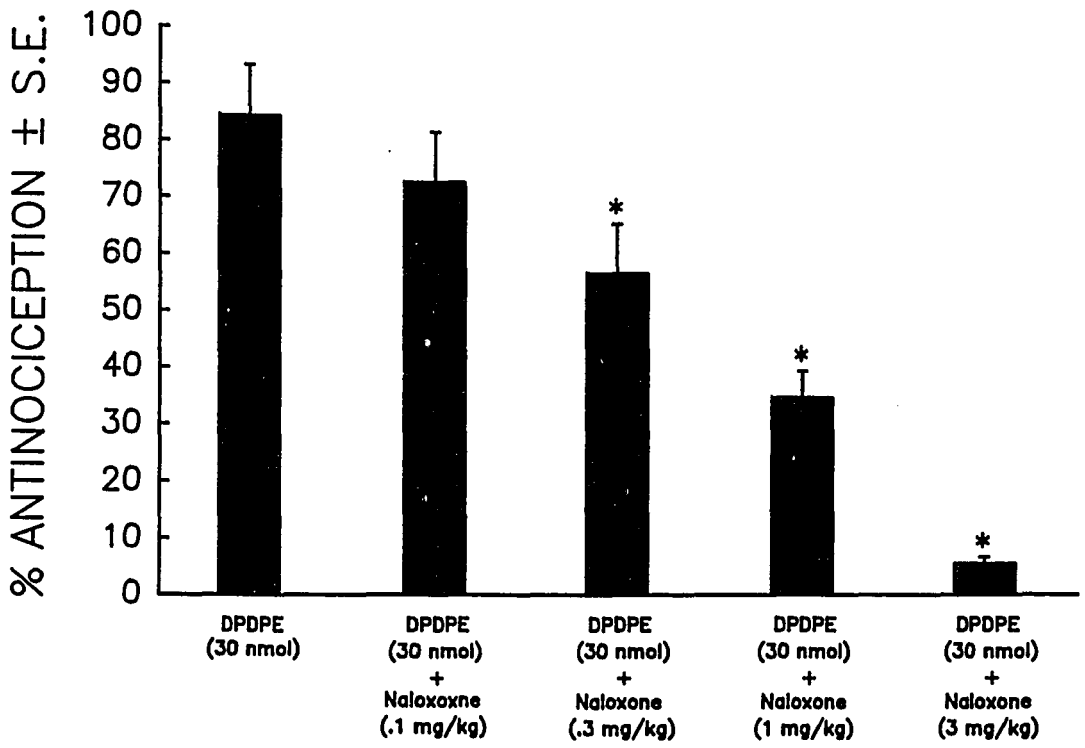

Figure 8. The antinociceptive effects of an A_{90} dose of the δ agonist DPDPE in control mice, or in seperate groups of mice pretreated with different doses of the opioid antagonist naloxone. Naloxone by itself produces no antinociception. Each bar represents a group of ten seperate mice.

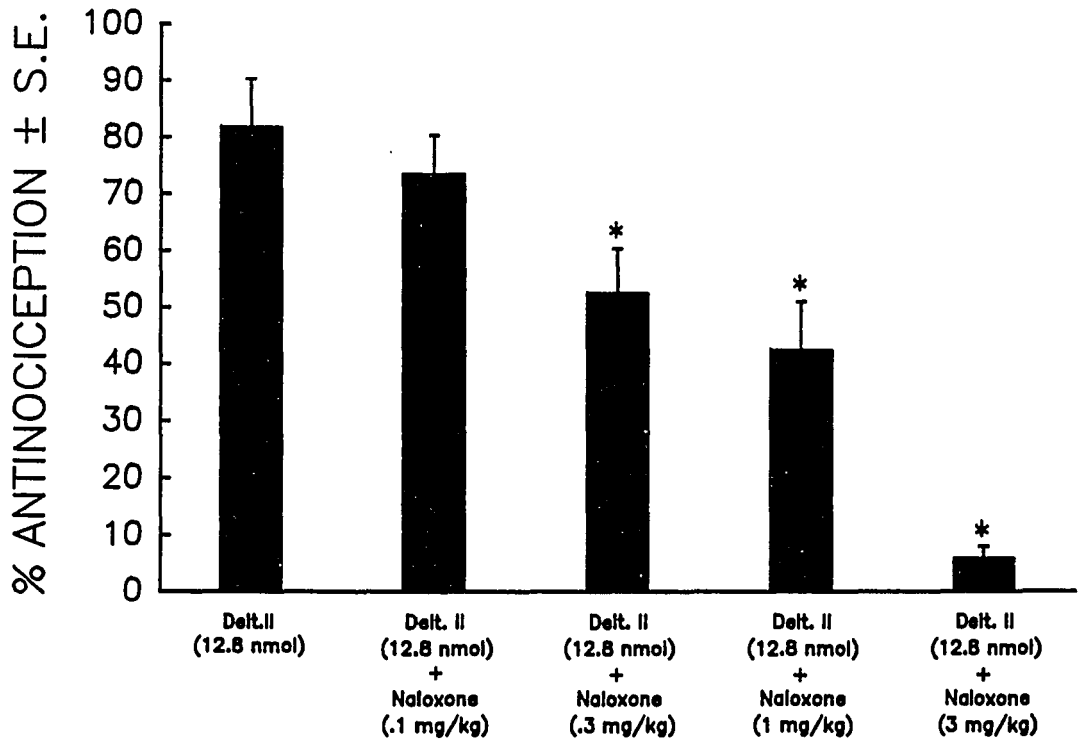

Figure 9. The antinociceptive effects of an A_{90} dose of the δ agonist [D-Ala^2, Glu^4]deltorphin in control mice, or in seperate groups of mice pretreated with different doses of the opioid antagonist naloxone. Naloxone by itself produces no antinociception. Each bar represents a group of ten seperate mice.

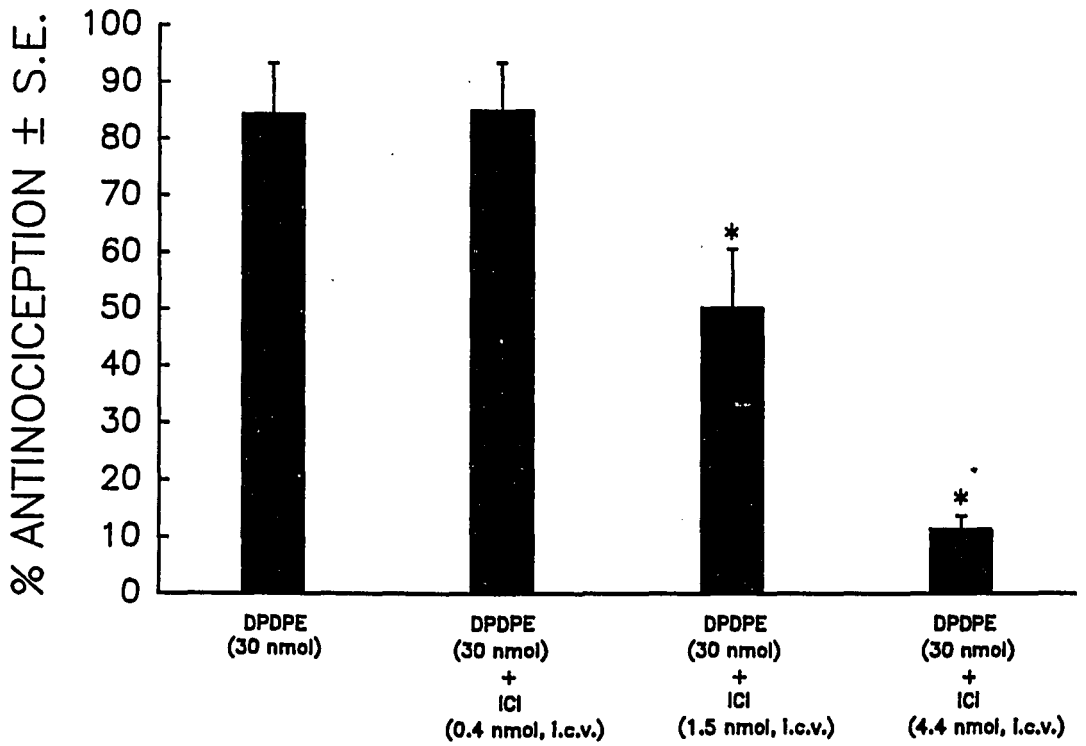

Figure 10. The antinociceptive effects of an A_{90} dose of the δ agonist DPDPE in control mice, or in seperate groups of mice coadministered with different doses of the δ opioid antagonist ICI 174,864. ICI 174,864 by itself produces no antinociception. Each bar represents a group of ten seperate mice.

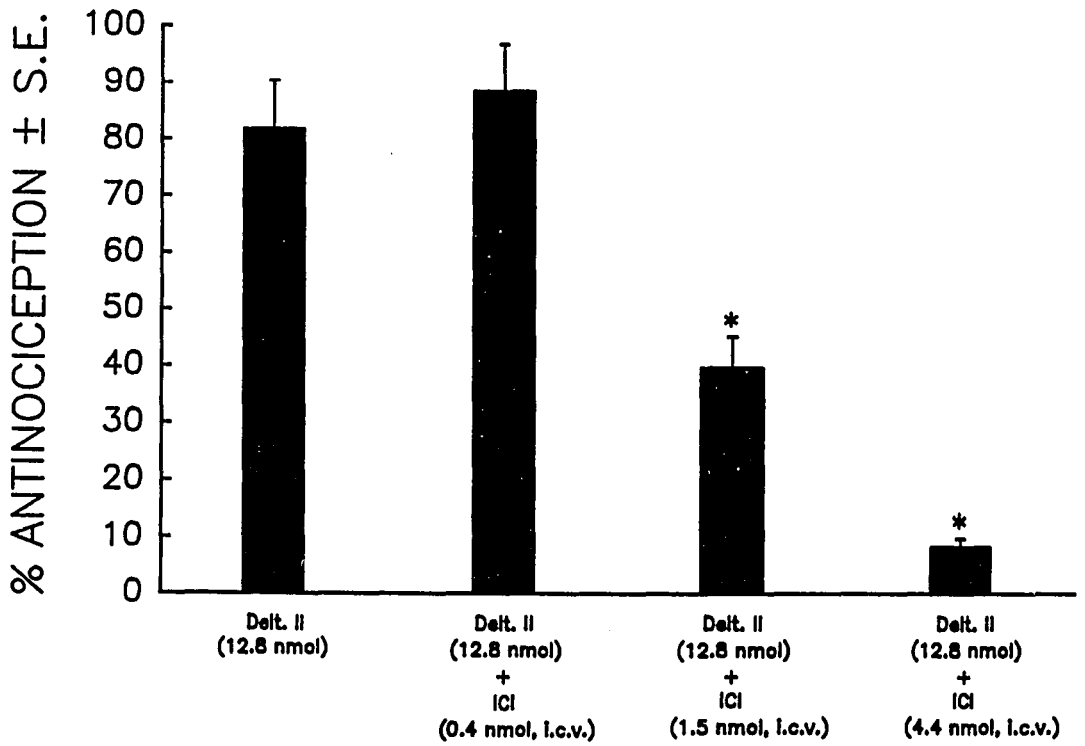

Figure 11. The antinociceptive effects of an A_{90} dose of the δ agonist [D-Ala^2, Glu^4]deltorphin in control mice, or in seperate groups of mice coadministered with different doses of the δ opioid antagonist ICI 174,864. ICI 174,864 by itself produces no antinociception. Each bar represents a group of ten seperate mice.

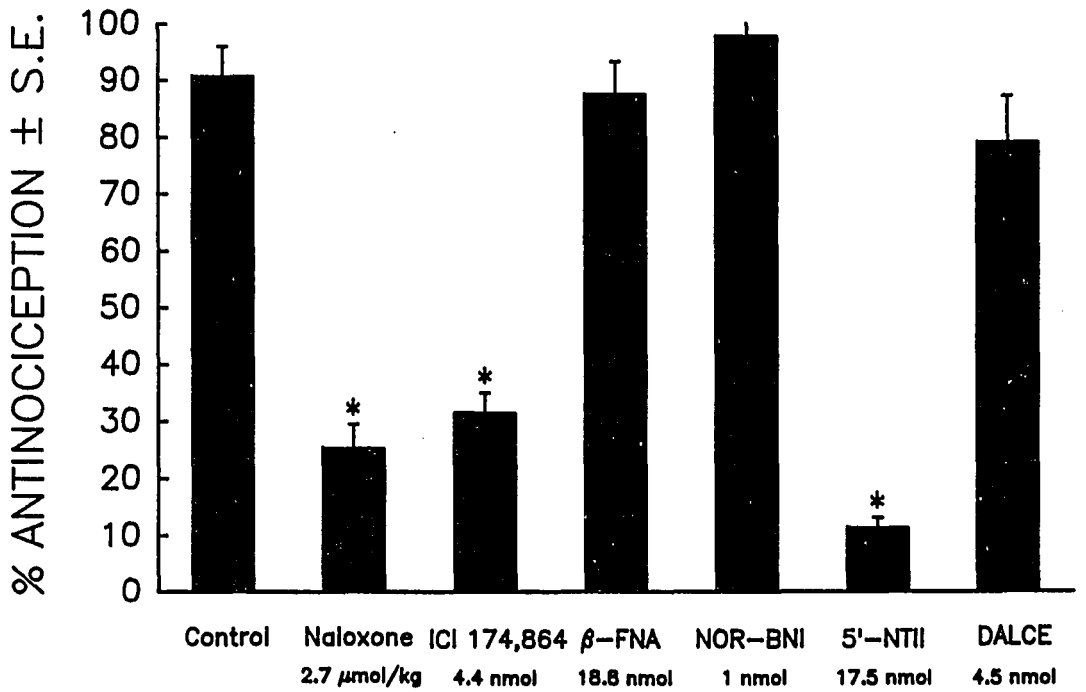

Figure 12. Effects of selective opioid antagonists on CWSS-induced antinociception, ten min after exposure, in the mouse tail-flick test. Naloxone was given *s.c.* 7 min prior to CWSS, ICI 174,864 was given *i.c.v.* 2 min prior to CWSS, nor-BNI was given *i.c.v.* 7 min prior to CWSS and β-FNA, 5'-NTII and DALCE were given *i.c.v.* 24 hr prior to CWSS. Antinociceptive testing took place 10 min after CWSS exposure using 10 mice per group.

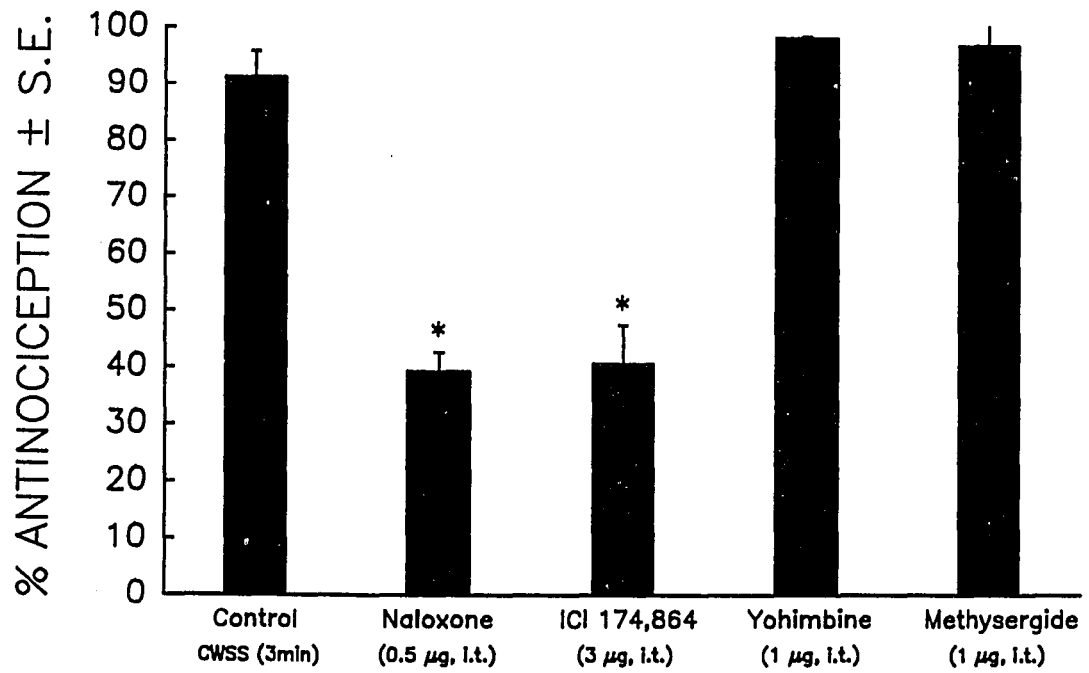

Figure 13. Effects of selective opioid antagonists on CWSS-induced antinociception, ten min after exposure, in the mouse tail-flick test. Naloxone was given *i.th.* 2 min prior to CWSS, ICI 174,864 was given *i.th.* 2 min prior to CWSS, yohimbine and methysergide were given *i.th.* 2 min prior to CWSS. Antinociceptive testing took place 10 min after CWSS exposure using 10 seperate mice per group.

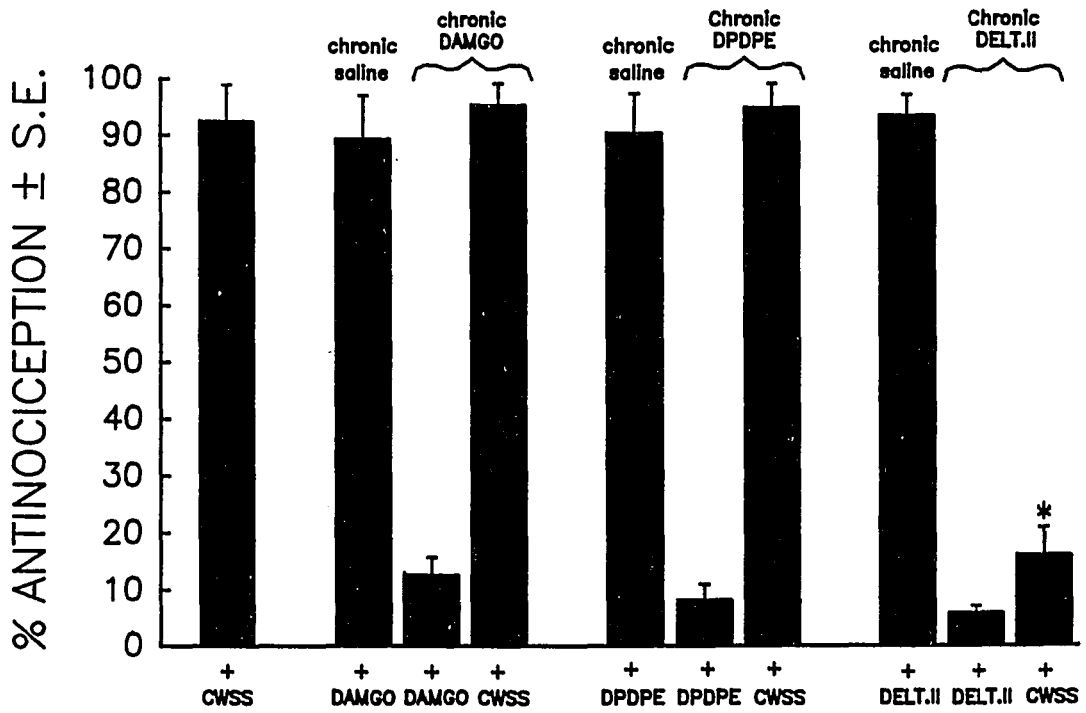

Figure 14. Effects of CWSS-exposure or selective *i.c.v.* agonists in mice pretreated with *i.c.v.* saline or with DAMGO, DPDPE or [D-Ala^2, Glu^4]deltorphin for 3 days in the mouse tail-flick test. Groups of 10 mice were tested on the morning of day 4 with either CWSS, or with DAMGO, DPDPE or [D-Ala^2, Glu^4]deltorphin. Effects of CWSS- exposure in non-pretreated mice are seen in the first bar. All antinociceptive testing took place 10 min after the *i.c.v.* agonist or the CWSS exposure.

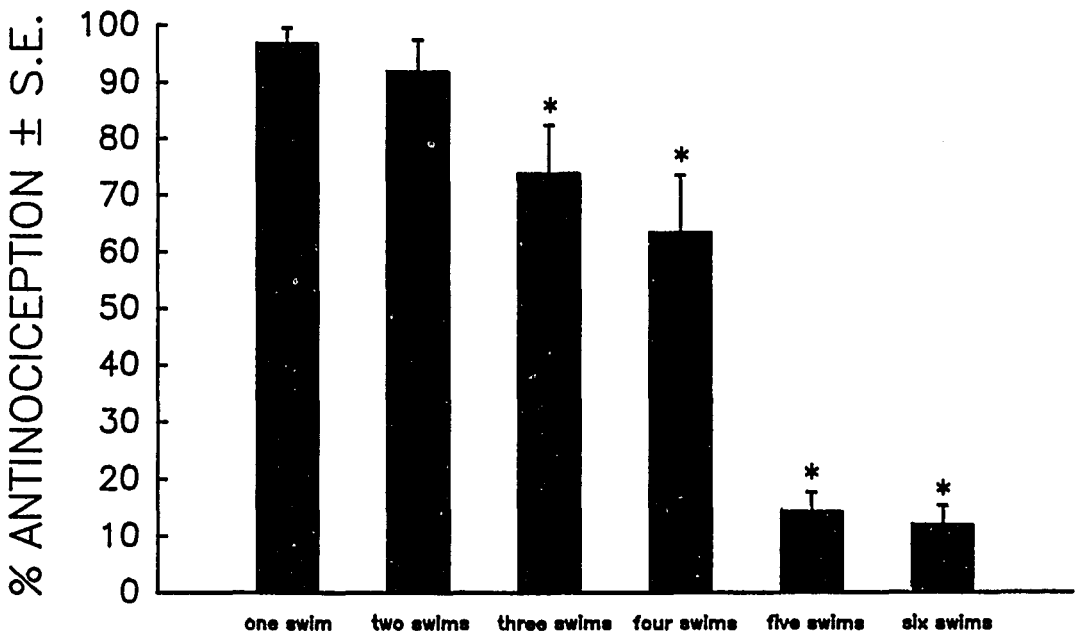

Figure 15. Effects of repeated exposure to CWSS in separate groups of 10 mice over a period of 3 days. Mice were subjected to CWSS at 0800 hr and again at 1800 hr up to a maximum of six times and tested 10 min after CWSS. Each group of mice was tested only once after the appropriate number of exposures.

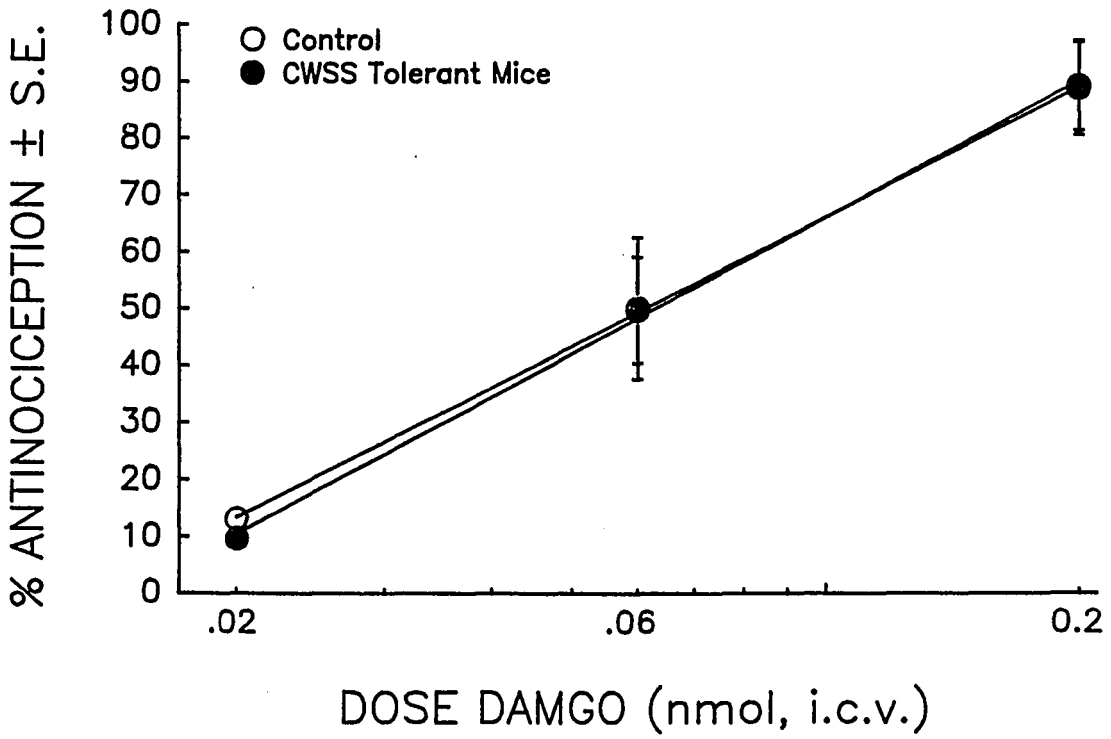

Figure 16. Dose-response lines for *i.c.v.* DAMGO in control or in CWSS-tolerant mice; 10 mice were used per group.

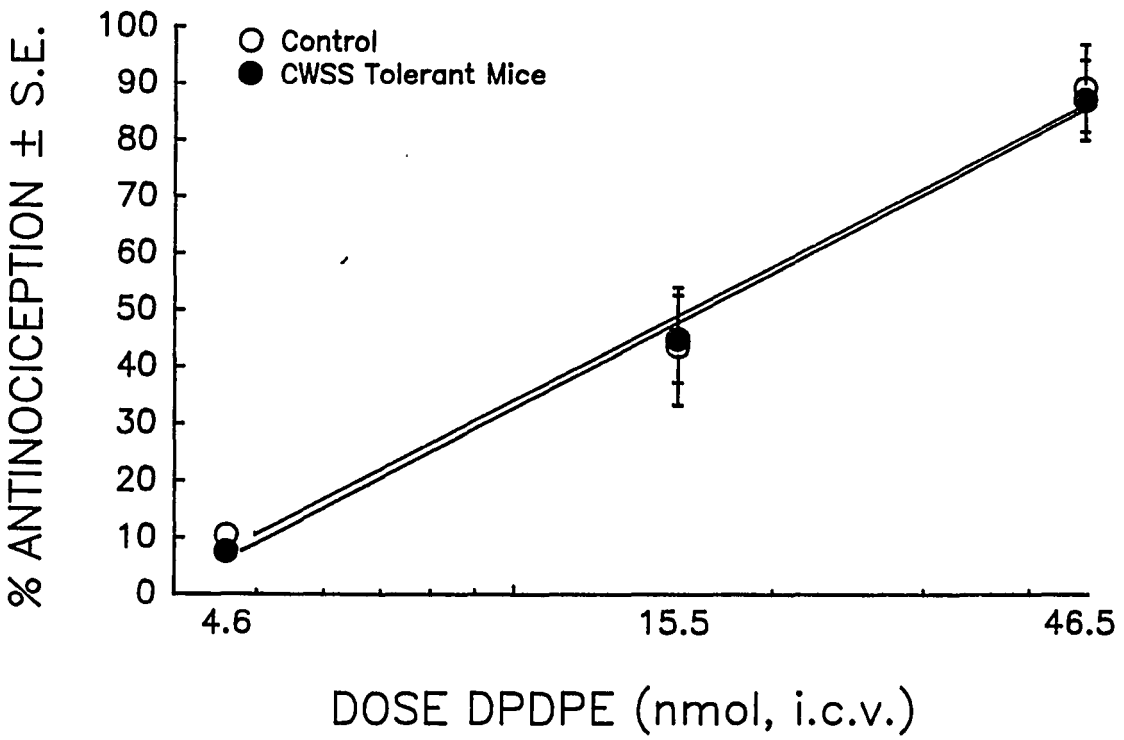

Figure 17. Dose-response lines for *i.c.v.* DPDPE in control or in CWSS-tolerant mice; 10 mice were used per group.

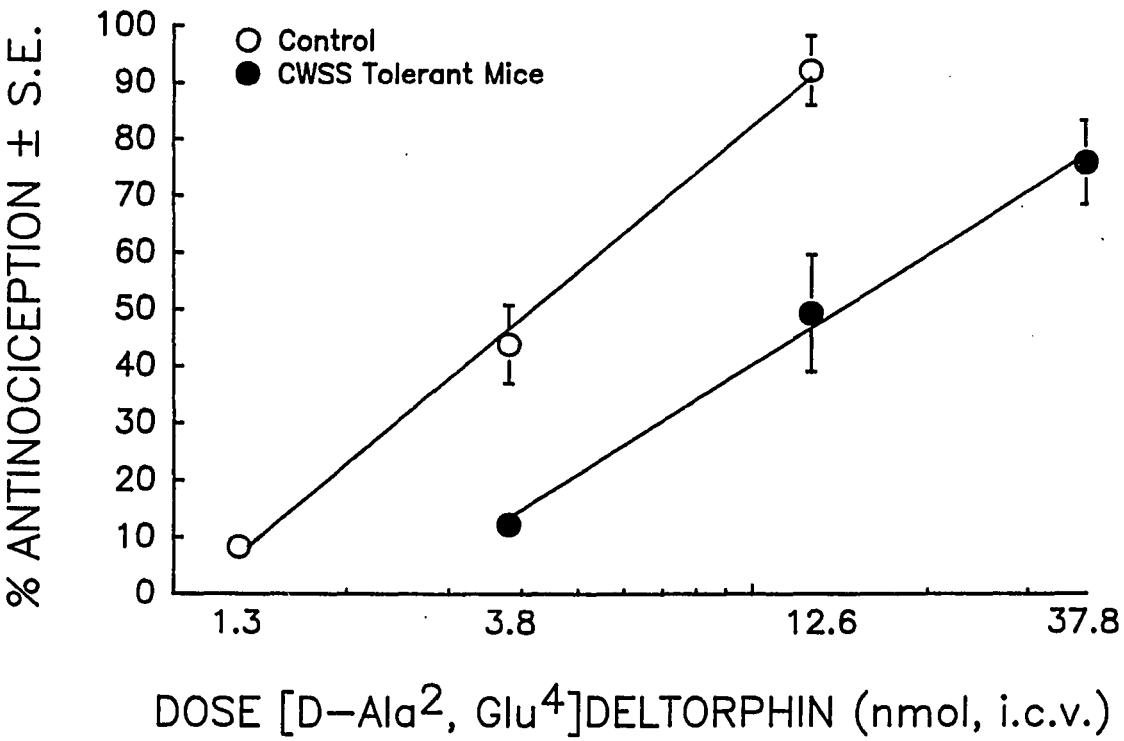

Figure 18. Dose-response lines for *i.c.v.* [D-Ala^2, Glu^4]deltorphin in control or in CWSS-tolerant mice; 10 mice were used per group.

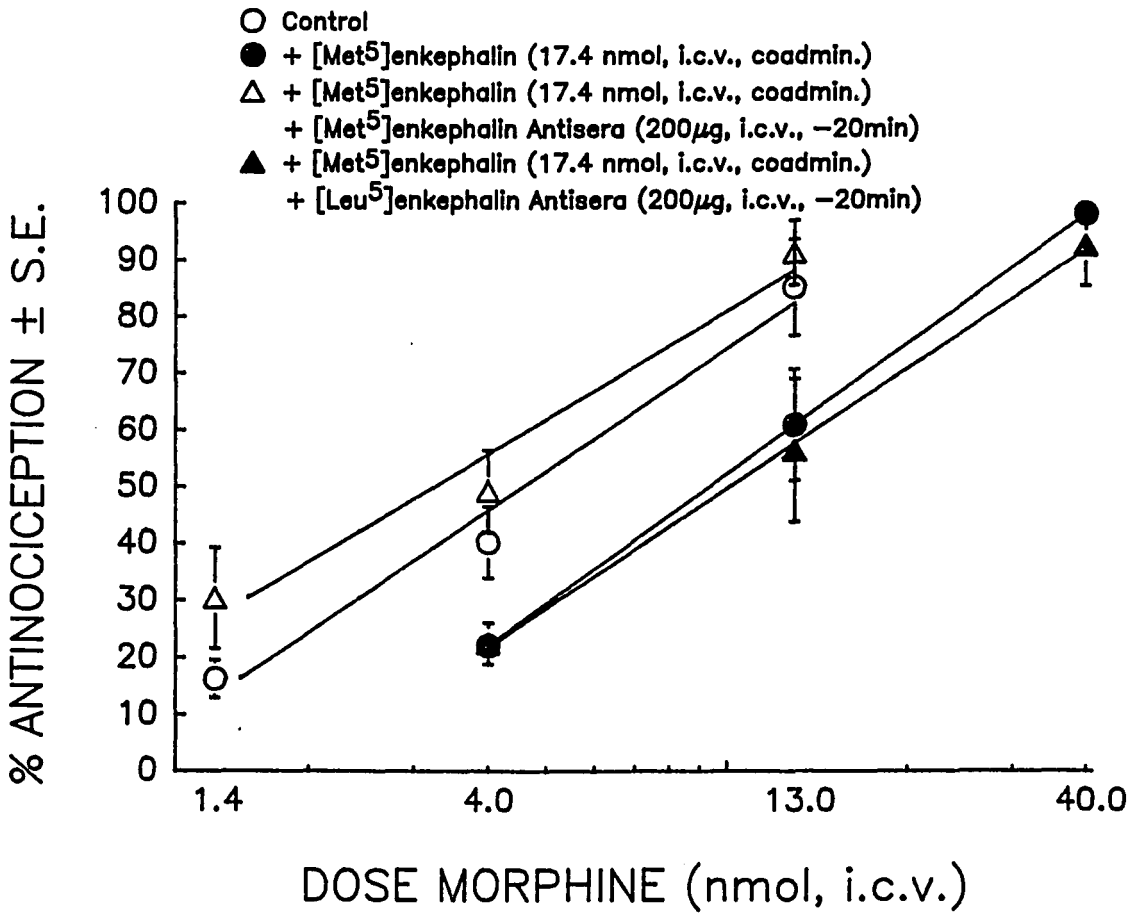

Figure 19. *I.c.v.* morphine dose-response lines in control (5 μl saline *i.c.v.*) mice, or with co-administration of [Met^5]enkephalin 10 min before testing in the warm-water tail-flick test. Dose-response lines for *i.c.v.* morphine are shown in the absence or presence of *i.c.v.* [Leu^5]enkephalin antisera or [Met^5]enkephalin antisera. Neither [Leu^5]enkephalin antisera, [Met^5]enkephalin antisera or [Met^5]enkephalin alone produced any behavioral or antinociceptive effect. [Met^5]enkephalin antisera, but not [Leu^5]enkephalin antisera, blocked the rightward displacement of the morphine dose-response line produced by *i.c.v.* [Met^5]enkephalin, confirming the specificity of the antibodies in this assay.

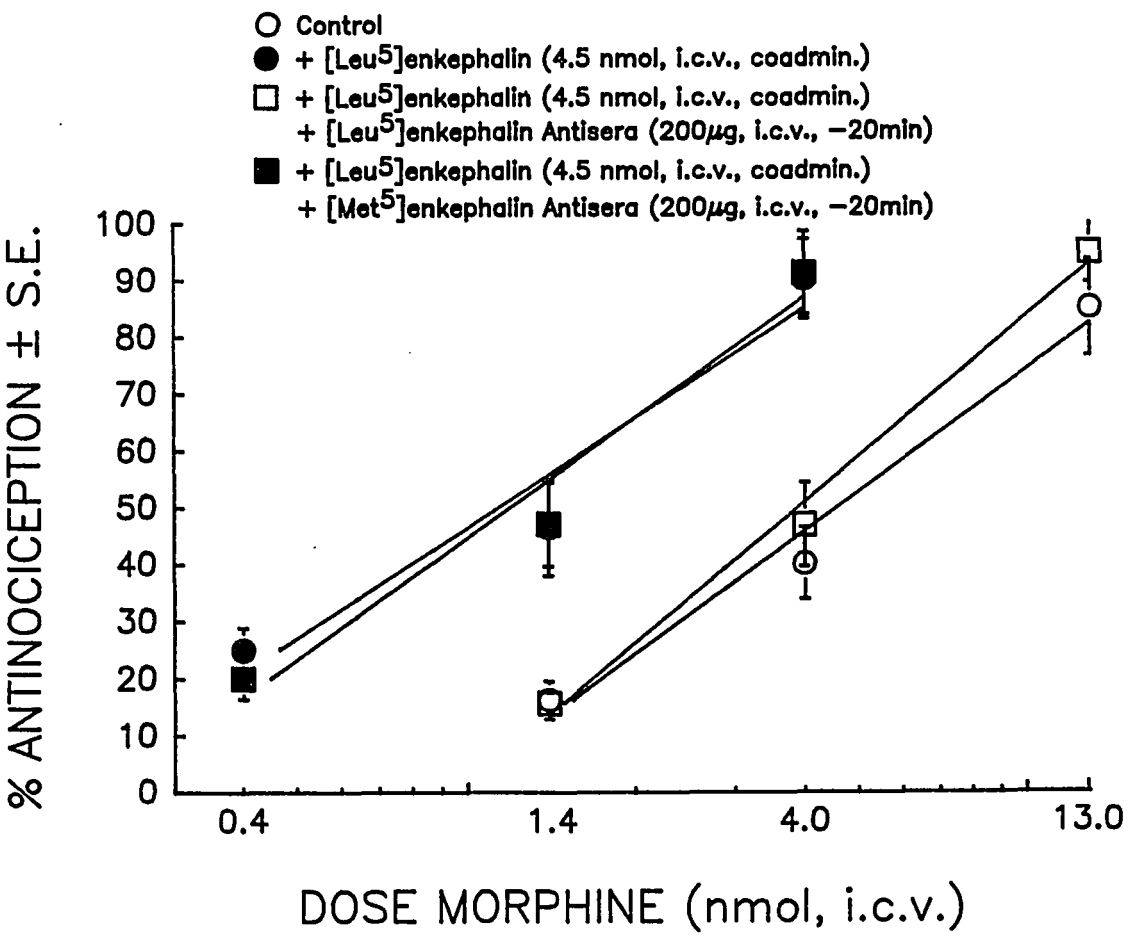

Figure 20. *I.c.v.* morphine dose-response lines in control (5 μl saline *i.c.v.*) mice, or with co-administration of [Leu^5]enkephalin 10 min before testing in the warm-water tail-flick test. Dose-response lines for *i.c.v.* morphine are shown in the absence or presence of *i.c.v.* [Leu^5]enkephalin antisera or [Met^5]enkephalin antisera. Neither [Leu^5]enkephalin antisera, [Met^5]enkephalin antisera or [Leu^5]enkephalin alone produced any behavioral or antinociceptive effect. [Leu^5]enkephalin antisera, but not [Met^5]enkephalin antisera, blocked the leftward displacement of the morphine dose-response line produced by *i.c.v.* [Leu^5]enkephalin, confirming the specificity of the antibodies in this assay.

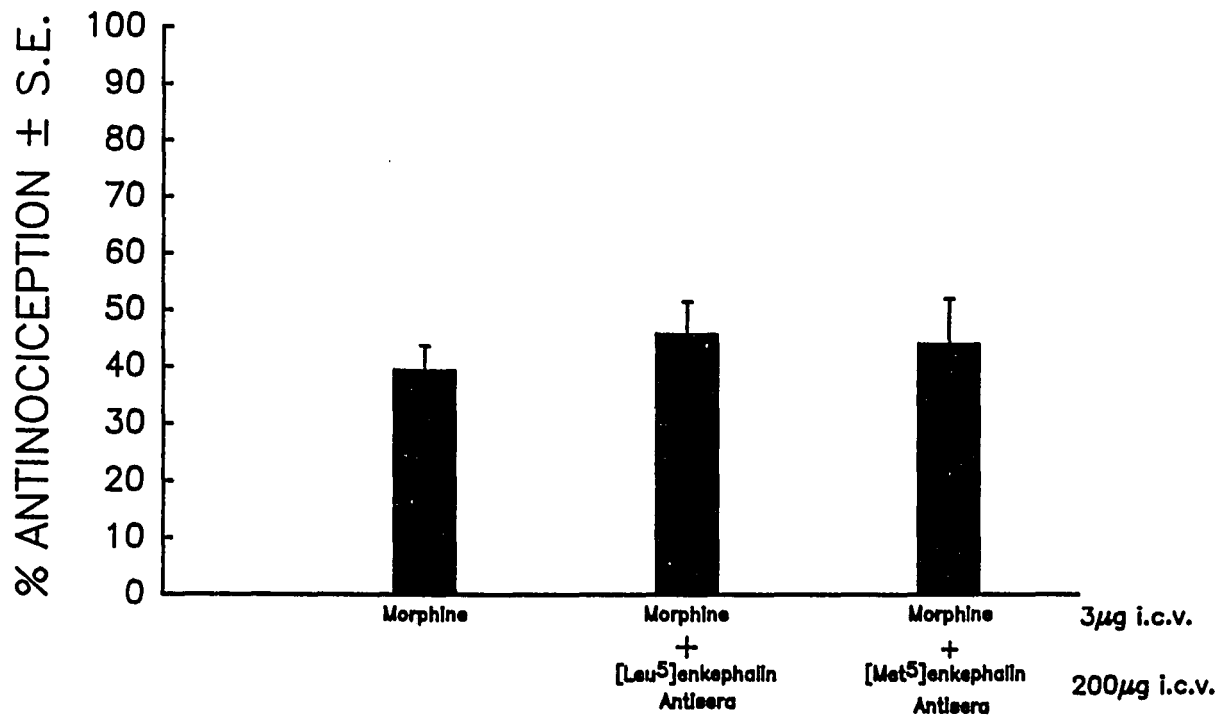

Figure 21. The antinocicpetive effects of an A_{50} dose of morphine in control mice, or in mice pretreated 10 min prior to morphine with antisera (*i.c.v.*, 200 μg/5μl) to either [Leu^5]- or [Met^5]enkephalin. The antisera themselves produce no antinociception or hyperalgesia.

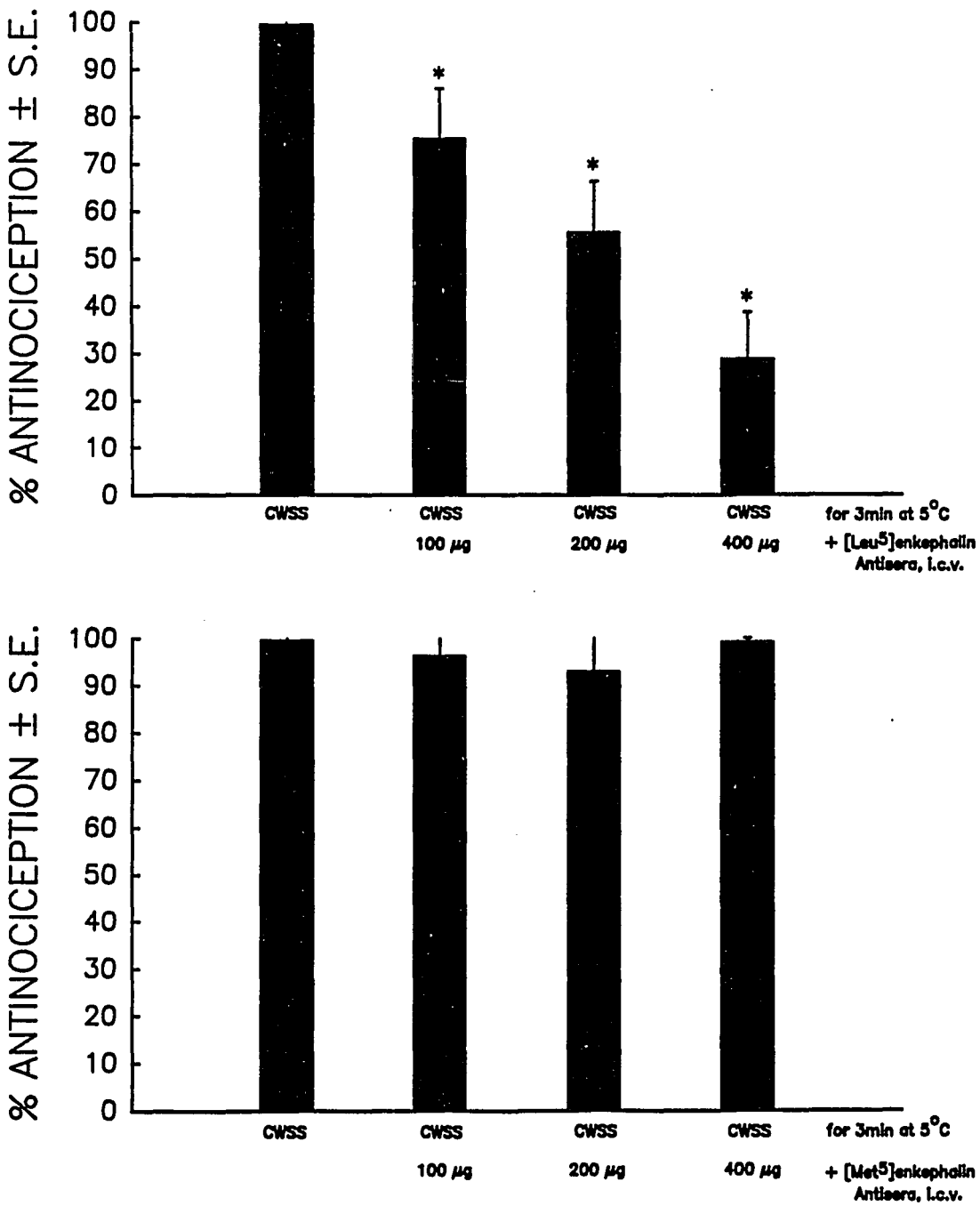

Figure 22. The antinociceptive effects of a 3 min CWSS tested 10 min after exposure in control mice, or in mice pretreated 7 min, *i.c.v.* with increasing concentrations of antisera to either [Leu^5]- or [Met^5]enkephalin. Each bar represents a group of ten seperate animals.

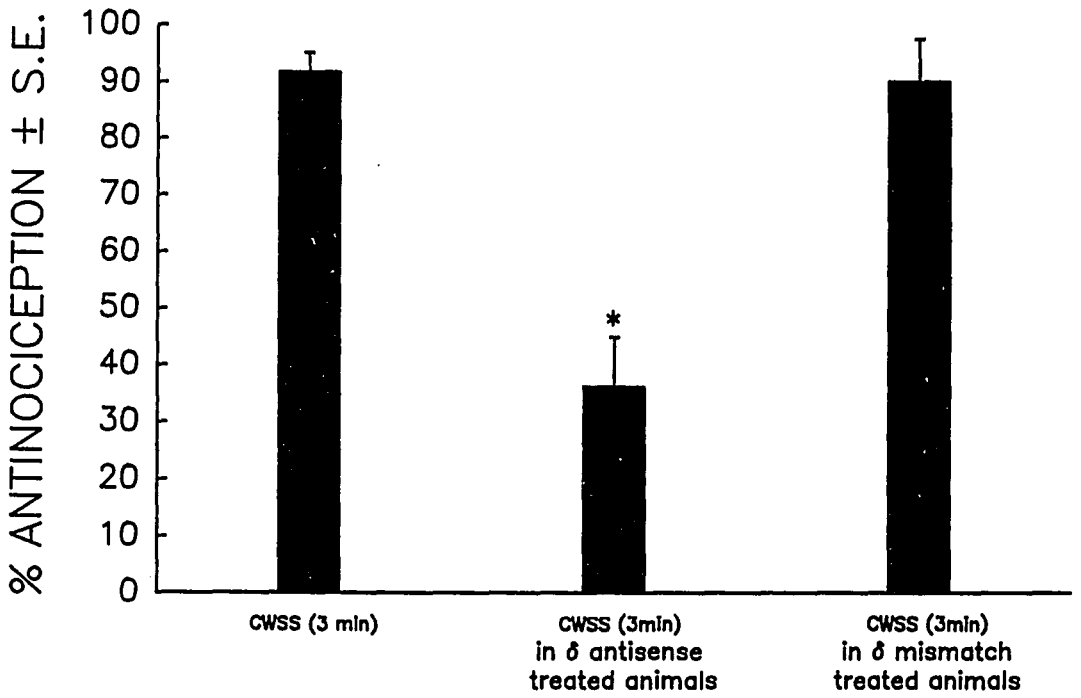

Figure 23. CWSS antinocicpetion from a 3 min exposure in control mice, or in mice treated twice a day for three days with either a δ antisense oligo (*i.c.v.*, 12.5 μg/5 μl) or a δ sense oligo (*i.c.v.*, 12.5 μg/5 μl). Mice were examined in the 55°C tail-flick test on the day after the last antisense or sense injection. Each bar represents 10 seperate animals.

SUMMARY OF SWIM-STRESS STUDIES

The present study has investigated the possibility of activation of the opioid systems using a stress paradigm in mice. Recently, Tierney et al. (1991) have reported that exposure of mice to swim-stress for 3 min in water at 20°C results in a significant degree of antinociception when evaluated in the tail-flick test using water at 47°C as the noxious stimulus. In our experiments, exposure of mice to swim-stress using the same conditions for the same time resulted in a modest antinociceptive response, possibly due to the more severe nature of the antinociceptive test in which water at 55°C was employed as the noxious stimulus. For this reason, the severity of the stress was also increased with mice being exposed to water at 5°C for a 3 min period. Under these conditions, a nearly maximal antinociceptive response was observed which persisted a period of approximately 15-20 min. As exposure to this water temperature was expected to produce a significant decrease in the animals' body temperature, the possibility that the observed antinociceptive response might be related to changes in temperature was investigated in three ways. First, mice were treated either with vehicle or with naloxone (at a dose which was shown to antagonize the antinociceptive response resulting from CWSS), and rectal temperature was monitored prior to and 10 min after CWSS. Though no differences were observed in body temperature between the control and naloxone treated groups, antinociception could be elicited only in the

control groups. Second, rectal temperature was monitored before and 10 min after CWSS successively for 6 swims over a 3 day period. While tolerance was observed to the antinociceptive response elicited by CWSS, no changes in body temperature were observed over the six swim period. That is, body temperature decreased consistently after each CWSS exposure, but significant antinociception was not observed after the fourth CWSS exposure. The almost complete reduction in antinociceptive effect in animals subjected to 5 or 6 CWSS episodes, together with the consistent reduction in body temperature seen after the first CWSS exposure, suggests that the antinociceptive effect is not related to changes in temperature. Finally, animals were pretreated 24 hr prior to testing with either β-FNA, DALCE or 5'-NTII given by the *i.c.v.* route; no differences in body temperature were observed in these animals 24 hr after pretreatment and, as expected, a decrease in body temperature resulted after exposure to CWSS. Nevertheless, the CWSS-induced antinociceptive response was not altered in the β-FNA or DALCE groups, but was antagonized in the 5'-NTII group. Collectively, these observations suggest that the observed antinociceptive response is not the result of non-specific alteration in body temperature regulation, but is the result of activation of endogenous systems, as previously suggested by several authors (e.g., Lewis et al., 1980; Watkins and Mayer, 1982; Terman et al., 1984).

Characterization of the CWSS-induced antinociceptive response using antagonists revealed that this effect was opioid in nature, as it was antagonized by naloxone. Additionally, the opioid effect appeared to be mediated through δ receptors in that the selective δ antagonist, ICI 174,864, but not the μ selective antagonist, β-FNA, nor the κ antagonist, nor-BNI, antagonized this response. Furthermore, the δ_2 antagonist 5'-NTII, but not the δ_1 antagonist DALCE, effectively blocked the CWSS-induced antinociceptive response suggesting mediation via this subtype of the δ receptor. This possibility was evaluated further using tolerance and cross-tolerance approaches.

Previous work from our laboratory has demonstrated that pretreatment of mice with selective agonists for a 3 day period produces an effective development of antinociceptive tolerance, without cross-tolerance between DAMGO, DPDPE or [D-Ala^2, Glu^4]deltorphin (Mattia et al., 1991a). This procedure was thus employed in order to evaluate the possibility of cross-tolerance between CWSS-induced antinociception and the μ selective DAMGO, the δ_1 selective DPDPE and the δ_2 selective [D-Ala^2, Glu^4]deltorphin (Jiang et al., 1991; Mattia et al., 1991a). Following pretreatment of mice with these agonists, antinociceptive tolerance was demonstrated to each. In addition, no cross-tolerance was observed between the CWSS-induced antinociception and DAMGO or DPDPE. In contrast, the CWSS-induced antinociceptive response was greatly reduced in mice tolerant to the antinociceptive effects of [D-Ala^2,

Glu^4]deltorphin. This result is consistent with the observations demonstrating sensitivity of the CWSS-induced response to antagonism by 5'-NTII.

Subsequent experiments were undertaken in order to determine if the observed cross-tolerance between CWSS-induced antinociception and [D-Ala^2, Glu^4]deltorphin could be characterized as a two-way phenomenon. For this purpose, and to support the concept that the CWSS-induced response was indeed an opioid mediated phenomenon, mice were subjected to repeated CWSS exposures and antinociception was monitored. The data demonstrate decreasing antinociceptive responses following repeated exposures, consistent with the development of tolerance to the CWSS-induced effect, and with the opioid nature of the observed antinociceptive response. In these CWSS-tolerant mice, antinociceptive dose-response lines were generated for each of the selective agonists, DAMGO, DPDPE and [D-Ala^2, Glu^4]deltorphin. No displacement in potency was observed for the antinociceptive effects of the μ selective DAMGO, nor for the δ_1-selective DPDPE in CWSS-tolerant mice, supporting previous interpretations asserting a lack of cross-tolerance between these agonists and CWSS-induced antinociception. In contrast, however, the antinociceptive dose-response line for *i.c.v.* [D-Ala^2, Glu^4]deltorphin was displaced significantly to the right, indicating cross-tolerance between the CWSS-induced effect and [D-Ala^2, Glu^4]deltorphin, and the supporting results showing a diminution of effect of CWSS-induced antinociception in [D-Ala^2,

Glu^4]deltorphin-tolerant mice. Thus, two-way and selective cross-tolerance has been demonstrated between the CWSS-induced antinociceptive effect and the antinociceptive actions of [D-Ala^2, Glu^4]deltorphin. Similarly, a two-way lack of cross-tolerance has been shown for the the CWSS-induced antinociceptive effect and the actions of either DAMGO or DPDPE.

The suggestion that [D-Ala^2, Glu^4]deltorphin and CWSS induce antinociception via the same receptor mechanism while DPDPE and DAMGO act at different receptors might be subject to alternative explanations. For example, recent work by Yaksh and colleagues (e.g., Mjanger and Yaksh, 1991) has suggested that differences in agonist efficacy can often explain data which apparently suggest differences in receptor mechanisms. Such differences in efficacy can have important implications, particularly in studies of cross-tolerance or with findings based on the use of non-competitive antagonists. For example, the demonstration of apparent differential cross-tolerance between two compounds using only one of the compounds to induce tolerance might be better explained by the concept of differential efficacy. That is, if one were to induce tolerance using a compound with low efficacy followed by a challenge with a compound of high efficacy, then no, or little, cross-tolerance might be observed between these two compounds, even if they were acting on the same receptor. This finding would also apply to the situation of differential antagonism using a non-competitive antagonist. If such

a non-competitive antagonist were employed and one compound was blocked while a second was not, it would be possible that the unblocked agonist had greater efficacy. Thus, it would be important to consider the possible implications of differences in efficacy of the compounds used in the present study. These two situations, however, would not apply to the experiments in this study since the cross-tolerance paradigm employed a two-way or symmetrical design. The data demonstrate cross-tolerance to CWSS-induced antinociception following induction of antinociceptive tolerance to [D-Ala^2, Glu^4]deltorphin, and similarly, cross-tolerance to [D-Ala^2, Glu^4]deltorphin-induced antinociception following induction of tolerance to CWSS-antinociception. Furthermore, the data indicate a two-way lack of cross-tolerance between antinociception produced by DPDPE and the CWSS-induced effect. Previous work from our laboratory has also demonstrated a two-way lack of cross-tolerance between the antinociception produced by DPDPE, [D-Ala^2, Glu^4]deltorphin and DAMGO (Mattia et al., 1991a). Additionally, in terms of the results using non-competitive antagonists, our previous work has shown a two-way differential antagonism of the antinociceptive actions of DPDPE and [D-Ala^2, Glu^4]deltorphin by DALCE and 5'-NTII, respectively. The use of a two-way paradigm in either the cross-tolerance experiments, or in experiments employing irreversible antagonists, rules out the possibility of erroneous interpretation of differences in receptors due to possible differences in efficacy.

Finally, it should be noted that while direct measurement of agonist efficacy is difficult *in vivo*, there is no evidence for differences in the efficacy of DPDPE and [D-Ala^2, Glu^4]deltorphin. In this regard, direct comparison of the efficacy of a series of opioid δ agonists in the mouse isolated vas deferens bioassay by plotting receptor occupation vs. response, showed no significant differences (Kramer et al., 1991). Such results, *in vitro*, clearly may not correlate with results *in vivo*, but nevertheless suggest that the relative efficacy of these two opioid δ receptor agonists is similar.

In conclusion, this investigation has employed a swim-stress model in the mouse which produced an antinociceptive effect under conditions of severe nociceptive stimuli. This test was chosen to reflect the conditions under which several selective opioid receptor agonists have been evaluated and by which subtypes of opioid δ receptors have been pharmacologically identified. Using the criteria of selective antagonism by naloxone and by ICI 174,864, together with a lack of antagonism by the μ antagonist, β-FNA, and the κ-antagonist, nor-BNI, the CWSS-effect is suggested to be mediated via opioid δ receptors. The receptors involved in this effect have been further identified as involving a subtype of opioid δ receptor on the basis of criteria previously established in our laboratory (Jiang et al., 1991; Mattia et al., 1991a). On this basis, the CWSS-induced antinociceptive response was sensitive to antagonism by 5'-NTII, but not by DALCE, suggesting activity at a δ_2 receptor. This finding

suggested that the CWSS-induced antinociceptive response should be mimicked by exogenous application of a selective δ_2 agonist, [D-Ala^2, Glu^4]deltorphin. In order to evaluate this possibility, two-way antinociceptive cross-tolerance was demonstrated between the CWSS-induced antinociception and [D-Ala^2, Glu^4]deltorphin. This antinociceptive cross-tolerance was selective in that the CWSS-induced effect was not altered in mice tolerant to the antinociceptive actions of DAMGO or DPDPE, and furthermore, the antinociceptive effect of these agonists was not altered in mice tolerant to the CWSS-induced antinociceptive effect. These data support the interpretation of involvement of a subtype of opioid δ receptor in the CWSS-induced antinociceptive response and indicate that these opioid δ_2 receptors can be implicated in response to activation of endogenous systems.

RESULTS OF CWSS MODULATION STUDIES

Effects of CWSS exposure alone

Exposure of mice to CWSS for a 30 sec period did not produce any significant (i.e., < 10%) antinociceptive response when tested after 10 min (Figure 24).

Morphine antinociception in control and CWSS-exposed mice

A comparison of the morphine dose-effect curve in control mice to that in CWSS-exposed mice showed that the antinociceptive potency of this compound was significantly increased (Figure 24). A_{50} values in control and in CWSS-exposed mice were 3.9 (3.42 - 4.47) and 1.1 (0.9 - 1.3) nmol, respectively.

Studies with antagonists

Co-administration of the δ antagonist, ICI 174,864, with morphine failed to antagonize the antinociceptive effects of this compound in control mice (Figure 25) in agreement with previous results (Heyman et al., 1987; 1989a; Jiang et al., 1990a; 1991; Porreca et al., 1992). However, the leftward displacement of the morphine dose-response line in CWSS-exposed mice was not observed in mice treated with ICI 174,864 and morphine (Figure 25), indicating that the modulatory effect of CWSS was antagonized by this δ antagonist. The A_{50} value (and 95% C.L.) for morphine in mice treated with ICI

174,864 or ICI 174,864 and exposure to CWSS were 3.7 (3.1 - 4.4) and 3.6 (2.8 - 4.6) nmol, respectively.

Pretreatment of mice with the putative δ_1 antagonist, DALCE, did not alter morphine antinociceptive potency in control mice (Figure 26) in agreement with previous results (Jiang et al., 1990c; 1991); the morphine A_{50} value in DALCE pretreated mice was 4.0 (3.2 - 5.1) nmol. Similar to controls, exposure of DALCE-pretreated mice to CWSS resulted in a leftward displacement of the morphine dose-effect curve (Figure 26), suggesting that this δ_1 antagonist did not block the modulatory actions of CWSS on morphine. The A_{50} value for morphine in DALCE-pretreated mice exposed to CWSS was 1.2 (0.9 - 1.5) nmol.

Pretreatment of mice with the putative δ_2 antagonist, 5'-NTII, did not alter morphine antinociceptive potency in control mice (Figure 27) in agreement with previous results (Jiang et al., 1991); the morphine A_{50} value in 5'-NTII pretreated mice was 4.2 (3.6 - 4.9) nmol. However, exposure of 5'-NTII pretreated mice to CWSS did not result in the expected leftward displacement of the morphine dose-effect curve (Figure 27) suggesting that this δ_2 antagonist blocked the modulatory actions of CWSS; the morphine A_{50} value in 5'-NTII-pretreated mice exposed to CWSS was 4.2 (3.8 - 4.8) nmol.

Pretreatment of mice with the μ antagonist, β-FNA, produced a rightward displacement of the morphine dose-response curve (Figure 28) consistent with

previous results (Heyman et al., 1987; 1989b); the morphine A_{50} in β-FNA pretreated mice was 35.2 (29.0 - 42.6) nmol. Exposure of β-FNA pretreated mice to CWSS did not result in a leftward displacement of the morphine dose-response curve, suggesting that β-FNA pretreatment prevented the modulatory actions of CWSS. The morphine A_{50} value in β-FNA pretreated mice exposed to CWSS was 27.1 (22.9 - 31.2) nmol.

Pretreatment of mice with graded doses of *i.c.v.* antibody to either [Leu^5]enkephalin or [Met^5]enkephalin had no direct antinociceptive or observable behavioral effects and did not alter morphine antinociceptive potency (Figure 21). Administration of morphine (4 nmol) alone, in the presence of antibodies against [Leu^5]enkephalin (200 μg), or in the presence of antibodies against [Met^5]enkephalin (200 μg), resulted in 40.2 $\pm$ 6.3, 45.7 $\pm$ 5.8 and 43.9 $\pm$ 8.1% antinociception. Additionally, antibodies to [Met^5]enkephalin had no effect on the leftward displacement of the *i.c.v.* morphine dose-response line produced by CWSS (Figure 29); the morphine A_{50} value following CWSS in mice treated with antibodies to [Met^5]enkephalin was 1.2 (0.9 - 1.5) nmol. In contrast, pretreatment with *i.c.v.* antibodies to [Leu^5]enkephalin blocked the leftward displacement in the *i.c.v.* morphine dose-response line produced by exposure to CWSS (Figure 29); the morphine A_{50} value following CWSS in mice treated with antibodies to [Leu^5]enkephalin was 3.7 (2.9 - 4.7) nmol.

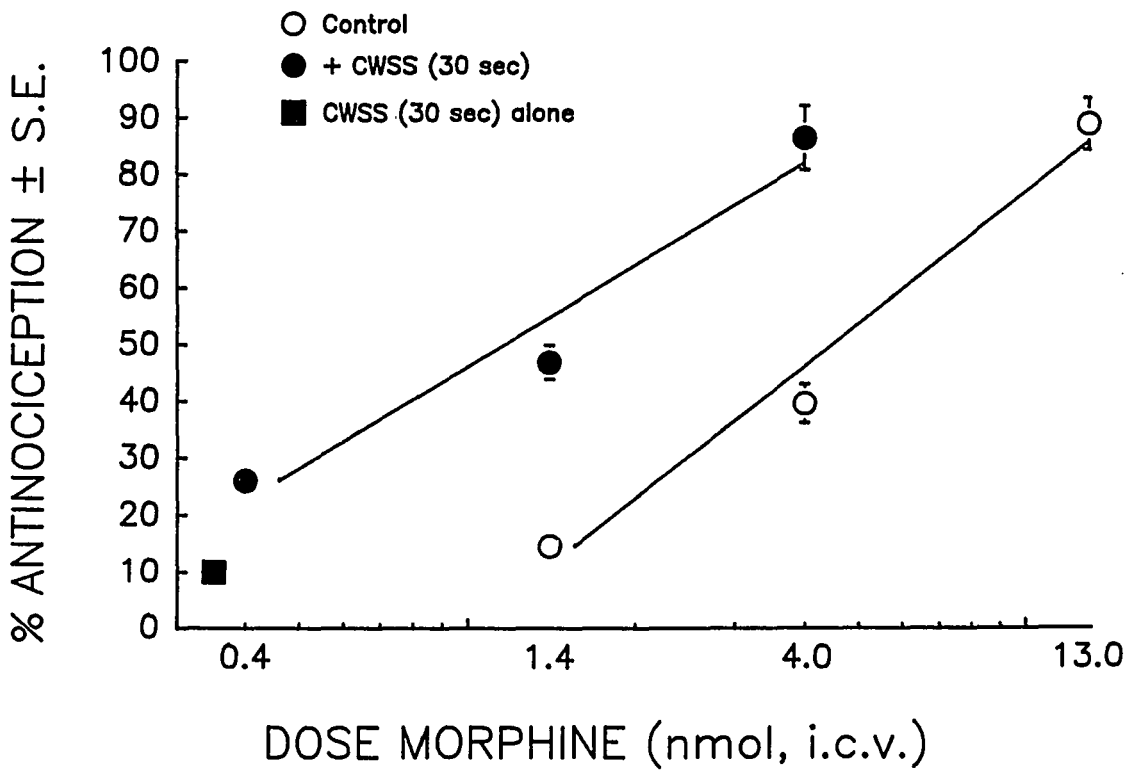

Figure 24. Antinociceptive effect of CWSS-exposure alone, and *i.c.v.* dose-response lines for morphine in control mice and in mice exposed to CWSS. Testing took place 12 min after morphine administration and 10 min after CWSS.

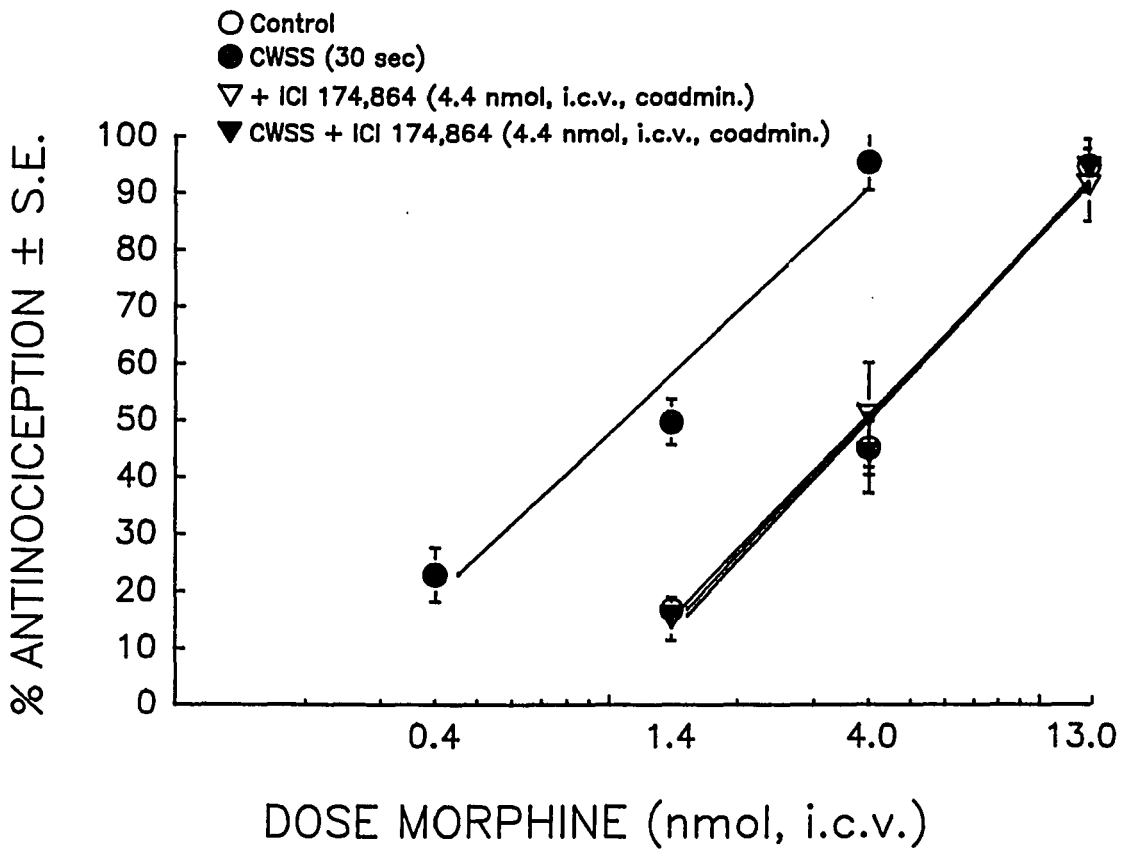

Figure 25. Antinociceptive dose-effect curves for *i.c.v.* morphine in control mice, or in mice exposed to CWSS, alone or in the presence of ICI 174,864. For purposes of comparison, the dose-response lines for morphine in the control groups and in groups exposed to CWSS are repeated from Figure 24.

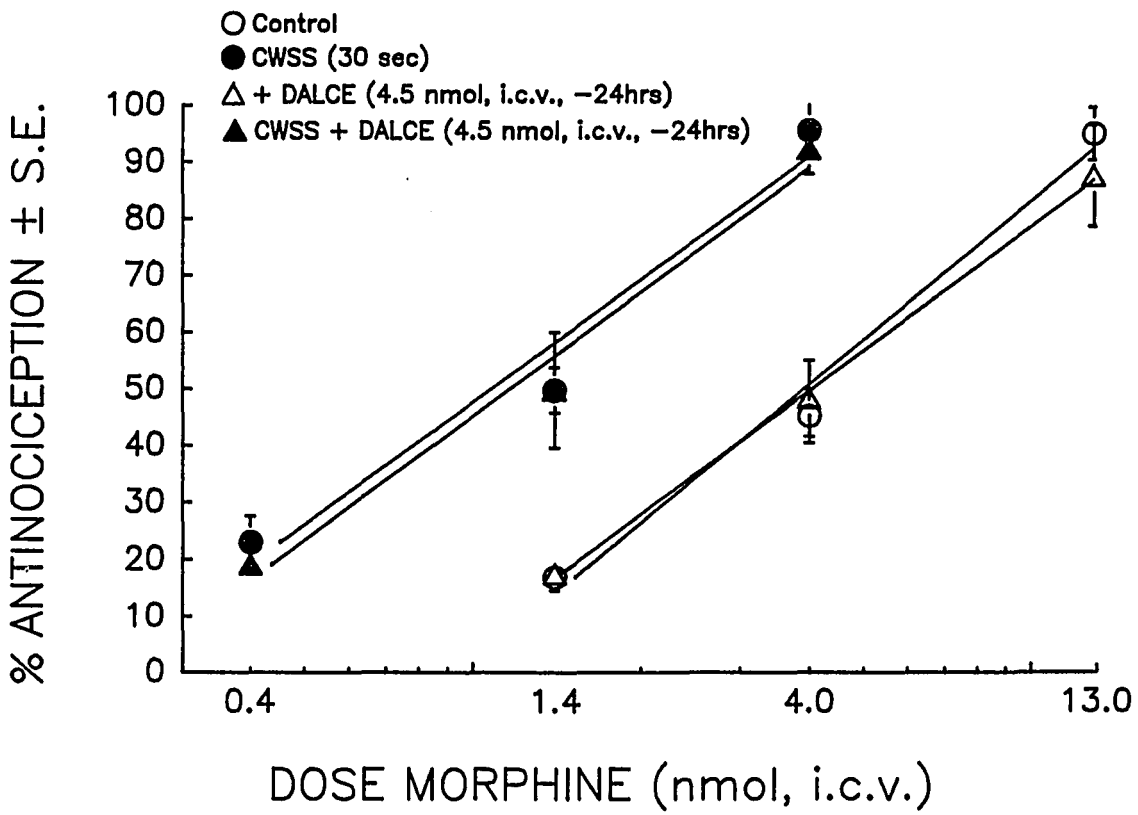

Figure 26. Effect of pretreatment with DALCE on the antinociception of *i.c.v.* morphine in control mice, or in mice exposed to CWSS. For purposes of comparison, the dose-response lines for morphine in the control groups and in groups exposed to CWSS are repeated from Figure 24.

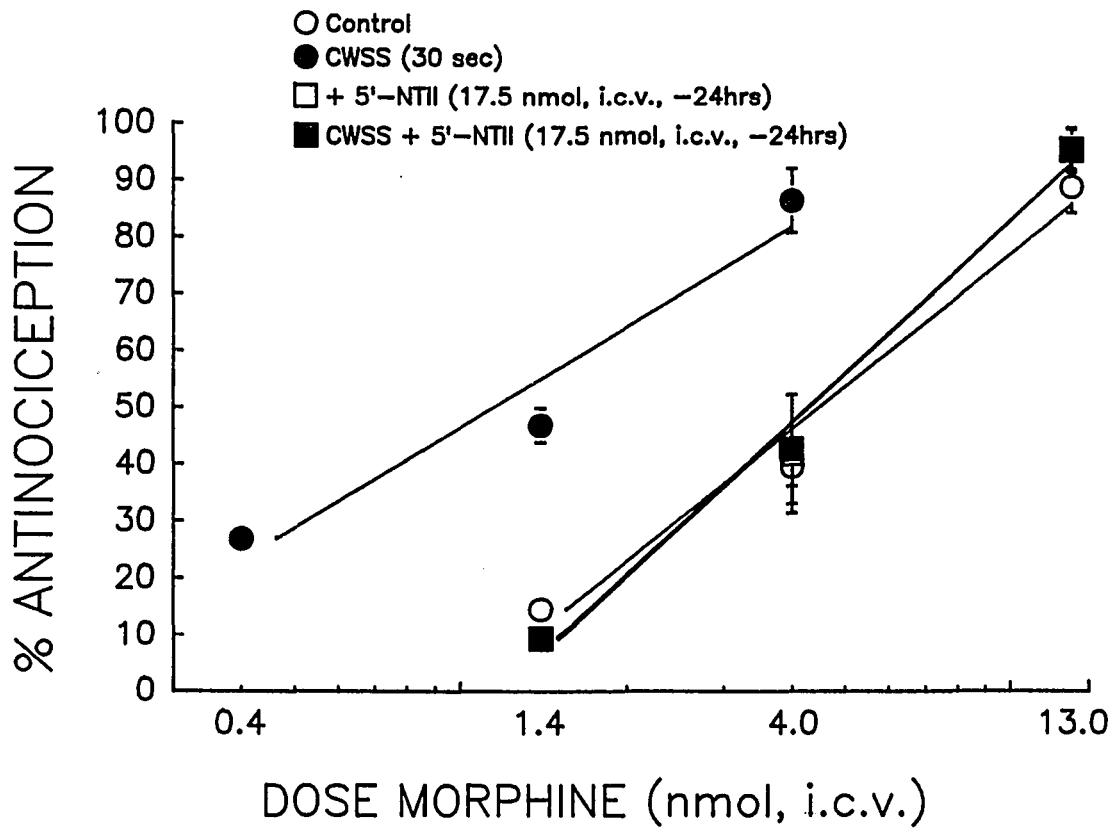

Figure 27. Effect of pretreatment with 5'-NTII on the antinociception of *i.c.v.* morphine in control mice, or in mice exposed to CWSS. For purposes of comparison, the dose-response lines for morphine in the control groups and in groups exposed to CWSS are repeated from Figure 24.

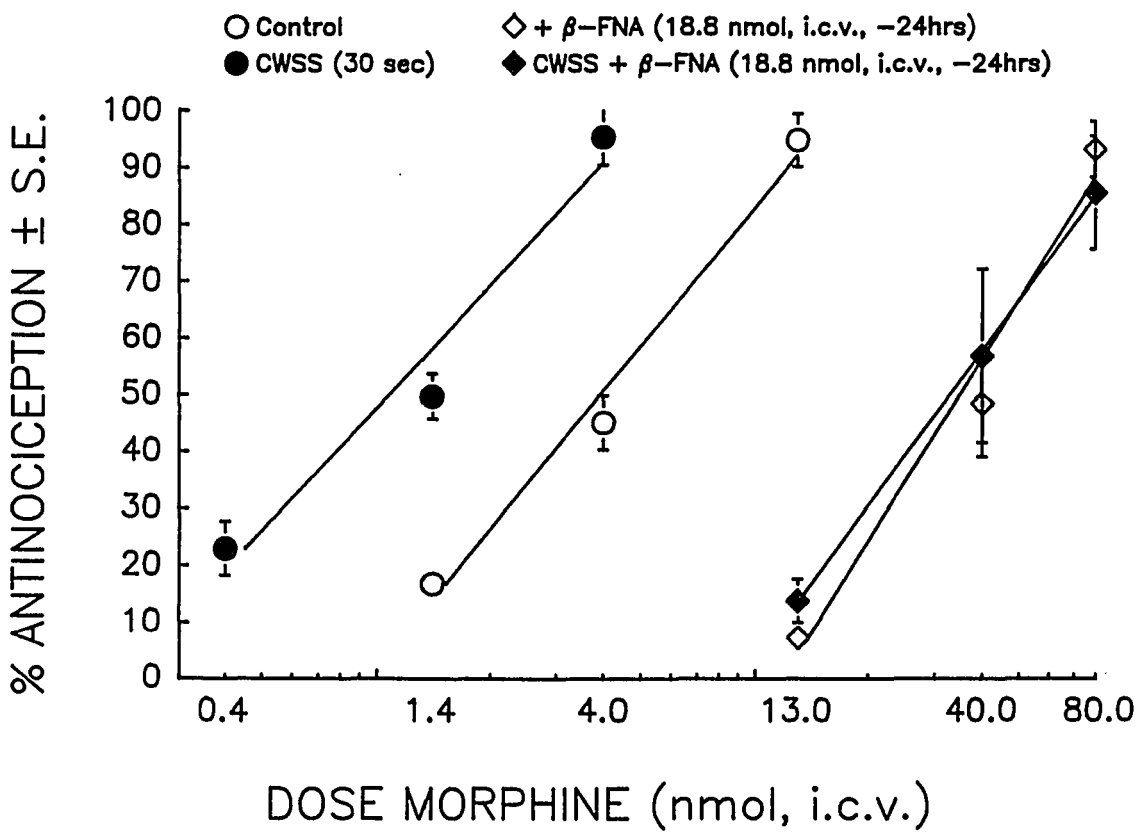

Figure 28. Effect of pretreatment with β-FNA on the antinociception of *i.c.v.* morphine in control mice, or in mice exposed to CWSS. For purposes of comparison, the dose-response lines for morphine in the control groups and in groups exposed to CWSS are repeated from Figure 24.

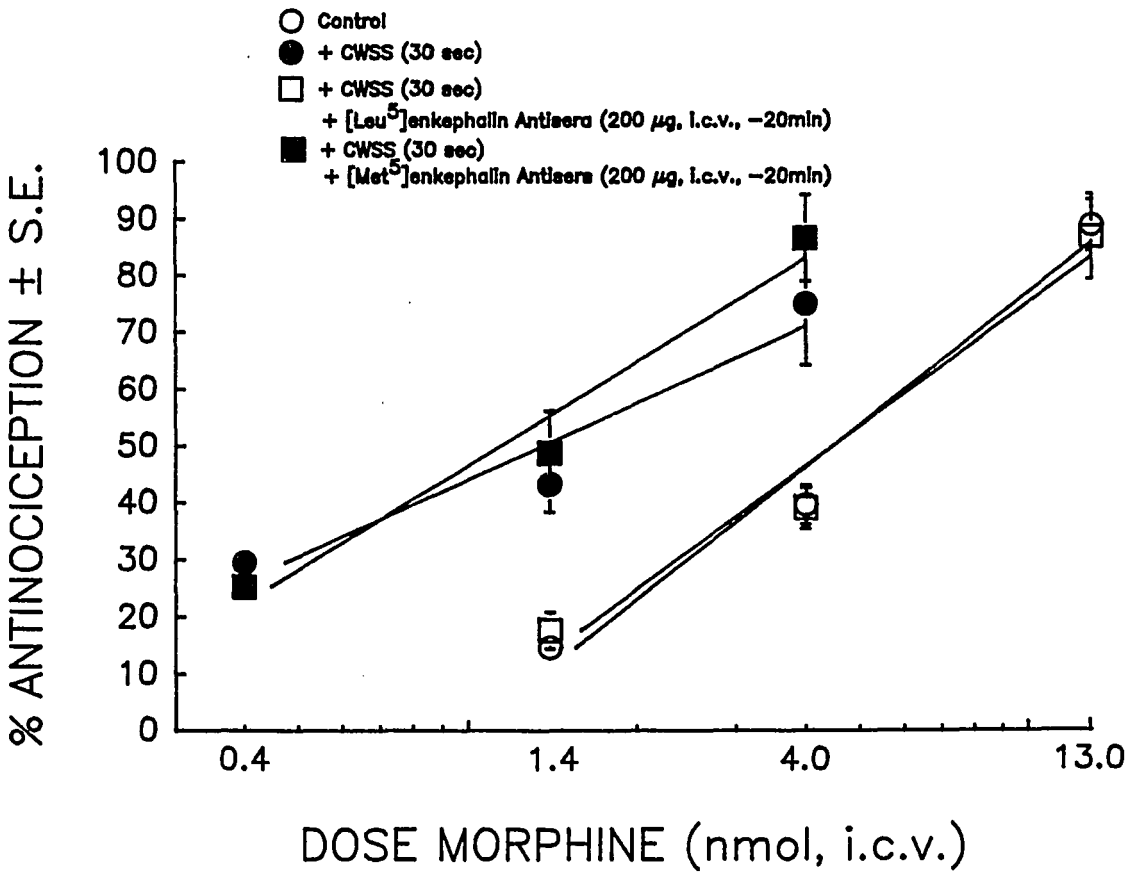

Figure 29. *I.c.v.* dose-response lines for morphine in control mice, or in mice exposed to CWSS, in the presence of antisera raised against either [Met^5]enkephalin or [Leu^5]enkephalin. For purposes of comparison, the dose-response lines for morphine in the control groups and in groups exposed to CWSS are repeated from Figure 24.

SUMMARY OF SWIM-STRESS MODULATION

The recent identification of opioid δ receptor subtypes (Sofuoglu et al., 1991; 1992; Jiang et al., 1991; Mattia et al., 1991a), termed δ_1 and δ_2 (Mattia et al., 1992), has allowed the investigation of the nature of the δ receptor involved in a variety of pharmacological effects. The direct antinociception resulting from supraspinal administration of [D-Ala^2, Glu^4]deltorphin or [D-Ser^2, Leu^5]enkephalin-Thr^6 (DSLET) to mice has been suggested to occur via activation of an opioid δ_2 receptor, while the direct antinociceptive effects associated with *i.c.v.* DPDPE appears to be mediated primarily by the δ_1 receptor. Paradigms have also been developed by which endogenous systems can be activated (e.g., exposure of mice to CWSS) to produce antinociception via the δ_2 receptor (Vanderah et al., 1992). Given that administration of opioid δ agonists to mice can result in an enhancement of the potency of opioid μ agonists in a variety of endpoints, including antinociception, via action at an opioid δ_2 receptor (Porreca et al., 1992), it was of interest to determine whether stimulation of endogenous systems resulting in activation of this receptor subtype could similarly result in a modulatory effect. The present data suggest that this is the case.

The approach used in the present study was similar in nature to the strategy used in the identification of δ subtypes in the mediation of direct antinociception, and was founded by demonstrating a two-way differential

antagonism of the direct effects of exogenous δ agonists or CWSS (Jiang et al., 1991; Vanderah et al., 1992) or modulatory (Porreca et al., 1992) effects of exogenous δ agonists by selective δ antagonists including DALCE (Bowen et al., 1987) and 5'-NTII (Portoghese et al., 1990). In the present experiments, the pharmacological endpoint was the modulation of morphine antinociceptive potency resulting from a brief exposure to CWSS. Such exposure did not produce antinociception alone, but significantly increased the antinociceptive potency of morphine.

The increase in morphine antinociceptive potency by CWSS was not predictable in that administration of opioid δ agonists has previously been demonstrated to either increase or decrease the antinociceptive potency of this μ agonist depending on the specific δ agonist involved (e.g., Vaught and Takemori, 1979; Lee et al., 1980) and furthermore, this modulatory action has been demonstrated to be either synergistic (Jiang et al., 1990b; Horan et al., 1992a) or sub-additive (Horan et al., 1992a). In such studies, both fixed subantinociceptive doses of opioid δ agonists, as well as a fixed-ratio approach, have been employed to produce the modulatory action on μ-mediated antinociception. On this basis, then, it was unclear whether exposure of mice to CWSS for a brief period, which did not directly result in antinociception, would be sufficient to produce a modulatory action on μ-mediated antinociception, and whether such modulation would be negative (i.e., decrease

in morphine potency) or positive (i.e., increase in morphine potency) as was, in fact, observed.

On the basis of the modulatory profiles previously observed with exogenous [Leu^5]enkephalin (i.e., positive modulatory effect)(Vaught and Takemori, 1979; Heyman et al., 1986b,1989a,b; Jiang et al., 1990c) and [Met^5]enkephalin (i.e., negative modulatory effect)(Lee et al., 1980; Heyman et al., 1989a,b), the positive modulatory action of a brief exposure to CWSS is suggestive of the effect of a [Leu^5]enkephalin-like molecule in the brain. On this basis, the present studies employed antibodies against [Leu^5]enkephalin and [Met^5]enkephalin in an attempt to block the modulatory actions of CWSS on morphine antinociception. Although antibodies against [Met^5]enkephalin and [Leu^5]enkephalin did not alter morphine antinociceptive potency directly, the modulatory action of CWSS was antagonized by [Leu^5]enkephalin-antibody. These observations suggest the release of [Leu^5]enkephalin or a [Leu^5]enkephalin-like substance in the brain which can alter morphine antinociceptive potency.

It should be noted that, although the direct antinociceptive actions of exogenous δ agonists have been distinguished on the basis of sensitivity to DALCE and 5'-NTII (Jiang et al., 1991), the δ antagonist, ICI 174,864 (Cotton et al., 1984), was effective in all cases, suggesting that this compound is a selective antagonist for the opioid δ receptor which does not distinguish

between the δ subtypes. The finding that both the positive and negative modulatory effects of δ agonists (Heyman et al., 1989a,b; Jiang et al., 1990c) could be antagonized by ICI 174,864 together with the identified subtypes of δ receptors suggested that these positive and negative modulatory effects might be mediated through different δ subtypes. This question was open to investigation by use of the antagonists selective for opioid δ receptor subtypes, DALCE and 5′-NTII. Previous results with exogenous δ agonists have shown that this possibility was not the case in that both the positive and negative modulatory effects were sensitive only to δ_2 antagonists (Porreca et al., 1992). The present results, with CWSS-induced modulation of morphine antinociceptive potency, likewise, suggest that the effect is mediated via opioid δ_2 (i.e., 5′-NTII-sensitive), rather than δ_1 (i.e., DALCE-sensitive) opioid receptors. It is also interesting that the modulatory actions of CWSS on morphine antinociceptive potency were not evident in animals pretreated with the μ antagonist, β-FNA. This result is consistent with previous observations in which β-FNA pretreated animals did not show susceptibility to modulation via exogenous δ agonists (Heyman et al., 1989a,b) and with studies *in vitro* which have suggested that β-FNA binds irreversibly to the hypothesized opioid μ-δ complex (Rothman et al., 1988).

The suggestion of a functional μ-δ receptor complex is based on studies *in vivo* which demonstrated both positive and negative changes in the

antinociceptive potency (Vaught and Takemori, 1979; Lee et al., 1980; Heyman et al., 1989a,b) of μ agonists such as morphine. Such modulatory actions *in vivo* showed that the modulation of μ agonist effects could be extended to other models including the reversal of endotoxic shock in the rat (Holaday and D'Amato, 1983; D'Amato and Holaday, 1984), and in the μ receptor mediated urinary bladder motility (Sheldon et al., 1987;1989). Furthermore, both the positive and negative modulatory actions of these δ agonists appear to be mediated through opioid δ receptors since their effects, but not the direct μ-mediated effect, can be antagonized by opioid δ antagonists. In addition, on the basis of a variety of experimental approaches, Rothman and colleagues have suggested that opioid δ receptors can be classified on the basis of their identification within or outside of a hypothesized opioid μ-δ complex (i.e., δ_{cx} and δ_{ncx}, respectively)(see Rothman et al., 1988 for review). The present results suggest that the opioid δ receptor involved in these observed modulatory effects can be identified as the δ_2 receptor, but it is unclear whether the opioid δ_2 receptor, identified on the basis of studies *in vivo* (Jiang et al., 1991; Mattia et al., 1991; 1992; Sofuoglu et al., 1991; 1992), is the same as the δ_{cx} receptor postulated on the basis of studies *in vitro*. Further experimentation will be required to resolve this issue, and may depend upon whether the opioid μ-δ receptor complex is identified as a physical or functional association of μ and δ receptors.

The question of whether or not the hypothesized functional μ-δ complex exists in a physically associated form cannot be addressed by the present studies using the *in vivo* approaches, but the present studies suggest that exposure of mice to a brief period of stress can result in the activation of opioid δ_2 receptors, presumably through the release of an endogenous [Leu^5]enkephalin-like substance in the brain. This finding is of considerable general interest in the context of analgesic activity of opioid μ agonists in situations of stress. In this regard, it is noteworthy that the analgesic effectiveness (i.e., potency and efficacy) of morphine has been consistently observed to be considerably greater in situations of obvious stress, such as in men wounded in battle (Beecher, 1946; 1956) or in other conditions of trauma (Carlen et al., 1978). While the unquestioned role of psychological interpretation of pain obviously plays a major factor in these findings, it appears possible that the observed analgesic actions of morphine under conditions of stress may also be related, in part, to a physiological mechanism involving the concurrent activation of opioid δ receptor systems.

RESULTS OF MODULATION IN CHRONICALLY TREATED ANIMALS

Administration of sub-effective doses of opioid δ agonists [D-Ala^2, Glu^4]deltorphin or DPDPE resulted in an enhanced antinociceptive effect of the μ agonist morphine (Figure 30). The activation of endogenous opioid δ systems by a brief (i.e., 30 sec) exposure to CWSS, results in a leftward displacement of the *i.c.v.* morphine dose-response line (shown for example with CWSS in Figure 24) and antagonized by the δ_2 antagonist 5'-NTII (Figure 27) but not the δ_1 antagonist DALCE (Firgure 26). Unlike the enhancement of subeffective doses of delta agonists on the μ agonists, morphine at a subeffective dose was unable to enhance either the δ_1 agonist DPDPE (Figure 31) or the δ_2 agonist [D-Ala^2, Glu^4]deltorphin (Figure 32). Production of tolerance to morphine (i.e., chronic morphine group) is shown by the rightward displacement in the *i.c.v.* morphine dose-response line, relative to control (Figure 33). When exogenous opioid δ agonists (i.e., DPDPE or [D-Ala^2, Glu^4]deltorphin) were given at sub-antinociceptive doses, or activation of endogenous opioid δ systems was achieved with CWSS in morphine-tolerant mice, a leftward displacement of the morphine dose-response line was seen. Modulation occurred regardless of the development of morphine tolerance resulting in no cross-tolerance between morphine and the modulatory mechanism. Pretreatment with DAMGO resulted in cross-tolerance to the antinociceptive effects of morphine as shown by the rightward displacement in the *i.c.v.* morphine dose-response line, relative to

control (Figure 34). When exogenous opioid δ agonists (i.e., DPDPE or [D-Ala^2, Glu^4]deltorphin) were given at sub-antinociceptive doses, or activation of endogenous opioid δ systems was achieved with CWSS in DAMGO-tolerant mice, a leftward displacement of the morphine dose-response line was observed. Modulation occurred regardless of the development of DAMGO tolerance (no cross-tolerance between DAMGO and the modulatory mechanism). Surprisingly, pretreatment with DPDPE was found to result in cross-tolerance to morphine (though this pretreatment did not result in cross-tolerance to DAMGO - data not shown), as shown by the rightward displacement in the *i.c.v.* morphine dose-response line, relative to control (Figure 35). When exogenous opioid δ agonists (i.e., DPDPE or [D-Ala^2, Glu^4]deltorphin) were given at sub-antinociceptive doses, or activation of endogenous opioid δ systems was achieved with CWSS, in DPDPE-tolerant mice, a leftward displacement of the morphine dose-response line was seen. Modulation occurred regardless of the development of DPDPE tolerance (no cross-tolerance between DPDPE and the modulatory mechanism). Pretreatment with [D-Ala^2, Glu^4]deltorphin did not result in cross-tolerance to morphine as shown by the lack of rightward displacement in the *i.c.v.* morphine dose-response line, relative to control (Figure 36). When exogenous opioid δ agonists (i.e., DPDPE or [D-Ala^2, Glu^4]deltorphin) were given at sub-antinociceptive doses, or activation of endogenous opioid δ systems was

achieved with CWSS, in [D-Ala^2, Glu^4]deltorphin-tolerant mice, a leftward displacement of the morphine dose-response line did not occur. The expected modulation of morphine potency was prevented by the development of tolerance to [D-Ala^2, Glu^4]deltorphin (cross-tolerance between [D-Ala^2, Glu^4]deltorphin and the modulatory mechanism). Repeated exposure to CWSS did not result in cross-tolerance to morphine as shown by the lack of rightward displacement in the *i.c.v.* morphine dose-response line, relative to control (Figure 37). When exogenous opioid δ agonists (i.e., DPDPE or [D-Ala^2, Glu^4]deltorphin) were given at sub-antinociceptive doses, or activation of endogenous opioid δ systems was achieved with CWSS, in CWSS-tolerant mice, a leftward displacement of the morphine dose-response line did not occur. The expected modulation of morphine potency was prevented by the development of tolerance to CWSS (cross-tolerance between CWSS and the modulatory mechanism).

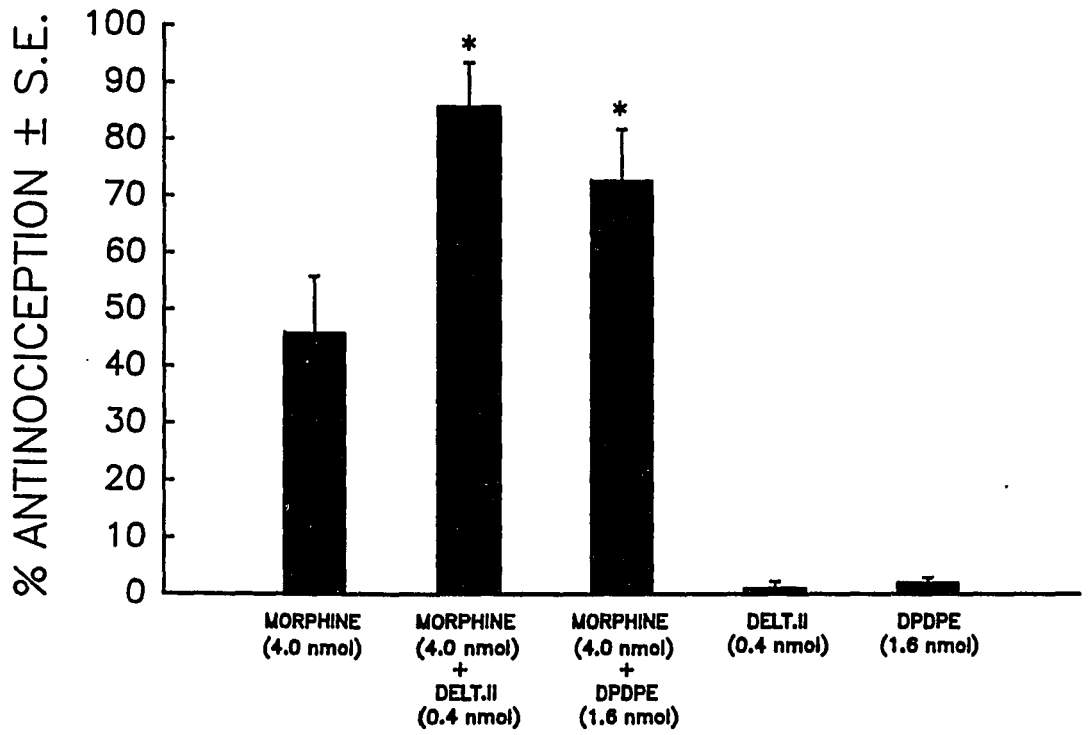

Figure 30. Enhanced antinociceptive effect of a single dose of morphine in the presence of a sub-effective dose of either [D-Ala$_2$, Glu$_4$]deltorphin or DPDPE. Each bar represents a group of 10 seperate mice.

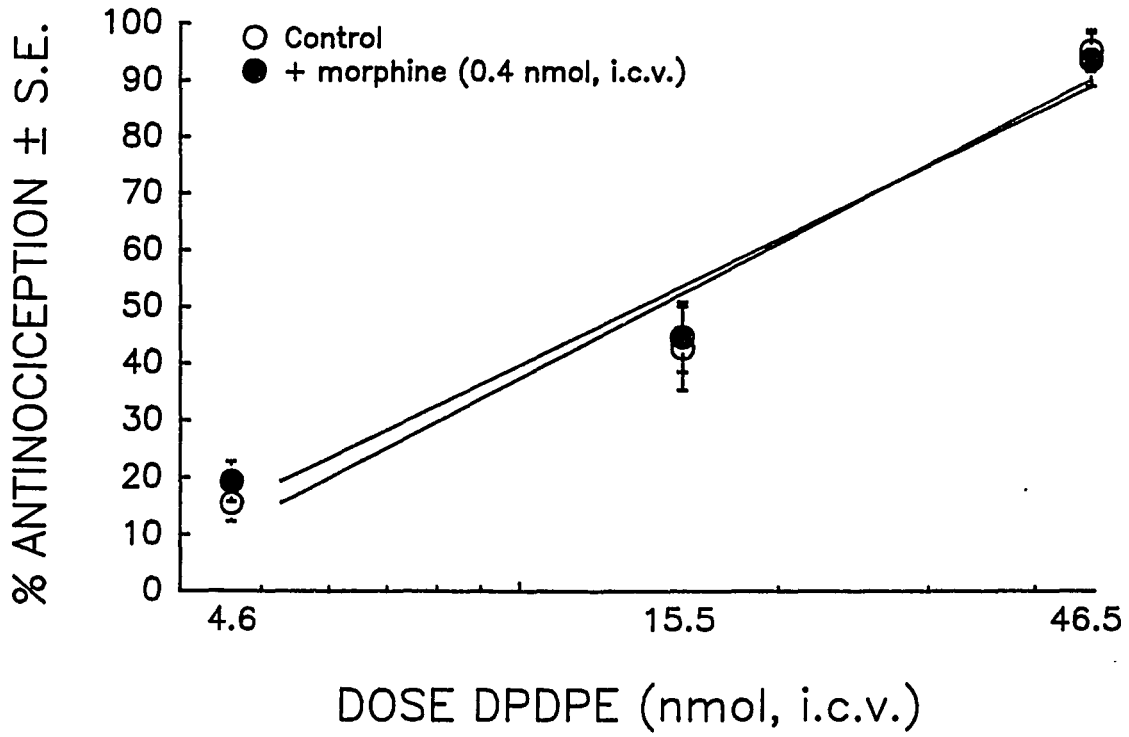

Figure 31. DPDPE dose-response lines in control mice, or in mice coadministered a subeffective dose of morphine.

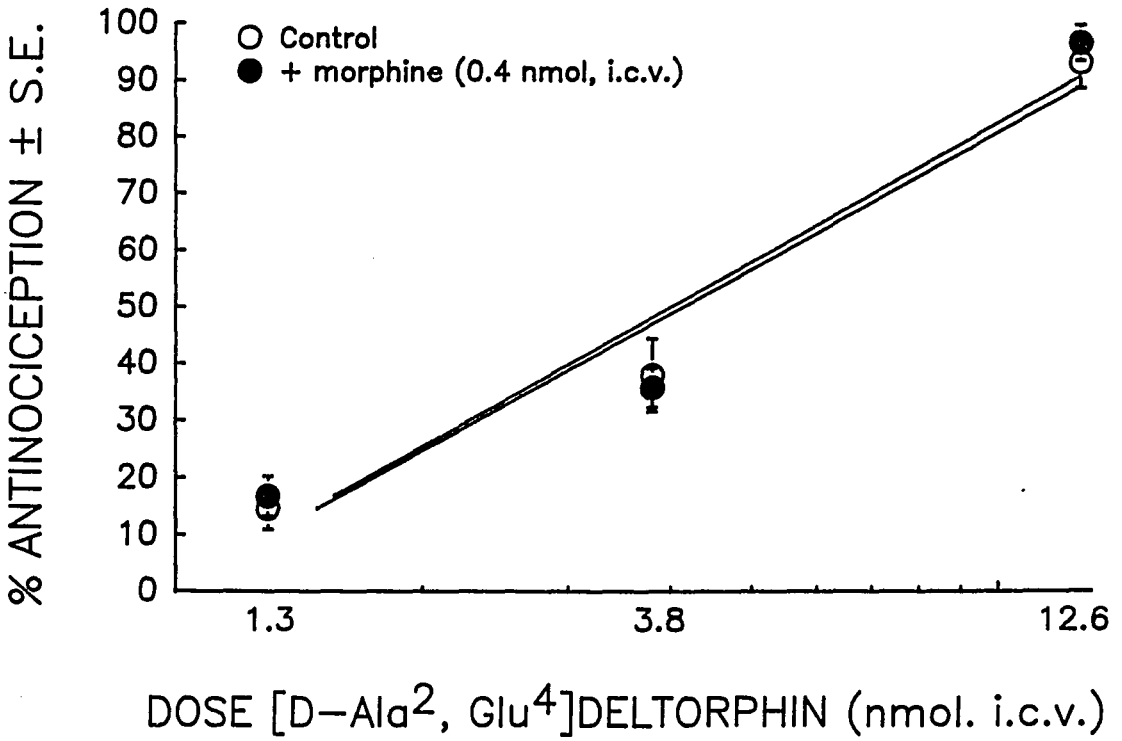

Figure 32. [D-Ala$_2$, Glu$_4$]deltorphin dose-response lines in control mice, or in mice coadministered a subeffective dose of morphine.

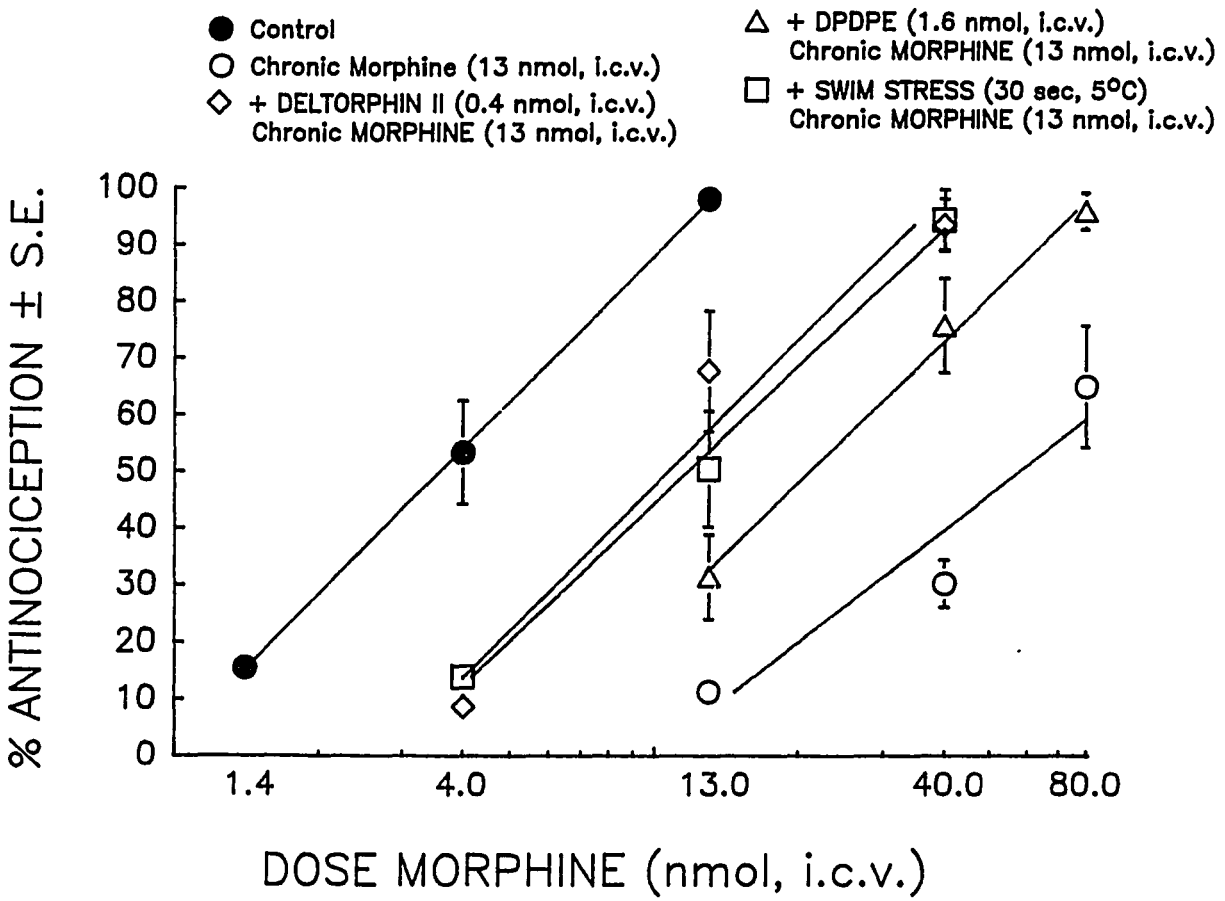

Figure 33. Morphine dose-response lines in control mice, or in mice pretreated with morphine. In morphine-pretreated mice, the morphine dose-response line was constructed in the presence of saline, [D-Ala^2, Glu^4]deltorphin, DPDPE or following a 30 sec exposure to cold water swim-stress (CWSS).

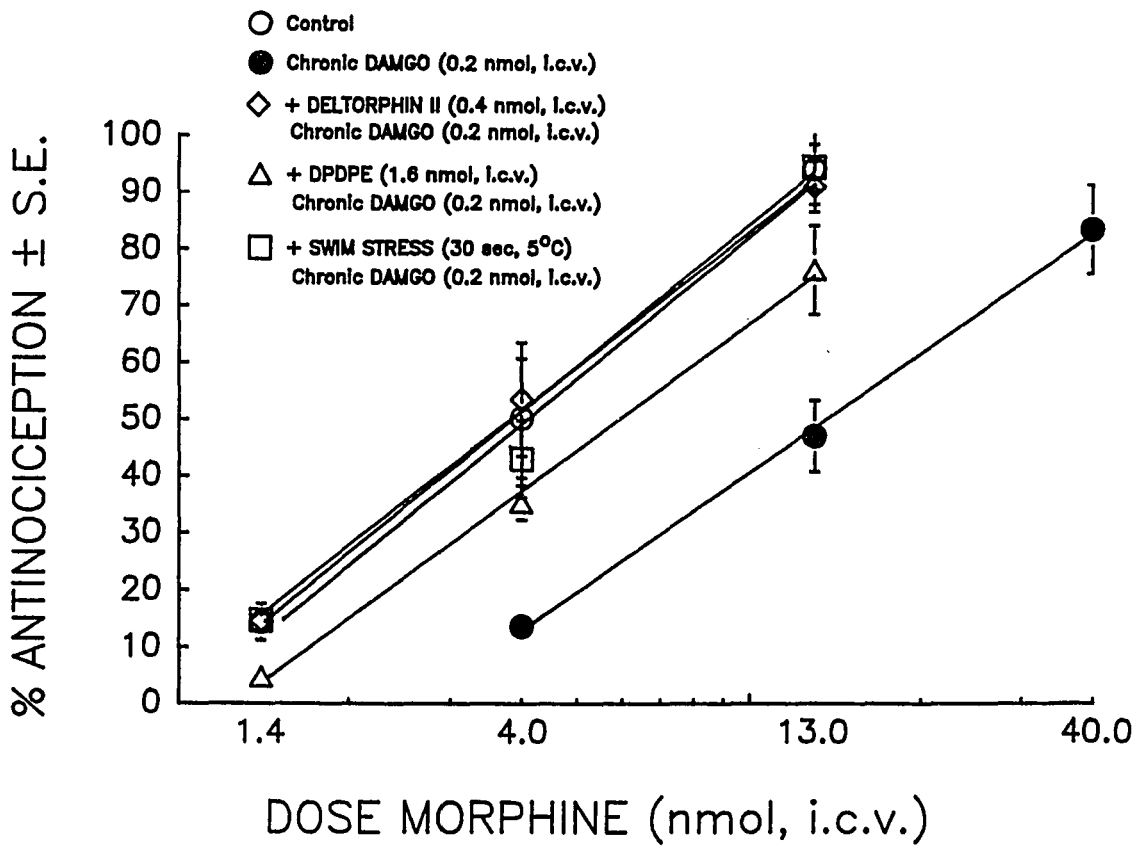

Figure 34. Morphine dose-response lines in control mice, or in mice pretreated with DAMGO. In DAMGO-pretreated mice, the morphine dose-response line was constructed in the presence of saline, [D-Ala^2, Glu^4]deltorphin, DPDPE or following a 30 sec exposure to cold water swim-stress (CWSS).

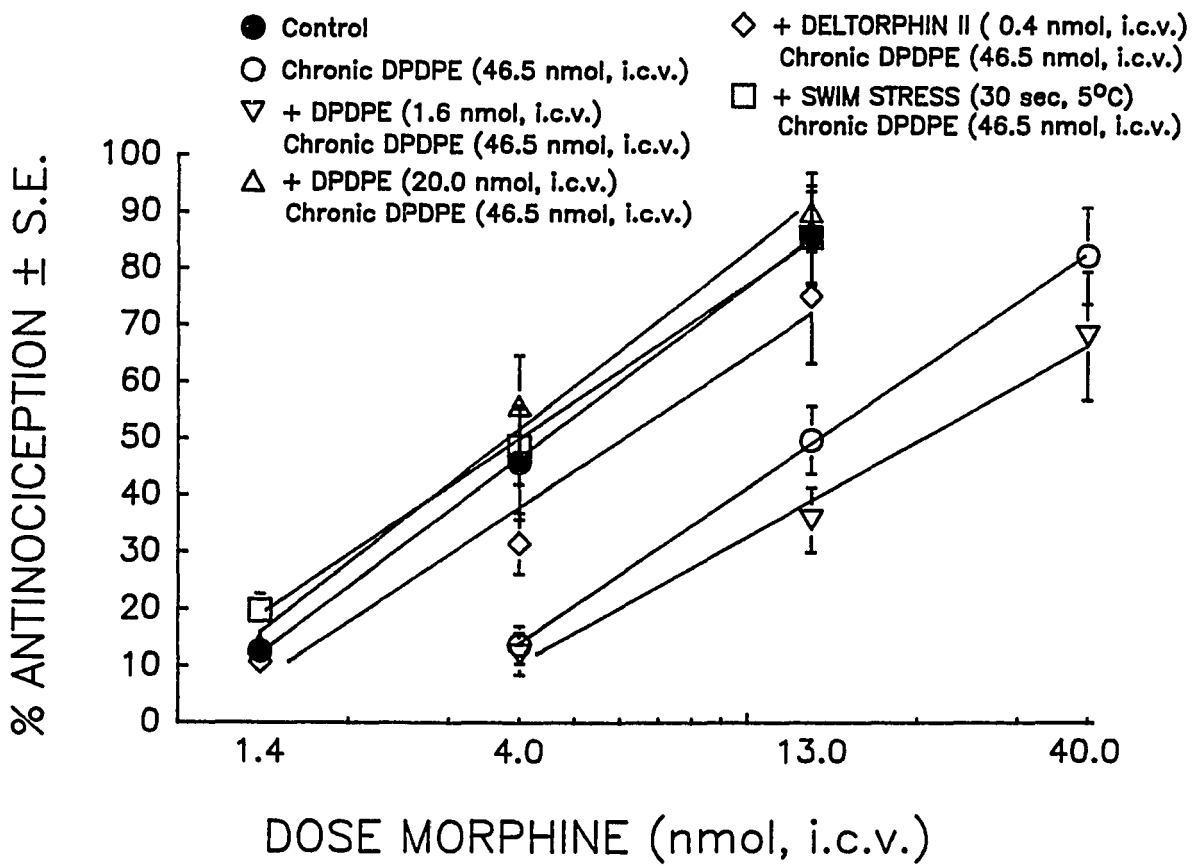

Figure 35. Morphine dose-response lines in control mice, or in mice pretreated with DPDPE. In DPDPE-pretreated mice, the morphine dose-response line was constructed in the presence of saline, [D-Ala^2, Glu^4]deltorphin, DPDPE or following a 30 sec exposure to cold water swim-stress (CWSS).

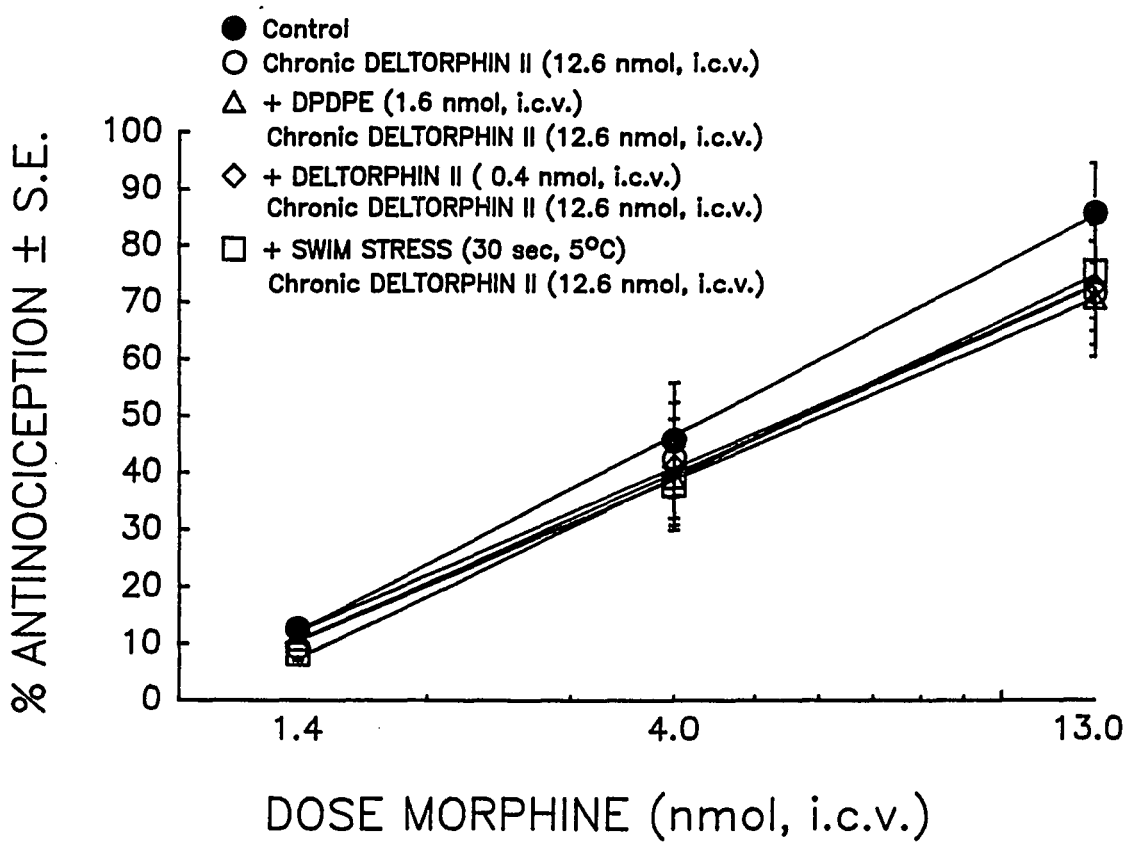

Figure 36. Morphine dose-response lines in control mice, or in mice pretreated with [D-Ala^2, Glu^4]deltorphin. In [D-Ala^2, Glu^4]deltorphin-pretreated mice, the morphine dose-response line was constructed in the presence of saline, [D-Ala^2, Glu^4]deltorphin, DPDPE or following a 30 sec exposure to cold water swim-stress (CWSS).

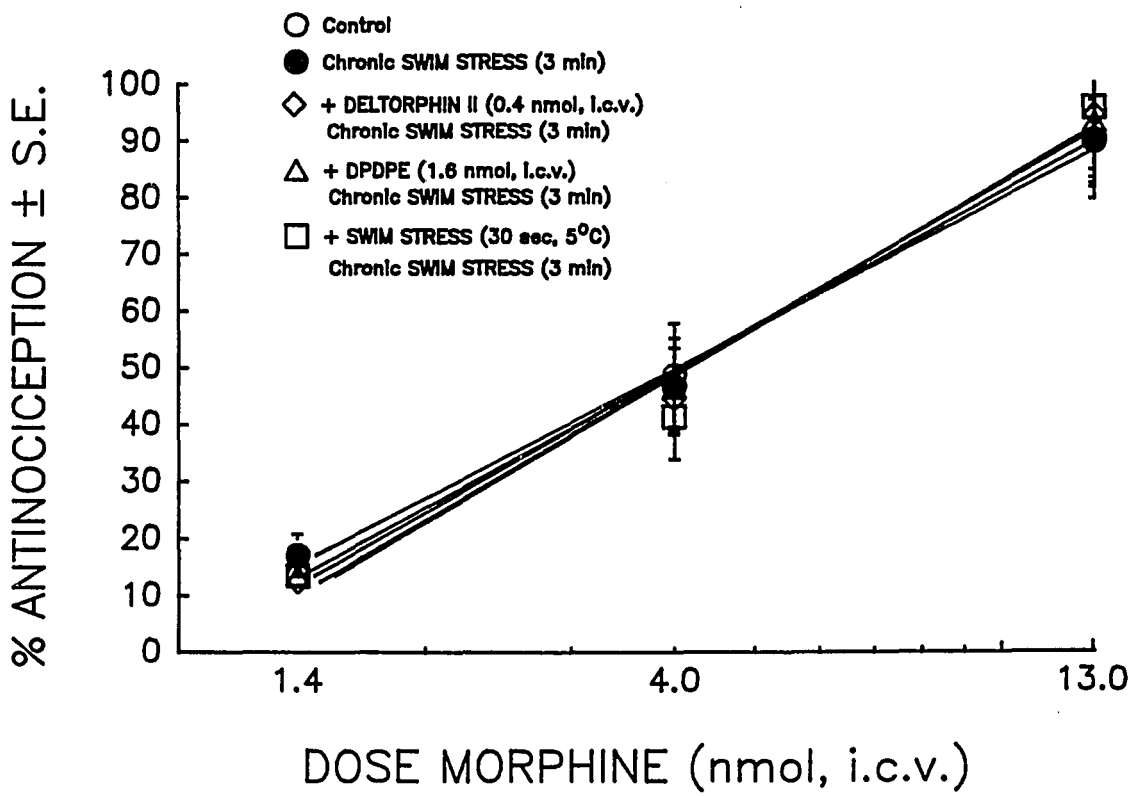

Figure 37. Morphine dose-response lines in control mice, or in mice exposed to repeated CWSS. In CWSS-exposed mice, the morphine dose-response line was constructed in the presence of saline, [D-Ala^2, Glu^4]deltorphin, DPDPE or following a 30 sec exposure to cold water swim-stress (CWSS).

SUMMARY OF MODULATION IN CHRONICALLY TREATED ANIMALS

Activation of endogenous opioid systems by limited exposure to CWSS, or by exogenous opioid δ agonists including DPDPE and [D-Ala^2, Glu^4]deltorphin, produces a modulatory action on morphine antinociception in the mouse. The modulatory effect resulting from endogenous or exogenous opioid δ agonists is mediated via an opioid δ receptor; this δ receptor is sensitive to 5'-NTII but not to DALCE, suggesting that it can be classified as an opioid δ_2 receptor. The results suggest the involvement of an opioid μ-δ receptor complex which represents either a functional or a physically associated state of μ and opioid δ_2 receptors. Note that DPDPE (an opioid δ_1 agonist) also produces a modulatory action. This is thought to be due to some degree of non-selectivity of DPDPE as its modulatory, but not direct antinociceptive actions are sensitive to antagonism by 5'-NTII (opioid δ_2 antagonist).

Production of tolerance at the opioid μ receptor (i.e., morphine- or DAMGO-pretreatment) did not alter the modulatory actions of opioid δ agonists, confirming that the modulatory effect is mediated via the δ receptor. Production of tolerance to DPDPE resulted in a rightward displacement in the morphine, but not DAMGO, antinociceptive dose-response line. This finding suggests that morphine may activate opioid δ receptors, in part, to produce its observed antinociceptive effects.

Production of tolerance to δ_2 agonists (i.e., [D-Ala^2, Glu^4]deltorphin or CWSS) prevented the modulatory effects of an opioid δ agonist (i.e., cross-tolerance was observed between opioid δ_2 agonists and the modulatory mechanism).

Tolerance develops to the μ-modulatory actions of opioid δ agonists and, induction of tolerance at the opioid δ_2 receptor results in cross-tolerance to the modulatory mechanism. This modulatory mechanism involves the opioid δ_2 receptor but not the δ_1 or the μ opioid receptors.

RESULTS OF THE INFLAMMATION STUDIES

Administration of Freund's Complete Adjuvant (FCA) in the right hind paw (5 μl) 60 minutes prior to testing produces a hyperalgesic response compared to control that was measured by the 50°C hot-plate or in the warm-water tail-flick tests (Figure 38). Under the same conditions, mice were coadministered cocaine and the FCA in the right hind paw (10 μl of a 5% solution). In both the hot-plate and tail-flick, the cocaine significantly blocked the hyperalgesic response due to FCA administration (Figure 38).

Morphine administration, *i.c.v.*, in mice produced a dose-response antinociceptive effect; the A_{50} value (and 95% CL) was calculated to be 4.90 (1.80-13.37) nmol. The morphine dose-response was reconstructed in mice that were pretreated with a non-inflammatory solution termed Freund's Incomplete Adjuvant (FIA), which lacks the mycobacterium (5 μl), in the right hind paw. The FIA was administered to mice 2 hr prior to the tail-flick test and morphine was given to the same mice 10 min prior to testing. The morphine dose-response curve did not differ from control in mice pretreated with the FIA; the A_{50} value (and 95% CL) was calculated to be 4.70 (3.59-6.15) nmol (Figure 39).

Mice were administered the FCA, which contains the mycobacterium and causes inflammation, in the right hind paw either 1, 2 or 4 hrs prior to testing along with morphine in a dose-response fashion. A rightward displacement of

the morphine dose-response curve appeared in mice pretreated 1 and 2 hrs with FCA, but not in the mice administered FCA 4 hrs before testing (Figure 40). The A_{50} values for the 1 and 2 hrs FCA pretreatment group are as follows; 0.94 (0.84-1.06) 1.03 (0.53-2.03), respectively with a shift ratio of 3.4 and 3.1. The A_{50} value for the 4 hr FCA pretreatment group was 4.31 (2.53-12.27). Several extended time points were done; 8 hr, 24 hr, 48 hr, 72 hr, 96 hr showing no significant change of the morphine dose-response (Figure 41).

In order to determine whether the modulatory effect of morphine by FCA could be blocked in the paw, cocaine (10 μl of a 5% solution), a local anesthetic, was coadministered with the FCA in the right hind paw. Figure 42 shows that cocaine was able to inhibit the leftward displacement of the morphine dose-response curve by FCA.

In order to determine whether the morphine modulation was acting at a δ opioid receptor in the central nervous system, mice were administered the FCA 2 hr prior to the tail flick test in the right hind paw and coadministered *i.c.v.* morphine 10 min prior to testing with the general δ opioid antagonist ICI 174,864 (4.4 nmol)(Figure 43). Control mice were coadministered *i.c.v.* morphine and ICI 174,864 10 min prior to testing. The FCA positive modulation was antagonized by ICI 174,864, identifying the receptor of morphine modulation by FCA as a δ opioid receptor (Figure 43). ICI 174,864 had no effect on the primarily μ opioid agonist morphine.

Next, in order to identify whether the leftward displacement of the morphine dose response curve by FCA was acting at a selective δ opioid receptor, mice were pretreated (24 hr prior to test) with either the selective δ_1 opioid receptor antagonist DALCE or with the selective δ_2 opioid receptor antagonist [Cys^4]deltorphin. Figure 44 shows that the δ_1 antagonist DALCE was unable to block the leftward displacement of the morphine dose-response curve by FCA, yet in figure 45 it is demonstrated that the δ_2 antagonist [Cys^4]deltorphin was able to inhibit the leftward displacement of the morphine dose-response curve. As a control, mice were pretreated separately with both DALCE and [Cys^4]deltorphin and given morphine 10 min prior to testing. In both of these groups the morphine dose-response was unchanged.

The leftward modulation of morphine by FCA was then tested in animals administered antisera raised against either [Leu^5]- or [Met^5]enkephalin. The leftward displacement of the morphine dose-response curve by FCA was antagonized by *i.c.v.* administration of antisera raised aginst [Leu^5]enkephalin 20 min prior to testing (Figure 46). Yet, the [Met^5]enkephalin antisera was unable to block the modulatory effects of FCA on morphine (Figure 46).

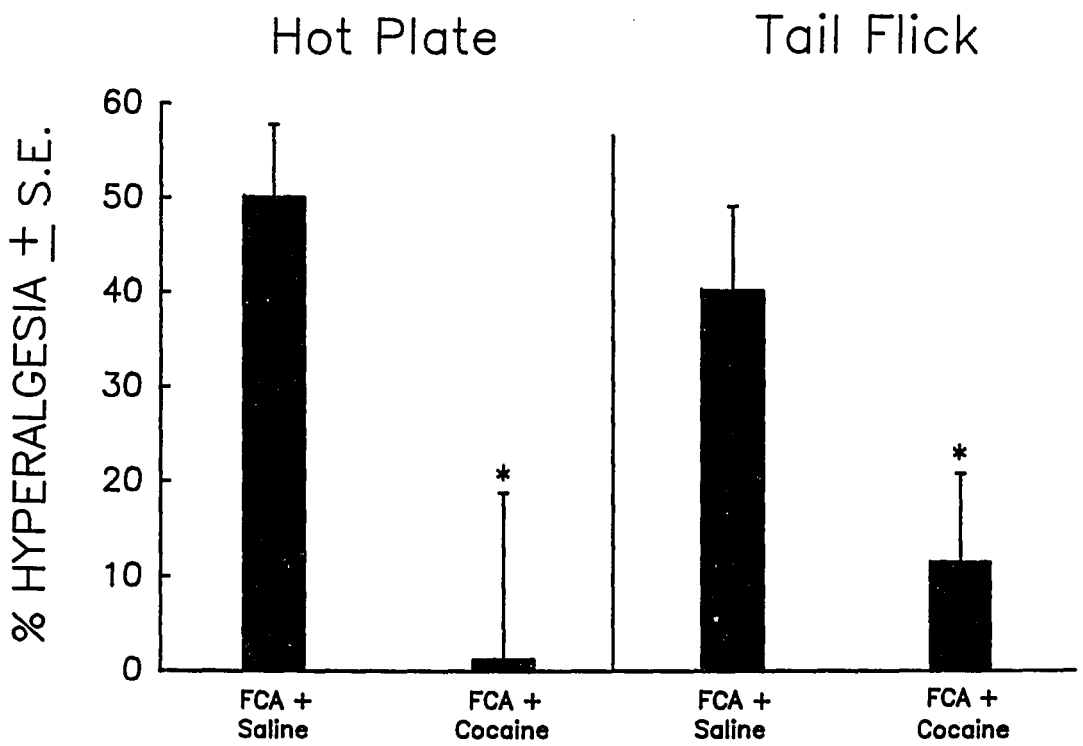

Figure 38. Hyperalgesic response in mice pretreated 60 min prior to testing with FCA (5 μl in the right hindpaw) as evaluated by an *increase* in response latency (compared to control) in the 50°C hot-plate or tail-flick tests in control mice, or in mice given cocaine in the paw (10 μl of a 5% solution). Cocaine was used as a local anesthetic to block the hyperalgesic response.

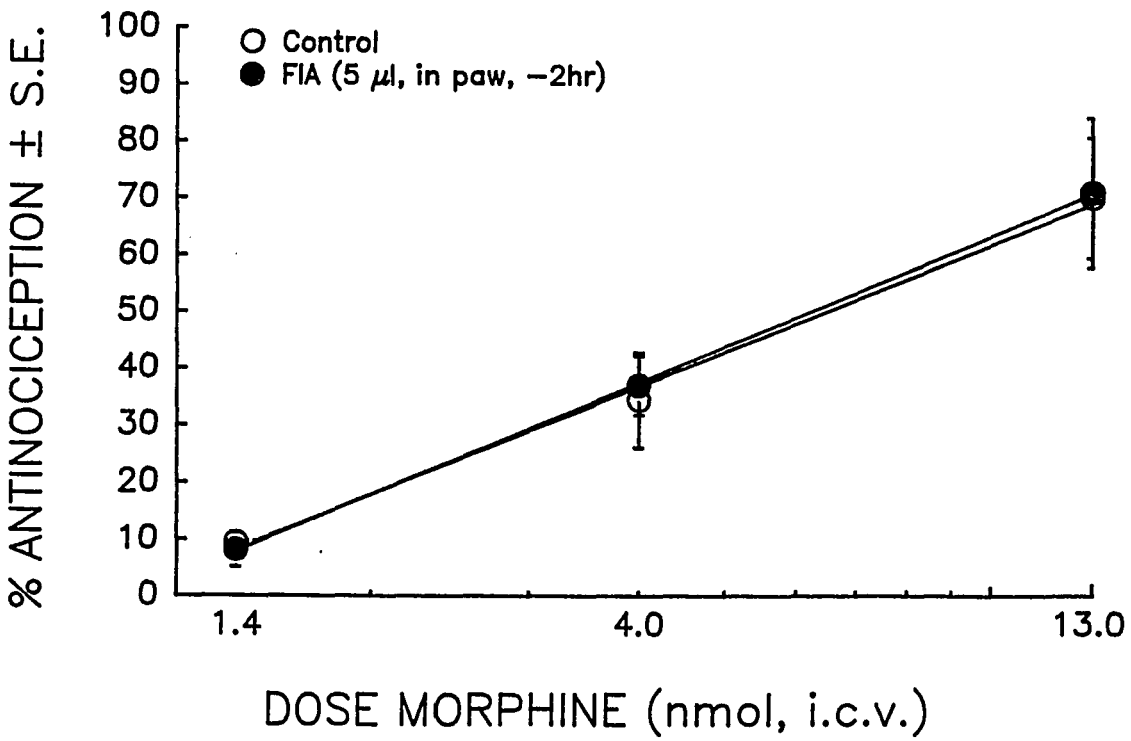

Figure 39. *I.c.v.* morphine dose-response line in control (5 µl saline in the right hindpaw) mice, or in mice pretreated with Freund's Incomplete Adjuvant (FIA) administered into the right hindpaw. FIA was given 2 hr prior to testing in the warm-water tail flick test while morphine was given 10 min prior to testing.

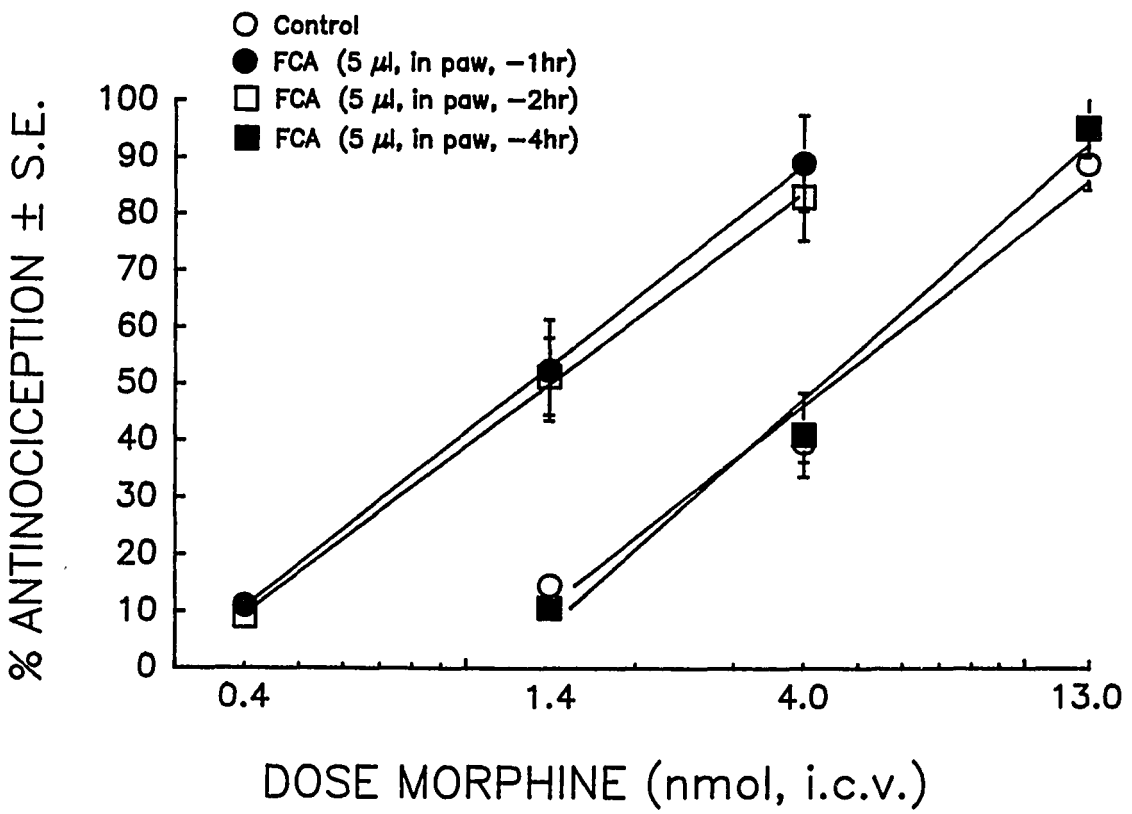

Figure 40. *I.c.v.* morphine dose-response line in control (5 µl saline in the right hindpaw) mice, or in mice pretreated 1, 2 or 4 hr prior to testing with Freund's Complete Adjuvant (FCA) administered into the right hindpaw (5 µl). Antinociception was evaluated using the warm-water (55°C) tail flick test 10 min following morphine.

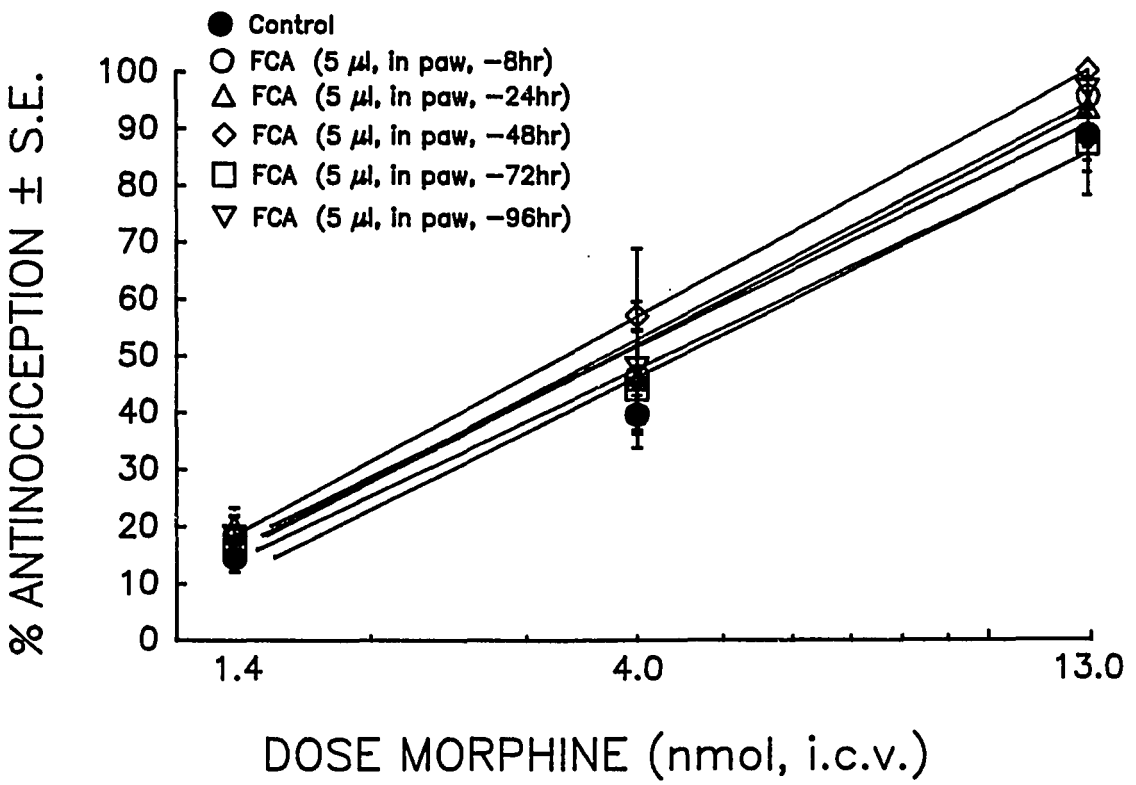

Figure 41. *I.c.v.* morphine dose-response line in control (5 µl saline in the right hindpaw) mice, or in mice pretreated 8, 24, 48, 72 or 96 hr prior to testing with Freund's Complete Adjuvant (FCA) administered into the right hindpaw (5 µl). Antinociception was evaluated using the warm-water (55°C) tail flick test 10 min following morphine.

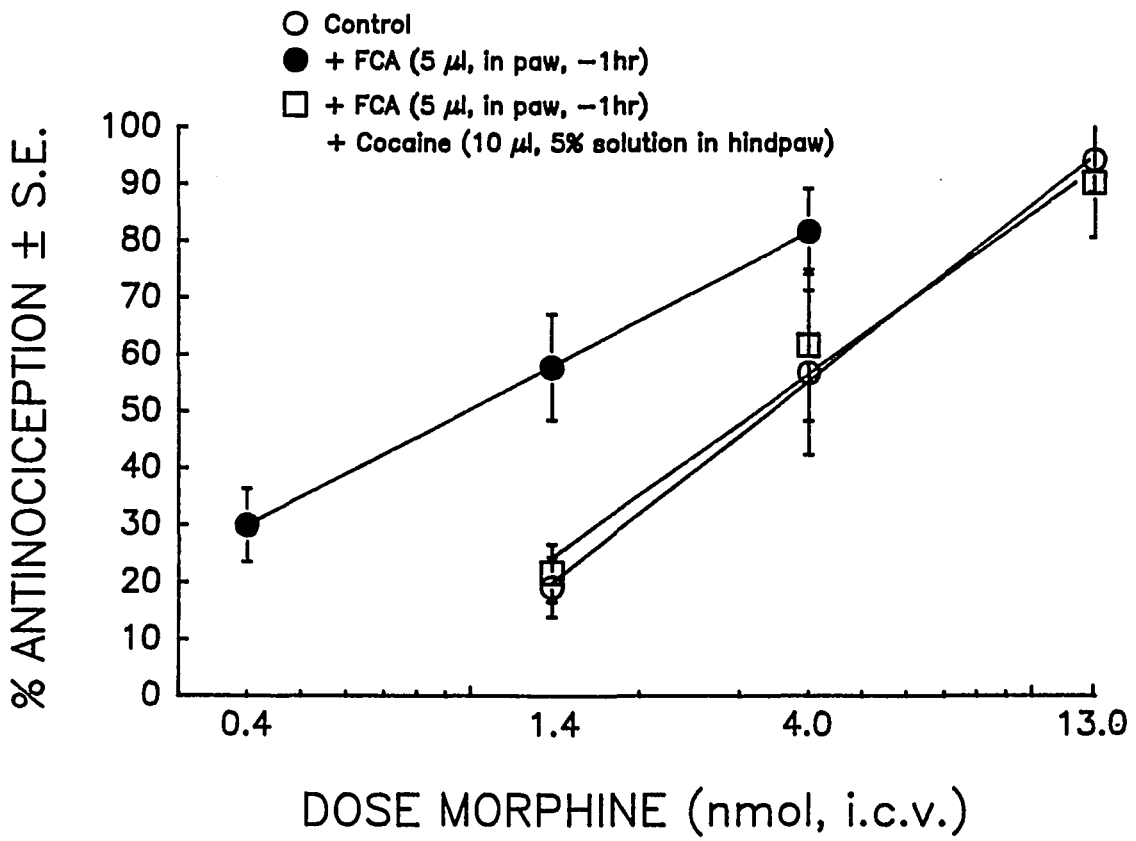

Figure 42. Effect of co-administration of cocaine (10 µl of 5% solution, in the paw) and Freund's Complete Adjuvant (FCA) on the antinociceptive potency of morphine (*i.c.v.*) in the mouse tail-flick test. Cocaine blocks the leftward displacement of the morphine dose-response line seen with FCA, apparently because of its local anesthetic actions.

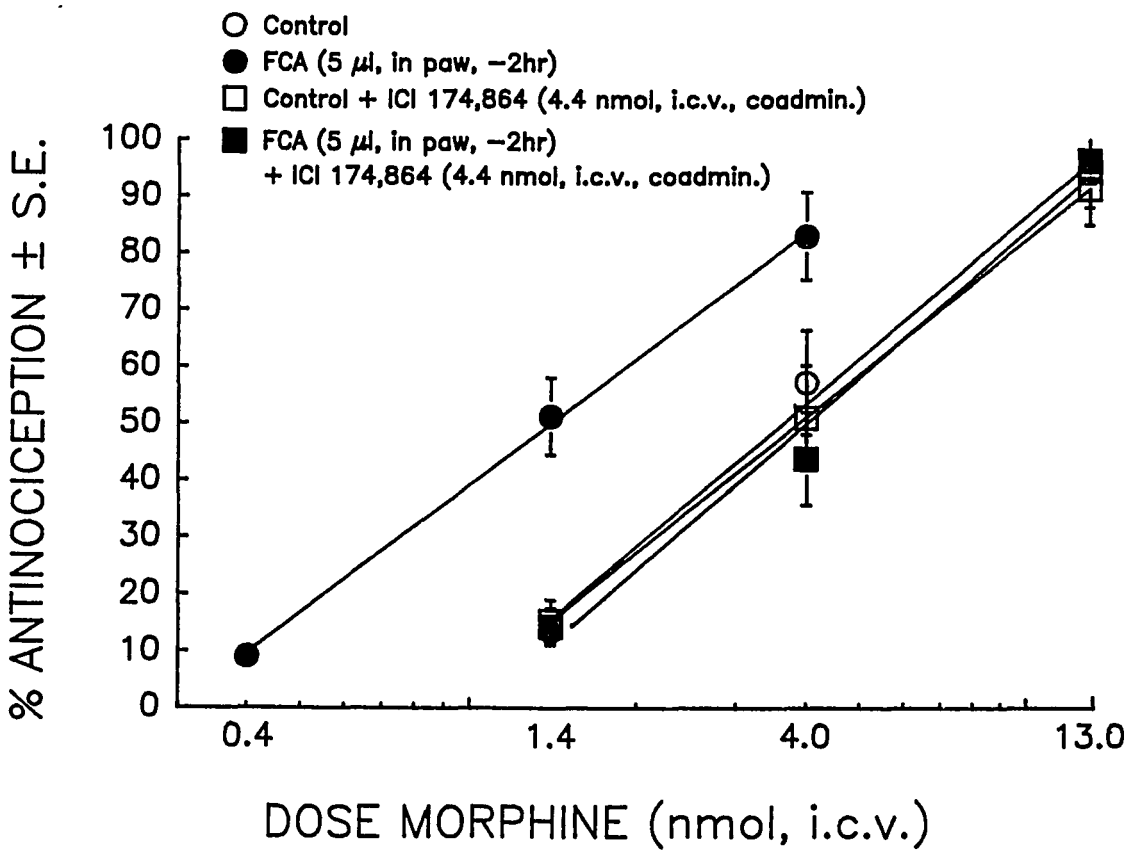

Figure 43. *I.c.v.* morphine dose-response lines in control (5 µl saline in the right hindpaw) mice, or in mice pretreated with Freund's Complete Adjuvant (5 µl in the right hindpaw) in the absence or presence of *i.c.v.* ICI 174,864. FCA was given 2 hr prior to testing, while morphine and ICI 174,864 were given concurrently, 10 min prior to testing in the warm-water tail flick test.

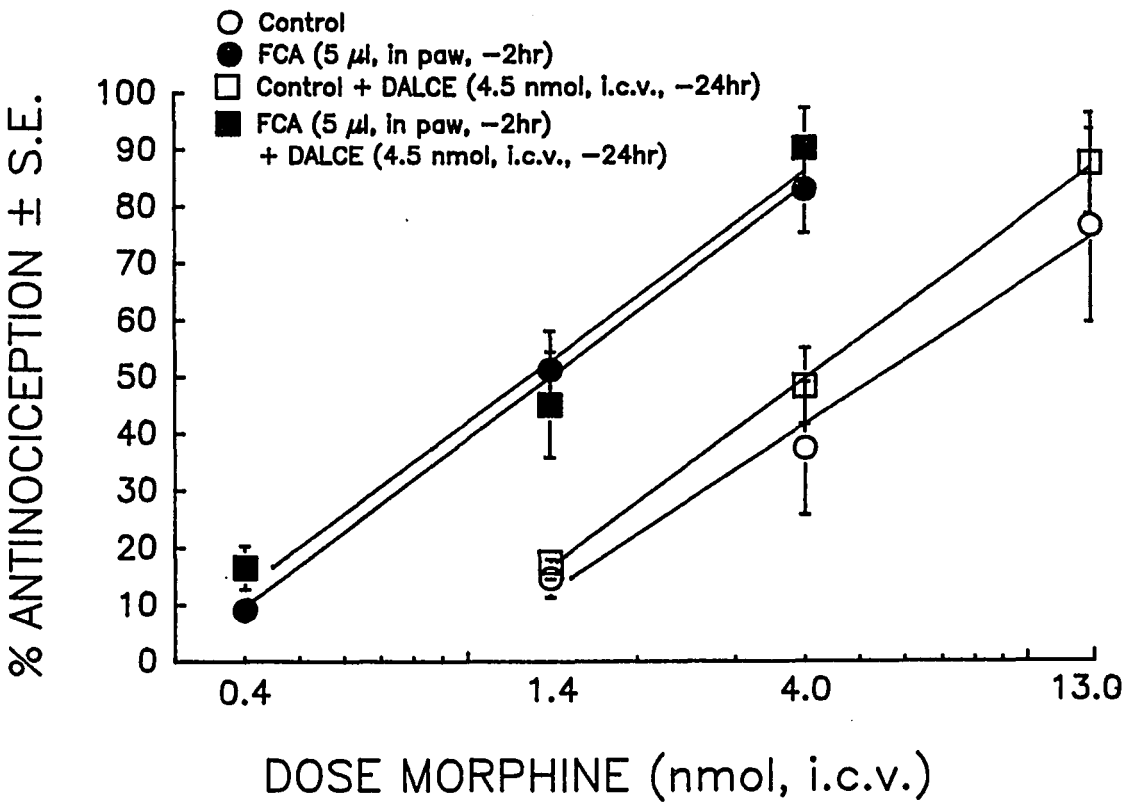

Figure 44. *I.c.v.* morphine dose-response line in control (5 μl saline in the right hindpaw) mice, or in mice pretreated with FCA (5 μl in the right hindpaw, 2 hr prior to testing) in the absence or presence of [D-Ala^2, Leu^5, Cys^6]enkephalin (DALCE). Morphine was given, 10 min prior to testing. DALCE was given *i.c.v.* 24 hr prior to testing.

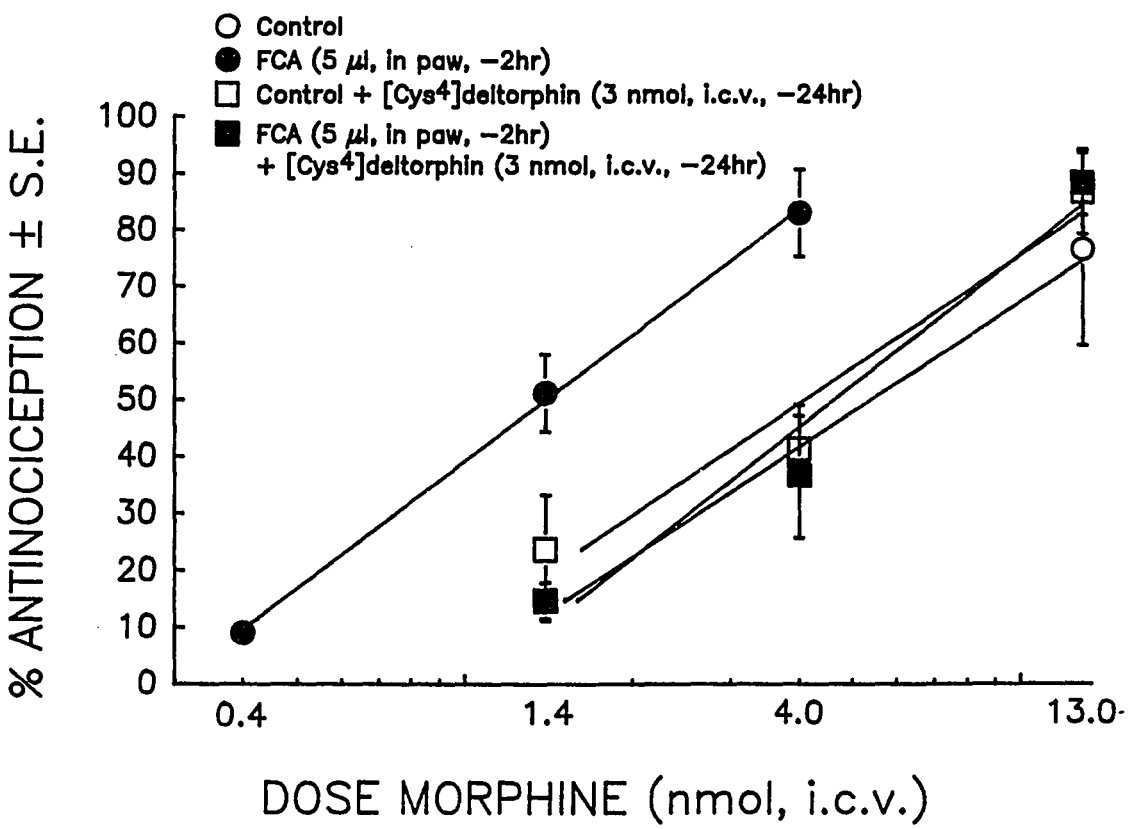

Figure 45. *I.c.v.* morphine dose-response line in control (5 µl saline in the right hindpaw) mice, or in mice pretreated with FCA (5 µl in the right hindpaw, 2 hr prior to testing) in the absence or presence of [D-Ala^2, Cys^4]deltorphin (Cys-DELT). Morphine was given 10 min prior to testing. Cys-DELT was given *i.c.v.* 24 hr prior to testing.

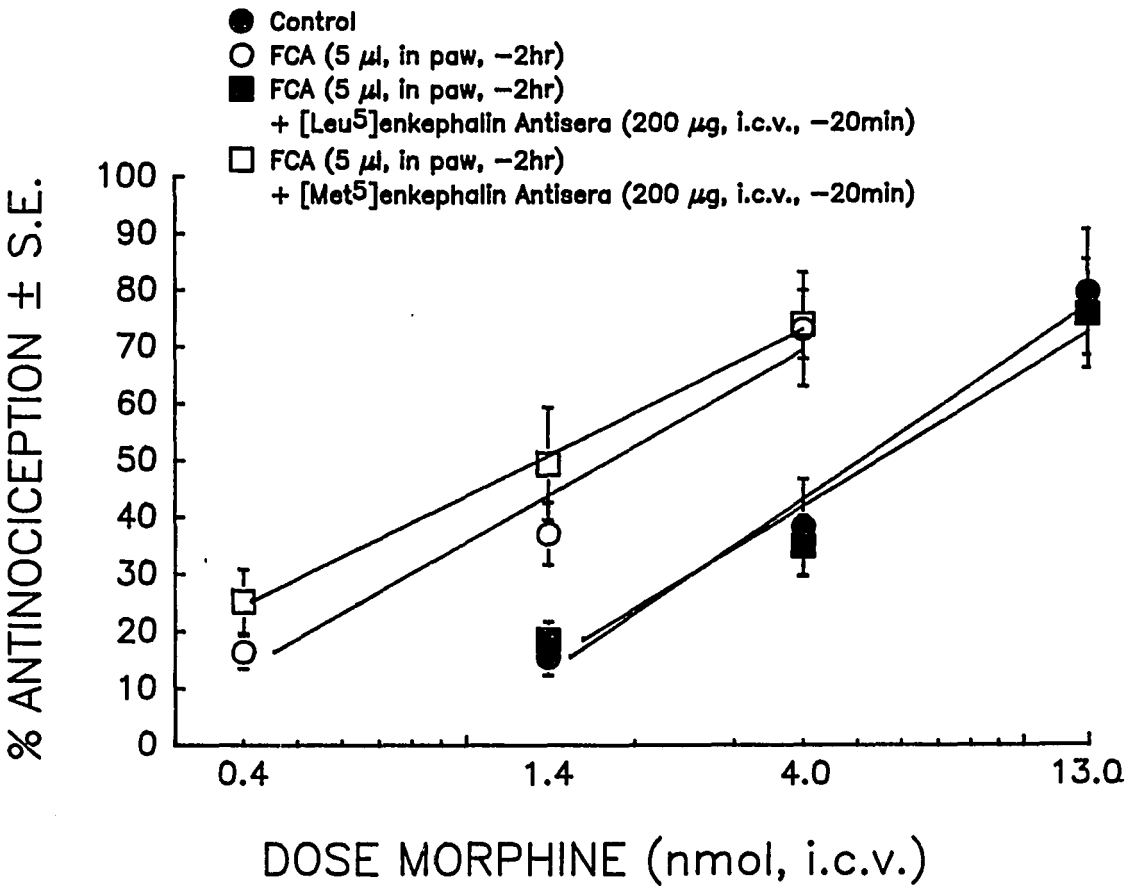

Figure 46. *I.c.v.* morphine dose-response lines in control (5 μl saline in the right hindpaw) mice, or in mice pretreated with Freund's Complete Adjuvant (5 μl in the right hindpaw) in the absence or presence of *i.c.v.* [Leu^5]enkephalin-antibody or [Met^5]enkephalin-antibody. Neither [Leu^5]enkephalin-antibody or [Met^5]enkephalin-antibody produced any behavioral or antinociceptive effect alone. FCA was given 2 hr prior to testing, the antibodies were given 20 min and morphine 10 min prior to testing in the warm-water tail flick test.

SUMMARY OF THE INFLAMMATION STUDIES

Production of a localized inflammatory (nociceptive) response results in a time-related hyperalgesic response as measured in the hot-plate or tail-flick test which is significant at 120 min after FCA. No hyperalgesia was observed following administration of saline of FIA into the right hind paw. Cocaine administration in the right hind paw acted as a local anesthetic by blocking the hyperalgesic effect of FCA.

The production of localized nociception can apparently result in the activation of the central endogenous opioid systems as shown by the leftward displacement of the *i.c.v.* morphine dose-response line. The modulatory effect is specific to the inflammatory response as it was produced by administration of Freund's Complete Adjuvant (FCA), but not by Freund's Incomplete Adjuvant (FIA) or by injection of saline into the hind paw under the same experimental conditions. Activation of the central endogenous opioid system was blocked by administering the local anesthetic cocaine into the same hind paw receiving the FCA.

The modulation of morphine antinociception potency resulting from preinjection of FCA is significant up to 4 hr following administration. At later times, the effect is not seen suggesting a diminution of the hyperalgesic response or, alternatively, a rapid development of tolerance to the modulatory

effect. The former possibility appears more likely as the hyperalgesic time course appears to be over by 4 hr following FCA injection.

The modulation of morphine antinociception potency resulting from preinjection of FCA is mediated *via* an opioid δ receptor as shown by the inhibition of the modulatory action (but not the direct morphine antinociceptive effect) by the general opioid δ antagonist ICI 174,864. The opioid δ receptor involved in the morphine modulatory effect of FCA was sensitive to the antagonism by [D-Ala^2, Glu^4]deltorphin, but not to DALCE, suggesting that the endogenous opioid activated by FCA acts at an opioid δ_2 receptor.

The opioid δ_2 receptor in the mouse brain is activated by [Leu^5]enkephalin administered *i.c.v.* and results in an increase in potency of morphine antinociception (Heyman et al., 1989a,b; Jiang et al., 1990c; Porreca et al., 1992). This leftward modulatory effect seen previously by CWSS and here by FCA can be selectively blocked by [Leu^5]enkephalin antisera but not by [Met^5]enkephalin antisera. The supraspinal opioid δ_2 receptor is activated by the presence of endogenous [Leu^5]enkephalin, or a [Leu^5]enkephalin-like substance after the peripheral administration of FCA.

In conclusion, the administration of FCA but not FIA in the hind paw of a mouse results in inflammation and stress that results in a [Leu^5]enkephalin-like substance which interacts with opioid δ_2 receptors to produce antinociception in the mouse.

RESULTS OF CCK_B ANTAGONISM OF MORPHINE ANTINOCICEPTION

I.c.v. administration of morphine produced dose- and time-related antinociception while *i.c.v.* administration of L365,260 did not alter nociceptive threshold. Pretreatment with L365,260 resulted in a parallel leftward displacement of the morphine dose-response curve BY approximately 7-fold (Table 1, Figure 47). Naltrindole did not produce any measureable antinociceptive effect alone and did not affect the potency of morphine. However, naltrindole blocked the leftward displacement of the morphine dose-effect curve resulting from pretreatment with L365,260 (Table 1, Figure 48). In order to confirm that the dose of naltrindole employed was selective for δ opioid receptors, this treatment time and dose were also evaluated against *i.c.v.* [D-Ala^2, Glu^4]deltorphin or DPDPE, two compounds whose antinociceptive actions have been shown to be mediated via δ receptors following *i.c.v.* administration in this assay (Jiang et al., 1990b). Naltrindole pretreatment reduced the antinociceptive effect of a 10 μg dose of *i.c.v.* [D-Ala^2, Glu^4]deltorphin from 79.5 ± 7.4% to 11.7 ± 2.9% and for DPDPE from 91.7 ± 5.7% to 19.5 ± 5.4%. In contrast, the *i.c.v.* effect of morphine was not changed by s.c. naltrindole (Table 1, Figure 49).

Similarly, *i.th.* administration of morphine results in dose- and time-related antinociception that is enhanced by *i.th.* pre-administration of the CCK_B antagonist L365,260. Left-ward displacement of the morphine dose-response

by L365,260 was approximately 3.8-fold (Table 1, Figure 50). The enhanced *i.th.* morphine effect by *i.th.* L365,260 was blocked by the *s.c.* pretreatment of the δ-selective antagonist naltrindole (Figure 50). *I.th.* administration of L365,260 produced no significant antinociception by itself.

S.c. administration of morphine also produced dose- and time-related antinociception. The morphine effect was similarly enhanced by pretreatment with L365,260, with the dose-effect curve being displaced to the left by approximately 3-fold (Table 1, Figure 51). Naltrindole pretreatment failed to alter the morphine dose-effect curve, but blocked the leftward displacement resulting from pretreatment with L365,260 (Table 1, Figure 51). The enhanced morphine antinociception by L365,260 administered *i.c.v.* was attenuated by an *i.c.v.* dose (4.4 nmol) of the δ antagonist ICI 174,864 that selectively blocks δ receptors (Figure 52). this dose of ICI 174,864 has no effect on morphine antinociception by itself (Figure 52).

Pretreatment of mice with the μ antagonist, β-FNA, resulted in a rightward displacement of the morphine dose-response curve with an A_{50} value of 26.3 (22.8-30.2) nmol (Figure 53). Animals pretreated with β-FNA and administered L365,260 shifted the new morphine dose response curve to the left by approximately 3-fold (Figure 53). The A_{50} for the morphine dose response in β-FNA and L365,260 treated animals was 9.1 (6.7-12.2).

i.c.v. administration of [Leu^5]enkephalin at 2.5 μg did not produce any measureable antinociceptive effects alone, but when co-administered with morphine produced a leftward shift of the morphine dose-effect curve. Antisera raised against [Leu^5]enkephalin or [Met^5]enkephalin did not affect the response to *i.c.v.* morphine (data not shown). Antisera to [Leu^5]enkephalin, but not to [Met^5]enkephalin, blocked the leftward displacement of the morphine dose-effect curve produced by *i.c.v.* [Leu^5]enkephalin (Vanderah et al., 1993). Similarly, antisera to [Leu^5]enkephalin, but not [Met^5]enkephalin, blocked the leftward displacement of the morphine dose-effect curve produced by *i.c.v.* L365,260 (Figure 54).

Animals treated chronically with [D-Ala^2, Glu^4]deltorphin by *i.c.v.* administration were cross-tolerant to the enhanced antinociceptive effect of *i.c.v.* administered L365,260 on morphine at three different doses of morphine (Figure 55). Repeated *i.c.v.* administration of L365,260 in different groups of mice resulted in a progressive diminution of the enhanced antinociceptive response of *i.c.v.* administered morphine observed after the first administration (Figure 56). A significant decrease in antinociception was seen after two injections of L365,260 and the antinociceptive response continued to decrease so that both administrations on day 2 resulted in virtually no change from morphine antinociception alone (Figure 56). Antinociceptive dose-response lines to [D-Ala^2, Glu^4]deltorphin were generated in either control animals or in mice

subjected to four *i.c.v.* injections of L365,260. The dose-response line of [D-Ala^2, Glu^4]deltorphin in chronically treated L365,260 animals was significantly displaced to the right by 8.2-fold (Figure 57). The calculated A_{50} values (and 95% C.L.) in control and in L365,260-tolerant mice were 8.3 (6.2 to 11.3) and 68.2 (41.1 to 113.4) for [D-Ala^2, Glu^4]deltorphin.

[Leu^5]enkephalin, when administered *i.c.v.* at sub-effective doses (2.5 μg), could enhance the antinocicetive effects of *i.c.v.* morphine, but was unable to enhance morphine antinociception in mice pretreated chronically with [leu^5]enkephalin (Figure 58). Similarly, *i.c.v.* administered L365,260 could no longer enhance the antinociceptive effects of morphine in animals pretreated with [Leu^5]enkephalin (Figure 58). Animals administered chronic [Leu^5]enkephalin exhibited cross tolerance to the enhanced antinociceptive effect of morphine by CCK_B receptor blockade.

I.c.v. administration of thiorphan (100 μg) did not produce any measureable effect alone (i.e., <5% response) in the 55°C. When thiorphan was given 5 min after *i.c.v.* L365,260 (i.e., 15 min prior to test), approximately a 35.8 ± 11.0% antinociceptive effect was observed (Figure 59). Pretreatment with naltrindole blocked this observed enhanced antinociceptive effect of L365,260 and thiorphan resulting in 11.0 ± 4.0% antinociception. ANOVA followed by Student's t-test revealed a significant difference between the groups, indicating that naltindole had antagonized the thiorphan/L365,260

antinociceptive response (Figure 59). The possible antinociceptive effects of thiorphan and L365,260 were also explored using the warm water tail-flick test with water maintained at 50°C, whereas, at the same doses, neither compound produced any measureable antinociceptive effect alone (Figure 59). When thiorphan was given 5 min after *i.c.v* L365,260 in the 50°C warm-water tail-flick test, a significant degree of antinociception was observed (Figure 59). This antinociceptive response was blocked by *i.c.v.* naltrindole and antisera to [Leu^5]enkephalin, but not by antisera to [Met^5]enkephalin (Figure 59). Both thiorphan and L365,260, administered *i.th.*, produced no significant antinociception by themselves in the 50°C warm-water tail-flick test (Figure 60). However, the *i.th.* coadministration of thiorphan and L365,260 resulted in significant antinociception in the tail-flick test, and was antagonized by the *s.c.* administration of a δ-selective dose of naltrindole (Figure 60). Naltrindole had no effect by itself on *i.c.v.* or *i.t.* thiorphan and L365,260 by themselves.

In animals made tolerant to the opioid δ receptor agonist [D-Ala^2, Glu^4]deltorphin, *i.c.v.* coadministration of L365,260 and thiorphan 12 hrs after the last [D-Ala^2, Glu^4]deltorphin injection, no longer resulted in significant antinociception (Figure 61).

Table 1. Morphine A_{50} (and 95% C.L.) following pretreatment with L365,260 or naltrindole.			
Pretreatment	**Intracerebro-ventricular**	**Intrathecal**	**Subcutaneous**
None	3.2 (2.5-4.0)	3.0 (2.07-4.31)	8.7 (7.3-10.5)
L365,260	0.45 (0.3-0.6)	0.79 (0.5-1.1)	2.7 (2.1-3.3)
Naltrindole	3.1 (2.3-4.2)	4.2 (2.7-6.7)	9.1 (7.3-11.3)
L365,260 plus naltrindole	2.9 (1.6-5.3)	3.0 (2.3-4.1)	8.9 (6.8-11.6)

Table 1. The A_{50} and 95% confidence intervals for morphine, morphine with L365,260, morphine with naltrindole, or morphine with both naltrindole and L365,260 administered by *i.c.v., i.th.* or by *s.c.* routes.

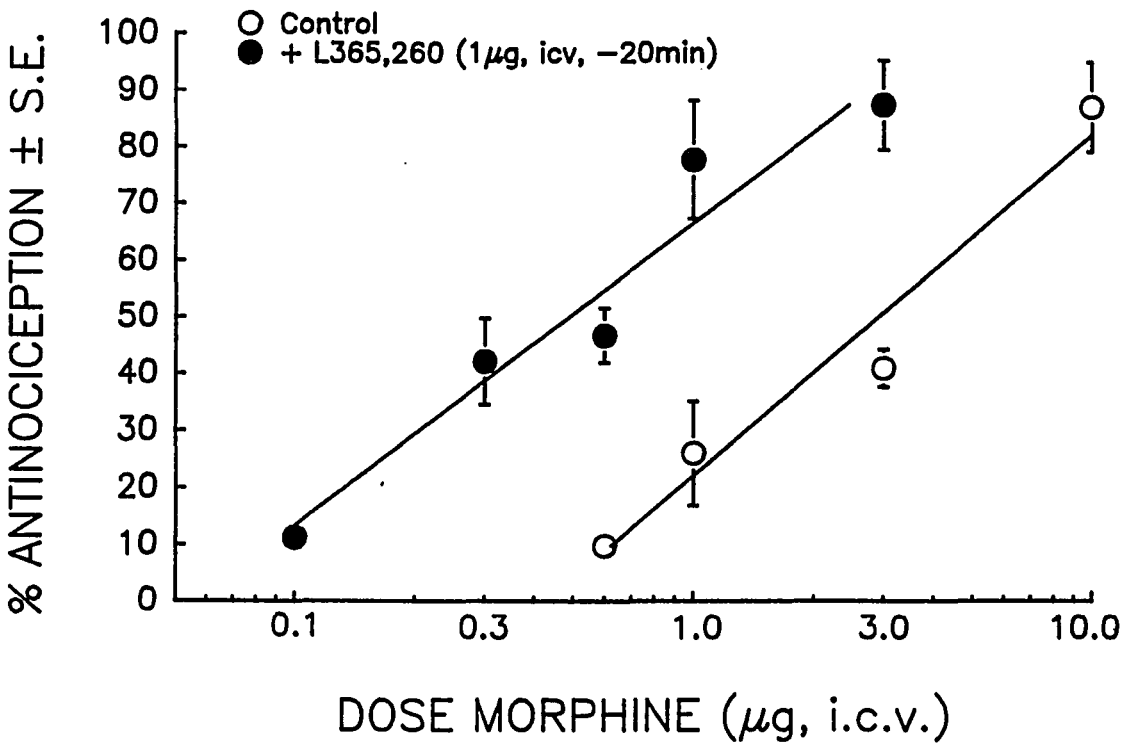

Figure 47. Morphine dose-response in control mice 10 min after *i.c.v.* aministration, or in mice pretreated with the CCK_B antagonist L365,260 (1 μg, *i.c.v.*) at 20 min prior to test (i.e., 10 min prior to morphine administration).

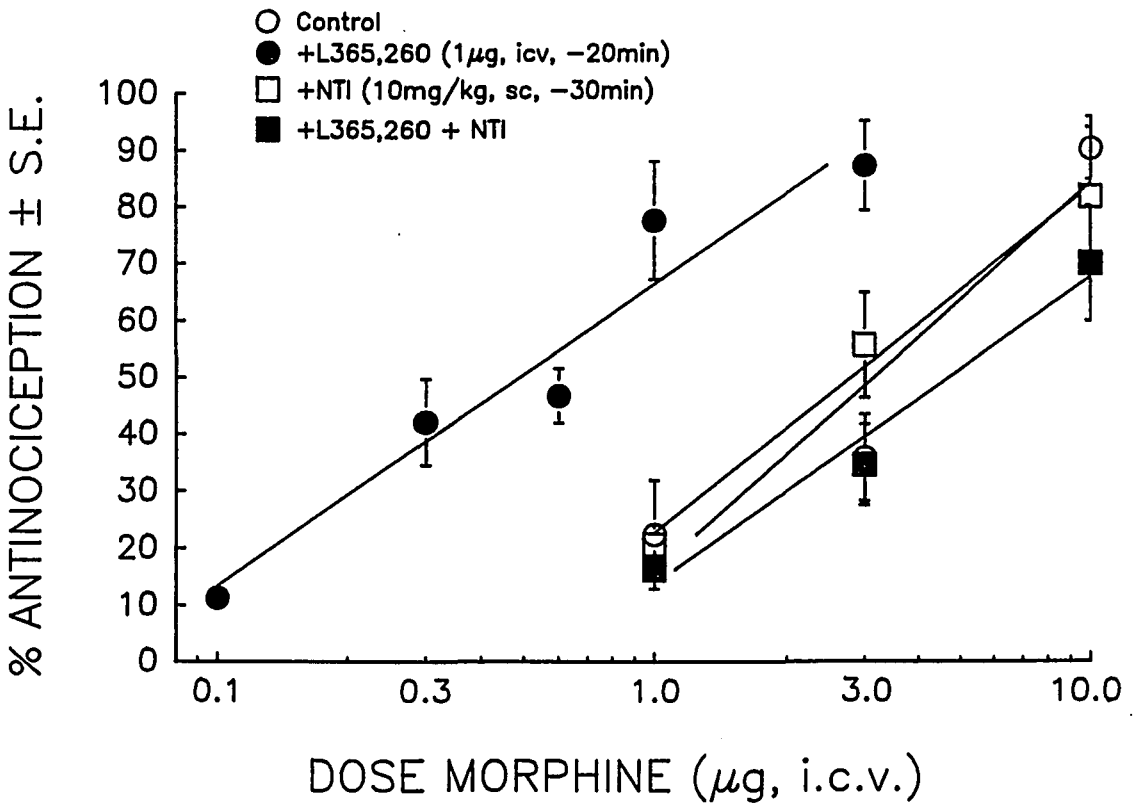

Figure 48. Morphine dose-response in control mice , or in mice pretreated with L365,260 in the presence or absence of naltrindole. Morphine was given *i.c.v.*, 10 min prior to testing. L365,260 was given *i.c.v.*, 20 min prior to testing. In experiments with naltrindole, this compound was given *s.c.*, at 10 mg/kg, 30 min prior to testing.

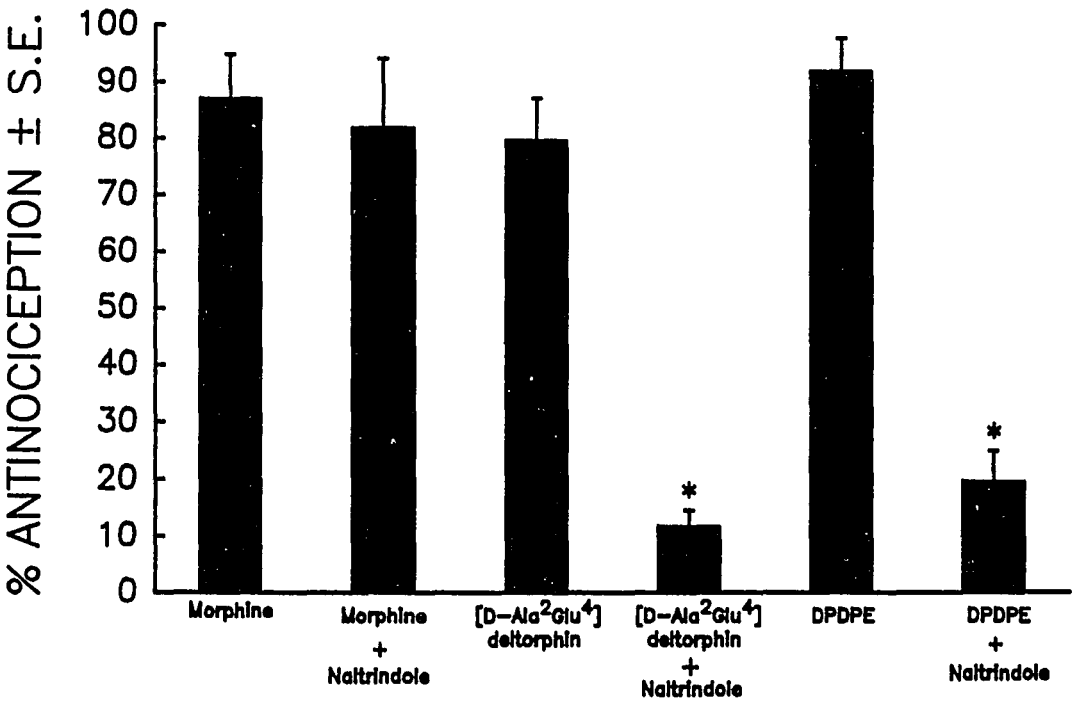

Figure 49. Morphine (*i.c.v.*, 10 μg), [D-Ala^2, Glu^4]deltorphin (*i.c.v.*, 12 nmol), or [D-Pen^2, D-Pen^5]enkephalin (*i.c.v.*, 30 nmol) administered in the presence or absence of naltrindole given *s.c.*, 10 mg/kg, 30 min prior to testing. Morphine, [D-Ala^2, Glu^4]deltorphin, and [D-Pen^2, D-Pen^5]enkephalin were administered 10 min prior to testing. Each bar represents 10 seperate groups of animals.

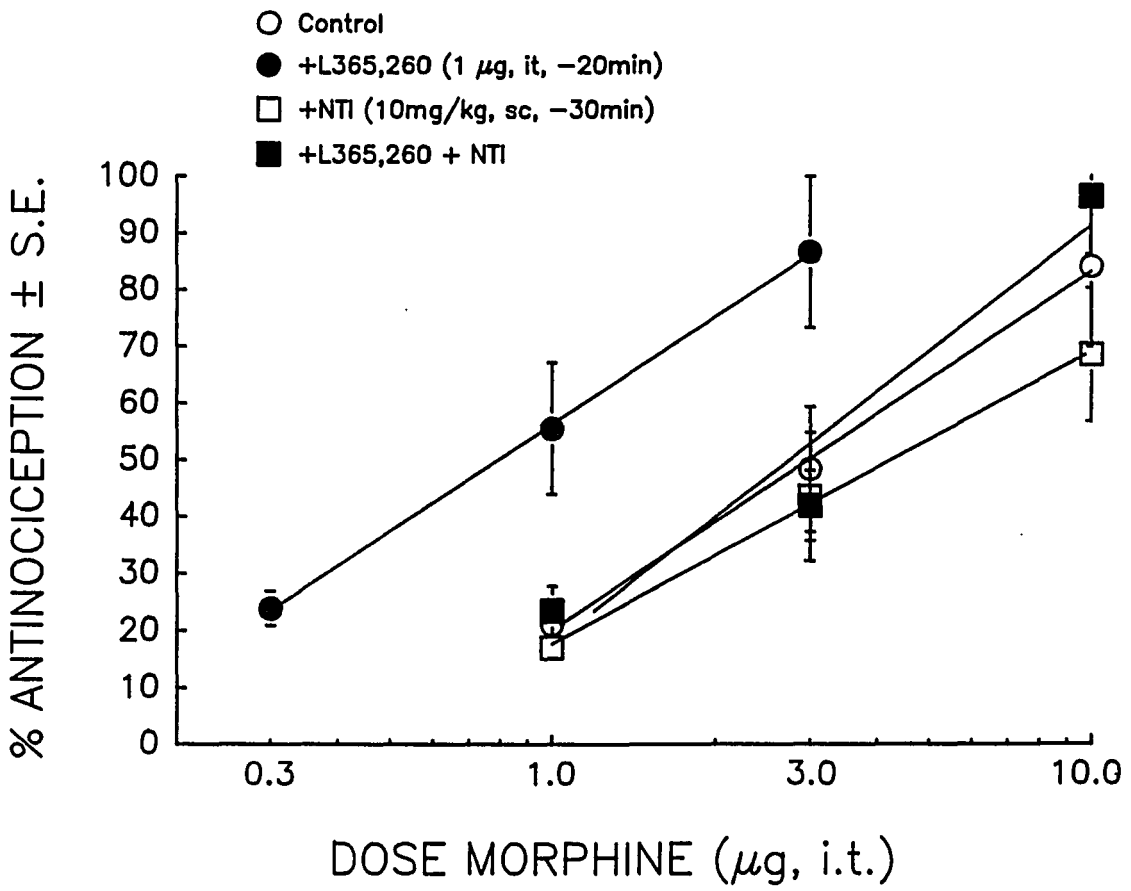

Figure 50. Morphine dose-response in control mice , or in mice pretreated with L365,260 in the presence or absence of naltrindole. Morphine was given *i.th.*, 10 min prior to testing. L365,260 was given *i.th.*, 20 min prior to testing. In experiments with naltrindole, this compound was given *s.c.*, at 10 mg/kg, 30 min prior to testing.

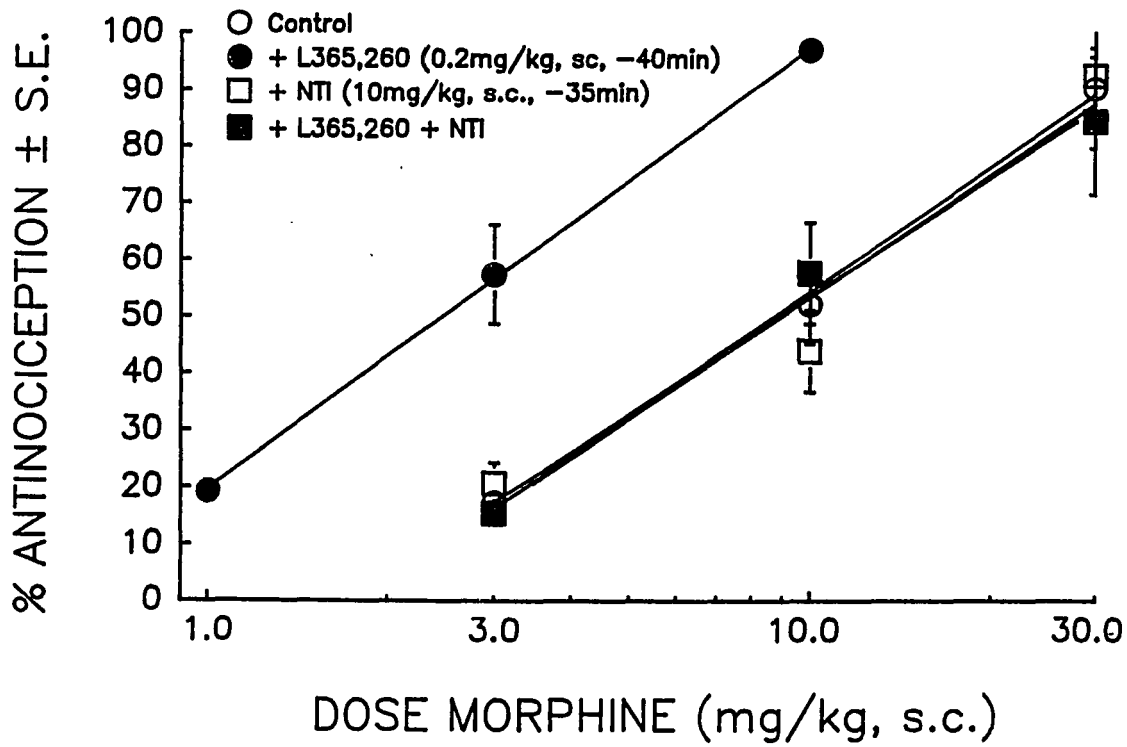

Figure 51. Morphine dose-response in control mice , or in mice pretreated with L365,260 in the presence or absence of naltrindole. Morphine was given *s.c.*, 30 min prior to testing. L365,260 was given *s.c.*, at 0.2 mg/kg, 40 min prior to testing. In experiments with naltrindole, this compound was given *s.c.*, at 10 mg/kg, 35 min prior to testing.

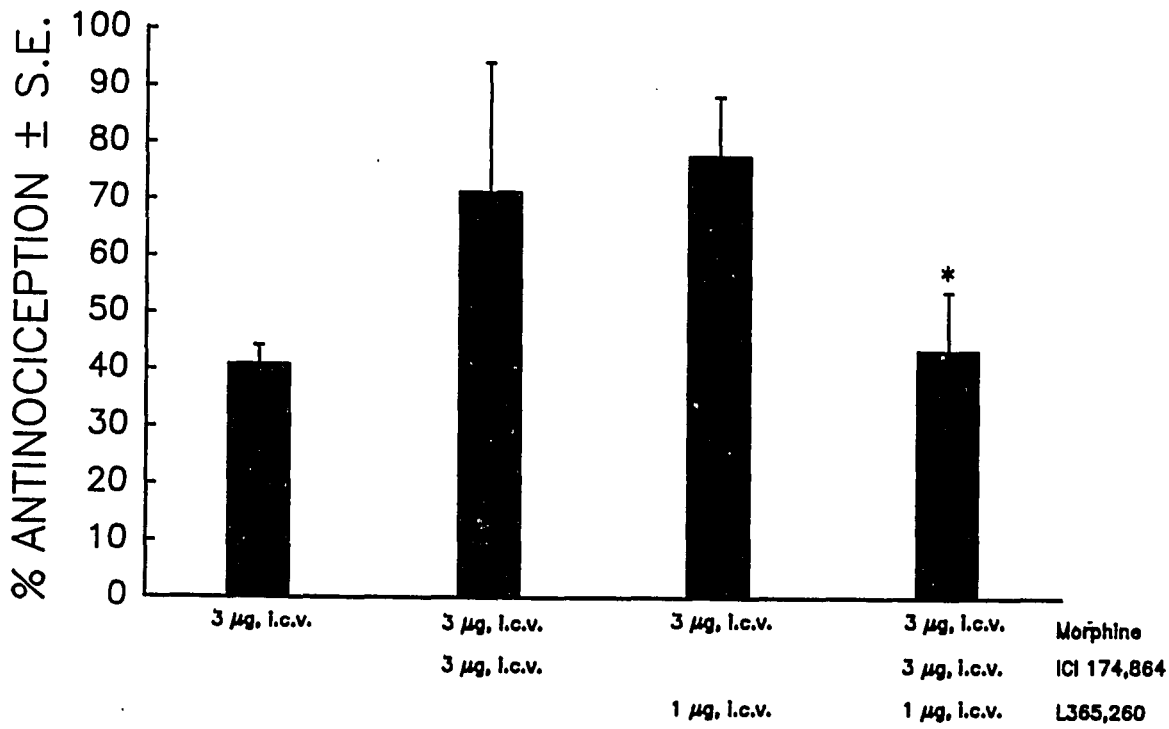

Figure 52. Morphine antinociception (3 μg, *i.c.v.*) is enhanced in the presence L365,260 (1 μg, *i.c.v.*) administered 10 min prior to morphine and tested in the mouse warm-water tail-flick test 10 min after morphine. Coadministration of ICI 174,864 (3 μg, *i.c.v.*) with morphine blocked the enhanced antinociceptive effect of L365,260, yet ICI 174,864 had no effect on morphine alone.

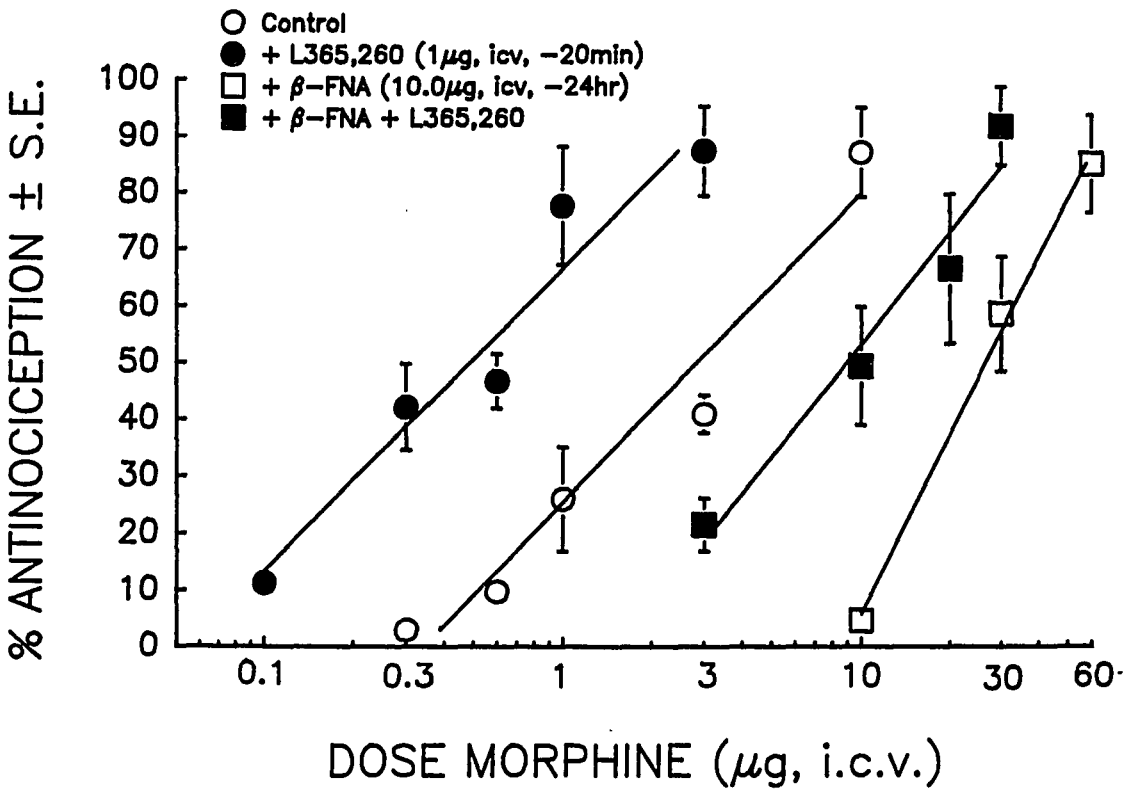

Figure 53. Effect of pretreatment with β-FNA (18.8 nmol, *i.c.v.*, -24 hr) on the antinociception of *i.c.v.* morphine in control mice, or in mice administered L365,260.

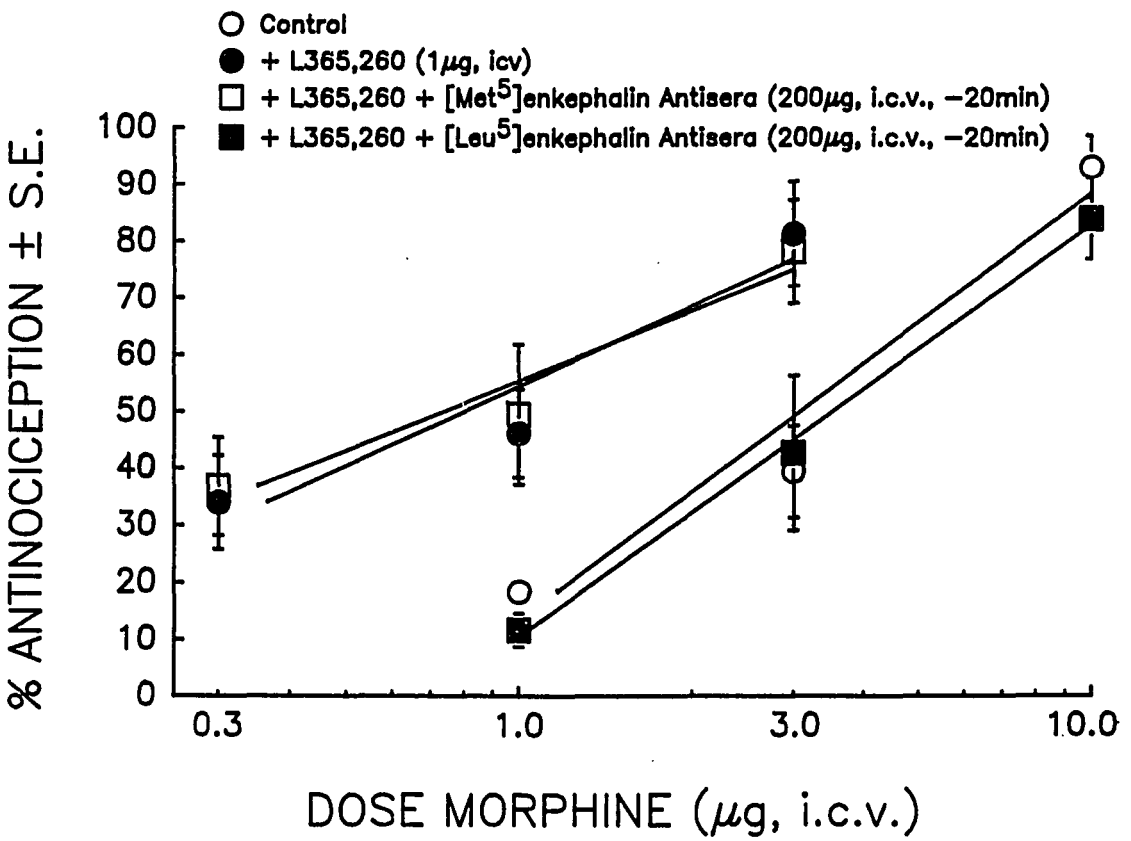

Figure 54. *I.c.v.* dose-response lines for morphine in control mice, or in mice administered L365,260, in the presence of antisera (200 μg/5 μl, *i.c.v.*, -20 min) raised against either [Met^5]enkephalin or [Leu^5]enkephalin.

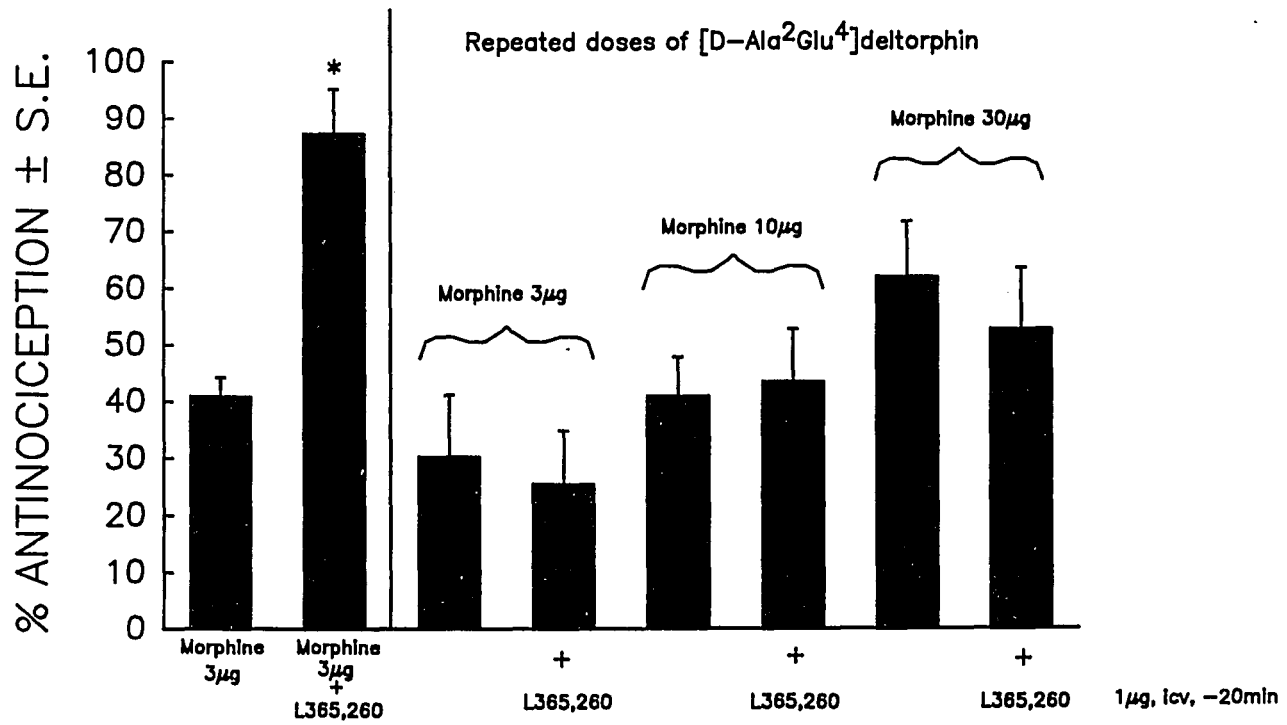

Figure 55. *I.c.v.* morphine dose response in the absence or presence of L365,260 (1 μg, *i.c.v.*) in animals pretreated chronically with [D-Ala^2, Glu^4]deltorphin for three days twice a day. Morphine was tested 10 min after injection, and L365,260 was administered 10 min before morphine (i.e., 20 min before test) on day four after chronic treatment. Each bar represents a minimum of 10 animals.

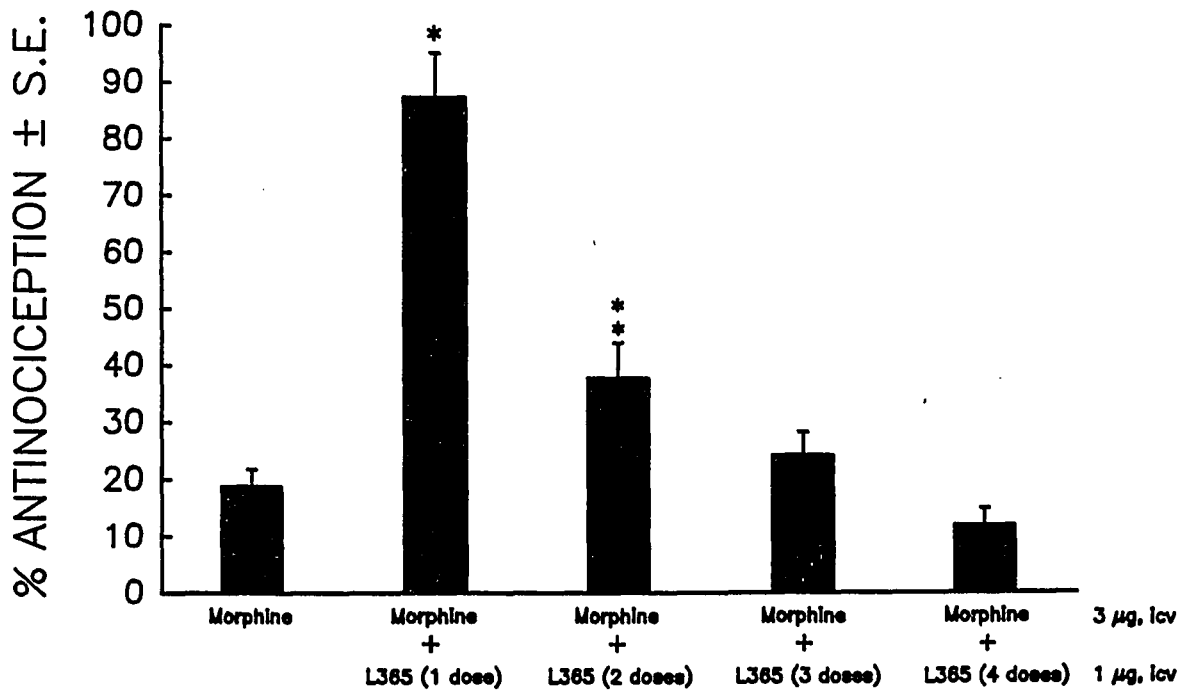

Figure 56. Time course for the development of tolerance to the enhaced antinociceptive effects of L365,260 (1 μg, *i.c.v.*) and morphine (3 μg, *i.c.v.*). Data are expressed as the means ± S.E.M. for pecentage of antinociception in seperate groups of mice injected *i.c.v.* twice daily for 3 days with L365,260. Testing was done 10 min after morphine administration, and 20 min after L365,260 administration.

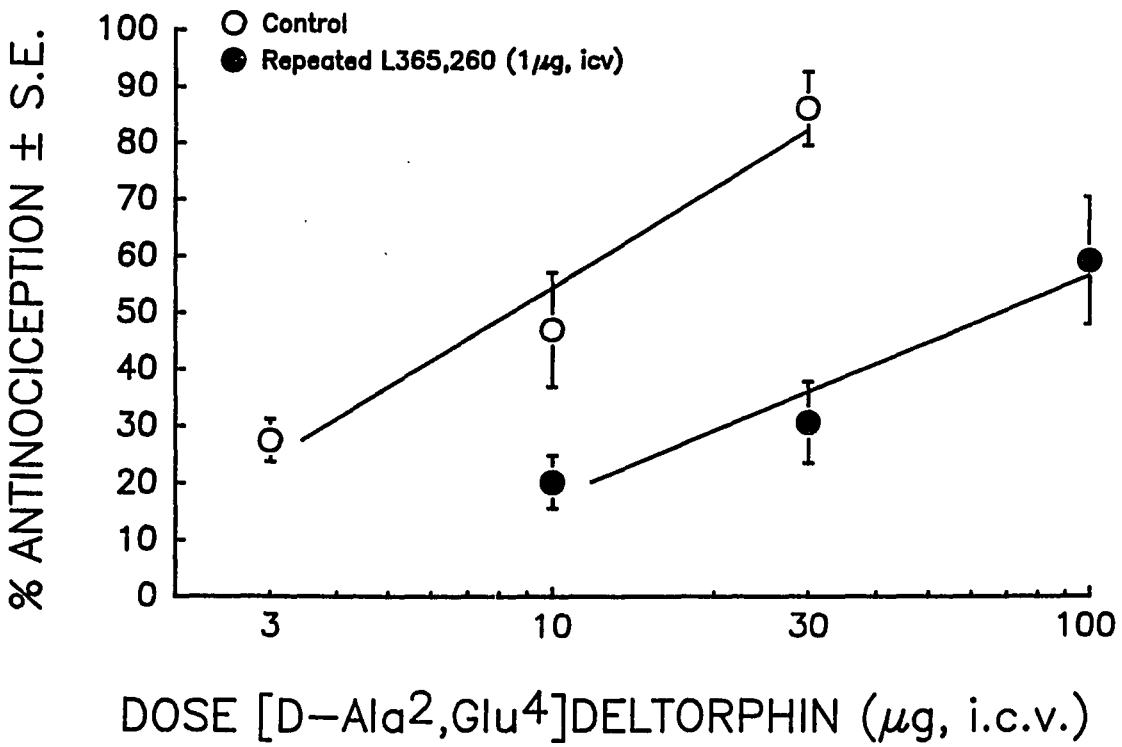

Figure 57. [D-Ala^2, Glu^4]deltorphin-induced antinociception after *i.c.v.* administration in naive mice or mice chronically given L365,260 (1 μg, *i.c.v.*). Each point represents ten mice.

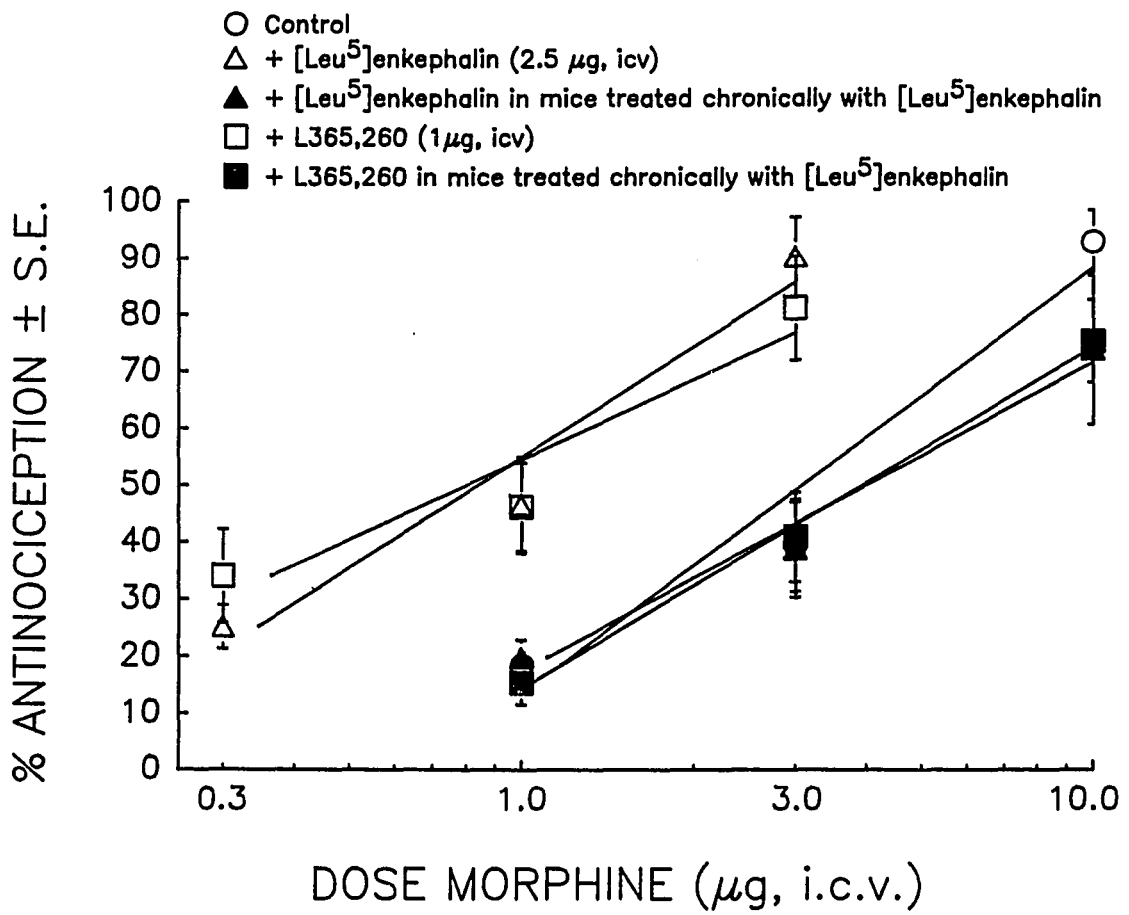

Figure 58. *I.c.v.* morphine antinociception in naive mice, in mice administered a modulating dose of [Leu^5]enkephalin (2.5 μg, *i.c.v.*), or in mice administered L365,260 (1 μg, *i.c.v.*). Enhanced morphine antinociception by [Leu^5]enkephalin or by L365,260 in animals treated chronically with [Leu^5]enkephalin (2.5 μg, *i.c.v.*) for 2 days twice a day.

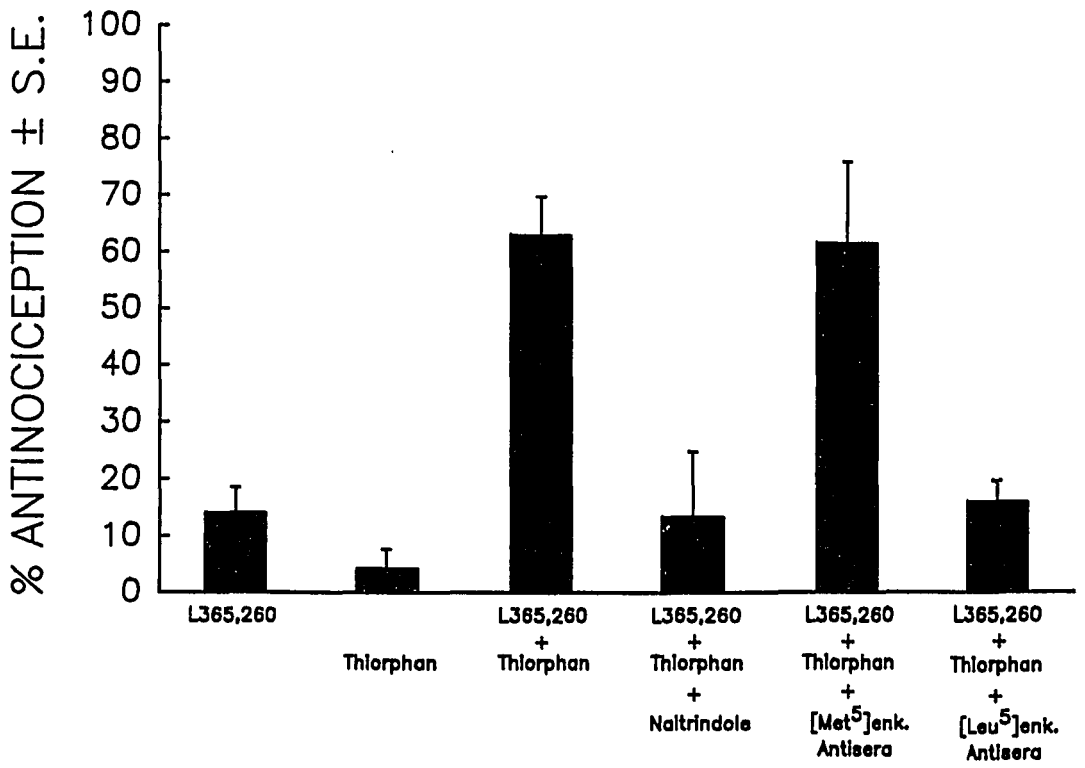

Figure 59. The antinociceptive effects of L365,260 (1 μg, *i.c.v.*) or thiorphan (100 μg, *i.c.v.*) alone or coadministered 20 min prior to test in the presence ar absence of naltridole (10 mg/kg, *s.c.*), [Met^5]enkephalin antisera (200 μg, *i.c.v.*), or [Leu^5]enkephalin antisera (200 μg, *i.c.v.*). Antisera administration was 25 min prior to testing (i.e., 5 min prior to coadministration of thiorphan and L365,260). Testing took place in 50°C water, with each bar representing a minimum of ten animals.

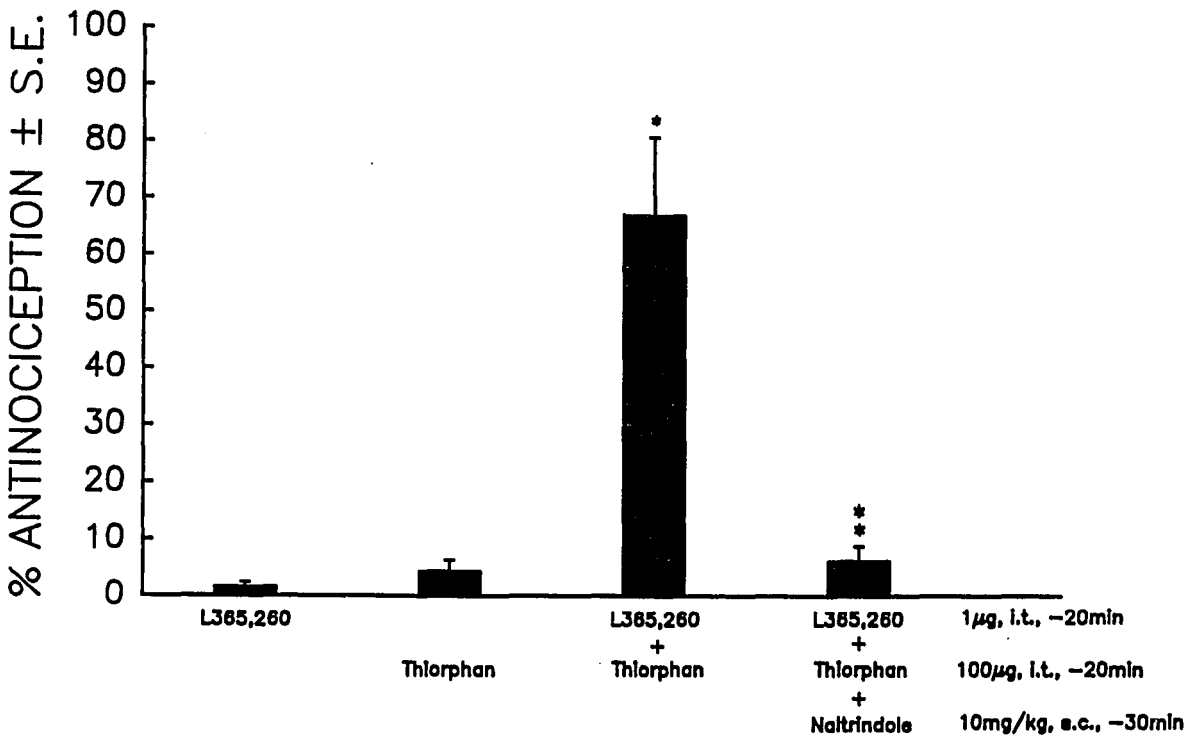

Figure 60. The antinociceptive effects of L365,260 (1 μg, *i.th.*) or thiorphan (100 μg, *i.th.*) alone or coadministered 20 min prior to test in the presence ar absence of naltridole (10 mg/kg, *s.c.*). Testing took place in 50°C water, with each bar representing a minimum of ten animals.

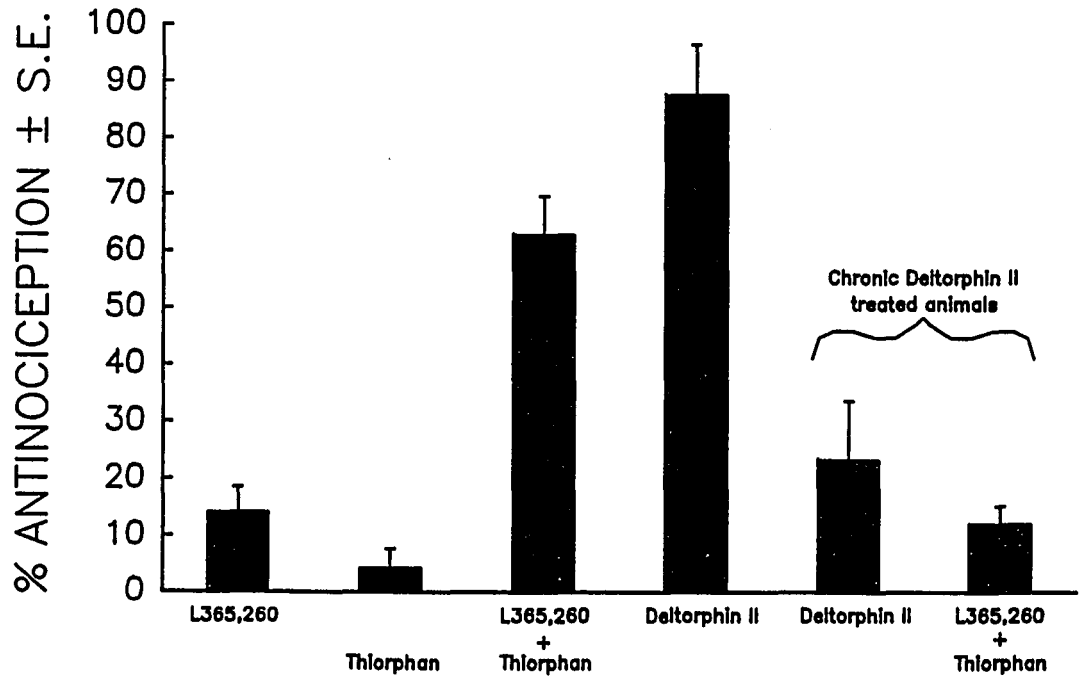

Figure 61. *i.c.v.* administration of L365,260 (1 μg, *i.c.v.*) or thiophan (100 μg, *i.c.v.*) in the naive animals or in animals pretreated chronically with [D-Ala^2, Glu^4]deltorphin (10 μg, *i.c.v.*) twice a day for three days. The antinociceptive effects of [D-Ala^2, Glu^4]deltorphin (10 μg, *i.c.v.*) was tested in naive animals or in animals pretreated chronically with [D-Ala^2, Glu^4]deltorphin. Testing took place on day four after chronic treatment, and each bar represents a minimum of 10 animals.

SUMMARY OF CCK_B RECEPTOR BLOCKADE

The present investigation has explored the possible involvement of opioid δ receptors in the well known modulatory actions of a CCK_B antagonist on morphine antinociception. Our data suggest that enkephalin release may be regulated tonically by CCK via CCK_B receptors. This conclusion is based on evidence related to blockade of the CCK_B antagonist modulatory actions by (a) a δ opioid receptor antagonist, (b) antisera raised against [Leu^5]enkephalin, but not [Met^5]enkephalin, (c) cross-tolerance with selective opioid δ-receptor agonists, and (d) the production of significant antinociception by a combination of an "enkephalinase-inhibitor" and CCK_B antagonist which was sensitive to an opioid δ receptor antagonist, chronic δ-receptor agonists, and to antisera directed at [Leu^5]enkephalin, but not [Met^5]enkephalin. Further, the observed effects were characterized by administration of these compounds either systemically, spinally or by direct injection into the lateral ventricles, suggesting that the observed modulatory actions and possible regulation of enkephalin may be occurring at both supraspinal and spinal sites. The latter suggestion is also supported by seperate experiments in the rat which demonstrate that *i.th.* administration of NTI blocks the modulatory action of *i.th.* L365,260 and morphine (Ossipov et al., 1994).

In the present study, administration of L365,260 at doses which have previously been reported to be selective for the CCK_B receptor (Dourish et al.,

1990) did not produce any measureable antinociception when evaluated in the tail flick test using water at 50 or 55°C as the nociceptive stimulus. This CCK_B receptor antagonist, however, produced a leftward displacement of the *i.c.v.* or *s.c.* morphine dose-effect curve, in accordance with several previous reports (Dourish et al., 1990, Lavigne et al., 1992, Stanfa and Dickenson, 1993). The observation reported here that the blockade of the CCK_B receptor produced an enhancement of morphine antinociceptive potency following *s.c.* administration of these compounds suggests that stress is not a necessary component in forming the modulatory effect. NTI was also shown to have no antinociceptive effect alone, and to selectively antagonize the antinociceptive actions of *i.c.v.* DPDPE and [D-Ala^2, Glu^4]deltorphin, without affecting the antinociceptive response of either *i.c.v.*, *i.th.* or *s.c.* morphine. These peptides have previously been demonstrated to produce their antinociceptive effects following *i.c.v.* administration via opioid δ receptors in this assay (Jiang et al., 1990a,b,c). These data suggest that NTI, at the dose chosen for these experiments, was acting via opioid δ receptors .

NTI pretreatment was able to block the leftward displacement of the *i.c.v.*, *i.th.* or *s.c.* morphine dose-effect curve produced by L365,260, suggesting that this modulatory action of L365,260 was occurring via opioid δ receptors. L365,260 is not known to bind to opioid receptors and so the observed interaction suggests an indirect mechanism involving an opioid δ

receptor agonist. Such a mechanism would be consistent with the observed modulatory effects produced by exogenous application of opioid δ agonists, such as [Leu^5]enkephalin (Figure 5, and Barrett and Vaught, 1982; Heyman et al., 1986b,1987,1989a,b; Jiang et al., 1990c).

RESULTS FROM CCK_B ANTISENSE STUDIES

Oligodeoxynucleotide of the CCK_B receptor (Figure 62) treatments alone had no behavioral effects on the mice and did not alter their weights. There was no difference in baseline antinociception between animals pretreated with water, mismatch or antisense oligos. *i.c.v.* administration of morphine in animals pretreated for three days with water produced dose-related antinociception with an A_{50} value of 3.8 (2.9 - 5.1 μg) (Figure 63). Evaluation of *i.c.v.* morphine antinociception in mice pretreated with *i.c.v.* antisense, but not mismatch, oligo to the CCK_B receptor demonstrated a significant enhancement in morphine potency as shown by the 5.9-fold leftward shift in the dose-effect curve. The A_{50} for morphine in animals pretreated with CCK_B antisense oligo was 0.6 (0.4 - 1.2 μg) while the morphine A_{50} value in animals treated with mismatch oligo was 4.0 (2.6 - 6.3 μg); the latter was not significantly different than that seen in animals treated with water (Figure 63).

Naltrindole had no antinociceptive effect alone in this assay, and did not significantly alter the antinociceptive effect of morphine. The A_{50} value for morphine in mice pretreated with naltrindole was 3.1 (2.3 - 4.2 μg)(data not shown). Pretreatment with naltrindole shifted the enhanced morphine dose response curve in CCK_B antisense pretreated animals to the right with an A_{50} of 6.3 (4.1 - 9.3 μg) (Figure 64). The antiserum raised against [Leu^5]enkephalin also shifted the enhanced morphine dose response curve in

CCK_B antisense treated animals to the right with an A_{50} of 5.4 (3.4 - 8.7 μg)(Figure 64). The morphine dose response curve in antisense pretreated animals in the presence of naltrindole or [Leu^5]enkephalin antiserum was not significantly different than the morphine dose response curve in control animals.

CCKB Receptor Antisense

ATG GAG CTG CTC AAG CTG AAC AGC AGC GTG CAG

GGA CCA GGA CCC GGG TCG GGG TCT TCT TTG TGC

CAC CCG GGT GTC TCC CTC CTC AAC AGC AGT AGT

GCG GGC AAC CTC AGC TGC GAG CCC CCT CGT ATC

CGC GGA ACC GGG ACC AGA GAA CTG GAG TTG GCC

ATT AGA ATC ACC CTT TAT GCG GTG ATC TTT CTG

ATG AGC ATT GGC GGA AAC ATG CTC ATC ATT GTG

GTC CTG GGA CTG AGC CGA CGC CTA AGA ACA GTC

ACC AAC GCC TTC CTG CCT TCC TGC TCT CCC TGG

CAG TCA GTG ACA TCC TGC 343

Figure 62. Partial sequence of the N-terminal of the mouse cloned CCK_B receptor as reported by Nakata et al. (1992). The 20 nucleotides underlined constitute the unique site chosen to design an antisense/mismatch for these studies.

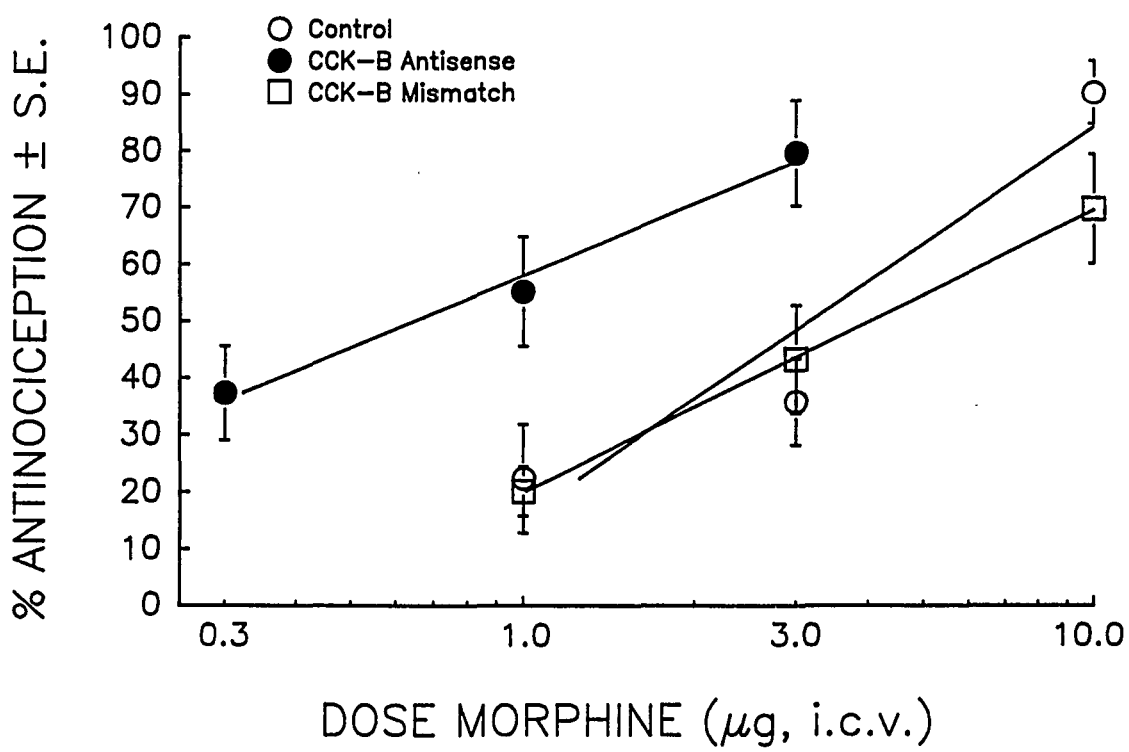

Figure 63. *I.c.v.* morphine dose-response lines in animals treated twice daily for 3 days with either a CCK_B antisense, CCK_B mismatch or vehicle. Treatments were given *i.c.v.* twice per day for three days and animals were tested on day four.

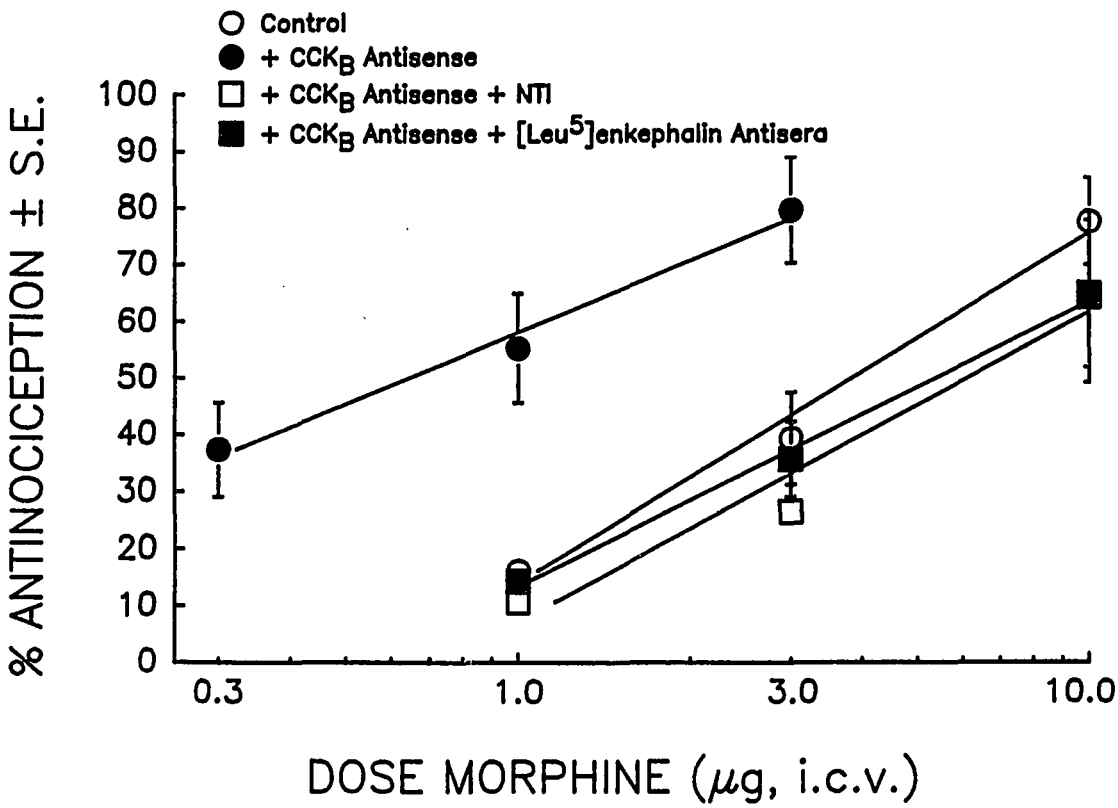

Figure 64. *I.c.v.* morphine dose-response lines in control mice or in mice treated twice daily for 3 days with the CCK_B antisense in the absence or presence of either naltrindole or the [Leu^5]enkephalin antiserum. Naltrindole was given *s.c.* 30 min before testing, and [Leu^5]enkephalin antiserum was given *i.c.v.* 20 min prior to testing.

SUMMARY OF CCK_B ANTISENSE STUDIES

Previous investigations have shown that endogenous CCK may act physiologically to attenuate opioid function (Weisenfeld-Hallin et al., 1990). A number of studies using CCK_B antagonists such as proglumide, lorglumide and L365,260 have shown enhancement of the antinociceptive effects of morphine (Lavigne et al., 1992). It has also been demonstrated that administration of CCK(*s*) octapeptide attenuates the antinociceptive effects of morphine at both spinal and supraspinal sites (Nobel et al., 1993), and that opioid agonists such as morphine and β-endorphin can induce the release of endogenous CCK in rodents (Tseng and Yuang, 1992). In addition to the modulation of opioid function, which has been reported with CCK agonists and antagonists, opioid δ ligands such as [Leu^5]enkephalin have been shown to produce a qualitatively similar modulatory action following exogenous administration, or by stimulation of release with stress paradigms (Barrett and Vaught, 1982; Jiang et al., 1990c; Vanderah et al., 1993; Watkins et al., 1985). The modulation of morphine antinociception by [Leu^5]enkephalin was blocked by δ, but not by μ (Heyman et al., 1989a) or κ antagonists (unpublished observations), suggesting that [Leu^5]enkephalin produces these modulatory effects via a δ opioid receptor (Heyman et al., 1989a; Jiang et al., 1990c). Similar findings resulted with synthetic and highly selective opioid δ agonists (Jiang et al., 1990c).

The present study using an antisense oligodeoxynucleotide strategy directed at the cloned CCK_B receptor (Nakata et al., 1992) supports previous suggestions that CCK acts via CCK_B receptors to negatively modulate endogenous opioid function (Lavigne et al., 1992; Weisenfeld-Hallin et al., 1990). Additionally, however, our data suggest that the modulatory effect of CCK involves the regulation of enkephalin levels and that the observed enhancement of morphine antinociception is the result of occupation of opioid δ receptors by [Leu^5]enkephalin or a [Leu^5]enkephalin-like peptide.

Evaluation of the morphine antinociceptive effect following treatment of mice with antisense, but not mismatch, oligos showed that the morphine dose-effect curve was displaced approximately 6-fold to the left. The magnitude of enhancement of the morphine antinociceptive effect with the antisense oligo treatment was similar to that observed following *i.c.v.* administration of L365,260 in mice (Vanderah et al., 1995). These data suggest that interference with CCK actions at a CCK_B receptor by use of an antisense oligo produced a modulatory effect on morphine antinociception, presumably by disrupting the synthesis of the CCK_B receptor protein (Albert and Morris, 1994). The involvement of opioid δ receptors in this modulatory action stems from the observation of antagonism of the morphine antinociceptive effect by naltrindole, an opioid δ receptor antagonist (Ossipov et al., 1994; Vanderah et al., 1995). Critically, naltrindole did not antagonize the antinociceptive effects of morphine

in control (water injected) or mismatch oligo treated animals, and the observed degree of antagonism of the morphine dose-effect relation in antisense oligo-treated animals was approximately 6-fold. That is, naltrindole blocked the enhancement of, but not the direct, morphine antinociceptive effect which was elicited by inhibition of CCK effects via CCK_B receptors.

In addition to the observed antagonism of the enhancement of morphine antinociceptive potency in antisense oligo treated animals, a similar effect could be elicited using antisera raised against [Leu^5]enkephalin, but not [Met^5]enkephalin. These antisera have been previously characterized by our laboratory in the same species and pharmacologic endpoint (Vanderah et al., 1993), and do not directly produce measurable antinociceptive or behavioral effects or alter the antinociceptive actions of morphine. Additionally, previous experiments have shown that antisera to [Leu^5]enkephalin, but not [Met^5]enkephalin, can prevent the increase in morphine antinociceptive potency produced by exogenous application of [Leu^5]enkephalin (Vanderah et al., 1995). The findings of the present study show that the increase in morphine antinociceptive potency observed after treatment with an antisense oligo to the CCK_B receptor is blocked by antisera to [Leu^5]enkephalin, but not to [Met^5]enkephalin, a finding which would be consistent with the potential inhibition of enkephalin release resulting from CCK actions via a CCK_B receptor.

It is also noteworthy that Weisenfeld-Hallin and colleagues have shown (Weisenfeld-Hallin et al., 1990) that PD134308 (CI-988), a selective CCK_B antagonist, directly produced a naloxone-sensitive antinociceptive effect when evaluated in depression of the rat flexor reflex. These observations led to the suggestion that antagonism of opioid analgesia by CCK may be tonically-mediated through a spinal CCK_B receptor, and that inhibition of this tonic activity leads to a hypothesized increase in opioid peptide release. It, therefore, seems reasonable to suggest, in view of the present findings with naltrindole and with antisera raised against [Leu^5]enkephalin, that the opioid peptides proposed to be released after blockade of CCK_B receptors might be [Leu^5]enkephalin, or a [Leu^5]enkephalin-like peptide and that the enhanced action of morphine under these conditions is related to the interactions of these peptides with opioid δ receptors.

RESULTS OF CLONING AN ORPHAN RECEPTOR

Degenerate primers were designed from the second and seventh transmembrane domain of three aligned mouse receptors: δ opioid, somatostatin and angiotensin. Polymerase chain reaction using the degenerate primers and mouse brain cDNA library resulted in a single product approximately 700 base pairs in length. When comparing this product to all of the gene products cloned thus far in the literature, a phylogenetic tree was constructed and found that the PCR product was more homologous with the delta opioid receptor, at 65% homology, than any other gene product (Figure 65A). Plaque hybridization screening using the 700 base pair PCR product resulted in three clones that were different upon enzyme digestion. These products were numbered 19, 20 and 21. All three products were 2,000 to 2,500 base pairs in size. Five different enzymes (Bam HI, Hind III, Not I, Ava I and Eco RI) were used on each clone which resulted in a number of different sized fragments from each clone, signifying that all three clones may be heterologous. Sequencing and identity comparison of clone number 19 was very homologous to the published δ opioid receptor gene, at 63% (Evans et al., 1992; Kieffer et al., 1992), whereas clone number 20 and 21 were less homologous to the δ opioid receptor, at approximately 40%.

Transiently transfected COS-7 cells with clone #19 were prepared for radioligand binding along with both positive and negative controls. Although

the cells transfected with reporter gene actively expressed the protein and acted as a good positive control, the cells transfected with clone #19 expressed no specific binding using several compounds such as; δ-selective [^{3}H]naltrindole and [^{3}H][4'-CL-Phe^4]DPDPE, μ-selective [^{3}H]CTOP, κ-selective [^{3}H]U69,593, σ-selective [^{3}H]SKF-10,047 and [5-^{3}H]DTG, as well as [^{125}I]-somatostatin analogues SS14 and SMS on crude membrane preparations.

A Northern blot was performed using a Multiple Tissue Northern Blot produced by CLONTECH with mRNA from mouse whole brain, skeletal muscle, heart, liver, spleen, kidney and testicles (Figure 65B). We found that the [^{32}P] labeled PCR product hybridized with the mRNA from the mouse whole brain but not any other tissue. This indicates that the PCR product that was amplified from the mouse brain cDNA library that is 65% homologous to the cloned δ opioid receptor is located in the mouse central nervous system and produces mRNA. Preliminary *In situ* studies have also shown that the PCR product lies within the hippocampus, striatum and cerebral cortex of the mouse brain.

Recent identity searches have determined that clone #19 is >95% homologous to the rat orphan gene reported by Wang et al., 1994a and Fukuda et al., 1994. This mouse orphan receptor clone is approximately 80% homologous in the transmembrane domain regions when comparing the amino acids to the other mouse cloned opioid receptors (Figure 66).

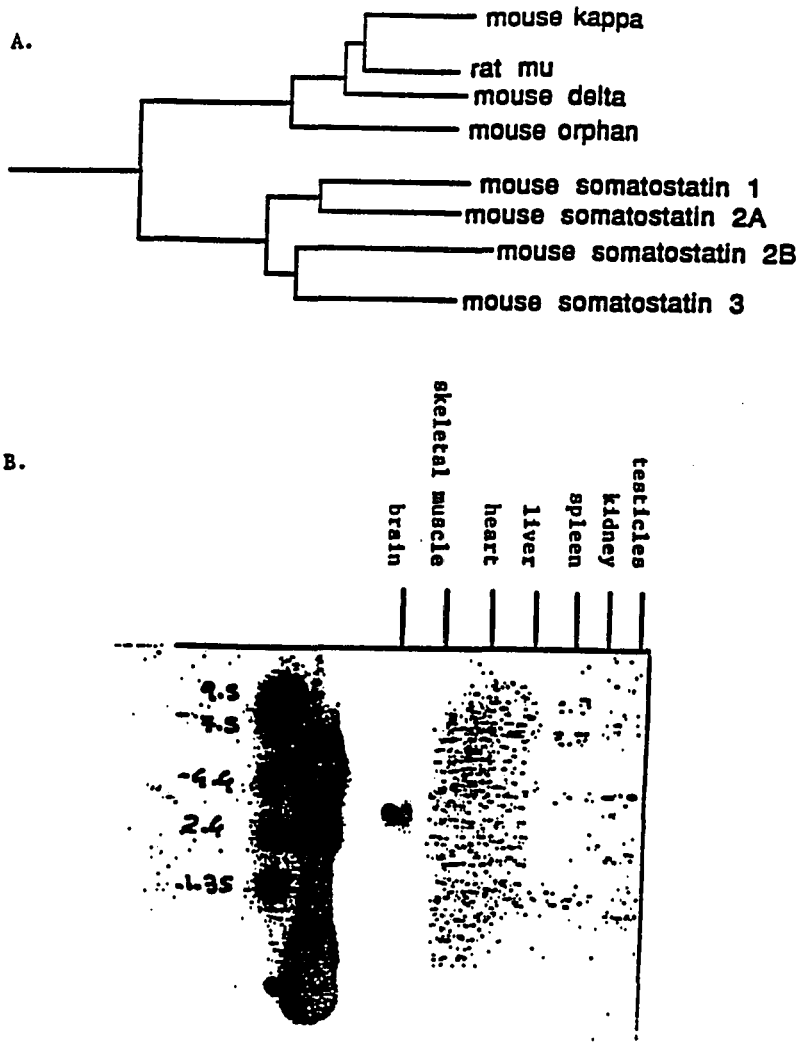

Figure 65A. Phylogenetic tree of the orphan receptor #19, the opioid receptors and the somatostatin receptors. On the basis of the distance matrix between aligned amino acid sequences, the tree was inferred by the neighbor-joining method. The deepest root was determined by including distantly related subfamilies in comparison. Branch lengths are proportional to the number of amino acid substitutions. The members of the opioid and somatostatin receptors include clones from the mouse unless it was unavailable as in the μ opioid receptor. The rat clone was used for the μ receptor. B. Northern blot analysis of the mRNA distribution in mouse tissue including brain, skeletal muscle, heart, liver, spleen, kidney and testicles. Each lane contained approximately 10 μg of mRNA from various tissues hybridized with 700 base pair [^{32}p]CTP PCR probe.

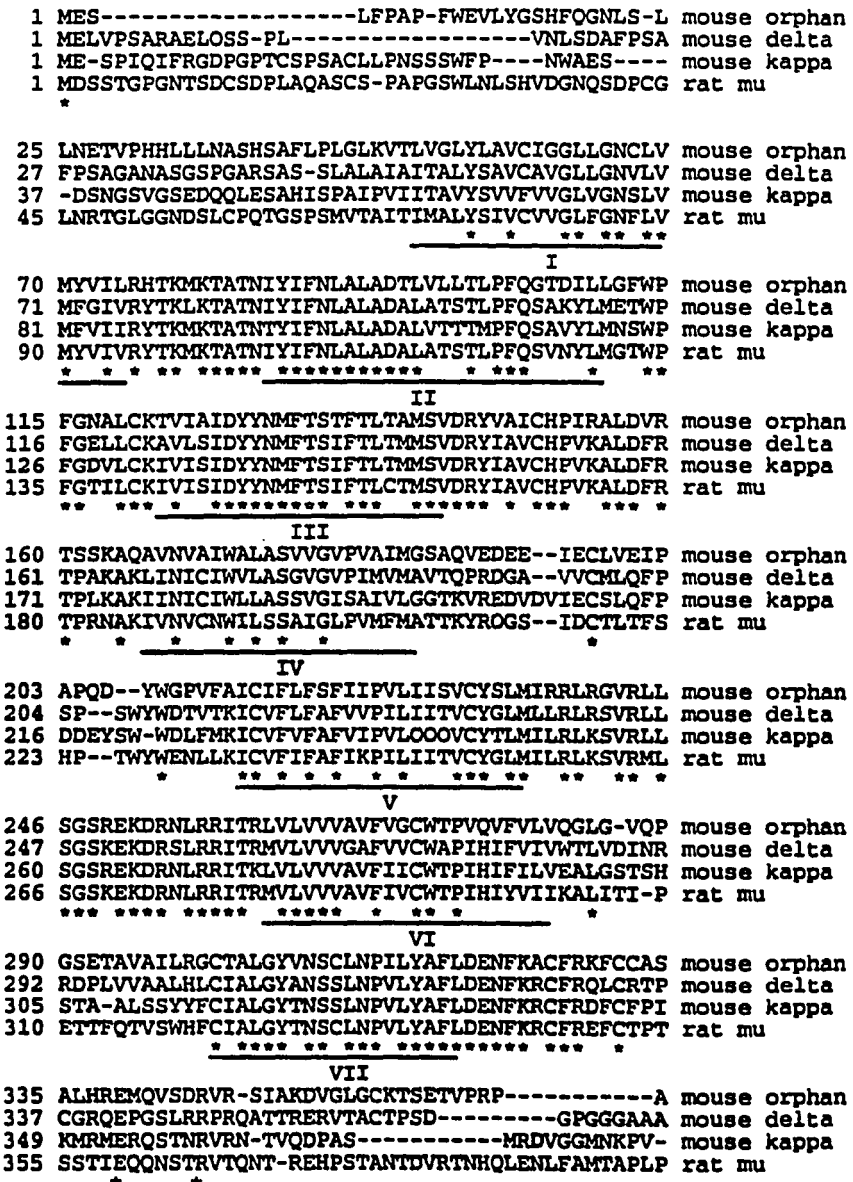

Figure 66. Deduced amino acid sequence of the mouse orphan clone #19 (first line) and its alignment with those of the mouse δ opioid receptor (second line), the mouse κ opioid receptor (third line) and the rat μ opioid receptor (last line). The one-letter amino acid notation and the open reading frame is used. Gaps (-) have been inserted to achieve maximal homology. Amino acid residues are numbered from the initiating methionine and numbers of the residues at the left-hand end of the individual lines are given. The predicted transmembrane segments (I-VII) are indicated. The asterisk (*) indicates consensus of an amino acid throughout all four receptors.

SUMMARY OF CLONING AN ORPHAN RECEPTOR

A number of recent reports have identified a receptor-like gene in the rat and human genome that are homologous to other G protein-coupled receptors including the opioid and somatostatin receptors. Although these genes have been categorized as G protein-coupled receptors, they do not actively bind any known ligand with high affinity, and are referred to as orphans (Wang et al., 1994a; Fukuda et al., 1994; Chen et al., 1994; Mollereau et al., 1994). Reported here, is the cloning of, yet, another orphan receptor from the mouse genome with over 80% homology to the cloned opioid receptors in transmembrane regions, however the amino- and carboxyl-terminal were very different in identity and length with less that 20% homology. This particular orphan is slighltly more homologous to the δ opioid receptor than the μ or the κ opioid receptors. The transiently transfected COS-7 cells with the orphan clone were unable to bind δ, μ, κ, σ or somatostatin compounds, therefore identifying the clone as an orphan until further characterization of the receptor is known. Both a Northern blot and *in situ* hybridization have demonstrated that mRNA for the mouse orphan clone is expressed in mouse brain tissue and not in other non-opioid tissues such as the heart, spleen, skeletal muscle, kidney, testicles and liver. The cloning of the orphan in several species only increases the urgency of discovering the natural agonist(s) of this new G protein-coupled receptor.

GENERAL SUMMARY

The goals set out in this dissertation were to investigate supraspinal and spinal δ opioid receptor subtypes in direct mediation and indirect modulation of antinociception in the mouse. The data reported within the dissertation suggest that: (1) δ opioid receptors enhance the antinociceptive effects of morphine, (2) cold-water swim-stress and inflammation in the paw produces significant antinociception via δ receptors by releasing an [Leu^5]enkephalin-like peptide and (3) that CCK tonically inhibits the release of [Leu^5]enkephalin or a [Leu^5]enkephalin-like substance, and that the observed increase in morphine antinociceptive potency may be associated with occupation of opioid δ receptors. This effect appears to be generalized at the supraspinal and spinal sites. Therefore, endogenous [Leu^5]enkephalin, as well as exogenous opioid delta agonists produce direct and modulatory antinociceptive actions that may be acting presynaptically on pain fibers (Figure 67). Opiate receptors, especially δ receptors, have been localized presynaptically on primary afferent neurons (Dado et al., 1993), and the co-localization of mRNA for CCK_B receptors and [Leu^5]enkephalin (Ghilardi et al., 1992; Stengaard-Pedersen et al., 1981), likewise, suggest the interaction between CCK and opiates. Other local or descending pathways that may be activated during stress and enhance the release of [Leu^5]enkephalin which produces direct and modulatory antinociceptive actions at δ opioid receptors (Figure 67).

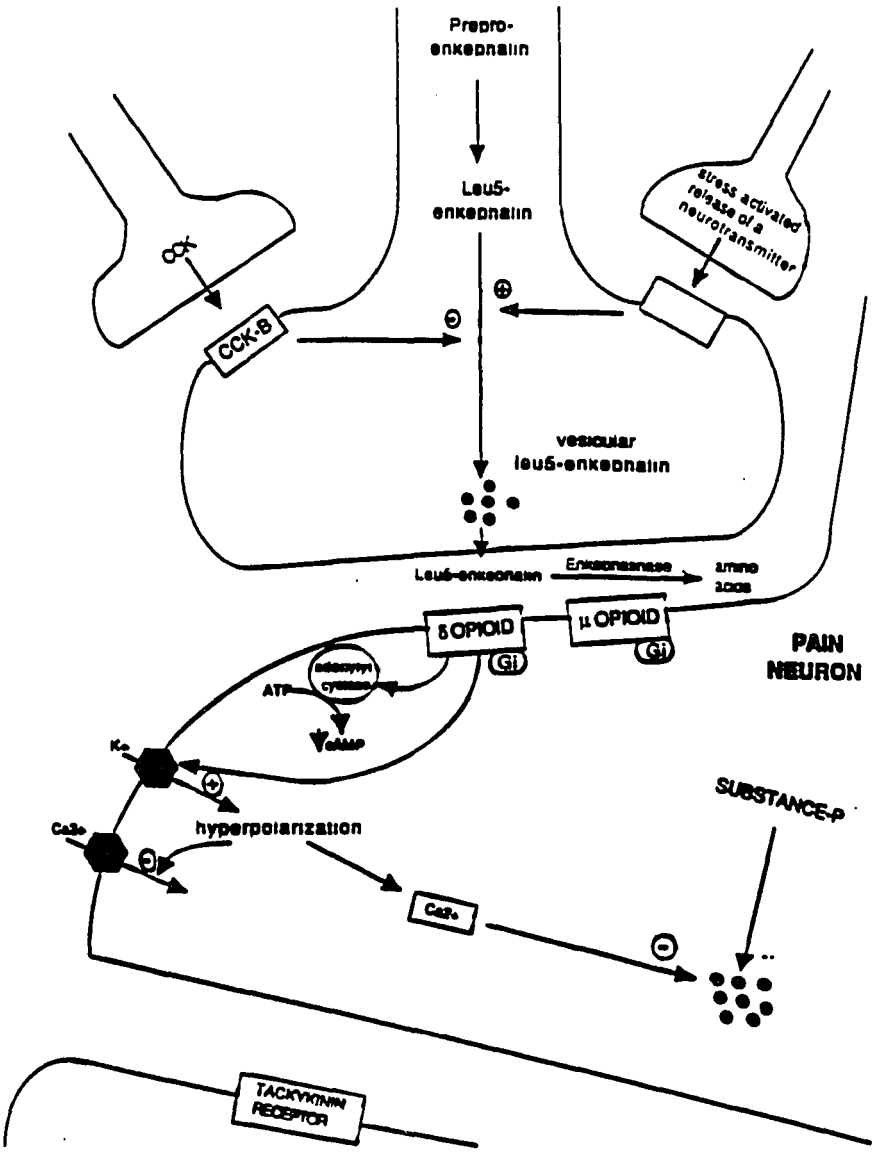

Figure 67. Diagram showing how cholecystokinin (CCK), by acting at CCK_B receptors, may reduce the analgesic effects of morphine. CCK, via CCK_B receptors, may tonically inhibit the release of [Leu^5]enkephalin or a [Leu^5]enkephalin-like peptide that would normally interact with δ opioid receptors presynaptically, enhancing the antinociceptive effects of the μ opioid agonist morphine. Opioid receptor activation by both morphine and [Leu^5]enkephalin result in hyperpolarization or the nerve terminal and a decrease in intracellular ca^{2+} accumulation, counter-acting the rise in intracellular ca^{2+} by depolarization of the pain fiber by a noxious stimuli. Other descending or local pathways may enhance the release of [Leu^5]enkephalin and produce direct and modulatory antinociception via δ opioid receptors.

Although subtypes of δ opioid receptors exist, the cloning of the receptor reported here does not bind any known opioid ligand with high affinity. This orphan clone resembles a G-protein coupled receptor and hybridizes with mRNA from mouse brain. Comparing the deduced amino acid sequence of the orphan with all proteins cloned and sequenced thus far using gene bank resulted in its highest homology to the opioid receptors. Specifically, the orphan was more homologous to the mouse cloned δ opioid receptor than the rat μ or the mouse κ opioid receptors.

These findings may have direct implications for the synthesis of new opioids, as well as for the use of analgesics that act at both δ and μ receptors or even at CCK_B sites. The clinical importance of analgesic combination therapy is such that tolerance will not develop for long-term narcotic use as seen when treating chronic pain. Based on previous reports, δ opioid agonist therapy may result in less dependence and less respiratory depression. The synthesis of new δ agonists and further clarification of interactions between the opioid and CCK receptor systems will inevitably lead to an improvement in pharmacologic agents for the clinical management of pain.

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