Opioids and Their Complicated Receptor Complexes

Bryen A. Jordan, Ph.D., Svetlana Cvejic, Ph.D., and Lakshmi A. Devi, Ph.D.

No field more eagerly awaits a molecular clarification for G-protein coupled receptor (GPCR) dimerization than the opioid receptor field. Extensive evidence of pharmacological and functional interactions between opioid receptor types has primed this field for such a resolution. In retrospect, much of the data collected on synergy between different opioid receptor types may represent the functional correlate for the newly found opioid receptor dimerization. While previous reports of functional synergy have been, for the most part, consistent in demonstrating cross-regulation between two receptor types, the lack of highly receptor-selective ligands allowed skeptics to remain doubtful over the interpretations of these results. Today, two important developments in the opioid receptor field help reinvigorate the hypothesis of functional, cross-modulating opioid receptor complexes: (1) The existence of highly selective ligands which eliminate any possibility of cross-reactivity between receptor types, and (2) the discovery that opioid receptors and a number of other GPCRs exist as dimers in biochemical, functional and pharmacological assays. It is with these new tools that we seek to understand the mechanisms and implications of dimerization. Initial results of these studies have demonstrated that the dimerization of opioid receptors may help consolidate several pharmacological findings that have remained unanswered. In this review we present biochemical, pharmacological and functional evidence for opioid receptor complexes and add evidence from our recent studies on opioid receptor dimerization. We believe a thorough understanding of receptor dimerization is crucial in clarifying the mechanism of action of opioids and other drugs and may serve a more practical purpose in aiding the development of novel therapeutic drugs.
[Neuropsychopharmacology 23:S5–S18, 2000] © 2000 *American College of Neuropsychopharmacology. Published by Elsevier Science Inc.*

Ancient civilizations used opium for its medicinal use. Current research has shown that the administration of opioids results in a variety of biological effects. These include analgesia, miosis, bradycardia, general sedation, hypothermia, insensitivity and depression of flexor reflexes. However, the best studied effect is in that of pain control, where opioids are known to inhibit neurotransmitter release from dorsal root ganglion projections in the dorsal horn of the spinal cord (MacDonald and Nelson 1978; Mudge et al. 1979; Yaksh 1993). Opioids can modulate endocrine processes (Genazzani and Petraglia 1989; Maggi et al. 1995; Schafer and Martin 1994) and can also affect the immune response (Brown et al. 1974; Roy and Loh 1996). All these effects are mediated by the interactions of opioids with members of the opioid receptor family.

Opioid binding sites were first discovered in mammalian brain (Pert and Snyder 1973; Simon et al. 1973; Terenius 1973). Subsequent pharmacological studies using different ligands uncovered three opioid receptor types, kappa, delta and mu (Chang and Cuatrecasas 1979; Lord et al. 1977) (reviewed in Paterson et al. 1983) which differed in their distribution, ligand binding and

From the Department of Pharmacology, New York University School of Medicine, 550 First Avenue, New York, NY 10016.

Address correspondence to: Lakshmi A. Devi, Ph.D., Department of Pharmacology, New York University School of Medicine, MSB 411, 550 First Avenue, New York, NY 10016. Tel.: (212) 263-7119; Fax: (212) 263-7133; E-mail: Lakshmi.Devi@med.nyu.edu

Received April 6, 2000; accepted May 1, 2000.

NEUROPSYCHOPHARMACOLOGY 2000–VOL. 23, NO. S4
© 2000 American College of Neuropsychopharmacology
Published by Elsevier Science Inc.
655 Avenue of the Americas, New York, NY 10010

0893-133X/00/$–see front matter
PII S0893-133X(00)00143-3

function. The endogenous ligands were found in the mid-1970s and were shown to be peptides (Bradbury et al. 1976; Cox et al. 1976; Goldstein et al. 1981; Hughes et al. 1975; Pasternak et al. 1976). These include enkephalins, dynorphins and endorphins which are derived from three larger precursors: proenkephalin A (Noda et al. 1982), prodynorphin (Kakidani et al. 1982) and pro-opioimelanocortin, respectively (Nakanishi et al. 1979). Recently two new mu receptor selective peptides named endomorphin-1 and endomorphin-2 have been reported although the presence of a precursor for these has not yet been determined (Zadina et al. 1997).

The search for novel opioids resulted in the identification of a large number of opioid ligands which exhibit a variety of efficacies, potencies, affinities and selectivities for the opioid receptor types. The use of these led to the identification of similar yet distinct receptor subtypes. The pharmacological differences observed led to the proposal of receptor subtypes (kappa1, kappa2, kappa3; delta1, delta2; mu1, mu2, mu3) (for review see Traynor 1989; Traynor and Elliot 1993). However to date, despite large-scale efforts to clone the opioid receptor subtypes, no cDNA corresponding to a receptor subtype has been found.

The cloning of the opioid receptors (Evans et al. 1992; Kieffer et al. 1992; Yasuda et al. 1993; Chen et al. 1993) revealed that they are members of the GPCR superfamily. The amino acid sequence revealed no more than a 30% homology to other GPCRs whereas a comparison between kappa, delta and mu receptors revealed a 65–70% homology between the three with highest homology among the transmembrane domains, intracellular loops and a small portion of the C-terminal tail near the 7th transmembrane region (TM7). The most divergent areas are the second and third extracellular loops as well as the N- and C-terminal tail which are extracellular and intracellular, respectively.

OPIOID RECEPTOR COMPLEXES

One of the principal goals in opioid receptor research is to dissociate the analgesic potential of opioid ligands from their liability to be abused. While an understanding of the molecular nature of receptor regulation is crucial for this undertaking, the discovery of ligands with a decreased addicitive component has remained the simplest approach. In spite of an enormous effort, such a compound has yet to be found. However, this search has resulted in a large library of compounds that exhibit varying degrees of efficacy, affinities, potencies and selectivities for the three opioid receptor types. Standard ligand binding assays using these compounds were crucial in the classification of opioid binding sites. The stereospecific binding of the universal opioid agonist, etorphine, to rat brain membranes identified the first opioid binding sites (Pert and Snyder 1973; Simon et al. 1973; Terenius 1973). Other agonists, ketocyclazocine, morphine and DADLE, were subsequently shown to compete against three different fractions of total opioid receptor binding (see Table 1 for ligands and selectivities). This resulted in the classification of opioid receptors into three types: kappa (for ketocyclazocine), mu (for morphine) and delta (for DADLE) (Chang and Cuatrecasas 1979; Lord et al. 1977). As the number of opioid specific ligands grew, so did the list of apparent opioid receptor subtypes found in pharmacological assays. For example, the new delta receptor selective compounds deltorphin II, DALCE, BNTX and naltrindole isothiocyanante (NTII) could differentially compete with selective binding to delta receptors in spinal cord and brain, suggesting the presence of subpopulations within the delta receptor type. There are now several opioid receptor subtypes and the list may grow as novel compounds are identified. However, a central concern pertaining to this subject remains the lack of cDNAs corresponding to these subtypes. The cloning of the opioid receptors revealed only three cDNAs corresponding to the original mu, delta and kappa receptor types. A mu receptor isoform named MOR1b has also been cloned and differs in the c-terminal tail (Zimprich et al. 1995). However, the pharmacological profile of this isoform appears to be identical to that of the previously cloned mu receptor. Therefore the pharmacological entities may not have a genetic correlate and thus warrant other explanations. Along with the question of receptor subtypes and prior to the postulation of opioid receptor subtypes, another pharmacological phenomenon had many researchers perplexed. The apparent interaction of receptor type selective compounds in both pharmacological and functional assays led to one of the first discussions of receptor "complexing," or more recently known as receptor "dimerization." From pharmacological assays, functional complexes of mu and delta receptors were proposed to exist. Interestingly, characterization of these "receptor complexes" yielded information linking them to certain opioid receptor subtypes and led some to propose that a mu-delta complex may in fact represent one of the delta receptor subtypes. Our work with kappa/delta receptor heterodimers suggests that these may represent a kappa receptor subtype (Jordan and Devi 1999). The following is the evidence collected from several labs to support the premise of opioid receptor dimerization.

FUNCTION

The administration of morphine results in a powerful analgesic response that is mediated primarily by mu opioid receptors. Although morphine exhibits high affinity for both delta and mu receptors, a recent study has

Table 1. Ligands and Selectivities

Ligand	Selectivity	Notes	Reference
Etorphine	μ, δ, κ	Universal agonist	(Blane et al. 1967)
Bremazocine	μ, δ, κ	Agonist/antagonist	(Romer et al. 1980)
Diprenorphine	μ, δ, κ	Antagonist	(Biscoe et al. 1972)
EKC	μ, δ, κ	Agonist	(Martin et al. 1976)
Leucine-Enkephalin	δ, μ	Endogenous	(Hughes et al. 1975)
Methionine-Enkephalin	δ, μ	Endogenous	(Hughes et al. 1975)
Dynorphin A	$\kappa > \mu > \delta$	Endogenous	(Goldstein et al. 1981)
β-endorphin	$\mu, \delta > \kappa$	Endogenous	(Bradbury et al. 1976; Cox et al. 1976)
Morphine	$\mu > \delta > \kappa$	Opiate	Sertüner 1805
Naloxone	μ, δ, κ	Universal antagonist	—
DPDPE	$\delta_1, \delta_{cx}, \delta_{ncx}$	Selective agonist	(Mosberg et al. 1983)
DADLE	$\mu, \delta, \delta_{cx} > \delta_{ncx}$	Agonist	(Kosterlitz et al. 1980)
DSLET	δ_2, δ_{cx}	Agonist	(Gacel et al. 1980)
CP-OH	δ_{cx}	Agonist	(Shimohigashi et al. 1988)
Deltorphin I	δ_1	Agonist	(Erspamer et al. 1989)
Deltorphin II	δ_2, δ_{cx}	Agonist	(Erspamer et al. 1989)
Naltrindole	$\delta > \kappa > \mu$	Antagonist	(Portoghese et al. 1988)
Naltrindole 5′-isothiocyanate	δ_2, δ_{cx}	Antagonist	(Portoghese et al. 1992b)
Naltriben	δ_2	Antagonist	(Sofuoglu et al. 1991)
BNTX	δ_1	Antagonist	(Portoghese et al. 1992a)
ICI 174,864	δ, δ_{ncx}	Antagonist	(Cotton et al. 1984)
DALCE	δ_1, δ_{ncx}	Irreversible antagonist	(Jiang et al. 1990a)
DIPP-NH2ψ	$\delta, \mu\ (\delta_{cx}\ ?)$	Mixed mu agonist/Delta antagonist	(Schiller et al. 1999)
FIT	δ	Irreversible antagonist	(Rice et al. 1983)
BIT	μ	Irreversible antagonist	(Rice et al. 1983)
Oxymorphone	$\mu > \delta, \delta_{cx}$	Agonist	—
β-funaltrexamine	$\mu > \delta, \mu_1?$	Irreversible antagonist	(Portoghese et al. 1980)
DAMGO	μ	Agonist	(Handa et al. 1981)
U-69593	κ_1	Agonist	(Lahti et al. 1985)

shown that mu receptor knockout mice no longer exhibit morphine-induced analgesia (Matthes et al. 1996). The first evidence for interactions between opioid receptor types were observations that delta receptor selective agonists could modulate morphine-induced analgesia. Vaught and Takemori (Vaught and Takemori 1979) noticed that the administration of leucine-enkephalin, a peptide agonist moderately selective for delta receptors could potentiate morphine-induced analgesia. A dose of leucine enkephalin that was unable to elicit transient analgesia could cause a leftward shift in the morphine dose-response curve, resulting in nearly half the ED50 as compared to control animals. These results were confirmed by other groups (Barrett and Vaught 1982; Lee et al. 1980). More selective ligands for delta as well as mu receptors were subsequently tested. In agreement with the previous findings, administration of delta receptor selective ligands, in doses that were unable to elicit any transient analgesia, were found to potentiate morphine-induced analgesia while mu receptor selective compounds were unable to do so (Barrett and Vaught 1982). Functional interaction between these two receptor types was also observed in other assays such as in opioid-induced reversal of endotoxic shock (D'Amato and Holaday 1984;

Dixon et al. 1985). Both mu and kappa receptor selective ligands could inhibit delta receptor mediated reversal of endotoxioc shock. Functional synergy between receptor types was also observed in opioid-induced gut propulsion. The highly selective delta receptor agonist, DP-DPE, was found to potentiate morphine-induced bladder motility effects (Sheldon et al. 1989). Mu receptor selective agonists were unable to do so and the kappa receptor selective ligand, dynorphin, could actually inhibit these effects (Sheldon et al. 1989). Synergy has also been reported in the antitussive effects of the mu receptor selective agonist, DAMGO, by the delta receptor selective ligand, deltorphin II (Kamei et al. 1991).

Gathering evidence of cooperativity between opioid receptor types and a growing list of selective ligands led to further characterization of this phenomenon. A surprising result from one such study revealed that while delta receptor selective ligands could potentiate morphine-induced analgesia, they were unable to potentiate analgesia elicited by other mu receptor selective compounds such as, DAMGO or Sulfentanil (Heyman et al. 1989). Most of these studies on synergy had measured the effects of ligands on the potency (i.e., effective dose for 50% effect (ED50) of morphine analgesia). The maximal

nociceptive effects (i.e., the efficacy) of morphine was also found to increase in the presence of delta receptor ligands. Importantly, it was shown that this increase in efficacy could also be observed in morphine-tolerant mice (Jiang et al. 1990b, 1990c). An interesting observation was that no synergy was detected when the delta receptor selective antagonist, ICI 174,864, was coadministered with morphine, suggesting that functional synergy may require the activation of delta receptors (Heyman et al. 1989). The irreversible delta receptor selective antagonist, DALCE, could block delta receptor mediated antinociception but could not block the potentiation of delta receptor selective ligands on morphine analgesia (Jiang et al. 1990a; Porreca et al. 1992). These observations led to a proposal that a distinction be made between delta receptors involved in modulation of morphine analgesia and those that are not. This was an important proposal for the development of delta receptor subtypes.

The importance of this functional synergy cannot be overstated. Potentiation of the potency and efficacy of morphine-induced analgesia literally translates to a decreased morphine dosage in the presence of sub-analgesic doses of delta receptor selective ligands. Of great importance are the effects of lower doses on the development of tolerance and analgesia to morphine. Several groups have initiated studies to answer these questions with surprising results. The compound DIPP-NH2ψ, a mixed delta receptor antagonist/mu receptor agonist, is a novel compound designed to test such a question (Schiller et al. 1999). The synergy by a delta antagonist on a mu agonist demonstrated that delta receptors do not have to be activated in order to potentiate mu receptor induced analgesia (Schiller et al. 1999). Another interesting study has shown that the body may use the co-release of endogenous peptides to promote strong analgesia and decrease the likelihood of tolerance and dependence (Dawson-Basoa and Gintzler 1998). The pain threshold is increased during pregnancy. This is mediated by spinal kappa and delta receptors but not by mu receptors since the analgesia during gestation can be blocked by high doses of either kappa or delta receptor selective antagonists. However, the application of suboptimal doses of kappa and delta receptor selective compounds results in total inhibition of analgesia indicating that both kappa and delta receptors are activated and function synergistically. This pathway may be important in minimizing tolerance and dependence during the relatively long period of gestation and result in a powerful analgesic response (Dawson-Basoa and Gintzler 1998). The presence of both kappa and delta selective agonists were also shown to produce powerful non-additive analgesia in the rat (Miaskowski et al. 1990). While both compounds individually could induce analgesia, the presence of both compounds resulted in a substantial leftward shift in the potency of the agonist (Miaskowski et al. 1990).

BINDING PHARMACOLOGY

While there is a large amount of evidence for synergy between delta/mu, delta/kappa, and mu/kappa pathways, the mechanism of this phenomenon is still poorly understood. Interactions at a ligand binding level had been proposed and were strengthened by mounting pharmacological evidence. Binding assays were critical in assessing the hypothesis that opioid receptors could exist in complexes given that functional interactions could arise from a variety of factors. Synergy between pathways does not require receptor interactions. In fact, functional synergy could arise from cooperativity among any number of downstream signaling factors. Furthermore, synergy could arise from the interaction of neuronal pathways as is clearly seen by coadministration of the same compound into different areas of the brain or spinal cord. Thus pharmacological evidence was crucial to differentiate synergy due to interacting opioid receptor types from synergy arising from any number of other causes.

Some of the first pharmacological evidence supporting an association between mu and delta receptors came soon after the discovery of functional synergy between the two. Rothman and Westfall (Rothman and Westfall 1981, 1982) demonstrated that the moderately selective delta receptor agonist leucine enkephalin (Leu-Enk) was a noncompetitive inhibitor of the slightly mu receptor selective opioid antagonist naloxone. In saturation assays of radiolabeled naloxone, Leu-Enk was able to decrease maximum binding with no significant difference in the apparent affinity of the compounds. They proposed that Leu-Enk could bind to a population of delta receptors allosterically coupled to mu receptors, which could cause the complexed mu receptor to become insensitive to naloxone. It is interesting that these results were opposite to those expected from the synergy observed in functional assays. One would expect to observe an increase in binding in the presence of both mu and delta receptor selective ligands. Furthermore this allosteric coupling was seemingly influenced by the presence of monovalent and divalent ions. Variations in the binding of certain compounds such as DADLE, a compound proposed to bind both delta and mu receptors with high and low affinity, respectively, varied greatly according to assay conditions (Bowen et al. 1981; Demoliou-Mason and Barnard 1986a, 1986b; Rothman et al. 1984). One group demonstrated that mu receptors and delta receptors detected by binding with ^{3}H-morphine and ^{3}H-DADLE respectively, could be intereconverted (Bowen et al. 1981; Quirion et al. 1982). In the presence of divalent and monovalent ions and GTP there was a sharp decrease in ^{3}H-morphine binding (presumably to mu receptors) with a concomitant increase in ^{3}H-DADLE binding (presumably to delta receptors). This variability caused some researchers to propose the unireceptor hypothesis stating that the kappa, delta or mu

receptors were but a single receptor under different conditions (Bowen et al. 1981; Quirion et al. 1982). These results would later form a strong argument for the influence of assay conditions on receptor complexing.

Many ligand binding assays demonstrated interactions that were heterogeneous in nature and that could not be described by simple competition using a two-site model. However the interpretation of these results was complicated due to the lack of highly selective compounds used in the assays. Despite research to prove the selectivity of the compounds used, a critical assumption for the interpretation of their results, the cloning and pharmacological characterization of the opioid receptors revealed that most compounds were notoriously promiscuous (Herz 1993). The observed noncompetitive interactions could be the result of two very different mechanisms. An allosteric model suggests that the binding of a ligand to a population of delta receptors results in a modification of a population of mu receptors, those that are allosterically coupled (in the complex); naloxone would not be able to recognize such allosterically modified mu receptors. This could explain how in the presence of Leu-Enk, naloxone detects a reduced number of receptors. However a two-site model is equally able to explain these results. In this model, delta and mu receptors are two independent binding sites, both detectable by naloxone. The addition of Leu-Enk would selectively block one site which would result in an apparent loss of B_{max} seen in standard saturation curves. This complexity forced researchers to address this issue by plotting computer- generated curves expected from a two-site model and the allosteric model and comparing them to the observed data.

Opioid receptor pharmacology became "complicated" because two-site models could rarely describe the observed data. DADLE, the compound initially thought to label delta and mu receptors with high and low affinities, respectively, was instead suggested to bind with high affinity to delta receptors renamed type 2 or ncx (not in complex) and also to delta-mu complex receptors renamed type 1 or cx (complexed receptor) (Rothman et al. 1984). These results were interesting in that they could consolidate some of the evidence from proponents of the unireceptor hypothesis. The loss of mu receptors and apparent inter-conversion to delta receptors measured by DADLE binding in their assays could be explained in the following manner: under certain conditions mu receptors became complexed with delta receptors and lost affinity for morphine that could not bind delta/mu receptor complexes. The binding of DADLE, which was proposed to bind to delta/mu complexes, would now in turn be increased. Specifically, in the presence of Na^+ and Mn^{++}, DADLE binding could be up-regulated (thus under these conditions, mu receptors could be driven to interact with delta receptors) (Bowen et al. 1981; Rothman et al. 1984). DADLE binding became the preferred method for observing delta/mu complexed receptors.

Interactions between kappa/delta and kappa/mu were also reported and were shown to vary significantly in the presence of monovalent and divalent ions (Demoliou-Mason and Barnard 1986a, 1986b; Garzon et al. 1982; Miaskowski et al. 1990, 1993; Sheldon et al. 1989).

SUBTYPES OR COMPLEXES?

Along with pharmacological assays *in vitro*, there was growing evidence from functional studies of differences within the opioid receptor types. The above functional and pharmacological evidence demonstrated the existence of different or complexed delta receptors. The ability of certain delta receptor specific ligands such as DALCE to block delta receptor mediated analgesia but not delta receptor stimulation of morphine-induced analgesia was complemented by compounds such as CP-OH which could block delta receptor stimulation of mu receptor mediated analgesia but not delta receptor mediated analgesia (Jiang et al. 1990a; Shimohigashi et al. 1988). This evidence suggested that there were two different delta receptor subtypes. The development of novel delta receptor selective compounds such as deltorphin II, naltriben, BNTX and naltrindole further supported the existence of multiple delta receptor sites (Jiang et al. 1991; Mattia et al. 1991). Binding studies with several compounds has resulted in the classification of the delta receptor into two subtypes, delta1 and delta2. Several groups have nominated delta1 receptors as those activated by DPDPE and blocked by BNTX or DALCE and delta2 sites as those activated by deltorphin II or DSLET and blocked by naltriben or a naltrindole derivative naltrindole 5'-isothiocyanate (Jiang et al. 1991; Mattia et al. 1991). While there has been some work to try to consolidate the similarities and differences between delta ncx/delta cx and delta1/delta2 the relationship between the two groups remains unclear. One group has shown that the delta receptors involved in modulation of mu receptors may be delta2 receptors thus suggesting that these may be delta cx (delta/mu complexes) (Porreca et al. 1992). A different group, based on binding and pharmacological evidence has proposed that in fact delta ncx and delta cx are synonymous with the delta1 and delta2 receptor subtypes, respectively (Xu et al. 1993).

Other pharmacological evidence for receptor complexes, and specifically for receptor dimers, came from the observation that dimeric analogs of opioids had greatly increased affinities for the opioid receptors. Dimeric analogs of morphine and enkephalin exhibit higher affinities for delta and mu receptors in membranes (Hazum et al. 1982) These compounds also have greatly increased potencies in mouse vas deference and guinea-pig illeum assays (Hazum et al. 1982). The dimeric nature of these compounds and their increased potencies suggests that the receptors may exist as dimeric complexes.

BIOCHEMISTRY

Perhaps the largest gap in the further development of the opioid receptor oligomerization hypothesis lay in the lack of biochemical evidence of their physical association. While there is a large amount of evidence for the dimerization of other GPCRs (for review see Devi et al.) few studies have shown the existence of opioid receptor oligomers. The first evidence of potential opioid receptor oligomerization were demonstrated in a series of elegant studies with delta receptors expressed in neuroblastoma cells (Hazum et al. 1979). Rhodamine conjugated enkephalin analogs were used to label delta receptors and it was observed that these receptors appeared as clusters on the cell surface (Hazum et al. 1979). The importance of disulfide groups in these associations was demonstrated by the ability of low concentrations of the reducing agent DTT to convert the patchy distribution of delta receptors to a uniformly diffuse phenotype (Hazum et al. 1979). Both agonists and antagonists could rapidly induce the formation of these clusters but only agonist mediated clustering could be reversed by DTT. Interestingly, the peptide agonists had to be removed prior to DTT treatment in order to reverse clustering, suggesting that the ligand may have a protective effect on the disulfide bond. Another interesting study involved the crosslinking of [125I]beta-endorphin to membranes from rat striatal patches (Schoffelmeer et al. 1990). Binding of beta-endorphin to the ~80-kDa band could be inhibited by either the mu receptor selective compound DAMGO or the delta receptor selective compound DSTBULET. This led the authors to conclude that beta-endorphin was cross-linked to a delta/mu receptor complex. However it is unclear as to whether the 80-kDa band does indeed represent a mu/delta receptor complex bound by beta-endorphin or if it represents beta-endorphin bound to either mu or delta receptors. Perhaps the first direct evidence of dimerization of opioid receptors came following the cloning of opioid receptor cDNAs which allowed the heterologous expression of these receptors in a variety of cell lines and detailed analysis (the details of which are described below). Antisera directed against the endogenous mu receptor detects high molecular forms of the mu receptor in bovine brain; this has been suggested to represent mu receptor dimers (Garzon et al. 1995).

RECENT ADVANCES

Using crosslinking agents, we showed that the delta opioid receptor exists as dimers when expressed in heterologous cells (Cvejic and Devi 1997). On polyacrylamide gels, a band of approximately twice the molecular weight size expected for the delta receptor was observed. This band was confirmed to be the dimeric species of the receptor when a flag-epitope tagged delta receptor could be co-immunoprecipitated by immunoprecipitating a myc-epitope tagged delta receptor. We have also demonstrated that kappa receptors exist as dimers (Jordan and Devi 1999). A significant difference between the two dimers was observed in their stability to SDS. While delta receptor dimers were unstable in SDS and required a crosslinking agent, kappa receptor dimers were SDS stable (Figure 1). A monomerization of delta receptor dimers was observed in the presence of delta receptor selective agonists (Cvejic and Devi 1997). Interestingly, this monomerization preceded rapid agonist mediated sequestration of receptors providing a possible functional correlate between dimers and monomers and receptor sequestration. While monomerization might lead to internalization, it did not require internalization as either a treatment with sucrose or potassium depleted medium (that block clathrin coated pit formation) could not prevent receptor monomerization (Figure 2). On the other hand, in the case of kappa receptors, selective ligands were unable to modulate the levels of receptor dimers. Kappa receptors were shown to be stable in up to 10% SDS (Figure 3A) and the level of dimers was not dependent on the level of receptor expression since it is observed in cells expressing low ($<1 \times 10^5$), medium ($\sim5 \times 10^5$) or high ($>10^6$) number of receptors per cell (Figure 3B). The SDS stability of kappa receptor dimers suggested that covalent bonds may mediate this interaction. We found that these dimers are sensitive to reducing agents suggesting that disulfide bonds mediate these interactions (Figure 4).
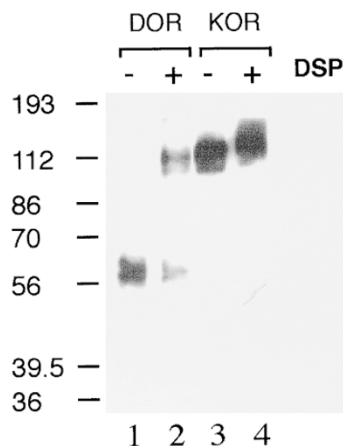

Figure 1. Characteristics of the kappa and delta homodimers. CHO cells expressing flag-tagged mouse delta or rat kappa receptors were treated with or without 2.5 mM DSP (crosslinking agent) and analyzed by SDS-PAGE followed by Western blotting as described previously (Jordan and Devi 1999). Delta receptors require crosslinking to enable visualization of the dimeric (~120 kDa) form (lanes 1 and 2). In contrast crosslinking is not required to observe the kappa receptor dimers (~130 kDa) (lanes 3 and 4).
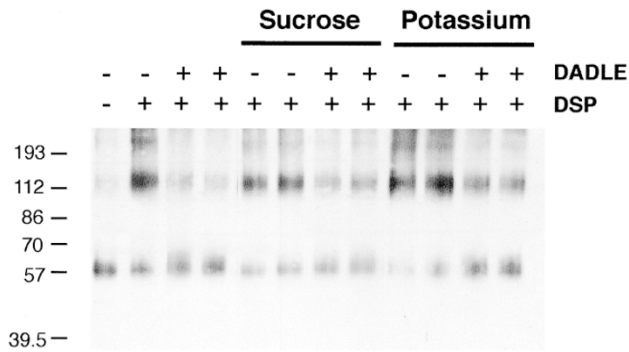
Figure 2. Inhibition of clathrin mediated endocytosis does not affect the agonist induced decrease in receptor dimerization. CHO cells expressing delta receptors were incubated in fresh media (control), 0.45 M sucrose (sucrose) or potassium-depleted medium (K$^+$ depletion) prior to agonist treatment. Cells were then treated for 30 min in the absense ($-$) or presence ($+$) of 100 nM DADLE. Each treatment (except lanes 1 and 2) is presented in duplicates. Following the treatment with ligand, cells were chilled, treated with 5 mM DSP and lysed. The lysates were subjected to SDS-PAGE under non-reducing conditions and to Western blotting.

Several studies discussed above had suggested functional and pharmacological interactions between delta and mu receptor types and also for kappa/delta and kappa/mu interactions (Dawson-Basoa and Gintzler 1998; Demoliou-Mason and Barnard 1986a, 1986b; Garzon et al. 1982; Miaskowski et al. 1990, 1993; Sheldon et al. 1989). Our work had identified that either kappa or
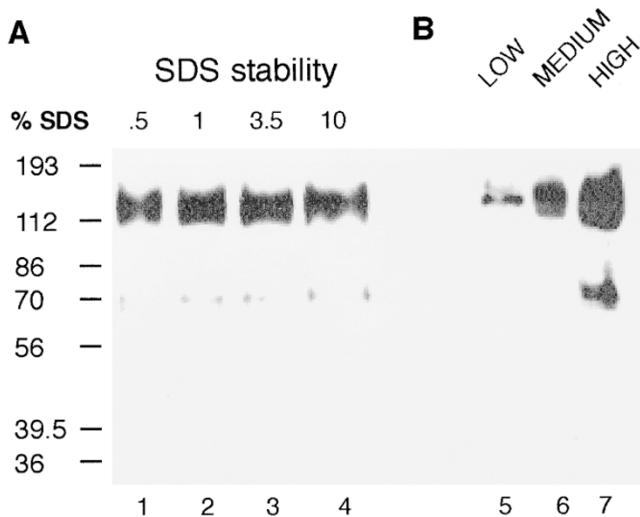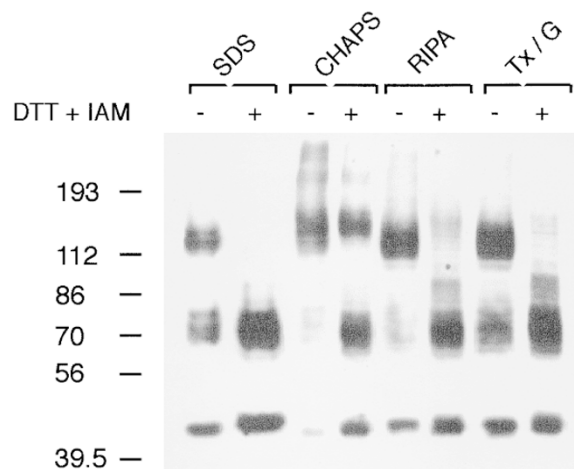

Figure 4. Kappa dimers are sensitive to DTT when solubilized in different detergents. **(A)** Cells expressing flag-tagged kappa receptors were plated in 6-well plates, treated with DTT (1 mM, 30 min, 37°C) followed by IAM (5 mM, 30 min, 37°C) and subsequently lysed in the buffers shown above: (SDS, 2% SDS in 50 mM Tris pH 6.8; CHAPS, 0.5% CHAPS in 50 mM Sodium Phosphate; RIPA, 1% NP-40, 0.5% SDS, 0.5% DOC, 100 mM NaCl in pH 8.0 Tris buffer; Tx/G- 1% Triton X-100, 10% Glycerol, 1 mM MgCl$_2$, 1 mM CaCl$_2$ 100 mM NaCl). 10 ug of total protein were then subjected to SDS-electrophoresis. Under all conditions, KOR is a dimer and exhibits DTT sensitivity.

Figure 3. Characteristics of kappa homodimers in CHO cells expressing flag-tagged kappa receptors. **(A)** Lanes 1–4: the ~130-kD molecular weight band demonstrates stability in up to 10% SDS. A lower ~65-kD band is also observed. **(B)** Lanes 5–7: the ~130-kD band is present in cells exhibiting different levels of expression (low = 2×10^5, medium = 5×10^5 and high = $>1 \times 10^6$ receptors per cell). The higher the levels of expression, the more of the ~65-kD band was observed.

delta receptors could form homodimers. We thus examined if kappa receptors could heterodimerize with either delta or mu receptors using differential epitope tagging followed by immunoprecipitation and Western blotting. We find that kappa receptors could heterodimerize with delta, but not with mu receptors (Jordan and Devi 1999). The biochemical nature of this interaction was examined and found to be similar to that of kappa receptor homodimers (i.e., kappa/delta heterodimers were SDS stable and sensitive to reducing agents, Jordan and Devi 1999). Heterodimers were also stable in a variety of detergents and were shown not to be artifacts of solubilization or extraction conditions. Interestingly, ligand binding in membranes from cells co-expressing kappa and delta receptors revealed opioid receptors sites that were distinct from either kappa or delta receptors (Figure 5A) (Jordan and Devi 1999). These sites could be labelled using lower concentrations of the radiolabeled nonselective opioid antagonist diprenorphine (Figure 5A). Interestingly, these sites were insensitive to kappa or delta receptor selective agonists and sensitive to the nonselective ligands such as bremazocine, ethylketocyclazocine or dynorphin A (Figure 5B). Remarkably a sensitivity to the selective ligands was restored only when both ligands (the kappa selective ligand and the delta selective ligand) were present in the binding assay, suggesting cooperativity in binding (Figure 6). Our results may explain the
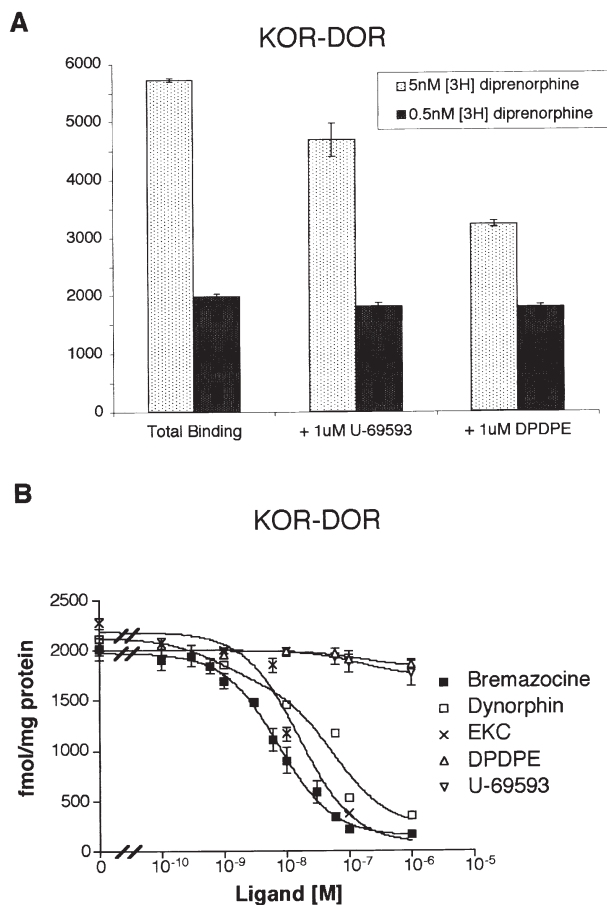
A

B

Figure 5. Displacement of [³H] diprenorphine from membranes expressing kappa-delta heterodimers (KOR-DOR). **(A)** Membranes from cells expressing kappa-delta heterodimers (10–15 ug of total protein) were labeled with either 5 nM or 0.5 nM [³H] diprenorphine and displaced by cold ligands as indicated. Note that 0.5 nM [³H] diprenorphine selectively labels a population of receptors that is insensitive to either DPDPE or U-69593; ~35% of total sites are not sensitive to either DPDPE or U-69593. Background values (approximately 450 fmols/mg protein when 5 nM diprenorphine is used and 50 fmols/mg protein when 0.5 nM diprenorphine is used) have been subtracted. This data represents the average ± S.E.M. from three independent experiments. **(B)** Displacement of [³H] diprenorphine binding by bremazocine, dynorphin, ethyketocyclazocine (EKC), DPDPE and U-69593 in cells expressing kappa-delta receptors. Membranes from cells expressing kappa-delta heterodimers were incubated with 0.5 nM [³H] diprenorphine. All three partially selective compounds (bremazocine, dynorphin and EKC) were able to displace diprenorphine binding in its entirety and with high affinities Ki 2 ± .34 nM for bremazocine, 11 ± 1 nM for dynorphin and 5 ± 1 nM for EKC. In contrast, neither DPDPE nor U-69593 are able to displace bound diprenorphine, even at a 1 uM concentration. This data represents the average ± S.E.M. from three independent experiments.

functional synergy observed *in vivo* between ligands selective for different opioid receptor types.

A functional correlate for our biochemical and pharmacological evidence was observed in the trafficking of these receptors and in their signaling properties (Jordan and Devi 1999). Delta receptors but not kappa receptors are known to rapidly internalize in response to universal opioid agonists such as, etorphine (Chu et al. 1997; Jordan et al. 2000; Li et al. 1999). Delta receptors in cells expressing kappa/delta heterodimers were unable to internalize in response to etorphine suggesting that heterodimerization affects the trafficking of these receptors (Jordan and Devi 1999). We also observed that in cells co-expressing kappa and delta receptors, stimulation of cells with selective agonists for both kappa and delta receptors resulted in a significant leftward shift in a dose-dependent inhibition of adenylyl cyclase when compared to either ligand alone (Jordan and Devi 1999). A similar potentiation of function was observed when the ability of a single agonist or a combination of agonists to affect the extent of mitogen activated protein kinase phosphorylation was examined (Figure 7). Therefore a cooperative and synergistic effect was observed in pharmacological and functional assays via receptor heterodimerization which may explain the observations *in vivo*.

ANATOMICAL DISTRIBUTION

The functional synergy and pharmacological evidence to suggest that a population of delta receptors interacts with a population of mu receptors has mostly been performed *in vivo* or in membranes prepared from tissues. It is therefore perhaps the best evidence that receptor interactions do occur within the organism. A demonstration of colocalization of two different receptor types within the same cell, and perhaps at an ultrastructural level would also be important to support this hypothesis. There are several lines of evidence to suggest that mu receptors and delta receptors exists within the same cell. Both by binding studies on dorsal root ganglia and measurement of the frequency of action potential firing in single neurons it has been shown that both receptors co-localize in a cell (Egan and North 1981; Fields et al. 1980; Zieglgansberger et al. 1982). There are also several different neuroblastomas which co-express delta and mu receptors (Kazmi and Mishra 1987; Palazzi et al. 1996; Polastron et al. 1994; Yu et al. 1986; Agarwal and Glasel 1993) or express all three opioid receptors (Baumhaker et al. 1993). The evidence at an ultrastructural level has been scarce. One group has shown that delta receptor immunoreactivity is observed in the plasmallema alongside mu receptor immunoreactivity (Cheng et al. 1997). However there are regions where distinct cellular localization was apparent. Only delta receptors were shown to be localized in large dense core vescicles (Cheng et al. 1997). From this evi-
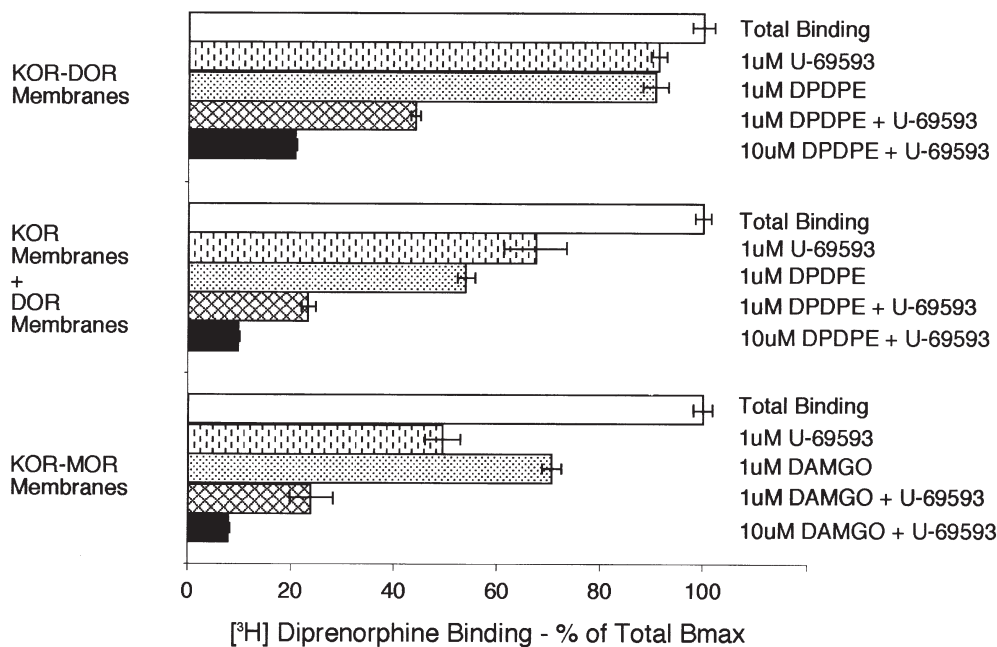
Figure 6. Characterization of kappa-delta receptor pharmacology. Displacement of [³H] diprenorphine binding by various selective agonists. Membranes from cells expressing kappa-delta receptors (KOR-DOR, top panel), a mixture of cells expressing kappa receptors (KORs) alone and delta receptors (DORs) alone (middle panel) or from cells expressing kappa-mu receptors (KOR-MOR, bottom panel) were labeled with 0.5 nM [³H] diprenorphine in the absence or the presence of the indicated ligands. Notice that the addition of 1uM U-69593 potentiates DPDPE binding and vice-a-versa only in cells expressing kappa-delta receptors. In contrast, in the mixture of membranes from cells expressing kappa receptor alone and delta receptors alone, there is no potentiation of this effect. Also, in cells expressing kappa-mu receptors there is no potentiation of binding between U-69593 and DAMGO.

dence it is apparent that there is a possibility for receptor oligomerization based on location.

PUTTING IT ALL TOGETHER

Delta receptor subtypes were not the only subtypes found. Subclassifications of kappa receptors and mu receptors have also been proposed (Audiger et al. 1982; Gintzler and Pasternak 1983; Nock et al. 1988; Zukin et al. 1988). Our observation that kappa receptors can heterodimerize with delta receptors prompted us to compare these novel binding sites with previously described kappa or delta receptor subtypes. Kappa/delta receptor complexes were seen to bind benzomorphans such as bremazocine and EKC with high affinity, but were unable to bind the highly selective kappa receptor agonist U69,593 (Figure 5). Not surprisingly, these characteristics make them pharmacologically identical to previously described kappa2 receptor subtypes described in guinea pig brain (Audiger et al. 1982; Nock et al. 1988; Zukin et al. 1988). Together with the evidence that delta/mu receptor complexes are in fact the delta2 subtypes described via functional and pharmacological studies, we believe that receptor subtypes may in fact be different opioid receptor complexes. It would clarify not only the lack of cDNAs for

opioid receptor subtypes but also, for example, why delta receptor knockout mice have lost both delta1 and delta2 receptor binding (Zhu et al. 1999). Therefore receptor oligomerization, of which dimers are suggested to play a prominent role, may represent the "complex" pharmacological nature of opioid receptor binding observed by many in brain membranes. Receptor subtypes have also been observed by selectively reducing the expression and function of opioid receptors using antisense oligonucleotides injected *in vivo*. Surprisingly, an injection of such molecules directed against the delta receptor results in a loss of deltorphin II mediated effect, but a retention of DPDPE induced effects (Sanchez-Blazquez et al. 1997). These results were also observed in mice in which exon-2 of mouse DOR was deleted (Zhu et al. 1999).

Functional synergy has also been observed in cells co-expressing opioid receptors which suggests again that this phenomenon might be attributed to receptor dimerization. We have been able to reproduce synergistic interactions in heterologous cells that do not endogenously express any opioid receptors. Ligand binding assays demonstrate a whole "new" population of opioid receptors in cells co-expressing kappa and delta receptors which can be activated in the presence of both ligands. Thus the functional synergy observed here might represent that observed *in vivo*. In preliminary
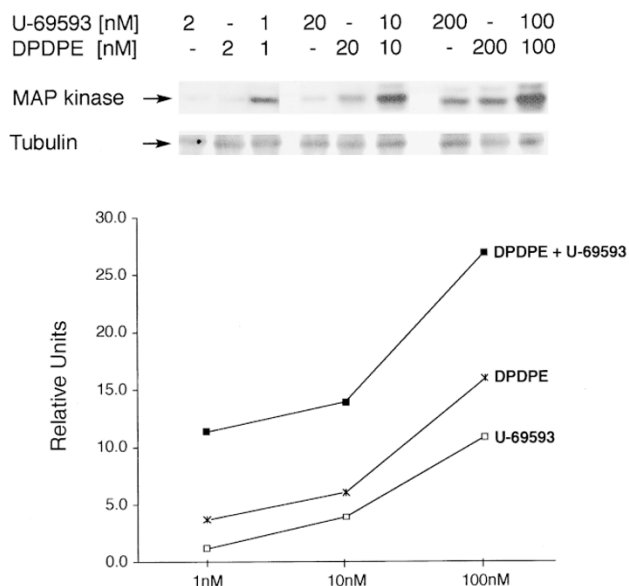
Figure 7. Synergistic activity on phosphorylation of mitogen activated protein kinase (MAP kinase). Cells expressing kappa-delta receptors were treated with U-69593 or DPDPE alone, or in combination, at the indicated concentrations followed by analysis by SDS-PAGE and Western blotting with Ab against phoshphorylated MAP kinase (New England Biolabs) as described previously (Jordan and Devi 1999). A large potentiation of map kinase phosphorylation was observed only when both ligands were present. Tubulin antibody was used to standardize loading (~5 ug). Shown below is a dose-dependance of this synergistic interaction obtained by quantification of phospho MAP kinase immunoreactivity. NIH Image 1.61 software was used to densitize and quantify the amount of phosphorylated map kinase from scaned film. The Y-axis represents the ratio of phophorylated map kinase over tubulin. The amount of phospho map kinase in cells treated with both ligands is greater than the sum of the level of phospho MAP kinase in DPDPE treated cells plus U-69593 treated cells. This is a representative of five independent experiments.

studies we find that delta and mu receptors are also able to form heterodimers and that they exhibit pharmacological and functional interactions; this could support previous work in this area.

Heyman and colleagues (Heyman et al. 1989) has suggested that receptor complexing may explain the puzzling lack of correlation between receptor affinity and potency for some compounds. Compounds such as DADLE, which have been shown to have similar affinities for the mu receptor as some mu receptor selective compounds, have a several-fold higher potency than others. The concept of "self-modulation" was used to describe compounds that could bind to "both" components of the delta/mu receptor complex and thus, by itself, create the allosteric interactions resulting in functional synergy (Heyman et al. 1989). Thus, a "self modulating" compound would perform a similar function as a mu receptor

selective compound, that is unable to bind the complex unless in the presence of a delta receptor selective compound. The novel compound DIPPψ would certainly fall under the description of a "self modulating" compound given its mixed mu agonist/delta antagonist properties (Schiller et al. 1999). It is therefore evident that only certain compounds are able to create synergistic effects; only some delta selective ligands are able to synergize with morphine but not DAMGO or sufentanyl (Heyman et al. 1989). It is therefore likely that receptors in a complex have altered pharmacological properties stemming from changes in their tertiary structure due to the complexing.

The significance of dimerization of G-protein coupled receptors has been gaining strength and while much work remains to be done, it is clear that understanding it will have an enormous impact on a wide variety of fields. New methodologies are being developed such as Fluorescent Resonant Energy Transfer, which have already demonstrated the existence of G-protein coupled receptor dimers *in vivo* and somatostatin receptor dimers and heterodimers in heterologous cells and brain tissue (Rocheville et al. 2000). While heterodimers illustrate a mechanism for the activation of receptors upon the co-release of selective endogenous peptides, as suggested by our work, they may also represent bona-fide receptors with a unique pharmacological profile for the endogenous opioid peptide family. The endogenous opioid ligands are far more numerous and notably redundant for the existing cloned opioid receptors. Thus, the existence of a large number of opioid-related receptors via heterodimerization with other GPCRs may explain both the large number of opioid receptor subtypes and the endogenous opioid ligands. There is long standing evidence of opioid receptor interactions with adenosine and perhaps adrenergic receptors (Aley and Levine 1997). It is possible that these may be mediated by a novel cross-family receptor heterodimerization. The heterodimerization of G-protein coupled receptors has enormous ramifications since it represents another mechanism that could modulate receptor function and points to additional targets for the development of drug therapies.

ACKNOWLEDGMENTS

REFERENCES

Agarwal D, Glasel J (1993): Co-localization of mu and delta opioid receptors on SK-N-SH cells detected by fluorescence microscopy using labeled anti-idiotypic antibodies. Life Sci 52:193–198

Aley K, Levine J (1997): Multiple receptors involved in peripheral alpha 2, mu, and A1 antinociception, tolerance, and withdrawal. J Neurosci 17:735–744

Audiger Y, Attali B, Mazsarguil H, Cros J (1982): Characterization of [3H]-etorphine binding in guinea pig striatum after blockade of mu and delta sites. Life Sci 31:1287–1290

Barrett R, Vaught J (1982): The effects of receptor selective opioid peptides on morphine-induced analgesia. Eur J Pharmacol 80:427–430

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

Biscoe T, Duggan A, Lodge D (1972): Effect of etorphine, morphine and diprenorphine on neurones of the cerebral cortex and spinal cord of the rat. Br J Pharmacol 46:201–212

Blane G, Boura A, Fitzgerald A, Lister R (1967): Actions of etorphine hydrochloride, (M99): a potent morphine-like agent. Br J Pharmacol 30:11–22

Bowen W, Gentleman S, Herkenham M, Pert C (1981): Interconverting mu and delta forms of the opiate receptor in rat striatal patches. Proc Natl Acad Sci USA 78:4818–4822

Bradbury A, Smyth D, Snell C, Birdsall N, Hulme E (1976): C-fragment of lipoprotein has a high affinity for brain opiate receptors. Nature (London) 260:793–795

Brown S, Stimmel B, Taub R, Kochwa S, Rosenfield R (1974): Immulogic dysfunction in heroin addicts. Arch Intern Med 134:1001–1014

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

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

Cheng P, Liu-Chen L, Pickel V (1997): Dual ultrastructural immunocytochemical labeling of mu and delta opioid receptors in the superficial layers of the rat cervical spinal cord. Brain Res 778:367–380

Chu P, Murray S, Lissin D, von Zastrow M (1997): Delta and kappa opioid receptors are differentially regulated by dynamin-dependent endocytosis when activated by the same alkaloid agonist. J Biol Chem 272:27124–27130

Cotton R, Giles M, Miller L, Shaw J, Timms D (1984): ICI 174864: a highly selective antagonist for the opioid delta-receptor. Eur J Pharmacol 97:331–332

Cox B, Goldstein A, Li C (1976): Opioid activity of a peptide, beta-endorphin (61-91), derived from beta-lipotropin. Proc Natl Acad Sci USA 73:1821–1823

Cvejic S, Devi L (1997): Dimerization of the δ opioid receptor: implication for a role in receptor internalization. J Biol Chem 272:26959–26964

D'Amato R, Holaday J (1984): Multiple opioid receptors in endotoxic shock: evidence for delta involvement and mu-delta interactions in vivo. Proc Natl Acad Sci USA 81:2898–2901

Dawson-Basoa M, Gintzler A (1998): Gestational and ovarian sex steroid antinociception: synergy between spinal kappa and delta opioid systems. Brain Res 794:61–67

Demoliou-Mason C, Barnard E (1986a): Characterization of opioid receptor subtypes in solution. J Neurochem 46:1129–1136

Demoliou-Mason C, Barnard E (1986b): Distinct subtypes of the opioid receptor with allosteric interactions in brain membranes. J Neurochem 46:1118–1128

Devi LA, Jordan BA, Trapaidze N, Nivarthi R, Nagy V, Gupta A, Bansinath M, Gomes I: G-protein couple receptor dimerization: a review. J Mol Med (in press)

Dixon W, Viveros O, Unsworth C, Diliberto EJ, Way E, Lewis J, Chandra A, Holaday J, Long J, Tortella F (1985): Multiple opiate receptors: functional implications. Fed Proc 44:2851–2862

Egan T, North R (1981): Both mu and delta opiate receptors exist on the same neuron. Science 214:923–924

Erspamer V, Melchiorri P, Falconieri-Erspamer G, Negri L, Corsi R, Severini C, Barra D, Simmaco M, Kreil G (1989): Deltorphins: a family of naturally occurring peptides with high affinity and selectivity for delta opioid binding sites. Proc Natl Acad Sci USA 86:5188–5192

Evans C, Keith D Jr, Morrison H, Magendzo K, Edwards R (1992): Cloning of a delta opioid receptor by functional expression. Science 258:1952–1955

Fields H, Emson P, Leigh B, Gilbert R, Iversen L (1980): Multiple opiate receptor sites on primary afferent fibres. Nature 284:351–353

Gacel G, Fournie-Zaluski M, Roques B (1980): D-Tyr–Ser–Gly–Phe–Leu–Thr, a highly preferential ligand for delta-opiate receptors. FEBS Lett 118:245–247

Garzon J, Jen M, Sanchez-Blazquez P, Lee N (1982): Dynorphin(1-13), a long-lasting inhibitor of opiate receptor binding in vitro. Life Sci 31:1789–1792

Garzon J, Juarros J, Castro M, Sanchez-Blazquez P (1995): Antibodies to the cloned mu-opioid receptor detect various molecular weight forms in areas of mouse brain. Mol Pharmacol 47:738–744

Genazzani A, Petraglia F (1989): Opioid control of leutinizing hormone secretion in humans. J Steroid Biochem 33:751–755

Gintzler A, Pasternak G (1983): Multiple mu receptors: evidence for mu2 sites in the guinea pig ileum. Neurosci Lett 39:51–56

Goldstein A, Fischli W, Lowney L, Hunkapiller M, Hood L (1981): Porcine pituitary dynorphin: complete amino acid sequence of the biologically active heptadecapeptide. Proc Natl Acad Sci USA 78:7219–7223

Handa B, Land A, Lord J, Morgan B, Rance M, Smith C (1981): Analogues of beta-LPH61-64 possessing selective agonist activity at mu-opiate receptors. Eur J Pharmacol 70:531–540

Hazum E, Chang K, Cuatrecasas P (1979): Role of disulphide and sulphydryl groups in clustering of enkephalin receptors in neuroblastoma cells. Nature 282:626–628

Hazum E, Chang K, Leighton H, Lever O Jr, Cuatrecasas P (1982): Increased biological activity of dimers of oxymorphone and enkephalin: possible role of receptor crosslinking. Biochem Biophys Res Commun 104:347–353

Herz A (1993): Opioids. Berlin, Springer-Verlag, Vol. 1

Heyman J, Vaught J, Mosberg H, Haaseth R, Porreca F

(1989): Modulation of mu-mediated antinociception by delta agonists in the mouse: selective potentiation of morphine and normorphine by [D-Pen2,D-Pen5]enkephalin. Eur J Pharmacol 165:1–10

Hughes J, Smith T, Kosterlitz H, Forthergill L, Morgan B, Morris H (1975): Identification of two related pentapeptides from the brain with potent opiate agonist activity. Nature (London) 258:577–579

Jiang Q, Bowen W, Mosberg H, Rothman R, Porreca F (1990a): Opioid agonist and antagonist antinociceptive properties of [D-Ala2,Leu5,Cys6]enkephalin: selective actions at the deltanoncomplexed site. J Pharmacol Exp Ther 255:636–641

Jiang Q, Mosberg H, Porreca F (1990b): Modulation of the potency and efficacy of mu-mediated antinociception by delta agonists in the mouse. J Pharmacol Exp Ther 254:683–689

Jiang Q, Mosberg H, Porreca F (1990c): Selective modulation of morphine antinociception, but not development of tolerance, by delta receptor agonists. Eur J Pharmacol 186:137–141

Jiang Q, Takemori A, Sultana M, Portoghese P, Bowen W, Mosberg H, Porreca F (1991): Differential antagonism of opioid delta antinociception by [D-Ala2,Leu5,Cys6]-enkephalin and naltrindole 5′-isothiocyanate: evidence for delta receptor subtypes. J Pharmacol Exp Ther 257: 1069–1075

Jordan B, Cvejic S, Devi L (2000): Kappa opioid receptor endocytosis by dynorphin peptides. DNA Cell Biol 19:19–27

Jordan B, Devi L (1999): G-protein coupled receptor heterodimerization modulates receptor function. Nature 399:697–700

Kakidani H, Furutani Y, Takahashi H, Noda M, Morimoto Y, Hirose T, Asai M, Inayama S, Nakanishi S, Numa S (1982): Cloning and sequence analysis of cDNA for porcine β-neo-endorphin/dynorphin precursor. Nature (London) 298:245–249

Kamei J, Tanihara H, Kasuya Y (1991): Modulation of mu-mediated antitussive activity in rats by a delta agonist. Eur J Pharmacol 203:153–156

Kazmi S, Mishra R (1987): Comparative pharmacological properties and functional coupling of mu and delta opioid receptor sites in human neuroblastoma SH-SY5Y cells. Mol Pharmacol 32:109–118

Kieffer B, Befort K, Gaveriaux-Ruff C, Hirth C (1992): The delta-opioid receptor: isolation of a cDNA by expression cloning and pharmacological characterization. Proc Natl Acad Sci USA 89:12048–12052

Kosterlitz H, Lord J, Paterson S, Waterfield A (1980): Effects of changes in the structure of enkephalins and of narcotic analgesic drugs on their interactions with mu- and delta-receptors. Br J Pharmacol 68:333–342

Lahti R, Mickelson M, McCall J, Von Voigtlander P (1985): [3H]U-69593 a highly selective ligand for the opioid kappa receptor. Eur J Pharmacol 109:281–284

Lee N, Leybin L, Chang J, Loh H (1980): Opiate and peptide interaction: effect of enkephalins on morphine analgesia. Eur J Pharmacol 68:181–185

Li J-G, Luo L-Y, Krupnick J, Benovic J, Liu-Chen L (1999): U50-488H-induced internalization of the human K opioid receptor involves B-arrestin and dynamin-dependent mechanism. J Biol Chem 274:12087–12094

Lord J, Waterfield A, Hughes J, Kosterlitz H (1977): Endogenous opioid peptides: multiple agonists and receptors. Nature 267:495–499

MacDonald RL, Nelson PG (1978): Specific-opiate-induced depression of transmitter release from dorsal root ganglion cells in culture. Science 199:1449–1451

Maggi R, Pimpinelli F, Martini L, Piva F (1995): Inhibition of leutinizing hormone-releasing hormone secretion by delta-opioid agonists in GT1-1 neuronal cells. Endocrinology 136:5177–5181

Martin W, Eades C, Thompson J, Huppler R, Gilbert P (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

Matthes H, Maldonado R, Simonin F, Valverde O, Slowe S, Kitchen I, Befort K, Dierich A, Le Meur M, Dolle P, Tzavara E, Hanoune J, Roques B, Kieffer B (1996): Loss of morphine-induced analgesia, reward effect and withdrawal symptoms in mice lacking the mu-opioid-receptor gene. Nature 383:819–823

Mattia A, Vanderah T, Mosberg H, Porreca F (1991): Lack of antinociceptive 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

Miaskowski C, Taiwo Y, Levine J (1990): Kappa- and delta-opioid agonists synergize to produce potent analgesia. Brain Res 509:165–168

Miaskowski C, Taiwo Y, Levine J (1993): Antinociception produced by receptor selective opioids. Modulation of supraspinal antinociceptive effects by spinal opioids. Brain Res 608:87–94

Mosberg H, Hurst R, Hruby V, Gee K, Yamamura H, Galligan J, Burks T (1983): Bis-penicillamine enkephalins possess highly improved specificity toward delta opioid receptors. Proc Natl Acad Sci USA 80:5871–5874

Mudge A, Leeman SE, Fischbach GD (1979): Enkephalin inhibits the release of substance P from sensory neurons in culture and decreases action potential duration. Proc Natl Acad Sci USA 76:526–530

Nakanishi S, Inoue A, Kita T, Nakamura M, Chang A, Cohen S, Numa S (1979): Nucleotide sequence of cloned cDNA for bovine corticotropin-beta-lipotropin precursor. Nature (London) 278:423–427

Nock B, Rajpara A, O'Connor L, Cicero T (1988): [3H]U-69593 labels a subtype of kappa opiate receptor with characteristics different from that labeled by [3H]ethylketocyclazocine. Life Sci 42:2403–2412

Noda M, Furutani Y, Takahashi H, Toyosato M, Hirose T, Inayama S, Nakanishi S, Numa S (1982): Cloning and sequence analysis of cDNA for bovine adrenal preproenkephalin. Nature (London) 295:202–206

Palazzi E, Ceppi E, Guglielmetti F, Catozzi L, Amoroso D, Groppetti A (1996): Biochemical evidence of functional interaction between mu- and delta-opioid receptors in SK-N-BE neuroblastoma cell line. J Neurochem 67:138–144

Pasternak G, Simantov R, Snyder S (1976): Characterization of an endogenous morphine-like factor (enkephalin) in mammalian brain. Mol Pharmacol 12:504–513

Paterson S, Robson L, Kosterlitz H (1983): Classification of opioid receptors. Br Med Bull 39:31–36

Pert C, Snyder S (1973): Opiate receptor: demonstration in nervous tissue. Science 179:1011–1014

Polastron J, Mur M, Mazarguil H, Puget A, Meunier J, Jauzac P (1994): SK-N-BE: a human neuroblastoma cell line containing two subtypes of delta-opioid receptors. J Neurochem 62:898–906

Porreca F, Takemori A, Sultana M, Portoghese P, Bowen W, Mosberg H (1992): Modulation of mu-mediated anti-nociception in the mouse involves opioid delta-2 receptors. J Pharmacol Exp Ther 263:147–152

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

Portoghese P, Sultana M, Nagase H, Takemori A (1992a): A highly selective delta 1-opioid receptor antagonist: 7-benzylidenenaltrexone. Eur J Pharmacol 218:195–196

Portoghese P, Sultana M, Nelson W, Klein P, Takemori A (1992b): Delta opioid antagonist activity and binding studies of regioisomeric isothiocyanate derivatives of naltrindole: evidence for delta receptor subtypes. J Med Chem 35:4086–4091

Portoghese P, Sultana M, Takemori A (1988): Naltrindole, a highly selective and potent non-peptide delta opioid receptor antagonist. Eur J Pharmacol 146:185–186

Quirion R, Bowen W, Herkenham M, Pert C (1982): Visualization and solubilization of rat brain opiate receptors with a "kappa" ligand selectivity pattern. Cell Mol Neurobiol 1982:333–346

Rice K, Jacobson A, Burke TJ, Bajwa B, Streaty R, Klee W (1983): Irreversible ligands with high selectivity toward delta and mu opiate receptors. Science 220:314–316

Rocheville M, Lange D, Kumar U, Sasi R, Patel R, Patel Y (2000): Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers. J Biol Chem 275:7862–7869

Romer D, Buscher H, Hill R, Maurer R, Petcher T, Welle H, Bakel H, Akkerman A (1980): Bremazocine: a potent, long-acting opiate kappa-agonist. Life Sci 27:971–978

Rothman R, Danks J, Pert C, Jacobson A, Burke TJ, Rice K (1984): Ionic conditions differentially affect 3H-DADL binding to type-I and type-II opiate delta receptors in vitro. Neuropeptides 4:261–268

Rothman R, Westfall T (1981): Allosteric modulation by leucine-enkephalin of [3H]naloxone binding in rat brain. Eur J Pharmacol 72:365–368

Rothman R, Westfall T (1982): Allosteric coupling between morphine and enkephalin receptors in vitro. Mol Pharmacol 21:548–557

Roy S, Loh H (1996): Effects of opioids on the immune system. Neurochem Res 21:1375–1386

Sanchez-Blazquez P, Garcia-Espana A, Garzon J (1997): Antisense oligodeoxynucleotides to opioid mu and delta receptors reduced morphine dependence in mice: role of delta-2 opioid receptors. J Pharmacol Exp Ther 280:1423–1431

Schafer M, Martin R (1994): Opioid peptides in the pituitary: a hormone, a panacrine modulator and a peptide in search of function. Biol Chem Hoppe Seyler 375:737–740

Schiller P, Fundytus M, Merovitz L, Weltrowska G, Nguyen T, Lemieux C, Chung N, Coderre T (1999): The opioid mu agonist/delta antagonist DIPP-NH(2)[Psi] produces a potent analgesic effect, no physical dependence, and less tolerance than morphine in rats. J Med Chem 42:3520–3526

Schoffelmeer A, Yao Y, Gioannini T, Hiller J, Ofri D, Roques B, Simon E (1990): Cross-linking of human [125I]beta-endorphin to opioid receptors in rat striatal membranes: biochemical evidence for the existence of a mu/delta opioid receptor complex. J Pharmacol Exp Ther 253:419–426

Sheldon R, Nunan L, Porreca F (1989): Differential modulation by [D-Pen2, D-Pen5]enkephalin and dynorphin A-(1-17) of the inhibitory bladder motility effects of selected mu agonists in vivo. J Pharmacol Exp Ther 249:462–469

Shimohigashi Y, Takano Y, Kamiya H, Costa T, Herz A, Stammer C (1988): A highly selective ligand for brain delta opiate receptors, a cyclopropyl(E)Phe(4)-enkephalin analog, suppresses mu receptor-mediated thermal analgesia by morphine. FEBS Lett 233:289–293

Simon J, Hiller J, Edelman I (1973): Stereospecific binding of the potent narcotic analgesic 3H etorphine to rat brain homogenate. Proc Natl Acad Sci USA 70:1947–1949

Sofuoglu M, Portoghese P, Takemori A (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

Terenius L (1973): Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. Acta Phamacol Toxicol 32:317–319

Traynor J (1989): Subtypes of k-opioid receptor: fact or fiction? Trends Pharmacol Sci 10:52–53

Traynor J, Elliot J (1993): δ-opioid receptor subtypes and cross talk with μ-receptors. Trends Pharmacol Sci 14:84–85

Vaught J, Takemori A (1979): Differential effects of leucine enkephalin and methionine enkephalin on morphine-induced analgesia, acute tolerance and dependence. J Pharmacol Exp Ther 208:86–94

Xu H, Partilla J, de Costa B, Rice K, Rothman R (1993): Differential binding of opioid peptides and other drugs to two subtypes of opioid delta ncx binding sites in mouse brain: further evidence for delta receptor heterogeneity. Peptides 14:893–907

Yaksh TL (1993): New horizons in our understanding of the spinal physiology and pharmacology of pain processing. Semin Oncol 20:6–18

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

Yu V, Richards M, Sadee W (1986): A human neuroblastoma cell line expresses mu and delta opioid receptor sites. J Biol Chem 261:1065–1070

Zadina J, Hackler L, Ge L, Kastin A (1997): A potent and selective endogenous agonist for the mu-opiate receptor. Nature 386:499–502

Zhu Y, King M, Schuller A, Nitsche J, Reidl M, Elde R, Unterwald E, Pasternak G, Pintar J (1999): Retention of supraspinal delta-like analgesia and loss of morphine tolerance in delta opioid receptor knockout mice. Neuron 24:243–252

Zieglgansberger W, French E, Mercuri N, Pelayo F, Williams J (1982): Multiple opiate receptors on neurons of the mammalian central nervous system. Life Sci 31:2343–2346

Zimprich A, Simon T, Hollt V (1995): Cloning and expression of an isoform of the rat mu opioid receptor (rMOR1B) which differs in agonist induced desensitization from rMOR1. FEBS Lett 359:142–146

Zukin R, Eghbali M, Oliver D, Unterwald E, Tempel A (1988): Characterization and visualization of rat and guinea pig brain κ opioid receptors: evidence for κ1 and κ2 opioid receptors. Proc Natl Acad Sci USA 85:4061–4065