BEHAVIORAL AND BINDING CHARACTERISTICS OF OPIOIDS IN *RANA PIPIENS*

By

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LIST OF ABBREVIATIONS

β-FNA	beta-funaltrexamine
BSA	bovine serum albumin
CNS	central nervous system
CHO	Chinese hamster ovary
DOR	cloned δ opioid receptor
δ	delta opioid receptor
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
i.s.	intraspinal
i.t.	intrathecal
KOR	cloned κ opioid receptor
κ	kappa opioid receptor
K_D	affinity constant
K_i	inhibition constant
k_{off}	off rate
k_{on}	on rate
MOR	cloned μ opioid receptor
μ	mu opioid receptor
NTI	naltrindole
nor-BNI	nor-binaltorphimine
PEI	polyethylenimine
s.c.	subcutaneous

Chapter I

INTRODUCTION

1.1 Background

The ability to alleviate pain through the use of substances derived from opium has been known for centuries. The desire to understand the mechanism by which exogenous compounds alter physiological states led to an intensive search for the site of action of opiate compounds and gave birth to the hypothesis of an endogenous opiate-like substance (Terenius and Whalstrom, 1975). This hypothesis was strengthened with the observation that electrical stimulation of certain brain regions produces powerful, sustained analgesia in the rat (Liebeskind *et al.*, 1974; Mayer *et al.*, 1971; Mayer and Liebeskind, 1974; Reynolds, 1969). The finding that naloxone readily reversed this electrically stimulated analgesia implied the existence of an endogenous opioid substance (Akil *et al.*, 1974). In the next several years, the endogenous opioids were discovered, beginning with the detection of peptide extracts with opiate-like properties in pig brain (Hughes, 1975b; Hughes, 1975a). The active components of these extracts, the enkephalins, were isolated by Hughes *et. al.* (1975). Next came the discovery of β-endorphin (Bradbury *et al.*, 1976; Cox *et al.*, 1976) followed by dynorphin (Chavkin *et al.*, 1982; Goldstein *et al.*, 1979). For an extensive review of the endogenous opioid peptides see cited review (Evans *et al.*, 1988). The discovery of the body's innate ability to elicit analgesia peaked interest in the study of opioid binding sites as the targets for endogenous opioid peptides and exogenously administered opioids.

Since opioid receptors were first postulated in 1954 (Beckett *et al.*, 1956; Beckett and Casy, 1954; Portoghese, 1965; Portoghese, 1966), extensive studies have since been undertaken to determine their location as well as their biochemical and pharmacological properties. These specific sites of opioid action were hypothesized from behavioral and clinical studies that revealed a remarkable degree of steric and structural specificity required for the pharmacological action of opiates and opioid peptides (Simon and Hiller, 1978). Initial radioligand binding studies to characterize opioid binding sites were complicated by problems of distinguishing specific from nonspecific binding (Simon and VanPraag, 1966). However, the discovery of opioid stereospecificity (Portoghese, 1966) was a significant advance and several years later Goldstein and colleagues (1971) utilized stereospecificity to aid in the identification of opioid receptor sites. This enabled the definition of specific binding, the portion of bound ligand that is displaced by unlabeled opioid but not its inactive enantiomer (Goldstein *et al.*, 1971). Consistent modifications of Goldstein's protocol, including the use of very low concentrations of labeled ligand made possible by higher specific activity and washing homogenates after incubation with ice-cold buffer to remove unbound radioligand, led to the formal identification and biochemical characterization of specific opioid binding sites in 1973 (Pert and Snyder, 1973a; Pert and Snyder, 1973b; Simon *et al.*, 1973; Terenius, 1973).

The concept of multiplicity of opioid receptors originated as early as 1954 with the finding that nalorphine failed to antagonize analgesics to the same extent (Cox and Weinstock, 1964; Houde and Wallenstein, 1956; Lasagna and Beecher, 1954; Veatch *et al.*, 1964). Martin interpreted these findings by introducing the theory of Receptor Dualism which predicted the existence of two types of opioid receptors: M (for

morphine) and N (for nalorphine) (Martin, 1967). Martin's studies using the chronic spinal dog provided evidence of opioid receptor multiplicity and he modified his postulate to include three distinct opioid receptors located at spinal and supraspinal levels (Mansour *et al.*, 1994; Martin *et al.*, 1976). These receptors were termed μ (for morphine), κ (for ketocyclazocine) and σ (for N-allylnormetazocine or SKF 10047). The fact that the general opioid antagonist, naloxone, was unable to inhibit the binding of opioid ligands to the σ receptor (Holtzman, 1980) and the discovery of a high affinity binding site for the enkephalins in mouse vas deferens that was termed the δ opioid receptor (Hughes, *et al.*, 1975; Lord *et al.*, 1977) led to what is now accepted as the three types of receptors that mediate the effects of opioids, μ, κ and δ . These distinct opioid receptors have been confirmed by molecular cloning and have been pharmacologically identified as μ, κ and δ (Chen *et al.*, 1993a; Chen *et al.*, 1993b; Evans *et al.*, 1992; Kieffer *et al.*, 1992; Raynor *et al.*, 1994; Yasuda *et al.*, 1993). Further studies have classified these receptors into subtypes using classical pharmacological techniques (Barnard and Demoliou-Mason, 1983; Mattia *et al.*, 1991; Pasternak *et al.*, 1983; Paul *et al.*, 1989; Sofuoglu *et al.*, 1991; Zukin *et al.*, 1988). Whether these subtypes can be attributed to distinct genes or different post-translational processing has yet to be distinguished by molecular cloning. The cloned opioid receptors possess similarities that are highly conserved across species. In addition to high sequence homology, genes encoding MOR (μ opioid receptor), KOR (κ opioid receptor) and DOR (δ opioid receptor) are present on different chromosomes (Miotto *et al.*, 1995) and possess similar intron-exon boundaries within the protein coding region (Kieffer, 1995). The presence of

introns in the opioid receptor genes suggests the potential for alternative splicing to give rise to receptor variants.

1.2 Properties of opioid receptors

Opioids interact with specific proteins (receptors) embedded in the cell membranes of opioid-sensitive neurons where they initiate physiological events in the cell (Paterson *et al.*, 1984). The interaction of an opioid with its receptor is characterized by two important properties: the ability and strength of binding (affinity) and the potential magnitude of the induced effect (efficacy). The cellular effects of opioids include inhibition of the enzyme adenylate cyclase and modulation of calcium and potassium conductances leading to an overall inhibition of nerve transmission (Barnard and Demoliou-Mason, 1983; Reisine and Bell, 1993; West and Miller, 1983). μ, κ and δ opioid receptors consist of seven transmembrane spanning domains, a schematic of which is shown in Figure 1, and transduce their signals through guanine nucleotide binding proteins (G-proteins) which are composed of α, β and γ subunits. Activation of G-proteins involves association of the G-protein with the receptor, substitution of GTP for GDP and the spontaneous hydrolysis of GTP by GTPase (Nestler and Duman, 1994; Standifer and Pasternak, 1997). Activation of opioid receptors can be studied using pertussis toxin (PTX), a protein derived from *Bordetella pertussis*, which interferes with signal transduction by catalyzing the ADP-ribosylation of a specific cysteine side chain on the α-subunit of inhibitory G-proteins (G_i). This covalent modification freezes G_i in its GDP form so that it cannot be activated by ligand-receptor complex formation (Abood *et al.*, 1985; Bodnar *et al.*, 1990; Costa *et al.*, 1983; Lujan *et al.*, 1984; Parolaro *et al.*,

1991; Sanchez-Blazquez and Garzon, 1991; Shah *et al.*, 1994a). Thus, abolishment of opioid effects with PTX is indicative of G-protein involvement.

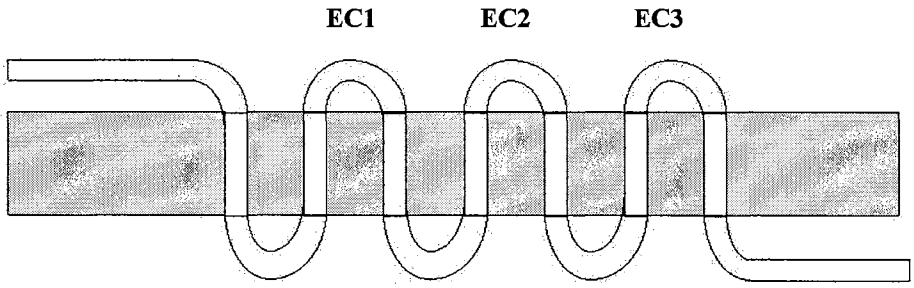


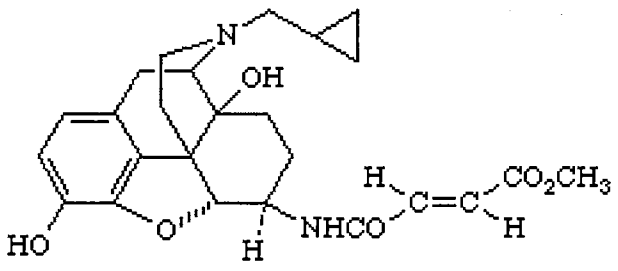
Figure 1. Schematic of a typical seven transmembrane spanning G-protein linked opioid receptor.

The μ, κ and δ opioid receptors have an overall homology of 60% with the most variation in the three extracellular loops. Site directed mutagenesis studies reveal that mutations in the extracellular loops of opioid receptors show dramatic effects in the binding of their respective ligands (Metzger and Ferguson, 1995). Specifically, mutation of the first extracellular loop (EC1) decreases the binding of μ opioid agents (Wang *et al.*, 1995), mutation in the second extracellular loop (EC2) diminishes κ ligand binding (Metzger and Ferguson, 1995) and interfering with the amino acid sequence in extracellular loop 3 (EC3) decreases δ opioid binding (Varga *et al.*, 1996). Thus, it is thought that each of the three extracellular loops confer specificity in binding and account for the signature ligand-binding profiles seen with μ, κ and δ opioid ligands in mammals (Probst *et al.*, 1992).

1.3 Selective antagonist studies of opioid analgesia in mammals

The study of systems with multiple receptor types is difficult in that most endogenous and synthetic ligands are not absolutely specific for a given receptor type but are selective for multiple receptors. This is further complicated by the fact that multiple receptor types can co-exist within a certain tissue or even within a single cell. For the most part, the definitive identification and classification of opioid receptor types and subtypes has been accomplished with the development of highly selective opioid ligands (Goldstein and Naidu, 1989). Selective opioid antagonists provide a novel pharmacological tool for the analysis of opioid receptor function by facilitating the analysis of individual receptor types which mediate the effects of exogenous opioids. The selective antagonists: β-funaltrexamine (β-FNA), μ selective, nor-binaltorphimine (nor-BNI), κ selective, and naltrindole (NTI), δ selective have been shown to exhibit high opioid receptor selectivity in mammals. These highly selective agents were used to study the organization of opioid receptors in *Rana pipiens* and for comparison to mammalian studies which will be discussed in Section 1.6.

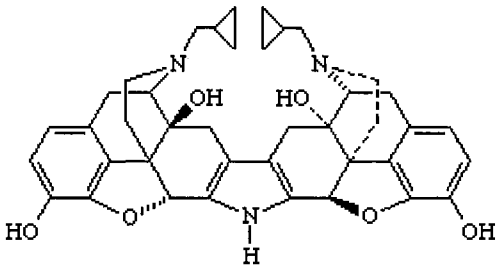
1.3.1 **β-FNA.** β-FNA, synthesized by Portoghese and colleagues (Portoghese *et al.*, 1980), is a naltrexone derivative which binds irreversibly to μ opioid receptors (Jiang *et al.*, 1990; Mjanger and Yaksh, 1991; Rothman *et al.*, 1988; Ward and Takemori, 1982; Zimmerman *et al.*, 1987).



β-FNA has been characterized to a great extent in rodents using various doses and injection routes (Martin *et al.*, 1993; Martin *et al.*, 1995; Ward *et al.*, 1982a). The effects of the spinal administration of β-FNA have been examined in several studies in rodents (Jiang, *et al.*, 1990; Mjanger and Yaksh, 1991; Russell *et al.*, 1987). The high selectivity of β-FNA for the μ opioid receptor has been shown both *in vitro*, where μ opioids are shown to be effective blockers of the specific binding of [^{3}H]-β-FNA, (Liu-Chen and Phillips, 1987; Tam and Liu-Chen, 1986; Ward *et al.*, 1985) and *in vivo*, where β-FNA reduced the analgesic effects of μ opioids but exhibited no effect on κ or δ opioid induced analgesia in rodents (Jiang, *et al.*, 1990; Martin, *et al.*, 1995; Ward, *et al.*, 1982a). In contrast to previous studies, acute spinal β-FNA administration has been shown both *in vitro* (Takemori *et al.*, 1981; Tam and Liu-Chen, 1986) and *in vivo* (Jiang, *et al.*, 1990; Ward, *et al.*, 1982a) to produce analgesia in the rodent which is blocked by naloxone. This analgesic effect can be further attributed to interaction with an opioid receptor, specifically the κ opioid receptor, since studies have shown that this analgesic effect of β-FNA is blocked by the κ opioid receptor antagonist, nor-BNI (Jiang, *et al.*, 1990; Takemori *et al.*, 1988). Additionally, the antagonism of the μ agonist, morphine, by β-FNA was not blocked by nor-BNI, suggesting that the antagonistic actions of β-FNA are not mediated by the κ opioid receptor as are its agonist properties (Jiang, *et al.*, 1990). Thus, β-FNA acts as a reversible κ agonist of short duration and as an irreversible μ antagonist of long duration at two separate opioid receptors. To avoid the complications of the analgesic agonist actions of β-FNA, studies have employed at least a 24 hour β-FNA pre-treatment to examine the effects of β-FNA antagonism alone (Jiang, *et al.*,

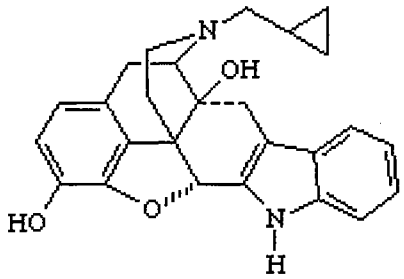
1990; Mjanger and Yaksh, 1991; Russell, *et al.*, 1987). The selectivity of spinally administered β-FNA for the μ opioid receptor has been shown in rats where β-FNA (i.t.) blocked the analgesic effects of μ ligands without affecting the production of analgesia by κ and δ ligands (Russell, *et al.*, 1987). The time course for β-FNA effectiveness as an antagonist has been addressed in several studies using rodents (Martin, *et al.*, 1995; Mjanger and Yaksh, 1991; Ward, *et al.*, 1982a). It has been shown that, in rats, after pre-treatment with β-FNA (i.t. 20 nmol/animal) there was a statistically significant recovery of activity beginning at 6 days post-treatment as noted by the animal response to μ agonists reaching control levels (Mjanger and Yaksh, 1991). This time course was also seen in mice by both s.c. (5 mg/kg) and i.c.v. (4.8 nmol/animal) injections of β-FNA in that it took 5-6 days for the animal's response to agonist to reach control levels (Ward, *et al.*, 1982a). The development of β-FNA is significant in that other widely used opioid antagonists such as naloxone and naltrexone are limited by their reversibility and overlapping receptor selectivity, although both appear to be more selective for the μ opioid receptor followed by the κ receptor and interacting least with the δ opioid receptor (Raynor, *et al.*, 1994). Thus, β-FNA, which selectively antagonizes the μ opioid receptor without irreversibly interacting with other receptor populations, holds great potential for enabling the characterization of physiological and pharmacological actions at opioid receptors.

1.3.2. nor-BNI. Nor-binaltorphimine is a long-lasting potent and selective κ opioid receptor antagonist (Horan *et al.*, 1992; Portoghese *et al.*, 1987).



Nor-BNI has been shown to antagonize κ opioid agonists *in vivo* (Broadbear *et al.*, 1994; Butelman *et al.*, 1993; Endoh *et al.*, 1992; Horan, *et al.*, 1992; Jones and Holtzman, 1992; Wettstein and Grouhel, 1996) as well as *in vitro* in mammals (Takemori, *et al.*, 1988). Studies with nor-BNI have shown a lack of antagonism of the antinociceptive effects as well as absence of inhibition of binding of μ or δ opioid agonists (Broadbear, *et al.*, 1994; Horan, *et al.*, 1992; Takemori, *et al.*, 1988). The production of analgesia by nor-BNI alone has not been demonstrated.

1.3.3 NTI. Naltrindole is a stable non-peptide antagonist which exhibits high selectivity and a potent affinity for the δ opioid receptor (Jackson *et al.*, 1989; Portoghese *et al.*, 1988; Takemori, 1985).



In mammals, the selectivity and affinity of naltrindole for the δ-opioid receptor has been shown both *in vitro* through binding studies where δ-opioid agonists inhibit the binding of [^{3}H] NTI while μ and κ-agents do not (Fang *et al.*, 1994; Portoghese *et al.*, 1988; Rogers *et al.*, 1990; Yamamura *et al.*, 1992) as well as *in vivo* where NTI was shown to

inhibit the antinociceptive actions of δ-opioid agonists but not those of μ or κ agonists (Calcagnetti and Holtzman, 1991; Drower *et al.*, 1991; Tiseo and Yaksh, 1993). Additionally, NTI has been shown to attenuate swim stress-induced antinociception, which is a model for endogenous δ-receptor activation, in a dose-dependent manner in the rat (Jackson and Kitchen, 1989). Agonistic properties of naltrindole have not been shown in mammals.

1.3.4. Schild Analysis. Further characterization of opioid receptors with selective antagonists can be made using Schild analysis which is a method for the characterization of functional receptors and is based on the equation developed by Gaddum for the competition of an antagonist and an agonist for a common binding site (Gaddum, 1937). This scale, developed by Schild, for antagonist affinity and potency was termed the pA scale (Schild, 1949). The term, pA_2, is an empirical parameter that defines the negative logarithm of the molar concentration of an antagonist which produces a two-fold shift to the right of a dose response curve. pA_2 represents the intercept of a Schild regression and can be considered an estimate of the equilibrium dissociation constant (K_B) for the antagonist of a receptor (Arunlakshana and Schild, 1959; Kenakin, 1982; Schild, 1947b; Schild, 1947a). The dextral displacements (dose ratios of agonist doses) are associated with the molar concentration of the antagonist producing the shifts and are related in the following known as the Schild equation (Arunlakshana and Schild, 1959):

$$\mathbf{\log(dr - 1) = n \log[B] - \log K_B}$$

The Schild equation is useful for the calculation of the K_B for a competitive antagonist. The x-axis of the Schild plot is -log[antagonist], M and the y-axis represents the log(dose ratio -1). Simple regression to the x-axis yields the apparent K_D for the antagonist (K_B)

for each selective opioid agonist. A Schild regression slope of unity is indicative of a one-to-one relationship between the antagonist and receptor with no substantial cooperative effects. Similar K_D values for antagonists may imply interactions at the same receptor. Schild analysis has been used extensively in the characterization of mammalian opioid receptors and is especially useful when a highly selective drug for a receptor is not available.

1.4 Binding studies of opioid receptors in mammals

Evidence for the molecular properties and multiplicity of opioid receptors in mammals has been obtained from radioligand binding studies using radiolabeled opioid drugs and peptides (Chang *et al.*, 1981; Gillan *et al.*, 1980; Lord, *et al.*, 1977; Pfeiffer *et al.*, 1982). Radioligand binding parameters are able to yield information about the structure and activity of compounds which transcends species and function. Binding studies provide a pharmacological correlate to the physiological information obtained in behavioral studies. Complementary binding studies are beneficial as behavioral experiments fail to yield parameters that reveal details of the ligand recognition site on opioid receptors while radioligand binding studies lack the physiological consequences mediated by receptor occupation. Unlike radioligand binding studies, behavioral analyses do not reflect pure drug-receptor phenomena but rather mirror the perilous journey a drug must overcome on its way to the receptor or the complex translation of the receptor events by the cell. The studies to be presented here involve examination of opioid receptor type(s) in the amphibian using radiolabeled nonselective antagonists as well as selective agonists in competition studies to compare with complementary behavioral studies.

1.4.1 [^{3}H]-Diprenorphine binding. Diprenorphine is a nonselective but potent opioid receptor antagonist that has been shown to label μ, κ and δ opioid receptor types with similar affinities *in vitro* (Chang, *et al.*, 1981; Iwamoto and Martin, 1981; Magnan *et al.*, 1982; Richards and Sadee, 1985). The high-affinity of [^{3}H]-diprenorphine makes it an ideal pharmacological tool for labeling opioid receptors. [^{3}H]-Diprenorphine has been used extensively in mammalian tissue models to characterize opioid receptor types. It has been shown to bind with considerable affinity to human neuroblastoma cell lines (Baumhaker *et al.*, 1993) as well as rat (Chang, *et al.*, 1981; Cunningham *et al.*, 1991; Wood and Traynor, 1989), guinea pig (Frances *et al.*, 1985; Traynor *et al.*, 1987; Wood and Traynor, 1989), rabbit (Farges *et al.*, 1988; Frances, *et al.*, 1985) and *Afghan pika* (Farges*et al.*, 1988) brain tissue. In addition, the above studies employed selective opioid agonists which effectively displaced [^{3}H]-diprenorphine binding to opioid receptors (Baumhaker, *et al.*, 1993; Chang, *et al.*, 1981; Farges, *et al.*, 1988; Frances, *et al.*, 1985; Wood and Traynor, 1989).

1.4.2. [^{3}H]-Naloxone binding. [^{3}H]-Naloxone is a nonselective opioid antagonist that has been used extensively for the characterization of opioid binding sites in mammals. Analyses using mammalian brain tissue shows that [^{3}H]-naloxone binds to two-sites with affinities ranging from 0.01 nM - 20 nM (Blurton *et al.*, 1986; Jacobson and Wilkinson, 1984; Pert and Snyder, 1973a; Pollack and Wooten, 1987). The density of receptor sites recognized by [^{3}H]-naloxone in the mammal are represented by B_{max} with values in mammals ranging from 13 - 177 fmol/mg protein (Schnittler *et al.*, 1990).

1.4.3. Radiolabeled selective opioid agonists. As previously mentioned, nonselective antagonists have played a large role in opioid receptor characterization in

numerous species. In the past, antagonists have been shown to be more selective for receptor types than agonists due to the larger, more flexible nature of antagonist molecules which allows them to bind accessory sites around the agonist binding site (Morley, 1983). However, the demonstration of the multiplicity of opioid receptors has ushered in the development of opioids selective for μ, κ and δ receptors. These selective opioid ligands have been examined in binding studies using CNS tissue from various species including rat, monkey, guinea pig, songbird, mongolian gerbil and frog. The highly selective opioid agonists, [^{3}H]-DAMGO (μ-selective) (Gillan and Kosterlitz, 1982) , [^{3}H]-DPDPE (δ-selective) (Akiyama *et al.*, 1985) and [^{3}H]-U65953 (κ-selective) (Kim *et al.*, 1996; Lahti *et al.*, 1985) have been used in numerous biochemical and pharmacological studies to define the nature of opioid receptors in CNS tissues (Clark *et al.*, 1988; Darlison *et al.*, 1997; Deviche *et al.*, 1993; Emmerson *et al.*, 1994; Gillan and Kosterlitz, 1982; Goldstein and Naidu, 1989; Niwa *et al.*, 1994). Selectivity profiles of these selective agonists allow the specific examination of opioid receptor types.

1.4.3. GTPγS^{35} binding. As mentioned previously, all known opioid receptors are coupled to G-proteins which in turn mediate biological responses by activating a number of second messenger systems or ion channels (Childers, 1991; Gilman, 1987). Activation of G-proteins after agonist occupation is determined as an increase in the binding of the radiolabeled nucleotide, GTPγS^{35}, to membranes. This technique affords the opportunity to examine the direct functional coupling of a receptor to the activation of G-proteins without preference to the types of G-proteins or effector systems involved. Stimulation of GTPγS^{35} is blocked completely by pretreatment with pertussis toxin confirming that the event is mediated through pertussis toxin-sensitive G-proteins (Traynor and Nahorski,

1995). Agonists stimulate GTPγS^{35} binding to receptor-coupled G-proteins in a concentration dependent manner in cell membranes in the presence of excess GDP (>1000 fold excess) which is required to decrease basal G-protein activity and reveal agonist-stimulated GTPγS^{35} binding (Sim *et al.*, 1995).

This technique distinguishes the ability of agonists to activate G-proteins and provides a functional correlate of radioligand binding experiments using simple membrane preparations. A rank order of potency can be obtained from these studies where EC50 values are defined as the concentration of the agonist producing 50% of the maximal response. These values should correlate with equilibrium dissociation constants from receptor binding experiments. Overall, this technique provides a measure of the functional response obtained by opioid agonists where the relative agonist efficacy is a function of the intrinsic ability of the ligand to activate the receptor as measured by GTPγS^{35} binding. The efficacies of different agonists can be compared in agonist concentration-effect curves through examination of their stimulation of GTPγS^{35} binding (Selley *et al.*, 1997; Traynor and Nahorski, 1995).

GTPγS^{35} stimulation has been examined in the amphibian (*Rana esculenta*) where κ opioid ligands were shown to cause a concentration-dependent stimulation of GTPγS^{35} binding which was completely inhibited by the κ-selective opioid antagonist, nor-BNI (Rottmann *et al.*, 1998).

1.5 Antinociception in the amphibian

When a stimulus threatens the homeostatic integrity of a tissue, steps occur to insure that the organism perceives the stimulus as harmful. Pain is a protective mechanism designed to alert an organism of impending environmental or internal damage. The

emotional response to pain cannot be evaluated in any animal and thus the term antinociception is used rather than analgesia and nociception rather than pain. The ability of the amphibian to experience pain is thought to be on a lower scale than that of phylogenetically higher species. Researchers have shown that the "pain potential" of an organism is correlated with phylogeny with respect to number of neurons, complexity and specialization (Dennis and Melzack, 1983; Herrick, 1948; Kicliter and Ebbesson, 1976; Northcutt, 1984; Stevens, 1995).

Nociceptive pathways in the amphibian are not as well characterized as those in mammals. In mammals, fibers lead to well-defined projections into the dorsal horn of the spinal cord to convey the "pain message" to defined populations of neurons. The dorsal horn of the spinal cord of mammals is divided into highly organized laminae based on the types of neurons and their organization. It has been shown that nociceptive nerve fibers in the amphibian are located in the dermis of the skin, consist of thinly myelinated (A_δ) or unmyelinated (C) primary afferent fibers and are similar to those found in mammals (Adrian, 1926; Spray, 1976). These fibers can be triggered by weak acid, intense mechanical stimuli, and strong heating. Like the mammalian system, nociceptive afferents terminate in the dorsal field of the spinal cord although second order neurons which receive afferent input have not been found in the dorsal field of the frog spinal cord and ascending nociceptive pathways have not been firmly established (Simpson, 1976). However, a higher central connection has been shown where electrical stimulation of the frog sciatic nerve was shown to evoke potentials in the posterior thalamic nuclei as well as the hypothalamus (Vesselkin *et al.*, 1971). It is known that there is no thalamus to cortex connection in the amphibian which would suggest that the

motivational-affective component of pain is lacking such that the amphibian may be expected to have a vastly diminished potential for the appreciation of pain. In humans, diminished activity of cortical neurons by anesthesia or surgical lesion results in a loss of a full appreciation for pain (Talbot *et al.*, 1991; White and Sweet, 1969). Overall, the decreased neural complexity of the amphibian offers a unique opportunity to study the mechanisms of opioids leading to antinociception using a comparative approach.

1.6 Behavioral studies of selective opioid antagonists in Rana pipiens

The study of opioid receptor expression in phylogenetically different species has played a significant role in the understanding of opioid receptor pharmacology (Buatti and Pasternak, 1981; Pert *et al.*, 1974; Simantov *et al.*, 1976). Pezalla was the first to show opioid antinociceptive activity specifically blocked by naloxone in an intact non-mammalian vertebrate (Pezalla, 1983). Since then, opioid antinociception has been well characterized in amphibians and several studies have depicted a significant correlation for opioid effects between amphibians and rodents (Brenner *et al.*, 1994; Stevens *et al.*, 1994; Stevens *et al.*, 1995; Stevens, 1996a). The amphibian brain displays a high level of opioid binding and in fact there are greater amounts of protein in the frog brain than in the mammalian brain (Ruegg *et al.*, 1981; Simon *et al.*, 1982). The presence of opioid receptors and the effects of opioids in amphibians have been studied in several species (Doerr-Schott J, 1981; Jackson *et al.*, 1980; Jegou *et al.*, 1983; Pezalla and Stevens, 1984; Yui, 1982). The amphibian represents a unique model for which there is a well-established behavioral assay (see Section 2.3) for testing antinociception produced by opioid agents. The antinociceptive effects of opioids in amphibians have been well characterized (Pezalla, 1983; Stevens, 1988; Stevens, *et al.*, 1994; Stevens, 1996a;

Stevens and Rothe-Skinner, 1997). Opioid agents have been shown to elicit consistent and potent antinociception by s.c, i.s. and i.c.v. routes of administration. The antinociception produced by these agonists was shown to be opioid receptor mediated as it was significantly blocked by the general opioid antagonist, naltrexone. Systemic administration of μ, κ and δ opioids was shown to produce antinociception in a dose-dependent manner in the amphibian where the order of antinociceptive potency among μ agents was similar to that produced in mammals as the ED_{50} values of opioid agonists tested were significantly correlated between the frog acetic acid test and the mouse hotplate and writhing test (Stevens, *et al.*, 1994). The i.c.v. administration of μ, κ and δ opioid agonists also elicits antinociception in amphibians and is blocked by naltrexone (Stevens and Rothe-Skinner, 1997). Moreover, amphibians have an endogenous opioid system with the highest abundance of opioid peptides and binding sites among vertebrates (Jackson, *et al.*, 1980; Simantov, *et al.*, 1976; Stevens, 1988). This endogenous system is involved in antinociception produced in response to stress in the amphibian (Pezalla and Dicig, 1984; Stevens, *et al.*, 1995). Previous binding studies in the amphibian have shown that at least 70% of opioid binding sites are associated with the benzomorphan preferring (κ) type with few sites characterized as μ or δ receptor binding sites (Benyhe *et al.*, 1990a; Benyhe *et al.*, 1990b; Mollereau *et al.*, 1988a; Simon*et al.*, 1982; Stevens and Paul, 1995). It has been determined that this opioid binding site in amphibians is so uniquely different from mammalian opioid receptors that some authors call it a "non-μ, non-δ, non-κ" opioid receptor (Mollereau, *et al.*, 1988a).

1.6.1. Relative potency of μ, κ and δ agents. The production of analgesia after spinal administration of μ, κ and δ opioids in the amphibian has been examined

previously. Spinal injection of morphine produces a potent analgesic response in the amphibian which is completely blocked by naloxone (Pezalla and Stevens, 1984; Stevens and Pezalla, 1983). Levorphanol (Stevens and Pezalla, 1984) and the endogenous opioid peptides dynorphin, β-endorphin and met-enkephalin (Stevens *et al.*, 1987) also increase nociceptive thresholds significantly in a dose dependent manner in the amphibian when administered spinally. Intraspinal (i.s.) administration of selective μ, κ and δ opioid agents in *Rana pipiens* results in the production of significant antinociception which is blocked by naltrexone pretreatment and the potency of μ and δ opioid agonists is found to be highly correlated with the potency data of the same opioid agonists in mammalian studies (Stevens, 1996a). The high correlation of potency data of μ and δ opioid agonists in the amphibian with the same opioid agonists in the mammal suggests that amphibians and mammals appear to possess common mechanisms of opioid analgesia at the level of the spinal cord.

1.6.2. Selectivity of β-FNA, nor-BNI and NTI. The spinal administration of opioid agents has been widely examined in rodents (Yaksh and Rudy, 1976; Yaksh and Rudy, 1977; Yaksh and Stevens, 1988). Thus far, the effects of the spinal administration of opioids have not been thoroughly characterized in a non-mammalian model. The analgesia produced by the spinal administration of μ, κ and δ opioids was examined in the amphibian after a 24 hour pre-treatment with β-FNA (i.s.) where the pain threshold was determined by the acetic acid test (AAT). These studies show that β-FNA effectively antagonizes the analgesic effects of μ agonists for a longer duration in the amphibian as compared to the rodent. In addition, unlike what is observed in rodents, β-FNA itself does not exhibit analgesia in the amphibian. This lack of antinociception by

β-FNA alone allowed for the examination of the effect of concurrent administration of β-FNA with μ, κ and δ opioid agonists. These results show that, with few exceptions, β-FNA pre-treatment as well as the co-administration of β-FNA with μ, κ and δ agonists significantly attenuated not only the effects of μ opioid agents (morphine, fentanyl and DAMGO) but also those of κ (bremazocine and CI977) and δ (DPDPE, DSLET and deltorphin) agonists (Stevens and Newman, 1999). These effects of β-FNA in *Rana pipiens* are unlike those seen in mammalian studies where β-FNA was shown to block solely the actions of μ agonists (Hayes *et al.*, 1986; Jiang, *et al.*, 1990; Mjanger and Yaksh, 1991; Ward *et al.*, 1982b; Ward, *et al.*, 1982a; Ward and Takemori, 1982; Zimmerman, *et al.*, 1987). The higher selectivity of β-FNA in the mammal versus the broad range of antagonism of β-FNA seen in the amphibian may suggest a different organization of opioid receptors in the amphibian. The selective antagonists at κ and δ opioid receptors were also studied for comparison to the unique results obtained with β-FNA.

The effect of nor-BNI on the antinociception produced by the aforementioned μ, κ and δ agonists was also examined in the amphibian. Remarkably, co-administration of nor-BNI with selective opioid agonists also produced the nonselective profile seen with β-FNA (Stevens and Newman, 1999). That is, nor-BNI blocked the κ agonists, GR89696 and U50488, as well as the μ agonists, morphine and fentanyl, and the δ agents, DPDPE and deltorphin.

The selectivity of naltrindole antagonism was explored in the amphibian by its co-administration with μ, κ and δ opioid receptor agonists. Naltrindole was co-administered

with the selective δ opioid agents, DPDPE, DSLET, DADLE and deltorphin (Stevens and Newman, 1999). The selectivity of naltrindole was also examined with non-δ opioid agents including the μ opioid receptor agonists, morphine, fentanyl and DAMGO and the κ opioid receptor agonists, GR89696, U50488 and CI977. The present results involving the co-administration of NTI spinally with μ, κ and δ opioid agonists suggest that naltrindole, like β-FNA is a nonselective opioid antagonist in *Rana pipiens* as it blocked not only the antinociception produced by the δ agents DPDPE, DSLET and deltorphin, but also that produced by morphine and CI977 (μ and κ agonists, respectively).

The cross-over of antagonism by β-FNA, nor-BNI and NTI with the μ, κ and δ selective opioid receptor agonists may provide credence to the existence of a single type of opioid receptor, termed the "unireceptor", which accommodates and mediates the effects of multiple opioid agonists (Stevens and Newman, 1999).

1.6.3. Schild Analysis. Some of the behavioral selective antagonist results need to be clarified. For example, pre-administration of β-FNA blocks CI977 but not U50488. NTI attenuates the μ-agonists, morphine and DAMGO, but appears to potentiate the effects of fentanyl and DADLE. Nor-BNI blocks the δ-agonists, DPDPE and deltorphin, but not DSLET and also appears to potentiate the antinociceptive effects of DADLE. Generation of full dose-response curves and using Schild analysis for the agonists in the presence of various doses of i.s. selective antagonist may help to clear up these discrepancies as only one dose of agonist was tested in these studies. Schild plot analysis provides an estimate of the apparent equilibrium dissociation constant for the selective antagonist for each μ, κ and δ opioid ligand. The apparent K_D values for the selective antagonist against each

type of opioid ligand may be similar if opioids are mediating antinociception through a single receptor site.

1.7 Binding studies of selective opioids in Rana pipiens

Characterization of the spinal sites mediating opioid antinociception can be made using highly selective opioid antagonists. As mentioned in Section 1.3, selective antagonists have been widely used in mammalian models to examine specific recognition sites of opioid agents (Takemori, 1985). β-FNA (μ-selective), nor-BNI (κ-selective) and naltrindole (δ-selective) were employed in studies to explore the mechanism of opioid action in *Rana pipiens*.

1.7.1. Radioligand binding. Radioligand receptor binding has been used extensively to characterize and identify both neurotransmitter and hormonal receptor systems in mammals. Saturation and kinetic analyses of selective and nonselective opioid radioligands in the amphibian were done for comparison to mammalian binding data. Further characterization of opioid binding sites in amphibian CNS tissue homogenates is possible using competition analyses with selective radiolabeled opioid agonists and antagonists. The potency of opioid agents in behavioral studies using selective opioid agonists in the amphibian show a high correlation to the potencies of opioid agents in the mammal. Thus, it is expected that opioid binding sites in the amphibian and mammal are not radically different and binding of opioid radioligands to CNS tissue homogenates in the amphibian should elicit similar affinity and density data. Behavioral spinal data allude to the presence of a single opioid receptor in the amphibian which has been termed the "unireceptor" (Stevens and Newman, 1999). Furthermore, interpretation of

parameters from radioligand binding studies using opioid agents to displace radiolabeled selective antagonists were done to either support the hypothesis of a single opioid binding site in the amphibian or to bring to light new results to expand the understanding of amphibian opioid receptors. The K_i value is one such parameter which provides information about types of binding sites. K_i values represent the affinity of the receptor for an unlabeled compound. It is a chemical term governed only by the molecular forces that control the rate of association and dissociation of the drug to and from the morphological and cognitive components of the receptor protein. Thus the K_i value is hopefully independent of receptor function, location and animal species. Similar K_i values for different opioid receptor ligands may indicate that agents are acting at one particular site. Binding competition experiments with selective agonists can also reveal the relative potencies for interpretation of the behavioral experiments with selective antagonists.

1.7.2. [^{3}H]-Diprenorphine binding. [^{3}H]-Diprenorphine has been used to pharmacologically characterize opioid receptors in non-mammalian models. Affinity and competition data have been determined in the amphibian, both in the frog (*Rana ridibunda*) and in the toad (*Bufo marinus*) (Brooks *et al.*, 1994; Mollereau, *et al.*, 1988a; Ruegg *et al.*, 1980; Ruegg, *et al.*, 1981) as well as the goldfish (Brooks, *et al.*, 1994). [^{3}H]-Diprenorphine was shown to bind with high affinity to both solubilized (Ruegg, *et al.*, 1980; Ruegg, *et al.*, 1981) and membrane-bound opioid receptors in the amphibian *in vitro* (Brooks, *et al.*, 1994; Mollereau, *et al.*, 1988a; Ruegg, *et al.* 1980; Ruegg, *et al.*, 1981). Competition studies in the toad and goldfish revealed specificity of μ and κ binding through the displacement of [^{3}H]-diprenorphine by selective μ and κ opioid

agents (Brooks, *et al.*, 1994). No studies thus far have examined opioid binding in the Northern grass frog, *Rana pipiens*. Experiments presented herein profile the characteristics of [^{3}H]-diprenorphine binding in *Rana pipiens* whole brain tissue as well as findings that specific binding is observed despite increased nonspecific binding (yielding negative specific binding) to the filter in the presence of 1 μM naltrexone as is shown in control studies. Given this observation of increased nonspecific binding, an artificially low B_{max} value would be expected. Moreover, the radiolabeled drug [^{3}H]-naloxone does not exhibit binding to the filter, either alone or with naltrexone, as does [^{3}H]-diprenorphine. These observations of [^{3}H]-diprenorphine filter binding in the presence of naltrexone, a general opioid antagonist used to determine nonspecific binding, and the lack of filter binding by [^{3}H]-naloxone suggest that caution should be taken when analyzing [^{3}H]-diprenorphine binding data. However, in the presence of tissue, saturation analysis demonstrated the binding of [^{3}H]-diprenorphine to be saturable with an apparent K_D value of 0.65 nM and a B_{max} value of 287.7 fmol/mg protein. Unlabeled diprenorphine dose-dependently displaced [^{3}H]-diprenorphine from a single non-interactive site in competition studies which yielded a K_i of 0.22 nM. However, in competition studies, selective opioid agonists were not able to compete with ^{3}H-diprenorphine for the opioid receptor. Given that [^{3}H]-diprenorphine presents filter binding properties that are exacerbated in the presence of the nonspecific drug, a reasonable hypothesis would be that the unlabeled nonspecific drug forms a complex with [^{3}H]-diprenorphine preventing it from being effectively washed through the filter or the unlabeled drug itself is blocking the flow of [^{3}H]-diprenorphine through the filter. The latter is unlikely however since other binding studies that will be discussed using the

radioligand [^{3}H]-naloxone with unlabeled naltrexone do not show significant binding to the filter (Newman *et al.*, 1999).

1.7.3. [^{3}H]-Naloxone binding in amphibian brain tissue. Radioligand binding techniques were employed to characterize the sites of opioid action in the amphibian, *Rana pipiens*. Naloxone is a general opioid antagonist which has not been characterized in *Rana pipiens*. Kinetic and saturation analyses for [^{3}H]-naloxone in the amphibian brain were done to determine affinity and density parameters. Additionally, K_i values were calculated in competition studies for various unlabeled μ, κ and δ competitors of [^{3}H]-naloxone to isolate their site of action. The highly selective antagonists for μ, κ and δ opioid receptors were also analyzed through competition analysis in amphibian brain.

1.7.4. [^{3}H]-Naloxone binding in amphibian spinal cord tissue. Kinetic and saturation analyses of [^{3}H]-naloxone in amphibian spinal cord tissue homogenates were also performed to determine affinity and density parameters for comparison to amphibian brain studies. Additionally, K_i values were calculated in competition studies for various unlabeled μ, κ and δ opioid ligands to determine their receptor recognition sites. The K_i values for the highly selective antagonists for μ, κ and δ opioid receptors were also determined.

1.7.5. Radiolabeled selective agonist binding. No studies thus far have examined radiolabeled selective opioid agonist binding in the Northern grass frog, *Rana pipiens*. Experiments presented herein profile the characteristics of the μ ([^{3}H]-DAMGO), κ ([^{3}H]-U65953) and δ ([^{3}H]-DPDPE) binding in *Rana pipiens* whole brain tissue homogenates. The binding parameters of these selective agonists as well as the affinity and selectivity profiles of various opioid agonists and antagonists at μ, κ and δ opioid

receptors were determined. Kinetic and saturation analyses for [^{3}H]-DAMGO, [^{3}H]-U65953 and [^{3}H]-DPDPE were performed in amphibian brain tissue homogenates. K_i values were calculated in competition studies against each selective agonist for various unlabeled μ, κ and δ opioid ligands to resolve their receptor preferences. Highly selective antagonists for μ, κ and δ opioid receptors were examined to determine K_i values against the selective agonists in competition studies.

1.7.6. GTPγS^{35} binding. Opioid agonists stimulate GTPγS^{35} binding to receptor-coupled G-proteins in a dose-dependent fashion in cell membranes (Sim *et al.*, 1995). Stimulation of GTPγS^{35} with selective agonists and their challenge with selective antagonists provides information about the intracellular events after ligand-receptor recognition. EC_{50} values from these experiments should correlate with equilibrium dissociation constants from receptor binding experiments and together these studies can reveal information about the differing efficacies of opioid agents (Selley *et al.*, 1997; Traynor and Nahorski, 1995). The potency of opioid agonists tested should parallel the potencies seen in behavioral and binding studies. In accordance with the unireceptor hypothesis, selective antagonists should exhibit the promiscuous binding profile seen in behavioral studies in the amphibian where each selective antagonist blocks μ, κ and δ opioid agents. GTPγS^{35} stimulation by opioid receptor agonists has been previously examined in *Rana esculenta* where κ opioid ligands were shown to cause a dose-dependent stimulation of GTPγS^{35} binding which was completely inhibited by the κ-selective opioid antagonist, nor-BNI (Rottmann *et al.*, 1998).

The present results are the first to document the binding characteristics of nonselective and selective radioligands in *Rana pipiens* brain and spinal cord tissue

homogenates. Additionally, these are the first data to analyze the competition of μ, κ, and δ opioid receptor agonists for opioid binding sites in *Rana pipiens*. Furthermore, the present data are the first to use highly selective opioid receptor antagonists in a competitive binding assay using central nervous system (CNS) tissue from a non-mammalian vertebrate species.

The main emphasis of this research was to employ behavioral analysis as well as radioligand binding techniques to elucidate opioid receptor type(s) in the amphibian as well as to gain a deeper understanding of opioid receptor pharmacology using a model with a simpler nervous system. Moreover, this research presents an intriguing hypothesis regarding the mechanisms of opioid antinociception in a non-mammalian model which suggests the presence of a single binding site for μ, κ and δ opioids. The amphibian represents a novel alternative non-mammalian model for opioid research.

Chapter II

RESEARCH DESIGN AND METHODS

2.1 Animals

Northern grass frogs, *Rana pipiens* (Sullivan, Nashville, TN) with a mean weight of 32 grams and were kept in groups of 48 in a flow-through, stainless steel enclosure at room temperature with running water. They were maintained with a 12 hour photoperiod and were fed live crickets twice a week. For behavioral studies the animals were transferred to the laboratory and placed in individual plastic pans with an adequate amount of tap water at least 2 days before experimental procedures. On the day of experimental study, frogs were randomly assigned to treatment groups and the water was adjusted to a depth such that the dorsal surface of the frog's thigh was exposed for testing. Each animal was used in only one experiment.

2.2 Drugs

Drugs used in these studies included beta-funaltrexamine (β-**FNA**), **morphine** and **fentanyl** which were obtained from National Institute on Drug Abuse Drug Supply Program (Mr. Robert Walsh of the Research Technology Branch). (D-Ala2, D-Leu5)-Enkephalin (**DADLE**), [D-Ala^2, NMePhe^4, Gly-ol]-enkephalin (**DAMGO**) and [D-Pen^2,D-Pen^5]-enkephalin (**DPDPE**) were obtained from commercial sources (Bachem Bioscience, Prussia, PA). (5R)-(544α,744α,845ß)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8yl]-4-benzofuranacetamide monohydrochloride (**CI977**, Enadoline) was obtained from Ms. Carol Germain of Parke-Davis, Ann Arbor, MI. T*rans*-(±)-3,4-

Dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide methane sulfonate (**U50488H**) was a gift from Dr. Philip VonVoigtlander of the Upjohn Company, Kalamazoo, MI. (D-Ser^2)-Leu-enkephalin-Thr^6 (**DSLET**), 4-[(3,4-Dichlorophenyl)acetyl]-3-(1-pyrrolidinylmethyl)-1-piperazinecarboxylic acid methyl ester fumerate (**GR89696**), (±)-6-Ethyl-1,2,3,4,5,6-hexahydro-3-[(1-hydroxycyclopropyl)methyl]-11,11-dimethyl-2,6-methano-3-benzazocin-8-ol hydrochloride (**bremazocine**), 17,17'-*bis*(Cyclopropylmethyl)-6,6',7,7'-tetrahydro-4,5,4',5'-diepoxy-6,6'-(imino)[7,7'-bimorphinan]-3,3',14,14'-tetrol dihydrochloride (nor-binaltorphimine, **nor-BNI**), **[D-Ala^2]deltorphin-II**, (5α, 7α)-17-(Cyclopropylmethyl)-4,5-epoxy-18,19-dihydro-3-hydroxy-6-methoxy-α,α-dimethyl-6,14-ethenomorphinan-7-methanol (**diprenorphine**) and 17-Cyclopropylmethyl-6,7-dehydro-4,5-epoxy-3,14-dihydroxy-6,7,2',3'-indolomorphinan hydrochloride (**naltrindole, NTI**) were obtained from Research Biochemicals International (Natick, MA). (+)-4-[(αR)-α-((2S,5R)-4-Allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide (**SNC-80**) was obtained from Tocris Cookson (Ballwin, MO). **[^{3}H] Diprenorphine** (30 Ci/mmol), **[N-allyl-2,3-^{3}H]-naloxone** (40-60 Ci/mmol) and **[^{3}H]-U65953** (63 Ci/mmol) were obtained commercially (Amersham, Arlington Heights, IL). **[^{3}H]-DAMGO** (51 Ci/mmol) and **[^{3}H]-DPDPE** (48 Ci/mmol) were a generous gift from the National Institute on Drug Abuse Drug Supply Program (Mr. Robert Walsh of the Research Technology Branch) and were generated by Multiple Peptide Systems (San Diego, CA).

For behavioral studies, drugs were mixed in saline to give nanomolar/microliter solutions of the free base. Opioid agonists and antagonists were administered by intraspinal (i.s.) injection into the lumbar region of the spinal cord with a microsyringe

fitted with a 26-gauge needle (Stevens and Pezalla, 1983). Injections were made percutaneously via the articulation between the seventh and eighth vertebrae. All injections, including co-run saline vehicle controls, were given in a volume of 5 μl/animal. Doses for selective antagonist behavioral studies were chosen based on previous work in the amphibian (Stevens, 1996a). The antagonist was administered concurrently with the agonist in all cases except for β-FNA pretreatment (24 hour) studies. All treatment groups consisted of a random control group which were given agonist in the absence of antagonist (saline controls). The spinal administration of all three selective antagonists were without overt behavioral or motor effects. Motor function was assessed by testing the animals for hindlimb withdrawal, corneal reflexes and their ability to right themselves. Animals that, on the rare occasion, displayed any of the above motor function abnormalities were eliminated from the experiment.

2.3 Acetic acid test (AAT)

The nociceptive threshold (NT) was determined by the acetic acid test (AAT) (Pezalla, 1983; Stevens, 1988; Stevens, 1992; Stevens, 1995b; Stevens, 1995a; Stevens, 1996b; Stevens and Pezalla, 1983; Stevens and Willenbring, 1996; Pezalla, 1983; Pezalla, 1983). Glacial acetic acid (17.5 M) was diluted serially to produce 11 concentrations. Code numbers were assigned from 0 to 10 with the code number 10 representing glacial acetic acid. Testing was performed by placing a single drop of the lowest concentration of acetic acid on the dorsal surface of a frog's thigh with a Pasteur pipette and then proceeding with increasing concentrations on alternate hind limbs until the animal responded with a wiping response. The wiping response is defined as a vigorous wipe by the frog of the treated leg with either hindlimb. To prevent tissue damage, the acetic acid

was washed off with a gentle stream of distilled H_2O when the animal responded or if the animal failed to respond within 5 seconds. The lowest code number of the acetic acid solution to which an animal responded with a wiping response was recorded as the nociceptive threshold. Baseline nociceptive thresholds were obtained 2 hours after the water level was adjusted on the morning of the experiment and post-treatment nociceptive thresholds at 1, 3 and 5 hours after drug administration.

2.4 Tissue preparation for radioligand binding studies

Crude membrane fractions from untreated frogs were prepared by decapitation followed by rapid excision of whole brain and storage at -70°C until use. Brains had a wet weight average of 80 mg and spinal cords weighed an average of 75 mg. Spinal cord preparations were obtained by expulsion out the rostral end of the vertebral column using a saline filled syringe inserted into the caudal end. Spinal cord tissue was also immediately stored at -70°C until the day of the experiment. CNS tissue was thawed and homogenized in approximately 100 volumes/weight of 50 mM Tris HCl with sodium EDTA, pH 7.4. Pellets were obtained by centrifugation of the homogenate at 400 rpm at 4°C for 15 min to remove cell debris followed by 14,500 rpm at 4°C for 15 min. The resulting pellet was suspended in 5 ml of 50 mM Tris HCl with 100 mM NaCl (for antagonist studies, 5 mM $MgCl_2$ (for agonist studies) pH 7.4 (working buffer) and rehomogenized for immediate use in the binding assay. Protein analysis was determined according to the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the standard (BioRad, Richmond, CA).

2.5 Binding assay

Receptor binding was performed in triplicate by incubating 400 µl of tissue (brain: 80 µg - 150 µg of protein, spinal cord: 90 µg - 200 µg of protein) with the labeled drug of interest (50 µl) and with 50 µl of unlabeled ligand (to determine non-specific binding) or without unlabeled ligand (50 µl buffer) for the determination of total binding. The components were incubated at room temperature for 60 minutes to attain binding equilibrium. The reaction was terminated by rapid filtration under vacuum using a Brandel 24-sample tissue harvester (Gaithersburg, MD) followed by a vigorous but brief washing (3 x 5 ml, approximately 15 seconds) with cold buffer onto Whatman GF/B glass-fiber filters which were pre-soaked for 1 hour in 0.3% polyethylenimine (PEI) (to decrease nonspecific binding to the filter). Radioactivity trapped in the filters was counted using a Beckman LS1801 scintillation counter (40-50% efficiency) with Scintiverse scintillation fluid (Fisher, Pittsburgh, PA). Specific binding was defined as the difference between nonspecific binding (measured in the presence of excess concentrations (10 µM) of unlabeled drug to block opioid receptor sites) and total binding.

2.5.1 Saturation and competition analyses. For saturation binding and subsequent Scatchard analysis, increasing concentrations of the radioligand (0.5 nM - 30 nM) were used to determine receptor density (B_{max}) and affinity (K_D). Together, kinetic and saturation experiments approximate the determined K_D of the radioligand which is an essential factor in obtaining accurate K_i values. Experiments on the displacement of radioligand binding by various opioids measure the binding of a certain concentration of radioligand (approximately equal to its K_D) in the presence of various concentrations of

unlabeled ligand (0.01 nM - 10 μM). The concentration of the unlabeled drug that binds to half of the binding sites at equilibrium (K_i) can be calculated using the equation of Cheng and Prusoff: (1973).

$$K_i = \frac{IC_{50}}{1 + \frac{[radioligand]}{K_D}}$$

2.5.2. Kinetic analyses. Association studies are performed to establish the length of time required to reach steady state for saturation and competition experiments and to calculate the association rate constant (k_{obs}). The radioligand is added to a mixture of tissue in a total volume of 0.5 ml and specific binding is measured at various time points (1 - 120 minutes). This data is analyzed using nonlinear regression to fit the specific binding data to the one phase exponential association equation: **($Y = Y_{max} [1 - e^{-kobs*t}]$).**

Dissociation studies are conducted to measure the off rate (k_{off}) for radioligand dissociation from the receptor. These studies are performed by allowing the radioligand and tissue to attain equilibrium, at which point excess unlabeled ligand (10 μM) is added at various time points (1 - 120 min) to interfere with binding of the radioligand. This measures how rapidly the radioligand dissociates from the receptors. Data are analyzed by fitting the data to the exponential decay equation, using nonlinear regression to determine the rate constant:

(Total Binding = NS + (Total – NS) x $e^{-koff*t}$)

Once the on rate (k_{on}), calculated using k_{obs}, and the off rate (k_{off}) constants have been determined, they can be combined to calculate the kinetically derived K_D of receptor

binding for comparison to the apparent K_D value obtained from saturation analysis. For detailed calculations and formulas see Tables V, X and XII.

2.5.3. ^{35}SGTPγS binding assay. Membranes (400 μl) are incubated with 50 μl of 0.05 nM ^{35}SGTPγS (guanosine-5'-O-(3-[^{35}S]thio)triphosphate) and with opioid ligands (agonists for stimulation and antagonists for inhibition) in the presence of 50 μM GDP in a total volume of 0.5 ml for 60 minutes at room temperature. Nonspecific binding is determined in the presence of unlabeled GTPγS (10 μM). Bound and free ^{35}SGTPγS are separated by rapid vacuum filtration through Whatman GF/B filters as before. Basal activity is determined by the absence of agonist and presence of GDP.

2.6 Data Analysis

2.6.1. Behavioral studies. All agonist effects were examined by three nociceptive threshold determinations over a 5 hour period. The raw nociceptive threshold data (code number of acetic acid solution) is converted to maximum percent effect (MPE) by the following formula:

$$\text{MPE} = \frac{\text{Posttreatment NT - Baseline NT}}{\text{Cutoff value (11) - Baseline NT}} \text{X} 100$$

Selectivity data are plotted as MPE versus opioid agonists used. The Students t-test, ANOVA and the *post-hoc* Newman-Keuls test were used for the analysis of data. Significant effects were considered at the $P < 0.05$ level.

2.6.2. Binding studies. Nonlinear regression analysis was used to fit the data to equations that minimize the sum of the squares of the distances of the data points to the curve in order to obtain binding parameters (De Lean *et al.*, 1981; Motulsky and Ransnas,

1987). Competition analysis data are fit to two equations to compare one and two site models to determine best fit and are based on the statistical F-ratio test which compares the weighted residual sum of squares with $P > 0.05$. K_i values are calculated by GraphPad using the method of Cheng and Prusoff. The Student's t-test is used to compare total and nonspecific binding data. Data are considered significant at $P < 0.05$. Binding selectivity was expressed as a selectivity index which represents the ratio of K_i values of a drug to inhibit the specific binding of two selective radiolabeled opioids, for example, [^{3}H]-DAMGO (μ) and [^{3}H]-U65953 (κ).

Chapter III

RESULTS

3.1 In vivo selective antagonist studies

3.1.1. Dosage of β-FNA. Pilot studies were performed to determine the dose of β-FNA that most effectively blocked opioid agonist antinociception. The doses of β-FNA tested in amphibian studies were based upon those used in intrathecal (i.t.) rodent studies (Jiang, *et al.*, 1990; Mjanger and Yaksh, 1991). Agonist doses chosen for behavioral studies were based on antinociceptive responses obtained in previous work in *Rana pipiens* (Stevens, 1996a). β-FNA, or saline as a control, was given 24 hours prior to injection of agonist as a comparison to β-FNA studies in the mammal. Figure 2 depicts the effects of β-FNA throughout a 5 hour time course. As seen in Figure 3, a dose of 20 nmol/frog of β-FNA was statistically significant from that of saline as well as β-FNA at 2 nmol/frog while β-FNA at 2 nmol/frog was not statistically significant from saline in diminishing the analgesia produced by the potent μ opioid agonist, fentanyl (30 nmol/frog). Therefore, β-FNA at 20 nmol/frog was used in all studies.

3.1.2. Long-term effects of intraspinal (i.s.) β-FNA. Figure 4 depicts the extended effects of β-FNA (20 nmol/frog) after spinal administration where the effect of β-FNA on fentanyl (30 nmol/frog) was examined for 28 days post-injection. Fentanyl was injected i.s. on each experimental day and the animals were tested at 1, 3 and 5 hours after injection by the acetic acid test. Antinociception was measured by the maximum percent effect (MPE). The effect of β-FNA on the analgesia produced by fentanyl at 1, 2, 3 and 6

days post-injection was significantly different compared to animals pretreated with saline. The ability of fentanyl to elicit analgesia returned at approximately 10 days after injection of β-FNA where the production of analgesia was not significantly different than that of animals pretreated with saline.

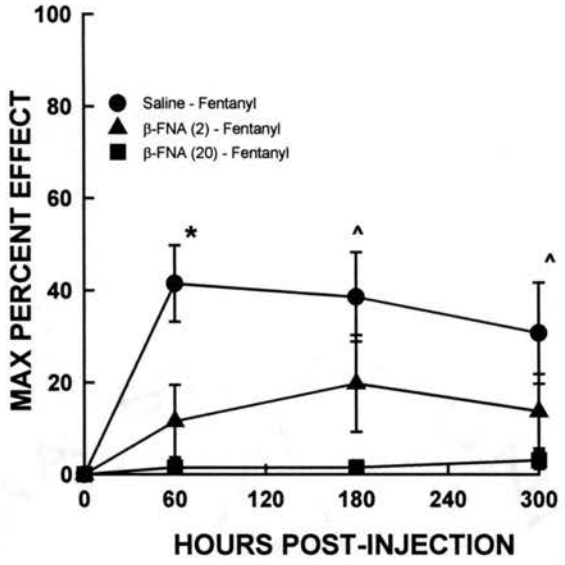


Figure 2. Antagonism of i.s. fentanyl by pretreatment with β-FNA. Fentanyl (30 nmol/frog) was administered 24 h after the i.s. administration of 2.0 or 20 nmol β-FNA. Data plotted as mean (+ s.e.m.) of the MPE observed over a 5 hour time course. N= 6 animals per treatment group. Asterisk denotes significance from saline and β-FNA (2 nmol/frog). Arrow (^) indicates significance of β-FNA (20 nmol/frog) from saline ($P < 0.05$).

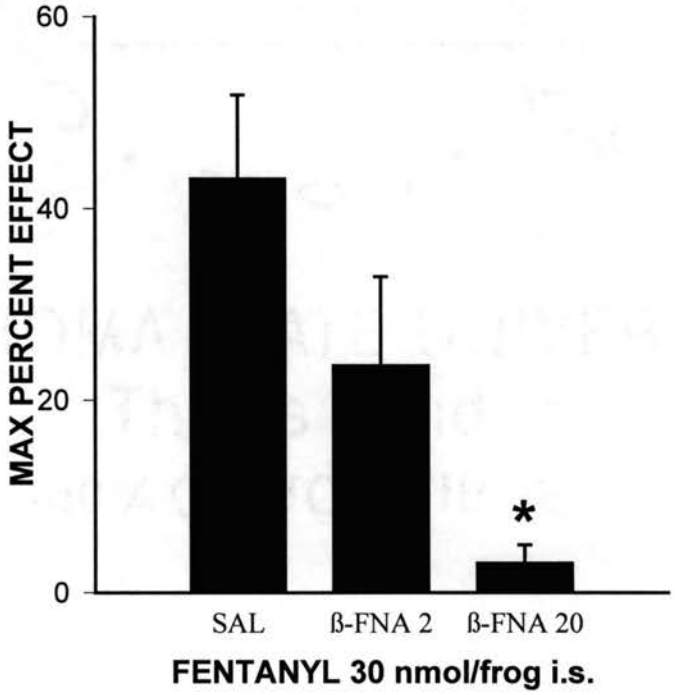


Figure 3. Antagonism of i.s. fentanyl by pretreatment with β-FNA. Fentanyl (30 nmol/frog) was administered 24 h after the i.s. administration of 2.0 or 20 nmol β-FNA. Data plotted as mean (+ s.e.m.) of the maximum MPE observed over a 5 hour time course. N= 6 animals per treatment group. Asterisk denotes significantly different from the saline pretreatment group and βFNA (2 nmol/frog) ($P < 0.05$).

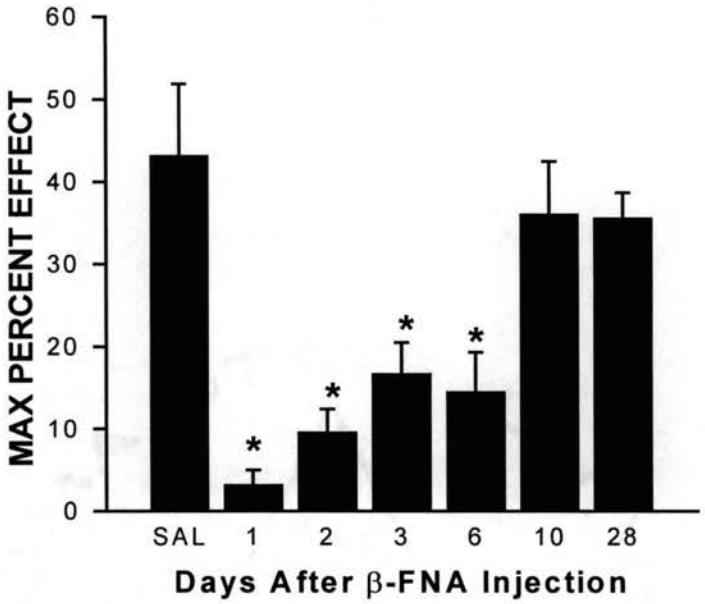


Figure 4. Time course of β-FNA antagonism of fentanyl antinociception. β-FNA (20 nmol/frog, i.s.) was administered on day 0, fentanyl (30 nmol/frog) tested on days 1, 2, 3, 6, 10, 28. Separate groups of animals (N = 6 - 12) were used for each treatment group. Asterisks denote significant difference from saline pretreated animals ($P < 0.05$).

3.1.3. Selectivity of β-FNA in *Rana pipiens* spinal cord - 24-hour pretreatment.

The selectivity of β-FNA (20 nmol/frog) as an antagonist was examined using μ, κ and δ opioid agonists. Figure 5 shows the results of these experiments with the maximum percent effect compared to selective opioid agonist after saline or β-FNA pretreatment. The μ agonists, morphine, fentanyl and DAMGO elicited a sharp rise in the pain threshold of the animals which were pretreated with saline. However, in animals pretreated with β-FNA, the antinociception of all three μ opioid agonists was, as expected, significantly reduced. In examining the effect of β-FNA on δ ligands, DADLE and DPDPE, no statistical difference was observed in the antinociceptive effect between saline and β-FNA pretreatment for DADLE while β-FNA appeared to significantly attenuate the antinociceptive effects of DPDPE. Additionally, the effect of β-FNA on κ opioid receptor agonists was studied. As shown in Figure 5, the κ opioid agonist,

U50488H did not appear to be affected by β-FNA pretreatment as the MPE for saline pretreated animals was not significantly different from the β-FNA pretreated group while the antinociception of CI977 was significantly diminished by β-FNA. A control group which received a 24 hour pretreatment with i.s. saline was tested 24 hours later with saline. No significant difference was seen in these groups, indicating that the spinal injections alone did not produce a significant amount of analgesia compared to the injections of agonists.

3.1.4. Selectivity of β-FNA in *Rana pipiens* spinal cord – Concurrent treatment.

The selectivity of β-FNA (20 nmol/frog) as an antagonist was examined using μ, κ and δ opioid agonists. Figure 6 shows the results of these experiments with the maximum percent effect compared to opioid agonist with or without β-FNA given concurrently. As is shown in the figure, concurrent administration of β-FNA shows an even more nonselective profile as it blocks not only the μ agonists, morphine, fentanyl and DAMGO, but also significantly blocks the δ agonists, DADLE , DSLET, deltorphin and DPDPE, as well as the κ agonists, bremazocine and CI977.

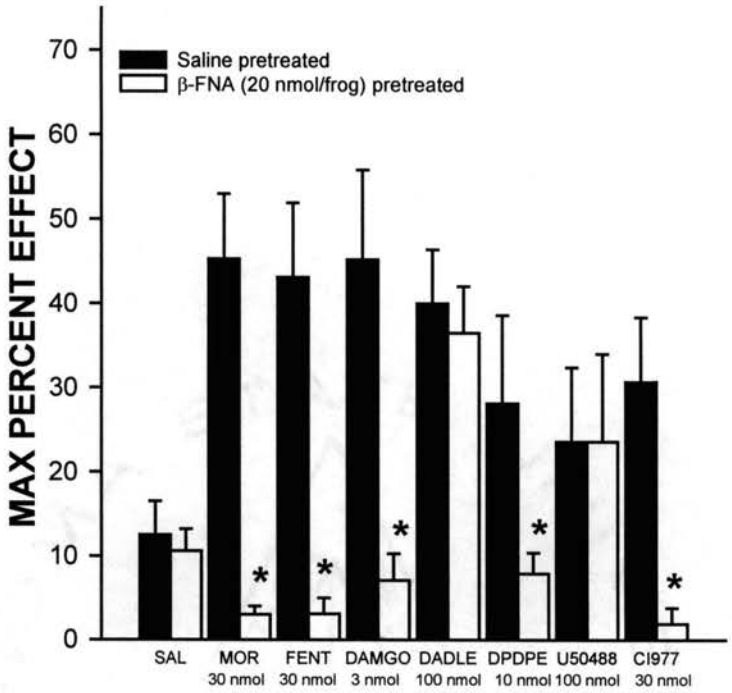


Figure 5. Selectivity of β-FNA opioid antagonism after 24 h pretreatment. Test opioids and doses are given in the figure. N = 6 - 12 animals per treatment group. Asterisks denote significant difference between the respective saline-pretreated group of each opioid agonist ($P < 0.05$).

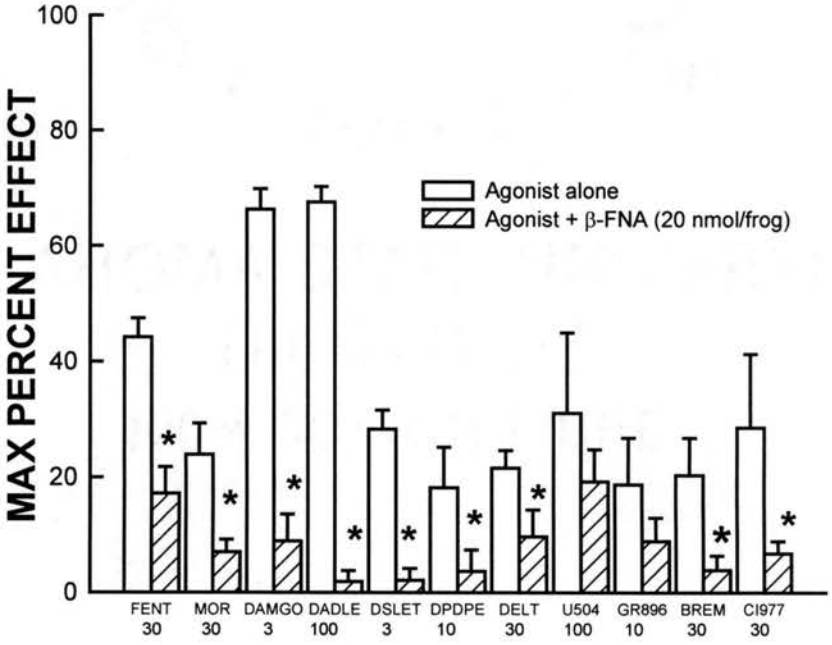


Figure 6. Selectivity of β-FNA opioid antagonism after concurrent β-FNA administration. Test opioids and doses are given in the figure. N = 6 - 12 animals per treatment group. Asterisks denote significant difference between the respective group of each opioid agonist given alone ($P < 0.05$).

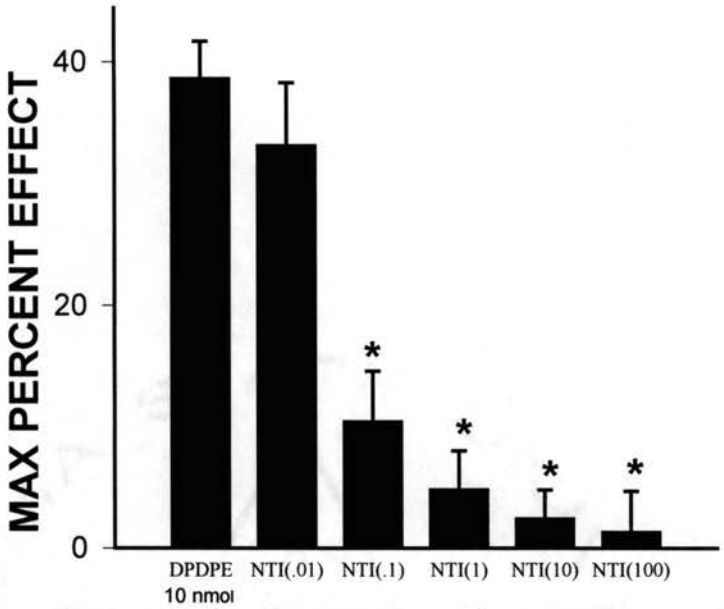


Figure 7. Dose response of NTI antagonism of DPDPE (10 nmol/frog). NTI doses are given in the figure. N = 6 - 12 animals per treatment group. Asterisks denote significant difference from DPDPE alone ($P < 0.05$).

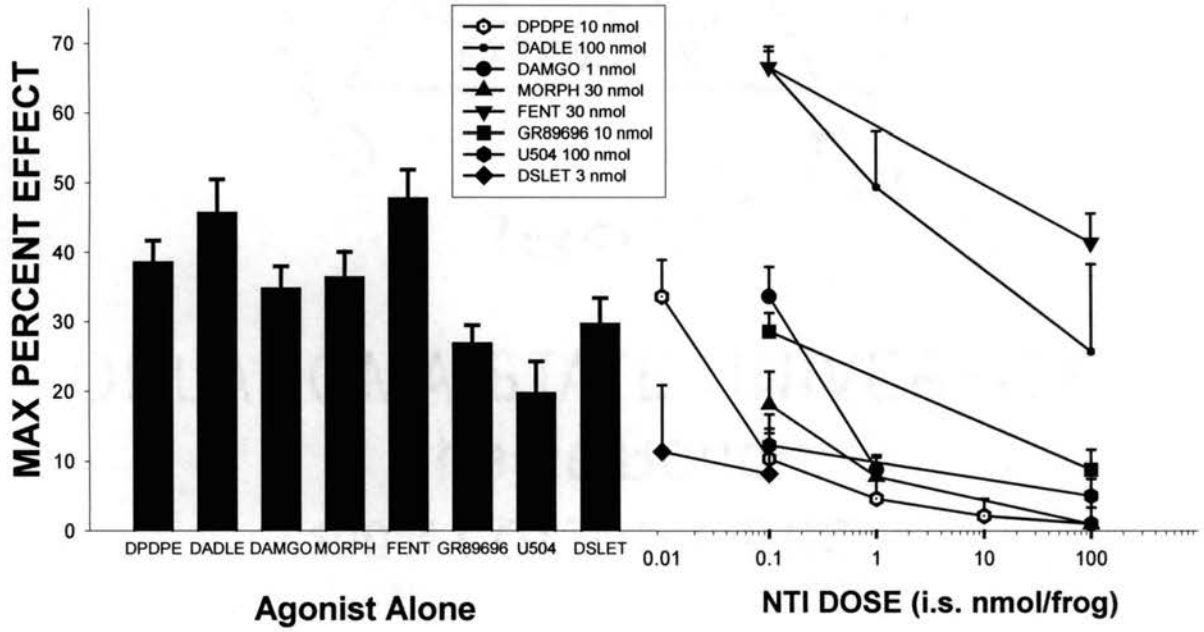


Figure 8. Dose response of NTI antagonism of select opioid agonists. NTI doses are given in the figure. N = 6 - 12 animals per treatment group. Left panel shows the MPE of the agonists. The right panel illustrates the effect of increasing concentrations of NTI on the antinociception produced by the selective agonists.

3.1.5. Effects of NTI on the highly selective δ opioid receptor agonist, DPDPE.

Figure 7 illustrates the MPE produced by the highly selective δ opioid agonist, DPDPE (10 nmol/frog) versus increasing doses of NTI administered concurrently to determine the lowest dose of NTI which produced effective antagonism. As Figure 7 shows, NTI dose-dependently attenuated the effect of DPDPE alone at 10 nmol/frog with the lowest significant dose being 0.1 nmol/frog. Thus an NTI dose of 0.1 nmol/frog was used in all studies.

3.1.6. Effect of NTI on various selective agonists. Figure 8 depicts the effects of NTI on selective opioid agonists. On the left the MPE versus agonist antinociception is shown. The right graph illustrates the MPE versus increasing concentrations of NTI. Overall, increasing NTI doses shows a downward trend in the antinociceptive action of all opioids tested. Lower doses of NTI appear to potentiate the effects of both DADLE and fentanyl with 100 nmol/frog of NTI bringing the MPE back to the original level elicited by the agonist alone.

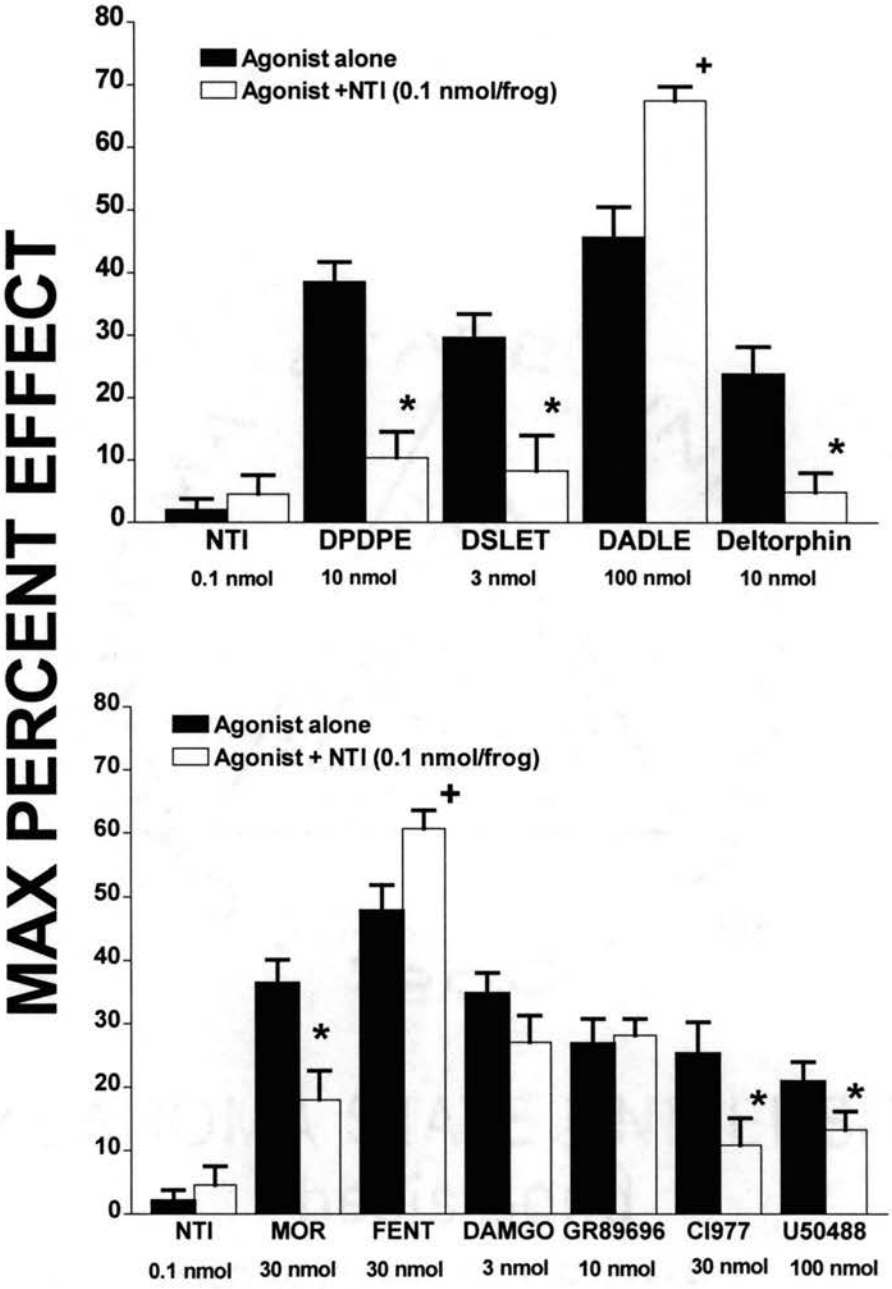


Figure 9. Selectivity of NTI opioid antagonism. Test opioids and doses are given in the figure. N = 6 - 12 animals per treatment group. Asterisks denote significant difference between agonist alone. The plus sign is indicative of significantly greater effects as compared to agonist alone ($P < 0.05$).

3.1.7. Examination of NTI selectivity in *Rana pipiens*. To examine the selectivity of NTI, various selective opioid agonists were used. The top panel in Figure 9 depicts the use of NTI co-administered with δ-selective agents. As is shown, no antinociception was produced with the spinal administration of NTI alone (0.1 nmol/frog, i.s.) or NTI co-administered with saline. NTI (0.1 nmol/frog, i.s.) significantly attenuated the antinociceptive effects of DPDPE (10 nmol/frog, i.s.), DSLET (3 nmol/frog, i.s.) and deltorphin (10 nmol/frog, i.s.) but not that of DADLE (100 nmol/frog, i.s.). Moreover, NTI not only fails to block the antinociceptive actions of DADLE but appears to significantly potentiate its antinociceptive effect.

The bottom panel in figure 9 shows the effects of the co-administration of NTI with μ and κ agonists. NTI (0.1 nmol/frog, i.s.) significantly blocked the actions of the μ agonist, morphine (30 nmol/frog, i.s.) but had no effect on the antinociception produced by DAMGO (3 nmol/frog, i.s.). Moreover, the antinociception produced by fentanyl (30 nmol/frog, i.s.) appears to be potentiated. NTI significantly blocked the antinociceptive effects of the κ agonists, CI977 (30 nmol/frog, i.s.) and U50488 (100 nmol/frog, i.s.) but not that of GR89696 (10 nmol/frog, i.s.).

3.1.8. Examination of nor-BNI selectivity in *Rana pipiens*. Figure 10 shows the effect of increasing concentrations of nor-BNI on the antinociceptive effects of fentanyl (30 nmol/frog) and GR89696 (10 nmol/frog). 0.01 nmol/frog nor-BNI was effective at blocking the κ agonist, GR89696 but not the μ agonist fentanyl. Increasing doses of nor-BNI up to 10 nmol/frog blocked the effects of both agents. Figure 11 depicts the results of the selectivity of nor-BNI in the amphibian where nor-BNI not only blocks the antinociceptive effects of the κ-agonists, GR89696 and U50488H, shown in the top panel, but it also blocks the effects of

the δ-agents, DPDPE and deltorphin, but not DSLET. Again the apparent potentiation of the antinociceptive effects of DADLE are observed as with the selective antagonist naltrindole. Nor-BNI also appears to block the antinociceptive activity of the μ-opioid agonists, morphine and fentanyl as is also shown in the lower panel of Figure 11.

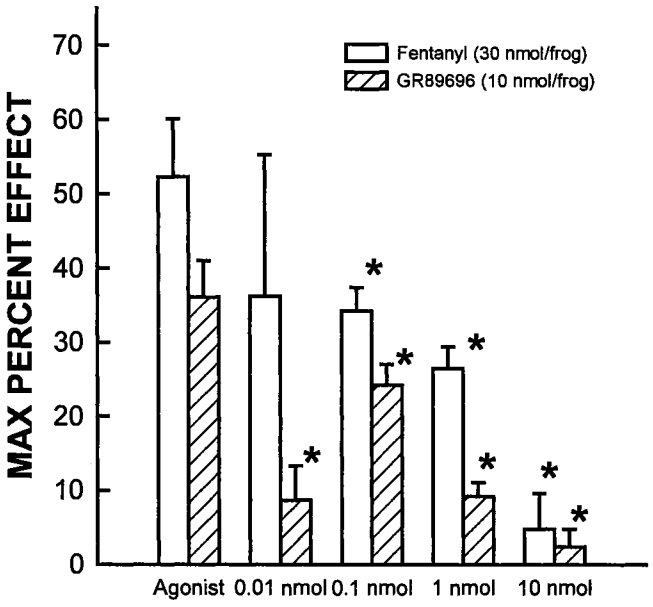


Figure 10. Antagonism of fentanyl (μ agonist) and GR89696 (κ agonist) by increasing concentrations of i.s. nor-BNI. Antinociception measured is shown as the maximum percent effect (MPE). Asterisks denote a significant decrease in antinociception compared to the agonist given alone ($P < 0.05$).

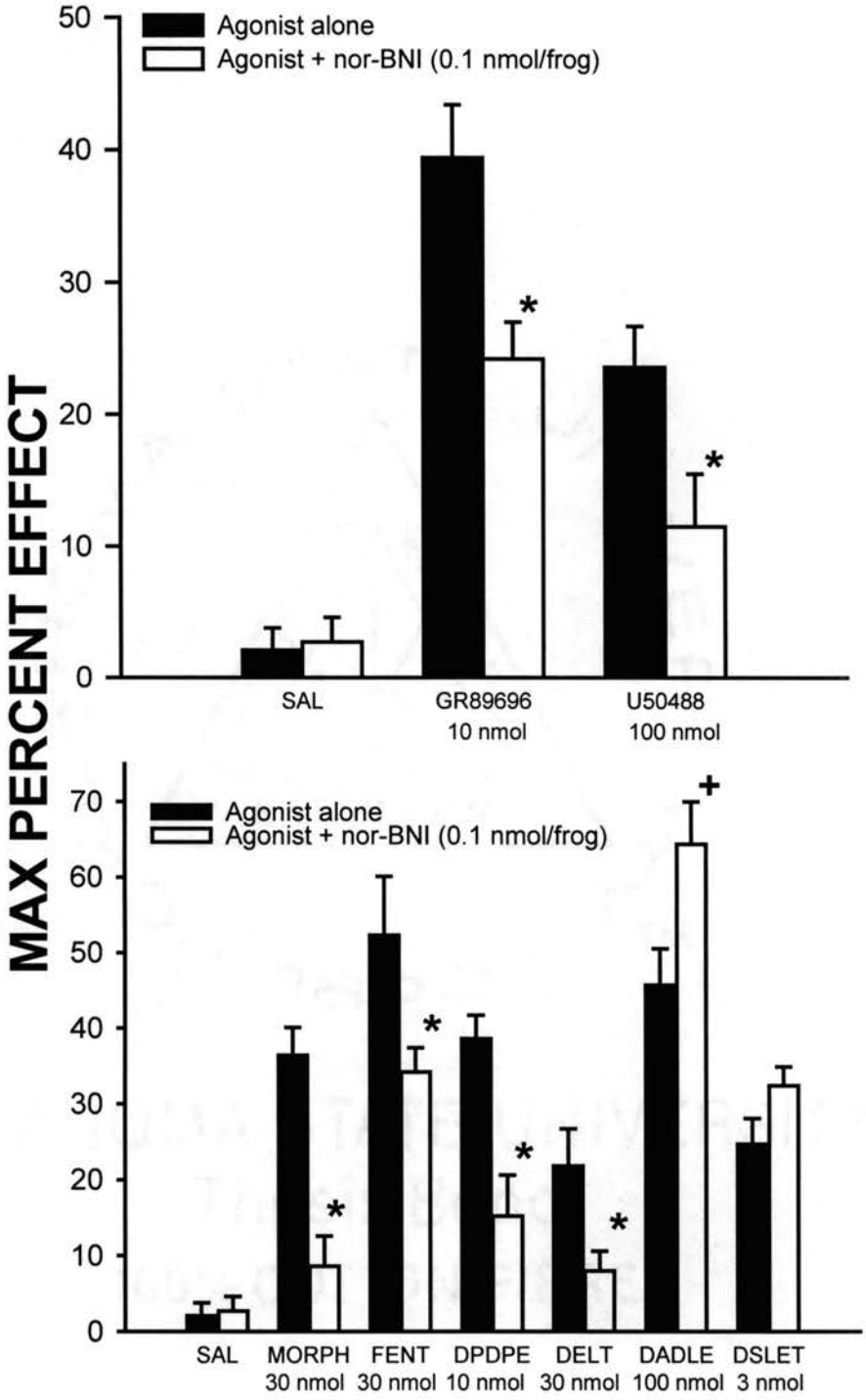


Figure 11. Antagonism of μ, κ and δ opioid agonists by i.s. nor-BNI (0.1 nmol/frog). Antinociception measured is shown as the maximum percent effect (MPE). Asterisks denote a significant decrease in antinociception compared to the agonist alone. The plus sign indicates significantly greater effects compared to agonist alone ($P < 0.05$).

3.1.9. Schild analysis. To further analyze the effects of nor-BNI on the antinociceptive effects of selective opioid agonists, the effect of increasing concentrations of nor-BNI on the full dose-response curves of selective agonists were generated. Figure 12 shows the effects of increasing doses of nor-BNI on varying concentrations of the κ agonist, GR89696. 0.1 nmol/frog nor-BNI appears to be the most effective at blocking antinociception produced by GR89696 while 1 and 10 nmol/frog were similar in their effects at inhibiting GR89696 antinociception. Figure 13 shows dextral shifts of the μ agonist, fentanyl by increasing concentrations of nor-BNI. Nor-BNI dose-dependently blocked fentanyl antinociception where higher concentrations of nor-BNI were most effective. Figure 14 illustrates the effects of nor-BNI on DADLE. Potentiation of antinociception produced by DADLE occurred at low doses of nor-BNI and DADLE while higher doses of nor-BNI show pure antagonism of DADLE. Figure 15 depicts the effects of nor-BNI on DSLET. 1 and 10 nmol/frog nor-BNI blocks the antinociceptive effect of DSLET. Only DSLET concentrations of 0.1 and 0.3 nmol/frog were antagonized by 0.1 nmol/frog nor-BNI. Figure 16 shows the results of Schild analysis with the pA_2 values for fentanyl, DADLE and DSLET summarized in Table I.

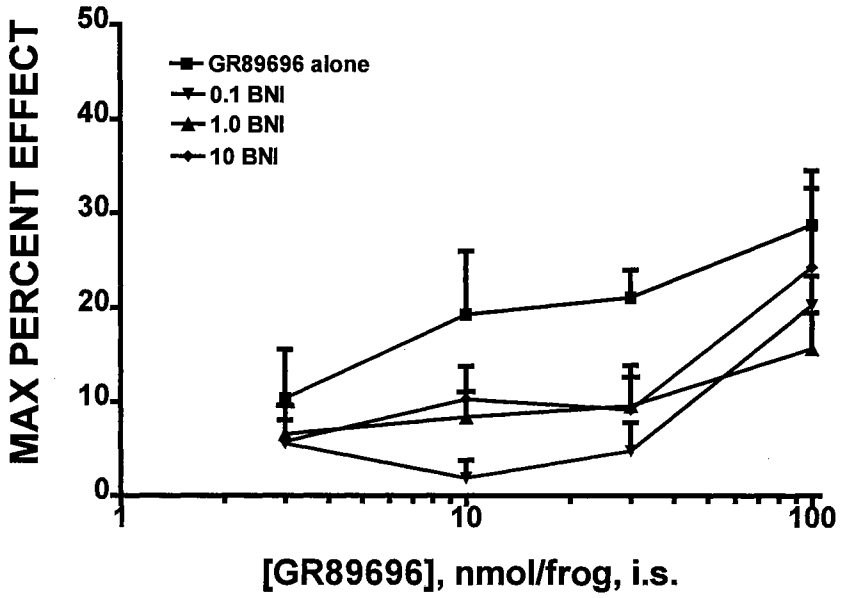


Figure 12. Dose response of the κ opioid agonist, GR89696 with different concentrations of nor-BNI. Antinociception measured is shown as the maximum percent effect (MPE). Nor-BNI doses are expressed as nmol/frog.

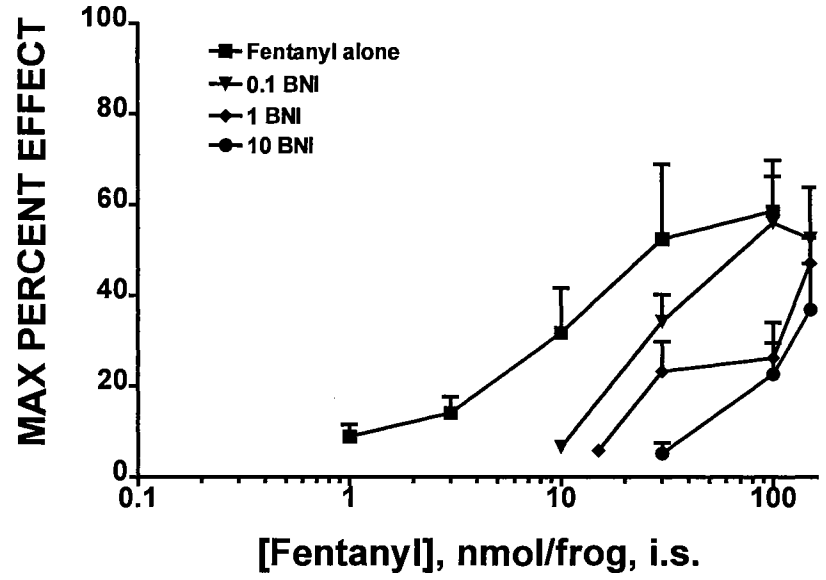


Figure 13. Dose response of the μ opioid agonist, fentanyl with different concentrations of nor-BNI. Antinociception measured is shown as the maximum percent effect (MPE). Nor-BNI doses are expressed as nmol/frog.

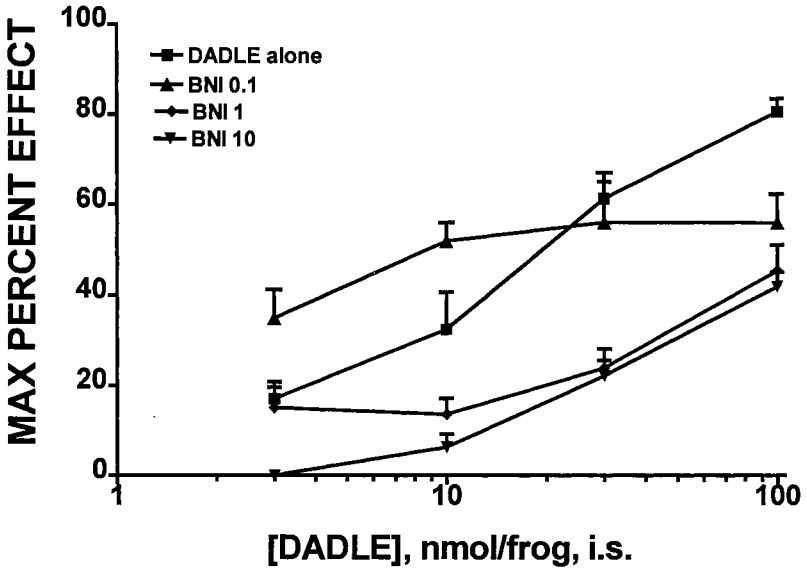


Figure 14. Dose response of the δ opioid agonist, DADLE with different concentrations of nor-BNI. Antinociception measured is shown as the maximum percent effect (MPE). Nor-BNI doses are expressed as nmol/frog.

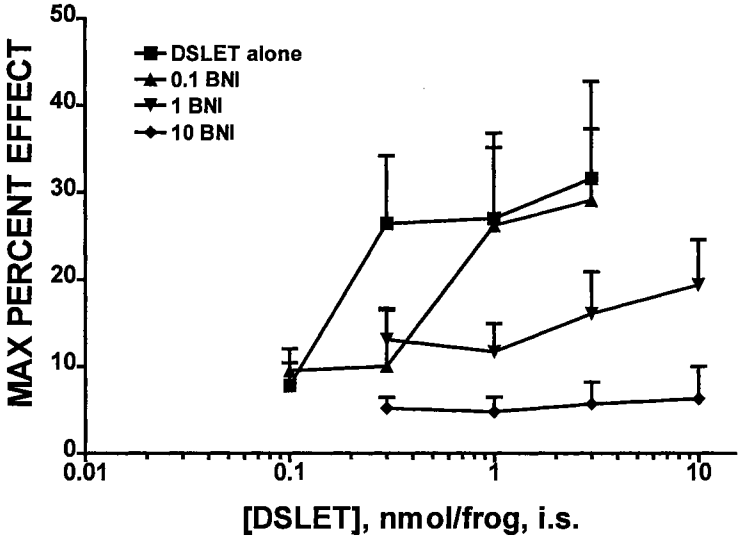


Figure 15. Dose response of the δ opioid agonist, DSLET with different concentrations of nor-BNI. Antinociception measured is shown as the maximum percent effect (MPE). Nor-BNI doses are expressed as nmol/frog.

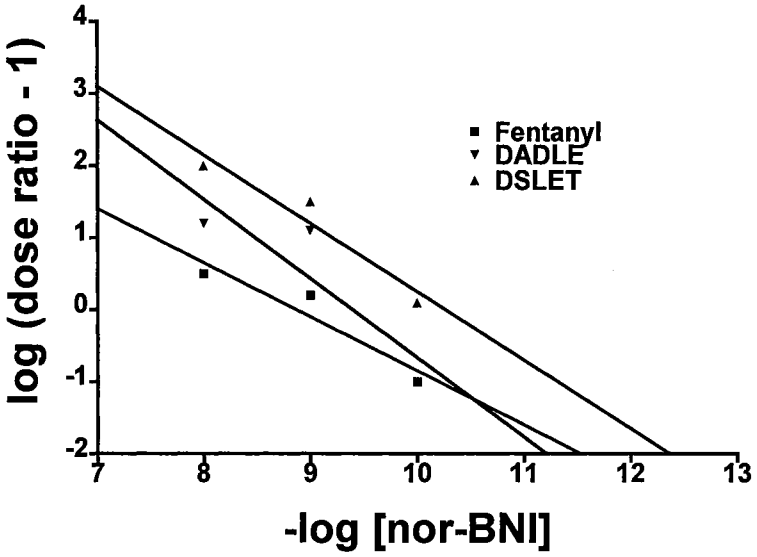


Figure 16. Schild plot analysis of fentanyl, DADLE and DSLET against nor-BNI. Linear regression to the x-axis yields the pA_2 value for the antagonist and is shown in Table I.

Table I. Results of Schild analysis for fentanyl, DADLE and DSLET against nor-BNI

Drug	pA_2	95% CI
Fentanyl	8.87	(4.75 – 13.20)
DADLE	10.26	(6.49 – 14.00)
DSLET	9.39	(2.82 – 16.12)
GR89696	ND[a]	-

[a]ND – Not determined due to insufficient dose-response curves

3.2 [^{3}H]-Diprenorphine radioligand binding studies

3.2.1. Optimization of conditions for [^{3}H]-diprenorphine binding. Figure 17 shows the effect of increasing sodium concentrations in the working buffer on the binding of [^{3}H]-diprenorphine (1 nM). As the figure shows, the binding of [^{3}H]-diprenorphine saturates at approximately 25 mM NaCl. A concentration of 100 mM NaCl (middle of the plateau) was used for all binding studies with [^{3}H]-diprenorphine.

3.2.2. Saturation analyses for [^{3}H]-diprenorphine. The nonselective opioid antagonist, diprenorphine, was characterized in amphibian brain tissue homogenates. Saturation analysis of [^{3}H]-diprenorphine is shown in Figure 18. Naltrexone (1 μM) was used to define nonspecific binding. The affinity and capacity data obtained in the amphibian with [^{3}H]-diprenorphine is similar to that observed in mammalian binding studies (see Table II) with [^{3}H]-diprenorphine as well as in other non-mammalian binding studies (see Table III).

The specific binding (fmol/mg protein) of increasing concentrations of the non-selective opioid antagonist [^{3}H]-diprenorphine (0.25 nM – 10 nM) was found to be saturable as is shown in Figure 18. Analysis indicated a B_{max} value of 287.7 fmol/mg protein and an apparent K_D value of 0.65 nM. Scatchard analysis is shown in the inset.

The specific binding of [^{3}H]-diprenorphine (1 nM) was significantly displaced with increasing concentrations of unlabeled diprenorphine (0.1 nM – 1 μM) as is shown in Figure 19. The inhibition constant (K_i) was found to be 0.22 nM.

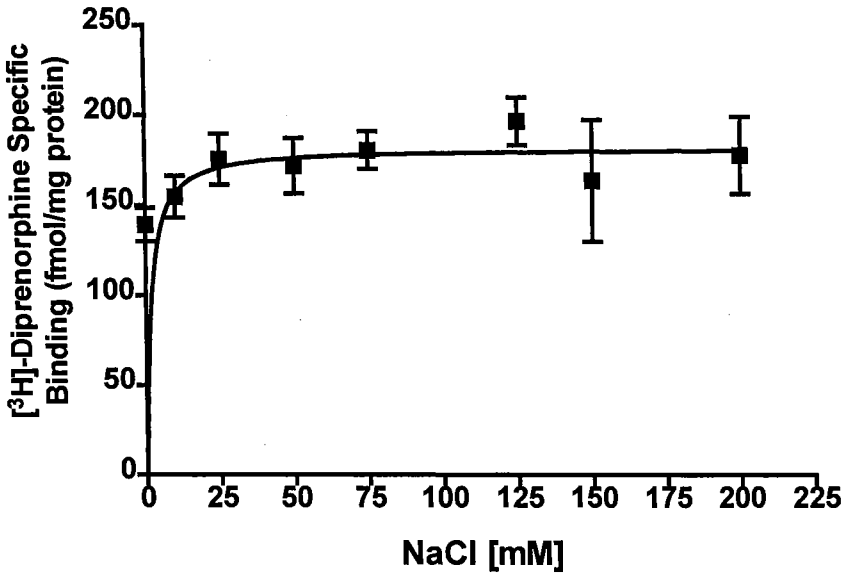


Figure 17. Saturation analysis of [^{3}H]-diprenorphine (1 nM) binding to amphibian brain tissue homogenates over increasing concentrations of NaCl (0 mM – 200 mM). Data points represent the mean of three membrane preparations with individual experiments performed in triplicate.

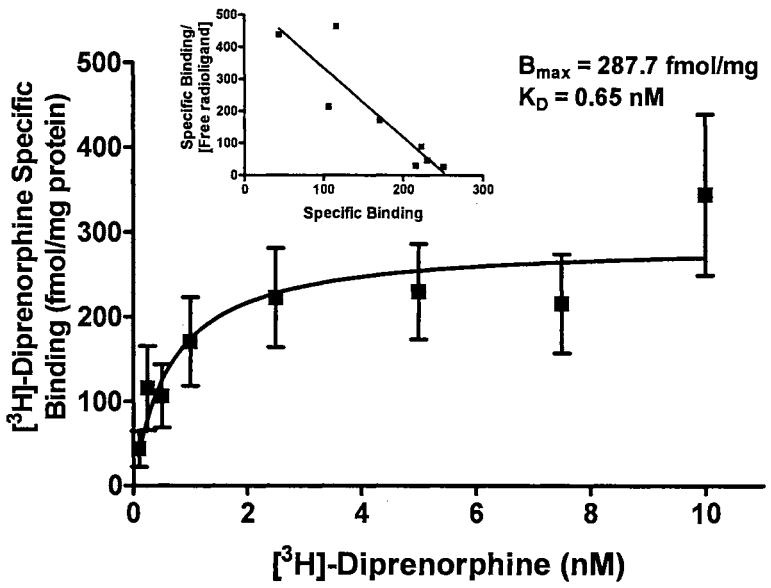


Figure 18. Saturation analysis of [^{3}H]-diprenorphine binding to amphibian brain tissue homogenates. *Inset:* Scatchard plot of saturation data. Non-linear regression analysis indicated a single site best fit as determined by the F-test. Data points represent the mean of three membrane preparations with individual experiments performed in triplicate.

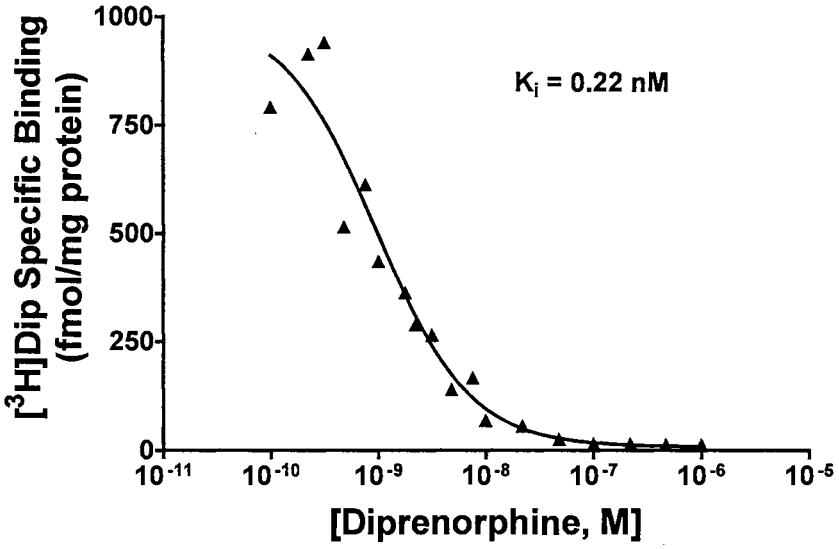


Figure 19. Competition analysis of diprenorphine with [^{3}H]-diprenorphine binding to amphibian brain tissue homogenates. Non-linear regression analysis indicated a single site best fit as determined by the F-test. Data points represent the mean of three membrane preparations with individual experiments performed in triplicate.

Values calculated for K_D and B_{max} in *Rana pipiens* were similar to those determined in both mammalian (Table II) and non-mammalian (Table III) models where the apparent K_D values ranged from 0.10 nM - 0.46 nM in mammals and from 0.10 nM - 1.0 nM in non-mammalian species.

Table II. Mammalian affinity and density binding data for [^{3}H]-diprenorphine.

Tissue	K_d[a]	B_{max}[b]	Nonspecific drug	Reference
Rabbit cerebellum	0.10	290	10 μM levorphanol	Frances, *et al.*, 1985
***Afghan pika* brain**	0.10	220	10 μM levorphanol	Farges, *et al.* 1988
Guinea-pig cerebellum	0.10	160	10 μM levorphanol	Frances, *et al.*, 1985
Rabbit	0.11	257	10 μM levorphanol	Farges, *et al.*1988
Rat	0.16	41,200	20 μM naloxone	Cunningham, *et al.*, 1991
Guinea-pig brain	0.20	172	10 μM naloxone	Wood and Traynor, 1989
Rat	0.23	530	1 μM diprenorphine	Chang, *et al.*, 1981
NMB cell line[c]	0.46	5340	0.2 mM naloxone	Baumhaker, *et al.*, 1993
Mongolian gerbil	0.56	135.8	1 μM diprenorphine	Niwa, *et al.*, 1994

[a]nM; [b]fmol/mg protein; [c]Human neuroblastoma

Table III. Non-mammalian affinity and density binding data for [^{3}H]-diprenorphine.

Tissue	K_d[a]	B_{max}[b]	Nonspecific drug	Reference
Rana ridibunda	0.1	3200	10 μM diprenorphine	Mollereau *et al.*, 1988b
Bufo marinus	0.17	21.7	1 μM levorphanol	Brooks, *et al.*, 1994
Carassius auratus	0.47	18.17	1 μM levorphanol	Brooks, *et al.*1994
***Bufo marinus* (solubilized)**	0.85	330	1 μM levorphanol	Ruegg, *et al.*, 1981
Solubilized toad brain *Bufo marinus*	1.0	350	1 μM levorphanol	Ruegg, *et al*, 1980
***Bufo marinus* (membrane bound)**	1.0	390	1 μM levorphanol	Ruegg, *et al.*, 1981

[a] nM; [b] fmol/mg protein

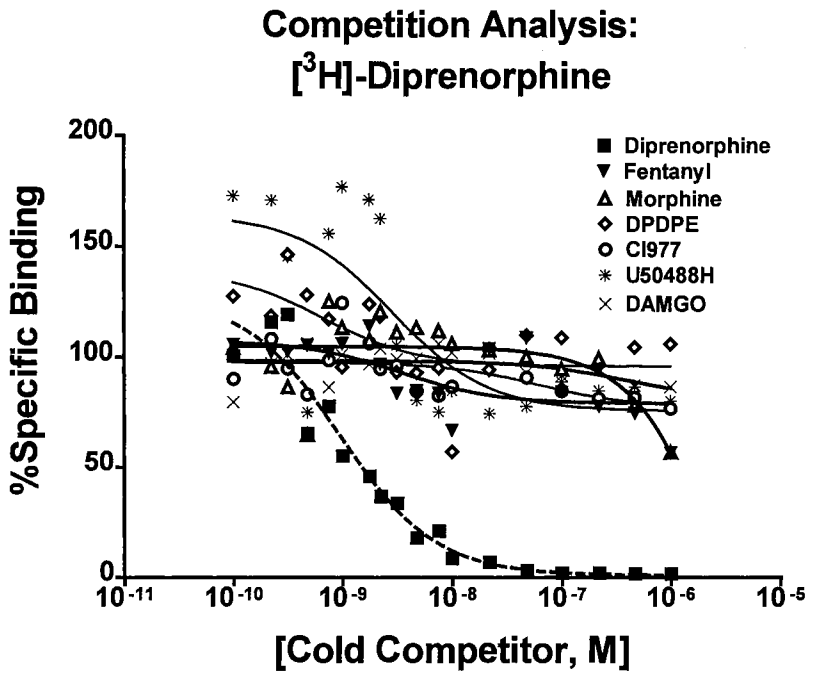


Figure 20. [^{3}H]-Diprenorphine (1 nM) competition analysis with various selective opioid agonists. K_i values are summarized in Table IV. Data points represent the mean of three membrane preparations with individual experiments performed in triplicate.

Table IV. Displacement of [^{3}H]-diprenorphine by selective opioid receptor agonists

Drug	Type	K_i (nM)	95% CI[a]
diprenorphine	μ, κ & δ	0.36	(0.20 – 0.64)
morphine	μ	2988	(958 – 4658)
fentanyl	μ	1333	(937 – 2569)
DAMGO	κ	1038	(500 – 37650)
DPDPE	κ	2389	(1267 – 4503)
CI977	δ	2322	(407 – 13220)
U50488	δ	1106	(512 – 8093)

[a] 95% confidence interval

3.2.3. Competition analysis with [^{3}H]-diprenorphine. Figure 20 illustrates attempts to compete with the nonselective opioid antagonist [^{3}H]-diprenorphine for binding to opioid ligands with selective opioid agonists. As is shown, unlabeled diprenorphine competes very well with itself yielding a K_i value of 0.36 nM. However, all other selective agonists tested were unable to effectively compete with diprenorphine for binding to opioid receptors in *Rana pipiens*. K_i values for these selective opioid agonists ranged from 1038 nM – 2988 nM as is shown in Table IV.

3.2.4. [^{3}H]-Diprenorphine control studies. Control studies, conducted to determine if observed binding of [^{3}H]-diprenorphine was solely to brain tissue, revealed significant binding to the filter. These control studies were done in the absence of tissue using various filter pretreatments with the results depicted in Figure 21. As shown, nonspecific binding (using 1 μM naltrexone) was significantly elevated over total binding in all pretreatment cases. The inset shows the negative specific binding obtained in these experiments. The elevation in binding due to the presence of a unlabeled 1 μM naltrexone was also seen with 1 μM naloxone but not with the opioid agonists, levorphanol (1 μM) and morphine (1 μM) or the selective opioid antagonists, β-FNA (1 μM) or NTI (1 μM) as is shown in Figure 22. Figure 23 shows the effects of varying concentrations of naltrexone on [^{3}H]-diprenorphine (1 nM) filter binding where 100 nM as well as 1 μM naltrexone showed significantly increased binding to the filter than with diprenorphine alone. Thus, problems with excess filter binding of [^{3}H]-diprenorphine limited further characterization of this compound in the amphibian. Virtually no binding to the filter was observed in control studies employing the nonselective radiolabeled antagonist, [^{3}H]-naloxone, using 1 μM naltrexone to determine nonspecific binding nor

was binding seen using 1 μM diprenorphine to define nonspecific binding (see Figure 24).

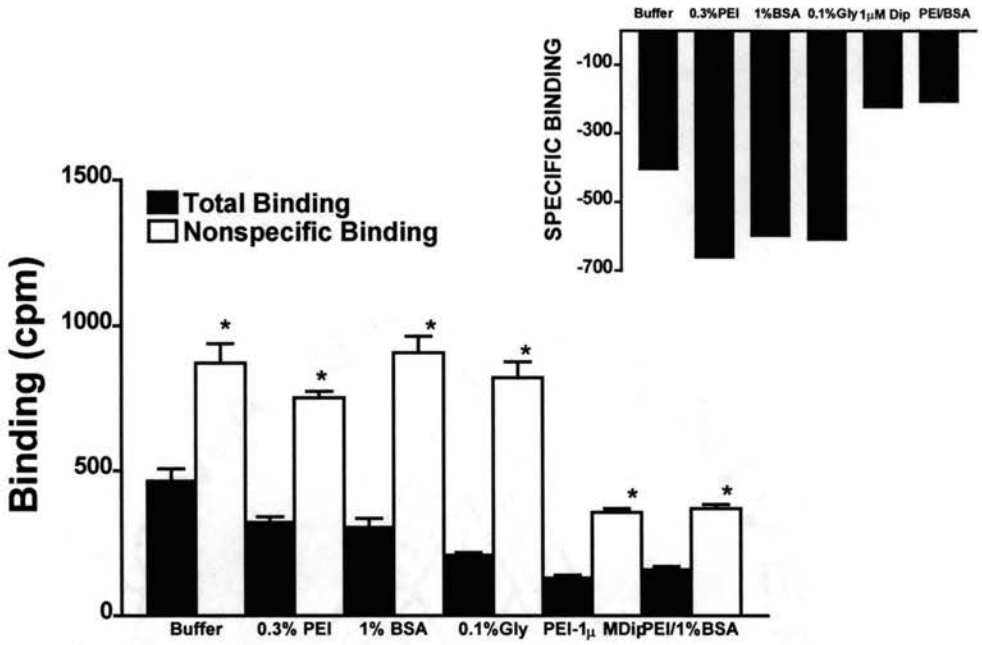


Figure 21. [^{3}H]-Diprenorphine (1 nM) total and nonspecific binding in cpm (counts per minute) in the absence of tissue showing various pretreatments of the filter on the x-axis. 1 μM naltrexone was used in all cases to define nonspecific binding. Buffer is 50mM Tris, pH 7.40. The concentration of PEI is 0.3%. Gly (glycoglycine), Dip (diprenorphine). Each bar represents the mean of 12 samples.

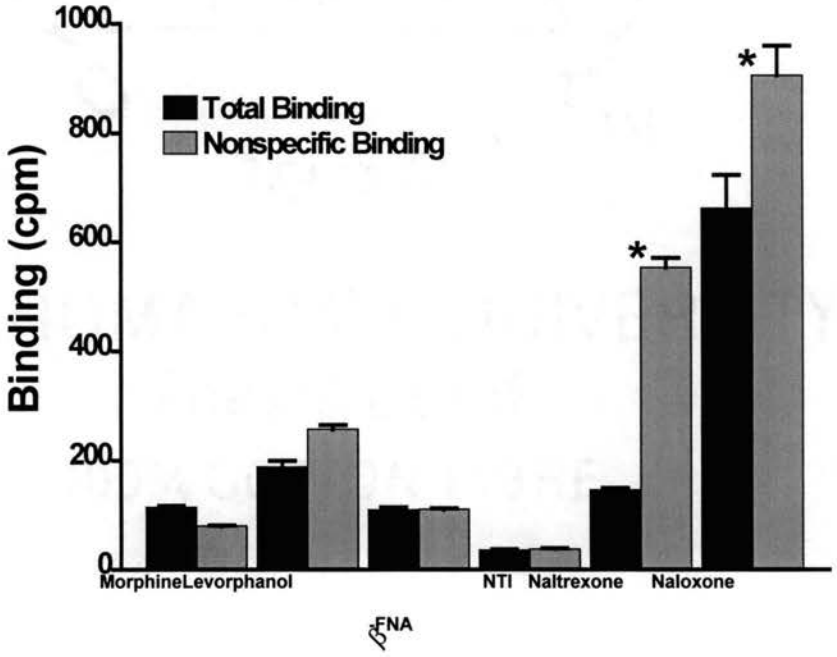


Figure 22. [^{3}H]-Diprenorphine (1 nM) total and nonspecific binding in cpm (counts per minute) in the absence of tissue. All nonspecific drugs concentrations were 1 μM. Each bar represents the mean of 12 samples.

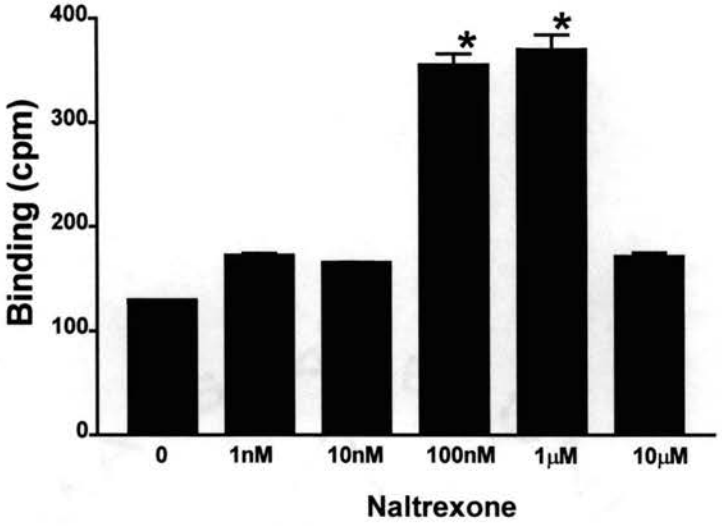


Figure 23. [^{3}H]-Diprenorphine (1 nM) filter binding expressed as cpm with various concentrations of naltrexone. Each bar represents the mean of 12 samples.

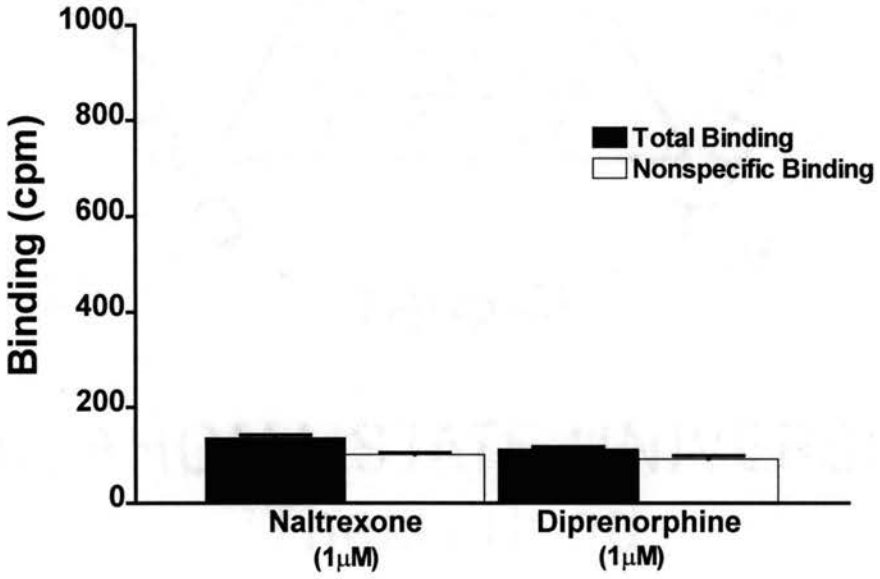


Figure 24. [^{3}H]-Naloxone (1 nM) total and nonspecific binding expressed as cpm in the absence of tissue using either 1 μM naltrexone or 1 μM diprenorphine to define nonspecific binding. Each bar represents the mean of 12 samples.

3.3 [^{3}H]-Naloxone binding

3.3.1. Kinetics of [^{3}H]-naloxone binding in *Rana pipiens* brain. Kinetic analysis was performed to determine the time needed to attain the condition of steady-state as well as the rate constants for association and dissociation. Kinetic analyses of [^{3}H]-naloxone (5 nM) binding in *Rana pipiens* brain homogenates are shown in Figure 25. Association studies (Figure 25A) in the brain yielded a k_{obs} (observed association rate) value of 0.4872 min^{-1} while dissociation (Figure 25B) results resulted in a k_{off} (dissociation rate constant) value of 0.2815 min^{-1}. These rate constants gave a n apparent K_D value of 6.84 nM. Nonspecific binding was defined by a parallel series of tubes containing 10 μM naltrexone and represented 38% of total binding. Statistical analysis of the comparison between one and two site models yielded a best fit for the one site model (see Table V for results of F test and significance).

3.3.2. [^{3}H]-Naloxone saturation studies. The properties of naloxone binding sites were studied over an extended range of concentrations of [^{3}H]-naloxone where affinity and density data for [^{3}H]-naloxone were determined. Saturation data for brain tissue is shown in Figure 26. As seen in the figure, specific binding was measured over increasing concentrations of [^{3}H]-naloxone (0.5 nM - 70 nM). Scatchard analysis of these data is shown in the inset. The experimentally derived K_D and B_{max} from saturation analysis were found to be 7.11 nM and 2170 fmol/mg protein, respectively. Kinetic and saturation data for [^{3}H]-naloxone are summarized in Table V. These data were best fit to a one site binding model as determined by the F test.

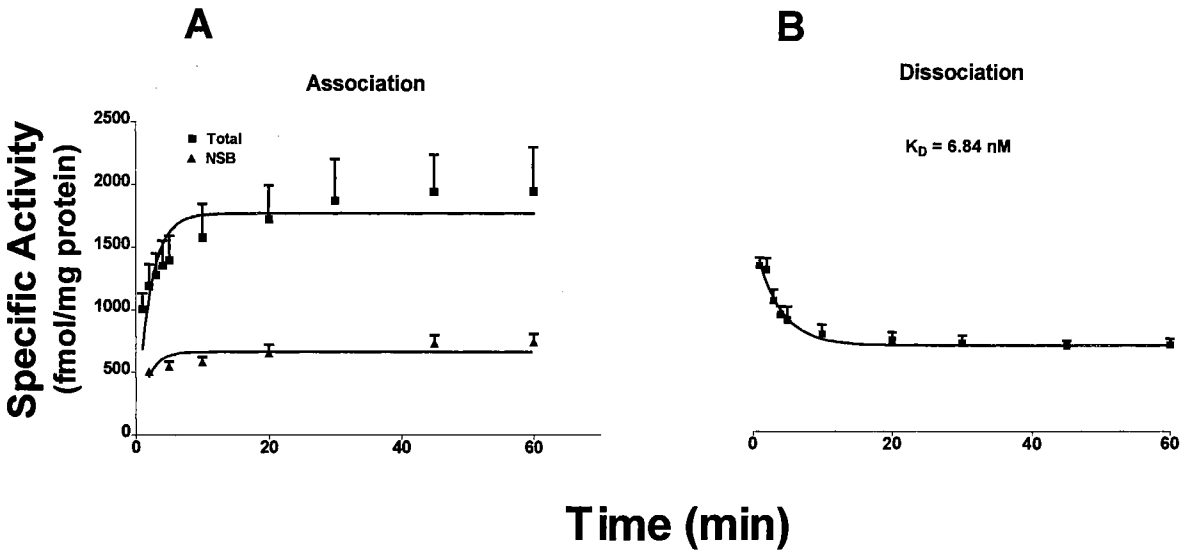


Figure 25. Association (A) and dissociation (B) kinetics of [^{3}H]-naloxone (5 nM) binding in *Rana pipiens* brain. In association studies the membrane preparation was incubated with radioligand in the absence (total binding) or in the presence (nonspecific binding) of 10μM naltrexone. Binding was measured at various time intervals. Data points represent the mean of three membrane preparations with individual experiments performed in triplicate. Dissociation studies involved the addition of unlabelled competitor (10 μM naloxone) at various time points. K_D values were calculated as described in Table III.

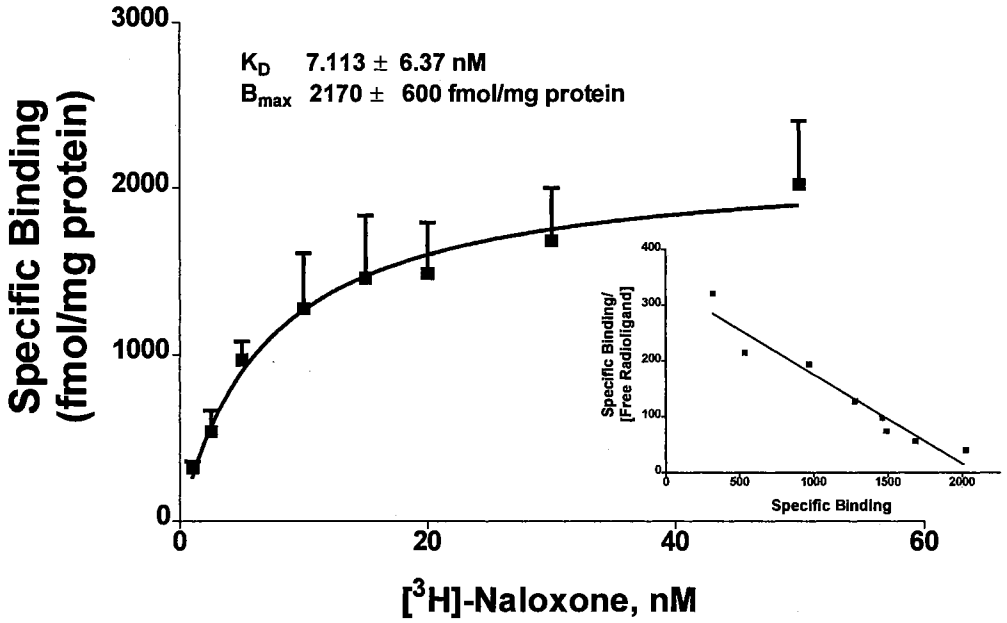


Figure 26. Saturation analysis of [^{3}H]-naloxone binding to amphibian brain tissue homogenates. *Inset:* Scatchard plot of saturation data. Non-linear regression analysis indicated a single site best fit as determined by the F-test. Data points represent the mean of three membrane preparations with individual experiments performed in triplicate.

Table V. Kinetically and experimentally derived affinity and density parameters for [^{3}H]-naloxone binding in brain

Kinetic Analysis		**Saturation Analysis**	
Parameters	Statistics	Parameters	Statistics
k_{obs}[a] 0.4872 ± 0.2353	F value 0.1532, p = 0.9451	K_D 7.113 ± 6.37 nM	F value 0.1911, p = 0.8275
k_{off}[a] 0.2815 ± 0.1157	F value 0.0743, p = 0.9357	B_{max}[d] 2170 ± 600	-
k_{on}[b] 0.0411	-		
K_D[c] 6.84 nM	-		
B_{max}[d] 1767 ± 211	-		

[a] min^{-1}

[b] $mol^{-1}min^{-1}$

[c] K_D values were calculated from rate constant on/off values where

$$k_{on} = \frac{k_{obs} - k_{off}}{[radioligand]} \text{ and } K_D = \frac{k_{off}}{k_{on}}$$

[d] fmol/mg protein

Affinity and density data for [^{3}H]-naloxone are comparable with data observed in other amphibian models as well as in the rat as is shown in Table VI.

Table VI. Affinity and density binding data for [^{3}H]-naloxone.

Tissue	K_d[a]	B_{max}[b]	Reference
***Rana catesbeiana* spinal cord**	7.5	1804.7	Suzuki *et al.*, 1987
***Rana esculenta* brain**	3.6	734	Benyhe, *et al.*, 1990a
***Rana esculenta* brain**	0.9	293	Simon *et al.*, 1984
Rat (PVG/C strain) brain	6.6	783	Benyhe, *et al.*, 1990a
***Rana pipiens* brain**	7.1	2725	Present Studies
***Rana pipiens* spinal cord**	18.8	2170	Present studies

[a]nM; [b]fmol/mg protein

3.3.3. Competition analysis using [^{3}H]-naloxone. In order to clarify drug interaction with particular receptor types, competition experiments were performed with selective opioid receptor ligands using [^{3}H]-naloxone as the label. Figure 27 shows these results with the top panel depicting competition with μ agonists, the middle panel showing competition with δ agents and the bottom panel, competition with κ agonists. Percent specific binding was measured over a range of concentrations (0.01 nM – 100 μM) of cold competitor. For each of these competitors the affinity constant (K_i) was calculated from the complete data set and is shown in Table VII. Additional competition studies with increasing concentrations (0.01 nM – 100 μM) of selective antagonists against [^{3}H]-naloxone (5 nM) were performed. These results are shown in Figure 28 with the complete K_i values shown in Table VIII. In the case of all competitive ligands, the data were best fit to a one site model as determined by the F-test.

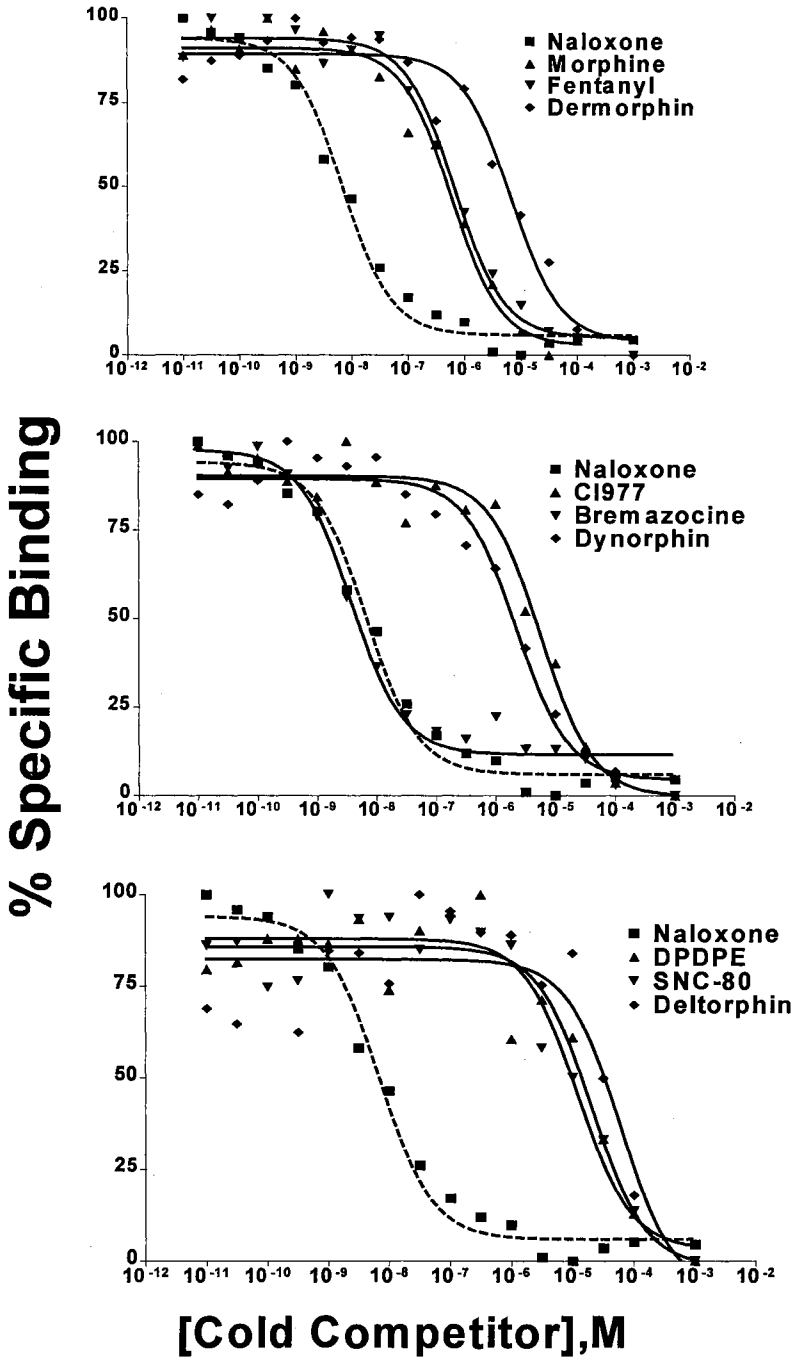


Figure 27. Inhibition of 5 nM [^{3}H]-naloxone binding with various unlabeled opioid ligands in *Rana pipiens* brain tissue homogenates. Aliquots of tissue homogenates were incubated for 60 minutes at room temperature with radioligand in the presence of various concentrations (0.01 nM – 100 μM) of cold competitor. Competitors included μ (top panel), κ(middle panel) and δ (lower panel) opioid agonists. Data were normalized to aid comparisons defining the smallest value in the data set as 0% and the largest value as 100% of specific binding. K_i values for these competitors are shown in Table V. Data points represent the mean of three brains with individual experiments performed in triplicate. Error bars are not shown for enhancement of clarity.

Table VII. Displacement of [^{3}H]-naloxone by selective opioid receptor agonists in brain

Drug	Type	K_i (nM)	95% CI[a]	Hill[b]	95% CI
bremazocine	κ	1.86	(1.12 – 3.09)	-0.8995	(-1.30 to -0.50)
naloxone	μ,δ,κ	3.92	(2.18 – 7.03)	-0.9437	(-1.43 to -0.45)
morphine	μ	445	(315 – 630)	-0.8685	(-1.16 to -0.67)
fentanyl	μ	520	(328 – 822)	-0.7462	(-1.04 to -0.44)
dynorphin	κ	2381	(1324 – 4282)	-0.9441	(-1.42 to -0.46)
dermorphin	μ	3025	(1636 – 5593)	-0.9389	(-1.46 to -0.42)
CI977	κ	5124	(1837 – 14300)	-1.155	(-2.29 to -0.02)
SNC-80	δ	14140	(7442 – 26860)	-0.8403	(-1.41 to -0.26)
DPDPE	δ	22570	(6345 – 80280)	-1.068	(-2.53 to 0.39)
deltorphin	δ	31010	(12610 – 76270)	-1.988	(-4.52 to 0.54)

[a] 95% confidence interval
[b] Hill slope

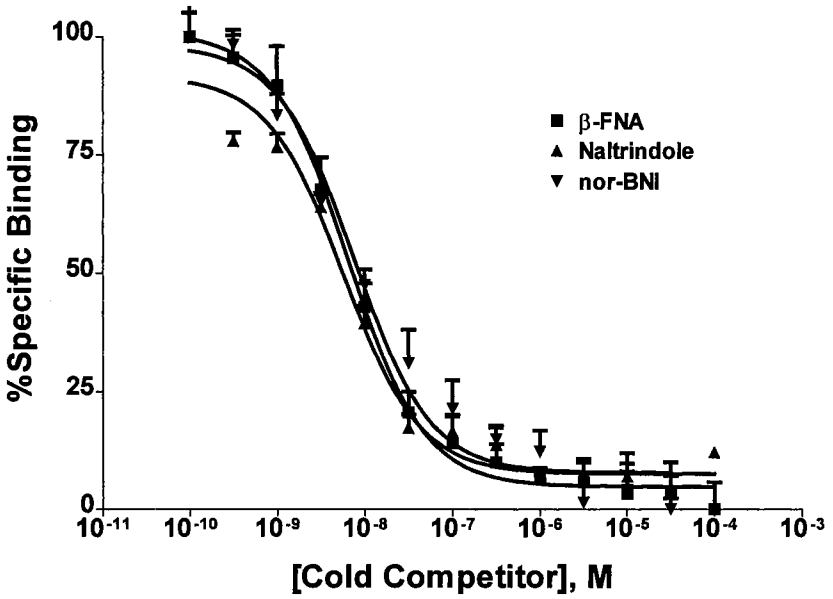


Figure 28. Displacement of 5 nM [^{3}H]-naloxone binding with increasing concentrations (0.01 nM – 10 μM) of selective antagonists in *Rana pipiens* brain. β-FNA is a μ-selective antagonist, naltrindole is δ-selective and nor-BNI is a κ-selective antagonist. Data was normalized to aid comparisons defining the smallest value in the data set as 0% and the largest value as 100% of specific binding. Data points represent the mean of three brains or spinal cords with individual experiments performed in triplicate.

Table VIII. Displacement of [^{3}H]-naloxone by selective antagonists in brain

Drug	Type	K_i (nM)[a]	95% CI[b]	Hill[c]	95% CI
β-FNA	μ	3.27	(2.33 to 4.60)	-0.9701	(-1.26 to -0.68)
nor-BNI	κ	3.55	(2.34 to 5.39)	-0.7947	(-1.09 to -0.50)
NTI	δ	3.22	(2.20 to 4.70)	-1.021	(-1.37 to -0.67)

[a]Apparent K_i value
[b]95% confidence interval
[c]Hill slope

3.3.4. Kinetics of [^{3}H]-naloxone binding in *Rana pipiens* spinal cord. Kinetic analysis was performed to determine the time needed to attain the condition of steady-state as well as the rate constants for association and dissociation. Kinetic analyses of [^{3}H]-naloxone (10 nM) binding in *Rana pipiens* spinal cord homogenates are shown in Figure 29. Association studies (Figure 29A) in the spinal cord yielded a k_{obs} (observed association rate) value of 0.3505 min^{-1} while dissociation (Figure 29B) results resulted in a k_{off} (dissociation rate constant) value of 0.2429 min^{-1}. Nonspecific binding represented 25% of total binding. These rate constants yielded an apparent K_D value of 11.29 nM. Statistical analysis of the comparison between one and two site models yielded a best fit for the one site model (see Table IX for results of F test and significance).

3.3.5. [^{3}H]-Naloxone saturation studies in spinal cord. The properties of naloxone binding sites were studied over an extended range of concentrations of [^{3}H]-naloxone (0.5 nM - 70 nM) where apparent affinity and density data for [^{3}H]-naloxone were determined in frog spinal cord. Saturation data for spinal cord tissue is shown in Figure 30. Scatchard analysis of these data is shown in the inset. The experimentally derived K_D and B_{max} from saturation analysis were found to be 18.75 nM and 2725 fmol/mg protein, respectively. Kinetic and saturation data for [^{3}H]-naloxone are summarized in Table IX. These data were best fit to a one site binding model as determined by the F test. Table VII relates these binding parameters to other non-mammalian and mammalian studies.

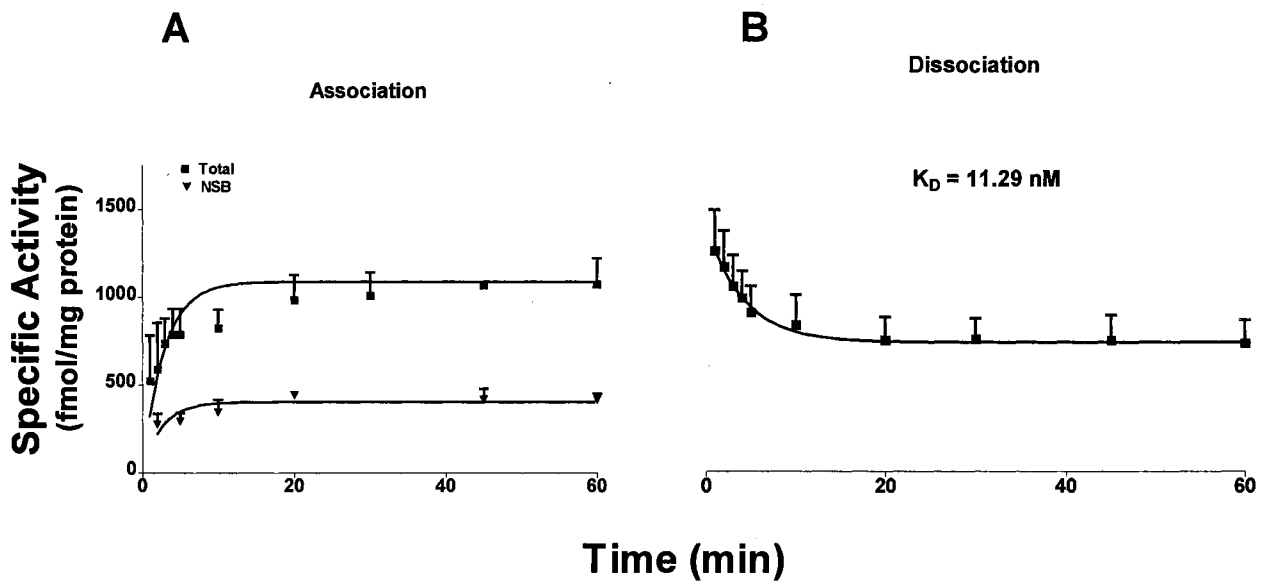


Figure 29. Kinetic analysis of [^{3}H]-naloxone (10 nM) binding in *Rana pipiens* spinal cord. In association studies (A) the membrane preparation was incubated with radioligand in the absence (total binding) or in the presence (nonspecific binding) of 10μM naltrexone. Dissociation (B) studies involved adding cold naltrexone at various timepoints to measure dissociation of [^{3}H]-naloxone. Binding was measured at various time intervals. Data points represent the mean of three membrane preparations with individual experiments performed in triplicate. The K_D values were calculated as described in Table III.

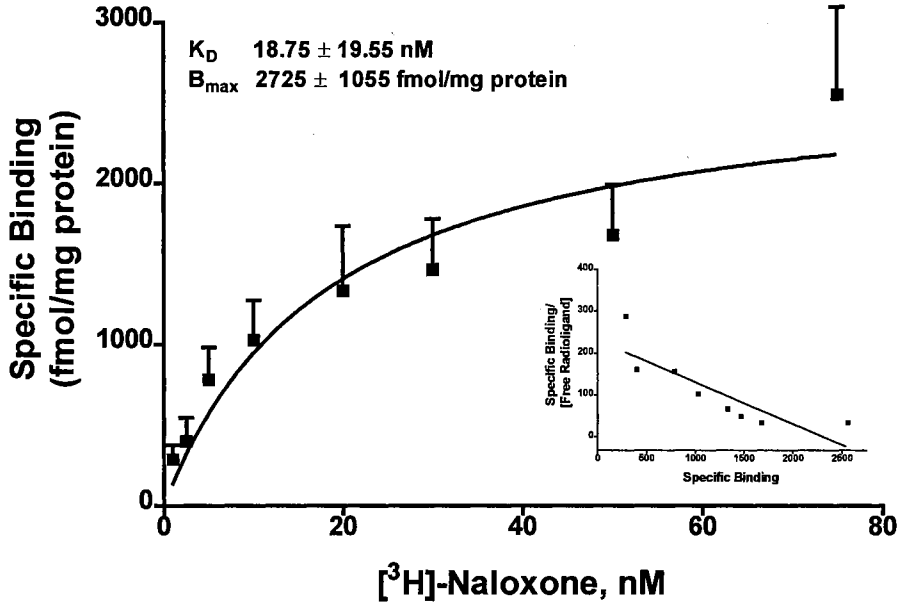


Figure 30. Saturation analysis of [^{3}H]-naloxone binding to amphibian spinal cord tissue homogenates. *Inset:* Scatchard plot of saturation data. Non-linear regression analysis indicated a single site best fit as determined by the F-test. Data points represent the mean of three membrane preparations with individual experiments performed in triplicate.

Table IX. Kinetically and experimentally derived affinity and density parameters for [^{3}H]-naloxone binding in *Rana pipiens* spinal cord

Kinetic Analysis		Saturation Analysis	
Parameters	**Statistics**	**Parameters**	**Statistics**
k_{obs}[a] 0.4581 ± 0.1822	F value 0.1467, p = 0.9523	K_D 18.75 ± 19.55 nM	F value 1.591, p = 0.2285
k_{off}[a] 0.2429 ± 0.1607	F value 0.06451, p = 0.9377	B_{max}[d] 2725 ± 1055	-
k_{on}[b] 0.02152	-		
K_D[c] 11.29 nM	-		
B_{max}[d] 1090 ± 145	-		

[a] min^{-1}

[b] $mol^{-1}min^{-1}$

[c] K_D values were calculated from rate constant on/off values

where $k_{on} = \frac{k_{obs} - k_{off}}{[radioligand]}$ and $K_D = \frac{k_{off}}{k_{on}}$

[d] fmol/mg protein

3.3.6. Competition analysis. In order to examine drug-receptor selectivity in amphibian spinal cord, competition studies were done with selective opioid ligands using [^{3}H]-naloxone as the label. Figure 31 shows these results with the top panel depicting competition with μ agonists, the middle panel showing competition with δ ligands and the bottom panel, competition with κ receptor agonists. Percent specific binding was measured over a range of concentrations (0.01 nM – 100 μM) of cold competitor. For each of these competitors the affinity constant (K_i) was calculated from the complete data set and is shown in Table X. Figure 32 depicts the correlation between brain and spinal cord K_i values in the amphibian where a correlation value of 0.786 was obtained. Additional competition studies with increasing concentrations (0.01 nM – 100 μM) of selective antagonists against [^{3}H]-naloxone (10 nM) were performed. These results are shown in Figure 33 with a summary of the K_i values shown in Table XI. In the case of all competitive ligands, the data were best fit to a one site model as determined by the F-test.

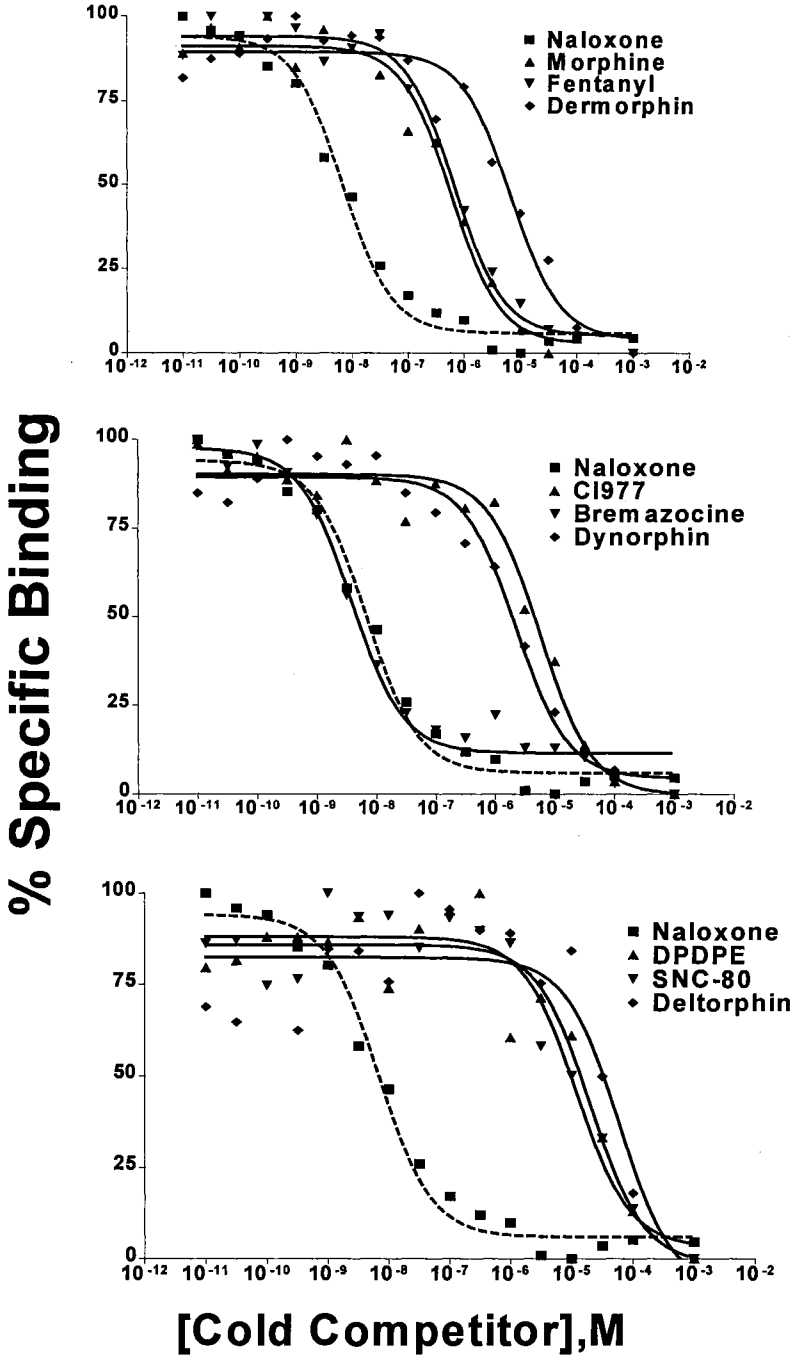


Figure 31. Inhibition of 5 nM [^{3}H]-naloxone binding with various unlabeled opioid ligands in *Rana pipiens* spinal cord tissue homogenates. Aliquots of tissue homogenates were incubated for 60 minutes at room temperature with radioligand in the presence of various concentrations (0.01 nM – 100 μM) of cold competitor. Competitors included μ (top panel), κ (middle panel) and δ (lower panel) opioid agonists. Data were normalized to aid comparisons defining the smallest value in the data set as 0% and the largest value as 100% of specific binding. K_i values for these competitors are shown in Table V. Data points represent the mean of three spinal cords with individual experiments performed in triplicate. Error bars are not shown for enhancement of clarity.

Table X. Displacement of [^{3}H]-naloxone by selective opioid receptor agonists in *Rana pipiens* spinal cord

Drug	Type	K_i (nM)	95% CI[a]	Hill[b]	95% CI
bremazocine	κ	2.58	(1.50 – 4.42)	-0.5461	(-0.78 to -0.31)
naloxone	μ,δ,κ	15.4	(7.97 – 29.95)	-0.2643	(-0.40 to -0.12)
morphine	μ	728	(515 – 1029)	-1.109	(-1.5 to -0.76)
fentanyl	μ	223	(137 – 362)	-0.9327	(-1.3 to -0.54)
dynorphin	κ	4252	(2005 – 9018)	-1.209	(-2.0 to -0.37)
dermorphin	μ	1870	(285 – 12250)	-0.3797	(-0.98 to 0.22)
CI977	κ	7755	(3207 – 18750)	-1.231	(-2.3 to -0.20)
SNC-80	δ	25050	(3064 – 204800)	-0.9404	(-1.4 to -0.66)
DPDPE	δ	12320	(4673 – 32470)	-0.6799	(-1.2 to -0.13)
deltorphin	δ	84910	(32000 – 225300)	-0.3432	(-0.90 to 0.21)

[a] 95% confidence interval
[b] Hill slope

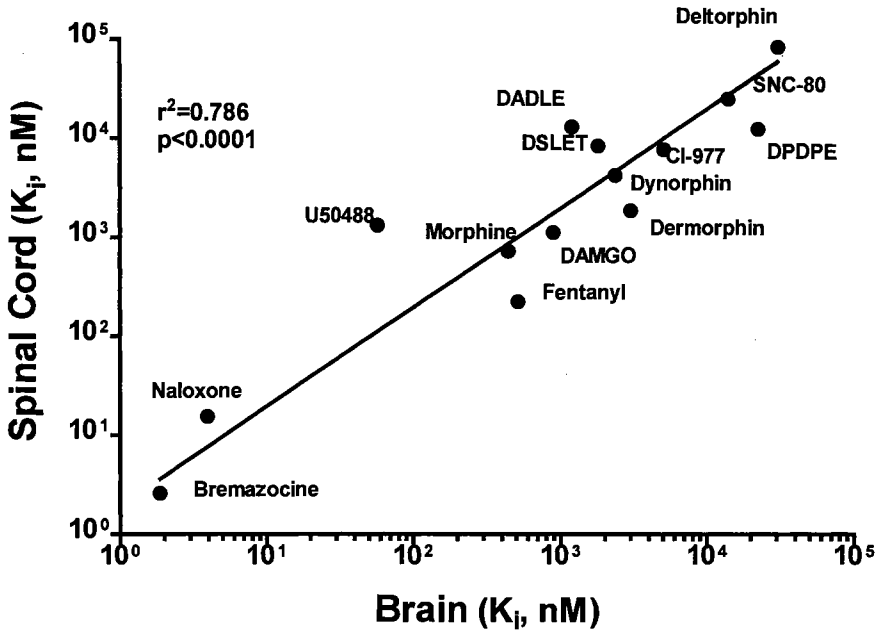


Figure 32. Correlation plot of *Rana pipiens* spinal cord K_i values versus brain K_i values against [^{3}H]-naloxone. Numerical results of regression analysis are shown in the figure.

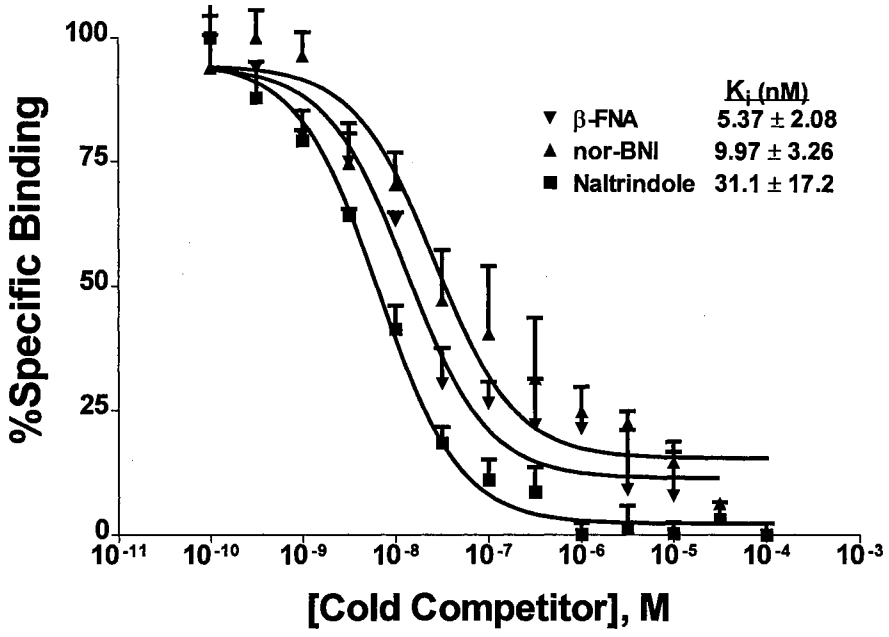


Figure 33. Displacement of 5 nM [^{3}H]-naloxone binding with increasing concentrations (0.01 nM – 10 μM) of selective antagonists in *Rana pipiens* brain. β-FNA is a μ-selective antagonist, naltrindole is δ-selective and nor-BNI is a κ-selective antagonist. Data was normalized to aid comparisons defining the smallest value in the data set as 0% and the largest value as 100% of specific binding. Data points represent the mean of three spinal cords with individual experiments performed in triplicate.

Table XI. Displacement of [^{3}H]-naloxone by selective antagonists

Drug	Type	K_i (nM)[a]	95% CI[b]	Hill[c]	95% CI
β-FNA	μ	5.37	(3.88 to 7.08)	-0.7632	(-0.96 to -0.56)
nor-BNI	κ	9.97	(5.31 to 20.48)	-0.4951	(-0.83 to -0.15)
NTI	δ	31.1	(10.15 to 42.90)	-0.3780	(-0.64 to -0.11)

[a]Apparent K_I value
[b]95% confidence interval
[c]Hill slope

3.3.7. [^{3}H]-Naloxone saturation and competition analysis in CHO cells. For an in-house comparison the saturability of [^{3}H]-naloxone was also examined in Chinese hamster ovary (CHO) cells expressing the human μ opioid receptor with the results shown in Figure 34. Saturation analysis in this preparation yielded an apparent K_D value of 4.4 nM and a B_{max} value of 911.5 fmol/mg protein. Naloxone and bremazocine show the lowest apparent K_i values in *Rana pipiens* brain and spinal cord tissue homogenates. This high competitiveness of bremazocine with [^{3}H]-naloxone was also seen in MOR1 in CHO cells as is shown in Figure 35. To test for partial agonist/antagonist properties of bremazocine, a behavioral study was conducted with fentanyl. As is shown in Figure 36, bremazocine, s.c. (30 nmol/g) was able to significantly diminish the antinociceptive effect of the potent agonist fentanyl, s.c. (30 nmol/g).

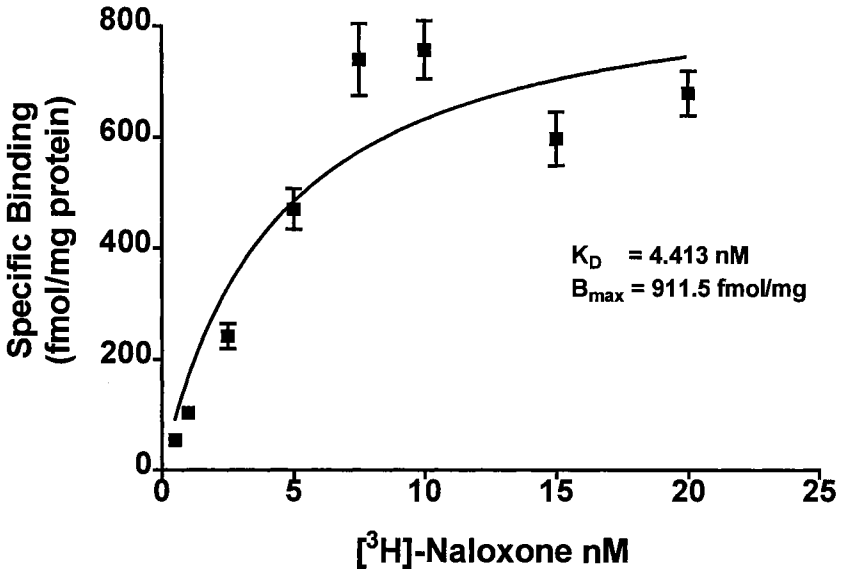


Figure 34. Saturation of [^{3}H]-naloxone binding to CHO membranes transfected with human MOR1 (μ-opioid receptors). Data points represent the mean of three experiments, each performed in triplicate.

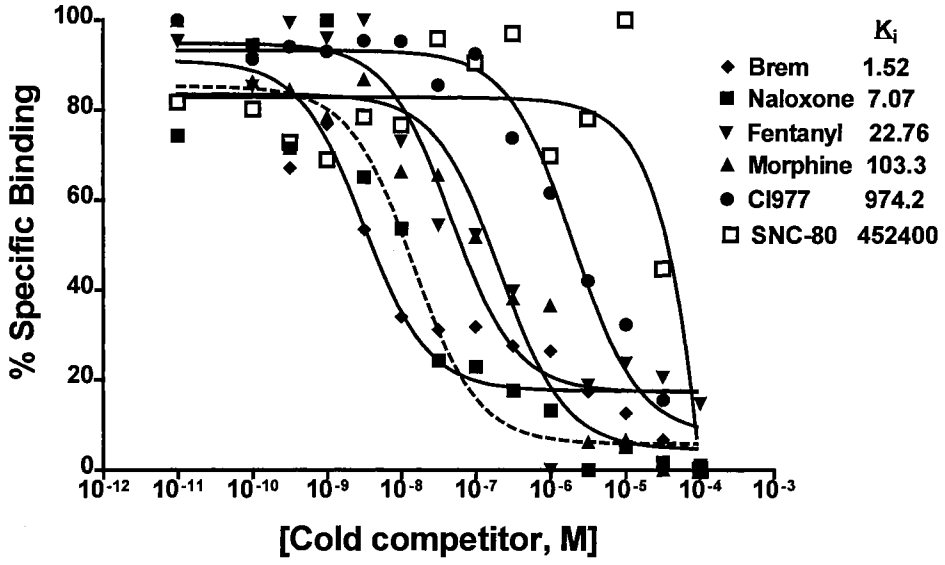


Figure 35. Displacement of [^{3}H]-naloxone (5 nM) binding to CHO membranes transfected with human MOR1 (μ-opioid receptors) by selective and non-selective opioid ligands. K_i values are shown in nM. Data points represent the mean of three experiments, each performed in triplicate.

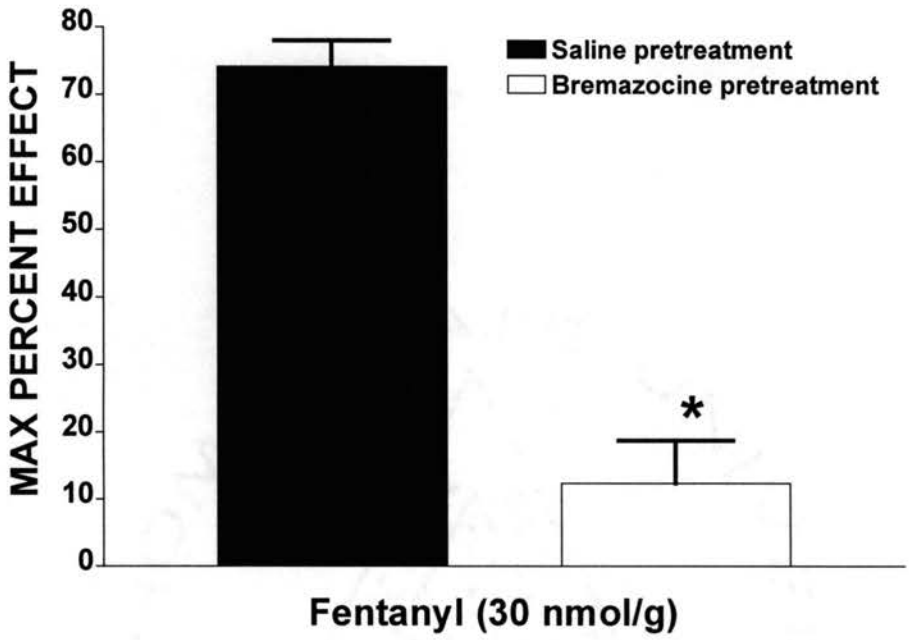


Figure 36. Effect of s.c. bremazocine (30 nmol/g) on the antinociception produced by s.c. fentanyl (30 nmol/g). Separate groups of animals (N = 6 - 12) were used for each treatment group. Asterisk denotes significant difference from saline-pretreated animals ($P < 0.05$).

3.4 G-protein analysis

3.4.1. Analysis of G-proteins in the amphibian. Pertussis toxin (PTX) was employed to demonstrate the involvement of G-proteins in the production of antinociception by opioid agonists in the amphibian. As depicted in Figure 37, PTX (100 ng/frog and 500 ng/frog) significantly attenuated the antinociception produced by DAMGO (3 nmol/frog) for at least up to 16 days post-treatment.

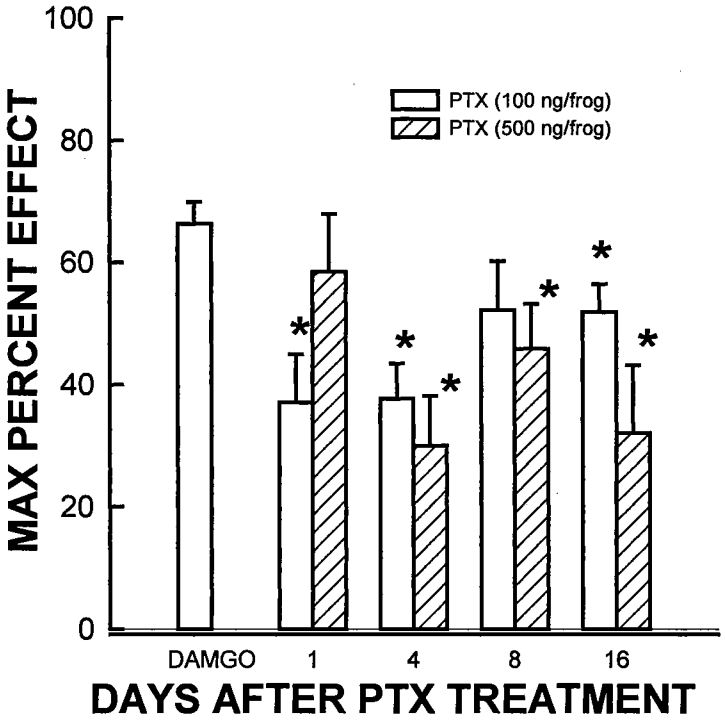


Figure 37. Pertussis toxin (100 ng/frog and 500 ng/frog) given i.s. followed by i.s. DAMGO (3 nmol/frog). Animals were tested using the acetic acid test and the maximum percent effect was determined at 1, 4, 8 and 16 days after PTX treatment. Data are plotted as mean (+ s.e.m.) of maximum MPE observed. N = 6 animals per treatment group. Asterisks denote significant difference from DAMGO alone ($P < 0.05$).

3.4.2. GTPγS^{35} binding. Figure 38 shows the effect of morphine (5 μM) stimulation of GTPγS^{35} (0.08 nM). Inhibition of the binding of GTPγS^{35} occurred using 1 μM naltrexone. Nonspecific binding was determined in the presence of 10 μM GTP. As the figure shows, morphine significantly stimulated GTPγS^{35} binding above basal levels and naltrexone significantly reduced this stimulation in rat nucleus accumbens. Figure 39 illustrates the same experiment done in *Rana pipiens* brain tissue. As shown, morphine did not significantly increase GTPγS^{35} binding above basal and 1 μM naltrexone likewise showed no significant difference from basal. Various experiments were performed to optimize conditions for GTPγS^{35} binding in the amphibian. Examination of the potent opioid agonist, fentanyl, to produce stimulation rather than morphine as well as using β-FNA instead of naltrexone did not produce optimal results in that no stimulation with fentanyl was observed (data not shown). A dose-response of GDP using concentrations ranging from 1 μM – 300 μM did show promising results where 300 μM GDP showed agonist stimulation of GTPγS^{35} which 1 μM naltrexone inhibited (data not shown). This is illustrated in Figure 40 which further illustrates attempts to optimize conditions for GTPγS^{35} binding in *Rana pipiens*. Changing the amount of GDP in the buffer from a final concentration of 50 μM (see Figure 39) to 300 μM yielded significant results where basal and stimulated were significantly different and inhibition was significantly different from stimulated.

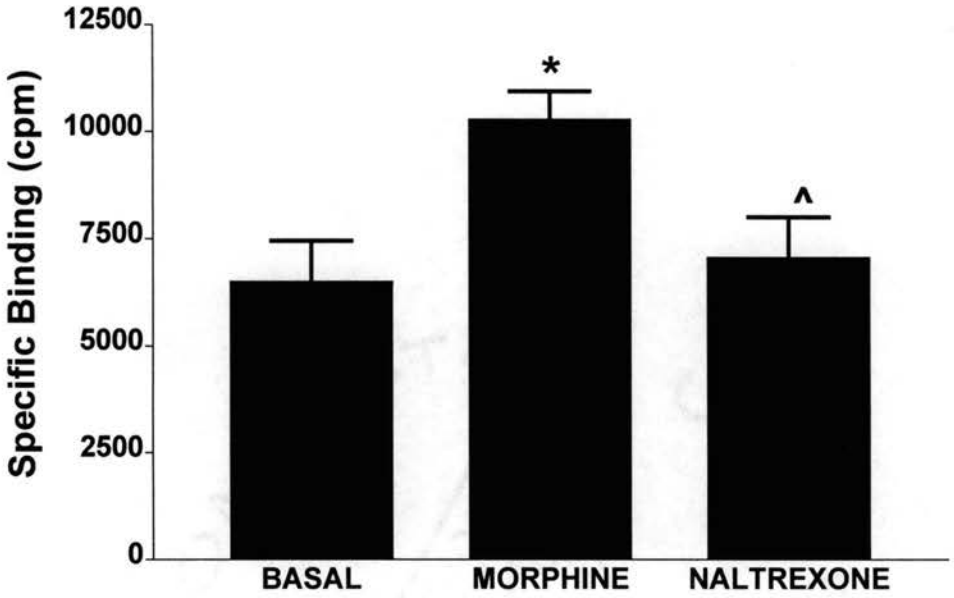


Figure 38. Morphine (5 μM) induced binding of GTPγS^{35} (0.08 nM) with inhibition by 1 μM naltrexone in rat nucleus accumbens. A concentration of 50 μM GDP was used in the buffer. Asterisk denotes significance from basal. Arrow (^) indicates significance from stimulated ($P < 0.05$).

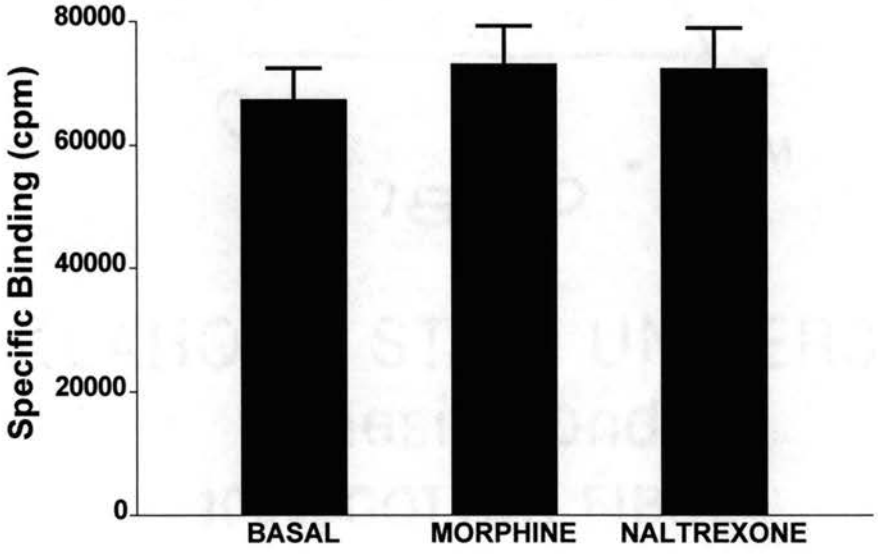


Figure 39. G-protein activation by morphine in *Rana pipiens* brain tissue. GTPγS^{35} binding using 5μM morphine for stimulation, 1 μM naltrexone for inhibition. Concentration of GDP in buffer is 50 μM.

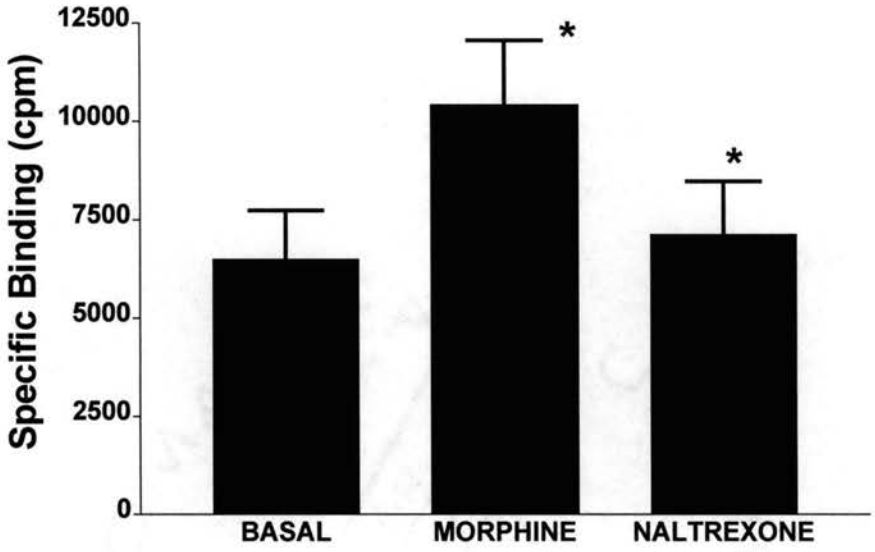


Figure 40. G-protein activation by morphine in *Rana pipiens* brain tissue. $GTP\gamma S^{35}$ (0.08 nM) binding using no drug for basal determination, morphine (5 μM) for stimulation and 1μM naltrexone for inhibition. GDP in buffer was 300 μM / tube. Asterisk denotes significant difference from basal and the arrow (^) denotes significant difference from stimulated.

3.5 Radiolabeled selective antagonist binding

3.5.1. [^{3}H]-Selective opioid antagonist binding. Figure 41 shows the saturation analysis of [^{3}H]-β-FNA (on the left) in *Rana pipiens* brain tissue homogenates and of [^{3}H]-NTI (on the right). β-FNA concentrations ranged from 0.5 nM – 20 nM. NTI concentrations ranged from 0.5 nM – 8 nM. Neither the binding of [^{3}H]-β-FNA nor [^{3}H]-NTI was saturable. Figure 42 shows the saturation analysis of [^{3}H]-NTI (0.5 nM – 15 nM) in rat nucleus accumbens where the apparent K_D value was found to be 7.8 nM and the B_{max} value 4156 fmol/mg protein.

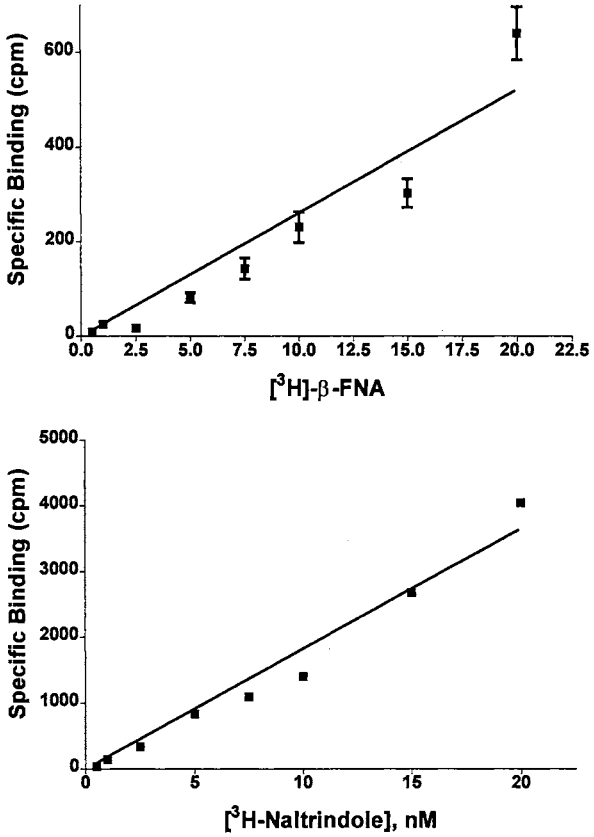


Figure 41. [^{3}H]-β-FNA (0.5 nM – 20 nM) and [^{3}H]-naltrindole (0.5 nM – 8 nM) saturation curves in *Rana pipiens* brain tissue. Data points represent the mean of three membrane preparations with individual experiments performed in triplicate.

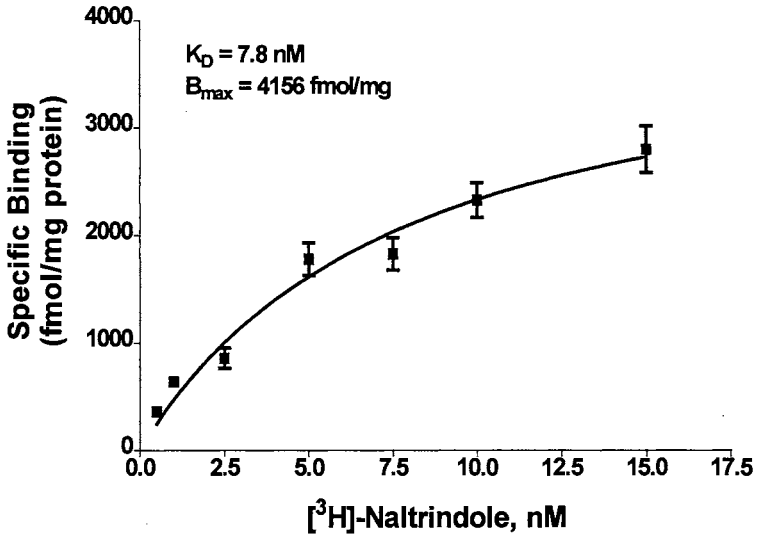


Figure 42. [^{3}H]-Naltrindole (0.5 nM – 15 nM) saturation curve in rat nucleus accumbens. Data points represent the mean of three membrane preparations with individual experiments performed in triplicate.

3.6 Radiolabeled selective agonist binding

3.6.1. Kinetics of [^{3}H]-DAMGO binding. Kinetic analysis was performed to determine the time needed to attain the condition of steady-state as well as the rate constants for association and dissociation. Kinetic analyses of [^{3}H]-DAMGO (5 nM) binding in *Rana pipiens* brain homogenates are shown in Figure 43. Association studies (left panel) in the brain yielded a k_{obs} (observed association rate) value of 0.247 min^{-1} while dissociation (right panel) results yielded a k_{off} (dissociation rate constant) value of 0.072 min^{-1}. These rate constants yielded a K_D value of 2.03 nM. Statistical analysis of the comparison between one and two site models yielded a best fit for the one site model (see Table XII for results of F test and significance).

3.6.2. Saturation studies. The properties of DAMGO binding sites were studied over an extended range of concentrations of [^{3}H]-DAMGO (0.5 nM - 20 nM) where apparent affinity and density data for [^{3}H]-DAMGO were determined. Saturation data for brain tissue is shown in Figure 44. Scatchard analysis of these data is shown in the inset. The experimentally derived K_D and B_{max} from saturation analysis were found to be 10.81 nM and 311.6 fmol/mg protein, respectively. Kinetic and saturation data for [^{3}H]-DAMGO are summarized in Table XII. These data were best fit to a one site binding model as determined by the F test.

3.6.3. Competition analysis. To analyze selectivity of [^{3}H]-DAMGO, competition experiments were performed with selective opioid ligands using [^{3}H]-DAMGO as the label. Figure 45 shows these results. Percent specific binding was measured over a range of concentrations (0.01 nM – 100 μM) of cold competitor. For each of these competitors the affinity constant (K_i) was calculated from the complete data set and is shown in Table

XIII. Additional competition studies with increasing concentrations (0.01 nM – 100 μM) of selective antagonists against [^{3}H]-DAMGO (5 nM) were performed. These results are shown in Figure 46 with a summary of the K_i values shown in Table XIII. In the case of all competitive ligands, the data were best fit to a one site model as determined by the F-test.

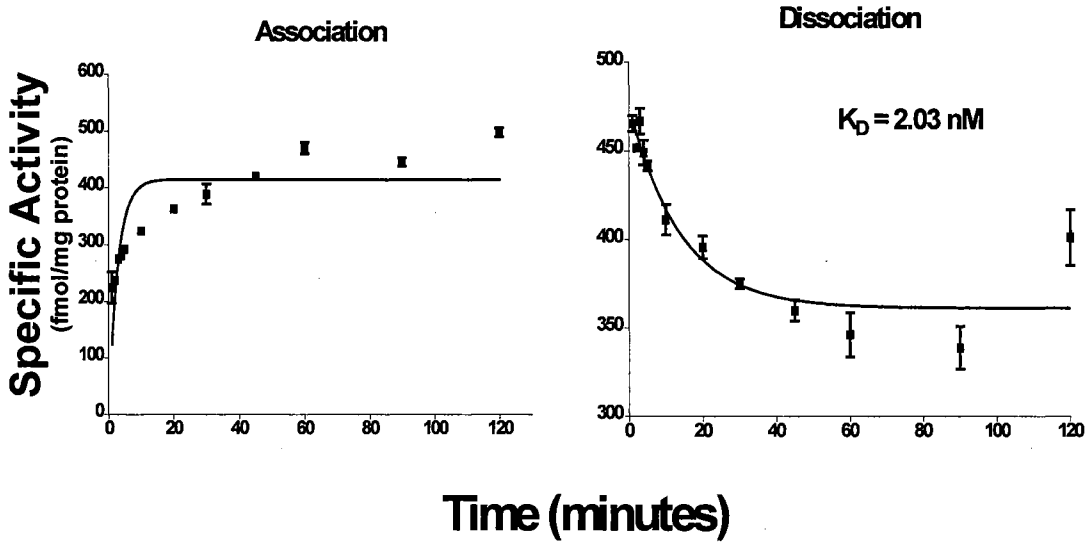


Figure 43. Association and dissociation kinetics of [^{3}H]-DAMGO (5 nM) binding in *Rana pipiens* brain. In association studies the membrane preparation was incubated with radioligand in the absence (total binding) or in the presence (nonspecific binding) of 10μM fentanyl. Binding was measured at various time intervals. Data points represent the mean of three membrane preparations with individual experiments performed in triplicate. Dissociation studies involved the addition of unlabelled competitor (10 μM naloxone) at various time points. K_D values were calculated as described in Table III.

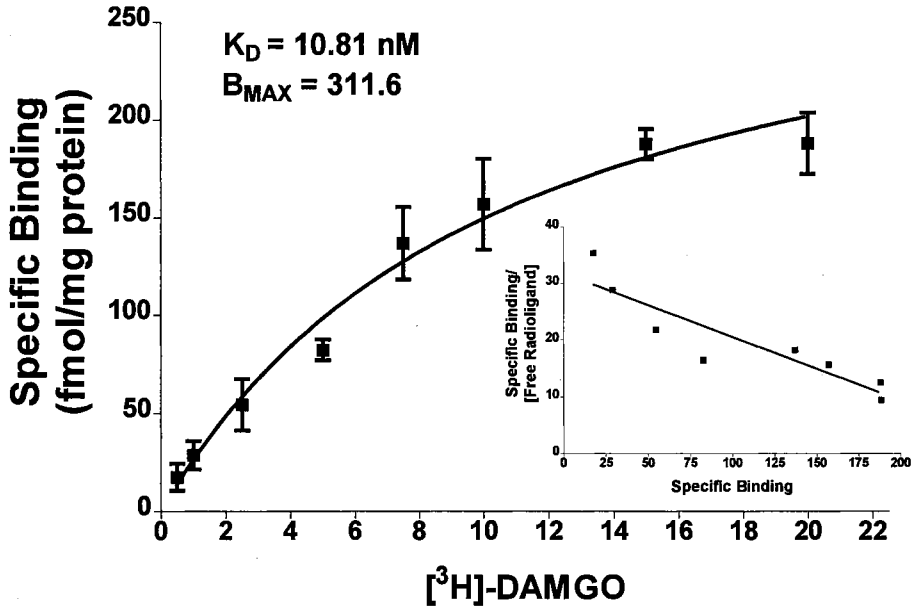


Figure 44. Saturation analysis of [^{3}H]-DAMGO binding to amphibian brain tissue homogenates. *Inset:* Scatchard plot of saturation data. Non-linear regression analysis indicated a single site best fit as determined by the F-test. Data points represent the mean of three membrane preparations with individual experiments performed in triplicate.

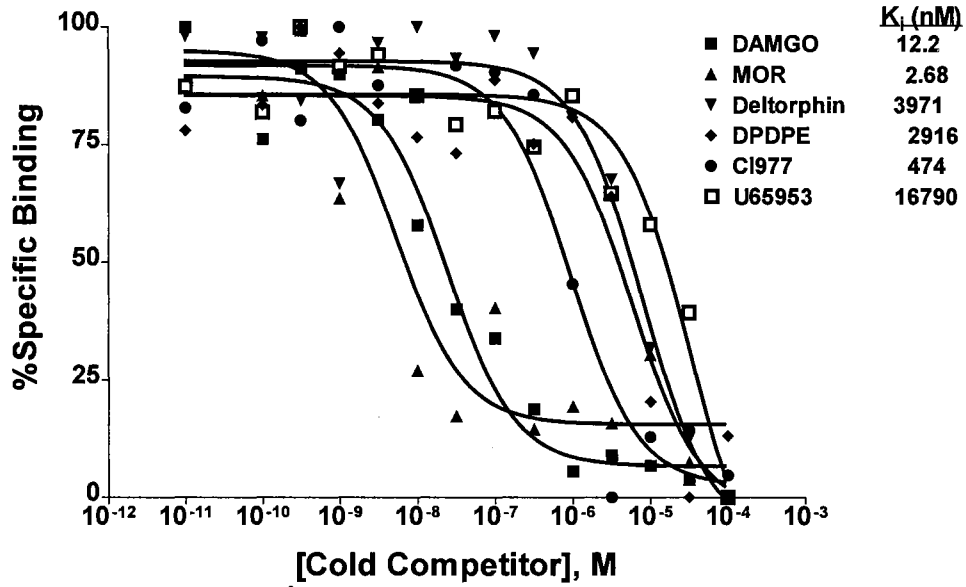


Figure 45. Inhibition of 5 nM [^{3}H]-DAMGO binding with various unlabeled opioid ligands in *Rana pipiens* brain tissue homogenates. Aliquots of tissue homogenates were incubated for 60 minutes at room temperature with radioligand in the presence of various concentrations (0.01 nM – 100 μM) of cold competitor. Competitors included μ , δ and κ opioid agonists. Data were normalized to aid comparisons defining the smallest value in the data set as 0% and the largest value as 100% of specific binding. K_i values for these competitors are shown in Table V. Data points represent the mean of three brains with individual experiments performed in triplicate. Error bars are not shown for enhancement of clarity.

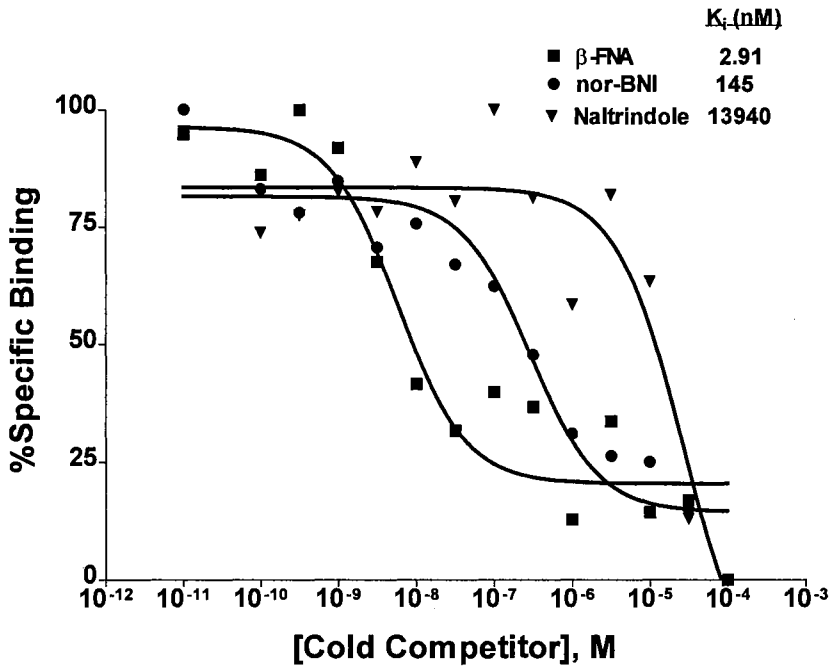


Figure 46. Displacement of 5 nM [^{3}H]-DAMGO binding with increasing concentrations (0.01 nM – 10 μM) of selective antagonists in *Rana pipiens* brain. β-FNA is a μ-selective antagonist, naltrindole is δ-selective and nor-BNI is a κ-selective antagonist. Data was normalized to aid comparisons defining the smallest value in the data set as 0% and the largest value as 100% of specific binding. Data points represent the mean of three brains or spinal cords with individual experiments performed in triplicate.

3.6.4. Kinetics of [^{3}H]-U65953 binding. Kinetic analysis was performed to determine the time needed to attain the condition of steady-state as well as the rate constants for association and dissociation. Kinetic analyses of [^{3}H]-U65953 (10 nM) binding in *Rana pipiens* brain homogenates are shown in Figure 47. Association studies (left panel) in the brain yielded a k_{obs} (observed association rate) value of 0.5744 min^{-1} while dissociation (right panel) results yielded a k_{off} (dissociation rate constant) value of 0.1994 min^{-1}. These rate constants yielded an apparent K_D value of 5.25 nM. Statistical analysis of the comparison between one and two site models yielded a best fit for the one site model (see Table XII for results of F test and significance).

3.6.5. Saturation studies. The properties of U65953 binding sites were studied over an extended range of concentrations of [^{3}H]-U65953 (0.5 nM - 15 nM) where apparent affinity and density data for [^{3}H]-U65953 were determined. Saturation data for brain tissue is shown in Figure 48. Scatchard analysis of these data is shown in the inset. The experimentally derived K_D and B_{max} from saturation analysis were found to be 20.76 nM and 184.1 fmol/mg protein, respectively. Kinetic and saturation data for [^{3}H]-U65953 are summarized in Table XII. These data were best fit to a one site binding model as determined by the F test.

3.6.6. Competition analysis. In order to clarify drug interaction with particular receptor types, inhibition experiments were performed with selective opioid ligands using [^{3}H]-U65953 as the label. Figure 49 shows these results. Percent specific binding was measured over a range of concentrations (0.01 nM – 100 μM) of cold competitor. For each of these competitors the affinity constant (K_i) was calculated from the complete data set and is shown in Table XIII. Additional competition studies with increasing

concentrations (0.01 nM – 100 μM) of selective antagonists against [^{3}H]-U65953 (10 nM) were performed. These results are shown in Figure 50 with a summary of the K_i values shown in Table XIII. In the case of all competitive ligands, the data were best fit to a one site model as determined by the F-test.

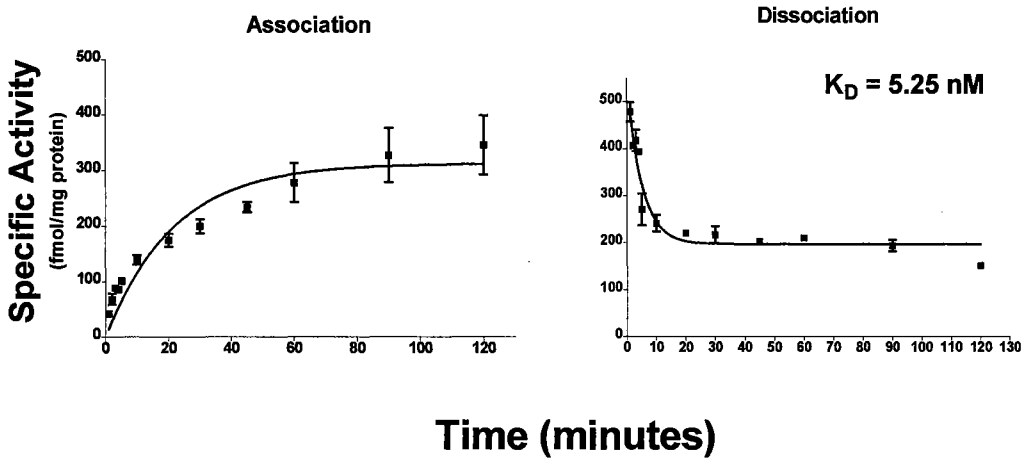


Figure 47. Association and dissociation kinetics of [^{3}H]-U65953 (10 nM) binding in *Rana pipiens* brain. In association studies the membrane preparation was incubated with radioligand in the absence (total binding) or in the presence (nonspecific binding) of 10μM fentanyl. Binding was measured at various time intervals. Data points represent the mean of three membrane preparations with individual experiments performed in triplicate. Dissociation studies involved the addition of unlabelled competitor (10 μM naloxone) at various time points. K_D values were calculated as described in Table III

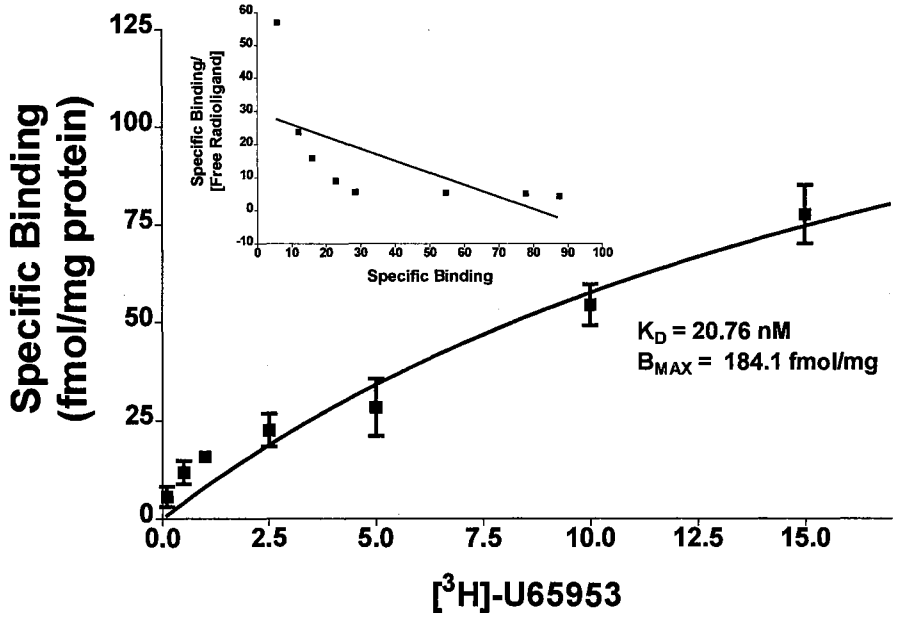


Figure 48. Saturation analysis of [^{3}H]-U65953 binding to amphibian brain tissue homogenates. *Inset:* Scatchard plot of saturation data. Non-linear regression analysis indicated a single site best fit as determined by the F-test. Data points represent the mean of three membrane preparations with individual experiments performed in triplicate.

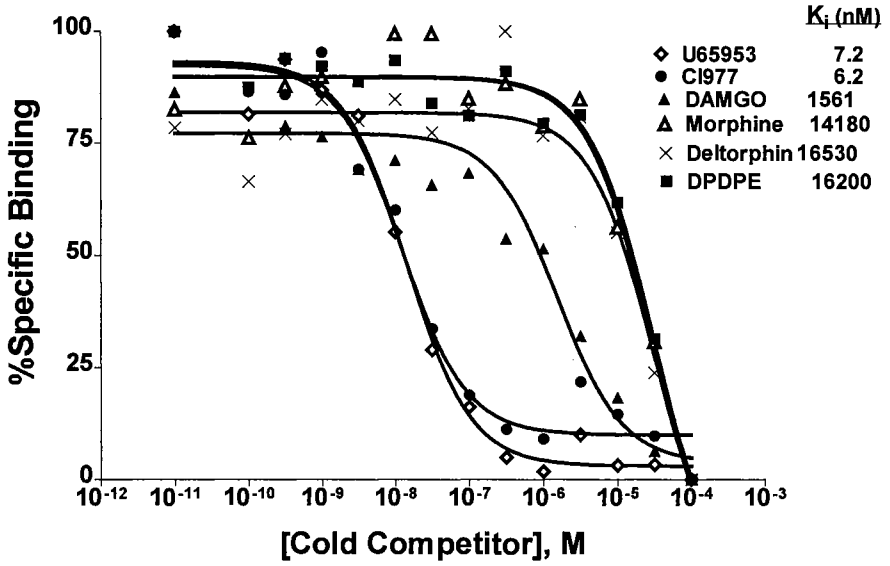


Figure 49. Inhibition of 10 nM [^{3}H]-U65953 binding with various unlabeled opioid ligands in *Rana pipiens* brain tissue homogenates. Aliquots of tissue homogenates were incubated for 60 minutes at room temperature with radioligand in the presence of various concentrations (0.01 nM – 100 μM) of cold competitor. Competitors included μ , δ and κ opioid agonists. Data were normalized to aid comparisons defining the smallest value in the data set as 0% and the largest value as 100% of specific binding. K_i values for these competitors are shown in Table V. Data points represent the mean of three brains with individual experiments performed in triplicate. Error bars are not shown for enhancement of clarity.

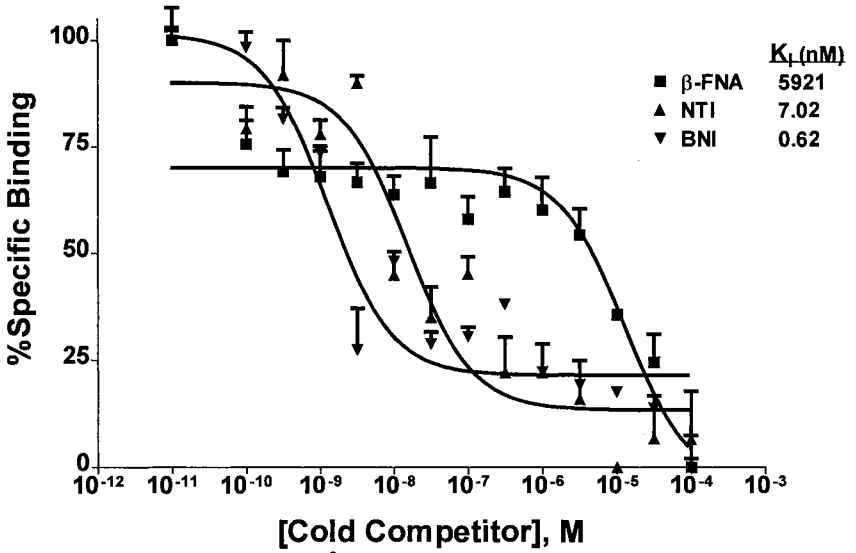


Figure 50. Displacement of 10 nM [^{3}H]-U65953 binding with increasing concentrations (0.01 nM – 10 μM) of selective antagonists in *Rana pipiens* brain. β-FNA is a μ-selective antagonist, naltrindole is δ-selective and nor-BNI is a κ-selective antagonist. Data was normalized to aid comparisons defining the smallest value in the data set as 0% and the largest value as 100% of specific binding. Data points represent the mean of three brains or spinal cords with individual experiments performed in triplicate.

3.6.7. Kinetics of [^{3}H]-DPDPE binding. Kinetic analysis was conducted to determine the time needed to attain the condition of steady-state as well as the rate constants for association and dissociation. Kinetic analyses of [^{3}H]-DPDPE (5 nM) binding in *Rana pipiens* brain homogenates are shown in Figure 51. Association studies (left panel) in the brain yielded a k_{obs} (observed association rate) value of 0.3582 min^{-1} while dissociation (right panel) results yielded a k_{off} (dissociation rate constant) value of 0.0707 min^{-1}. These rate constants yielded a K_D value of 1.23 nM. Statistical analysis of the comparison between one and two site models yielded a best fit for the one site model (see Table XII for results of F test and significance).

3.6.8. Saturation studies. The properties of DPDPE binding sites were studied over an extended range of concentrations of [^{3}H]-DPDPE (0.5 nM - 15 nM) where apparent affinity and density data for [^{3}H]-DPDPE were determined. Saturation data for brain tissue is shown in Figure 52. Scatchard analysis of these data is shown in the inset. The experimentally derived K_D and B_{max} from saturation analysis were found to be 3.541 nM and 425.2 fmol/mg protein, respectively. Kinetic and saturation data for [^{3}H]-DPDPE are summarized in Table XII. These data were best fit to a one site binding model as determined by the F test. Table XVI shows species and cell line comparisons for saturation parameters for the radiolabeled selective opioid agonists.

3.6.9. Competition analysis. In order to determine drug interaction with particular receptor types, inhibition experiments were performed with selective opioid ligands using [^{3}H]-DPDPE as the label. Figure 53 shows these results. Percent specific binding was measured over a range of concentrations (0.01 nM – 100 μM) of cold competitor. For each of these competitors the affinity constant (K_i) was calculated from the complete data

set and is shown in Table XIII. Additional competition studies with increasing concentrations (0.01 nM – 100 μM) of selective antagonists against [^{3}H]-DPDPE (5 nM) were performed. These results are shown in Figure 51 with a summary of the K_i values shown in Table XIII. In the case of all competitive ligands, the data were best fit to a one site model as determined by the F-test. A comparison of Ki values of various selective opioid agonists for the radiolabeled selective agonists is shown in Table XVII.

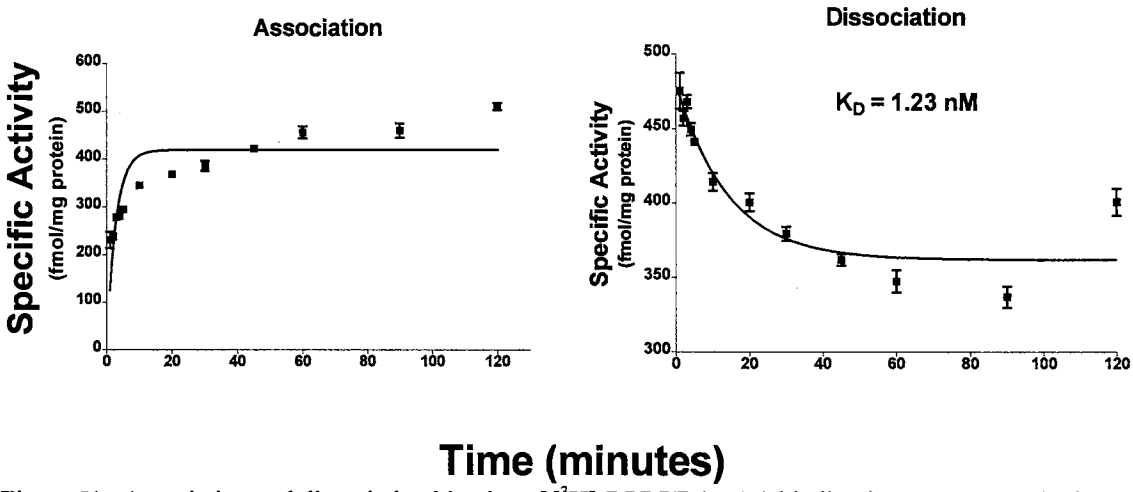


Figure 51. Association and dissociation kinetics of [^{3}H]-DPDPE (5 nM) binding in *Rana pipiens* brain. In association studies the membrane preparation was incubated with radioligand in the absence (total binding) or in the presence (nonspecific binding) of 10μM fentanyl. Binding was measured at various time intervals. Data points represent the mean of three membrane preparations with individual experiments performed in triplicate. Dissociation studies involved the addition of unlabelled competitor (10 μM naloxone) at various time points. K_D values were calculated as described in Table III.

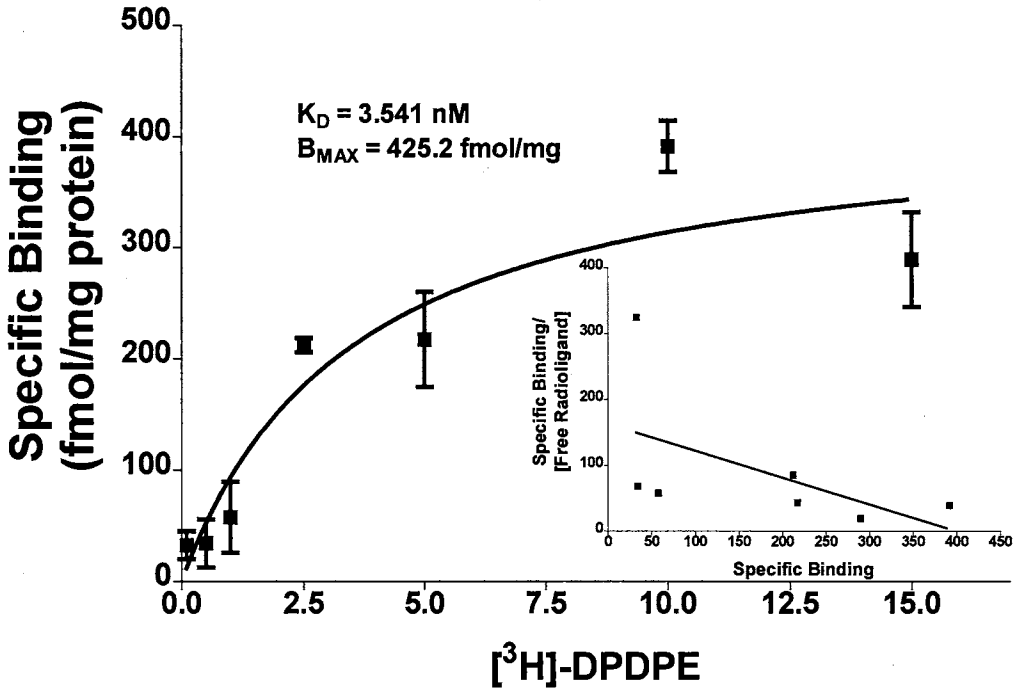


Figure 52. Saturation analysis of [^{3}H]-DPDPE binding to amphibian brain tissue homogenates. *Inset:* Scatchard plot of saturation data. Non-linear regression analysis indicated a single site best fit as determined by the F-test. Data points represent the mean of three membrane preparations with individual experiments performed in triplicate.

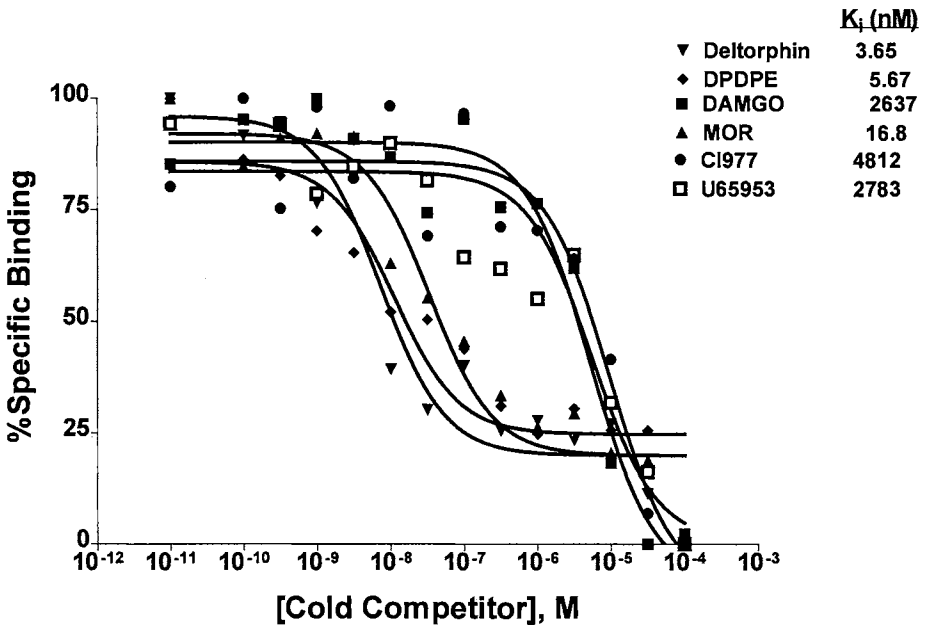


Figure 53. Inhibition of 10 nM [^{3}H]-DPDPE binding with various unlabeled opioid ligands in *Rana pipiens* brain tissue homogenates. Aliquots of tissue homogenates were incubated for 60 minutes at room temperature with radioligand in the presence of various concentrations (0.01 nM – 100 μM) of cold competitor. Competitors included μ , δ and κ opioid agonists. Data were normalized to aid comparisons defining the smallest value in the data set as 0% and the largest value as 100% of specific binding. K_i values for these competitors are shown in Table V. Data points represent the mean of three brains with individual experiments performed in triplicate. Error bars are not shown for enhancement of clarity.

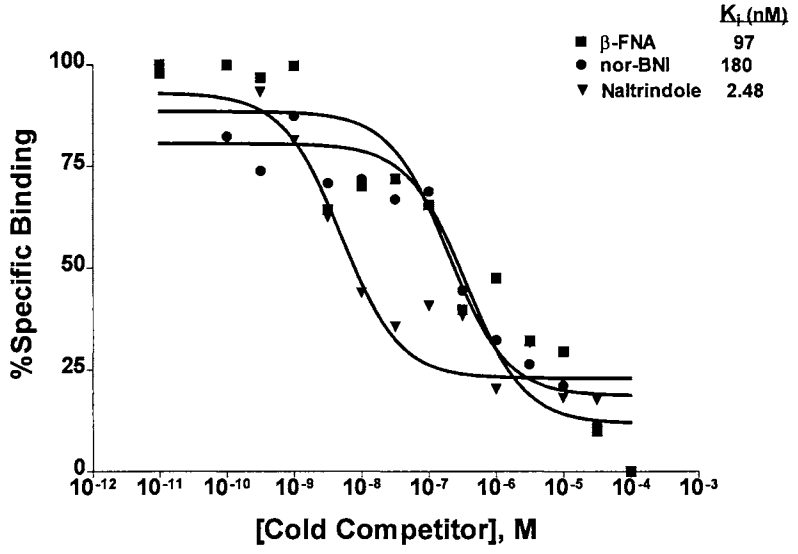


Figure 54. Displacement of 10 nM [^{3}H]-DPDPE binding with increasing concentrations (0.01 nM – 10 μM) of selective antagonists in *Rana pipiens* brain. β-FNA is a μ-selective antagonist, naltrindole is δ-selective and nor-BNI is a κ-selective antagonist. Data was normalized to aid comparisons defining the smallest value in the data set as 0% and the largest value as 100% of specific binding. Data points represent the mean of three brains or spinal cords with individual experiments performed in triplicate.

Table XII. Kinetically and experimentally derived affinity and density parameters for [^{3}H]-selective agonist binding

	[^{3}H]-DAMGO		[^{3}H]-U65953		[^{3}H]-DPDPE	
	Parameters (CI)[e]	**Statistics**	**Parameters (CI)**	**Statistics**	**Parameters (CI)**	**Statistics**
Kinetics						
k_{obs}[a]	0.247 (0.14-0.47)	F value 1.76 p = 0.98	0.5744 (0.30-0.63)	F value 0.34 p = 0.87	0.3582 (0.27-0.45)	F value 1.53 p = 0.95
k_{off}[a]	0.072 (0.03-0.12)	F value 0.82 p = 0.94	0.1994 (0.12-0.28)	F value 1.56 p = 0.92	0.070 (0.04-0.10)	F value 2.77 p = 0.99
k_{on}[b]	0.035		0.0380		0.0575	
K_D[c]	2.03		5.25		1.23	
B_{max}[d]	414.6 (382-447)		313.1 (276-349)		419.3 (393-445)	
Saturation						
K_D[c]	10.81 (4.12-17.55)	F value 1.87 p = 0.382	20.76 (0.90-44.8)	F value 2.04 p = 0.156	3.541 (1.49-4.57)	F value 0.93 p = 0.132
B_{max}[d]	311.6 (214-408)		184.1 (67.1-301)		425.2 (192-271)	

[a] min^{-1}

[b] $mol^{-1}min^{-1}$

[c] K_D (nM) values are calculated from the rate constant on/off values where

$$k_{on} = \frac{k_{obs} - k_{off}}{[radioligand]} \text{ and } K_D = \frac{k_{off}}{k_{on}}$$

[d] fmol/mg protein

[e] 95% confidence interval

Table XIII. Displacement of radiolabeled selective agonists by selective opioid receptor agonists and antagonists

	[^{3}H]-DAMGO		[^{3}H]-U65953		[^{3}H]-DPDPE	
Drug	**K_i (nM)**	**95% CI[a]**	**K_i (nM)**	**95% CI**	**K_i (nM)**	**95%CI**
μ-ligands						
DAMGO	12.2	(7.77 - 19.3)	1561	(374 - 1630)	2637	(1297 to 5363)
morphine	2.68	(1.28 - 5.60)	14180	(2683 - 74960)	16.8	(8.18 to 34.6)
β-FNA	2.91	(1.27 - 6.64)	5921	(2264 - 17820)	97	(43.6 to 219)
κ-ligands						
U65953	16790	(8223 - 34230)	7.2	(4.87 - 10.6)	2783	(571 to 13560)
CI977	474	(270 - 828)	6.2	(3.26 - 11.8)	4812	(1793 to 12910)
nor-BNI	145	(65.5 - 319)	0.62	(0.33 - 1.19)	180	(43.2 to 752)
δ-ligands						
DPDPE	2916	(1612 - 5246)	16200	(1232 - 21300)	5.67	(1.28 to 25.2)
deltorphin	3971	(2448 - 6443)	16530	(1236 - 22100)	3.65	(1.27 to 10.6)
NTI	13940	(4448 - 43720)	7.02	(3.65 - 15.9)	2.48	(1.07 to 5.79)

[a] 95% confidence interval

Table XIV displays the saturation analyses of opioid ligands in amphibian brain tissue where the B_{max} values for nonselective opioid antagonists are compared to the B_{max} values for selective opioid agonists both in total and as a percentage of nonselective antagonist binding.

Table XV illustrates a selectivity index for μ, κ and δ opioid agonists. The selectivity of each selective opioid agent for certain opioid receptors is expressed as the ratio of selective opioid K_i values in displacing two selective radiolabeled ligands. For example, [^{3}H]-DAMGO/[^{3}H]-U65953 (selectivity:μ/κ), [^{3}H]-DAMGO/[^{3}H]-DPDPE (selectivity:μ/δ), [^{3}H]-DPDPE/[^{3}H]-U65953 (selectivity:δ/κ), etc.

Table XIV. Saturation analyses of nonselective and selective opioid ligands in *Rana pipiens* brain tissue

Radioligand	B_{max}[a]	K_D[b]	% Binding of [^{3}H]-Diprenorphine	% Binding of [^{3}H]-Naloxone
Nonselective				
[^{3}H]-diprenorphine	287.7	0.65	100%	13%
[^{3}H]-naloxone	2170	7.1	754%	100%
Selective				
[^{3}H]-DAMGO	311.6	10.81	108%	14%
[^{3}H]-U65953	184.1	20.76	64%	8%
[^{3}H]-DPDPE	425.2	3.541	148%	19%
Total	920.9			

[a]fmol/mg protein, [b]nM

Table XV. Binding selectivity of μ, κ and δ opioid receptor agonists

	Selectivity Index	
μ-ligands	δ/μ	κ/μ
DAMGO	216	128
morphine	6.27	5291
β-FNA	33.3	2035
κ-ligands	μ/κ	δ/κ
U65953	2332	387
CI977	76.4	776.1
nor-BNI	233	290
δ-ligands	μ/δ	κ/δ
DPDPE	514.3	2857
deltorphin	1088	4529
NTI	5621	2.83

The selectivity index is expressed as the ratio of selective opioid K_i values in displacing two selective radiolabeled ligands: [^{3}H]-DAMGO/[^{3}H]-U65953 (selectivity:μ/κ), [^{3}H]-DAMGO/[^{3}H]-DPDPE (selectivity:μ/δ), [^{3}H]-DPDPE/[^{3}H]-U65953 (selectivity:δ/κ), etc.

Table XVI. Species and cell line comparisons of K_D and B_{max} values for radiolabeled selective opioid agonists.

Radioligand/Species	K_D[a]	B_{max}[b]	Reference
[^{3}H]-DAMGO			
Rana pipiens	10.81	311.6	Present studies
Rat	1.09	7300	Gillan and Kosterlitz, 1982
Junco hyemalis	<5	<120	Deviche *et al.*, 1993
Mongolian gerbil	1.55	127.8	Niwa, *et al.*, 1994
HEK 293 cells	0.63	-	Darlison, *et al.*, 1997.
COS-7 cells	0.57	444	Raynor, *et al.*, 1994
Rhesus monkey	0.57	44.9	Emmerson, *et al.* 1994
Guinea pig	0.14	1600	Goldstein and Naidu, 1989
Neuroblastoma cells	0.67	147	Standifer *et al.*, 1994a
[^{3}H]-U65953			
Rana pipiens	20.76	184.1	Present studies
Rana esculenta	15.3	258	Benyhe *et al.*, 1992
Rat	3.94	3300	Lahti *et al.*, 1985
Mouse	4.80	3300	Lahti *et al.*, 1985
Junco hyemalis	<5	<120	Deviche *et al.*, 1993
COS-7 cells	2.8	3346	Raynor, *et al.*, 1994
Human cortex	3.8	12.6	Kim, *et al.*, 1996
Rhesus monkey	0.95	39.2	Emmerson, *et al.* 1994)
Guinea pig	2.88	3300	Lahti, *et al.*, 1985
Mouse	4.8	3300	Lahti, *et al.*, 1985
Rat	3.94	1500	Lahti, *et al.*, 1985
[^{3}H]-DPDPE			
Rana pipiens	3.54	425.2	Present studies
Rat	3.3	252	Akiyama *et al.*, 1985
Junco hyemalis	<5	<120	Deviche *et al.*, 1993
Guinea pig	2	5000	Goldstein and Naidu, 1989
Rhesus monkey	2.05	55.9	Emmerson, *et al.* 1994
Neuroblastoma cells	1.12	59	Standifer *et al.*, 1994a

[a]nM
[b]fmol/mg protein

Table XVII. Species and cell line comparisons between the K_i values for radiolabeled selective opioid agonists.

	K_i (nM)		
Competitor	**[^{3}H]-DAMGO**	**[^{3}H]-U65953**	**[^{3}H]-DPDPE**
Rana pipiens[a]			
DAMGO	12.2	1561	2637
morphine	2.68	14180	16.8
β-FNA	2.91	5921	97
U65953	16790	7.2	2783
CI977	474	6.2	4812
nor-BNI	145	0.62	180
DPDPE	2916	16200	5.67
deltorphin	3971	16530	3.65
NTI	13940	7.02	2.48
Rhesus Monkey[b]			
DAMGO	0.65	534	634
fentanyl	1.48	387	403
U65953	260	0.98	>2500
nor-BNI	8.02	0.06	12.1
DPDPE	457	>2500	1.86
deltorphin	502	>2500	1.29
NTI	3.72	5.78	0.04
Cloned receptors[c]			
DAMGO	2	>1000	
fentanyl	0.39	255	
β-FNA	0.33	2.8	
U65953	>1000	0.59	
nor-BNI	2.2	0.027	
DPDPE	>1000	>1000	
deltorphin	>1000	>1000	
NTI	64	66	
Neuroblastoma cells[d]			
DAMGO	0.51		>200
morphine	1.02		>100
nor-BNI	38.2		16.3
DPDPE	423		0.87
deltorphin	57.5		0.49
NTI	22.9		1.04

[a]**Present studies,** [b]**Emmerson,** ***et al.*** **1994,** [c]**Raynor,** ***et al.*****, 1994,** [d]**Standifer** ***et al.*****, 1994a**

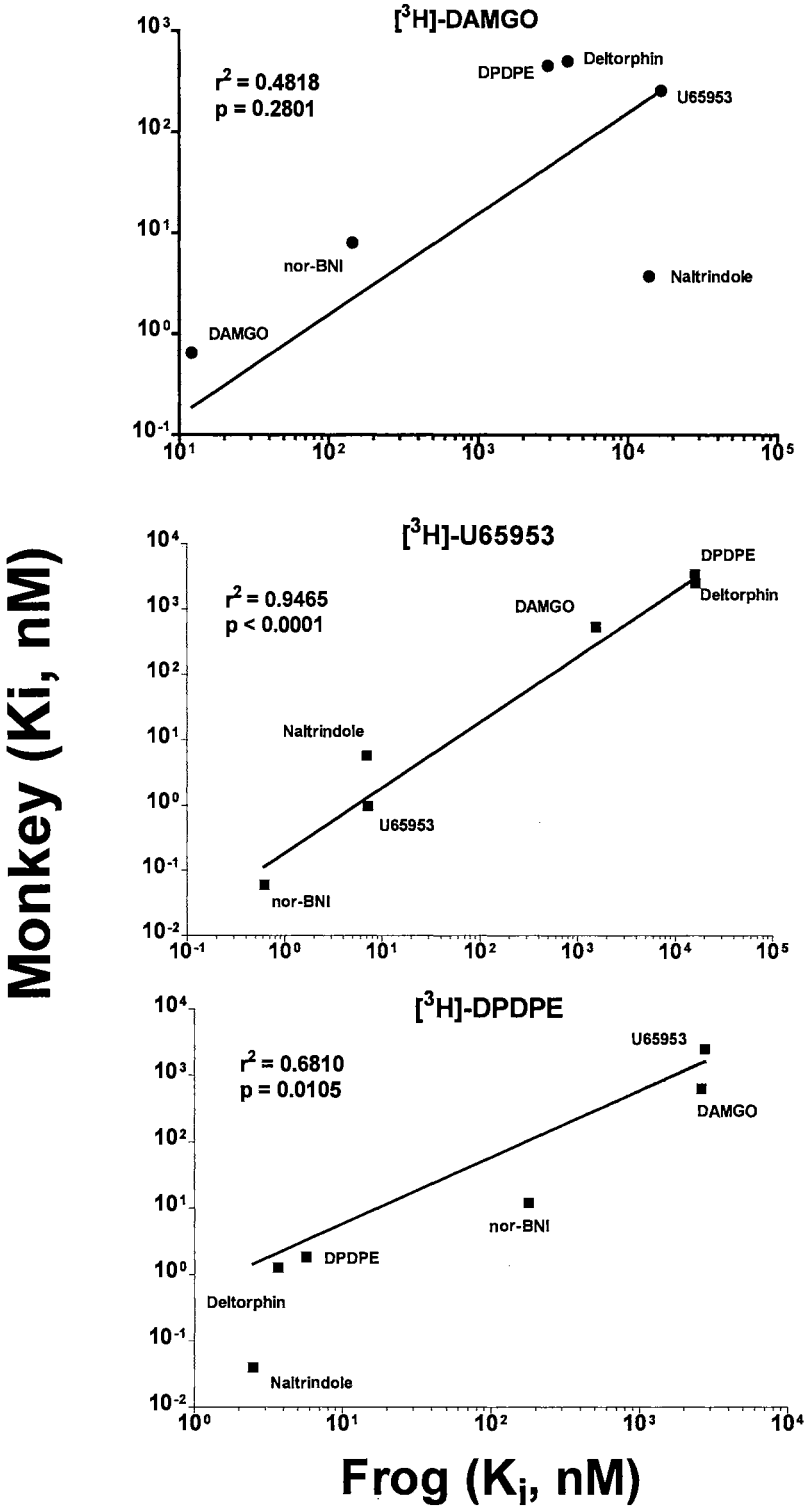


Figure 55. Correlation of K_i values between Rhesus monkey and *Rana pipiens* brain tissue for [^{3}H]-DAMGO, [^{3}H]-U65953 and [^{3}H]-DPDPE. Numerical results of regression analysis are shown in the figure.

Chapter IV

DISCUSSION

4.1 Antinociception in amphibians

Opioid receptors are found to be expressed in species ranging from hagfish to humans (Pert, *et al.*, 1974; Simantov, *et al.*, 1976). The organization and precise role of opioid receptors in lower vertebrates has not been established. However, it has been shown that the same analgesics that produce analgesia in humans also elicit antinociception in amphibians (Stevens, 1988; Stevens, 1995b; Stevens, 1995a; Stevens and Pezalla, 1983). Moreover, several studies have demonstrated a correlation between mammals and amphibians in their responses to opioid agonists (Stevens, *et al.*, 1994; Stevens, 1996a; Stevens and Rothe-Skinner, 1997). Thus, it would not be expected that the opioid binding sites or intracellular mechanisms leading to antinociception would be radically different between mammals and amphibians. Additionally, the selectivity of opioid-receptor interaction, although presumed to be similar to the mammal, has not been examined in the amphibian. The selectivity of opioids in the amphibian was examined through behavioral studies using highly selective opioid antagonists as well as through complementary radioligand binding studies to determine affinity binding parameters for receptor-selective opioid ligands. The ability to displace the same ligand with the same affinity may imply action at a single receptor site.

The amphibian nervous system displays high levels of opioid binding (Ruegg, *et al.*, 1981). *Rana pipiens* has proven to be an effective alternative model for opioid research

in numerous studies (Brenner, *et al.*, 1994; Stevens, 1988; Stevens, 1992; Stevens, 1995a; Stevens, 1996a). Agents selective for μ, κ and δ opioid receptors are shown to elicit potent and sustained antinociception in *Rana pipiens* by any route of administration (Stevens, *et al.*, 1987; Stevens, 1988; Stevens *et al.*, 1994; Stevens, 1996a; Stevens and Pezalla, 1983). This antinociceptive effect was demonstrated with systemic and i.c.v. opioid administration (Stevens and Rothe-Skinner, 1997) as well as with spinal administration using the acetic acid test to measure the nociceptive threshold (Stevens, *et al.*, 1987; Stevens and Pezalla, 1983; Stevens and Pezalla, 1984). The spinal administration of opioid agents from the μ, κ, and δ opioid classes were examined where time courses of effect and dose-response curves were generated for twelve selective opioid agonists (Stevens, 1996a). This study revealed a significant production of antinociception in the amphibian by all agents.

Initially the fundamental question first posed in mammals was tendered for the amphibian model: "Do the different types of opioid ligands recognize distinct and independent receptor molecules or do they simply reflect different conformations of a single opioid receptor with interconvertibility between multiple forms that generate different binding properties?" This fundamental question can be addressed through the analysis of the effect of selective antagonists on opioid antinociception as well as through interpretation of radioligand binding parameters. Selective antagonists used to examine opioid receptors in *Rana pipiens* included β-FNA (μ selective), nor-BNI (κ selective) and naltrindole (δ selective).

4.2 β-FNA

In mammals, the highly selective antagonist, β-FNA, has been shown to be a valuable research tool in studying opioid receptor function in its ability to selectively and irreversibly bind to the μ opioid receptor. β-FNA's agonist and antagonist properties have been investigated to a great extent in rodents (Jiang, *et al.*, 1990; Tam and Liu-Chen, 1986; Ward, *et al.*, 1982a; Ward, *et al.*, 1985). The studies presented herein involve the examination of the acute effects of β-FNA as well as the result of β-FNA administration with opioid agonists in *Rana pipiens*. Intraspinal administration of β-FNA (20 nmol/frog) did not elicit significant antinociception in the amphibian. This is in contrast to mammals in which studies have shown an antinociceptive agonist effect of β-FNA of short duration in rodents due to the interaction of β-FNA with the κ opioid receptor (Jiang, *et al.*, 1990; Takemori, *et al.*, 1981; Ward, *et al.*, 1982a). The acute production of analgesia by β-FNA has caused researchers to employ a 24 hour pre-treatment with β-FNA to avoid the conflicting effect of antinociception produced by β-FNA in order to isolate the analgesia elicited by opioid agonists. β-FNA itself does not exhibit antinociception in *Rana pipiens* and thus the pure antagonistic properties of β-FNA can be taken advantage of to study opioid action. The effectiveness of β-FNA in mammals has been shown in several studies to last for up to 5 - 6 days (Mjanger and Yaksh, 1991; Ward, *et al.*, 1982a). However, in the amphibian, the inhibition of the analgesic effects of fentanyl by β-FNA did not begin to wane until 10 days post-injection as is depicted in Figure 4. In the amphibian, the effect of β-FNA can be examined concurrently with agonist since any agonist action detected can be attributed solely to the agonist. The selectivity of β-FNA as an antagonist for the μ opioid receptor has been well established in rodents where it has been found that β-FNA blocks the analgesic

effects of μ agonists without affecting the antinociception produced by κ or δ opioid agonists (Jiang, *et al.*, 1990; Martin, *eet al.*, 1995; Tam and Liu-Chen, 1986; Ward, *et al.*, 1982a). Figure 5 depicts the examination of β-FNA selectivity in the amphibian using a 24 hour pretreatment for comparison to mammalian studies. As shown in the figure, β-FNA significantly blocks the effects of the μ agonists, morphine, fentanyl and DAMGO as would be expected. Surprisingly, β-FNA also blocked the antinociceptive effects of the κ agonist CI977 and the δ agonist, DPDPE. Examination of the concurrent administration of β-FNA is shown in Figure 6 and reveals an even greater nonselective inhibitory profile for β-FNA where β-FNA not only blocks the μ agonists, morphine, fentanyl and DAMGO but also the κ agonists, bremazocine and CI977 as well as the δ agonists, DAMGO, DADLE, DSLET, DPDPE and deltorphin. The only agonist not affected by concurrent β-FNA administration was the κ agonist, GR89696 which is interesting in light of the fact that β-FNA has been shown to have effects at the κ opioid receptor in mammals.

The only differences between 24 hour pretreatment with β-FNA and concurrent β-FNA is that DADLE is significantly inhibited by concurrent administration but not with β-FNA pretreatment. This may reflect the fact that DADLE has been shown to possess δ opioid properties as well as μ. Full dose-response curves using various doses of agonist may be beneficial since only one dose of agonist, based on previous behavioral studies in the amphibian was used in these experiments. This may shed light on the differences seen with DADLE as well as the fact that some agonists (GR89696 and U50488) were not affected by either concurrent or pretreatment with β-FNA. Overall, β-FNA appears

to be nonselective for amphibian opioid receptors, blocking representative μ, κ and δ selective ligands.

4.3 nor-BNI

In mammals, like β-FNA for the μ opioid receptor and NTI for the δ opioid receptor, nor-BNI has been shown to be highly selective for the κ opioid receptor. Administration of nor-BNI by s.c. as well as by i.c.v. routes have shown effective inhibition of κ opioid agonists that lasts for as long as 28 days without any effect on the antinociception produced by μ or δ opioid agents (Butelman, *et al.*, 1993; Endoh, *et al.*, 1992; Horan, *et al.*, 1992; Takemori, *et al.*, 1988; Wettstein and Grouhel, 1996). In the amphibian, nor-BNI dose-dependently diminishes the effects of the μ agonist, fentanyl and the κ agonist GR89696 with a dose of 0.1 nmol/frog nor-BNI being the lowest dose to block both as is shown in Figure 10. Additionally, nor-BNI not only blocks the antinociceptive effects of the κ agonists, GR89696 and U50488, but also the effects of μ agonists (morphine and fentanyl) and δ agonists (DPDPE and deltorphin) as is shown in Figure 11. In the mammal, nor-BNI was shown to block the effects of U50488 but had no effect on morphine or DPDPE (Broadbear, *et al.*, 1994; Horan, *et al.*, 1992; Takemori, *et al.*, 1988). The only opioids tested that were not affected by nor-BNI were DADLE, which appeared to be potentiated, and DSLET. Thus as seen with β-FNA in the amphibian and in contrast to the mammal, nor-BNI exhibits a nonselective profile of inhibition with regard to μ, κ and δ agonists.

4.4 NTI

Naltrindole possesses a high selectivity and a potent affinity for the δ opioid receptor. The spinal administration of NTI alone did not produce significant antinociception in the amphibian. In mammals, antinociception is seen only after i.c.v. administration of NTI at

very high doses, 1000 times those used to block δ agonist effects (Stapelfeld *et al.*, 1992). This antinociception by NTI is δ opioid receptor mediated since it can be blocked by the δ peptide antagonist, ICI-174,864, but not by β-FNA, the μ opioid antagonist, or by nor-BNI, a κ opioid antagonist. In mammals, NTI has been shown to block the binding of δ opioid agonists in *in vitro* studies (Fang, *et al.*, 1994; Portoghese, *et al.*, 1988; Rogers, *et al.*, 1990; Yamamura, *et al.*, 1992). In whole animal studies, NTI was shown to inhibit the antinociceptive actions of δ opioid agents but not those of μ or κ opioid agonists when administered i.s., i.c.v. or s.c. (Calcagnetti and Holtzman, 1991; Drower, *et al.*, De Costa, 1991; Tiseo and Yaksh, 1993).

In the amphibian, NTI was shown to dose-dependently block the antinociceptive actions of DPDPE (Figure 9), a highly selective δ agonist, after spinal co-administration. This dose dependent inhibition was mirrored after intrathecal co-administration of NTI and DPDPE in the rat (Drower, *et al.*, 1991). In the amphibian, spinal co-administration of NTI antagonized the antinociceptive effects of DSLET also shown in Figure 9. In the mouse, antagonism of DSLET occurred with i.t. administration of NTI (Shah *et al.*, 1994b) and when NTI was administered subcutaneously (Calcagnetti and Holtzman, 1991; Portoghese, *et al.*, 1988). This antagonism of DSLET was also seen in the rat with i.c.v., i.t. and s.c. administration of NTI (Malmberg and Yaksh, 1992; Sofuoglu, *et al.*, 1991) as well as with i.p. injection (Crook *et al.*, 1992). The δ opioid agonist, deltorphin was also blocked by NTI in the amphibian. This too was observed in the rat where spinally administered NTI completely abolished deltorphin's antinociceptive effects (Improta and Broccardo, 1992). Surprisingly, DADLE was not antagonized by NTI in *Rana pipiens* and in fact the antinociceptive effect of DADLE appears to be potentiated by concurrent NTI as was seen with nor-BNI. Binding studies have

shown a potent antagonism of DADLE by NTI in mammalian tissue (Portoghese, *et al.*, 1988). However, rodent *in vivo* studies demonstrate inhibition of antinociception produced by DPDPE with i.s. administration of NTI but not inhibition of the antinociceptive effects of DADLE (Tiseo and Yaksh, 1993). The fact that DADLE is known to have cross-reactivity with the μ opioid receptor as well as the δ receptor may explain its profile of inhibition seen with the co-administration of NTI and nor-BNI in the amphibian (Corbett *et al.*, 1984).

The μ opioid receptor selective agonist, morphine but not DAMGO was significantly antagonized by i.s. co-administration with NTI. This is in contrast to that observed in most mammalian studies where no antagonism of i.t. morphine or DAMGO antinociception was seen even at the highest doses of i.t. NTI. (Calcagnetti and Holtzman, 1991; Drower, *et al.*, 1991; Malmberg and Yaksh, 1992). In addition, i.c.v. (Calcagnetti and Holtzman, 1991) or s.c. (Portoghese, *et al.*, 1988) administration of NTI did not affect the antinociceptive effects of morphine or DAMGO. In binding studies, NTI was unable antagonize DAMGO or morphine as evidenced by their 100 fold greater affinity constant values than for δ agonists (Portoghese, *et al.*, 1988). However, one study did observe antagonism of morphine without antagonism of DAMGO (Tiseo and Yaksh, 1993). The antagonism of morphine by NTI might be expected since morphine exhibits some selectivity for the δ opioid receptor as well as μ while DAMGO is more selective for the μ opioid receptor (Heyman *et al.*, 1987; Takemori and Portoghese, 1987). In the amphibian, the μ opioid agonist, fentanyl, was not antagonized by NTI and in fact its antinociceptive effect, like that of DADLE, appears to be potentiated. Fentanyl has not been examined with NTI in the mammal.

The effects of NTI with κ opioid agents has been examined to some extent in mammals. Binding studies as well as the s.c. administration of NTI have shown that NTI does not

antagonize the effects of U50488 (Portoghese, *et al.*, 1988; VonVoigtlander *et al.*, 1983). In the amphibian, the antinociceptive effects of U50488 and CI977 were attenuated by i.t. NTI but not the antinociceptive effects of GR89696.

In summary, NTI antagonized only the antinociceptive effects of δ opioid agonists without affecting the antinociceptive actions of μ or κ opioid agonists in mammals. These findings would imply that δ opioid agonists are mediating their analgesic effect by a different receptor mechanism than μ or κ opioid receptor agonists. However, in the amphibian a broader spectrum of antagonism was observed with inhibition of μ and κ opioid receptor mediated antinociceptive action as well as that of δ opioid receptor ligands. These results suggest that NTI, like β-FNA and nor-BNI, is a nonselective opioid antagonist in the amphibian. The inability of NTI to antagonize the effects of DAMGO, GR89696, fentanyl and DADLE may be explained by examining additional agonist doses.

4.5 Schild analysis

In order to clarify outlying data in selective antagonist studies, full dose-response curves for the agonists were generated to analyze the affinity of the antagonist, nor-BNI and its ability to antagonize the antinociceptive effects of selective opioid agonists. The Schild pA_2 values, determined from linear regression shown in Figure 16 and summarized in Table I, for fentanyl, DADLE and DSLET, were not significantly different from each other which may suggest that nor-BNI is acting at the same receptor site as each of the selective opioid agonists. This supports the intraspinal data with nor-BNI where this highly selective antagonist for κ opioid receptors in mammals blocks not only the antinociception produced by κ agents but also that of μ and δ opioids as is shown in Figure 11. Otherwise, nor-BNI may be exhibiting the same affinity for distinct

opioid receptors in the amphibian. GR89696 yielded insufficient dose-response curves for proper analysis. With regard to the potentiation of the antinociceptive effect produced by DADLE, it appears that higher concentrations of nor-BNI significantly suppress the antinociception of DADLE whereas 0.1 nmol/frog of nor-BNI shows an initial potentiation of DADLE's effect while DADLE at 100 nmol/frog seems to be suppressed by 0.1 nmol/frog nor-BNI (see Figure 14). This is in contrast to what was seen in Figure 11 where 100 nmol/frog DADLE was potentiated by 0.1 nmol/frog nor-BNI. Nonetheless, the Schild analysis of DADLE with nor-BNI illustrates the unique ability of nor-BNI at 0.1 nmol/frog to potentiate the antinociceptive effects of DADLE. It will be of interest to examine Schild plots for β-FNA and naltrindole especially since naltrindole shows the same potentiation of DADLE as well as fentanyl as is shown in Figure 9. Figure 12 depicts the Schild analysis of GR89696. 0.1 nmol/frog nor-BNI is the most effective at diminishing the antinociceptive effect of GR89696. The effect of increasing concentrations nor-BNI on DSLET is shown in Figure 15. DSLET is not blocked by 0.1 nmol/frog nor-BNI except at 0.3 nmol/frog DSLET. However, nor-BNI at 1 and 10 nmol/frog does appear to suppress DSLET antinociception. This is in accordance with Figure 11 where nor-BNI given i.s. at 0.1 nmol/frog had no effect on the antinociception produced by 3 nmol/frog DSLET.

4.6 Summary of selective antagonist studies

In mammalian studies, β-FNA, nor-BNI and naltrindole inhibit the antinociceptive effects of their respective μ, κ or δ opioid agonists without affecting the antinociceptive actions of the other selective agonists. These findings would imply that the different selective agonists mediate their analgesic effects through different receptor mechanisms.

However, in the amphibian, a wider range of antagonism was observed with these highly selective antagonists. Overall, the promiscuousness of these "selective" antagonists in the amphibian may be explained by their interaction with the same receptor or by their similar affinity for distinct receptors. Schild analysis of nor-BNI, fentanyl, DADLE and DSLET revealed similar pA_2 values, further suggesting similarity in binding affinity of nor-BNI at multiple sites or interaction at a single site. Moreover, binding studies with [^{3}H]-naloxone reveal nearly identical K_i values for the three selective antagonists which will be discussed in Section 4.9 and the K_D values of the three selective opioid agonists do not show a significant difference (see Section 4.12). Overall, it is undeniable that the opioid receptor system in the amphibian is unique. Determining the exact number and type of opioid receptors in the amphibian will require molecular cloning.

4.7 Radioligand binding studies

Major progress in understanding the action of opioids has been gained by correlating the molecular mechanisms, induced by opioid-receptor interactions, with the physiological effects observed in the whole animal. In comparing data from behavioral and radioligand binding studies, it is important to show that the relative potencies of the ligands correlate with their biological effects. In general, the *in vitro* affinity of opioids for receptor sites has been shown to closely parallel their pharmacological potency *in vivo* (Pert *et al.*, 1973; Pert and Snyder, 1973b; Pert and Snyder, 1973a). Additionally, species differences in the distribution and properties of opioid receptors in brain, either as a result of multiple receptor types (McLean *et al.*, 1987; Robson *et al.*, 1985) or subtypes of a receptor (Benyhe, *et al.*, 1990b; Benyhe, *et al.*, 1990a; Benyhe *et al.*, 1992; Nock *et al.*, 1988; Rothman *et al.*, 1992; Simon *et al.*, 1985; Wollemann *et al.*, 1993; Zukin, *et al.*,

1988) makes it difficult to compare opioid action between species *in vivo*. However, parameters obtained in radioligand binding studies reflect the isolation of drug and protein and appear to transcend species and function. Information on the receptor binding affinity and selectivity of a range of opioids provides a unique opportunity to evaluate and interpret physiological and behavioral parameters of opioid action in the amphibian.

4.8 [^{3}H]-Diprenorphine binding studies

[^{3}H]-Diprenorphine is a potent, nonselective opioid antagonist which has been shown to be a novel tool in the study of opioid receptor types in mammals. The binding profile of [^{3}H]-diprenorphine in *Rana pipiens* revealed saturable binding. Figure 18 depicts the saturation analysis for [^{3}H]-diprenorphine which yielded a K_D value of 0.65 nM and a B_{max} value of 287.7 fmol/mg protein. The K_D and B_{max} values for [^{3}H]-diprenorphine in *Rana pipiens* fall within the range of those seen with other amphibian studies examining [^{3}H]-diprenorphine binding (see Table III) as well as in other mammalian studies (Table II). In competition studies, increasing concentrations of unlabeled diprenorphine (0.1 nM – 1 μM) dose dependently displaced [^{3}H]-diprenorphine (1 nM) binding yielding a K_i of 0.22 nM as is illustrated in Figure 2. However, attempts to displace the binding of [^{3}H]-diprenorphine using other selective opioid agonists (fentanyl, morphine, DAMGO, DPDPE, CI977 & U50488) were unsuccessful in *Rana pipiens* (see Figure 20 with corresponding K_i values shown in Table IV). Other studies utilizing an amphibian model were able to demonstrate significant competition for binding of [^{3}H]-diprenorphine using the selective opioid agents, DAMGO and EKC (Brooks, *et al.*, 1994). Control studies were conducted to ensure that [^{3}H]-diprenorphine was binding solely to brain tissue

(Newman, *et al.*, 1999). Control studies performed in the absence of tissue demonstrated significant binding of [^{3}H]-diprenorphine to the filter which surprisingly was enhanced by 1 μM naltrexone. These results are illustrated in Figure 21 where different filter pretreatments were used to isolate the mechanism by which filter binding occurred. As is shown in the figure, soaking the filters in 0.3% PEI with 1 μM diprenorphine or 0.3% PEI with 1% BSA were the only treatments that appeared to reduce both total and nonspecific binding. However, a significant increase in the nonspecific portion was still observed. This increase in binding of [^{3}H]-diprenorphine to the filter was also seen with 1 μM naloxone but not with 1 μM levorphanol, morphine, β-FNA or naltrindole, as the nonspecific drug as is illustrated in Figure 22. Other researchers have demonstrated labeled opioid binding to glass fiber filters stereospecifically with selective levorotatory binding as in opioid receptors (Snyder *et al.*, 1975).

[^{3}H]-Naloxone does not exhibit the same filter binding profile as [^{3}H]-diprenorphine (shown in Figure 24) where total and nonspecific binding in the absence of tissue for [^{3}H]-naloxone are minimal. In addition unlabeled diprenorphine does not increase [^{3}H]-naloxone binding to the filter, also shown in Figure 24. This is interesting in light of the fact that [^{3}H]-diprenorphine filter binding is enhanced by unlabeled naloxone. This unidirectional phenomenon of increased filter binding by naloxone may reflect some unknown property in the labeling of [^{3}H]-naloxone or [^{3}H]-diprenorphine.

Specific binding was observed in the presence of tissue despite the absence of specific binding shown in control studies which occurred due to increased nonspecific binding to the filter. It is possible that binding to the filter does not occur in the presence of tissue. The drugs employed in these studies possess a high affinity for opioid receptors

and would be expected to interact with those receptors in the presence of tissue. This is highly probable since affinity and density data correlate with those observed in other experiments and saturable binding with [^{3}H]-diprenorphine was obtained. In conclusion, these observations suggest that the nonselective drug, levorphanol, would be a better agent than naltrexone or naloxone to define nonspecific binding when using [^{3}H]-diprenorphine as the labeling agent.

4.9 [^{3}H]-Naloxone binding affinity and density

Kinetic analysis of [^{3}H]-naloxone in *Rana pipiens* brain tissue resulted in a kinetically derived K_D value of 6.84 nM (Newman, *et al.*, 2000a). [^{3}H]-Naloxone binding was saturable in amphibian brain tissue, yielding an apparent K_D value of 7.11. The experimentally derived K_D value was similar to the apparent K_D value derived from kinetic analysis. Like [^{3}H]-diprenorphine, binding to a single site was seen in *Rana pipiens* brain tissue homogenates for [^{3}H]-naloxone (Newman, *et al.*, 1999). Additionally, examination of [^{3}H]-naloxone binding in CHO membranes transfected with human MOR1 yielded a K_D value of 4.41 nM and a B_{max} value of 911.5 fmol/mg tissue and displayed best fit to a single site (see Figure 34). Mammalian studies using brain homogenates show that [^{3}H]-naloxone binds to two sites (either 2 different affinity states or 2 or more separate receptor populations) with affinities ranging from 0.01 nM - 20 nM (Blurton, *et al.*, 1986; Jacobson and Wilkinson, 1984; Pert and Snyder, 1973a; Pollack and Wooten, 1987). Density data was also determined in amphibian brain tissue homogenates where the B_{max} was found to be 2170 fmol/mg protein. This B_{max} value should theoretically represent all opioid receptor populations since naloxone is nonselective in nature. This density value in *Rana pipiens* as well as values in other

amphibian species are higher than those in the mammal where B_{max} values range from 13–177 fmol/mg protein (Jacobson and Wilkinson, 1984; Schnittler, *et al.*, 1990; Szucs *et al.*, 1987). A higher B_{max} in the amphibian would be expected considering that the amphibian brain has been shown to contain higher amounts of total protein / mg tissue than mammalian brain. Higher levels of protein / mg tissue in the amphibian was also demonstrated at the level of G-protein-receptor interaction. As is shown in Figures 38 and 39, The amount of specific binding in the amphibian (Figure 39) is almost 7-fold higher than in rat nucleus accumbens (Figure 38).

Using the non-selective opioid antagonist, [^{3}H]-naloxone, opioid binding sites were also characterized in amphibian spinal cord (Newman, *et al.*, 2000b). As was done in amphibian brain tissue, competitive binding assays were done using selective opioid agonists and highly-selective opioid antagonists. Kinetic analyses of [^{3}H]-naloxone in amphibian spinal cord tissue homogenates yielded a K_D of 11.29 nM while the experimentally derived K_D value from saturation experiments was found to be 18.75 nM. Both kinetic and saturation analyses showed naloxone binding best to a single site. Density data were also determined from saturation analyses which yielded a B_{max} of 2725 fmol/mg.

4.10 Competitive binding with selective μ, κ, and δ opioid receptor ligands

At mammalian opioid receptor binding sites, naloxone preferentially interacts with expressed μ binding sites (K_D = 3.9 nM, cloned rat receptor) but also has significant affinity for κ opioid receptors (K_D = 16 nM, cloned rat receptor) and a lesser affinity for δ opioid receptors (K_D = 95 nM, cloned rat receptor) (Raynor, *et al.*, 1994; Satoh and Minami, 1995). [^{3}H]-Naloxone, through competition analysis, has been useful in the

determination of receptor affinities of type-selective opioids in mammals (Pfeiffer and Herz, 1982; Schnittler, *et al.*, 1990; Szucs, *et al.*, 1987). Unlabeled naloxone effectively displaced itself with a K_i of 3.92 nM. The K_i values for [^{3}H]-naloxone binding in frog brain ranged from 1.86 nM for bremazocine to 31 μM for deltorphin. As is shown in Table VII, δ receptor ligands and most κ receptor drugs were weak displacers of [^{3}H]-naloxone binding.

The competition constants (K_i) of unlabeled μ, κ and δ opioids in amphibian spinal cord ranged from 2.58 nM for bremazocine to 84 μM for deltorphin as is shown in Table X. As was seen in amphibian brain tissue, bremazocine yields the lowest K_i value in competition with [^{3}H]-naloxone versus all selective agonists tested.

The strong displacement of [^{3}H]-naloxone by bremazocine is interesting as it is classified as a κ selective agonist in mammalian studies (Horan *et al.*, 1993), but has been considered a nonselective antagonist in previous binding studies (Broadbear, *et al.*, 1994; Wood and Traynor, 1989). This potent displacement of [^{3}H]-naloxone by bremazocine was also seen in other amphibian binding studies using [^{3}H]-naloxone (Deviche *et al.*, 1990; Simon, *et al.*, 1984) as well as in this lab with CHO membrane preparations expressing the human μ opioid receptor where bremazocine yielded a K_i value of 1.52 nM (see Figure 35). Additionally, in behavioral studies, systemically administered bremazocine showed partial agonist/antagonist properties as it significantly blocked the antinociception produced by fentanyl following systemic administration (see Figure 36).

In examining average K_i values, the overall trend of binding in *Rana pipiens* shows an affinity series of $\mu > \kappa > \delta$. This affinity profile is consistent with the relative affinity of naloxone for μ, κ and δ receptors (Raynor, *et al.*, 1994; Satoh and Minami, 1995).

4.11 Competition binding with highly selective opioid receptor antagonists

The finding that naloxone bound to a single high affinity site in amphibian brain and that μ, κ and δ opioids could displace naloxone may be suggestive of a single type of opioid receptor binding site. To further test this hypothesis, selective opioid receptor antagonists were used. In mammals these highly selective μ, κ and δ antagonists affect the binding of opioid receptor agonists only at their respective receptors (Takemori, 1985; Takemori and Portoghese, 1992). As mentioned above, behavioral studies revealed a lack of selectivity of these antagonists in *Rana pipiens*. Interestingly, the three selective antagonists yielded nearly identical K_i values in *Rana pipiens* brain tissue (Figure 28, Table VIII). Overall, the selective antagonists have not been examined in a non-mammalian model. However, nor-binaltorphimine against [^{3}H]-naloxone was examined in another species of frog, *Rana esculenta*, which was shown to have an apparent K_i value of 3.1 nM and is nearly identical to that observed in the present studies in *Rana pipiens* where nor-BNI yielded a K_i value of 3.55 nM (Benyhe, *et al.*, 1990a; Benyhe, *et al.*, 1990b).

Highly selective antagonists for μ, κ and δ opioid receptors yielded similar K_i values (5.37 nM – 31.1 nM) against [^{3}H]-naloxone in competition studies in amphibian spinal cord (Newman *et al.*, 2000b). These studies are the first to examine opioid binding in amphibian spinal cord. In conjunction with previous behavioral and binding data, these results suggest that non-mammalian vertebrates express a unique opioid receptor which mediates the action of selective μ, κ and δ opioid agonists. A correlation plot was compiled to compare the K_i values between brain and spinal cord of selective opioid agonists against [^{3}H]-naloxone. Figure 32 depicts this correlation where an r^2 value of

0.786 was obtained. It is interesting to note that the peptides and alkaloid compounds appear to be demarcated where the peptides are located at high K_i for both brain and spinal cord. This may indicate that peptides are not as effective at inhibiting the binding of labeled ligands. However, this is unlikely because these same peptides have been shown to elicit potent and sustained analgesia in the amphibian. The lack of robust competition with the opioid peptides may be due to the absence of peptidase inhibitors in the assay procedure to prevent breakdown. Alternatively, peptides and alkaloid opioids may bind to different sites on opioid receptors, as was previously suggested (Ward, *et al.*, 1986, Kong, *et al.*, 1994).

K_i values for the selective antagonists in mammals have been determined using selective ligands for the μ, κ and δ cloned opioid receptors. In cell lines expressing the μ opioid receptor, selective antagonists had K_i values of 0.33 (β-FNA), 2.2 (nor-BNI) and 64 nM (NTI). At κ receptors, K_i values were 2.8 (β-FNA), 0.027 (nor-BNI) and 66 nM (NTI) and for δ receptors, 48 (β-FNA), 65 (nor-BNI) and 0.02 nM (NTI). Thus, unlike the amphibian, each selective antagonist possesses a high affinity binding to its respective receptor and a much lesser affinity for the other two opioid receptors in mammals (Raynor, *et al.*, 1994). The similar affinities of the selective antagonists in *Rana pipiens* in their displacement of [^{3}H]-naloxone would suggest that β-FNA, NTI, and nor-BNI may not bind to separate sites. This similarity in K_D values, together with the linear transformation of the binding data, may suggest binding to a single, noninteractive site but does not rule out binding to several different sites with similar affinity. However, analysis of the data show a best fit to a single site as indicated by the F test. The present results, together with the previous behavioral data (see above) suggest that opioids may

act on a single receptor binding site in amphibians, that has been termed the unireceptor (Stevens and Newman, 1999). This may be the case since the amphibian has a much simpler nervous system and thus may be expected to have a simpler organization of receptors. Additionally, the endogenous opioid ligands have been shown to be the same in all vertebrates throughout the animal kingdom (Pert, *et al.*, 1974). Thus, opioid receptors may be similar across various species due to the need for a good steric fit. This similarity between species provides a feasible explanation for the similar potency seen between mammals and amphibians as well as for the obvious receptor differences observed in behavioral selective antagonist studies. A useful analysis to determine the exact number and types of opioid receptors in the amphibian would be binding analysis with highly selective radiolabeled opioid antagonists. Attempts were made in the course of these studies to characterized these selective antagonists in the amphibian. However as shown in Figure 41, saturable binding was not obtained with either the μ selective antagonist, β-FNA or with the δ selective antagonist, naltrindole. Problems with the saturation of these selective antagonists has been encountered in other studies (INRC-L discussion list, personal communications). However, assay conditions were validated with rat nucleus accumbens tissue in that saturable binding was obtained wherein a K_D value of 7.8 nM and a B_{max} of 4156 fmol/mg protein were determined.

4.12 Radioligand binding with selective agonists

The availability of the highly receptor selective opioid ligands, [^{3}H]-DAMGO (Gillan and Kosterlitz, 1982), [^{3}H]-DPDPE (Akiyama, *et al.*, 1985) and [^{3}H]-U65953 (Kim, *et al.*, 1996; Lahti, *et al.*, 1985), has made possible the characterization of opioids selectively interacting with μ, κ and δ opioid receptors. These studies involve

examination of nine frequently used opioids to determine their binding selectivity. Kinetic analysis of [^{3}H]-DAMGO, [^{3}H]-U65953 and [^{3}H]-DPDPE yielded K_D values that were in the same order of magnitude as the experimentally derived K_D values obtained from saturation analysis. Both kinetic and saturation analyses revealed a best fit to a single site as determined by the F-ratio test. Studies with the cloned μ, κ and δ opioid receptors also showed binding to a single site with [^{3}H]-DAMGO, [^{3}H]-U65953 and [^{3}H]-naltrindole (Raynor, *et al.*, 1994) as did binding of [^{3}H]-DAMGO, [^{3}H]-U65953 and [^{3}H]-DPDPE for μ, κ and δ receptors expressed in neuroblastoma cells (Standifer, *et al.*, 1994a). The affinity value for [^{3}H]-DAMGO was found to be 10.81 nM while that of [^{3}H]-DPDPE for the amphibian opioid receptor was 3.54 nM. The apparent K_D value for [^{3}H]- U65953 (20.76 nM) was similar to that seen in *Rana esculenta* which yielded a K_D value of 15.3 nM (Benyhe, *et al.*, 1992).

Density values in *Rana pipiens* were calculated for [^{3}H]-DAMGO (311.6 fmol/mg protein), [^{3}H]-U65953 (184.1 fmol/mg protein) and [^{3}H]-DPDPE (425.2 fmol/mg protein). The fact that [^{3}H]-U65953 showed the lowest density value is surprising considering that many researchers have deemed the amphibian opioid receptor "κ-like" (Benyhe, *et al.*, 1990a; Benyhe, *et al.*, 1990b; Mollereau, *et al.*, 1988a; Simon, *et al.*, 1982). Other studies have shown [^{3}H]-U65953 to have a B_{max} value of 258 ± 41 fmol/mg protein (Benyhe, *et al.*, 1992) which falls within the 95% confidence interval in *Rana pipiens* for [^{3}H]-U65953 (see Table XII). Theoretically, the sum of the B_{max} values for the three radiolabeled selective agonists should approximate the B_{max} obtained with a radiolabeled nonselective ligand. The sum of B_{max} values for [^{3}H]-DAMGO, [^{3}H]-U65953 and [^{3}H]-DPDPE equals 920.9 fmol/mg protein. B_{max} values for the nonselective

opioids, [^{3}H]- diprenorphine (287.7 fmol/mg protein) and [^{3}H]- naloxone (2170 fmol/mg protein) are not similar to the summed B_{max} values (see Table XIV). Other studies in the mammal have shown similarities between the B_{max} value of [^{3}H]-diprenorphine (383 fmol/mg protein) and the sum of the B_{max} values in selective binding assays (370 fmol/mg protein) (Niwa, *et al.*, 1994; Standifer, *et al.*, 1994a). The results of kinetic and saturation analysis of selective agonists in *Rana pipiens* are summarized in Table XII.

Competition analyses of selective opioid agonists for the selective radiolabeled agonists revealed similarities to mammalian studies. That is, among the μ, κ and δ opioids tested against [^{3}H]-DAMGO, only those with established μ opioid actions effectively blocked the binding of [^{3}H]-DAMGO where DAMGO itself yielded a K_i value of 12.2 nM. Morphine and β-FNA had Ki values of 2.68 nM and 2.91 nM, respectively. The highly selective antagonists for μ, κ and δ opioid receptors yielded K_i values ranging from 2.91 nM for β-FNA to 13 μM for naltrindole against [^{3}H]-DAMGO in competition studies. Among the μ, κ and δ opioids tested against [^{3}H]-U65953, only those with noted κ opioid actions effectively blocked the binding of [^{3}H]-U65953 where U65953 itself yielded a K_i value of 7.2 nM. CI977 and nor-BNI had Ki values of 6.2 nM and 0.62 nM, respectively. Highly selective antagonists for μ, κ and δ opioid receptors yielded K_i values ranging from 0.62 nM for nor-BNI to 6 μM for β-FNA against [^{3}H]-U65953 in competition studies. Among the μ, κ and δ opioids examined with [^{3}H]-DPDPE, only those with established δ opioid actions effectively blocked the binding of [^{3}H]-DPDPE where DPDPE itself yielded a K_i value of 5.67 nM. Deltorphin and naltrindole had Ki values of 3.65 nM and 2.48 nM, respectively. Highly selective

antagonists for μ, κ and δ opioid receptors yielded K_i values ranging from 2.48 nM for NTI to 180 nM for nor-BNI against [^{3}H]-DPDPE in competition studies.

A summary of a comparison among species of K_D and B_{max} values for the radiolabeled selective agonists is shown in Table XVI. The B_{max} values among the different species varied considerably for the three agonists while the apparent K_D values were very similar for [^{3}H]-DPDPE and differ approximately an order of magnitude from *Rana pipiens* for [^{3}H]-U65953 and [^{3}H]-DPDPE. The selectivity profiles for [^{3}H]-DAMGO, [^{3}H]-U65953 and [^{3}H]-DPDPE were determined in *Rana pipiens* brain tissue to study the organization of receptors. The K_i values of selective opioid agonists and antagonists are summarized in Table XIII with comparisons to other species shown in Table XVII. A direct comparison between the K_i values of selective opioid agonists and antagonists for the Rhesus monkey and *Rana pipiens* was made and is depicted in a correlation plot in Figure 55. The brain cortex of the Rhesus monkey is unique in that it contains μ, κ and δ opioid receptors which are found in similar proportions (Mansour *et al.*, 1988). [^{3}H]-U65953 showed near perfect correlation of K_i values between the Rhesus monkey and *Rana pipiens* yielding an r^2 value of 0.9465. [^{3}H]-DPDPE and [^{3}H]-DAMGO had r^2 values of 0.6810 and 0.4818, respectively. Only the correlations for [^{3}H]-U65953 ($p < 0.0001$) and [^{3}H]-DPDPE ($p = 0.0105$) were significant. The fact that [^{3}H]-U65953 showed the highest correlation is logical in that researchers have shown the amphibian opioid receptor to be "κ-like" and thus it would be expected to resemble the [^{3}H]-U65953 selective profile of inhibition. One strategy to compare the selectivity profile of these highly selective agonists is to compile a selectivity index which is expressed as the ratio of selective opioid K_i values in displacing two selective

radiolabeled ligands, for example: [^{3}H]-DAMGO/[^{3}H]-U65953 (selectivity:μ/κ), [^{3}H]-DAMGO/[^{3}H]-DPDPE (selectivity:μ/δ), [^{3}H]-DPDPE/[^{3}H]-U65953 (selectivity:δ/κ), etc. In the μ series the most remarkable finding was the μ selectivity of β-FNA which was higher compared to κ binding (>2000-fold) but was only 33-fold higher relative to δ binding. This is interesting considering that in mammals, β-FNA is known to have κ agonist action while in the amphibian no κ agonist action was seen for β-FNA. However, no agonist action of β-FNA has been observed in the amphibian. Indeed, β-FNA shows the greatest inhibition of [^{3}H]-DAMGO in selective agonist studies over [^{3}H]-U65953 and [^{3}H]-DPDPE as is shown in Figure 46 and Table XIII. DAMGO displays similar selectivity profiles with respect to δ (216) and κ (128) binding. Morphine shows a dramatic 5000-fold higher μ selectivity relative to κ and is only 6-fold that of δ binding. This makes sense considering morphine is shown to be somewhat δ selective in mammals. Examining the κ series of selectivity, U65953 exhibits κ selectivity of 387- and 2332-fold compared with its affinity at κ and δ sites. CI977 shows 700-fold selectivity for κ binding relative to δ and 70-fold the selectivity compared to μ binding. A δ selectivity profile was also done and most notable is naltrindole which shows 5000-fold selectivity for δ relative to μ and only 2.83-fold for δ compared to κ. Overall, as was observed in the Rhesus monkey, each selective agonist shows superior selectivity for its own opioid receptor relative to the other two opioid receptors. Additionally the κ and δ opioids are in general agreement with the selectivity of opioids in monkey cortex as is depicted in Figure 55.

The characterization of the binding affinity, density and receptor selectivity of [^{3}H]-DAMGO, [^{3}H]- U65953 and [^{3}H]-DPDPE in *Rana pipiens* brain tissue, for which much data exists on the spectrum of action of opioids in this model *in vivo*, contributes to the assessment of opioid ligand binding at opioid receptors as the primary process underlying opioid function in the amphibian. Whether these opioid binding sites in the amphibian are separate, distinct receptors or simply different sites on one unique receptor remains to be elucidated through the use of molecular cloning techniques.

4.13 The Unireceptor hypothesis of opioid antinociception in the amphibian

Obvious receptor differences between the mammal and amphibian exist even though potency data have been shown to be similar for the three selective agonists. Receptor differences have been shown to exist even within the mammalian species. For example, the differences in analgesia observed in mammals among the different opioid receptors has been attributed in several studies to different associations of opioid receptors for distinct stimuli where spinally administered μ and δ opioids are able to produce analgesia in thermal, noxious stimuli, while spinal μ and κ opioid agonists are effective against visceral-chemical, noxious stimuli (Hunter *et al.*, 1990; Porreca *et al.*, 1987; Schmauss and Yaksh, 1984; Stevens and Yaksh, 1986). Other studies have shown a possible species difference for the actions of κ opioids against thermal stimuli where spinal administration of κ opioids produces analgesia in mice (Piercey *et al.*, 1982) but has no effect in rats (Leighton *et al.*, 1988). Species differences in the binding characteristics of some opioids has been shown even among rodents. Large variations in the density of μ, κ and δ opioid receptors were shown in the subregions of the hippocampus of squirrel, guinea pig, hamster and rat (McLean, *et al.*, 1987). Additionally the affinity and density of opioid binding sites selective

for DPDPE was significantly greater in brain homogenates of mouse than in the rat. This *in vitro* observation paralleled those measured as dose-responses of antinociception in the two species (Yoburn *et al.*, 1991).

The best feasible explanation for the data presented herein to date is that the simpler nervous system of the amphibian possesses one unique opioid receptor that recognizes and mediates the antinociception of all types of selective opioids. This concept has been hypothesized for receptors in phylogenetically lower vertebrates and has been termed a universal or primordial receptor (Moyle *et al.*, 1994).

As shown in Figure 56, it is hypothesized that phylogenetically lower vertebrates contain an opioid receptor which can bind μ, κ and δ ligands. Through gene duplication and mutations which block two of the three sites, selective and distinct μ, κ and δ opioid receptors exist in mammals. This mechanism has been implicated for peptide hormone receptors and was termed negative determination (Moyle, *et al.*, 1994). Further support of such a mechanism can be found in the data of site directed mutagenesis studies where mutations in the extracellular loops of opioid receptors show dramatic effects in the binding of their respective ligands (Metzger and Ferguson, 1995). Specifically, mutation of the first extracellular loop (EC1) decreases the binding of μ opioid agents (Wang, *et al.*, 1995), mutation in the second extracellular loop (EC2) diminishes κ ligand binding (Metzger and Ferguson, 1995) and interfering with the amino acid sequence in extracellular loop 3 (EC3) decreases δ opioid binding (Varga, *et al.*, 1996). Thus, the unireceptor model supports the behavioral data where highly selective opioid antagonists were not selective in amphibians (Stevens and Newman, 1999). For example, it is thought that β-FNA binds to the μ site on the unireceptor and, due to steric hindrance,

also blocks the binding of κ and δ selective agonists. The same may occur with naltrindole and nor-BNI, explaining their similar nonselectivity in amphibians. The present binding data also supports the

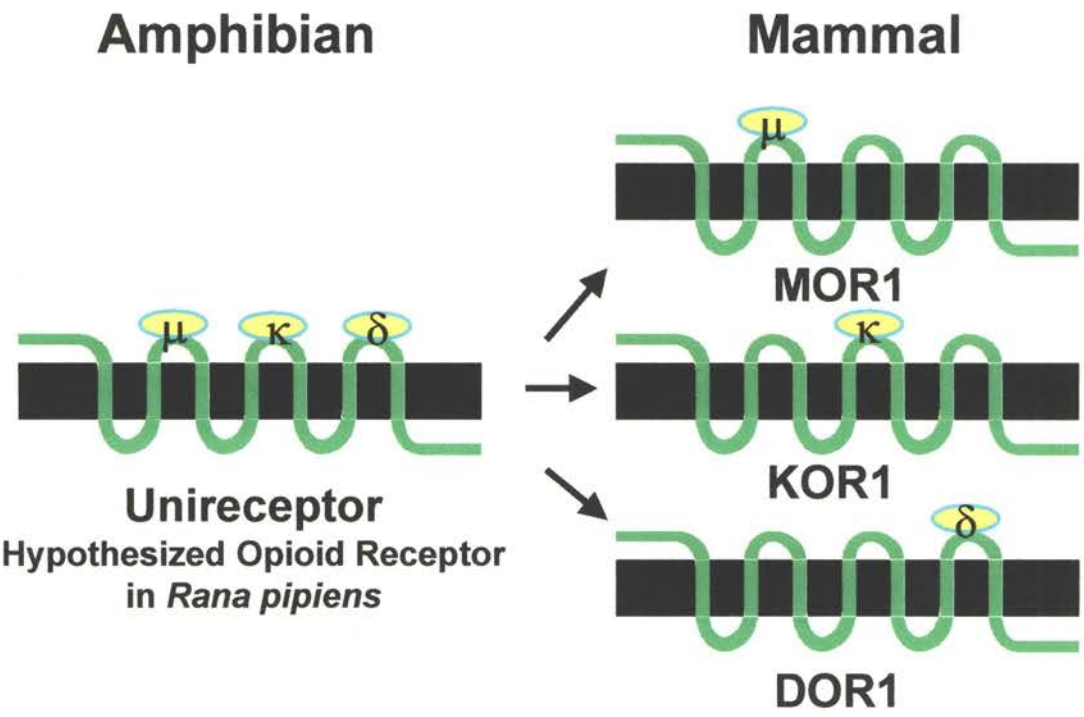


Figure 56. Schematic of the Unireceptor, the hypothesized opioid receptor in the amphibian. This hypothesis posits that whereas the mammal posseses three distinct opioid receptors for μ, κ and δ opioid receptors, antinociception in the amphibian is thought to occur through the interaction of μ, κ and δ opioids at one unique receptor.

unireceptor model by [^{3}H]-naloxone binding to a single site, and the nearly identical potency of β-FNA, nor-BNI and naltrindole in blocking naloxone binding in amphibian brain and spinal cord tissue homogenates. That the binding of all opioids tested in these studies show binding to a single site is significant in that the use of agonist ligands for the characterization of G-protein coupled receptors by radioligand binding studies is complicated by their selectivity for high affinity (G-protein couple) states of a single receptor that cannot be easily distinguished from receptor multiplicity (Molinoff *et al.*, 1981). If the unireceptor model is correct, conservation of the μ, κ and δ sites throughout vertebrate phylogenesis would also suggest conservation of the "molecular efficacy" of selective agonists interacting at those sites. Thus, μ opioids have relatively similar intrinsic activity or efficacy at the μ site on the unireceptor and the μ opioid receptor in mammals, δ opioids relatively less efficacy at their respective sites in amphibians and mammals and κ opioids the least efficacious.

Initial studies in the amphibian revealed that the relative antinociceptive potency of μ, κ and δ agents between *Rana pipiens* and mammals was highly correlated, regardless of the route of administration, indicating some degree of similarity between the receptor proteins (Stevens, 1996a). Surprisingly, results of behavioral studies employing selective antagonists in the amphibian suggest the presence of a single opioid binding site which recognizes μ, κ and δ opioid ligands (Stevens and Newman, 1999). Thus, the less complex nervous system of the amphibian may possess a simpler opioid receptor organization where all three opioid agonists recognize the same receptor but the receptor protein itself is not necessarily different, hence the similar relative potencies of the opioid ligands. Most importantly, the unireceptor model can explain the surprisingly high

correlation between the relative antinociceptive potency of selective μ, κ and δ opioid agents in amphibians and mammals.

4.14 Future directions

4.14.1 GTPγS^{35} binding. The beginning of the intracellular cascade leading to the physiological effects of opioids can be investigated using GTPγS^{35}. As mentioned earlier, GTPγS^{35} binding is indicative of G-protein activation which occurs upon agonist binding to the receptor. Thus, a measure of not only the binding of the opioid (affinity) can be obtained but also the efficacy that the opioid imparts. Attempts were made in these studies to examine GTPγS^{35} binding in *Rana pipiens*. However, problems with optimization of conditions were encountered such that GTPγS^{35} was not effectively stimulated and an antagonist was not able to significantly inhibit binding from basal in *Rana pipiens*. The details of steps to optimize conditions for GTPγS^{35} binding are explained in Section 3.4.2. Examination of GTPγS^{35} in rat nucleus accumbens validated initial assay conditions where stimulation by morphine was observed and this stimulation in turn was inhibited by 1 μM naltrexone as is shown in Figure 38. However, these same conditions in *Rana pipiens* did not produce as ideal results as is shown in Figure 39. The increase in GDP concentration from 50 μM to 300 μM yielded more significant results as is shown in Figure 40 with 5 μM morphine as the stimulating drug but not with fentanyl (5 μM). Thus further optimization of the conditions for GTPγS^{35} binding in the amphibian needs to be done in order to take advantage of this valuable analytical tool.

4.14.2. Antisense strategies. The physiological and pharmacological significance of opioid receptors can be evaluated by antisense oligonucleotide strategy. These powerful selective molecular pharmacological tools can be employed to test the underlying

hypothesis proposed herein. If a single binding site that recognizes μ, κ and δ opioid receptors does indeed exist then the proposed "knock-down" of this receptor would eliminate all antinociception produced by these agents. This technique is useful for the characterization of opioid drug selectivity. An antisense strand is complementary to the mRNA of a target protein. The antisense strand inhibits the synthesis of receptor protein by interference with information flow from gene to protein, blocking transcription (Wahlestedt, 1994). Spinal injection of antisense oligonucleotides can affect the expression of specific receptor proteins *in vivo* altering the pharmacology of a target response. This approach is particularly useful for the study of receptors for which there are no selective ligands. This concept is especially intriguing in light of the fact that no ligand tested in behavioral studies so far appears to be selective in the amphibian. Injection (icv, i.t.) of antisense oligodeoxynucleotides, using mismatch or sense strands as the control, against cloned opioid receptors (μ, κ and δ) has abolished the antinociceptive effects of agents for these receptors. Intrathecal or i.c.v. injection of antisense oligonucleotides have been shown to selectively block the antinociceptive effects of opioid agonists selective for the same receptors without altering the action of opioids acting at other receptors. This effect is prominent after 24 hours and diminished at about 5 days (Knapp *et al.*, 1995). In addition, treatment of NG 108-15 cells with δ opioid receptor antisense oligonucleotide significantly reduced δ receptor density as measured by radioligand binding experiments. This would be expected in the inhibition of δ receptor transcription. Other studies employing δ receptor antisense oligonucleotides *in vivo* include i.c.v. administration of δ receptor antisense oligonucleotides which selectively blocked i.c.v. administration of deltorphin (Lai *et al.*,

1994). Another group observed selective loss of i.t. DPDPE analgesia with intrathecal δ antisense oligonucleotides but not with the i.t. administration of the μ agonist, DAMGO, or the κ agonist, U50488 (Standifer *et al.*, 1994b). μ antisense oligonucleotides have also been examined *in vivo* using where injection into PAG blocked the antinociception produced by morphine (Rossi *et al.*, 1994). κ antisense oligonucleotides have likewise been studied where the i.t. administration of κ antisense oligonucleotides blocked the antinociceptive effects of U50488H (Adams *et al.*, 1994; Chien *et al.*, 1994). The previous studies used either sense or mismatch strands as the control. Treatment with either of these strands exhibited no effect on the antinociceptive actions of opioid agents. The application of DNA antisense oligonucleotide neuropharmacology in *Rana pipiens* is a reality now that a unique opioid receptor-like nucleotide sequence from *Rana pipiens* has been partially cloned in the lab of Dr. Sri Nagalla. This proposed technique will produce the first "knock-down" model of a neurotransmitter receptor in a non-mammalian species. From the *Rana pipiens* oprc1 partial clone provided by Dr. Nagalla, base pairs 20 - 35 yield the antisense oligonucleotide as: 5'- GCGTGAAGGTGCTGG-3'. The sense oligonucleotide (5'- CCAGCACCTTCACGC-3') or a missense strand (replacing 3-4 bases) will be used as a control. These sequences have no known homology to any Genbank listed clones as determined by a search. Both sense and control oligonucleotides will be injected intraspinally as well as μ, κ, and δ opioids administered at 24 hours to determine the effects if any of decreasing the translation of the *Rana pipiens* oprc1. The oprc1 clone is the major opioid receptor-like sequence expressed in amphibian CNS. This method of opioid receptor "knock-down" has shown the loss of functional opioid receptors and antinociception produced by the respective

opioid agonist in cell lines and rodents (Lai *et al.*, 1997; Pasternak and Standifer, 1995). In addition, amphibian CNS tissue homogenates can be analyzed through binding assays following *in vivo* receptor "knock down". In attempts to show the presence of a single opioid receptor by "knock-down" with the proposed sense strand, a potential hitch would arise if multiple receptors exist in the amphibian which contain the complementary bases of the antisense strand. This would produce a "knock-down" of potentially all receptors and would not clearly identify the presence of a single opioid receptor. After "knock-down" with the sense strand, if agonists still elicit antinociception while other types do not, this would provide evidence for multiple opioid receptors. Thus, this proposed technique will be potentially more useful in rejecting the single site hypothesis than supporting it. A full clone of the *Rana pipiens* opioid receptor is needed to definitively study this hypothesis and to accurately determine the number and type of opioid receptors in the amphibian. Cloning will facilitate the development of novel compounds for use in further detailed structural analysis of the receptors for new clinically useful opioids. Work is in progress to clone the opioid receptor(s) in *Rana pipiens* and the opioid receptors in other non-mammalian species are being examined. Partial sequences of three different members of the opioid receptor family in the frog genome as well as two members in the hagfish genome have been identified by PCR technology (Li *et al.*, 1995). Additionally, in *Catostomus commersoni*, a teleost fish, the cDNA for the μ opioid receptor as well as five partial clones that encode distinct opioid receptor-like proteins have been cloned and seem to most closely resemble mammalian opioid receptors (Darlison, *et al.*, 1997). It is important to note that these sequences are only

partial clones and therefore do not validate the presence of multiple receptor types in non-mammals.

In mammals, before the much celebrated cloning of the opioid receptors, distinct binding and activity profiles of highly selective ligands, variable sensitivity to naloxone antagonism and selective protection and inactivation of opioid receptors strongly suggested that μ, κ and δ receptors represented discrete molecular entities. The lack of a distinct binding profile and variable sensitivity for [^{3}H]-naloxone would suggest that the amphibian may not have distinct sites of opioid action but perhaps one unique site that has been termed the unireceptor. The use of a phylogenetically lower species is not only ethically appealing but also the availability of a species with a limited number of opioid binding site types may provide a method for more extensive investigations of these binding subtypes. Deciphering the organization of receptors in the amphibian is important in that science still lacks the knowledge to harness the unique pain relieving potential of opioids without the complication of their addictive and sometimes lethal side effects. Clarification of the physiological and pharmacological roles of opioid receptors will be important for the development of novel analgesics more selective for specific conditions.

Chapter V

SUMMARY

These studies comprehensively employed a well-established behavioral assay using selective antagonists to examine the properties of opioid receptors in the amphibian and indeed the results of these experiments allude to the working hypothesis which suggests the presence of a single type of opioid receptor in the amphibian that has been termed the unireceptor. Schild analyses of fentanyl, DADLE and DSLET show identical pA_2 values for nor-BNI, further suggesting interaction of this highly selective κ antagonist at a single site although it has not been ruled out that these agents may act at multiple sites with similar affinity. Additionally, complementary radioligand binding studies using [^{3}H]-naloxone in both amphibian brain and spinal cord tissue homogenates show nearly identical K_i values for the three highly selective antagonists, β-FNA (μ), nor-BNI (κ) and NTI (δ). Radiolabeled selective agonist analyses show that, with agonists selective for μ, κ and δ opioid receptors, each has a higher affinity for its own recognition site however the K_D values exhibited at their respective receptors are not significantly different. Together these behavioral and pharmacological data undeniably point to the mediation of antinociception in amphibians via interaction at a unique opioid receptor. The simplicity of the amphibian nervous system as well as the incessant suggestion of binding of nonselective and selective opioid ligands to a single site imply that a single receptor with

multiple ligand recognition sites that mediates the antinociceptive effects of μ, κ and δ opioids in amphibians.

It is interesting to note that our opioid agonist data, both behavioral and binding, strongly resembles mammalian data. That is, the relative antinociceptive potencies of μ, κ and δ opioids in the amphibian is highly correlated to that of the mammal. Moreover, competition studies with [^{3}H]-naloxone show that μ agonists compete best with [^{3}H]-naloxone followed by κ and then δ which is expected based on the affinities of naloxone for μ, κ and δ receptors. And finally, in competition studies with highly selective agonists, only competitors with similar selectivities block their respective radiolabeled selective agonist. Thus, notable differences between the opioid receptors and intracellular mechanisms between amphibians and mammals is improbable. However, selective antagonist data, both behavioral and binding, show considerable differences between the amphibian and the mammal. This leads to the Unireceptor hypothesis as the best possible explanation for these data. The Unireceptor hypothesis supports the similar antinociceptive potencies between mammals and amphibians but also the obvious receptor differences highlighted by selective antagonist data. Additionally, the simpler nervous system of the amphibian supports the notion that a simpler receptor organization such as a single receptor mediating the effects of multiple ligands exists. Only molecular techniques will decipher whether these selective agonists are interacting with three distinct receptors or with three different sites on one unique opioid receptor in the amphibian.

REFERENCES

Abood, M. E., Law, P-Y., and Loh, H. H. Pertussis toxin treatment modifies opiate action in the rat brain striatum. (1985) *Biochem. and Biophys. Res. Comm.* **127:**477-483.

Adams JU, Chen X, DeRiel JK, Adler MW, and Liu-Chen LY (1994) Intracerebroventricular treatment with an antisense oligodeoxynucleotide to κ-opioid receptors inhibited κ-agonist-induced analgesia in rats. *Brain Res.* **667**:129-132.

Adrian ED (1926) The impulses produced by sensory nerve endings. Part 4: impulses from pain receptors. *J. Physiol.* **62**:33-51.

Akil, H., Mayer, D. J., and Liebeskind, J. C. (1974) Antagonism of stimulation-produced analgesia by naloxone, a narcotic antagonist. *Science* **191**:961-962.

Akiyama K, Gee KW, Mosberg HI, Hruby VJ, and Yamamura HI (1985) Characterization of [^{3}H][2-D-penicillamine, 5-D-penicillamine]-enkephalin binding to delta receptors in the rat brain and neuroblastoma-glioma hybrid cell line (NG 108-15). *Proc. Natl. Acad. Sci. U.S.A.* **82**:2543-2547.

Arunlakshana O and Schild HO (1959) Some quantitative uses of drug antagonists. *Br. J. Pharmacol.* **14**:48-58.

Barnard EA and Demoliou-Mason C (1983) Molecular properties of opioid receptors. *Br. Med. Bull.* **39**:37-45.

Baumhaker Y, Wollman Y, Goldstein MN, and Sarne Y (1993) Evidence for mu-, delta- and kappa-opioid receptors in a human neuroblastoma cell line. *Life Sci.* **52**:PL-205-PL-210.

Beckett, A. H. and Casy, A. F. (1954) Synthetic analgesics: stereochemical considerations. *J. Pharm. Pharmacol.* **6**:986-1001.

Beckett, A. H., Casy, A. F., Harper, N. J., and Phillips, P. M. (1956) Analgesics and their antagonists: some steric and chemical considerations. The influence of the basic group on biological response. *J. Pharm. Pharmacol.* **8**:874-889.

Benyhe S, Szucs M, Borsodi A, and Wollemann M (1992) Species differences in the stereoselectivity of kappa opioid binding sites for [^{3}H]U-69593 and [^{3}H] ethylketocyclazocine. *Life Sci.* **51**:1647-1655.

Benyhe S, Varga E, Borsodi A, and Wollemann M (1990b) The occurrence of different kappa opioid receptors (K_1 and K_2) in frog (*Rana esculenta*) brain membranes. *Acta. Physiologica. Hungarica.* **76**:291-294.

Benyhe S, Varga E, Hepp J, Magyar A, Borsodi A, and Wollemann M (1990a) Characterization of kappa1 and kappa2 opioid binding sites in frog (*Rana esculenta*) brain membrane. *Neurochem. Res.* **15**:899-904.

Blurton PA, Broadhurst AM, Wood MD, and Wyllie MG (1986) Is there a common, high-affinity opioid binding site in rat brain? *J. Recept. Res.* **6**:85-93.

Bodnar, R. J., Paul, D., Rosenblum, M., Liu, L., and Pasternak, G. W. (1990) Blockade of morphine analgesia by both pertussis and cholera toxins in the periaqueductal gray and locus coeruleus. **529**:324-328.

Bradbury, A. F., Smyth, D. G., Snell, C. R., Birdsall, N. J. M., and Hulme, E. C. (1976) C fragment of lipotropin has a high affinity for brain opiate receptors. *Nature* **260**:793-795.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* **72**:248-54.

Brenner GM, Klopp AJ, Deason LL, and Stevens CW (1994) Analgesic potency of alpha adrenergic agents after systemic administration in amphibians. *J. Pharmacol. Exp. Ther.* **270**:540-545.

Broadbear JH, Negus SS, Butelman ER, De Costa BR, and Woods JH (1994) Differential effects of systemically administered nor-binaltorphimine (nor-BNI) on kappa-opioid agonists in the mouse writhing assay. *Psychopharmacology* **115**:311-319.

Brooks AI, Standifer KM, Cheng J, Ciszewska GR, and Pasternak GW (1994) Opioid binding in giant toad and goldfish brain. *Receptor* **4**:55-62.

Buatti MC and Pasternak GW (1981) Multiple opiate receptors: phylogenetic differences. *Brain Res.* **218**:400-405.

Butelman ER, Negus SS, Al Y, De Costa BR, and Woods JH (1993) Kappa opioid antagonist effects of systemically administered nor-binaltorphimine in a thermal

antinociception assay in Rhesus monkeys. *J. Pharmacol. Exp. Ther.* **267**:1269-1276.

Calcagnetti DJ and Holtzman SG (1991) Delta opioid antagonist, naltrindole, selectively blocks analgesia induced by DPDPE but not DAGO or morphine. *Pharmacol. Biochem. Behav.* **38**:185-190.

Chang KJ, Hazum E, and Cuatrecasas P (1981) Novel opiate binding sites selective for benzomorphan drugs. *Proc. Natl. Acad. Sci. U.S.A.* **78**:4141-4145.

Chavkin C, James IF, and Goldstein A (1982) Dynorphin is a specific endogenous ligand of the kappa opioid receptor. *Science* **215**:413-415.

Chen Y, Mestek A, Liu J, Hurley J, and Yu L (1993b) Molecular cloning and functional expression of a mu-opioid receptor from rat brain. *Mol. Pharmacol.* **44**:8-12.

Chen, Y., Mestek, A., Liu, J., and Yu, L. (1993) Molecular cloning of a rat kappa opioid receptor reveals sequence similarities to the mu and delta opioid receptors. *Biochem. J.* **295**:625-628.

Cheng Y-C and Prusoff WH (1973) Relationship between the inhibition constant (K_I) and the concentration of inhibitor which causes 50 percent inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099-3108.

Chien C-C, Brown G, Pan YX, and Pasternak GW (1994) Blockade of U50488H analgesia by antisense oligoeoxynucleotides to a κ-opioid receptor. *Eur. J. Pharmacol.* **253**:R7-R8.

Childers SR (1991) Opioid receptor-coupled second messenger systems. *Life Sci.* **48**:1990-2003.

Clark MJ, Carter BD, and Medzihradsky F (1988) Selectivity of ligand binding to opioid receptors in brain membranes from the rat, monkey and guinea pig. *Eur. J. Pharmacol.* **148**:343-351.

Corbett, A. D., Gillan, M. G. C., Kosterlitz, H. W., Mcknight, A. T., Paterson, S. J., and Robson, L. E. (1984) Selectivities of opioid peptide analogues as agonists and antagonists at the delta-receptor. *Br. J. Pharmacol.* 83: 271-279.

Costa, T., Aktories, K., Schultz, G., and Wuster, M. (1983) Pertussis toxin decreases opiate receptor binding and adenylate cyclase inhibition in a neuroblastoma X glioma hybrid cell line. *Life Sci.* **33**: 219-222.

Cox, B. M., Goldstein, A., and Li, C. H. (1976) Opioid activity of a peptide, β-lipotropin-(61-91), derived from β-lipotropin. *Proc. Natl. Acad. Sci. USA* **73**:1821-1823.

Cox, B. M. and Weinstock, M. (1964) Quantitative studies of the antagonism by nalorphine of some of the actions of morphine-like analgesic drugs. *Br. J. of Pharm.* **22**:289-300.

Crook TJ, Kitchen I, and Hill RG (1992) Effects of the delta-opioid receptor antagonist naltrindole on antinociceptive responses to selective delta-agonists in post-weanling rats. *Br. J. Pharmacol.* **107**:573-576.

Cunningham VJ, Hume SP, Price GR, Ahier RG, Cremer JE, and Jones AKP (1991) Compartmental analysis of diprenorphine binding to opiate receptors in the rat in vivo and its comparison with equilibrium data in vitro. *J. of Cereb. Blood Flow and Metab.* **11**:1-9.

Darlison MG, Greten FR, Harvey RJ, Kreienkamp H, Stuhmer T, Zwiers H, Lederis K, and Richter D (1997) Opioid receptors from a lower vertebrate (*Catostomus commersoni*): Sequence, pharmacology, coupling to a G-protein-gated inward-rectifying potassium channel (GIRK1), and evolution. *Proc. Natl. Acad. Sci. USA* **94**:8214-8219.

De Lean A, Hancock AA, and Lefkowitz RJ (1981) Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol. Pharmacol.* **21**:5-16.

Dennis SG and Melzack R (1983) Perspectives on Phylogenetic Evolution of Pain Expression, in *Animal Pain: Perception and Alleviation* (Kitchell RL and Erickson HM eds) pp 151-160, Waverly Press, Baltimore,M.D.

Deviche, P., Cotter, P., and Gulledge, C. C. (1993) Identification, partial characterization, and hypothalamic distribution of kappa, mu, and delta opioid receptors in a passerine songbird (*Junco hyemalis*). *Brain Res.* **614**:220-226.

Deviche P, Murray TF, and Moore FL (1990) Effects of sodium and temperature on naloxone binding in brain tissues of a urodele amphibian. *Comp. Biochem. Physiol.* **96C**:393-398.

Doerr-Schott J, Dubois MP Lichte C. (1981) Immunohistochemical localizaton of substances reactive to antisera against alpha- and beta-endorphin and met-enkephalin in the brain of *Rana temporaria. Cell Tissue Res.* **217:**79-92.

Drower EJ, Stapelfeld A, Rafferty MF, De Costa BR, Rice KC, and Hammond DL (1991) Selective antagonism by naltrindole of the antinociceptive effects of the

delta opioid agonist cyclic[D-Penicillamine2-D-Penicillamine5] enkephalin in the rat. *J. Pharmacol. Exp. Ther.* **259**:725-731.

Emmerson PJ, Liu M-R, Woods JH, and Medzihradsky F (1994) Binding affinity and selectivity of opioids at mu, delta and kappa receptors in monkey brain membranes. *J. Pharmacol. Exp. Ther.* **271**:1630-1637.

Endoh T, Matsuura H, Tanaka C, and Nagase H (1992) Nor-binaltorphimine: a potent and selective kappa-opioid receptor antagonist with long-lasting activity *in vivo*. *Arch. Int. Pharmacodyn. Ther.* **316**:30-42.

Evans CJ, Hammond DL, and Frederickson RCA (1988) The opioid peptides, in *The opiate receptors* (Pasternak GW ed) pp 23-71, Humana Press, Clifton Park.

Evans CJ, Keith DEJr, Morrison H, Magendzo K, and Edwards RH (1992) Cloning of a delta opioid receptor by functional expression. *Science* **258**:1952-1955.

Fang L, Knapp RJ, Horvath R, Matsunaga TO, Haaseth RC, Hruby VJ, Porreca F, and Yamamura HI (1994) Characterization of [3H] naltrindole binding to delta opioid receptors in mouse brain and mouse vas deferens: evidence for delta opioid receptor heterogeneity. *J. Pharmacol. Exp. Ther.* **268**:836-846.

Farges RC, Puget A, Moisand C, and Meunier JC (1988) Opioid receptor types in the brain of the afghan pika (*Ochotona rufescens*), a species which is naturally tolerant to morphine. *Life Sci.* **43**:659-664.

Frances B, Moisand C, and Meunier JC (1985) Na^+ ions and Gpp(NH)p selectively inhibit agonist interactions at μ- and κ-opioid receptor sites in rabbit and guinea-pig cerebellum membranes. *Eur. J. Pharmacol.* **117**:223-232.

Gaddum JH (1937) The quantitative effects of antagonistic drugs. *J. Physiol.* **89**:7P-9P.

Gillan MCC and Kosterlitz HW (1982) Spectrum of the mu, delta and kappa binding sites in homogenates of rat brain. *Br. J. Pharmacol.* **77**:461-469.

Gillan MGC, Kosterlitz HW, and Paterson SJ (1980) Comparison of the binding characteristics of tritiated opiates and opioid peptides. *Br. J. Pharmacol.* **70**:481-490.

Gilman AG (1987) G proteins: Transducers of receptor-generated signals. *Ann. Rev. Biochem.* **56**:649.

Goldstein A, Lowney LI, and Pal BK (1971) Stereospecific and nonspecific interactions of the morphine congener levorphanol in subcellular fractions of mouse brain. *Proc. Natl. Acad. Sci. U.S.A.* **68**:1742-1747.

Goldstein, A. and Naidu, A. (1989) Multiple opioid receptors: ligand selectivity profiles and binding signatures. *Mol. Pharm.* **36**:265-272.

Goldstein A, Tachibana S, Lowney LI, Hunkapiller M, and Hood L (1979) Dynorphin-(1-13), an extraordinarily potent opioid peptide. *Proc. Natl. Acad. Sci. U.S.A.* **76**:6666-6670.

Hayes AG, Skingle M, and Tyers MB (1986) Reversal by beta-funaltrexamine of the antinociceptive effect of opioid agonists in the rat. *Br. J. Pharmacol.* **88**:867-872.

Herrick, C. J. The brain of the tiger salamander. 1-409. 1948. Chicago, IL, University of Chicago Press.

Heyman JS, Mulvaney SA, Mosberg HI, and Porreca F (1987) Opioid delta receptor involvement in supraspinal and spinal antinociception in mice. *Brain Res.* **420**:100-108.

Holtzman, S. G. (1980) Phencyclidine-like discriminative effects of opioids in the rat. *J. Pharmacol. Exp. Ther* **214**, 614-615.

Horan P, Taylor J, Yamamura HI, and Porreca F (1992) Extremely long-lasting antagonistic actions of nor-binaltorphimine (nor-BNI) in the mouse tail-flick test. *J. Pharmacol. Exp. Ther.* **260**:1237-1243.

Horan PJ, De Costa BR, Rice K, Haaseth RC, Hruby VJ, and Porreca F (1993) Differential antagonism of bremazocine and U69,593-induced antinociception by quadazocine: further functional evidence of opioid kappa receptor multiplicity in the mouse. *J. Pharmacol. Exp. Ther.* **266**:926-933.

Houde, R. W. and Wallenstein, S. L. (1956) Clinical studies of morphine-nalorphine combinations. *Fed. Proc.* **15**:440-441.

Hughes, J. (1975) Isolation of an endogenous compound from the brain with properties similar to morphine. *Brain Res.* **88**:295-308.

Hughes, J. (1975) Search for the endogenous ligand of the opiate receptor. *Neurosci. Res. Program Bull.* **13**:55-58.

Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A., and Morris, H. R. (1975) Identification of two related pentapeptides from the brain with potent opiate agonist activity. *Nature* **258**:577-579.

Hunter JC, Leighton GE, Meecham KG, Boyle SJ, Horwell DC, Rees DC, and Hughes J (1990) CI-977, a novel and selective agonist for the kappa-opioid receptor. *Br. J. Pharmacol.* **101**:183-189.

Improta G and Broccardo M (1992) Spinal antinociceptive effects of [D-Ala2] deltorphin II, a novel and highly selective delta-opioid receptor agonist. *Peptides* **13**:1123-1126.

Iwamoto ET and Martin WR (1981) Multiple opioid receptors. *Med. Res. Rev* **4**:411-439.

Jackson HC and Kitchen I (1989) Swim-stress-induced antinociception in young rats. *Br. J. Pharmacol.* **96**:617-622.

Jackson HC, Ripley TL, and Nutt DJ (1989) Exploring delta-receptor function using the selective opioid antagonist naltrindole. *Br. J. Pharmacol.* **28**:1427-1430.

Jackson IMD, Bolaffi JL, and Guillemin R (1980) Presence of immunoreactive beta endorphin and enkephalin-like material in the retina and other tissues of the frog, *Rana pipiens*. *Gen. Comp. Endocrinol.* **42**:505-508.

Jacobson W and Wilkinson M (1984) Opiate ([^{3}H]-naloxone) binding to hypothalamic and cerebral cortical slices of mouse brain. *Brain Res. Bull.* **13**:481-485.

Jegou S, Tonon MC, Leroux P, Delarue C, Leboulenger F, Pelletier G, Cote J, Ling N, and Vaudry H (1983) Immunological characterization of endorphins, adrenocorticotropin, and melanotropins in frog hypothalamus. *Gen. Comp. Endocrinol.* **51**:246-254.

Jiang QI, Heyman JS, Sheldon RJ, Koslo RJ, and Porreca F (1990) Mu antagonist and kappa agonist properties of β-funaltrexamine (β-FNA) *in vivo*: long-lasting spinal analgesia in mice. *J. Pharmacol. Exp. Ther.* **252**:1006-1011.

Jones DN and Holtzman SG (1992) Long term kappa-opioid receptor blockade following nor-binaltorphimine. *Eur. J. Pharmacol.* **215**:345-348.

Kenakin TP (1982) The Schild regression in the process of receptor classification. *Can. J. Physiol. Pharmacol.* **60**:249-265.

Kicliter E and Ebbesson SOE (1976) Organization of the "Nonolfactory" Telencephalon, in *Frog Neurobiology: A Handbook* (Llinas R and Precht W eds) pp 946-972, Springer-Verlag, Berlin.

Kieffer, B. L. (1995) Recent advances in molecular recognition and signal transduction of active peptides: receptors for opioid peptides. *Cell. Mol. Neurobiology* **15**:615-635.

Kieffer BL, Befort K, Gaveriaux-Ruff C, and Hirth CG (1992) The delta-opioid receptor: Isolation of a cDNA by expression cloning and pharmacological characterization. *Proc. Natl. Acad. Sci. U.S.A.* **89**:12048-12052.

Kim KW, Eun YA, Soh SM, Eun JS, and Cho KP (1996) Ligand binding profiles of U-69,593-sensitive and -insensitive sites in human cerebral cortex membranes: Evidence of kappa opioid receptors heterogeneity. *Life Sci.* **58**:1671-1679.

Knapp RJ, Malatynska E, Collins N, Fang L, Wang JY, Hruby VJ, Roeske WR, and Yamamura HI (1995) Molecular biology and pharmacology of cloned opioid receptors. *FASEB J.* **9**:516-525.

Kong H, Raynor K, Yano H, Takeda J, Bell GI, and Reisine T (1994) Agonists and antagonists bind to different domains of the cloned κ opioid receptor. *Proc. Natl. Acad. Sci. USA.* **91**:8042-8046.

Lahti RA, Mickelson MM, Mccall JM, and VonVoigtlander PF (1985) [^{3}H]U-69593 a highly selective ligand for the opioid kappa receptor. *Eur. J. Pharmacol.* **109**:281-284.

Lai J, Bilsky EJ, Rothman RB, and Porreca F (1994) Treatment with antisense oligodeoxynucleotide to the opioid delta receptor selectively inhibits delta 2-agonist antinociception. *Neuro. Report.* **5**:1049-1052.

Lai J, Crook TJ, Payne A, Lynch RM, and Porreca F (1997) Antisense targeting of delta opioid receptors in NG 108-15 cells: Direct correlation between oligodeoxynucleotide uptake and receptor density. *J. Pharmacol. Exp. Ther.* **281**:589-596.

Lasagna, L. and Beecher, H. K. (1954) Analgesic effectiveness of nalorphine and nalorphine-morphine combinations in man. *J. Pharmacol. Exp. Ther* **112**, 356-363.

Leighton GE, Rodriguez RE, Hill RG, and Hughes J (1988) Kappa-opioid agonists produce antinociception after i.v. and i.c.v. but not intrathecal administration in the rat. *Br. J. Pharmacol.* **93**:553-560.

Li X, Keith DEJr, and Evans CJ (1995) Identification of opioid receptor-like sequences among different species. *Society for Neuroscience Abstracts* **21**:1843.

Liebeskind, J. C., Mayer, D. J., and Akil, H. (1974) Central mechanisms of pain inhibition: studies of analgesia from focal brain stimulation. *Adv. Neurol.* **4**:261-268.

Liu-Chen LY and Phillips CA (1987) Covalent labeling of mu opioid binding by site by [3H]Beta-funaltrexamine. *Mol. Pharmacol.* **32**:321-329.

Lord JAH, Waterfield AA, Hughes J, and Kosterlitz HW (1977) Endogenous opioid peptides: multiple agonists and receptors. *Nature* **267**:495-499.

Lujan, M., Lopez, E., Ramirez, R., Aguilar, H., Martinez-Olmedo, M. A., and Garcia-Sainz, J. A. (1984) Pertussis toxin blocks the action of morphine, norepinephrine and clonidine on isolated guinea-pig ileum. *Eur. J. of Pharmacol.* **100**:377-380.

Magnan J, Paterson SJ, Tavani A, and Kosterlitz HW (1982) The binding spectrum of narcotic analgesic drugs with different agonist and antagonist properties. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **319**:197-205.

Malmberg AB and Yaksh TL (1992) Isobolographic and dose-response analyses of the interaction between intrathecal mu and delta agonists: effects of naltrindole and its benzofuran analog (NTB). *J. Pharmacol. Exp. Ther.* **263**:264-275.

Mansour A, Fox CA, Burke S, Meng F, Thompson RC, Akil H, and Watson SJ (1994) Mu, delta, and kappa opioid receptor mRNA expression in the rat CNS: an *in situ* hybridization study. *J. Comp. Neurol.* **350**:412-438.

Mansour A, Khachaturian H, Lewis ME, Akil H, and Watson SJ (1988) Anatomy of CNS opioid receptors. *Trends Neurosci.* **11**:308-314.

Martin TJ, Dworkin SI, and Smith JE (1993) Effects of intracerebroventricular administration of β-funaltrexamine on [^{3}H]DAMGO binding to rat sections. *J. Pharmacol. Exp. Ther.* **267**:506-514.

Martin TJ, Dworkin SI, and Smith JE (1995) Alkylation of mu opioid receptors by beta-funaltrexamine *in vivo*: comparison of the effects on *in situ* binding and heroin self-administration in rats. *J. Pharmacol. Exp. Ther.* **272**:1135-1140.

Martin, W. R. (1967) Opioid antagonists. *Pharmacological Rev.* **19**: 463-521.

Martin WR, Eades CG, Thompson JA, Huppler RE, and Gilbert PE (1976) The effects of morphine-and nalorphine-like drugs in the nondependent and morphine-dependent chronic spinal dog. *J. Pharmacol. Exp. Ther.* **197**:517-532.

Mattia A, Vanderah T, Mosberg HI, and Porreca F (1991) Lack of antinociception cross-tolerance between [D-Pen2, D-Pen5] enkephalin and [D-Ala2]deltorphin II in mice: evidence for delta receptor subtypes. *J. Pharmacol. Exp. Ther.* **258**:583-587.

Mayer, D. J. and Liebeskind, J. C. (1974) Pain reduction by focal electrical stimulation of the brain: an anatomical approach. *Brain Res.* **68**:73-93.

Mayer DJ, Wolfle TL, Akil H, Carder B, and Liebeskind JC (1971) Analgesia from electrical stimulation in the brainstem of the rat. *Science* **174**:1351-1354.

McLean, S., Rothman, R. B., Jacobson, A. E., Rice, K. C., and Herkenham, M. (1987) Distribution of opiate receptor subtypes and enkephalin and dynorphin

immunoreactivity in the hippocampus of squirrel, guinea pig, rat and hamster. *J. Comp. Neurology* **255**:497-510.

Metzger TG and Ferguson DM (1995) On the role of extracellular loops of opioid receptors in conferring ligand selectivity. *FEBS L.* **375**:1-4.

Miotto K, Magendzo K, and Evans CJ (1995) Molecular characterization of opioid receptors, in *The Pharmacology of Opioid Peptides* (Tseng L ed) Harwood Acad., Singapore.

Mjanger E and Yaksh TL (1991) Characteristics of dose-dependent antagonism by beta funaltrexamine of the antinociceptive effects of intrathecal mu agonists. *J. Pharmacol. Exp. Ther.* **258**:544-550.

Molinoff, P. B., Wolfe, B. B., and Weiland, G. A. (1981) Quantitative analysis of drug-receptor interactions: II. Determination of the properties of receptor subtypes. *Life Sciences* **29**:27-443.

Mollereau C, Pascaud A, Baillat G, Mazarguil H, Puget A, and Meunier JC (1988b) 5'-guanylylimidodiphosphate decreases affinity for agonists and apparent molecular size of a frog brain opioid receptor in digitonin solution. *J. Biol. Chem.* **263**:18003-18008.

Mollereau C, Pascaud A, Baillat G, Mazarguil H, Puget A, and Meunier JC (1988a) Evidence for a new type of opioid binding site in the brain of the frog *Rana ridibunda*. *Eur. J. Pharmacol.* **150**:75-84.

Morley, J. S. (1983) A common opiate receptor? *Trends Pharmacol. Sci.* **4**:370-371.

Motulsky HJ and Ransnas LA (1987) Fitting curves to data using nonlinear regression: a practical and nonmathematical review. *FASEB J.*365-374.

Moyle WR, Campbell RK, Myers RV, Bernard MP, Han Y, and Wang X (1994) Co-evolution of ligand-receptor pairs. *Nature* **368**:251-254.

Nestler EJ and Duman RS (1994) G proteins and cyclic nucleotides in the nervous system, in *Basic Neurochemistry: Molecular, Cellular, and Medical Aspects* (Siegel GJ ed) pp 429-447, Raven Press, New York.

Newman, L. C., Wallace, D. R., and Stevens, C. W. (1999) Characterization of 3H-diprenorphine binding in *Rana pipiens*: Observations of filter binding enhanced by naltrexone. *J. Pharmacol. Toxicol. Meth.* **41**:43-48.

Newman, L. C., Wallace, D. R., and Stevens, C. W. (2000a) Selective opioid agonist and antagonist displacement of [^{3}H]-naloxone binding in amphibian brain. *Eur. J. Pharmacol.*, **397**:255-262.

Newman, L. C., Wallace, D. R., and Stevens, C. W. (2000b) Selective opioid receptor agonist and antagonist displacement of [^{3}H]-naloxone binding in amphibian spinal cord. *Brain Res.*, (submitted).

Niwa, M., Iwai, T., Luay, A. E., Nozaki, M., and Tsurumi, K. (1994) Characterization of opioid receptors in the Mongolian gerbil cerebellum. *Life Sci.* **55**:1277-1283.

Nock B, Rajpara A, O'Connor LH, and Cicero TJ (1988) Autoradiography of [3H]U-69593 binding sites in rat brain: evidence for kappa opioid receptor subtypes. *Eur. J. Pharmacol.* **154**:27-34.

Northcutt RG (1984) Evolution of the vertebrate central nervous system: patterns and processes. *Amer. Zool.* **24**:701-716.

Parolaro D, Massi P, Patrini G, Rubino T, Parenti M, and Gori E (1991) Pertussis toxin pretreatment affects opiate/nonopiate and stress-induced analgesia differently. *Pharmacol. Biochem. Behav.* **38**:569-573.

Pasternak GW, Gintzler AR, Houghten RA, Ling GSF, Goodman RR, Spiegel K, Nishimura S, Johnson N, and Recht LD (1983) Biochemical and pharmacological evidence for opioid receptor multiplicity in the central nervous system. *Life Sci.* **33**:167-173.

Pasternak GW and Standifer KM (1995) Mapping of opioid receptors using antisense oligodeoxynucleotides: correlating their molecular biology and pharmacology. *Trends Pharmacol. Sci.* **16**:344-350.

Paterson SJ, Robson LE, and Kosterlitz HW (1984) Opioid Receptors, in *The Peptides* pp 147-189, Academic Press.

Paul D, Bodnar RJ, Gistrak MA, and Pasternak GW (1989) Different mu receptor subtypes mediate spinal and supraspinal analgesia in mice. *Eur. J. Pharmacol.* **168**:307-314.

Pert CB, Aposhian D, and Snyder SH (1974) Phylogenetic distribution of opiate binding. *Brain Res.* **75**:356-361.

Pert CB, Pasternak G, and Snyder SH (1973) Opiate agonists and antagonists discriminated by receptor binding in brain. *Science* **182**:1359-1361.

Pert CB and Snyder SH (1973b) Opiate receptor: demonstration in nervous tissue. *Science* **179**:1011-1014.

Pert CB and Snyder SH (1973a) Properties of opiate-receptor binding in rat brain. *Proc. Natl. Acad. Sci. U.S.A.* **70**:2243-2247.

Pezalla PD (1983) Morphine-induced analgesia and explosive motor behavior in an amphibian. *Brain Res.* **273**:297-305.

Pezalla PD and Dicig M (1984) Stress-induced analgesia in frogs: evidence for the involvement of an opioid system. *Brain Res.* **296**:356-360.

Pezalla PD and Stevens CW (1984) Behavioral effects of morphine, levorphanol, dextrorphan and naloxone in the frog *Rana pipiens*. *Pharmacol. Biochem. Behav.* **21**:213-217.

Pfeiffer A and Herz A (1982) Discrimination of three opiate receptor binding sites with the use of a computerized curve-fitting technique. *Mol. Pharmacol.* **21**:266-271.

Pfeiffer A, Pasi A, Mehraein P, and Herz A (1982) Opiate receptor binding sites in human brain. *Brain Res.* **248**:87-96.

Piercey MF, Lhati RA, and Schroeder LA (1982) U-50488H, a pure kappa receptor agonist with spinal analgesic loci in the mouse. *Life Sci.* **31**:1197-1200.

Pollack AE and Wooten GF (1987) Effects of sodium on cell surface and intracellular ^{3}H-naloxone binding sites. *Life Sci.* **41**:385-390.

Porreca F, Mosberg HI, Omnaas JR, Burks TF, and Cowan A (1987) Supraspinal and spinal potency of selective opioid agonists in the mouse writhing test. *J. Pharmacol. Exp. Ther.* **240**:890-894.

Portoghese, P. S. (1965) A new concept on the mode of interaction of narcotic analgesics with receptors. *J. Med. Chem.* **8**:609-611.

Portoghese, P. S. (1966) Stereochemical factors and receptor interactions associated with narcotic analgesics. *J. Pharm. Sci.* **55**:865-887.

Portoghese, P. S., Larson, D. L., Sayre, L. M., Fries, D. S., and Takemori, A. E. (1980) A novel opioid receptor site directed alkylating agent with irreversible narcotic antagonistic and reversible agonistic activities. *J. Med. Chem.* **23**:233-234.

Portoghese PS, Lipkowski AW, and Takemori AE (1987) Binaltorphimine and nor-binaltorphimine, potent and selective κ-opioid receptor antagonists. *Life Sci.* **40**:1287-1292.

Portoghese PS, Sultana M, and Takemori AE (1988) Naltrindole, a highly selective and potent non-peptide delta opioid receptor antagonist. *Eur. J. Pharm* **146**:185-186.

Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J., and Seaflon, S. C. (1992) Sequence alignment of the G-protein-coupled receptor superfamily. *DNA Cell Biol* **11**:1-20.

Raynor K, Kong H, Chen Y, Yasuda K, Yu L, Bell GI, and Reisine T (1994) Pharmacological characterization of the cloned κ–,δ–,and μ-opioid receptors. *Mol. Pharmacol.* **45**:330-334.

Reisine T and Bell GI (1993) Molecular biology of opioid receptors. *Trends Neurosci.* **16**:506-510.

Reynolds, D. V. (1969) Surgery in the rat during electrical analgesia induced by focal brain stimulation. *Science* **164**:444-445.

Richards ML and Sadee W (1985) In vivo opiate receptor binding of oripavines to μ, δ, and κ sites in rat brain as determined by an ex vivo labeling method. *Eur. J. Pharmacol.* **114**:343-353.

Robson LE, Gillan MGC, and Kosterlitz HW (1985) Species differences in the concentrations and distributions of opioid binding sites. *Eur. J. Pharmacol.* **112**:65-71.

Rogers H, Hayes AG, Birch PJ, Traynor JR, and Lawrence AJ (1990) The selectivity of the opioid antagonist, naltrindole, for delta-opioid receptors. *J. Pharm. Pharmacol.* **42**:358-359.

Rossi G, Pan YX, Cheng J, and Pasternak GW (1994) Blockade of morphine analgesia by an antisense oligonucleotide against the mu receptor. *Life Sci.* **54**:PL375-PL379.

Rothman RB, Bykov V, Xue BG, Xu H, De Costa BR, Jacobson AE, Rice K, Kleinman JE, and Brady LS (1992) Interaction of opioid peptides and other drugs with multiple kappa receptors in rat and human brain. Evidence for species differences. *Peptides* **13**:977-987.

Rothman RB, Long JB, Bykov V, Jacobson AE, Rice KC, and Holaday JW (1988) β-FNA binds irreversibly to the opiate receptor complex: *In vivo* and *in vitro* evidence. *J. Pharmacol. Exp. Ther.* **247**:405-416.

Rottmann M, Fabian G, Spicher K, Offermanns S, and Szucs M (1998) Receptor-mediated activation of G-proteins by κ opioid agonists in frog (*Rana esculenta*) brain membranes. *Brain Res. Bull.* **45**:467-474.

Ruegg UT, Cuenod S, Hiller JM, Gionnini T, Howells RD, and Simon EJ (1981) Characterization and partial purification of solubilized active opiate receptors from toad brain. *Proc. Natl. Acad. Sci. U.S.A.* **7**:4635-4638.

Ruegg UT, Hiller JM, and Simon EJ (1980) Solubilization of an active opiate receptor from *Bufo marinus*. *Eur. J. Pharmacol.* **64**:367-368.

Russell RD, Leslie JB, Su YF, Watkins WD, and Chang KJ (1987) Continuous intrathecal opioid analgesia: tolerance and cross-tolerance of mu and delta spinal opioid receptors. *J. Pharmacol. Exp. Ther.* **240**:150-158.

Sanchez-Blazquez P and Garzon J (1991) Cholera toxin and pertussis toxin on opioid- and alpha$_2$-mediated supraspinal analgesia in mice. *Life Sci.* **48**:1721-1727.

Satoh M and Minami M (1995) Molecular pharmacology of the opioid receptors. P*harmac. Ther.* **68**:343-364.

Schild HO (1947a) $_p$A, a new scale for the measurement of drug antagonism. *Br. J. Pharmacol.* **2**:189-206.

Schild, H. O. (1947) The use of drug antagonists for the identification and classification of drugs. *Br. J. Pharmacol.* **2**:251-258.

Schild, H. O. (1949) pAx, and competitive drug antagonism. *Br. J. Pharmacol.* **4**:277-280.

Schmauss C and Yaksh TL (1984) *In vivo* studies on spinal opiate receptor systems mediating antinociception. II. Pharmacological profiles suggesting a differential association of mu, delta and kappa receptors with visceral chemical and cutaneous thermal stimuli in the rat. *J. Pharmacol. Exp. Ther.* **228**:1-12.

Schnittler M, Liebmann C, Schrader U, Schulze HP, Neubert K, and Repke H (1990) [^{3}H]-Naloxone as an opioid receptor label: Analysis of binding site heterogeneity and use for determination of opioid affinities of casomorphin analogues. *Biomed. Biochim. Acta.* **49**:209-218.

Selley DE, Sim LJ, Xiao R, Liu Q, and Childers SR (1997) μ-opioid receptor-stimulated guanosine-5'-O-(γ-thio)-triphosphate binding in rat thalamus and cultured cell lines: signal transduction mechanisms underlying agonist efficacy. *Mol. Pharmacol.* **51**:87-96.

Shah S, Davis T, and Yoburn BC (1994b) The effect of naltrindole on spinal and supraspinal delta opioid receptors and analgesia. *Life Sci.* **55**:1451-1458.

Shah S, Duttaroy A, Davis T, and Yoburn BC (1994a) Spinal and supraspinal effects of pertussis toxin on opioid analgesia. *Pharmacol. Biochem. Behav.* **49**:773-776.

Sim LJ, Selley DE, and Childers SR (1995) *In vitro* autoradiography of receptor-activated G proteins in rat brain by agonist-stimulated guanylyl 5'-[γ[^{35}S]thio]-triphosphate binding. *Neurobiol.* **92**:7242-7246.

Simantov R, Goodman R, Aposhian D, and Snyder SH (1976) Phylogenetic distribution of a morphine-like peptide enkephalin. *Brain Res.* **111**:204-211.

Simon EJ and Hiller JM (1978) The opiate receptors. *Ann. Rev. Pharmacol. Toxicol.* **18**:371-394.

Simon EJ, Hiller JM, and Edelman I (1973) Stereospecific binding of the potent narcotic analgesic [^{3}H] etorphine to rat-brain homogenate. *Proc. Natl. Acad. Sci. U.S.A.* **70**:1947-1949.

Simon EJ, Hiller JM, Groth J, Itzhak Y, Holland MJ, and Beck SG (1982) The nature of opiate receptors in toad brain. *Life Sci.* **31**:1367-1370.

Simon, E. J. and VanPraag, D. (1966) Studies on the intracellular distribution and tissue binding of dihydromorphine-7,8-3H in the rat. *Proc. Soc. Exp. Biol.Med.* **122**:6-11.

Simon J, Benyhe S, Borsodi A, Szucs M, and Wollemann M (1985) Separation of κ-opioid receptor subtype from frog brain. *FEBS L.* **183**:395-397.

Simon, J., Szucs, M., Benyhe, S., Borsodi, A., Zemlan, F. P., and Wollemann, M. (1984) Solubilization and characterization of opioid binding sites from frog (*Rana esculenta*) brain. *J. Neurochem.* **43**:957-963.

Simpson JI (1976) Functional Synaptology of the Spinal Cord, in *Frog Neurobiology: A Handbook* (Llinas R and Precht W eds) pp 728-749, Springer-Verlag, Berlin.

Snyder SH, Pasternak GW, and Pert CB (1975) in *Handbook in Psychopharmacology* (Iversen LL, Iversen SD, and Snyder SH eds) pp 329-329, Plenum, New York.

Sofuoglu M, Portoghese PS, and Takemori AE (1991) Differential antagonism of delta opioid agonists by naltrindole and its benzofuran analog (NTB) in mice: Evidence for delta opioid receptor subtypes. *J. Pharmacol. Exp. Ther.* **257**:676-680.

Spray DC (1976) Cutaneous Receptors: pain and temperature receptors of anurans, in *Frog Neurobiology: a handbook* (Llinas R and Precht W eds) pp 607-628, Springer-Verlag, Berlin.

Standifer KM, Cheng J, Brooks AI, Honrado CP, Su W, Visconti LM, Biedler JL, and Pasternak GW (1994a) Biochemical and pharmacological characterization of mu, delta and kappa 3 opioid receptors expressed in BE(2)-C neuroblastoma cells. *J. Pharmacol. Exp. Ther.* **270**:1246-1255.

Standifer KM, Chien C-C, Wahlestedt C, Brown GP, and Pasternak GW (1994b) Selective loss of delta opioid analgesia and binding by antisense oligodeoxynucleotides to a delta opioid receptor. *Neuron* **12**:805-810.

Standifer KM and Pasternak GW (1997) G proteins and opioid receptor-mediated signaling. *Cell. Sig.* **9**:237-248.

Stapelfeld A, Hammond DL, and Rafferty MF (1992) Antinociception after intracerebroventricular administration of naltrindole in the mouse. *Eur. J. Pharmacol.* **214**:273-276.

Stevens CW (1988) Opioid antinociception in amphibians. *Brain Res. Bull.* **21**:959-962.

Stevens CW (1992) Alternatives to the use of mammals for pain research. *Life Sci.* **50**:901-912.

Stevens CW (1995a) An amphibian model for pain research. *Lab Animal* **24**:32-36.

Stevens CW (1995b) An amphibian model for the assessment of opioid analgesia: systemic and spinal studies. *Analgesia* **1**:683-686.

Stevens CW (2000) Amphibian models of nociception and pain, in *Animal Models of Nociception and Pain* (Kavaliers MK, Ossenkopp KP, and Sanberg PR eds) R.G. Landes Co., Austin. (in press).

Stevens CW (1996a) Relative analgesic potency of mu, delta and kappa opioids after spinal administration in amphibians. *J. Pharmacol. Exp. Ther.* **276**:440-448.

Stevens CW, Klopp AJ, and Facello JA (1994) Analgesic potency of mu and kappa opioids after systemic administration in amphibians. *J. Pharmacol. Exp. Ther.* **269**:1086-1093.

Stevens CW and Newman LC (1999) Spinal administration of selective opioid antagonists in amphibians: evidence for an opioid unireceptor. *Life Sci.* **64**:PL125-PL130.

Stevens, C. W. and Paul, D. Opioid analgesia after spinal administration in amphibians: binding and behavioral studies. Harris, L. S. 1995. *NIDA Research Monograph.*

Stevens CW and Pezalla PD (1983) A spinal site mediates opiate analgesia in frogs. *Life Sci.* **33**:2097-2103.

Stevens CW and Pezalla PD (1984) Naloxone blocks the analgesic action of levorphanol but not of dextrorphan in the leopard frog. *Brain Res.* **301**:171-174.

Stevens CW, Pezalla PD, and Yaksh TL (1987) Spinal antinociceptive action of three representative opioid peptides in frogs. *Brain Res.* **402**:201-203.

Stevens CW and Rothe-Skinner K (1997) Supraspinal administration of opioids with selectivity for μ–, δ–, and κ–opioid receptors produces analgesia in amphibians. *Eur. J. Pharmacol.* **331**:15-21.

Stevens CW, Sangha S, and Ogg BG (1995) Analgesia produced by immobilization stress and an enkephalinase-inhibitor in amphibians. *Pharmacol. Biochem. Behav.* **51**:675-680.

Stevens, C. W. and Willenbring, S. (1996) Pain sensation and analgesia in amphibians and reptiles, in: *The Biology, Husbandry and Health Care of Reptiles and Amphibians* (L Ackerman ed.) T.F.H. Publications, Neptune City, NJ. (in press).

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

Suzuki T, Oka J, and Fukuda H (1987) In vitro studies of the effects of naloxone on the root potentials in the frog spinal cord: enkephalin-like effect on the recurrent presynaptic inhibition. *Comp. Biochem. Physiol.* **87C**:221-225.

Szucs M, Borsodi A, Bogdany A, Gaal J, Batke J, and Toth G (1987) Detailed analysis of heterogeneity of [^{3}H]-naloxone binding sites in rat brain synaptosomes. *Neurochem. Res.* **12**:581-587.

Takemori AE (1985) Affinity labels for opioid receptors. *Ann. Rev. Pharmacol. Toxicol.* **25**:193-223.

Takemori AE, Ho BY, Naeseth JS, and Portoghese PS (1988) Nor-binaltorphimine, a highly selective kappa-opioid antagonist in analgesic and receptor binding assays. *J. Pharmacol. Exp. Ther.* **246**:255-258.

Takemori AE, Larson DL, and Portoghese PS (1981) The irreversible narcotic antagonistic and reversible agonistic properties of the fumarate methyl ester derivative of naltrexone. *Eur. J. Pharmacol.* **70**:445-451.

Takemori AE and Portoghese PS (1987) Evidence for the interaction of morphine with kappa and delta opioid receptors to induce analgesia in beta-funaltrexamine treated mice. *J. Pharmacol. Exp. Ther.* **243**:91-94.

Takemori AE and Portoghese PS (1992) Selective naltrexone-derived opioid receptor antagonists. *Ann. Rev. Pharmacol. Toxicol.* **32**:239-269.

Talbot JD, Marrett S, Evans AC, Meyer E, Bushnell MC, and Duncan GH (1991) Multiple representations of pain in human cerebral cortex. *Science* **251**:1355-1358.

Tam SW and Liu-Chen LY (1986) Reversible and irreversible binding of beta-funaltrexamine to mu, delta and kappa opioid receptors in guinea pig brain membranes. *J. Pharmacol. Exp. Ther.* **239**:351-357.

Terenius L (1973) Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. *Acta. Pharmacol. Toxicol.* **32**:317-320.

Terenius, L. and Whalstrom, A. (1975) Search for an endogenous ligand for the opiate receptor. *Acta Physiologica Scandinavica* **94**:74-81.

Tiseo PJ and Yaksh TL (1993) Dose-dependent antagonism of spinal opioid receptor agonists by naloxone and naltrindole: additional evidence for delta-opioid receptor subtypes in the rat. *Eur. J. Pharmacol.* **236**:89-96.

Traynor JR, Corbett AD, and Kosterlitz HW (1987) Diprenorphine has agonist activity at opioid κ-receptors in the myenteric plexus of the guinea-pig ileum. *Eur. J. Pharm* **137**:85-89.

Traynor JR and Nahorski SR (1995) Modulation by μ-opioid agonists of guanosine-5'-O-(3-[^{35}S]thio)triphosphate binding to membranes from human neuroblastoma SH-SY5Y cells. *Mol. Pharmacol.* **47**:848-854.

Varga E, Li X, Stropova D, Zalewska T, Landsman RS, Knapp RJ, Malatynska E, Kawai K, Mizusura A, Nagase H, Calderon SN, Rice K, Hruby VJ, Roeske WR, and Yamamura HI (1996) The third extracellular loop of the human δ-opioid receptor determines the selectivity of δ-opioid agonists. *Mol. Pharmacol.* **50**:1619-1624.

Veatch, R. M., Adler, T. K., and Way, E. L. (1964) The importance of steric configuration in certain morphine-mimetic actions of synthetic analgesics. *J. Pharmacol. Exp. Therap.* **145**:11-19.

Vesselkin NP, Agayan AL, and Nomokonova LM (1971) A study of thalamo-telencephalic afferent systems in frogs. *Brain Behav. Evol.* **4**:295-306.

VonVoigtlander PF, Lahti RA, and Ludens JH (1983) U-50,488: A selective and structurally novel non-mu (kappa) opioid agonist. *J. Pharmacol. Exp. Ther.* **224**:7-12.

Wahlestedt C (1994) Antisense oligonucleotide strategies in neuropharmacology. *Trends Pharmacol. Sci.* **15**:42-46.

Wang WW, Shahrestanifar M, Jin J, and Howells RD (1995) Studies on μ and δ opioid selectivity using chimeric and site-mutagenized receptors. *Proc. Natl. Acad. Sci. U.S.A.* **92**:12436-12440.

Ward SJ, Fries DS, Larson DL, Portoghese PS, and Takemori AE (1985) Opioid receptor binding characteristics of the non-equilibrium mu antagonist, β-funaltrexamine (β-FNA). *Eur. J. Pharmacol.* **107**:323-330.

Ward SJ, LoPresti D, and James DW (1986) Activity of mu- and delta-selective opioid agonists in the guinea pig ileum preparation: differentiation into peptide and nonpeptide classes with β-funaltrexamine. *J. Pharmacol. Exp. Ther.* **238**:625-631.

Ward SJ, Portoghese PS, and Takemori AE (1982a) Pharmacological characterization *in vivo* of the novel opiate, β-funaltrexamine. *J. Pharmacol. Exp. Ther.* **220**:494-498.

Ward SJ, Portoghese PS, and Takemori AE (1982b) Pharmacological profiles of β-funaltrexamine (β-FNA) and β-Chlornaltrexamine (β-CNA) on the mouse vas deferens preparation. *Eur. J. Pharmacol.* **80**:377-384.

Ward SJ and Takemori AE (1982) Relative involvement of mu, kappa and delta receptor mechanisms in opiate-mediated antinociception in mice. *J. Pharmacol. Exp. Ther.* **224**:525-530.

West, R. E. and Miller, R. J. (1983) Opiates, second messengers and cell response. *Br. Med. Bull.* **39**:53-58.

Wettstein JG and Grouhel A (1996) Opioid antagonist profile of sc nor-binaltorphimine in the formalin paw assay. *Pharmacol. Biochem. Behav.* **53**:411-416.

White JC and Sweet WH (1969) *Pain and the Neurosurgeon.* Thomas, Springfield.

Wollemann M, Benyhe S, and Simon J (1993) The kappa opioid receptor: evidence for the different subtypes. *Life Sci.* **52**:599-611.

Wood MS and Traynor JR (1989) [3H]diprenorphine binding to kappa sites in guinea-pig and rat brain: evidence for apparent heterogeneity. *J. Neurochem.* **53**:173-178.

Yaksh TL and Rudy TA (1976) Analgesia mediated by a direct spinal action of narcotics. *Science* **192**:1357-1358.

Yaksh TL and Rudy TA (1977) Studies on the direct spinal action of narotics in the production of analgesia in the rat. *J. Pharmacol. Exp. Ther.* **202**:411-427.

Yaksh TL and Stevens CW (1988) Properties of the modulation of spinal nociceptive transmission by receptor-selective agents, in *Proceedings of the 5th World Congress on Pain* (Dubner R, Gebhart GF, and Bond MR eds) pp 417-435, Elsevier Science Publishers BV, Amsterdam.

Yamamura MS, Horvath R, Toth G, Otvos F, Malatynska E, Knapp RJ, Porreca F, Hruby VJ, and Yamamura HI (1992) Characterization of [^{3}H] naltrindole binding to delta opioid receptors in rat brain. *Life Sci.* **50**:119-124.

Yasuda, K., Raynor, K., Kong, H., Breder, C., Takeda, J., Reisine, T., and Bell, G. I. (1993) Cloning and functional comparison of kappa and delta opioid receptors from mouse brain. *Proc. Natl. Acad. Sci. U.S.A* **90**:6736-6740.

Yoburn BC, Lutfy K, and Candido J (1991) Species differences in μ- and δ-opioid receptors. *Eur. J. Pharmacol.* **193**:105-108.

Yui R (1982) Immunohistochemical studies on peptide neurons in the hypothalamus of the bullfrog *Rana catesbeiana. Gen. Comp. Endocrinol.* **49**:195-209.

Zimmerman DM, Leander JD, Reel JK, and Hynes MD (1987) Use of beta-funaltrexamine to determine mu opioid receptor involvement in the analgesic activity of various opioid ligands. *J. Pharmacol. Exp. Ther.* **241**:374-377.

Zukin, R. Z., Eghali, M., Olive, D., Unterwald, E. M., and Tempel, A. (1988) Characterization and visualization of rat and guinea pif brain κ opioid receptors: evidence for κ_1 and κ_2 opioid receptors. *Proc. Natl. Acad. Sci. U.S.A* **85**:4061-4065.

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