# ELUCIDATING THE ROLE OF OPIOID RECEPTOR HETEROMERS AS TARGETS FOR ANALGESIC DRUG DESIGN

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## ACKNOWLEDGEMENTS

MATHRUDEVO BHAVA, PITHRUDEVO BHAVA, ACHARYADEVO BHAVA
*I salute thee dearest Mother, Father, and Mentor*

As I near the end of this long and arduous sojourn, I would like to take a pause and reflect on how it was made possible. I think it quite apt to liken finishing a PhD to a difficult hike up a mountain. A good hike needs a challenging mountain, all the ropes and climbing gear for support, and good company to be memorable. Hence, I will take a step back and skim through a collage of memories for that wonderful, once-in-a-lifetime journey, and of the people who made it memorable in the first place.

First, let me describe the mountain. A tall and wondrous peak that would dwarf the Alps, nay, the Everest! The sun dazzles on its peak making it look like a lighthouse showing the way to the heavens. As you stand at the foothills, you will be dazzled and look up in awe! Such is the sheer stature and brilliance of my mentor, and academic father, Dr. Portoghese. Standing in the shadow of this giant, I have learned the methods of scientific exploration, that oft it is the question that may be more important than the answer. A stalwart, he leads by example on how one should be humble in the pursuit of science, to question everything, and that fact supersedes the hypothesis. His child-like exuberance and passion has been contagious and have contributed to several exhilarating conversations and discoveries. I am proud of my association with him and he will forever be a role model to me.

To have been able to come this far, I have to thank my family for being my support system. Mom, dad and Swami have always been there for me, loving, nurturing and guiding me. They created a cocoon of affection and peace in which I could fashion myself into whatever I wanted to be. As early as my tenth birthday, dad revealed to me the dichotomy of life where I got to experience an affluent birthday party and a trip to a nearby orphanage to spend time with kids my age. He taught me by experience that the simple things in life like sharing, kindness, and being true to your roots were a greater currency than any gift I could imagine. I may never know all the sacrifices they had to make to keep me going, but I am forever grateful for having such wonderful parents.

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Portoghese and helped spawn a life-long association. Indeed, all of them have been mentors and I am forever in their debt.

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No journey is memorable without a close band of fellow travelers. Several come to my mind. Erick Leggans, Sadiya Addo, John Giraldes, Ranga Balasubramaniam, Amber Onorato, Annie Jackson, Mike Peterson, David Hermanson, Katie Pietch, Irfan Javed, Matthew Metcalf, Eyup Akgun, Jignesh Doshi, and Morgan LeNour in the Department of Medicinal Chemistry have been constant companions. In the Department of Pharmacology, Andrea Yoder, Kate Cornish, Matt Bess, Kevin Allen, David Lin, Jeff Brazuinas, Tiffany Mackenzie, and Hemanth Joshi were fellow partners in crime. I am blessed to have so many wonderful people I can call friends and will always cherish the great times we spent together.

I have to especially thank my fiancé, Sonia for being my closest friend. She has uncomplainingly supported me through my myriad of emotions, failure and

success. We are both finishing our theses at the same time and I take tremendous comfort in having her love, support, and advice.

I have been fortunate to make so many associations here that everyone will be sorely missed. The life lessons that I have learnt will be instructive for the rest of my life. As I contemplate on the future, I know there are more journeys to take and people to meet. In spite of that, I know that I have to just look back at this journey, my friends, and the radiant mountain in the background to know which way is home.

## DEDICATION

**Dearest Mom, Dad and Swami,**

**None of this would have been even remotely possible without your support, guidance and love. I humbly dedicate this work to you.**

## ABSTRACT

**Ajay S. Yekkirala**

**346 words**

Opioid receptors are class A members of the G protein coupled-receptor (GPCR) superfamily. There is high amino acid homology (~60%) within the opioid receptor family that constitutes a group of four receptor types: MOP (mu), DOP (delta), KOP (kappa), and NOP (nociceptin, orphanin FQ, ORL1). Opioid receptors are present in the central nervous system and peripherally, including immune cells, and are believed to function as neuromodulators or immunomodulators. Morphine is among the best known clinically employed analgesics that activate opioid receptors.

Although the concept of opioid receptor dimers was proposed nearly 30 years ago, classical models of GPCRs, including opioid receptors, were generally based on the assumption that they are organized and function as monomers. However, in view of burgeoning evidence for the existence of heteromeric GPCRs, that includes at least twelve different heteromeric opioid receptors in cultured cells, it seems likely that constitutive oligomerization of GPCRs may be the general rule rather than the exception. Among the many heteromers reported in the opioid receptor family are mu/kappa, kappa/delta and mu/delta. However, the *in vivo* physiological and behavioral relevance for the proposed heteromers have not yet been rigorously established.

The greatest drawback in studying the signaling and trafficking properties of heteromers pertains to the lack of selective ligands targeting opioid

heteromers. It is, therefore, necessary to first develop tools that can be used as probes to address the shortcomings. Hence, we evaluated standard opioids agonists and antagonists, novel ligands synthesized in our lab, and clinically used opioid analgesics to establish a ligand selectivity profile that takes into account the existence of heteromers.

Utilizing mu-delta agonist/antagonist bivalent ligands we have provided direct evidence for bridging of opioid receptor heteromers using immunofluorescent methods. Moreover, we performed studies to elucidate how the individual protomers constituting a heteromer modulate the trafficking and functional properties of each other. The results have painted an intriguing picture suggesting that the effects are dependent on both protomer composition and the ligands used. Those studies have given us valuable information on the role of opioid heteromers in physiology, and as unique targets in drug discovery.

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## ABBREVIATIONS

| | |
|---|---|
| Å | angstroms |
| β-FNA | β-funaltrexamine |
| BNTX | benzylidenenaltrexone |
| BRET | bioluminescence resonance energy transfer |
| $CCK_B$ | cholecytokinin receptor B |
| $CCR_5$ | chemokine CC Receptor 5 |
| CHO | Chinese hamster ovary |
| CTAP | D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-$NH_2$ |
| CTOP | D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-$NH_2$ |
| DADLE | Tyr-D-Ala-Gly-Phe-D-Leu |
| DALCE | [D-$Ala^2$-D-$Leu^5$]enkephalyl-Cys |
| DAMGO | D-$Ala^2$-$MePhe^4$-$Glyol^5$ enkephalin |
| Deltorphin-II | Tyr-D-Ala-Phe-Glu-Leu-Met-Asp-$NH_2$ |
| DORKO | delta opioid receptor knockout mice |
| DPDPE | D-$Pen^2$D-$Pen^5$enkephalin |
| EKC | ethyketocyclazocine |
| FRET | fluorescence resonance energy transfer |
| GABA | γ-aminobutyric acid |
| GPCR | G protein coupled receptor |
| GPI | guinea pig ileum |
| GNTI | guanidinylnaltrindole |
| HEK | human embryonic kidney |

| | |
|---|---|
| i.c.v. | intracerebroventricular |
| i.t. | intrathecal |
| MDAN | mu-delta agonist antagonist bivalent ligands |
| $mGluR_5$ | metabotropic glutamate receptor 5 |
| MORKO | mu opioid receptor knockout mice |
| MVD | mouse vas deferens |
| NTB | naltriben |
| NTI | naltrindole |
| norBNI | norbinaltorphimine |
| TM | transmembrane |

# CHAPTER 1

## 1. CURRENT KNOWLEDGE ON OPIOID RECEPTOR HETEROMERS

### 1.1. Opioid ligands and their receptors – a brief history

It has been known for over five millennia that the alkaloids obtained from the juice of opium poppy seeds proffered analgesic and euphoric properties[1]. The various ligands in the opium alkaloid cocktail were termed 'opiates', of which morphine is the major component[2]. Decades of concerted research have expanded the list of compounds that have similar pharmacological effects, and the armamentarium as a whole is designated as 'opioid' ligands[1, 2]. Even with the advent of several classes of analgesic drugs, opioids agonists such as, morphine and codeine are the analgesics of choice to treat pain in the clinic.

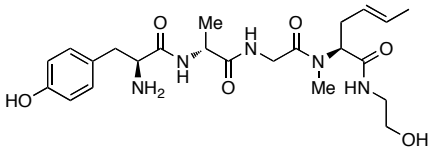
The major problems with managing pain using morphine are respiratory depression, tolerance, constipation, and physical dependence associated with its use. In 1929, it was proposed that making structural modifications to the morphine scaffold would result in a molecule devoid of the side effects while retaining its more salutary attributes[3, 4]. Unfortunately, none of the synthetic molecules showed any reduction in addictive potential. This was a considerable blow to the opioid field as the prevailing thought at the time was that it would be impossible for a non-morphine scaffold to have potent analgesic properties. Hence, the discovery that the rather simple piperidine, meperidine, could also elicit potent analgesic effects was a departure that revitalized the field[4]. In time, other analgesics such as methadone, fentanyl, morphinans and

benzomorphans were synthesized. However, the ideal opioid that would produce potent analgesia without deleterious side effects remained elusive.

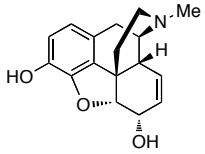
By the early 1950s, structure-activity relationship (SAR) studies with the prevailing opioid ligands suggested that ligand structure, size and shape were all important for analgesic activity. This led Beckett and Casy to first propose a unique opioid receptor that followed the lock and key mechanism to interact with opioids[5]. To simplify their hypothesis, they proposed that all opioids adopt a morphine-like structure within the receptor, which allowed for the similarity of activity for a diverse group of molecules. However, there were several anomalies that did not fit such a structurally rigid receptor[1, 4].

In an effort to address the inconsistencies in the Beckett and Casy model, Portoghese suggested an alternate hypothesis in 1965[6]. Again using SAR, he showed that parallel changes of the N-substituent of rigid scaffolds (morphine, morphinan or benzomorphan) produced similar effects on the analgesic activity. This suggested that the rigid parent structures were all interacting with the active site in a simi[1]lar fashion. However, for non-rigid scaffolds (methadone, meperidine, etc) similar modification did not produce parallel changes in activity indicating the rigid and non-rigid molecules were binding differently to the opioid receptor. This was hence called the bimodal binding model of opioids. A prescient interpretation by Portoghese was that instead of different modes of interaction with the same active site, the data also indicated the possible existence of multiple opioid receptors[6].

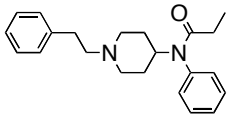
Around the same time, Goldstein and colleagues also used structural determinants in opioids to propose the existence of a unique opioid receptor[7]. As the radioligand binding assays gained prevalence[8], three independent laboratories simultaneously described the first opioid receptor sites in rat brain membranes[9-11]. Shortly thereafter, Hughes and coworkers[12] isolated the first endogenous opiate-like factors, methionine enkephalin (Met-enkephalin) and leucine enkephalin (Leu-enkephalin). The discovery of the biological aspects of the opioid system was well underway.



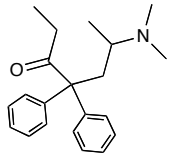

DAMGO




Morphine




Fentanyl




Methadone

**Tyr-Pro-Trp-Phe-$NH_2$**

Endomorphin-1 (EM-1)

**Tyr-Pro-Phe-Phe-$NH_2$**

Endomorphin-2 (EM-2)

**Figure 1.1. Selective mu agonists**

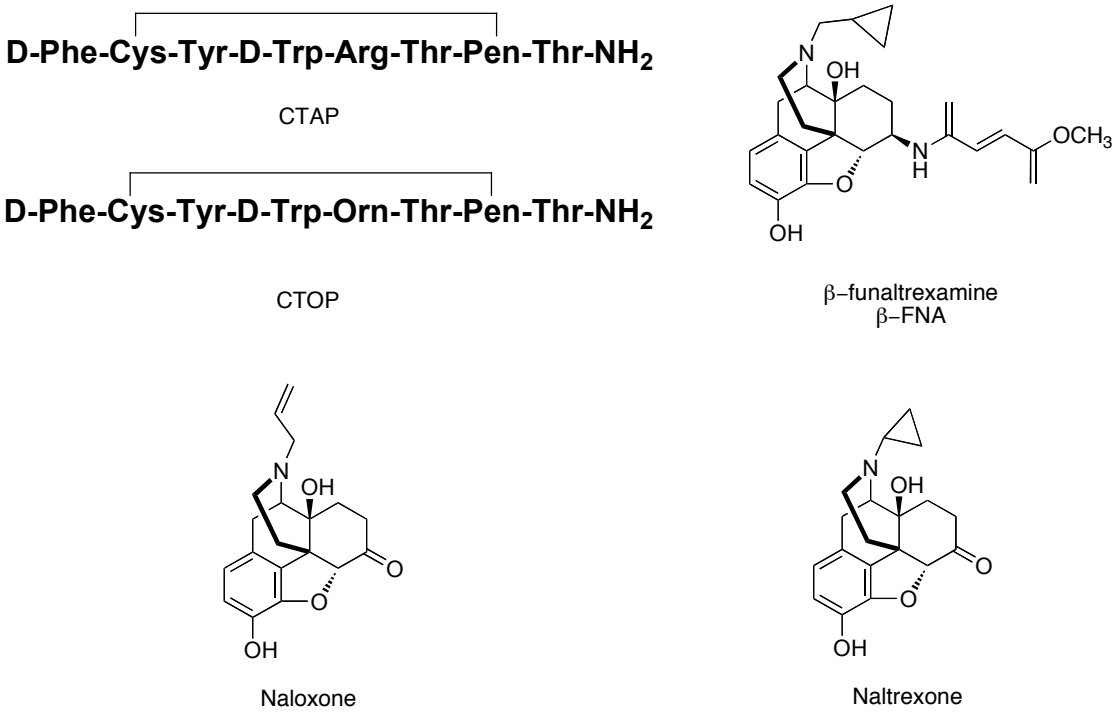
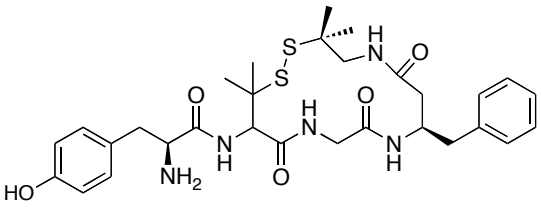
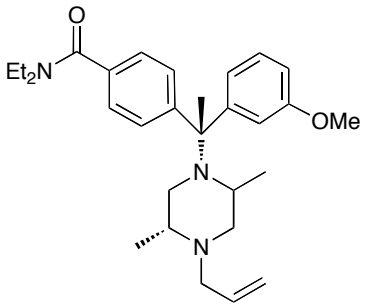



**Figure 1.2 Selective mu antagonists**



DPDPE



SNC80

**Tyr-D-Ala-Phe-Glu-Leu-Met-Asp-$NH_2$**

Deltorphin-II
DELT-II

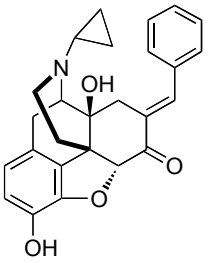
**Tyr-Gly-Gly-Phe-Leu**

[$Leu^5$]Enkephalin
L-enk

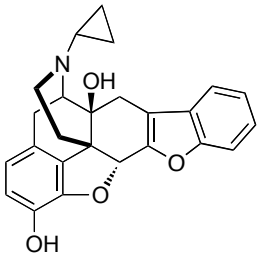
**Tyr-Gly-Gly-Phe-Met**

[$Met^5$]Enkephalin
M-enk

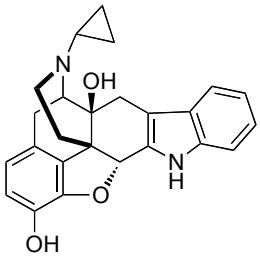
**Figure 1.3 Selective delta agonists**




Benzylidenenaltrexone
BNTX




Naltriben
NTB




Naltrindole
NTI

**[D-Ala$^2$-D-Leu$^5$]enkephalyl-Cys**

DALCE

**Figure 1.4. Selective delta antagonists**

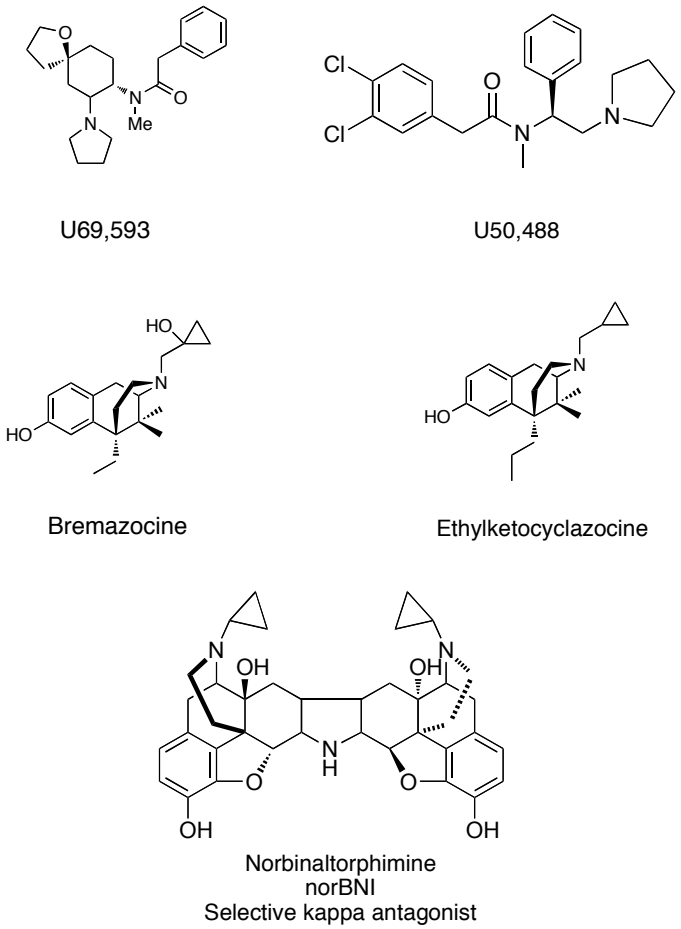



**Figure 1.5. Selective kappa ligands**

To add to the initial suggestion by Portoghese[6] that the diversity in opioid ligand structure would require multiple opioid receptor sites, the experiments by Martin and coworkers[13-16] in dogs led to suggestion of mu ($\mu$), kappa ($\kappa$) and sigma ($\sigma$) opioid receptors. The activity of sigma receptors is not naloxone-reversible and these receptors are no longer considered as opioid receptors[17].

An observation that the mouse vas deferens showed greater affinity for enkephalins than morphine led to the suggestion of the delta (δ) opioid receptor[18]. Two studies by Cuatrecasas and colleagues showed the existence of the enkephalin-preferring delta site in the brain, and they attempted to describe opioid receptor distribution in various brain regions[19, 20]. However, to perform such detailed analyses it was paramount to develop ligands selective for the various suggested opioid receptors types (Fig 1.1 – 1.5).

## 1.2. Selective opioid ligands

The development of high specific activity tritiated ligands in the 1970s and 1980s opened up the field for receptor characterization and localization studies. After the discovery of enkephalins, synthetic efforts were centered on making substitutions that would render stability towards hydrolysis by enkephalinase [21]. These efforts led to the synthesis of the enkephalin analogs, DADLE and DSLET, that were shown to be delta-selective peptides[18-21]. However, they still had affinity for mu receptors. The synthesis of conformationally restricted bis-pencillamine enkephalins afforded the highly delta-selective analog DPDPE[22].

The isolation of the endogenous linear heptapeptides from frog skin extracts led to the discovery of deltorphins[23, 24]. The first ligand that was isolated had the sequence: Tyr-D-Met-Phe-His-Leu-Met-Asp-NH2. Two more peptides were later reported that had D-Alanine as the second residue, with either aspartate or glutamate residue in position 4, and were named [D-Ala$^2$]-deltorphin I (DELT-I) and II (DELT-II), respectively. These linear peptides

showed the highest affinity for delta receptors compared to any of the ligands at the time. Indeed, the two [D-Ala$^2$] deltorphins had ~200-fold greater affinity for delta receptors than DPDPE.

For kappa receptors, ethylketocyclazocine (EKC, Fig 1.5) stood as the prototypic ligand throughout the 1970s. However, Von Voightlander and colleagues used binding and in vivo behavioral procedures to show that the benzeneacetamide, U50,488, is a kappa ligand with greater selectivity[25]. They showed that U50,488 displaced [$^3$H]EKC and the displacement was not blocked by high doses of dihydromorphine. In addition, U50,488 did not produce cross-tolerance to morphine tolerance suggesting that U50,488 was mediating its effects via a non-mu opioid receptor. $pA_2$ studies with naloxone further implicated the involvement of kappa receptors in the activity of U50,488 and bremazocine.

At the time, there still wasn't a highly selective tritiated ligand available for kappa receptors. The introduction of [$^3$H]U69593, an analog of U50,488, provided the opportunity to determine the distribution and expression levels of kappa receptors in various tissues[26]. U69395 was shown to be 484-fold more selective at kappa, when compared with mu or delta receptors, making it the most selective kappa ligand. This study also showed that bremazocine was less selective and could bind to all the three opioid receptors. Thus, in early studies, morphine [27, 28] and [D-Ala$^2$-MePhe$^4$-Glyol$^5$] enkephalin (DAMGO; [21, 29]) have been described and used widely as selective ligands for mu receptors, DADLE, D-Pen$^5$D-Pen$^5$enkephalin (DPDPE; [22]) and deltorphin-II (DELT-II) [30, 31]

for delta receptors, and EKC, U69593 [26, 32], and bremazocine [32] for kappa receptors.

### 1.3. Early attempts to tackle morphine's adverse effects in the clinic: Mixed agonist-antagonist ligands

The development of tolerance, physical dependence, and respiratory depression are the major side effects associated with morphine pharmacotherapy. The design of improved ligands with reduced deleterious effects and efforts to elucidate mechanisms that lead to tolerance and dependence remain a major focus of opioid research. Nalorphine was an early opioid antagonist that was used in the clinic for opioid overdose. However, Lasagna and coworkers were surprised to observe that nalorphine could produce potent analgesic activity by itself[33]. Since nalorphine was shown to reverse morphine-induced withdrawal, it was deemed that a mixture of kappa agonist opioid with mu antagonist properties would be a preferred opioid analgesic. The hypothesis was that μ antagonism would ensure that the ligand would mitigate mu opioid side effects like respiratory depression, tolerance, and dependence, while the kappa agonism would confer the antinociceptive ability[2]. With this premise a number of kappa agonist/mu antagonist ligands were developed.

Pentazocine, a benzomorphan-derived analgesic, was one of the first drugs in this class that was initially considered to have the right attributes and was used extensively in the clinic[33, 34]. Depending on the route of

administration, pentazocine was considered to be one-half to one-fifth as potent as morphine[35-38]. Pentazocine could attenuate abstinence syndrome in patients dependent on low-dose morphine (30 mg/kg), but not at higher doses of morphine. Indeed, pentazocine has been shown to precipitate withdrawal-like syndrome in morphine-addicted patients which may be due to its mu antagonistic activity[39]. Pentazocine has also been shown to precipitate physical dependence, and naloxone administration in these patients can produce moderate withdrawal symptoms[39, 40]. In addition, pentazocine can also produce dysphoric and psychotomimetic effects that limited its use[39, 41-43]. It was also contraindicated in patients with coronary problems[44].

Nalbuphine is a noroxymorphone analogue that shows effects that are very much like pentazocine. However, nalbuphine has been shown to produce substantially fewer psychotomimetic effects[45]. In postoperative pain management, nalbuphine was equipotent to morphine, but at least 3 to 5-fold more potent than pentazocine. Physical dependence was observed only with chronic administration of ~200 mg of nalbuphine per day. Interestingly, nalbuphine did not produce any abstinence syndrome in morphine-dependent patients, unlike naloxone[46].

Butorphanol is an opioid ligand of the morphinan class of compounds and has been used extensively in the clinic. Butorphanol produces pharmacological effects that are also very much like pentazocine. By the intramuscular route of administration, butorphanol has been found to be >15-fold more potent than pentazocine and 5-8 times more potent than morphine[47-51]. Similar difference in

potency was also observed when both morphine and butorphanol were administered by the intravenous route[52]. Butorphanol was reported to be fast acting with limited side effects. Indeed, psychotomimetic effects were only observed in patients who had a history of using narcotic drugs which suggested that these effects may represent mild withdrawal syndrome[53].

In spite of the largely favorable pharmacological profile (limited tolerance and abuse potential when compared with morphine) of the above analgesics, the psychotomimetic and dysphoric effects made them eventually unpopular in the clinic. Indeed, even today, the issue of limiting the abuse potential of opioids remains the central focus of most opioid investigators.

### 1.4. Opioid antagonists

The introduction of naloxone as a potent narcotic antagonist remains a watershed moment in the history of opioid research[54, 55]. It is a potent and non-selective opioid antagonist that has been used so often that those ligands whose effects are not naloxone-reversible are not considered to act via opioid receptors. It has been most useful in the clinic as an antidote for narcotic overdose and has helped save many lives. Even though naltrexone was more potent than naloxone, the lack of intrinsic activity has helped naloxone remain as the prototypic opioid antagonist[56].

The elucidation of effects of the different opioid receptors was greatly facilitated by the synthesis of selective opioid antagonists. In the early 1980s, Portoghese and coworkers described the synthesis, pharmacological

properties, and mechanism of action of β-funaltrexamine (β-FNA) as an affinity label for mu opioid receptors[57-60]. β-FNA produced long-acting and irreversible antagonism of mu agonists in the guinea pig ileum (GPI) preparation, mouse vas deferens (MVD), and in vivo[59, 60]. Indeed, β-FNA produced long-acting antagonism of morphine, even 120 hrs after i.c.v. or s.c. administration in mice. Interestingly, β-FNA produced short-acting agonism that was reversible and was attributed to activity at kappa receptors[58].

In search of a reversible antagonist for mu opioid receptors, Hruby and colleagues developed D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-$NH_2$ (CTOP) and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-$NH_2$ (CTAP), cyclic derivatives of somatostatin, as highly selective mu ligands[61, 62]. They attributed the mu selectivity by determining the ability of CTOP in displacing [$^3$H]naloxone and [$^3$H]DPDPE. The results showed that CTOP displaced [$^3$H]naloxone ~ 4,829-fold greater than [$^3$H]DPDPE. In 1994, CTOP was shown to be highly selective for the cloned mu opioid receptor[63].

A major step in the quest to develop selective antagonists for kappa receptors occurred with the discovery of TENA, a bivalent ligand containing two naltrexone-derived pharmacophores tethered by a spacer of 10 atoms[64]. Portoghese and colleagues reported that shorter spacers promoted greater selectivity for kappa receptors, while increased spacer length promoted mu selectivity[65]. With this in mind, bivalent ligands with short spacers were developed. Pyrrole ring was chosen as a spacer to conformationally restrict the orientation of the molecule in an effort to facilitate better binding to the kappa

receptors. This led to the development of norbinaltorphimine (norBNI, Fig 3) that possessed the highest affinity and selectivity for kappa receptors. Indeed, even with the synthesis of several other kappa selective antagonists, to date norBNI has remained the standard kappa antagonist in opioid research[66, 67].

A few years before the synthesis of norBNI, the "message-address concept" was proposed by Schwyzer as a way to reconcile peptide ligand selectivity for receptors[68]. Based on specific sequence identifiers in opioid peptides, this model was shown to be applicable to non-peptide opioid ligands as well[69, 70]. For instance, it was suggested that adding Phe-Leu to mu ligands conferred delta selectivity[71]. This was used as the design strategy to convert naltrexone into a non-peptide delta antagonist. It was hypothesized that the benzene ring in $Phe^4$ of Leu-enkephalin will need to be incorporated into the morphinan core of naltrexone via a rigid spacer. While a pyrrole ring was chosen as the spacer for synthetic ease, it led to the development of the prototypic delta antagonist, naltrindole (NTI, Fig 2)[72]. NTI was 240-fold more selective for delta receptors than naltrexone, giving credence to the incorporation of the delta address-mimic into a non-selective ligand. Using a furan ring instead of the pyrrole led to the synthesis of naltriben (NTB, Fig 2)[73] which is less potent, but with greater affinity than NTI.

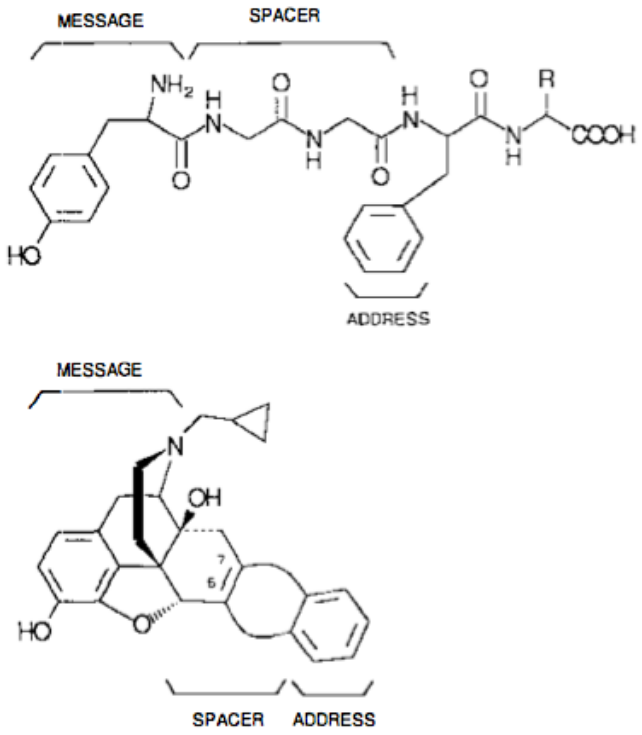



**Figure 1.4. "Message-address" concept applied to naltrexone to develop NTI. Pyrole ring was used as the spacer. (Figure adapted with permission from ref [70])**

## 1.5. Genetic receptors vs pharmacological receptors

For years investigators had been piecing together the complex physiological effects of opioid receptors with the help of ligands. However, given that most ligands are not specific for a single receptor, it was near impossible to determine the receptor effects in isolation. The advent of molecular biology changed this scenario when two groups independently cloned the delta opioid receptor[74, 75]. Within a couple of years other

investigators cloned the kappa[76] and mu opioid[77, 78] receptors and genetically mapped the gene sequences to specific chromosomal locations[79-81].

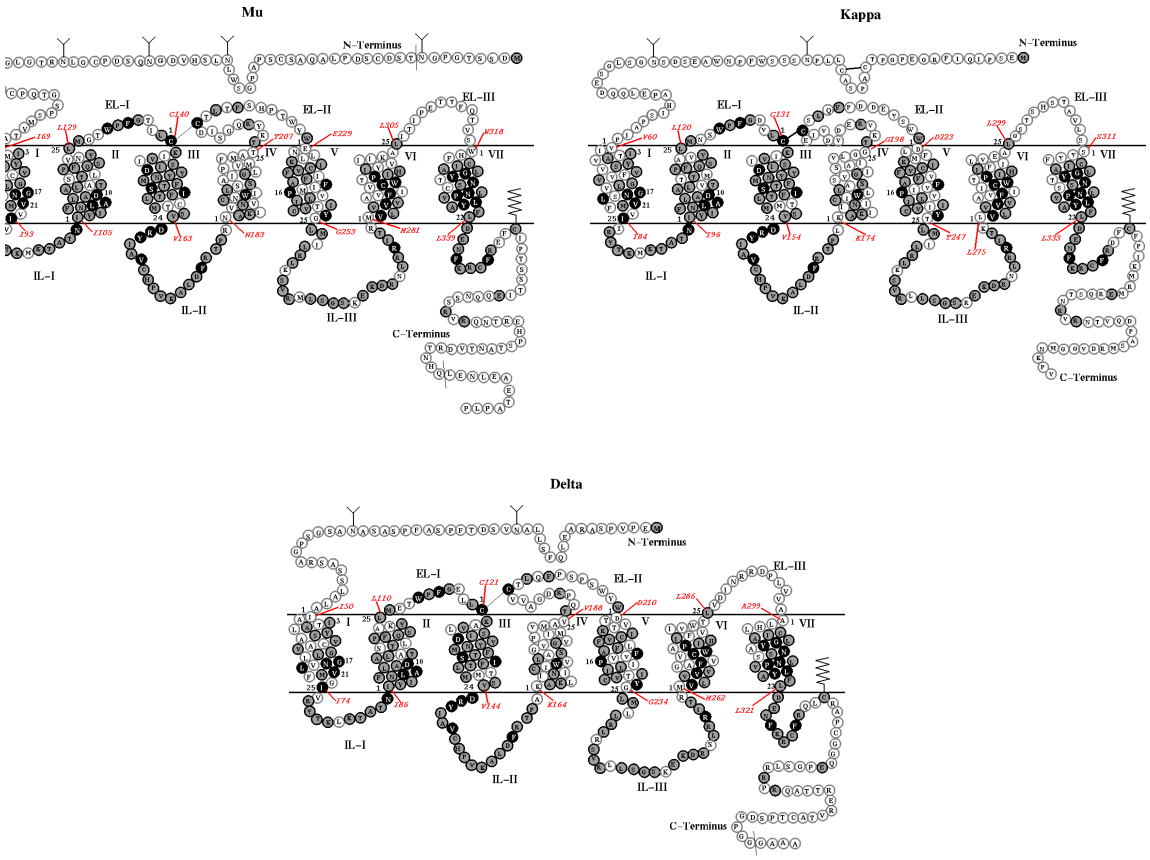



**Figure 1.5. Snake diagrams for opioid receptors (Figures adapted with permission from the University of Minnesota CORD website)**

The studies showed that opioid receptors are class A members of the G protein coupled-receptor (GPCR) superfamily. The general GPCR structure consists of seven transmembrane (7TM) domains linked by three alternating intracellular and extracellular loops. There is high amino acid homology (~60%)

within the opioid receptor family that constitutes a group of four receptor types (Fig 5): MOP (mu), DOP (delta), KOP (kappa) and ORL-1 (nociceptin)[82]. Though ORL-1 was originally placed within the opioid family due to sequence homology, it is not known to interact with any of the non-selective opioid ligands and produces downstream effects that are unlike the other three opioid receptors. We have, therefore, focused our research efforts on the mu, kappa and delta opioid receptors.

Most of the homology between the opioid receptors occurs in the TM domains, intracellular loops and in the C-terminus[17]. However, it was surprising that all the cloning studies pointed to just three different gene products when the literature at the time suggested the existence of three mu ($\mu_1$, $\mu_2$, $\mu_3$), two delta ($\delta_1$ and $\delta_2$), and three kappa ($\kappa_1$, $\kappa_2$ and $\kappa 3$) receptor subtypes based on distinctive pharmacological effects.

### 1.5.1. Opioid receptor subtypes

In the early 1990s, several reports emerged that showed that the delta opioid agonists, DPDPE and deltorphin II (DELT-II), were inhibited to different extents by delta antagonists[83-85]. For instance, it was shown that the DALCE and BNTX selectively inhibited DPDPE, but not DELT-II. On the other hand NTB selectively antagonized DELT-II, but did not antagonize DPDPE. This led to the postulation of two distinct delta subtypes: $\delta_1$ which is selective for DPDPE and BNTX, and $\delta_2$ which is selective for DELT-II and NTB.

Genetic manipulation of delta receptors revealed tissue specific effects of ligands targeting delta opioid receptors. Using antisense oligos that were administered intrathecally (i.t.), Pasternak and colleagues showed that the spinal activity of both DPDPE ($\delta_1$) and DELT-II ($\delta_2$) was completely abolished[86]. However, Bilsky and coworkers showed that the antisense molecules inhibited the antinociceptive activity of DELT-II ($\delta$2), but not DPDPE ($\delta$1), when administered supraspinally[87]. In both studies the effects of DAMGO ($\mu$) or U69,593 ($\kappa$) were unaffected by the delta-selective oligos.

Since molecular cloning only identified a single gene for the delta receptor, it was speculated that different splice variants could lead to the expression of the $\delta_1$ and $\delta_2$ receptor subtypes. There are three exons contained in the delta receptor gene, which prompted Pasternak and coworkers to design oligonucleotides for all the three exons to gain a comprehensive understanding of delta receptor expression[88]. In all, five oligonucleotides were designed. All the oligos inhibited the antinociception of DPDPE and DELT-II when administered i.t. However, while the antinociception mediated by DELT-II was abolished by i.c.v. administration of all the oligos, only oligos targeting exon 3 attenuated the i.c.v. activity of DPDPE. This led to the suggestion that $\delta_1$ and $\delta_2$ are distinct receptors that are expressed due to splice variants that contain either all three exons ($\delta_2$), or only the 3$^{rd}$ exon ($\delta_1$). While the study had tremendous implications, no such splice variants have been isolated to date.

One of the criticisms of antisense oligo methods is that the level of knockdown is rarely homogenous across all the cells in the tissue of interest.

This can lead to challenges in interpretation, as some tissues may be more permeable to both the oligos and ligands than other tissues. To get around this problem, knockout mouse models were developed that allowed for the entire genes to be abolished. In an ineteresting study, Pintar and coworkers developed a delta knockout (DORKO) mouse model and studied the antinociceptive activity of the two subtype selective ligands, DPDPE and DELT-II[89]. In these knockout animals, neither [$^3$H]DPDPE or [$^3$H]DELT-II showed any binding in the brain. In addition, the spinal activity of both ligands was attenuated. In contrast to the antisense studies, the supraspinal antinociceptive activity of both DPDPE and DELT-II remained intact. The authors summarized that the lack of binding indicated that both $\delta_1$ and $\delta_2$ were expressed by the same $\delta$ receptor gene, but the supraspinal activity of these ligands was mediated by delta-like receptor that is different from the cloned delta receptor.

But this interpretation has several inconsistencies. The authors suggest that the supraspinal activity of these ligands is mediated by a delta-like receptor[89]. However, they also showed that there was no binding of the tritiated ligands in the brain. If there was a different receptor that was mediating the antinocieption, the authors should have still observed residual binding in the brain. In other words, if the ligands are not binding to any receptor in the brain, where is the supraspinal activity coming from?

Several studies also suggested pharmacological subtypes for kappa opioid receptors[90-93]. Such an idea stemmed from the fact that benzenomorphan ligands like bremazocine consistently showed greater amount of binding than

arylacetamide ligands[90]. The receptors that arylacetamides bound were considered $\kappa_1$, while the residual binding for bremazocine that was observed in the presence of excess $\delta$, $\mu$ and $\kappa_1$ selective ligands was considered to be $\kappa_2$. However, genetic knockout studies suggested that bremazocine is a non-selective opioid ligand that binds all three opioid receptors. In a sequential knockout study by Simonin and colleagues[90] in mu, delta, double mu/delta, and triple mu/kappa/delta knockout animals, [$^3$H]bremazocine binding was shown to represent 68% from mu, 27% from delta and only 14.5% from kappa receptor genes. Indeed all of the labeling due to [$^3$H]bremazocine was abolished in the triple knockout mice. Thus, the putative $\kappa_2$ receptor, for which bremazocine has been considered to be the prototype agonist, may just stem from the simultaneous occupancy of all the three receptors.

While there have also been suggestions for the existence of multiple mu opioid receptor subtypes[94], as in the case of delta and kappa receptors, there is little genetic evidence to support it. Though numerous mu opioid splice variants have been isolated, further studies are needed to elucidate their importance in modulating physiological and pharmacological effects.

There is an additional consideration that had been consistently gaining ground to explain the evidence for pharmacological receptor subtypes. Even in the early 1980s, independent studies by Portoghese and Rothman groups suggested the possibility of adjacent and interacting opioid receptors that are organized as complexes[65, 95]. For instance, studies by Vaught et al., suggested that mu and delta receptors interact in the spinal cord and promote distinct

downstream effects[96]. This provocative idea, led to the suggestion that the multiple pharmacological subtypes may be distinct receptor complexes expressed in specific tissue regions.

## 1.6. Oligomerization of opioid and other GPCRs

### 1.6.1. Early evidence

In the early 1980s, much of the opioid field was focused on elucidating the function and physiological relevance of the different opioid receptors. This was still a nascent field then, with the existence of multiple opioid receptors established in just the previous decade (1970s)[9-11]. Even in such a time there were two groups that considered the possibility that opioid receptors may interact and allosterically modulate each other.

Portoghese and coworkers conducted elegant experiments in the Guinea Pig Ileum (GPI) and mouse vas deferens (MVD) to show that two pharmacophores separated by a spacer of varying length can bridge proximal receptor active sites[64, 65]. They used the narcotic antagonist, β-naltrexamine, as the pharmacophore at either end that were separated by a glycolic spacer. The shortest spacer bivalent ligand (spacer of ~ 9Å) blocked activation at kappa receptors to the greatest extent, while a slightly longer spacer ligand (spacer of ~20Å) blocked delta opioid receptors. The changing length did not have any effect on the activity at mu opioid receptors. The data was also consistent with the idea of bridging proximal sites as increasing spacer length beyond the optimal range reduced efficacy at both kappa and delta receptors[65, 70].

In an attempt to elucidate the ligand recognition sites in opioid receptors, Rothman and coworkers performed competition binding studies using tritiated opioid ligands. In one such study, they utilized [$^3$H]Leu-enkephalin (L-enk) and showed that low-dose morphine non-competitively inhibits [$^3$H]L-enk binding[97]. Reciprocally, they later showed that L-enk also non-competitively inhibits ligands at mu opioid receptors[98]. Since, at lower concentrations, morphine and L-enk have been shown to bind selectively to mu and delta receptors, respectively, the authors suggested that there was allosteric coupling between these two receptors[99]. While these results are intriguing, they need to tempered with the knowledge that later studies have shown that these ligands are not as selective as they were deemed to be at that time. Still, these studies opened up the field to the possibility that two receptors within a complex may modulate each other.

### 1.6.2. Experimental evidence for oligomers

If we survey the literature on receptor organization, the persistent dogma for a number of years has been that GPCRs exist and function as monomeric receptor units. Indeed, it has been shown for many receptors that single receptors are coupled to second messengers and can activate the full complement of downstream signaling. However, several studies have questioned the idea and shown that receptors can, and do, form higher order complexes[100, 101]. In some cases oligomerization is a pre-requisite for receptor function and they are termed ‘obligate oligomers’. The classical case for such

obligate heteromers are the $GABA_B$ receptors. The GABAB heteromers are constituted by $GABA_{B1}$ and $GABA_{B2}$ receptors. Neither receptor is active when expressed by itself, and therefore need each other to be functional[102]. In addition, obligate homomers have been commonly seen for other class C GPCRs such as metabotropic glutamate receptors[101]. For other GPCRs, however, oligomerization may not be necessary for normal receptor function. In many cases, oligomerization offers the possibility of novel downstream effects that may be relevant in maintaining the physiological integrity of organisms. To better understand the conceptual aspects of this organization of receptors, let us first examine some key terms and definitions.

## 1.7. Homomers vs heteromers

Receptors of the same kind can oligomerize to form a homogenous complex and are called 'homomers'. There is still considerable debate about what constitutes such a 'minimal functional unit'. Until the exact organization of GPCR complexes is identified, it is recommended to use 'homomer' instead of thinking of them as dimers[103]. Some of the most compelling evidence for higher-order GPCR complexation came from a study utilizing atomic-force microscopy to study the organization of rhodopsin in retinal tissues[104-106]. Fotiadis et al., showed that in native membranes rhodopsin receptors were arranged in arrays.

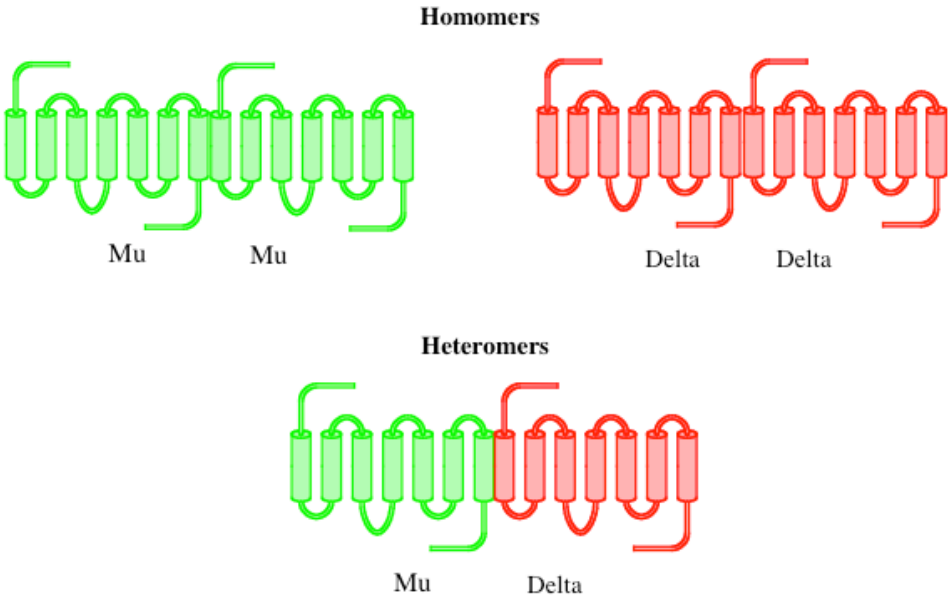



**Figure 1.6. Schematic representation of homomers and heteromers of mu and delta opioid receptors**

In opioid research, Cvejic and Devi were the first to biochemically identify delta receptor homomers[107]. The Devi group transfected heterologous cell lines with c-myc and FLAG- epitope tagged delta receptors and used co-immunoprecipitation to identify the delta homomer. Later, they also isolated the kappa homomers using a similar strategy[108]. An interesting observation was that ligands for delta receptors reduced delta homo-oligomerization, while kappa ligands did not affect the oligomeric state of kappa receptors. The identification of mu opioid receptor homomers[109, 110], rounded off the full complement of opioid receptor homomers. It should be mentioned that around the same time period, several laboratories reported the isolation of various

GPCR homomers, including, β2-adrenergic[111], calcium sensing[112], and Muscarinic M3[113] receptors.

When different types of receptors associate they form complexes that are called 'heteromers'. In the opioid field, the Devi group was again instrumental in co-immunoprecipitating the delta-kappa heteromer[114], which was the first opioid heteromer to be identified. In this study, the authors unsuccessfully tried to immunoprecipitate mu-kappa heteromers. They suggested that either mu and kappa do not associate, or that the solubilization techniques may have been too harsh. Subsequently, the mu-delta heteromer was identified independently by the research groups of Devi and George[109, 115]. By this time, several investigators recognized that co-immunoprecipitation studies lead to artifacts that could confound data interpretation. Resonance energy transfer experiments allowed for monitoring complex formation in living cells, and quickly became the preferred method to study heteromerization. Sadee and coworkers utilized the bioluminescence resonance energy transfer (BRET) technique to show that even mu and kappa receptors can associate to form heteromers[116].

An obvious question at the time centered on the propensity of receptors to form homomers vs heteromers. It was initially argued that homology between receptors was directly proportional to the formation of receptor complexes. Hence, in a given pair of GPCRs (R1 and R2), the probability to form homomers (R1 – R1 or R2 – R2) would be greater than that of heteromers (R1 – R2) as the homology would be highest for similar GPCRs. However, several

studies have suggested that GPCRs, including opioid receptors, can form homomers and heteromers to similar extent[100, 101, 117]. In fact, there have reports for the heteromerization of opioid receptors (which are class A, rhodopsin-like GPCRs) with several non-opioid GPCRs[109, 114, 115, 118-126], including class C receptors like mGluR5[127]. All of these examples suggest that homology between receptors may have no relationship with the oligomerization of GPCRs.

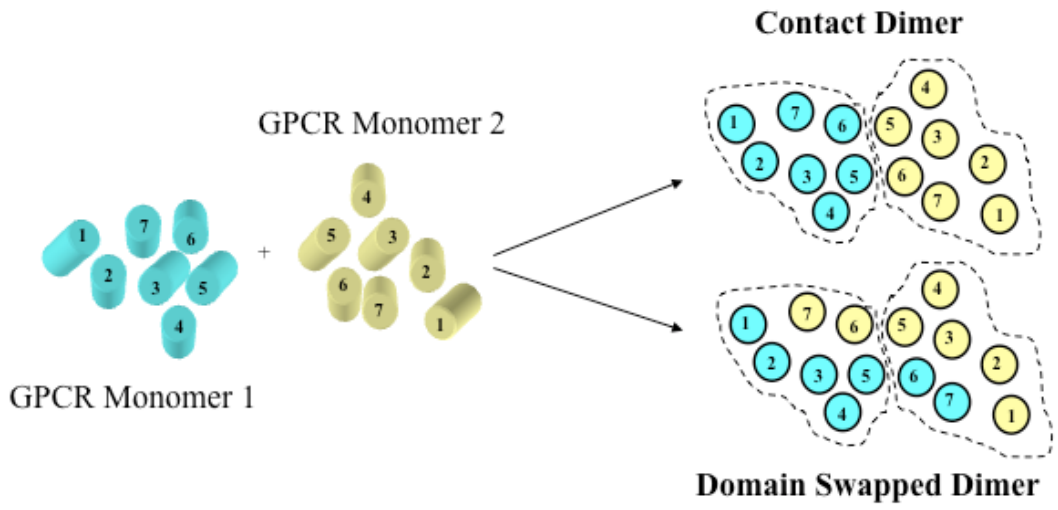



**Figure 1.7. Possible models for dimerization (Adapted with permission from Dr. Philip Portoghese)**

When we think of oligomeric receptors, an important question pertains to how the interaction happens in the first place? There are two schools of thought regarding this (Fig 1.7):

a) Contact oligomerization: complementary regions between two receptors may promote electrostatic or even covalent interactions between them, leading to the formation of a complex.

b) Domain-swap oligomerization: an entire transmembrane (TM) and/or loop domain may be swapped between two receptors early in their biosynthesis.

Several studies have shown that GPCRs that are known to exist as constitutive homomers or heteromers, are secreted to the cell surface as oligomerized complexes[100, 101, 128-130]. Cell fractionation, coupled with BRET/FRET, has been used to conclude that receptor oligomers exist in the ER, which are then packaged and secreted onto cell membranes. In spite of this, there has been little experimental evidence to show that domain-swap can occur. Indeed, the more popular hypothesis for oligomeric associations has been for contact via TM IV and/or V as the interface[100, 131].

## 1.8. Effect of ligands on oligomerization

There has been considerable debate about the effect of ligands on the oligomerization of GPCRs. Since many GPCRs have been shown to form oligomers during their biosynthesis itself, experiments to tease out the effect of ligands has been complicated. There have been a few early reports that showed that the effect may be predominantly receptor-specific. Both mu and delta opioid receptor homomers were shown to be reduced after ligand

administration, while kappa agonists were unable to modulate kappa homomerization[107, 108].

The advent of BRET and FRET to study oligomerization allowed for a quantifiable approach to monitor ligand effects. Portoghese and coworkers generated a compelling example to illustrate this concept[132]. They studied the ability of mu opioid receptors to associate with cholecystyokinin ($CCK_B$) receptors using BRET and showed that there was little association when they are coexpressed. However, administration of a mu agonist – $CCK_B$ antagonist bivalent ligand (MCCK-18), connected by an 18-atom spacer, induced strong BRET signals that indicated heteromer formation. The formation of such induced heteromers was specific for a particular spacer distance as ligands with shorter or longer spacers were less or ineffective in inducing complexation. There are many other studies utilizing BRET or FRET that have also suggested that ligands can play a role in oligomerization[100].

In this context, it is important to examine several caveats to using RET to look for induction/reduction of receptor complexation. As illustrated beautifully by Milligan and Bouvier, the energy transfer between the fluorescent species is dependent on both distance and dipole orientation[133]. This would mean that even small changes in conformation lead to large increases in signal[100, 133]. Moreover, signal intensity would depend on the exact location and orientation of the two fluorogenic species and has lead several investigators to speculate on whether the changes in signal suggest association/dissociation or simply, changes in receptor conformation induced by ligand binding.

## 1.9. The possibility of altered pharmacology via oligomers

A considerable element of the excitement with receptor oligomers stems from the possibility that such receptors may allow for novel pharmacological effects. If this is true, there will be profound ramifications to both understanding the physiological and behavioral role of such oligomers, and also lead to drug discovery.

One example for selectively targeting GPCR heteromers came from the elucidation of 6'-GNTI[134]. This ligand was developed by Portoghese and coworkers and shown to selectively activate kappa-delta heteromers in HEK-293 cells. In mice, the ligand produced antinociception only when administered intrathecally (i.t.), but not intracerebroventricularly (i.c.v.). This suggested the possibility that kappa-delta heteromers are expressed in a tissue-specific manner and exist in the spinal cord of rodents. This idea has further support from a study conducted by Wessendorf and coworkers, that showed that kappa and delta receptors are extensively colocalized in the rodent spinal cord[135].

Several studies have suggested that physical and/or functional interaction between mu and delta opioid receptors is instrumental in mediating some of the deleterious effects of opioid therapy. As far back as 1991, a collaboration between the laboratories of Portoghese and Takemori had shown that co-administration of a delta antagonist naltrindole (NTI) with morphine attenuated tolerance and dependence due to the agonist. Studies with delta receptor antisense knockdown [136] and delta receptor knockout [137] have also indicated

the possible relevance of interaction between mu and delta receptors in development of analgesic tolerance and dependence. These seminal observations, along with the discovery that mu and delta opioid receptors oligomerize to form heteromers[109, 115], led to the design of a series of MDAN bivalent ligands that contain mu agonist and delta antagonist pharmacophores tethered via spacers of varying length[138].

When we consider the rationale behind the design of bivalent ligands, it is suggested that a bivalent ligand with a spacer of optimal length can bridge proximal receptor active sites. Such bivalent ligands may serve as tools to selectively target dimeric GPCRs and lead to unique pharmacological effects[139]. Several peptide[140-143] and opioid alkaloid[65, 144-148] bivalent ligands have now been characterized to show effects that are consistent with bridging in vivo. Importantly, MDAN-21, the 21-atom spacer bivalent ligand was reported to produce potent antinociception devoid of tolerance, physical dependence, or place preference,[138, 149] which provided the strongest possible correlation yet between selectively targeting GPCR heteromers with the appropriate pharmacophores and therapeutic benefit. On the other hand, bivalent ligands with shorter spacers produced significant tolerance and dependence in the above study.

The possibility that physically coupled mu-delta heteromeric receptors could be responsible for these effects requires colocalization of these receptors in the same neuron. This was challenged by a recent study using delta-GFP knock-in mice which showed that mu and delta receptors do not colocalize in

nociceptor neurons in these mice[150]. However, Hökfelt and colleagues have recently rebutted this finding as an artifact of modified receptor trafficking due to the GFP fusion protein[151]. In other strains of mice with wild-type delta receptors, extensive colocalization was observed using selective antibodies for native mu and delta receptors[151]. Also, antibodies selective for mu-delta heteromers have been developed that show mu-delta heteromers to be extensively distributed in the CNS of rodents[152]. Moreover, the report that coexpressed mu and delta receptors are transported as mu-delta heteromers from the ER to the surface of cultured cells lends credence to the idea that this may occur in vivo[130].

## 1.10. How do GPCR heteromers traffic?

Several studies have shown that coexpressed receptors that form heteromers can co-internalize in the presence of ligands that have affinity for only one of the receptors[100, 153]. When delta opioid receptors and $\beta_2$-adrenergic receptors are coexpressed, the opioid ligand, etorphine, produces strong co-internalization of both receptors[154]. Reciprocally, isopraline (a $\beta_2$-AR agonist) also promotes co-internlization of both receptors[155]. In control cells expressing just $\beta_2$-AR, etorphine does not produce any internalization. The story with kappa receptors is quite the contrary. Kappa receptors are generally resistant to internalization in response to stimulation by agonists[117, 154]. When kappa receptors were coexpressed with $\beta_2$-AR, isopraline induced internalization was

greatly attenuated indicating that characteristics of the binding partner play a role in the behavior of the heteromer complex[154].

There have been a few studies that have examined the trafficking of mu-delta heteromers. However, the interpretation of the results has been complicated. An initial study reported that mu-delta heteromers did not internalize together, as selective ligands for one receptor did not cause concomitant internalization of the other protomer[108]. In a later study, George and coworkers showed that the co-internalization of mu-delta heteromers was ligand-dependent[130]. DAMGO and DELT-II produced strong co-internalization, while DPDPE and DSLET did not. An important observation pertained to the fact that the internalization profile depended on coexpression of both receptors at the same time. If the cDNA for the two receptors were transfected >6 hrs of each other, there was considerably less co-internalization due to DAMGO[130]. It was suggested that mu-delta heteromers associate in the ER itself and if the cDNA were transfected at times that were too far apart, there would be considerably lesser heteromers in the cells[130]. Recently, Zhang and coworkers have shown that both DELT-II and SNC80 can also promote co-internalization of both mu and delta receptors, when they are coexpressed[156].

## 1.11. Summary and conclusions

The greatest drawback in studying the signaling and trafficking properties of heteromers pertains to the lack of selective ligands targeting opioid heteromers. As has been outlined in this introductory chapter, historically it has

been necessary to first develop tools that can be used as probes to address the important questions. In the first five chapters, we have tried to do just that. We studied standard opioids agonists and antagonists, novel ligands synthesized in our lab, and clinically used opioid analgesics to establish a ligand selectivity profile that takes into account the existence of heteromers. Those studies have given us valuable insight on the role of opioid heteromers in physiology, and as unique targets in drug discovery.

Bivalent ligands were synthesized to achieve bridging of proximal receptors. As discussed earlier, several series of bivalent ligands have been shown to promote unique pharmacological effects that are consistent with bridging of heteromers. In chapter six, we have extended this hypothesis by providing direct evidence for bridging of opioid receptor heteromers using immunofluorescent methods.

Chapter seven deals with an extension of ligand selectivity that pertains to the existence of various classes of endogenous ligands. In many cases, there isn't much difference between the sequences of ligands within the same class. For instance, [Leu$^5$]enkephalin and [Met$^5$]enkephalin only differ at the C-terminal amino acid but are generally thought to activate delta opioid receptors. What is the need for such multiple ligands to exist if they target only similar receptors? Since their activities at opioid receptor heteromers has never been considered, we explored the possibility that they may be activating different opioid receptor heteromers. Such studies have also provided tremendous insights into the in vivo relevance of opioid receptor heteromers.

There are considerable gaps in the information regarding trafficking of opioid receptor heteromers. Hence, armed with new insights into ligand selectivity, we performed studies to elucidate how the individual protomers constituting a heteromer modulate the trafficking properties of each other. These studies are also discussed in chapter seven.

Mechanisms of allosteric regulation of GPCR heteromers may explain how the individual protomers interact with each other and affect downstream signaling. In chapter five, we performed experiments to determine whether allosterism between mu-delta heteromers exists in the mouse spinal cord and in rhesus monkeys. In chapter eight, we have utilized the intracellular calcium release assay to show that allosteric activation and inhibition is possible between mu-mGluR5 and mu-CCR5 receptors. Also the finding that both mu-mGluR5 and mu-CCR5 are allosterically coupled can lead to the design and development of analgesics devoid of deleterious effects.

The final chapter deals with a novel and paradoxical finding that the opioid antagonist, naloxone, can produce antinociception in mice with inflammation. Using several research tools and assays, we present data that naloxone antagonizes glutamate-induced activation of mGluR5 receptors. This study suggests that a novel scaffold devoid of affinity for opioid receptors can be developed for treatment of inflammatory pain.

# CHAPTER 2[1]

## 2. THE SELECTIVITY OF STANDARD OPIOID AGONISTS IN HEK-293 CELLS

### 2.1. Introduction

When we initially examined the literature, we realized that there were no reports on the selectivity of standard opioid ligands in activating both homomers and heteromers. The existence of opioid receptor heteromers in cultured cells may have significant ramifications concerning the interpretation of *in vivo* data, particularly if standard agonists (Fig 2.1) that are widely employed as tools in opioid research were to activate them differently or more efficiently than homomers. In order to investigate whether or not this is a possibility, we have evaluated standard μ-, κ-, and δ-opioid agonist ligands using HEK-293 cells that contain singly or doubly expressed opioid receptors and transfected chimeric $G_{iq}$ protein. Here we demonstrate that although the functional selectivity of these standard ligands is in harmony with their reported binding selectivity to homomeric opioid receptors, they often exhibit greater or equivalent activity at opioid receptor heteromers. In this regard, and most significantly, the standard agonists, morphine and DAMGO, selectively activate μ-δ heteromers rather than μ receptors as traditionally viewed. These studies call into question the conclusions derived from a multitude of published *in vivo*

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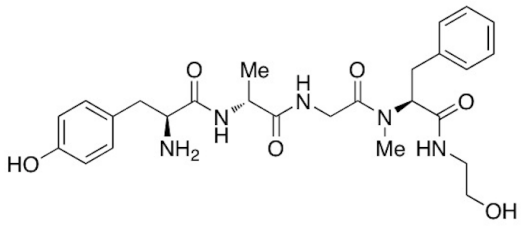
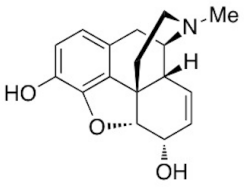
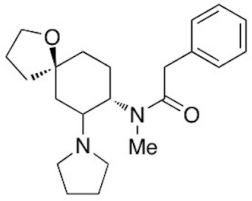
pharmacologic studies whose data was interpreted based on the assumption that these standard ligands selectively activate μ opioid receptor homomers.




DAMGO




Morphine

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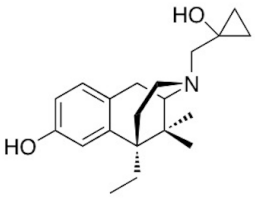
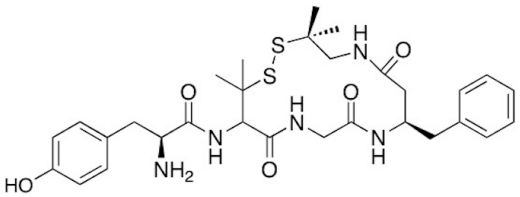
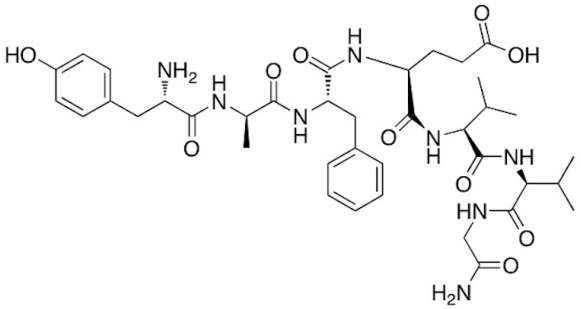



Bremazocine




DPDPE




Deltorphin II

**Figure 2.1 Structures for standard opioid ligands used in this study**

## 2.2. Results and Discussion

Researchers in the pharmacologic sciences have traditionally depended on selective ligands as tools to establish the identity of receptor targets. In this regard, over the years the field of opioid research has seen the development of an array of ligands that target opioid receptors. After the existence of multiple opioid receptors was suggested[157] and later established [13, 18], standard ligands were developed that were apparently selective for each of the opioid receptor types and these became cornerstones in the field [21, 22, 26-32, 158].

An additional layer of complexity has more recently been added with the numerous reports that GPCRs, including opioid receptors, can associate to form higher order structures that include heteromers in cultured cells[109, 114, 115, 118-126]. The existence of heteromers raises cogent questions concerning receptor recognition, activation and signal transduction. Since opioid receptor heteromers represent potential targets that have not been considered previously, we investigated frequently employed standard opioid ligands using the intracellular calcium release and [$^{35}$S]GTPγS assays on HEK-293 cells stably expressing homomeric and heteromeric opioid receptors.

### 2.2.1. MOP receptor selective agonists.

The HEK-293 cell lines stably co-expressing hemagglutinin (HA) or FLAG tagged opioid receptors which were characterized previously by co-immunoprecipitation and shown to contain heteromeric receptors [134, 159], were

employed in the present study. To determine the relative densities of the receptors in these dual transfected cells, two-color immunoflourescence was used. High-resolution confocal images obtained for HEK-293 cells stably coexpressing HA-μ and FLAG-δ opioid receptors show that both receptors were co-localized on the plasma membrane and similarly expressed (Fig 2.2).

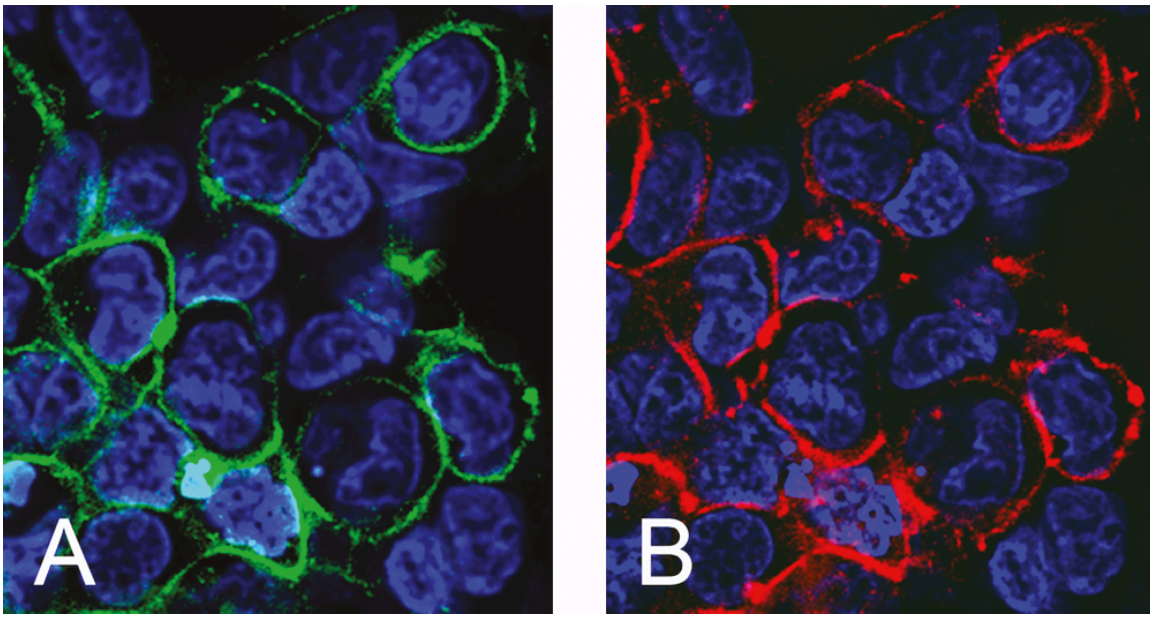



**Figure 2.2. High-magnification confocal images of double-labeling immunofluorescence for HA-μ and FLAG-δ opioid receptors.**

HEK-293 cells stably expressing both HA-μ and FLAG-δ opioid receptors are shown labeled for μ (A, green fluorescence) and for δ (B, red fluorescence). DAPI (blue fluorescence) has been used to stain the nuclei.

The functional selectivity of morphine and DAMGO that are considered to be μ-selective agonists [21, 27-29] were evaluated using the intracellular calcium release method with minor modifications [134]. A transiently transfected chimeric

G protein, $\Delta$6-$G_{qi4\text{-}myr}$ [160] whose activation is coupled to calcium release, was employed. Morphine was observed to have the highest potency and efficacy (curve height) in cells coexpressing $\mu$ and $\delta$ opioid receptors, relative to cells containing other singly or coexpressed receptors of equal density (Fig 2.3a). Since the coexpressed cells are known [109, 114, 115, 134, 159] to contain opioid receptor heteromers, the selective activation of $\mu$-$\delta$ receptors may be due to conformational differences when compared with homomeric $\mu$ receptors or other opioid heteromers. The potency of morphine was at least 5-fold greater at $\mu$-$\delta$ heteromers (16.4 nM) than at homomeric $\mu$-opioid receptors (89.4 nM) or $\mu$-k-opioid receptor heteromers (141.6 nM). There was little observable activation at the $\kappa$, $\delta$, or $\kappa$-$\delta$ heteromeric opioid receptors (Fig 2.3a).

DAMGO (Fig 2.3c) was 7-fold more potent at $\mu$-$\delta$ opioid receptor heteromers ($EC_{50}$ = 1.5 nM) relative to $\mu$ opioid receptors ($EC_{50}$ = 10.6 nM). Interestingly, unlike morphine DAMGO possessed similar efficacy at $\mu$, $\mu$-$\delta$ and $\mu$-$\kappa$ receptors. Again, there was little observable activation at the $\kappa$, $\delta$ or $\kappa$-$\delta$ heteromeric opioid receptors. The difference between DAMGO and morphine could be related to their divergent structures.

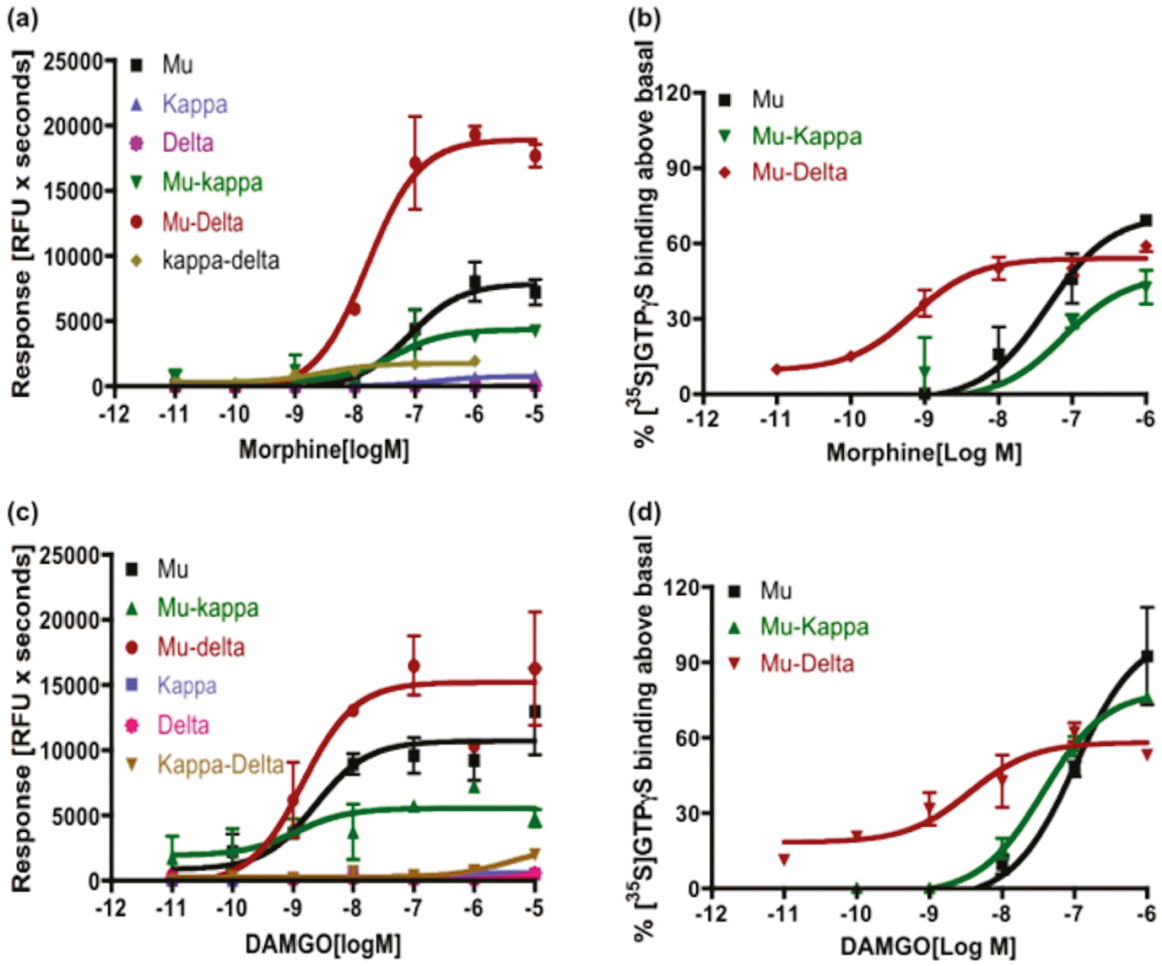



**Figure 2.3. Morphine and DAMGO are highly selective agonists for μ-δ opioid receptor heteromers.**

Intracellular $Ca^{2+}$ ion release mediated by increasing concentrations of the agonist (a) Morphine and (c) DAMGO were measured in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric G-protein. Response was measured as Relative Fluorescence Units (RFU) and time was measured in seconds. The Y-axis plots the Area Under Curve (AUC) values ± SEM (n = 12-16) which is represented as RFU x seconds. Concentration response curves for stimulation of [$^{35}$S]GTPγS by (b) Morphine and (d) DAMGO, as measured in HEK-293 cell membranes expressing various opioid receptors.

We also employed the [$^{35}$S]GTPγS assay in order to evaluate receptor activation at the Gα-protein level as opposed to a downstream signal transduction effect. Here also we observed that morphine and DAMGO both more potently activated μ-δ opioid receptor heteromers (Fig 2.3b and d). It is noteworthy to note that the [$^{35}$S]GTPγS and intracellular calcium assays differed in the observed output though giving similar results. The [$^{35}$S]GTPγS assay also revealed the greater selectivity of both morphine and DAMGO for activation of μ-δ heteromers, as manifested by the nearly 100-fold lower $EC_{50}$ when compared with the μ opioid receptors (leftward shift of the concentration-response curve, Fig 2.3b and d) while the calcium assay was characterized by an increased curve height or efficacy (higher $AUC_{peak}$ values, Fig 2.3a and c).

Though widely employed as an analgesic, the well-known side effects of morphine, such as tolerance and physical dependence limit its chronic use. Some insight into the origin of these side effects was obtained when it was reported that co-administration of morphine with δ-opioid antagonists abolished the development of tolerance and dependence in mice, with a concomitant increase in agonist potency [161]. These results are consistent with the absence of morphine-induced tolerance in mice lacking δ-opioid receptors [89] and both tolerance and dependence in δ receptor antisense experiments [136, 162]. Additionally, it is particularly noteworthy that bivalent ligands containing both μ agonist and δ antagonist pharmacophores produce potent antinociception without tolerance or dependence in mice, only when the linker length between the pharmacophores is long enough to presumably permit bridging of

receptors, suggesting that μ and δ receptors are in close proximity, perhaps as heterodimers [138, 149]. A possible mechanism for this effect is that the δ antagonist pharmacophore effectively blocks the development of tolerance and dependence by allosterically altering the signaling pathway normally induced by the activation of the μ recognition site of the μ-δ heteromer. The alteration in the signaling pathway could occur at the G protein level, in view of the report that $G_z$ rather than $G_i$ or $G_o$ is activated by interaction of morphine with μ-δ heteromers in cultured cells [130].

The observation that both morphine and DAMGO selectively activate μ-δ opioid receptor heteromers in the calcium release and [$^{35}$S]GTPγS assays (Fig 2.3) has important implications. When taken together with the literature suggesting interaction between μ and δ receptors [89, 95, 136, 138, 161, 162], the activation selectivity data for morphine suggests a major role for μ-δ heteromers in mediating morphine analgesia and the development of tolerance and dependence. Similarly, the central and peripheral tolerance and dependence by DAMGO [163-165] further implicates the role of μ-δ receptors in these side effects.

### 2.2.2. KOP receptor agonists

There have been suggestions of multiple subtypes for κ receptors based mainly on differences between the *in vivo* pharmacology [166-168] of the benzeneactamides (eg, U50488, U69593) and bremazocine. On this basis, the benzeneacetamides and bremazocine have been referred to as $\kappa_1$ and $\kappa_2$

opioid receptor agonists, respectively. However, since only a single κ receptor has been cloned [76], and in view of evidence for of κ receptor heteromers in cultured cells, it appears that the receptor "subtype" may actually represent a phenotypic κ receptor in the form of a heteromer [114, 124, 145, 168].

Based on the above considerations, we have investigated two κ-opioid receptor ligands, U69593 and bremazocine, at various opioid receptors in HEK-293 cells. Though U69593 has long been considered a highly selective κ-opioid agonist, the activation at κ-δ opioid receptors ($EC_{50}$ = 2.9 nM, $AUC_{peak}$ = 11803 RFUxSec) was at least 80% more efficacious and 11-fold more potent than at κ-opioid receptors alone ($EC_{50}$ = 33.7 nM, $AUC_{peak}$ = 6704 RFUxSec; Fig 2.4a). There was no significant activation observed at μ, δ and μ-δ receptors.

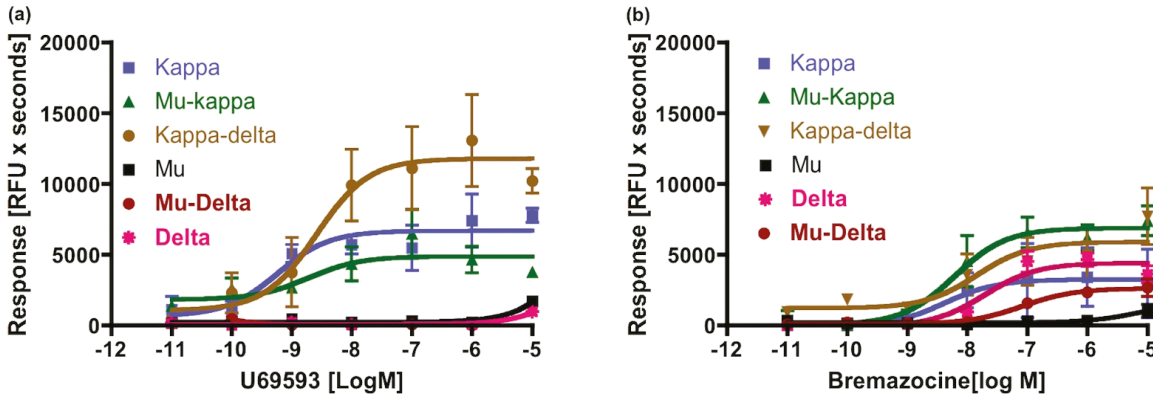



**Figure 2.4. U69593 and bremazocine are both non-selective for activation of κ-opioid receptor-containing homomers and heteromers.**

Intracellular $Ca^{2+}$ ion release was mediated by increasing concentrations of the agonist (a) U69593 and (b) bremazocine in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric G-protein. Response was measured as Relative Fluorescence Units (RFU) and time was measured in seconds. The Y-axis plots the AUC values ± SEM (n = 12-16) which is represented as RFU x seconds.

On the other hand, bremazocine produced comparable activity at all the receptors tested except for μ opioid receptors where it was inactive (Fig 2.4b). Interestingly, the activity of bremazocine at κ-δ receptors ($AUC_{peak}$ = 5916 RFUxSec) resembled that of a partial agonist with $AUC_{peak}$ values around half of that of U69593. Bremazocine also activated κ, δ and μ-δ receptors to the levels of a partial agonist thus showing no functional selectivity to κ opioid receptors. In this regard, bremazocine has been recently suggested to be a partial agonist at κ opioid receptors using a similar calcium release method [169]. It has been previously shown that bremazocine bound extensively to μ (68%), δ (27%) and κ receptors (15%) in μ, δ and μ/δ opioid receptor knockout mice, respectively [90]. The higher μ and δ binding could be explained by our finding that bremazocine has the highest functional activity at μ-κ and κ-δ receptors (Fig 2.4b) while also activating δ and μ-δ opioid receptors. When taken together with the reported *in vivo* binding [90], the results underscore the non-selective nature of bremazocine, making the interpretation of its *in vivo* pharmacology problematic. Thus, it appears likely that the difference in the *in vivo* pharmacological profiles observed between U69593 and bremazocine may in part be due to κ-opioid receptors that are heteromerized with μ, δ, or a non-opioid GPCR.

### 2.2.3. DOP receptor agonists

The two standard agonist ligands that are most frequently employed as research tools for investigating δ-opioid receptors are the peptides, DPDPE [22]

and deltorphin-II [30, 31]. They have been classified as $\delta_1$ and $\delta_2$ agonists, respectively. They are selectively antagonized *in vivo* by the δ antagonists, BNTX [85]($\delta_1$) and naltriben also called NTB [83]($\delta_2$). The *in vivo* selective antagonism by BNTX and NTB, has led to the proposal for δ opioid receptor subtypes [83, 85, 170, 171]. However, to date, genotypic $\delta_1$ and $\delta_2$ receptors have not been reported. In view of reports for the heterodimerization of δ receptors expressed in cultured cells [109, 114, 115, 124, 134, 159], it seems plausible that the $\delta_1$ and $\delta_2$ subtypes actually represent phenotypic receptors that display different sensitivity to the selective antagonists [145, 168].

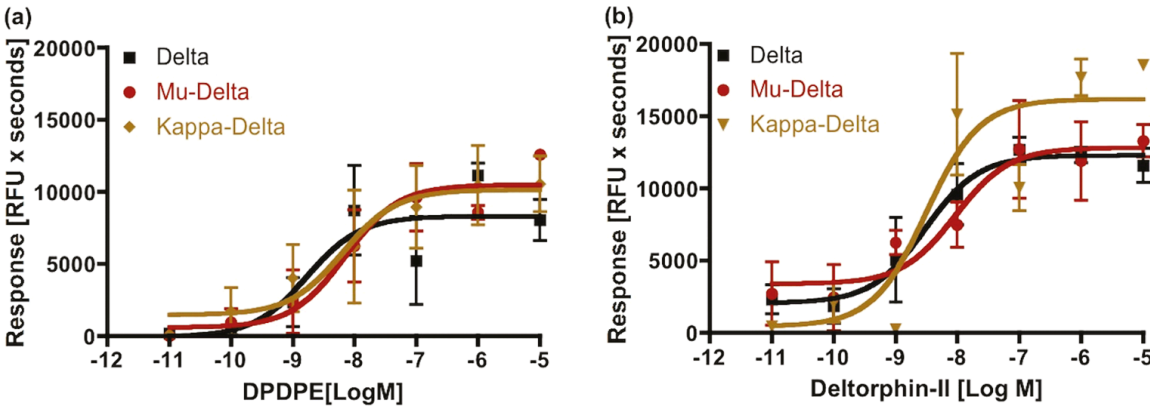



**Figure 2.5. DPDPE and Deltorphin-II are non-selective agonists for δ-opioid containing homomers and heteromers.**

Intracellular $Ca^{2+}$ ion release mediated by increasing concentrations of the agonist, (a) DPDPE and (b) deltorphin-II, was measured in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric G-protein. The Y-axis plots the AUC values ± SEM (n = 12-16) which is represented as RFU x seconds.

In order to address the aforementioned questions concerning the agonist selectivity of DPDPE and deltorphin-II, we have evaluated their ability to activate homomeric and heteromeric δ receptors. The calcium release data for DPDPE reveal little, if any, difference in the activation of homomeric and heteromeric δ-opioid receptors (Fig 2.5a). DPDPE potently elicited a calcium response in all the cell lines (Fig 2.5a). DPDPE was completely non-selective, as the response was equi-efficacious and equipotent in δ, μ-δ and κ-δ cell lines.

Similarly, deltorphin-II also activated δ, μ-δ and κ-δ opioid receptors to the same degree (Fig 2.5b). However, when compared with the other standard ligands, deltorphin-II was most active at the κ/δ opioid receptor heteromers. The apparent absence of selectivity in the activation by deltorphin-II suggests that it may produce antinociception via multiple phenotypic heteromers that contain δ and a non-opioid receptor. Moreover, the possibility that such phenotypic receptors may show tissue-specific expression increases the variability of ligand selectivity and potency *in vivo* and brings into focus the importance of route of administration [134, 145, 168]. If this is the case, the antagonist selectivity for $\delta_1$ and $\delta_2$ phenotypes observed for BNTX and naltriben may represent their ability to target different populations of δ receptor heteromers. In this connection, it has been reported that antagonist selectivity ratios (eg, $\delta_1$/ $\delta_2$) for BNTX and naltriben (NTB) differ as a function of the route of administration [168].

In the [$^{35}$S]GTPγS assay, however, DPDPE showed 60-fold lower $EC_{50}$ at κ-δ receptors when compared with κ receptors. As this is the only instance

where there is a quantitative difference in the observations between the intracellular calcium release and [$^{35}$S]GTPγS assays, it may be related to the fact that, the calcium release method employs a chimeric G protein, whereas the [$^{35}$S]GTPγS assay measures [$^{35}$S]GTPγS binding to endogenous G proteins. Also, since the calcium release assay is performed in whole cells, it measures a response that is downstream to receptor activation, while in the [$^{35}$S]GTPγS assay the change in binding affinity is directly coupled to receptor activation via the endogenous G protein. However, although the assay outputs are different, the general qualitative similarity in results between the two assays are reassuring. If the higher selectivity of DPDPE for κ-δ opioid receptors observed in [$^{35}$S]GTPγS experiments reflects its selectivity *in vivo*, the behavioral differences between DPDPE and deltorphin-II may indeed be due to their actions on different phenotypic receptors.

## 2.3. Conclusions

In order to assess the possible role of heteromers in mediating antinociception, the present study has evaluated the agonist selectivity of μ, κ, and δ standard opioid ligands that are currently widely employed in opioid research. The results reveal that all of the ligands exhibit no agonist selectivity for their presumed target homomeric opioid receptors. In fact, morphine and DAMGO selectively activate μ-δ heteromers, which is consistent with a variety of studies that implicate the involvement of μ- and δ-opioid receptors in analgesia, tolerance, and dependence [89, 136, 138, 149, 161, 162]. Given the multiplicity

of heteromeric μ receptors reported in cultured cells, perhaps many of the side effects associated with opioids can be traced to multiple opioid receptor heteromers. Opioid behavioral effects have been studied using standard ligands whose selectivity was based on binding studies and on the assumption that such tools identify the response of only homomeric receptors [172]. It is therefore imperative to confirm the functional selectivity of standard ligands on both homomeric and heteromeric GPCRs in order to confidently use them as tools for pharmacological studies. Moreover, in view of the results obtained for the standard opioid agonist ligands in the present study, the anatomical distribution of colocalized opioid receptors in the CNS should be investigated as a prelude to evaluating the possible involvement of heteromers. Finally, designing ligands that selectively target specific heteromers could represent a general approach to developing analgesics with minimal side effects.

## 2.4. Methods

**Materials.** Morphine, bramazocine, U69593, deltorphin-II in salt form and the peptide ligands DAMGO and DPDPE were provided as gifts from the National Institute on Drug Abuse .

**Cells.** Human embryonic kidney cells (HEK-293) stably expressing single opioid receptors (human μ, κ and mouse δ) were generated. HEK-293 cells co-expressing human opioid receptors in pairs were procured from Dr. Jennifer Whistler (University of California, San Fransisco). Their construction and characterization has been described previously [134]. Briefly, HEK-293 cells were

cotransfected with HA-μ and FLAG-δ (μ-δ), HA-δ and FLAG-κ (κ-δ), HA-μ and FLAG-κ (μ-κ) to make the different stable coexpression cell lines. The stably expressing cells were checked for the expression of μ-δ and κ-δ opioid receptor heteromers using co-immunoprecipitation [134, 159]. All the single and dual stable transfected cell lines were grown at 37°C and 10% $CO_2$ in Dulbelcco's modified medium (GIBCO) supplemented with 10% FBS and 1% Penicillin-streptomycin antibiotic solution. G418 alone was used for selecting cells expressing opioid receptors singly, while both G418 and Zeocin were used to select for dual coexpression cell lines.

**Immunocytochemistry.** Two-color immunofluorescence was employed to analyze co-expression of μ- and δ-opioid receptors. Briefly, HEK-293 cells stably coexpressing HA-μ and FLAG-δ opioid receptors were incubated in the presence of goat anti-HA (Abcam, Cat #ab9134) antibody mixed with rabbit anti-FLAG (Abcam, Cat #1162) antibody for 60 min at 4°C (final working dilution for both antibodies was 1:100). Cells were then rinsed with PBS and fixed with 2% formaldehyde for 10 min at room temperature. Then cells were washed (3 x 15 min) with 50 mM PBS (pH 7.2) and incubated 1 hour at room temperature with the mixture of fluorescent secondary antibodies: anti-goat NL-493 (Cat # NL003; R&D Systems, Inc.) and anti-rabbit NL-557 (Cat # NL004; R&D Systems, Inc.) both diluted 1:200. After that cells were washed in PBS (3 x 15 min), counterstained with DAPI and mounted under coverslips with anti-fade mounting media iBright Plus (Cat # SF40000-10; Neuromics, Inc.). Images

of labeled cells were collected using Olympus FluoView1000 confocal microscope.

**Intracellular $Ca^{2+}$ release assay.** HEK-293 cells stably expressing opioid receptors obtained from Dr. Jennifer Whistler [134] were transiently transfected with a chimeric $G_{\alpha}$-protein [160], $\Delta$6-$G_{qi4\text{-}myr}$ at a concentration of 200 ng/20,000 cells using Lipofectamine 2000 (Invitrogen, Carlsbad CA). The DNA for the chimeric G-protein was the only DNA that was transiently transfected. Cells were grown to a confluency of approximately 2 million cells in a petridish. The cells were then counted and DNA for the chimeric G-protein was added to a ratio of 200 ng/ 20,000 cells. Lipofectamine 2000 at a ratio of 1:2 wt/vol (DNA:Lipofectamine) was used for the transfection. The cells were then seeded 24 hours later into half area black 96 well plates (Corning) at 20,000 cells per well. The FLIPR calcium kit (Molecular devices) was used for the assay. Cells were incubated with a $Ca^{2+}$ ion chelating dye from the kit, 48 hours after transfection, and incubated for an hour. The plates were then assayed in a Flexstation-III apparatus (Molecular Devices) using a range of concentrations of the opioid ligand. The response was measured as Relative Fluorescence Units (RFUs) and the time of the response was measured in seconds. A response window of 33 seconds after ligand addition was used to measure the response before calcium ion reuptake mechanisms caused a drop in fluorescence. Area under the curve (RFU x seconds) was computed for each concentration which was then plotted as a concentration response curve using non-linear regression. To incorporate well-well variability, four well replications were

performed for each concentration of the ligand. Importantly, each ligand was tested in at least three independent replications where, each replicate experiment consists of cells transiently transfected with the chimeric G-protein on a separate day thus ensuring true biological replication. The representative curves, $EC_{50}$ and $AUC_{peak}$ values are all thus calculated from the four internal/dependent and three independent replications. Thus any variability due to transfection is contained in the error bars and has been accounted for.

**[$^{35}$S]GTPγS assay.** The [$^{35}$S]GTPγS assay as described previously [173] was used to measure the level of activation of endogenous $G_{i/o}$ proteins due to receptor activation. Briefly, using varying concentrations of ligand, HEK-293 cell membranes expressing opioid receptors and [$^{35}$S]GTPγS (Perkin-Elmer) were incubated together in a 96 well plate in membrane buffer (50mM Tris-HCl, 3mM $MgCl_2$, 0.2mM EGTA, 100mM NaCl and 0.5% BSA) for an hour at 37C. The incubated mixtures were then filtered onto a filter plate (Multiscreen HTS, Millipore) and counted for [$^{35}$S]. At least three replications were performed for each treatment.

# CHAPTER 3

## 3. SELECTIVITY OF CLINICALLY USED OPIOID ANALGESICS

### 3.1. Introduction

Morphine and related drugs (methadone and fentanyl) are widely employed as analgesics despite the side effects associated with their use. Although morphine, methadone, and fentanyl are thought to mediate analgesia through mu opioid receptors, delta opioid receptors have been implicated in mediating side effects such as tolerance and dependence. Historically, opioid mixed agonist-antagonists were developed so as to counter the tolerance and dependence seen with opioid analgesics. While some of these compounds did show reduced abuse potential, incidence of psychotomimetic side effects limited their use. Buprenorphine is a partial agonist-antagonist that has the least deleterious side effects and been shown to have promise in maintanence programs. Until now, the possibility that some of these ligands could be targeting opioid heteromers has not been considered. Our idea was to investigate their selectivity profile to elucidate patterns in ligand selectivity vs side effect profile. Such a study will be beneficial in providing clues towards designing drugs that are potent analgesics, devoid of tolerance or abuse potential.

The data are presented in three parts:

(a) The in vitro and in vivo evaluation of morphine, methadone, and fentanyl will be described first. In collaboration with Drs. Steve Negus and

Matthew Banks (Virginia Commonwealth University), we present evidence in rhesus monkeys that morphine, fentanyl and possibly methadone selectively activate mu-delta heteromers to produce antinociception that is potently antagonized by the delta opioid receptor antagonist, naltrindole (NTI). Studies with HEK293 cells expressing mu-delta heteromeric opioid receptors exhibit a similar antagonism profile with NTI. Taken together, these results strongly suggest that in primates, mu-delta heteromers are allosterically coupled and mediate antinociceptive effects of at least some opioid analgesics. Given the known involvement of delta receptors in morphine tolerance and dependence, our results implicate mu-delta heteromers in mediating these side effects.

(b) In the second section, we will present data on the in vitro evaluation of pentazocine, nalbuphine and butorphanol. While pentazocine and butorphanol strongly activated mu-kappa and mu-delta receptors, nalbuphine produced equivalent activation of mu-kappa, mu-delta, and kappa-delta heteromers. The various implications relating to ligand selectivity are presented and discussed.

(c) In the last section, the selectivity of buprenorphine that is currently being employed in the opioid addiction treatment clinics was evaluated. Given that buprenorphine produces limited tolerance and dependence when compared with morphine, we were surprised to observe that buprenorphine and morphine selectively activate mu-delta heteromers to the same extent. However, buprenorphine is also an antagonist at delta opioid receptors in the intracellular calcium release assay. Moreover, in contrast with DAMGO, buprenorphine does not produce endocytosis of mu or mu-delta receptors in

HEK293 cells. Taken together, the data suggest a unique activity profile for buprenorphine, which may be indicative of its behavioral effects in vivo.

## 3.2. Morphine, methadone and fentanyl activate mu-delta heteromers in HEK-293 cells, mice, and rhesus monkeys

The functional selectivity of methadone and fentanyl (Fig 3.1) was determined using the intracellular calcium release method as described previously ([174]). Given that the calcium release and [$^{35}$S]GTPγS assays give qualitatively similar results with respect to the selectivity of morphine for mu-delta heteromers, calcium release is a reasonable method for such studies.

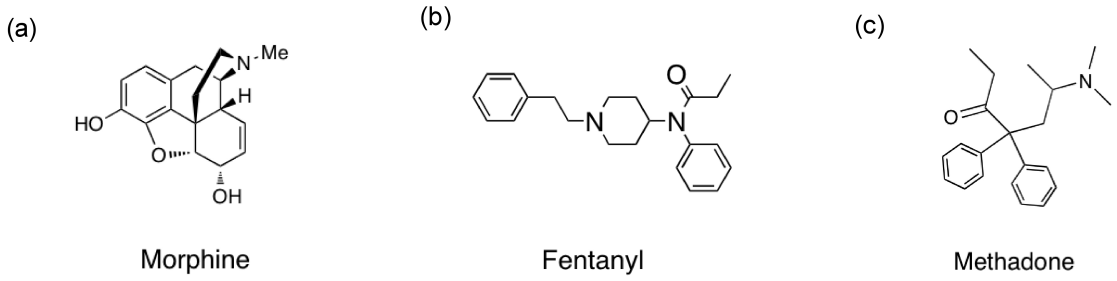



**Figure 3.1 Chemical structures of morphine (a), fentanyl (b) and methadone (c).**

HEK-293 cells stably expressing mu or delta receptors alone, mu and delta receptors in pairs, and mu and kappa receptors together were used for

this study. Both methadone and fentanyl, while activating mu receptors, produced more potent activation in cells coexpressing mu and delta opioid receptors (Fig 3.2). Methadone was ~12-fold more potent at mu-delta ($EC_{50}$ = 67.7 ± 1.9 nM) than at mu ($EC_{50}$ = 837.5 ± 23.5 nM) receptors, while fentanyl was ~6-fold more potent in cells expressing both mu and delta receptors ($EC_{50}$ = 54.7 ± 18.2 nM) when compared to cells containing only mu receptors ($EC_{50}$ = 326.6 ± 12.0 nM). Neither of these ligands produced significant activity in cells expressing only delta opioid receptors. Thus, when taken together with the morphine data ([174]) (Fig 3.2a), all three of these commonly used analgesics selectively activate mu-delta opioid receptor heteromers.

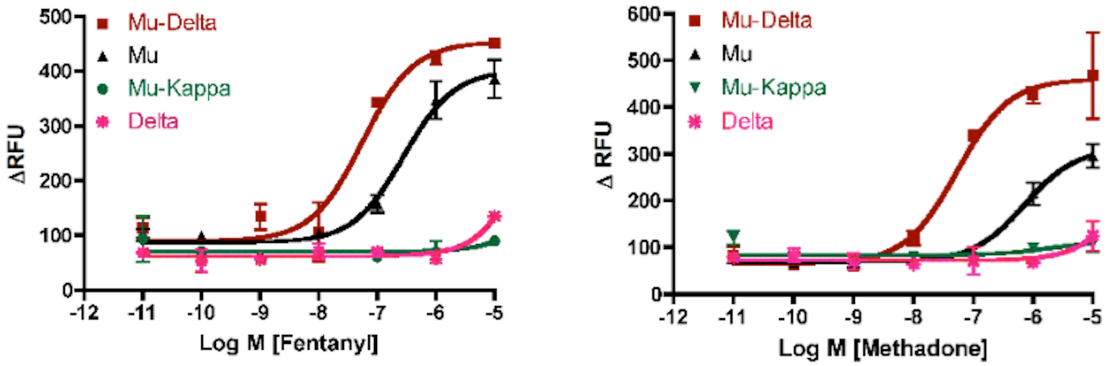



**Figure 3.2. Clinical ligands methadone and fentanyl selectively activate mu-delta opioid receptor heteromers.**

Intracellular Ca2+ ion release mediated by increasing concentrations of the agonist, were measured in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric G-protein ($\Delta$6-$G_{\alpha qi4\text{-}myr}$). Response was measured as Relative Fluorescence Units (RFU). The Y-axis plots the change in RFU for (a) fentanyl and (b) methadone.

To relate the contribution of the delta protomer in mu-delta heteromers to the agonist effect of morphine, methadone, and fentanyl in HEK293 cells, we examined the ability of the delta-selective antagonist, NTI ([72]), to affect the activation at homomeric and heteromeric opioid receptors (Fig 3.3). NTI did not produce any antagonism in cells expressing mu opioid receptors alone (Fig 3a-c). Significantly, NTI antagonized the activation of these analgesics at mu-delta heteromers (Fig 3.3d-f), strongly suggesting allosterism between the delta and mu protomers. Another distinction is that the NTI antagonism of methadone is surmountable by increasing the concentration of methadone (Fig 3.3f), whereas this is not the case with morphine and fentanyl (Fig 3.3d, e). That the antagonism was mediated via the delta protomer rather than its mu partner was suggested by the inability of NTI to antagonize the activation of singly expressed mu opioid receptors in HEK293 cells.

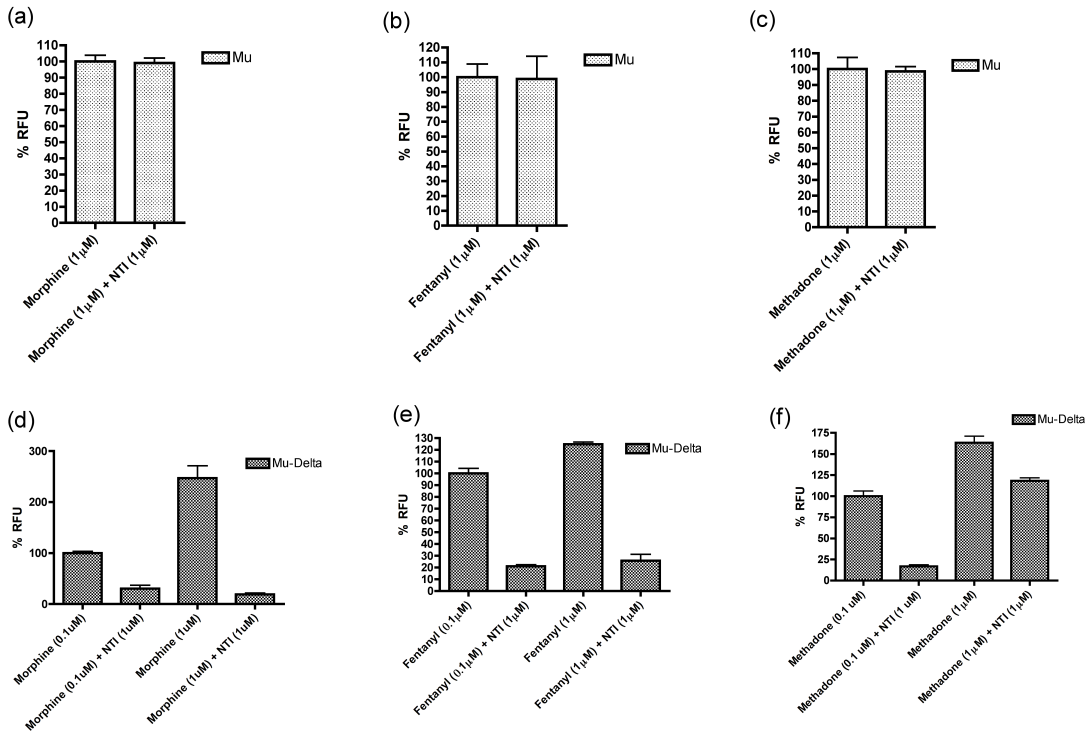



**Figure 3.3 Morphine, methadone and fentanyl selectively activate mu-delta opioid receptor heteromers.**

Intracellular $Ca^{2+}$ ion release mediated by increasing concentrations of either (a) morphine (adapted with permission from ref. 28), (b) fentanyl or (c) methadone, were measured in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric G-protein ($\Delta$6-$G_{\alpha qi4\text{-}myr}$). Response was measured either as (a) AUC = Relative Fluorescence Units * time (RFU*sec) or (b – c) change in Relative Fluorescence Units ($\Delta$ RFU = $RFU_{max}$ - $RFU_{min}$). All points in the panels represent mean ± SEM from triplicate biological replications.

In order to investigate the relevance of our cell-based results with respect to the antinociception produced by morphine, methadone and fentanyl, we conducted analogous studies in rhesus monkeys. All three ligands were observed to produce dose-dependent thermal antinociception ([175]), as shown

by the dose-effect curves for each opioid agonist administered alone (Figure 3.4a-c; Table 1).

To evaluate whether these agonists were producing antinocicpetion via mu-delta heteromers or homomeric mu receptors, NTI was employed to determine if it would antagonize their effects. Indeed, antagonism of the antinociceptive effects of morphine, methadone, and fentanyl was observed upon NTI pretreatment (0.1 – 3.2 mg/kg) (Figure 3.4a-c). Naltrindole produced a dose-dependent rightward shift in the morphine and fentanyl dose-effect curves with significant increases in morphine and fentanyl $ED_{50}$ values at NTI doses of 0.32 – 3.2 mg/kg. While NTI also shifted the methadone dose-effect curve to the right, the antagonist effect was significant only at the highest dose of NTI (3.2 mg/kg). Figure 3.4d and Supplemental Table S1 show NTI antagonism quantified as dose ratios. Doses of 1.0 and 3.2 mg/kg of NTI produce greater antagonism of morphine and fentanyl than of methadone. Overall, NTI was more potent as an antagonist of antinociception induced by morphine and fentanyl than by methadone.

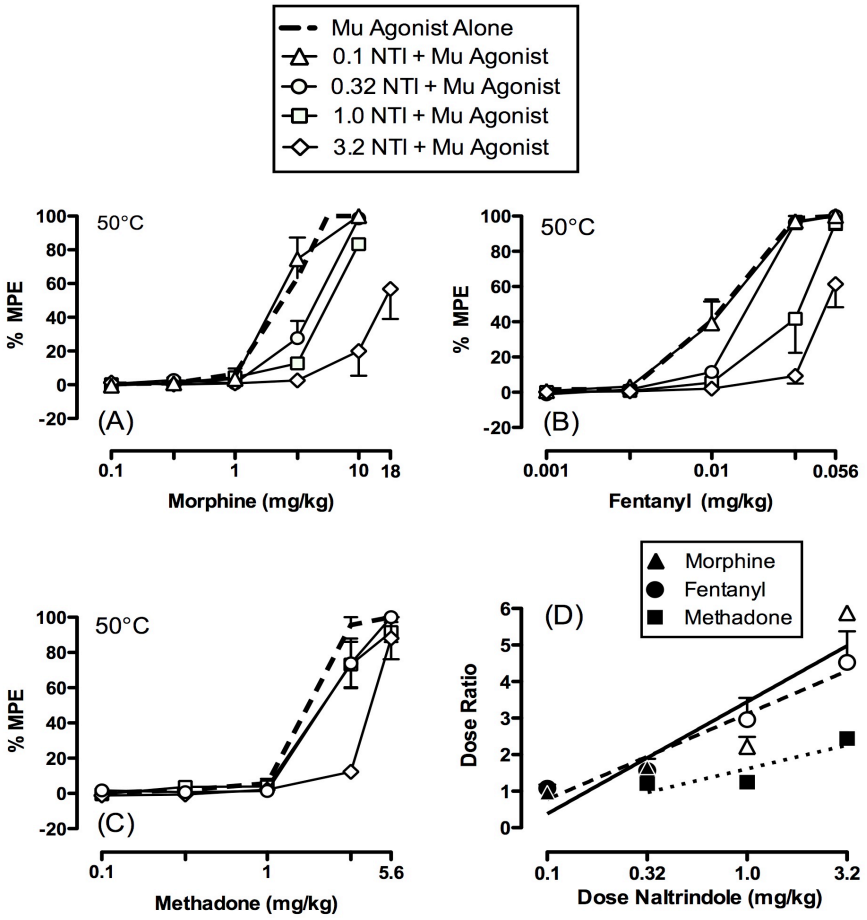



**Figure 3.4 Effects of pretreatment with NTI on morphine (a), fentanyl (b), and methadone (c)-induced antinociception in the assay of thermal nociception using a 50°C thermal stimulus in rhesus monkeys (n=3-4) (performed by Drs. Steve Negus and Matthew Banks at VCU).**

Naltrindole was administered 30 min before the start of the behavioral session. Abscissae for panels (a - c): Dose of test drug in mg/kg (log scale). Ordinates for panels (a – c): Percent maximal possible effect (%MPE). Dashed lines indicate the antinociceptive effects of each mu agonist alone. Panel (d) shows dose ratios (ED50 of mu agonist in the presence of naltrindole ÷ ED50 of the mu agonist alone) for morphine, fentanyl, and methadone as a function of the naltrindole dose. Open symbols in panel (d) represent points significantly different from methadone within a naltrindole dose as indicated by non-overlapping confidence limits shown in Table 2. All points in all panels represent mean ± SEM.

The potency of NTI in the antagonism of the antinociceptive effect of morphine and fentanyl bears a similarity to the antagonism of the behavioral effects of selective delta agonists in rhesus monkeys, in that the doses of NTI are in a similar range as the present study ([176-179]). In light of this and our antagonism studies in HEK293 cells, it is conceivable that NTI binds to the delta protomer, thereby allosterically inhibiting signaling by agonist bound to the mu protomer. Indeed, we have observed that morphine and fentanyl did not surmount the antagonistic effects of the highest dose of NTI (3.2 mg/kg) in the dose ranges tested in rhesus monkeys (Fig 3.4). [Note that higher morphine and fentanyl doses were not tested due to the emergence of severe sedative and/or respiratory depressant effects that were apparently insensitive to naltrindole antagonism. In these cases, monkeys were treated with 0.1 mg/kg IM naltrexone, which effectively reversed morphine- and fentanyl-induced untoward effects.]

That insurmountable antagonism was also observed in HEK-293 cells (Fig 3.3), suggests the possibility of such an allosteric mechanism. Insofar as NTI has relatively low affinity for isolated mu receptors, these results are consistent with the hypothesis that the thermal antinociceptive effects of morphine and fentanyl are not mediated by homomeric mu receptors, but rather by mu-delta heteromeric opioid receptors *in vivo*.

Unlike morphine and fentanyl, the antagonism of methadone by NTI appears to be surmountable (Fig 3.3f, Fig 3.4c). This supports the idea that the

cell-based studies represent a credible model for the role of mu-delta heteromers in rhesus monkeys. The lower sensitivity of methadone to NTI-induced antagonism of antinociception could arise as a consequence of a methadone-induced conformational perturbation of the mu-delta heteromer that is different from that induced by morphine or fentanyl.

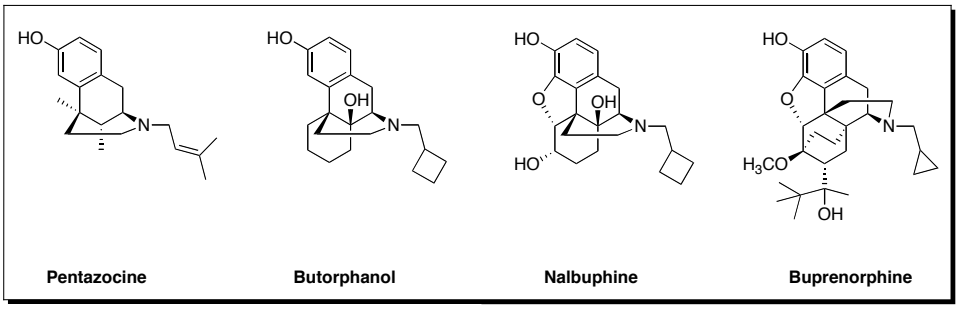



**Figure 3.5 Opioid mixed agonist-antagonists**

## 3.3. Opioid mixed agonist-antagonists

### 3.3.1. Pentazocine is equivalently active at both mu-kappa and mu-delta heteromers

Pentazocine (Fig 3.5) has been classified as a mu partial agonist, kappa agonist[2, 33]. To evaluate its selectivity, we utilized HEK293 cells that are stably expressing the various opiopid receptors singly or in pairs and transiently expressing $G_{qi4}$ chimeric protein. The intracellular calcium release assay as mentioned in chapter 3.1 was used for the analysis. Pentazocine was a partial agonist at mu receptors (Fig 3.6) with little to no activity in cells expressing either delta, kappa or kappa-delta receptors. Significantly, Pentazocine was potent and similarly active in cells that contained mu-kappa ($EC_{50}$ = 74.3 nM;

$AUC_{peak}$ = 15,004 RFUxSec) and mu-delta ($EC_{50}$ = 130.9 nM; $AUC_{peak}$ = 14,974 RFUxSec) receptors. Given that the ligand did not show much activity at kappa receptors, it is possible that it was mu-kappa activation that was erroneously deemed to kappa agonism in the past.

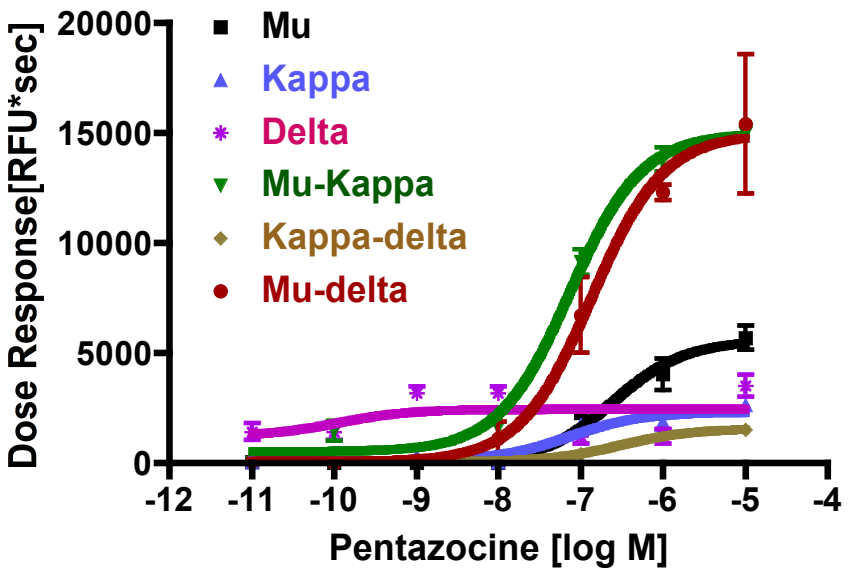



**Figure 3.6 Pentazocine activates mu-kappa and mu-delta opioid receptor heteromers.**

Intracellular Ca2+ ion release mediated by increasing concentrations of the agonist, were measured in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric G-protein ($\Delta$6-$G_{\alpha qi4\text{-}myr}$). Response was measured as Relative Fluorescence Units (RFU). The Y-axis plots the area under the curve (RFU x seconds) for pentazocine. All experiments were performed in triplicate with four internal replications.

### 3.3.2. Butorphanol preferentially activates mu-kappa heteromers in HEK293 cells

The clinical effects of butorphanol are very similar to pentazocine and it is hence classified as a partial mu agonist, full kappa agonist[33]. When butorphanol was tested in HEK293 cells expressing the various opioid receptors, it was a partial agonist at mu opioid receptors (Fig 3.7). Though the potency of butorphanol was ~ 6-fold higher at mu (EC50 = 26.8 nM) over mu-delta ($EC_{50}$ = 144.6 nM) receptors, it produced greater amount of calcium release at mu-delta ($AUC_{peak}$ = 13404 RFUxSec) when compared with mu ($AUC_{peak}$ = 7641 RFUxSec). Significantly, butorphanol was most potent and active at mu-kappa receptors ($EC_{50}$ = 17.2 nM; $AUC_{peak}$ = 17,343 RFUxSec), with ~9-fold greater $EC_{50}$ compared with mu-delta. Just like with pentazocine, the previously determined kappa activity may be mediated via mu-kappa heteromers.

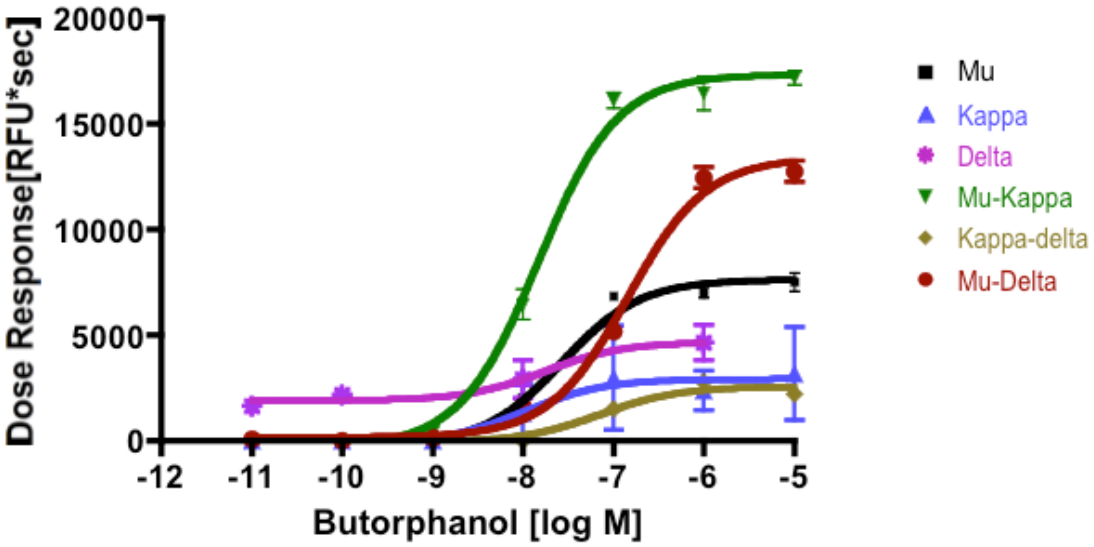



**Figure 3.7 Butorphanol activates mu-kappa and mu-delta opioid receptor heteromers.**

Intracellular Ca2+ ion release mediated by increasing concentrations of the agonist, were measured in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric G-protein ($\Delta$6-$G_{\alpha qi4\text{-}myr}$). Response was measured as Relative Fluorescence Units (RFU). The Y-axis plots the area under the curve (RFU x seconds) for butorphanol. All experiments were performed in triplicate with four internal replications.

### 3.3.3. Nalbuphine is a non-selective agonist for opioid receptors heteromers

Nalbuphine has been classified as a mu antagonist, kappa full agonist[33]. When tested in the HEK293 cell lines expressing opioid receptors, nalbuphine showed partial agonism at kappa and negligible activation at mu and delta opioid receptors. Interestingly, nalbuphine was most potent in cells expressing kappa-delta heteromers ($EC_{50}$ = 30.3 nM; $AUC_{peak}$ = 7121 RFUxSec) with ~ 2-

fold and ~ 5-fold greater $EC_{50}$ than at mu-kappa ($EC_{50}$ = 72.5 nM; $AUC_{peak}$ = 7341 RFUxSec) or mu-delta ($EC_{50}$ = 164.6 nM; $AUC_{peak}$ = 6948 RFUxSec) heteromers (Fig 3.8). However, nalbuphine was equivalently active in all these cell lines. Given the rather minimal differences in potency, the data suggests that nalbuphine is non-selective agonist the three opioid receptor heteromers.

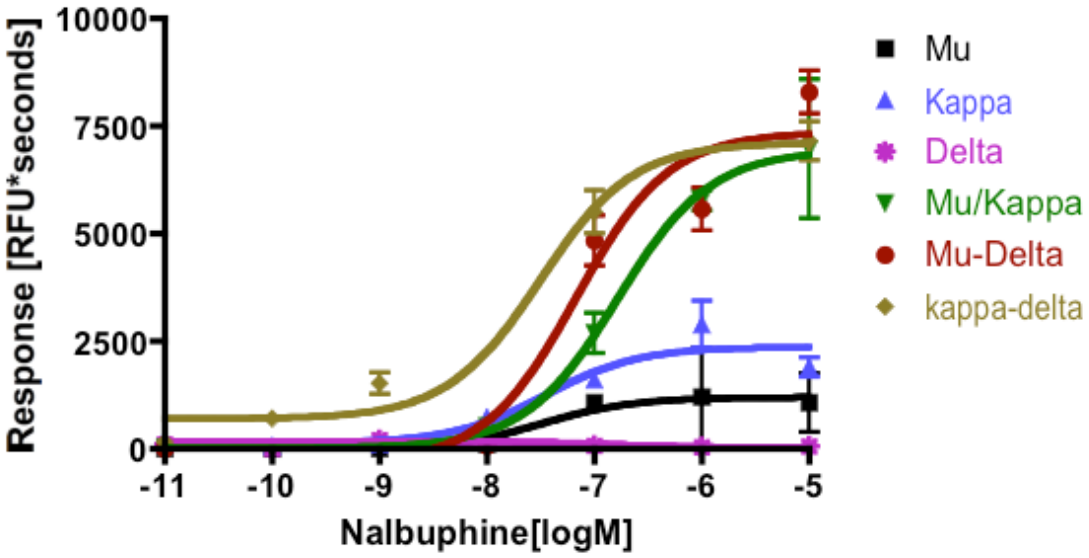



**Figure 3.8 Nalbuphine is a non-selective agonist for kappa-delta, mu-kappa, and mu-delta opioid receptor heteromers.**

Intracellular Ca2+ ion release mediated by increasing concentrations of the agonist, were measured in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric G-protein ($\Delta 6\text{-}G_{\alpha qi4\text{-}myr}$). Response was measured as Relative Fluorescence Units (RFU). The Y-axis plots the area under the curve (RFU x seconds) for nalbuphine. All experiments were performed in triplicate with four internal replications.

### 3.4. Buprenorphine is a mu-delta agonist/delta antagonist

Buprenorphine has gained a lot of clinical use as an analgesic for chronic pain and in opioid maintenance programs[180, 181]. It has been classified as a mu partial agonist with noticeable antagonism at kappa and delta opioid receptors. Buprenorphine also has attenuated tolerance and dependence liabilities, which has allowed for substitution in opioid addicts for long-term maintenance and use in chronic pain.

#### 3.4.1. Buprenorphine selectively activates mu-delta opioid heteromers

To identify the receptors targeted by buprenorphine, we utilized the intracellular calcium release assay in HEK-293 cells containing mu, kappa or delta opioid receptors singly, or in pairs. The dual transfected cell lines have been previously characterized and shown to contain opioid heteromers[134]. We have also shown that these cell lines express both receptors in similar densities and are extensively colocalized[174, 182].

Buprenorphine was highly selective for mu-delta heteromers as it showed the highest potency and efficacy (Fig 3.8, Table 1) in cells coexpressing mu and delta receptors (EC50 = 36.3 nM; $AUC_{peak}$ = 20111 RFU*Sec). It was a partial agonist with some activity at mu-kappa receptors (EC50 = 403 nM; $AUC_{peak}$ = 11342 RFU*Sec) and mu receptors (EC50 = 78.9 nM; $AUC_{peak}$ = 7890 RFU*Sec). Significantly, buprenorphine was completely inactive at delta opioid receptors (Fig 3.9).

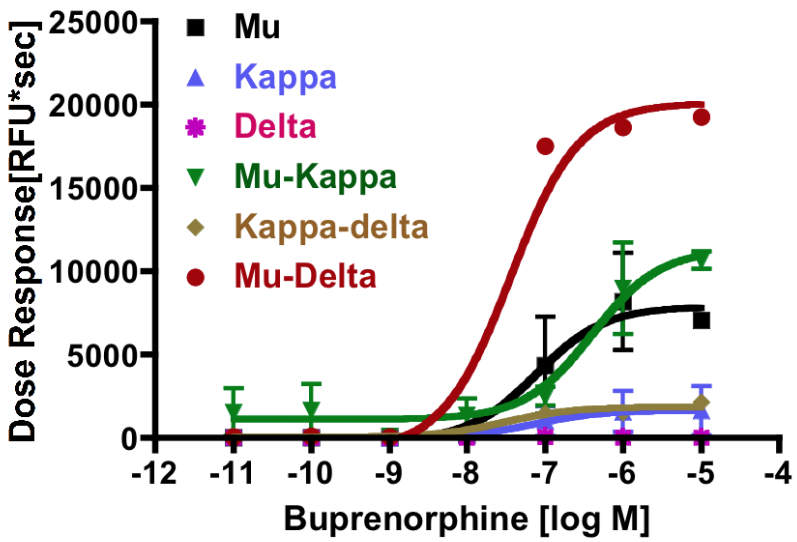



**Figure 3.9 Buprenorphine selectively activates mu-delta opioid receptor heteromers.**

Intracellular Ca2+ ion release mediated by increasing concentrations of the agonist, were measured in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric G-protein (Δ6-Gαqi4-myr). Response was measured as Relative Fluorescence Units (RFU). The Y-axis plots the area under the curve (RFU x seconds) for buprenorphine. All experiments were performed in triplicate with four internal replications.

### 3.4.2. Buprenorphine is a delta opioid antagonist

Given the fact that buprenorphine was not active in HEK cells expressing delta opioid receptors, we evaluated its ability to produce antagonism at delta receptors. In the intracellular calcium release assay, buprenorphine produced potent antagonism ($p<0.005$, t-test) of DPDPE in HEK-293 cells expressing delta receptors (Fig 3.10). Buprenorphine has been previously suggested to

have delta opioid antagonist activity in monkeys and is thus a mu-delta heteromer agonist with delta antagonist activity.

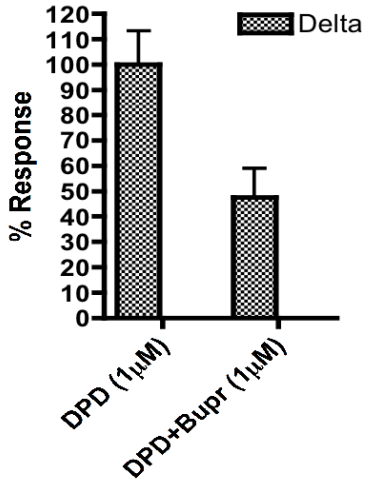



**Figure 3.10 Buprenorphine is an antagonist at mu-delta opioid receptor heteromers.**

Intracellular Ca2+ ion release mediated by the delta-selective agonist, DPDPE (1 μM), was measured in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric G-protein ($\Delta$6-$G_{\alpha qi4\text{-}myr}$) with and without buprenorphine (1 μM). Buprenorphine significantly antagonized the calcium fluxes stimulated by DPDPE. Response was measured as Relative Fluorescence Units (RFU). The Y-axis plots the area under the curve (RFU x seconds) for DPDPE. All experiments were performed in triplicate with four internal replications.

### 3.4.3. Buprenorphine does not produce internalization of mu or delta receptors

Internalization of receptors has been implicated as a possible mechanism for desensitization of receptors that can lead to tolerance. However, the fact that morphine does not produce any internalization of mu opioid receptors has

challenged this idea. To determine the internalization profile of buprenorphine, we performed immunofluorescence experiments in HEK-293 cells containing mu receptors alone or coexpressing mu and delta opioid receptors (Fig 3.11). The cells were grown in 8-well chamber slides overnight and then treated with buprenorphine (1 μM) for 30 minutes. Interestingly, we observed that buprenorphine did not produce any internalization of mu or delta opioid receptors, in either cell line (Fig 3.11).

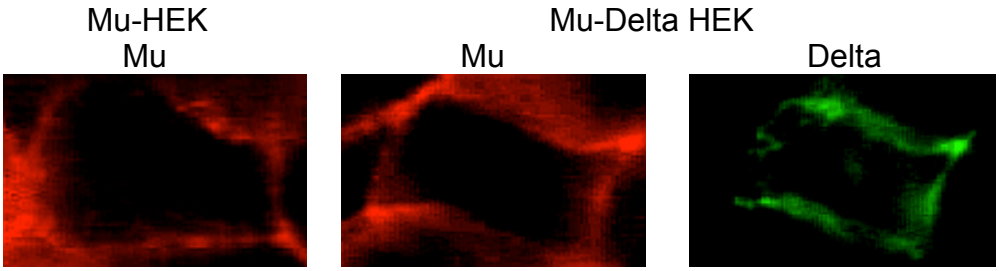



**Figure 3.11 Internalization studies with buprenorphine.**
Buprenorphine did not promote internalization of mu receptors when expressed alone. In HEK293 cells containing both mu and delta receptors, buprenorphine did not promote internalization of either receptor.

## 3.5. Conclusions

In conclusion, it appears that morphine, methadone, and fentanyl, which have been traditionally viewed as mu-selective ligands, selectively activate heteromeric mu-delta receptors in rhesus monkeys. Data supporting this conclusion were derived from studies involving the antagonism of activation of mu-delta heteromers in HEK293 cells and antagonism of antinociception in

monkeys using the delta opioid receptor antagonist, NTI. Evidence for allosterically mediated antagonism is presented in both cell-based and monkey studies. Given the homology between rhesus monkeys and humans, this work opens the possibility of heteromeric mu-delta receptor-mediated analgesia by morphine, fentanyl, and methadone in humans. While the extent of antinociception mediated by mu-delta heteromers *in vivo* remains unanswered due to paucity of knowledge about their presence and distribution, mu-delta heteromeric opioid receptors also may mediate some of the deleterious side effects as implicated in prior studies ([136-138, 149, 161]). Because delta-selective antagonists are widely employed as pharmacologic tools for characterization of delta opioid receptors *in vivo*, the results of present study reveal possible pitfalls in attributing antagonism of antinociception to delta receptors alone, if delta receptor-containing heteromers are also targeted. Consequently, the literature will require re-examination in light of the present results.

The data from the selectivity studies of the mixed agonist-antagonists showed that pentazocine, butorphanol, and nalbuphine strongly activated mu-kappa heteromers. Previous studies in our lab have shown that a mu-kappa selective agonist, NNTA, produced potent antinociception without any physical dependence or place preference. It is possible that the ability to activate mu-kappa heteromers may be responsible for the reduced clinical side effects observed for these ligands. In addition to mu-kappa heteromers, the ligan ds also activated mu-delta heteromers. Given the studies that implicated mu-delta heteromers in tolerance and physical dependence[89, 161], we speculate that their

activity at mu-delta heteromers may be responsible for the mild to moderate withdrawal syndrome precipitated by these ligands in man. It is telling that buprenorphine, which is the ligand with least propensity to precipitate tolerance and dependence in vivo, has a mu-delta agonist, delta antagonist profile. It has been previously shown[161] that co-administration of delta antagonist NTI with morphine attenuated tolerance and dependence. In addition, the MDAN series of bivalent ligands[138] have a similar profile where they bridge mu-delta heteromers and do not produce any supraspinal tolerance, dependence or place preference.

Finally, given the side effects associated with analgesics that act through mu-delta heteromers, the present work has implications with respect to development of new analgesics, as it clearly suggests a paradigm shift involving a greater reliance on coexpressed receptors in the screening effort.

## 3.6. Methods

***Subjects:*** Four adult rhesus monkeys (two female and two male) were used in studies of thermal nociception. Subjects weighed 4.5 to 12 kg during the course of these studies. All monkeys had prior exposure to central nervous system acting drugs (primarily opioid compounds) and to the behavioral procedures in which they were tested. The subjects were individually housed, and water was freely available. Their diet consisted of Lab Diet high protein monkey chow (Purina, Framingham, MA). This diet was supplemented with

fresh fruit twice daily. A 12-h light/12-h dark cycle was in effect (lights on from 7:00 AM to 7:00 PM).

Animal maintenance and research were conducted in accordance with the guidelines provided by the National Institutes of Health Committee on Laboratory Animal Resources. The facility was licensed by the United States Department of Agriculture, and accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care. Protocols were approved by the Institutional Animal Care and Use Committee. The health of the monkeys was monitored daily by technical staff and periodically by a veterinarian. Monkeys had visual, auditory, and olfactory contact with other monkeys throughout the study. Monkeys also had access to puzzle feeders, mirrors, and chew toys to provide environmental enrichment. Music was played daily in all housing rooms.

***Assay of thermal nociception:*** Monkeys were seated in acrylic restraint chairs so that their tails hung down freely. The bottom 10 cm of each monkey's shaved tail was immersed in a thermal container of warm water. If the subject did not remove its tail within 20 sec, the tail was removed by the experimenter, and a latency of 20 s was assigned to that measurement. During each cycle of measurements, tail-withdrawal latencies were measured from water heated to 38°C and 50°C. The order in which the temperatures were presented varied from one set of measurements to the next. Experiments were conducted no more than twice a week with at least two days between tests. A stopwatch was used to measure and record time intervals.

Each test session consisted of multiple 15-min cycles. Before the first drug dose was administered, baseline latencies to tail-withdrawal at 38 and 50 water were determined. Testing continued only if tail withdrawal from 38°C water did not occur before the 20 s cutoff, and if tail withdrawal occurred in ≤ 2 s from 50°C water. This criterion was met during every session with every monkey in this study. During cumulative dosing experiments, a single drug dose was administered at the start of each sequential 15 min cycles, and each dose increased the total cumulative dose by one-fourth or one-half log units. Starting 10 min after each injection, tail-withdrawal latencies were recorded as described above. Initially, complete dose-effect curves were determined for morphine (0.1 – 10 mg/kg), fentanyl (0.001 – 0.056 mg/kg), and methadone (0.1 – 5.6 mg/kg) alone. Subsequently, doses of naltrindole (0.1 – 3.2 mg/kg) were randomly administered 30 min as a pretreatment before redetermining the morphine, fentanyl, or methadone dose-effect curve.

***Data analysis:*** Drug effects were expressed as %Maximum Possible Effect (%MPE) using the following equation:

%MPE = (Test Latency – Baseline Latency)/(20 – Baseline Latency)*100

where test latency was the tail withdrawal latency from 50°C water obtained after drug administration, and control latency was the latency obtained at the beginning of the session prior to drug administration. ED50 for each drug was defined as the dose that produced 50%MPE, and these values were determined by interpolation when only two data points were available (one below and one above 50 percent control response rate) or by linear regression

when at least three data points were available on the linear portion of the dose-effect curve. Individual ED50 values were averaged to determine mean values and 95% confidence limits, and values were considered to be significantly different if confidence limits did not overlap.

To quantify antagonist effects of naltrindole, dose ratios were calculated for each subject as the ED50 of the mu agonist in the presence of naltrindole ÷ ED50 of the mu agonist alone. Individual dose ratios were averaged to determine mean values and 95% confidence limits, and values were considered to be significantly different if confidence limits did not overlap.

***Drugs:*** Morphine sulfate, fentanyl HCl, and (±)-methadone HCl were provided by the National Institute on Drug Abuse (Besthesda, MD) and dissolved in sterile water. Naltrindole HCl was generously provided by Dr. Kenner Rice (Chemical Biology Research Branch, National Institute on Drug Abuse and National Institute on Alcohol Abuse and Alcoholism). Morphine, fentanyl, methadone, and naltrindole were dissolved in sterile water. Drugs were administered intramuscularly in the thigh, and doses were determined based on the salt forms listed above.

**Cell Culture and Intracellular $Ca^{2+}$ release assay.** HEK-293 cells containing singly expressed opioid receptors were generated using pcDNA3 vectors (Invitrogen, Carlsbad, CA) encoding human kappa, mu and murine delta opioid receptors. HEK-293 cells stably co-expressing mu-kappa, kappa-delta and mu-delta receptors were obtained from Dr. Jennifer Whistler and their construction has been previously described and verified ([134, 159]). The chimeric

G-protein, $\Delta$6-$G_{\alpha qi4\text{-}myr}$, was graciously provided by Dr. Evi Kostenis and its construction has been previously described ([160]).

HEK-293 cells were cultured at 37°C in Dulbelcco's modified Eagle's medium supplemented with 10% bovine calf serum, Pen/Strep antibiotics. For cells singly expressing opioid receptors, G418 was used as the selection antibiotic; G418 and Zeocin were used for selecting for cells coexpressing two opioid receptors. The protocol for the intracellular calcium release has been previously described in detail ([174]). Briefly, the stable opioid receptor cell lines were transiently transfected with 200 ng/20,000 cells of the chimeric G-protein, $\Delta$6-$G_{\alpha qi4\text{-}myr}$, using OptiMEM medium (Invitrogen) and Lipofectamine 2000 (Invitrogen, Carlsbad CA) reagent according to manufacturer's protocol (1:2 wt/vol ratio for DNA:Lipofectamine). The cells were seeded into 96 well plates (half-area; Corning) at 20,000 cells/well after 24 hours and assayed 48 hours after transfection using the FLIPR calcium kit (Molecular devices) in a Flexstation-III apparatus (Molecular devices). The response was measured as area under the curve (Relative Fluorescence Units X seconds) and plotted using non-linear regression.

# CHAPTER 4[2]

# 4. THE DEVELOPMENT OF A SELECTIVE AGONIST FOR MU-KAPPA HETEROMERS

## 4.1. Introduction

Numerous GPCRs have been shown to form heteromeric receptors in cell-based assays. Among the many heteromers reported in the opioid receptor family are mu/kappa, kappa/delta and mu/delta. However, the *in vivo* physiological and behavioral relevance for the proposed heteromers have not yet been established. In the previous chapters, we have examined the selectivity of standard and clinical opioid ligands. An important conclusion that stemmed from those studies pertained to the possibility that mu-delta opioid heteromers may be playing a major role in opioid tolerance and dependence. With that in mind, we were interested elucidating the physiological and pharmacological role of mu-kappa opioid heteromers. In the current chapter, we describe the development of NNTA (Fig 4.1) as a selective agonist for mu-kappa heteromers.

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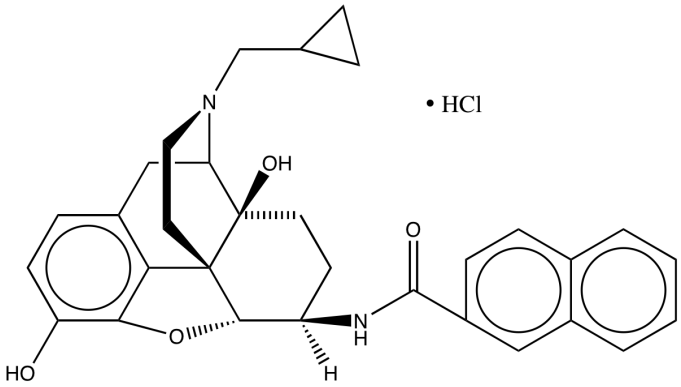



**Figure 4.1 Structure for *N*-Naphthoyl-β-naltrexamine (NNTA)**

## 4.2. Results

### 4.2.1. Immunoflourescence

HEK-293 cells stably expressing Hemagglutinin tagged mu (HA-mu) or FLAG tagged kappa (FL-kappa) receptors were used in the immunofluorescence assay to determine their relative densities. Cells were stained with anti-HA and anti-FLAG primary and respective secondary antibodies and imaged. High-resolution confocal images reveal both mu and kappa opioid receptors to be extensively co-localized and similarly expressed on the cell surface of HEK-293 cells (Fig 4.2).

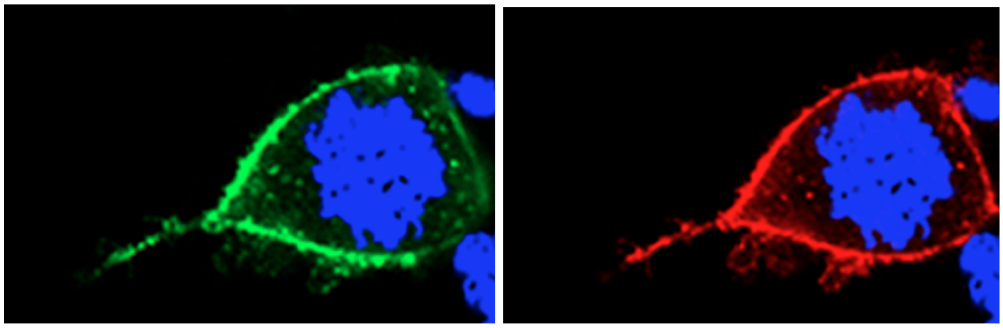


**Figure 4.2 High-magnification confocal images of double-labeling immunofluorescence for mu and kappa opioid receptors on HEK-293 cells.** The identical cells are shown labeled for mu (green fluorescence) and for kappa (red fluorescence). DAPI counterstaining is shown in blue.

### 4.2.2. Binding of NNTA in stably expressing HEK293 cells

The affinity of NNTA for the different opioid receptor types was investigated in cells stably transfected with the different types of opioid receptors. In a competition binding assay using [$^3$H]diprenorphine (Table 4.1a), NNTA was found to bind with very high affinity to cells that express mu ($K_i$ = 0.077 pM) or kappa ($K_i$ = 0.084 pM) opioid receptors. NNTA possessed substantially lower binding affinity for delta opioid receptors ($K_i$ = 1.39 nM).

The selective radioligands, [$^3$H]DAMGO and [$^3$H]U69593, were employed for mu and kappa opioid receptors, respectively, in cells containing mu or kappa receptors alone and in mu/kappa coexpressing cells (Table 4.1b). Competition binding with these radioligands afforded $K_i$ values that were essentially identical between the cells coexpressing mu/kappa receptors and cells singly expressing mu or kappa receptors.

**Table 4.1a. Competition binding of [3H]Diprenorphinea and NNTA in HEK-293 cells singly expressing opioid receptors.**

| Cell Type | $K_i$ (pM)[b] |
|---|---|
| mu | 0.0773± 0.0034 |
| kappa | 0.0843± 0.0387 |
| delta | 1390 ± 300 |

[a]Kd Values of [$^3$H]Diprenorphine: 0.16 nM (mu), 0.12 nM (kappa) and 0.36 nM (delta)

[b]Data shown as Mean ± SEM (n = 3)

**Table 4.1b. Competition binding of selective radioligands and NNTA in HEK-293 cells coexpressing mu/kappa receptors or individually expressing mu and kappa receptors.**

| Cell Type | Radioligands | |
|---|---|---|
| | [$^3$H]-DAMGO | [$^3$H]-U69593 |
| | $K_i$ (pM) | $K_i$ (pM) |
| mu/kappa coexpressed | 2.07 ± 1.18 | 3.39 ± 0.38 |
| mu | 1.25 ± 0.176 | - |
| kappa | - | 9.47 ± 5.75 |

Kd values:
[$^3$H]-DAMGO: 4.64 nM (mu) and 4.08 nM (mu/kappa)
[$^3$H]-U69593: 8.16 nM (kappa) and 5.62 nM (mu/kappa)

Data shown as Mean ± SEM (n = 3)

### 4.2.3. NNTA selectively activates mu/kappa heteromeric opioid receptors

The selectivity of NNTA for the different opioid receptors was determined using intracellular $Ca^{2+}$ release experiments in HEK-293 cells stably expressing opioid receptors. Six different cell lines containing singly expressed mu, kappa

and delta receptors or co-expressed as mu/kappa, kappa/delta and mu/delta were used for the experiments. It is important to note here that the same co-expressed cell lines used in our studies have been established to be expressing heterodimeric receptors using co-immunoprecipitation, previously[134, 159]. The six different stably expressing cell lines were transiently transfected with $\Delta$6-$G_{\alpha qi4\text{-}myr}$, a chimeric $G_{\alpha}$ subunit[160] to measure the extent of intracellular $Ca^{2+}$ ion release due to receptor activation. The chimeric G-protein has already been characterized previously and used for different $G_i/G_o$ G-protein coupled receptors including the opioid receptors[174]. Prior comparisons between the $\Delta$6-$G_{\alpha qi4\text{-}myr}$ method and [$^{35}$S]GTP$\gamma$S binding have given consistent results[174].

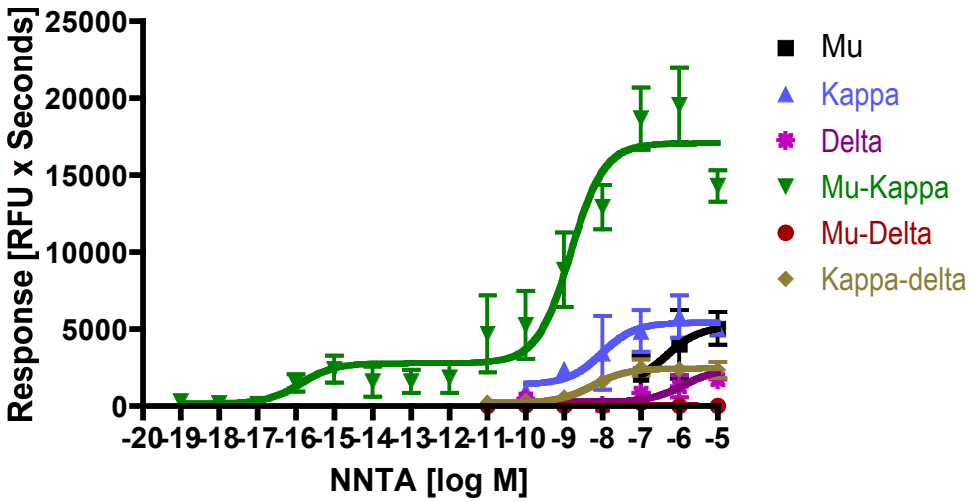



**Figure 4.3 NNTA is highly selective agonist at the mu-kappa opioid receptor heteromers in intracellular calcium release experiments.**

The concentration response curve showed a characteristic biphasic response in the HEK-293 cells coexpressing mu/kappa opioid receptor heteromers.

NNTA was substantially more efficacious in the cells coexpressing mu/kappa opioid receptors ($A_{peak}$ = 15947 RFU-Seconds) compared with other cell lines (Fig 4.3a, Table 4.2). Interestingly, the concentration response curve of NNTA at the mu/kappa opioid receptor was biphasic with activation observed at concentrations as low as $10^{-16}$ M (Fig 3a). The different cell lines were evaluated for consistent receptor expression and activation using the standard ligands, DAMGO (mu), U69593 (kappa) and DPDPE (delta), as controls (Table 4.2a and b).

**Table 4.2: (a) $EC_{50}$ and (b) [a]$AUC_{peak}$ values for $Ca^{2+}$ release from HEK-293 cells stably expressing opioid receptors**

(a) $EC_{50}$ (nM)[c]

| Cells | NNTA | DAMGO | U69593 | DPDPE |
|---|---|---|---|---|
| Mu/kappa[**] | I: 1.3E-07 ± 1.1E-07 II: 0.70± 0.33 | 13.4±10.8 | 6.5±4.1 | - |
| Kappa/delta | 17.9 ± 10.6 | - | 2.8 ± 1.3 | 21.0 ± 20.4 |
| Mu/delta | >1000 | 1.5 ± 1.4 | - | 17.6 ± 15.9 |
| Mu | >1000 | 10.6±0.4 | - | - |
| Kappa | 51.4 ± 11.0 | - | 33.7±22.1 | - |
| Delta | >1000 | - | - | 11.0 ± 10.5 |

(b) [a]$AUC_{peak}$ ($RFU^b$ X seconds)[c]

| Cells | NNTA | DAMGO | U69593 | DPDPE |
|---|---|---|---|---|
| Mu/kappa | 15947 ± 1087 | 5562 ± 668 | 4899 ± 566 | - |
| Kappa/delta | 2462 ±197 | - | 11803 ± 1291 | 10153 ± 1479 |
| Mu/delta | 57 ± 42 | 12054 ± 1281 | - | 10504 ± 969 |
| Mu | 5203 ±1622 | 9989 ± 702 | - | - |
| Kappa | 5548 ±1285 | - | 6452 ± 737 | - |
| Delta | 2441 ±409 | - | - | 8319 ± 1117 |

** *There are two $EC_{50}$ values for mu/kappa due to the biphasic concentration curve*

[a] *AUC = Area Under the Curve ($RFU^b$ x seconds)*

[b] *RFU = Relative Fluorescent Units* [c] *Values presented as Mean ± SEM (n=12-16)*

To ensure the reproducibility of the results obtained from the $Ca^{2+}$ release experiments, we evaluated NNTA using the [$^{35}$S]GTPγS assay in membranes isolated from HEK 293 cells stably expressing mu, kappa and mu/kappa opioid receptors. It is important to note here that for this experiment, the binding of

[$^{35}$S]GTPγS to the endogenous pool of G-proteins was measured because the chimeric G-protein was not transfected. Thus, the data observed was independent of the calcium release experiments in order to reassess NNTA's unique selectivity using a different experimental technique. NNTA was again most potent at the mu/kappa opioid heteromers ($EC_{50}1$ = 0.81 fM; $EC_{50}2$ = 22 pM) when compared with kappa (1.1 pM) and mu (0.3 nM). The biphasic concentration response was even more pronounced with greater activation in the sub-pM range (Fig 4.4) using this alternate method.

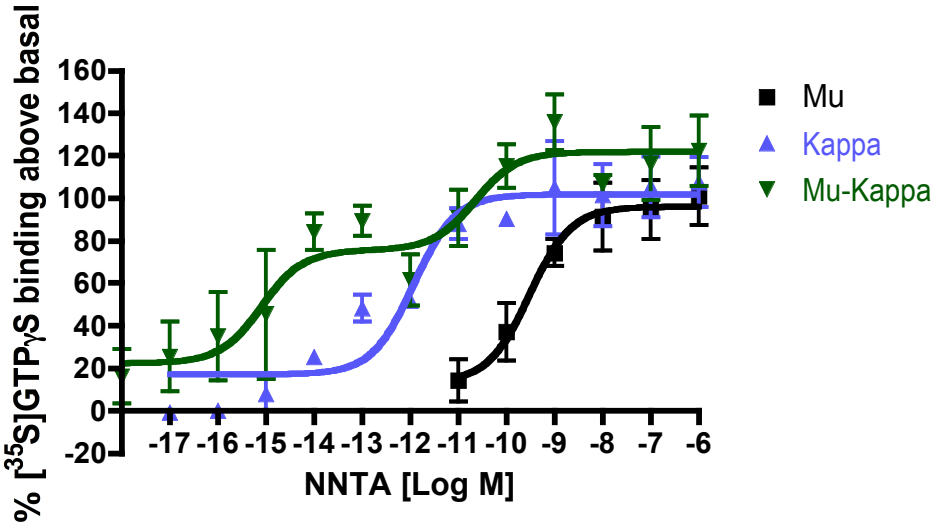



**Figure 4.4 NNTA is highly selective agonist at the mu/kappa opioid receptor heteromers in [$^{35}$S]GTPγS experiments.**

The concentration response curve showed a characteristic biphasic response in the HEK-293 cells coexpressing mu/kappa opioid receptor heteromers.

### 4.2.4. NNTA is a potent antagonist at homomeric mu opioid receptors

Given that NNTA contains a pharmacophore derived from the mu-selective opioid antagonist, naltrexone, we wished to evaluate the possibility of

antagonism at mu opioid receptors. We therefore evaluated NNTA antagonism of the standard mu agonist, DAMGO, in the mu opioid receptor cell line. NNTA (0.1 pM) was observed to significantly antagonize (~80%) the effect produced by 1 μM DAMGO (Fig. 4.5).

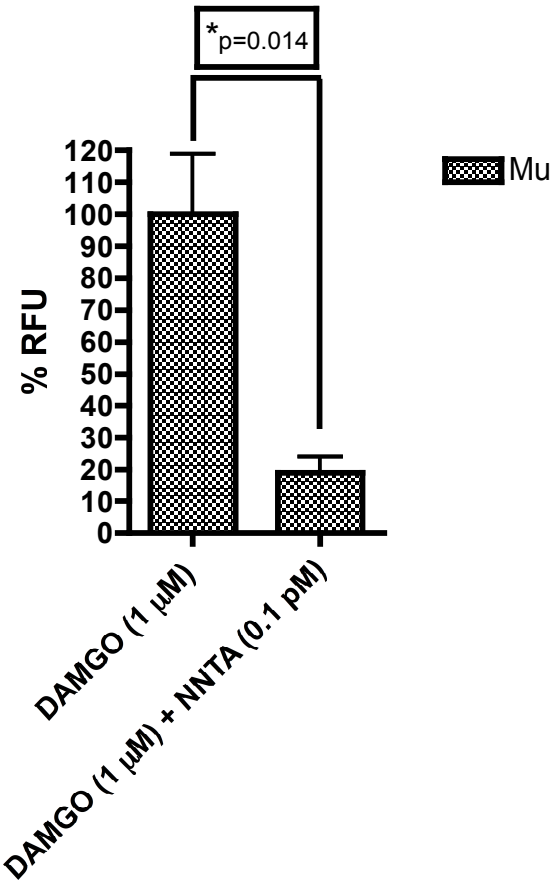



**Figure 4.5. NNTA antagonized the effect of DAMGO in HEK-293 cells expressing mu opioid receptors in the intracellular calcium release assay.** There was ~80% inhibition of DAMGO (1 μM) by NNTA (0.1 pM) that was determined to be significant by an unpaired t-test (two-tailed, $p < 0.05$).

### 4.2.5. NNTA is a highly potent agonist in the guinea pig ileum and mice

**(By Mike Powers and Mary Lunzer).**

NNTA was tested for agonistic activity in the Hartley guinea pig ileum (Charles River laboratory, Wilmington, MA). In comparison with morphine ($IC_{50}$ = 50.6 nM), NNTA ($IC_{50}$ = 0.038 nM) was 1225 times more potent (Fig 4.6a). In ICR-CD1 mice (Harlan labs, Madison, WI), NNTA produced potent antinociception through both intrathecal (i.t.) and intracerebroventricular (i.c.v.) routes when tested using the tail-flick assay (Fig 4.6b). Interestingly, NNTA produced a biphasic dose-response relationship when administered i.t. The antinociceptive $ED_{50}$ (95% CI) values were 18.7 (10.3-32.8) pmol/mouse and 101.7 (75.3-137.5) for the i.t. route and 2.06 (1.09-3.27) nmol/mouse for the i.c.v. injections. Thus, NNTA was at least 110-fold more potent when administered spinally than when given supraspinally.

NNTA is also a potent antinociceptive agonist when administered by the i.v. route (Fig 4.6c), with an $ED_{50}$ of 8.8 (6.8-11.5) nmol/mouse. By comparison, the i.v. morphine $ED_{50}$ value was 420 (378-469) nmol/mouse or about ~ 50-fold more potent than morphine after systemic administration. NNTA was also active via the oral route with an $ED_{50}$ of 2.68 mg/Kg (1.74-4.29).

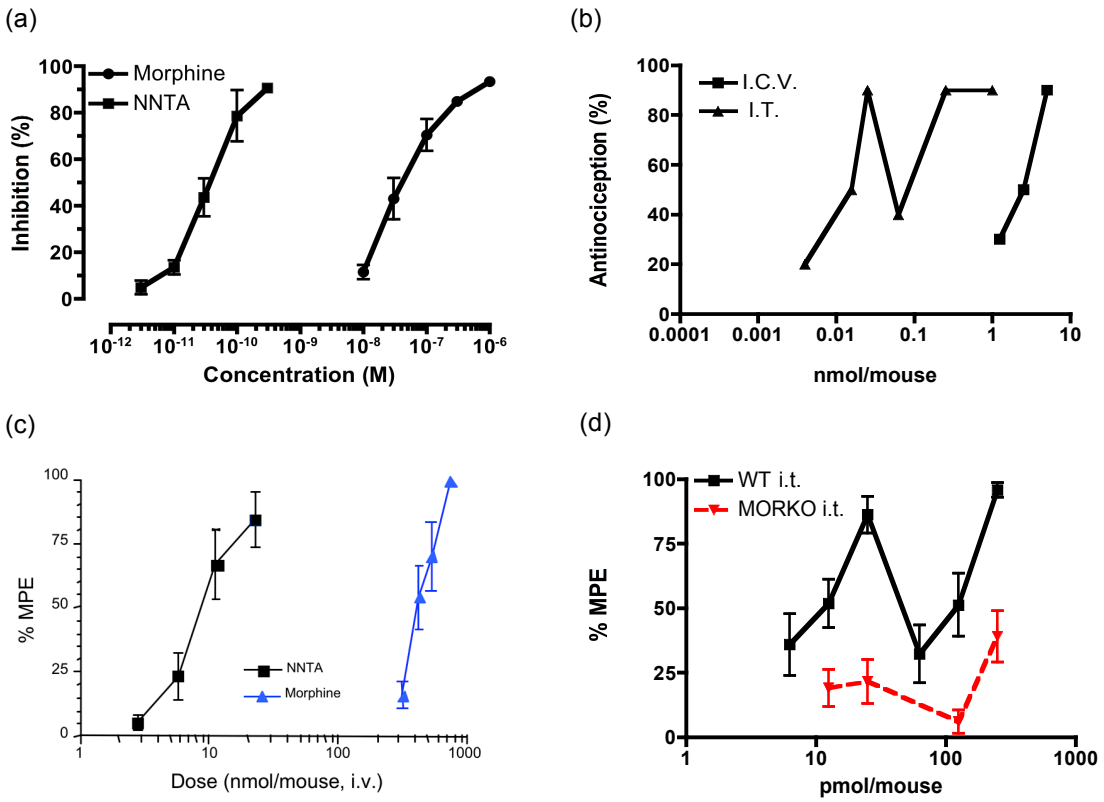



**Figure 4.6 NNTA is a potent agonist *in vivo* but lacks spinal activity in MORKO mice.**

(a) NNTA was ~ 1200-fold more potent agonist when compared to morphine in the guinea pig ileum. (b) In the ICR-CD1 mouse tail-flick experiments (Quantal method) a biphasic antinocicpetive dose-response was observed after intrathecal (i.t.) administration ($ED_{50}$ = 9.8 pmol/mouse and 101.7 pmol/mouse); NNTA was >200-fold more potent i.t. than i.c.v. ($ED_{50}$ = 2.1 nmol/mouse). (c) NNTA ($ED_{50}$ = 8.8 nmol/mouse, CI: 6.8-11.5) was at least 50-fold more potent when compared with morphine ($ED_{50}$ = 420 nmol/mouse, CI: 378-469) when administered i.v. (d) NNTA did not produce significant antinociception when administered intrathecally in the MORKO mice at doses that produced maximal antinociception in wild-type mice. (MPE = Maximal possible effect)

### 4.2.6. NNTA is not spinally active in mu knockout (MORKO) mice

Given the potent and biphasic spinal activity of NNTA in wild type mice, its cell-based antagonistic activity at mu opioid receptors and highly potent activity at mu/kappa receptors, these data suggested that the spinal activity was mediated via mu/kappa heteromeric receptors rather than homomeric kappa receptors in vivo. Hence, we determined the activity of NNTA in mu knockout mice (MORKO) at doses that produced maximal antinocicpetion (25 pmol/mouse and 250 pmol/mouse) in wild type mice. NNTA did not produce significant antinociception in MORKO mice for the dose ranges tested (Fig 4.6d).

### 4.2.7. NNTA is antagonized by the kappa-selective antagonist, norBNI, in mice and HEK-293 cells coexpressing mu and kappa receptors (By Mary Lunzer)

Selective antagonists were used to investigate possible mechanisms for the agonism of NNTA in ICR-CD1 mice. Antagonist $ED_{50}$ ratios were determined for selective mu and kappa antagonists β-funaltrexamine (β-FNA, 35) and norbinaltorphimine (norBNI, 36), respectively. While β-FNA did not significantly antagonize NNTA i.c.v., norBNI produced weak antagonism by that route ($ED_{50}$ ratio = 5.63). More potent antagonism was observed upon i.t. administration ($ED_{50}$ ratio = 38.26, Table 3).

**Table 4.3. $ED_{50}$ Ratios showing the shift in NNTA agonism caused by specific antagonists.**

| NNTA | $ED_{50}$ Ratio ( ± 95% C.I.)[a] | | | |
|---|---|---|---|---|
| | norBNI | | β-FNA | |
| | i.t. (2.5 nmol/mouse) | i.c.v. (5.0 nmol/mouse) | i.t. (1.0 nmol/mouse). | i.c.v. (1.0 nmol/mouse) |
| i.t. | 38.26 (21.10 – 70.08) | - | 1.48 (0.42 – 5.79) | - |
| i.c.v. | - | 5.63 (3.23 – 12.02) | - | 1.54 (0.63 – 3.25) |

[a] *$ED_{50}$ ratios are calculated as the fold shift of an agonist in the presence of a specific dose of antagonist. Formula: (Agonist $ED_{50}$ after antagonist treatment/Agonist $ED_{50}$ before antagonist treatment)*

Since β-FNA induced significant calcium release in HEK-293 cells coexpressing mu and kappa receptors, we used the mu antagonist D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-$NH_2$ (CTOP) and norBNI to antagonize NNTA (Fig 4.7). CTOP did not antagonize NNTA, while norBNI significantly antagonized NNTA corroborating the in vivo data.

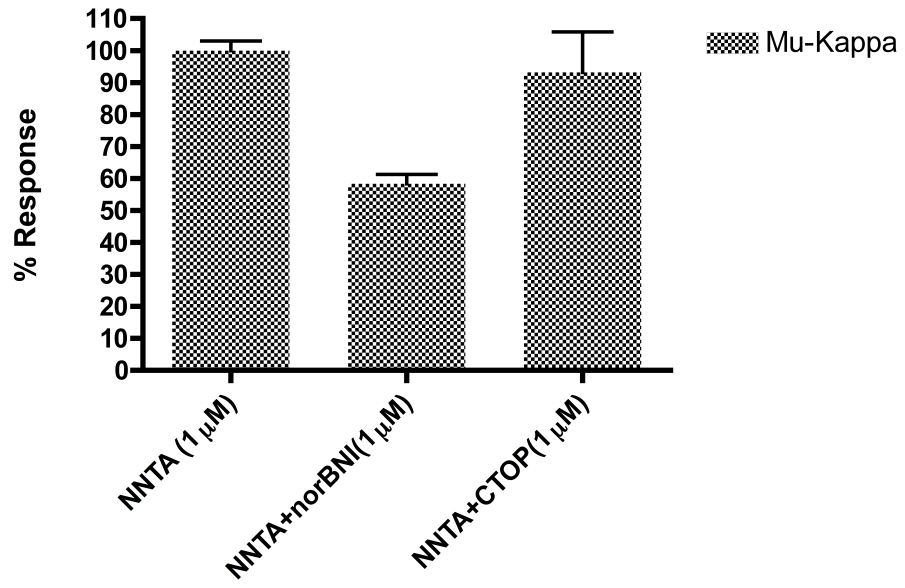


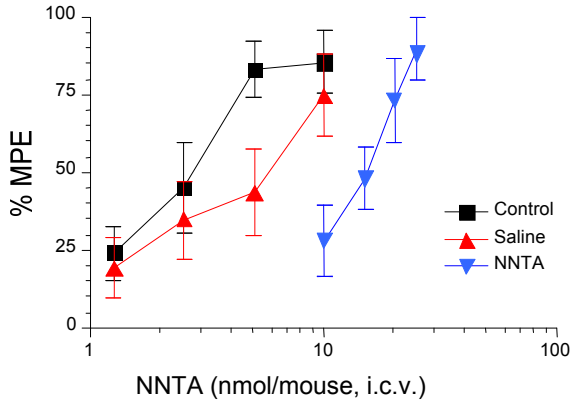

**Figure 4.7. NNTA is antagonized by norBNI in cells coexpressing mu and kappa receptors. norBNI (1 μM) significantly antagonized NNTA (1 μM) while CTOP (1 μM) did not antagonize NNTA.**

### 4.2.8. NNTA produces no intrathecal tolerance or physical dependence (By Sandra Roerig, LSU)

The i.c.v. tolerance and physical dependence experiments were conducted as described previously[149] using osmotic mini-pumps to infuse NNTA or saline i.c.v. for 3 days. Results shown in Figure 4.8a are the tail flick dose-response curves obtained for NNTA administered i.c.v. to naïve control mice, mice infused i.c.v. with saline, and mice infused i.c.v. with NNTA. The $ED_{50}$ values calculated from these curves are: control, 2.5 (1.8-3.6) nmol/mouse; saline infused, 4.8 (2.8-8.1) nmol/mouse; and NNTA infused, 14.1 (11.9-17.5) nmol/mouse. These results indicate that a 3-fold tolerance developed to NNTA-induced antinociception. Previous studies using morphine

in the same protocol showed a 6-fold development of tolerance to i.c.v. morphine.

(a)




(b)

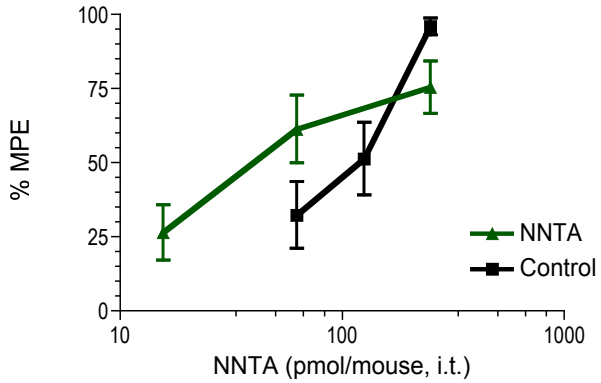



**Figure 4.8. NNTA i.c.v. and i.t. tolerance studies.**

(a) Saline or NNTA was infused for 3 days and the antinociceptive $ED_{50}$s were determined on the fourth day. The dose response curve for NNTA i.c.v. infusion ( ) is right-shifted (3-fold) compared to saline infused ( ) and acute NNTA treatment (control) in the mouse tail flick assay indicating the development of tolerance. (b) NNTA (250 pmol/mouse) was injected i.t. twice a day (5h gap between injections) for two days and once on the third day after which, the tail flick latencies were calculated. No significant tolerance was observed as the NNTA curve did not differ significantly from the acute NNTA treatment (control) after the repeated injections.

To determine i.t. tolerance, mice were injected i.t. with NNTA (250 pmol/mouse) twice daily (5h gap between injections) for two days and once on the third day, after which the antinociceptive $ED_{50}$ was determined. NNTA did not induce any significant tolerance (Figure 4.8b), as the control $ED_{50}$ of 101.7 pmol/mouse (75.27 - 137.4) did not significantly differ from the NNTA $ED_{50}$ of 46.3 pmol/mouse (22.91 - 93.42).

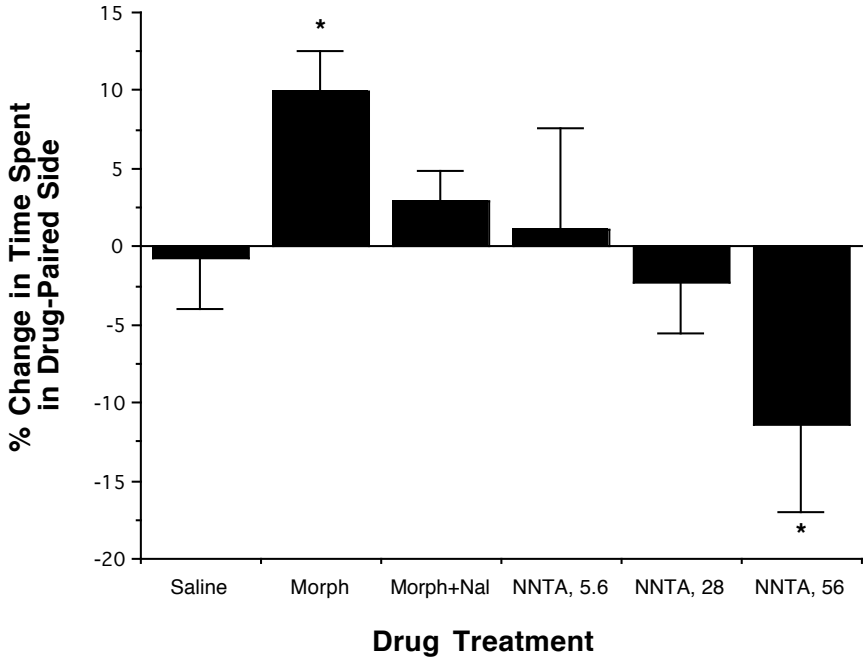



**Figure 4.9. NNTA does not produce dependence in mouse conditioned place preference studies.**

NNTA at the $ED_{50}$ dose (5.6 nmol/Kg, i.v.) and 5x $ED_{50}$ dose (28 nmol/Kg, i.v.) did not produce any significant place preference, suggesting a lack of reward due to chronic NNTA treatment. At the 10x $ED_{50}$ dose (56 nmol/Kg, i.v.) considerable place aversion was observed. On the other hand, morphine (1200 nmol/Kg) in the same protocol produced robust place preference that could be reversed by naloxone (300 nmol/Kg).

After three days of NNTA infusion, the naloxone-induced jumping method failed to reveal any physical dependence. Naloxone did not induce significant jumping in either saline (1.57 ± 0.7 jumps) or NNTA-infused mice (1.0 ± 0.7 jumps) over a 10 min period. Under identical conditions in prior studies (31), naloxone induced 100 jumps in i.c.v. morphine-infused mice, while only ~ 5 jumps were observed for the saline-infused mice. Thus, in contrast to the robust tolerance and physical dependence that develops after chronic i.c.v. morphine infusion (data not shown), no withdrawal syndrome was effected with naloxone after chronic i.c.v NNTA treatment.

Conditioned place preference studies corroborated the absence of reward learning and drug seeking behavior of NNTA. In the conditioned place preference experiments set up as described previously (27), morphine (1200 nmol/kg) produced a place preference that was blocked by naloxone (300 nmol/kg). The two lower doses of NNTA (5.6 and 28 nmol) did not produce a significant effect on the time that the mice spent in the drug-paired chamber (Fig 4.9). However, at the 10 X $ED_{50}$ dose (56 nmol), NNTA produced a significant place aversion effect ($p < 0.1$).

## 4.3. Discussion

The report by Wang et al. 2005[124] that demonstrated the association of mu and kappa opioid receptors as heteromers in cultured cells renewed our interest in a ligand, NNTA (Fig 4.1), prepared in our laboratory a decade ago. In

view of its extremely high potency (1200-fold greater than morphine) in the guinea pig ileum preparation (Fig 4.6a) and sub-pM binding affinity for mu and kappa receptors (Table 4.1), we entertained the possibility that NNTA might activate mu-kappa heteromeric receptors. In view of the pressing need for selective pharmacologic tools for the study of heteromeric opioid receptors *in vivo*, we investigated the properties of NNTA in cultured cells and mice.

Experiments with HEK-293 cells containing stably expressed homomeric (mu, kappa, delta) and heteromeric opioid receptors (mu-kappa, mu-delta, delta-kappa) using fluorometric $Ca^{2+}$ ion release methodology and [$^{35}$S]GTP$\gamma$S binding assays as an index of receptor activation revealed results that were consistent with selective activation of heteromeric mu-kappa (Fig 4.3 and 4.4). In this regard, treatment of the mu-kappa cell line with NNTA led to remarkably potent activation at concentrations consistent with the binding data. Significantly, interaction of NNTA with cell lines that contained other heteromeric opioid receptors or homomeric receptors afforded substantially lower activity.

The remarkable ~1000-fold enhancement of agonist potency exhibited in the mu-kappa cell line when compared to singly expressed kappa receptors conceivably may be due to allosterically-induced amplification of activation of the kappa receptor due to heteromer formation. From this perspective, and in view of the comparable affinity of NNTA for mu and kappa receptors, one possible scenario is that a conformational change induced in the mu receptor protomer leads to enhanced activation via its NNTA-bound kappa receptor

partner in the heteromeric mu-kappa receptors. The high mu receptor antagonist potency of NNTA in the mu receptor cell line would be consistent with this view (Fig 4.5).

Since the results of experiments with the HEK-293 cell lines were consistent with the selective activation of mu-kappa heteromers by NNTA, we investigated its antinociceptive properties using the mouse tail-flick assay (Fig 4.6b and 4.6c). By the intrathecal (i.t.) route, NNTA exhibited a biphasic dose-response relationship ($ED_{50}$ = 18.7 pmol and 101.7 pmol) which was over 100-fold greater than by i.c.v. administration. The large difference raised the possibility that NNTA might be activating phenotypic opioid receptors that are more responsive in the cord when compared to those in the brain.

Since a similar phenomenon has been reported previously[135] in connection with differential populations of putative heteromeric opioid receptors in the spinal cord and brain, we were interested in pharmacologically characterizing the receptors that were activated by NNTA. This involved determining the $ED_{50}$ ratios for antagonism using selective antagonists for kappa (norBNI) and mu (β-FNA) opioid receptors. These data revealed that only norBNI antagonized the effect of NNTA, which suggested involvement of kappa receptors in the antinociceptive response.

In view of reports for the colocalization of delta and kappa receptors in rodent spinal cord[135] and the presence of putative heteromeric delta/kappa receptors[114], taken together with our cell-based data that show NNTA to be substantially more efficacious in activating mu-kappa heteromers, the

exceptional potency of i.t. NNTA tends to support the concept that the observed spinal antinociception is mediated principally via mu-kappa heteromers. This concept was further supported by the lack of significant spinal antinociception in mu opioid receptor knockout mice (Fig 4.6d), demonstrating that kappa receptors alone are unable to mediate the potent spinal activity of NNTA. These data are also consistent with the significantly lower activity of NNTA in cells expressing only kappa opioid receptors in the intracellular calcium release assay (Fig 4.3).

In view of the unique properties of NNTA, it was evaluated for tolerance and physical dependence. Antinociceptive tolerance was dependent on the route of administration of NNTA. Some tolerance was observed after three days of infusion by the i.c.v. route (Figure 4.8a), although it was substantially less robust when compared to morphine[138]. Significantly, no tolerance was observed when NNTA was administered i.t. (Figure 4.8b). These results, coupled with the potent spinal antinociception of NNTA in wild type mice and lack of spinal activity in mu knockout mice, suggest that there may be a tissue-dependent localization of different phenotypic receptor populations as suggested from other studies[134, 135, 171]. Since the *in vitro* data showed NNTA to be highly selective and active at heteromeric mu/kappa opioid receptors, these receptors may be the predominant target for NNTA in the cord.

Our finding that naloxone did not precipitate naloxone-induced jumping in mice infused with NNTA for three days suggested the absence of physical dependence. Moreover, conditioned place preference (CPP) studies indicated

the absence of reward learning or drug-seeking behavior through the administration of NNTA (Fig 4.9). At the $ED_{50}$ dose (5.6 nmol) and 5X $ED_{50}$ dose (28 nmol) of NNTA, there was no significant CPP. However, there was a significant dose-related trend for aversion at doses of NNTA that produced antinociception in 100% of the mice. The aversion of NNTA at high dose is of interest because members of the clinically employed mixed agonist-antagonist class of opioids that have kappa agonist and mu antagonist components, are known to produce dysphoria in humans. Whether the dysphoria is mediated primarily through activation of mu-kappa heteromers, homomeric kappa receptors or other kappa receptor-containing heteromers remains to be investigated.

In conclusion, the present study has revealed that the highly potent opioid ligand, NNTA, selectively activates heteromeric mu-kappa in cultured cells, and therefore may be a useful pharmacological tool for investigating the role of such receptors in vitro and *in vivo*. As NNTA produces unusually potent, biphasic spinal (i.t.) antinociception that is two orders of magnitude greater than its supraspinal (i.c.v.) effect in mice, and given its very high potency in cultured cells, the mouse spinal cord may possibly contain mu-kappa heteromers. The potent spinal activity is abolished in MORKO mice further corroborating the high degree of functional coupling between mu and kappa receptors in the cord. Since the unusually high potency of NNTA was initially uncovered through screening on the electrically stimulated guinea pig ileum, this preparation also may possibly contain a subpopulation of mu-kappa heteromers. This is of

interest because the GPI has been widely employed in the screening of opioid agonists. The absence of physical dependence in mice, the low degree of antinociceptive tolerance upon chronic i.c.v administration, and the lack of significant CPP at its $ED_{50}$ dose, suggest that mu/kappa opioid receptors may be a viable target for the development of analgesics devoid of the undesirable side effects associated with morphine.

## 4.4. Materials & Methods

**Materials.** β-FNA and norBNI were synthesized as described previously[57, 66]. cDNAs encoding human kappa, mu and murine delta opioid receptors were inserted separately into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA) and these were used to generate the singly expressing stable cell lines. HEK-293 cells stably co-expressing mu-kappa, kappa-delta and mu-delta receptors were obtained from Dr. Jennifer Whistler and their construction has been previously explained and verified[134, 159]. The chimeric G-protein[160], Δ6-$G_{\alpha qi4\text{-}myr}$, was graciously provided by Dr. Evi Kostenis and its construction has been previously described.

**Synthesis of 17-Cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[(2'-naphthyl)carboxamido]morphinan (NNTA):**

*β*-Naltrexamine (400 mg, 1.16 mmol), naphthoic acid (399 mg, 2.32 mmol) and benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) (1.03 g, 2.32 mmol) were dissolved in methylene chloride (15 mL). To this solution, *N, N*-Diisopropylethylamine (DIPEA) (600

mL, 3.25 mmol) was added and the mixture was stirred at room temperature for 16 hours. The solution was concentrated under reduced pressure and the residue was taken up in MeOH (15 mL), and $K_2CO_3$ was added (1.2 g). After 1 hour at room temperature, the mixture was concentrated to dryness. The residue was purified by silica gel chromatography (eluted with EtOAc/Hexanes : 4/1) to afford the desired product (NNTA) as a white solid. NNTA was recrystallized from an acetone/hexanes (1/4 : v/v) mixture (473 mg, 82%). NNTA was subsequently converted into the HCl salt for biological testing.

The melting point was determined on a Thomas-Hoover melting point apparatus and are uncorrected. $^1$H NMR spectra and $^{13}$C NMR spectra were determined on a Bruker 400 MHz instruments and calibrated using an internal reference. ESI mode mass spectra were recorded on a BrukerBioTOF II mass spectrometer. Elemental analyses were performed by M-H-W Laboratories, Phoenix, AZ.

Base form :

$^1$H NMR (DMSO-$d_6$) δ : 0.12 (m, 2H) ; 0.47 (m, 2H) ; 0.86 (m, 1H) ; 1.27-1.63 (m, 4H) ; 1.85-2.03 (m, 2H) ; 2.09 (m, 1H) ; 2.22 (m, 2H) ; 2.37 (m, 2H) ; 3.01 (m, 2H) ; 3.75 (m, 1H) ; 4.76 (d, 1H, $J_{H5\text{-}H6}$= 7.8 Hz) ; 4.92 (bs, 1H, O<u>H</u>-14) ; 6.54 (d, 1H, $J_{H1\text{-}H2}$= 8.1 Hz) ; 6.60 (d, 1H, $J_{H2\text{-}H1}$= 8.1 Hz) ; 7.59-7.62 (m, 2H) ; 7.96-8.03 (m, 4H) ; 8.50 (s, 1H), 8.84 (d, 1H amide, $J_{N\underline{H}\text{-}H6}$= 8.2 Hz) ; 9.05 (bs, 1H, O<u>H</u>-3) ; $^{13}$C NMR (DMSO-$d_6$) δ : 3.52, 3.65, 9.21, 22.12, 24.69, 30.06, 30.27, 43.66, 47.01, 51.63, 58.35, 61.71, 69.58, 90.64, 116.93, 118.36, 123.45,

124.08, 126.68, 127.43, 127.55 (x2), 127.77, 128.80, 131.36, 131.58, 132.08, 134.05, 140.41, 142.06, 165.52 ; mp= 199-201 °C. Anal. Calcd. for $C_{31}H_{32}N_2O_4$ : C,74.98 ; H, 6.50 ; N, 5.64. Found : C, 73.85 ; H, 6.13 ; N, 5.52. ESI-TOF MS *m/z* : 497.2930 ($MH^+$), 993.5777 ($2xMH^+$)

Salt form :

$^1$H NMR salt form (DMSO-$d_6$) δ : 0.53-0.68 (m, 4H) ; 1.08 (m, 1H) ; 1.46 (m, 2H) ; 1.63 (m, 1H) ; 1.79-1.95 (m, 2H) ; 2.48 (m, 2H) ; 2,87 (m, 1H) ; 3.03-3.12 (m, 2H) ; 3.35 (m, 2H) ; 3.75 (m, 1H) ; 3.90 (m, 1H) ; 4.90 (d, 1H, $J_{H5\text{-}H6}$= 7.8 Hz) ; 6.29 (bs, 1H, O<u>H</u>-14) ; 6.65 (d, 1H, $J_{H1\text{-}H2}$= 8.1 Hz) ; 6.74 (d, 1H, $J_{H2\text{-}H1}$= 8.1 Hz) ; 7.59-7.62 (m, 2H) ; 7.97-8.03 (m, 4H) ; 8.52 (s, 1H), 8.90 (d, 1H amide, $J_{N\underline{H}\text{-}H6}$= 8.2 Hz) ; 9.38 (bs, 1H, O<u>H</u>-3) ; $^{13}$C NMR salt form (DMSO-$d_6$) δ : 2.57, 5.10, 5.70, 22.99, 23.80, 27.30, 29.32, 40.07, 45.56, 46.45, 51.23, 56.59, 61.56, 69.69, 89.86, 117.83, 119.23, 120.59, 124.05, 126.71, 127.49, 127.57 (x2), 127.81, 128.80, 129.66, 131.44, 132.07, 134.09, 141.28, 142.10, 165.59 mp> 260°C. Anal. Calcd. for $C_{31}H_{33}ClN_2O_4$ : C, 69.85 ; H, 6.24 ; N, 4.67. Found : C, 67.79 ; H, 6.44 ; N, 4.67. ESI-TOF MS *m/z* : 497.2930 ($MH^+$)

**Guinea Pig Ileum:** Longitudinal muscle strips were prepared for experiments using the method of Rang, 1964 (41) and assays were conducted as described previously (42).

**Competition Binding.** Experiments were performed using HEK 293 cells genetically modified to produce wild-type human mu, kappa, or delta opioid receptors and coexpressing mu/kappa opioid receptors. Ten concentrations of the tested compounds (50 μL) were added to test tubes containing 0.5 nM

[$^3$H]diprenorphine (≈ 1.0 x $K_D$) (50 μL) or selective radioligands [$^3$H]DAMGO and [$^3$H]U69593 (both 2.0 nM) and whole cells (75 mm$^2$ plate, 80-90 % confluent) suspended in 12 ml HEPES buffer (25 mM, pH=7.4) (400 μL). Final volume was 500 μL. Non-specific binding was measured in the presence of 10 μM naloxone. Assays were incubated at room temperature for 90 min. and then filtered using a Brandel M-48 tissue harvester through Whatman GF/C filter paper that was pre-soaked in 0.25 % poly(ethyleniminie). Filters were washed three times with ice cold HEPES buffer (see above), and the radioactivity counted using a LS 6500 liquid scintillation counter (Beckman, Fullerton, CA). All measurements were performed in triplicate. $IC_{50}$ values were calculated using PRISM software (GraphPad, San Diego, CA) utilizing non-linear regression of the data normalized to fit a sigmoidal dose-response curve with a variable slope (100% defined at concentration = 0 (total binding) and 0% defined at the value of non-specific binding). $K_i$ values were determined from the Cheng-Prusoff equation assuming a single site binding model. Values reported are mean $K_i$ ± standard error of the mean (SEM) of three or more independent experiments.

**Immunocytochemistry:** Two-color immunofluorescence was employed to analyze co-expression of mu- and kappa-opioid receptors as previously described (34). Briefly, HEK-293 cells coexpressing HA-mu and FL-kappa were incubated with goat anti-HA (Abcam, Cat #ab9134) antibody and rabbit anti-FLAG (Abcam, Cat #1162) antibody at a final working concentration of 1:100 for 60 min at 4°C. After rinsing thrice with 50 mM PBS (pH 7.2), cells were fixed

with 2% formaldehyde for 10 min at room temperature. Then cells were washed (3 x 15 min) with PBS and incubated at room temperature with the mixture of anti-goat NL-493 (Cat # NL003; R&D Systems, Inc.) and anti-rabbit NL-557 (Cat # NL004; R&D Systems, Inc.) fluorescent secondary antibodies (1:200 final dilutions). Cells were again rinsed with PBS (3 x 15 min) and mounted under coverslips with anti-fade mounting media iBright Plus (Cat # SF40000-10; Neuromics, Inc.) containing DAPI. Olympus FluoView1000 confocal microscope was used for image collection.

**Cell Culture and Intracellular $Ca^{2+}$ release assay.** HEK-293 cells were cultured at 37°C in Dulbelcco's modified Eagle's medium supplemented with 10% bovine calf serum, Pen/Strep antibiotics. For cells singly expressing opioid receptors, G418 was used as the selection antibiotic; G418 and Zeocin were used for selecting for cells coexpressing two opioid receptors. The protocol for the intracellular calcium release has been previously described in detail. Briefly, the stable opioid receptor cell lines were transiently transfected with 200 ng/20,000 cells of the chimeric G-protein, $\Delta$6-$G_{\alpha qi4\text{-}myr}$, using OptiMEM medium (Invitrogen) and Lipofectamine 2000 (Invitrogen, Carlsbad CA) reagent according to manufacturer's protocol (1:2 wt/vol ratio for DNA:Lipofectamine). The cells were seeded into 96 well plates (half-area; Corning) at 20,000 cells/well after 24 hours and assayed 48 hours after transfection using the FLIPR calcium kit (Molecular devices) in a Flexstation-III apparatus (Molecular devices). The response was measured as area under the curve (Relative Fluorescence Units X seconds) and plotted using non-linear regression.

**[$^{35}$S]GTPγS assay.** The assay was setup as described previously[174]. Briefly, membranes from mu, kappa and mu/kappa cells were incubated with varying concentrations of NNTA and [$^{35}$S]GTPγS (Perkin-Elmer) in a 96 well plate. The incubation was performed at 37°C for an hour in membrane buffer (50mM Tris-HCl, 3mM $MgCl_2$, 0.2mM EGTA, 100mM NaCl and 0.5% BSA), filtered and counted for radioactivity. Three replications were performed for each treatment.

**Animals.** For studies performed at the University of Minnesota, male ICR-CD1 mice (18-25 g or 30-35 g; Harlan, Madison, WI), were housed in groups of 8 in a temperature and humidity controlled environment with unlimited access to food and water and maintained on a 12 hour light/dark cycle. The mu opioid receptor knockout mice (BALB/C X C57BL/6 MORKO) were a generous gift from Dr. Sabita Roy and have been described previously. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota.

For studies performed at the Louisiana State University Health Sciences Center in Shreveport (LSUHSC-S), male ICR mice weighing 25 – 35 gm (Harlan Sprague Dawley) were used in the i.v. and chronic i.c.v. studies. They were housed in AALAC-accredited animal facilities with 12/12 hr light/dark cycles and food and water available ad lib. All procedures were approved by the Institutional Animal Care and Use Committee at LSUHSC-S.

Antinociceptive Studies.

All solutions were dissolved in distilled water. Controls with only distilled water showed no antinociception. All ligands were administered in a 5-µl volume in conscious mice according to the method Hylden and Wilcox[183] for i.t. and Haley and McCormick[184] for i.c.v. injections.

**Quantal method.** For measuring the antinociception of NNTA in mice, the drugs were administered so that the antagonist and agonist effects would peak simultaneously. The peak times for the antagonists were as follows: β-FNA and norBNI were both 20 minutes. The agonist peak times for NNTA, i.t. 5 minutes and i.c.v. 10 minutes. Antinociception was measured using the modified radiant heat tail-flick test . Briefly, a radiant heat source was applied to the dorsal side of the tail, and the latency to flick away from the heat source was recorded. The data were made quantal by designating a positive antinociceptive response of an animal as those that increased their latency to tail flick (after drug treatment) by at least three standard deviations above the mean of the baseline latency of the whole group. The light source was manually turned off if the mouse did not flick its tail after the three standard deviation criteria for a positive response. At least three groups of 8-10 mice were used for each drug paradigm, and each mouse was used only once. $ED_{50}$ values and 95% confidence intervals were calculated by using the parallel line assay. When $ED_{50}$ values were compared, all the data were analyzed together and values were considered significantly different if they did not lie in each other's 95% confidence limits at $p < 0.05$.

**%MPE method.** The tail flick assay was performed as described by D'Amour and Smith[52] and modified by Dewey et. al.[53]. For the measurement the latency of the tail-flick measurement, the mice were held gently in one hand with the tail positioned in the apparatus (Tail Flick Analgesia Meter, Columbus Instruments, Columbus, Ohio) for radiant heat stimulus. The tail-flick response was elicited by applying radiant heat to the dorsal side of the tail. The intensity of the heat was set at setting 8 so that the animal flicked its tail within 2 to 3 s. The test latency was measured once before drug treatment (control) and again after the drug treatment (test) at the peak time of the compound, a 10s maximum cut-off time was used to prevent damage to the tail. Antinociception was quantified according to the method of Harris and Pierson (54) as the percent maximal possible effect (%MPE) which is calculated as: %MPE = (Test - Control/10 – Control) x 100. At least three groups of eight to ten mice were used for each dose response curve, and each mouse was used only once. $ED_{50}$ values with 95% confidence intervals (C.I.) were computed with GraphPad Prism 4 by using nonlinear regression methods.

**i.v. and chronic i.c.v. studies.** For the i.v. and chronic i.c.v. studies, mice were tested using the %MPE radiant heat tail flick test as described above. For all experiments, baseline latency times were determined, drugs were injected either i.v. or i.c.v. and tail flick latency times were again determined at the time of peak antinociceptive response as determined from preliminary studies. For morphine i.v., the time of peak antinociception was 15 min, for i.v. NNTA, 20 min, and for i.c.v. NNTA, 10 min. Animals were sacrificed at the end of the

experiment and each animal was used only once, for one dose. The $ED_{50}$ (95% confidence interval) values were calculated using Prism software.

**Tail Flick Assay for intrathecal tolerance.** For measuring intrathecal tolerance, animals were injected according a modified methodology of Fairbanks et. al (55). The mice were injected twice on day 1 with the 80 – 90 MPE% dose (250 pmol/mouse) with a 5 hour gap between injections. The same regimen was repeated again on day 2. On the third day, the mice were injected once with an acute dose of NNTA (250 pmol/mouse) following which, tail flick latencies were measured and %MPE values were calculated to look for tolerance.

**Chronic infusion.** Mice were infused i.c.v. with saline or NNTA (7.1 nmol) for 3 days using osmotic mini-pumps as previously described (30). On the fourth day, animals were injected with 1 mg/kg naloxone (s.c.), placed in a 4 liter glass beaker, and number of jumps in 10 min was recorded. After testing, the mini pumps were removed and after 2 hr the animals were administered an acute dose of NNTA i.c.v. Tail flick latencies were measured and %MPE values were calculated as described above.

**Conditioned place preference (CPP).** Mice were tested for CPP as previously described[27]. Drugs were administered i.v. for 3 days of conditioning. On the fourth day, time spent on the drug-paired or saline-paired side was recorded. Doses of morphine and naloxone used were as previously described. Data were analyzed using students t-test.

# CHAPTER 5

# 5. A TALE OF TWO MU OPIOID ANTAGONIST SCAFFOLDS: PENCILLAMINE OCTAPEPTIDES AND β-NALTREXAMINES

## 5.1. Introduction

CTOP and β-funaltrexamine (β-FNA) have been used for decades as selective antagonists for mu opioid receptors. Given that opioid receptors have been shown to oligomerize to form homomers and heteromers, we investigated the selectivity of these antagonists in vitro and in vivo. Here we reveal observations that are consistent with CTOP selectively antagonizing mu-delta heteromers rather than mu homomers, while β-FNA antagonizes both mu and mu-delta receptors. In intracellular calcium release experiments utilizing HEK293 cells containing singly or coexpressed receptors, CTOP significantly blocked DAMGO-induced calcium release in cells containing mu-delta, but not mu-kappa or mu receptors. Moreover, CTOP inhibited DAMGO-induced mu opioid receptor endocytosis in cells containing mu-delta heteromers, but not mu homomers alone. On the other hand, β-FNA antagonized DAMGO-induced mu receptor endocytosis in both mu and mu-delta containing cells. Interestingly, β-FNA and the β-naltrexamine monovalent analog, MN-14, produced potent calcium release in HEK-293 cells containing kappa and mu-kappa receptors suggesting that either receptor complex may be involved in the short acting antinociceptive effects of β-naltrexamine ligands that has been reported in several studies.

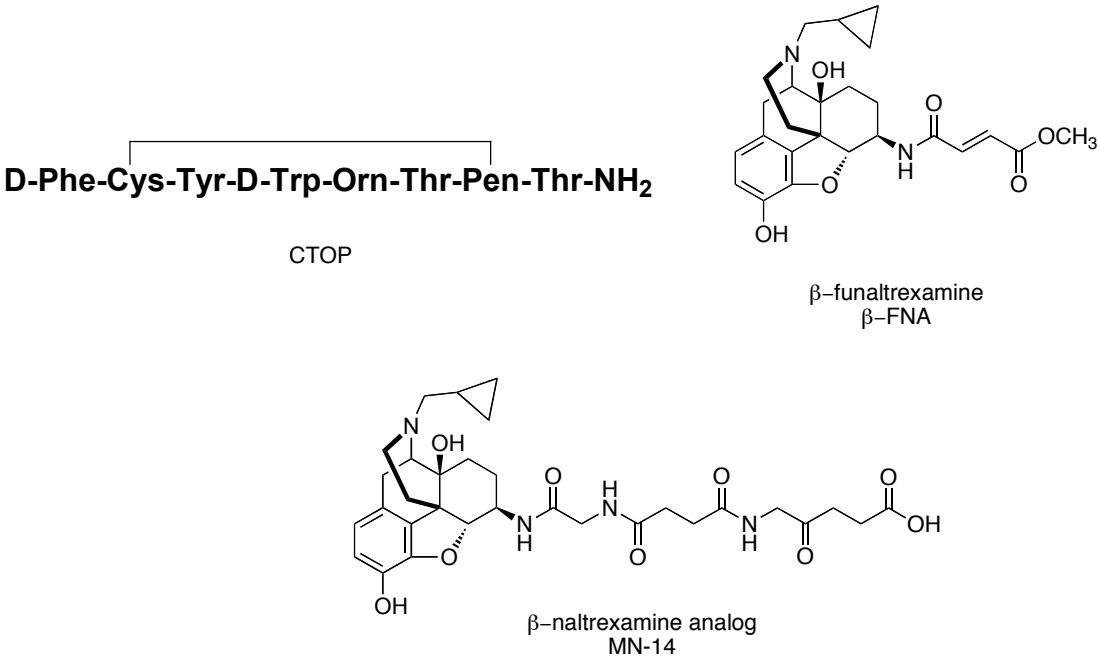



**Figure 5.1 Structures for the ligands used in this study**

## 5.2. Results

### 5.2.1. CTOP is a potent antagonist at mu-delta opioid heteromers

To evaluate the antagonistic ability of CTOP, we utilized the intracellular calcium release assay in HEK-293 cells stably expressing mu, mu-delta and mu-kappa opioid receptors. The origin and characterization of these cell lines have been previously published[134, 159, 174]. These cells were transiently transfected with $\Delta 6\text{-}_{G\alpha qi4\text{-}myr}$, a chimeric G protein that has been used to couple opioid receptors to the intracellular calcium release mechanisms[134, 159, 174]. CTOP (1 μM) significantly inhibited calcium currents induced by DAMGO (1 μM) in cells containing mu-delta heteromers (Fig 5.2). The inhibition observed

in cells expressing mu receptors was smaller (~25%) and was not significant using an unpaired t-test (two-tailed) with Welch's correction. Interestingly, CTOP did not produce any significant antagonism at mu-kappa heteromers or DPDPE induced calcium currents at delta opioid receptors (Fig 5.2).

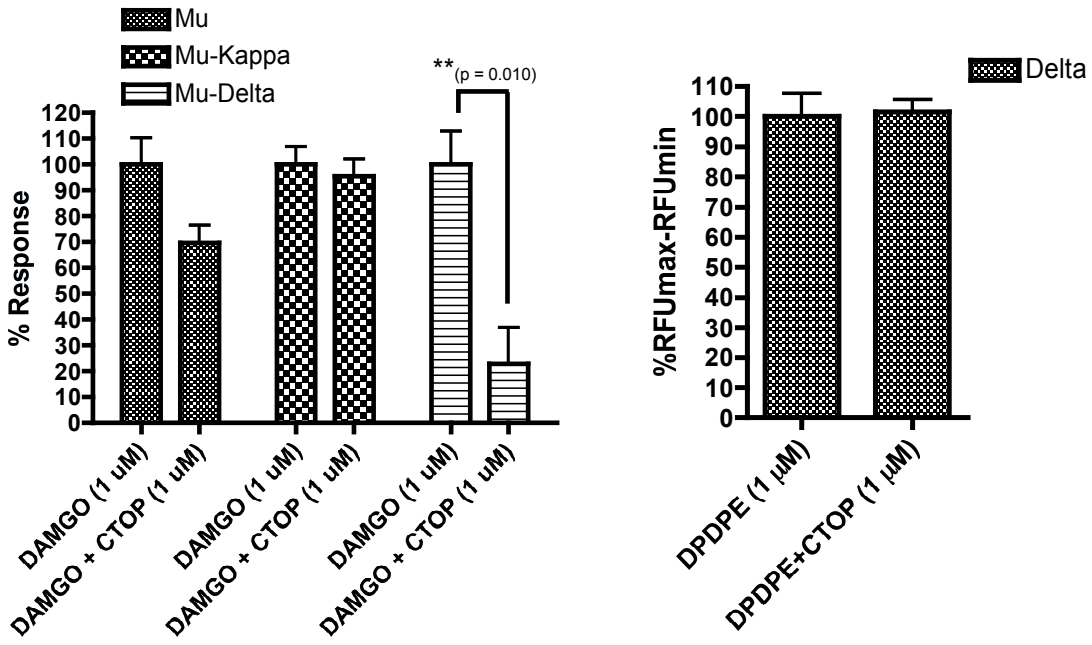



**Figure 5.2 CTOP selectively antagonizes DAMGO at mu-delta receptors.**
In intracellular calcium release experiments, CTOP (1 μM) produced weak antagonism of calcium currents induced by DAMGO (1 μM) in HEK293 cells expressing mu receptors. However, CTOP significantly antagonized DAMGO in cells expressing mu and delta receptors. CTOP did not have any effect on delta opioid receptors or mu-kappa heteromers. Data is represented as mean of %response of DAMGO without any antagonist.

### 5.2.2. CTOP inhibits DAMGO-induced endocytosis of mu-delta heteromers

Given that CTOP significantly antagonized mu-delta, but only weakly antagonized mu opioid receptors, we utilized immunocytochemistry to evaluate how CTOP would affect receptor endocytosis. In HEK-293 cells stably expressing mu receptors, DAMGO produced strong endocytosis that was not significantly affected by pretreatment of CTOP (Fig 5.3).

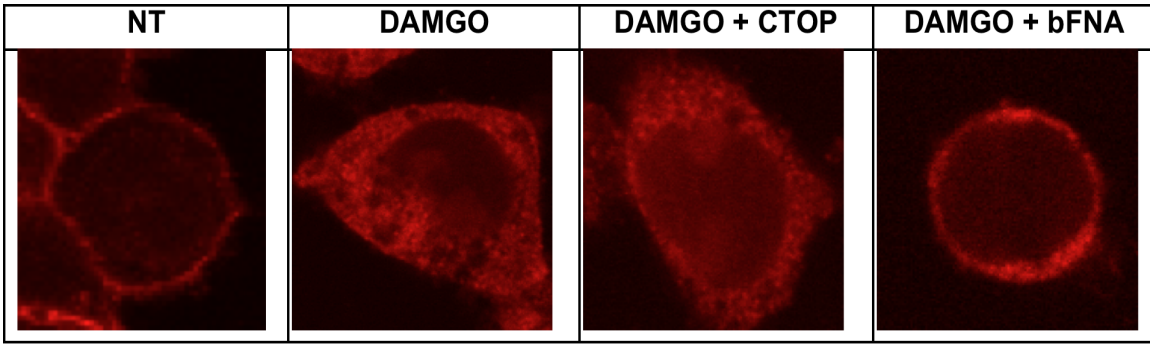



**Figure 5.3 β-FNA, but not CTOP, can inhibit the internalization of mu receptors induced by DAMGO.**

DAMGO (1 μM) produced strong internalization in HEK293 cells expressing mu opioid receptors. While β-FNA was able to inhibit the internalization of mu receptors, CTOP was unable to do so.

In cells expressing both mu and delta, DAMGO produced strong co-internalization of both receptors (Fig 5.4). Significantly a 10 min pretreatment with CTOP, before addition of DAMGO, blocked the endocytosis of both mu and delta (Fig 5.4). On the other hand, β-FNA blocked receptor endocytosis in cells that contained just mu, or both mu and delta receptors (Fig 5.3 and 5.4).

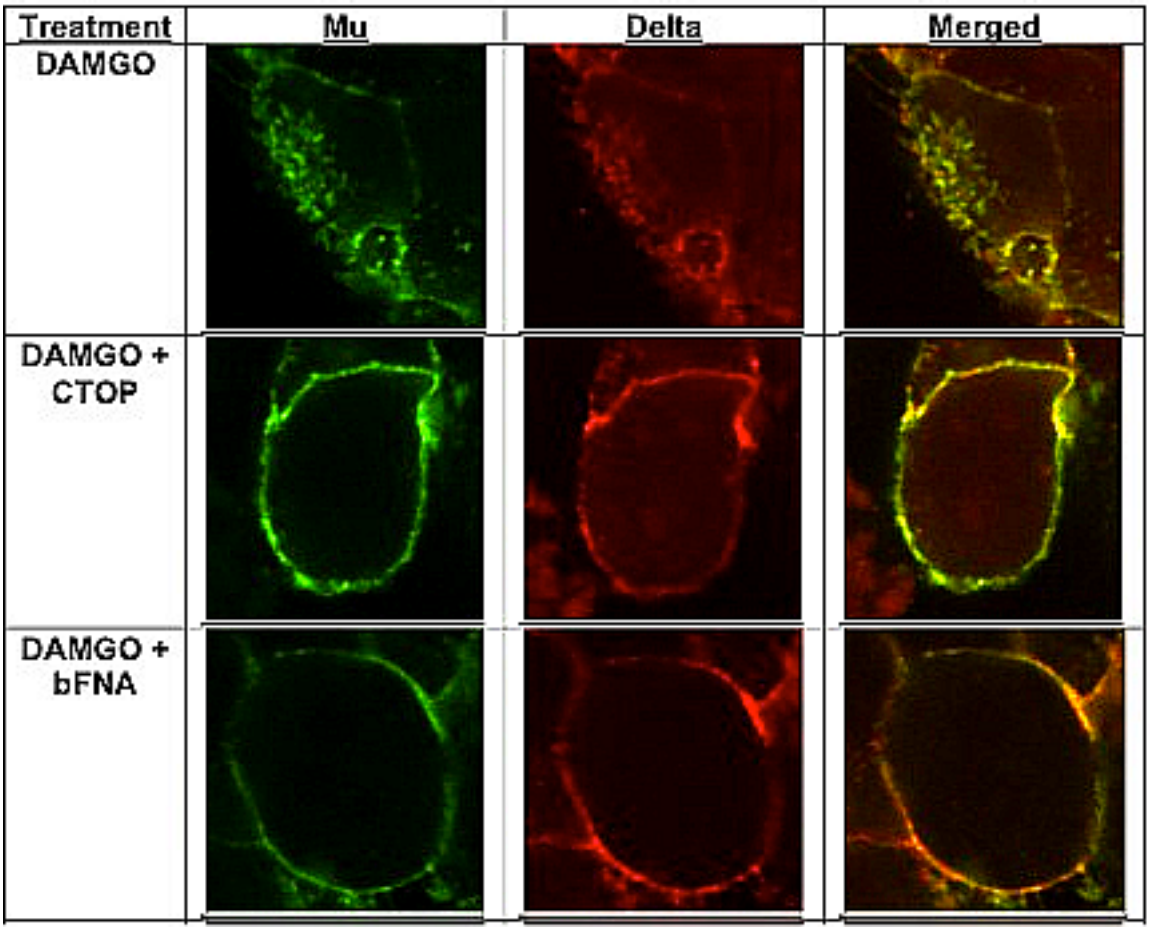



**Figure 5.4 CTOP and β-FNA inhibit the co-internalization of mu-delta heteromers in HEK293 cells.**

DAMGO (1 μM) produced strong co-internalization of mu-delta heteromers that was attenuated by a 10 min pre-incubation with either CTOP or β-FNA (both 1 μM).

**Effect of CTOP on DAMGO-induced internalization**

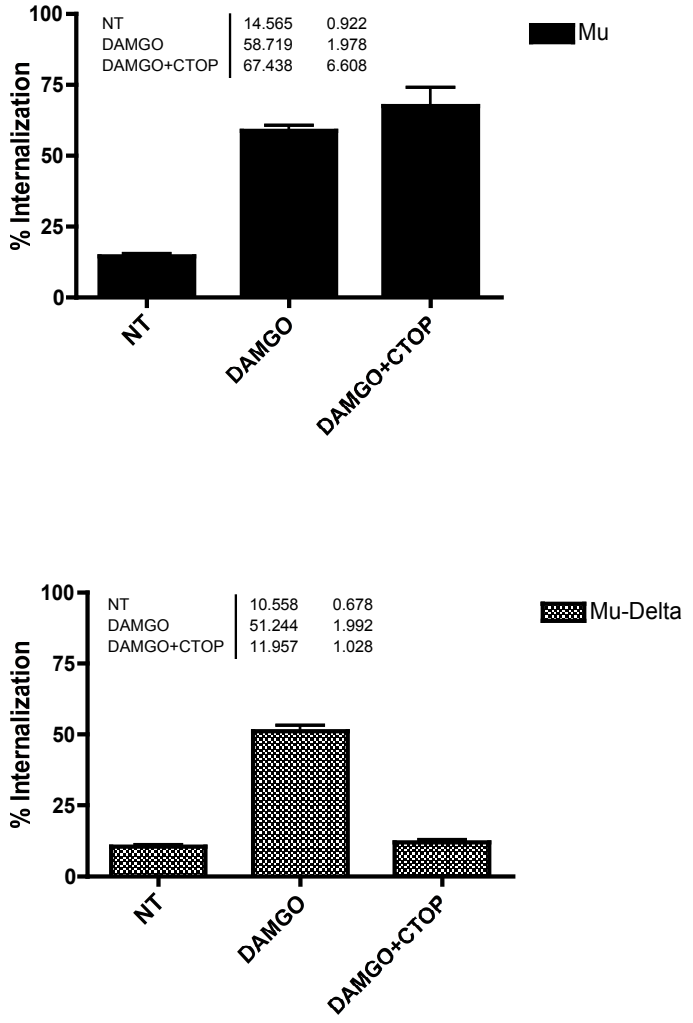



**Figure 5.5 Quantification of internalization due the administration of DAMGO and CTOP in HEK-293 cells.**

Fluorescent intensity of the cytoplasm was calculated as a percentage of cell surface intensity for at least 50 cells per treatment. The data shows that CTOP significantly inhibits the DAMGO-induced internalization of mu-delta receptors, but not mu opioid receptors.

### 5.2.3. CTOP inhibits antinociception of both DAMGO and DPDPE in mice

**(Performed by Mary Lunzer)**

We have previously shown that both DAMGO and DPDPE strongly activate mu-delta opioid receptor heteromers. From that study we also postulated that DAMGO and DPDPE would trigger mu-delta activation via mu and delta protomers respectively. Since CTOP potently inhibited the activation and endocytosis of mu-delta heteromers in vitro, we evaluated its antagonist activity against both DAMGO and DPDPE in mice. CTOP potently antagonized the i.c.v. dose response curves of both DAMGO and DPDPE (Fig 5.6). When administered i.c.v., DAMGO produced potent antinociception ($ED_{50}$ = 20.3 pmol/mouse) that was ~20-fold right-shifted by a 10 min i.c.v. pre-treatment with CTOP ($ED_{50}$ = 410.1). Similarly, the antinociceptive DRC of DPDPE ($ED_{50}$ = 5.8 nmol/mouse) was ~14-fold right-shifted by i.c.v administration of CTOP ($ED_{50}$ = 84.6 nmol/mouse).

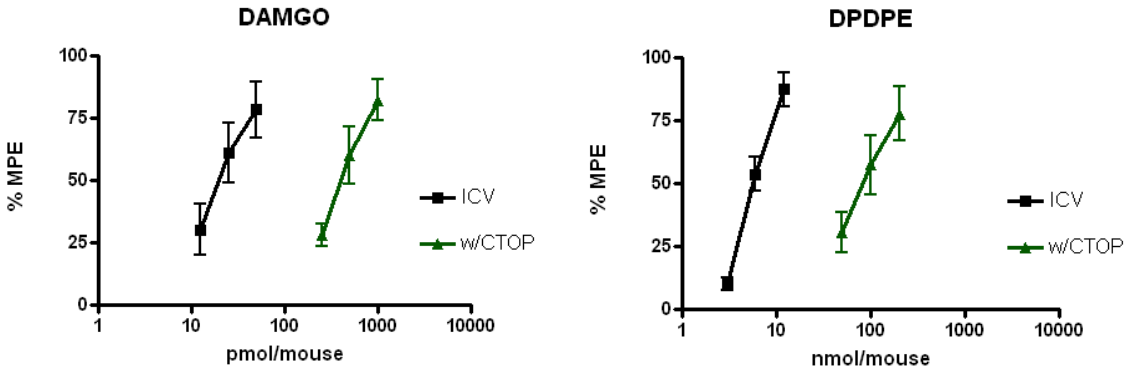



**Figure 5.6 CTOP antagonized both DAMGO and DPDPE in mice.**

The antinociceptive dose-response curves of both DAMGO and DPDPE (both i.c.v.) were significantly right-shifted by the administration of 100 pmol/mouse (i.c.v.) of CTOP.

We also determined the $AD_{50}$ of CTOP for DAMGO and DPDPE to compare the doses needed to antagonize them. We were surprised to observe that $AD_{50}$ for inhibiting DAMGO ($AD_{50}$ = 31.6 pmol/mouse) is very similar to the $AD_{50}$ for DPDPE ($AD_{50}$ = 39.5 pmol/mouse).

### 5.2.4. CTOP does not antagonize DAMGO in DOR-KO mice (Performed by Mary Lunzer at U of MN)

Given that our experiments in HEK-293 cells suggested that CTOP was unable to antagonize DAMGO in cells that stably expressed mu receptors, we utilized the DOR-KO mice to see if this would hold true in vivo. Since the gene for delta receptors is disrupted in these mice, there would be intact mu receptors, but no mu-delta heteromers. An $ED_{90}$ dose of DAMGO (50 pmol/mouse), as determined in WT control mice, produced similar antinociception (~90% MPE) even in the DORKO mice. However, even though 100 pmol/mouse of CTOP produced a ~20-fold shift in DAMGO-induced antinociception in WT mice (Fig 5.6), the same dose of CTOP did not produce significant antagonism of DAMGO in the DORKO mice.

### 5.2.5. β-naltrexamine analogs activate mu-kappa opioid heteromers

We have recently reported on a β-naltrexamine analog, NNTA, that is potent antinociceptive agent that selectively activates mu-kappa opioid heteromers[182]. Previous studies have shown that β-FNA and other β-

naltrexamine analogs produce immediate antinociception that wears off after some time to unmask the mu-antagonism[59, 60]. Hence, we investigated β-FNA and MN-14 (a β-naltrexamine analog with a 14-atom spacer) in the intracellular calcium release assay that has been described previously. Both β-FNA and MN-14 produced potent and selective activation of HEK293 cells containing mu and kappa receptors when compared with cells expressing only kappa or mu receptors (Fig 5.7a and b). As expected, neither ligand produced any activation in cells that contained singly expressed mu opioid receptors.

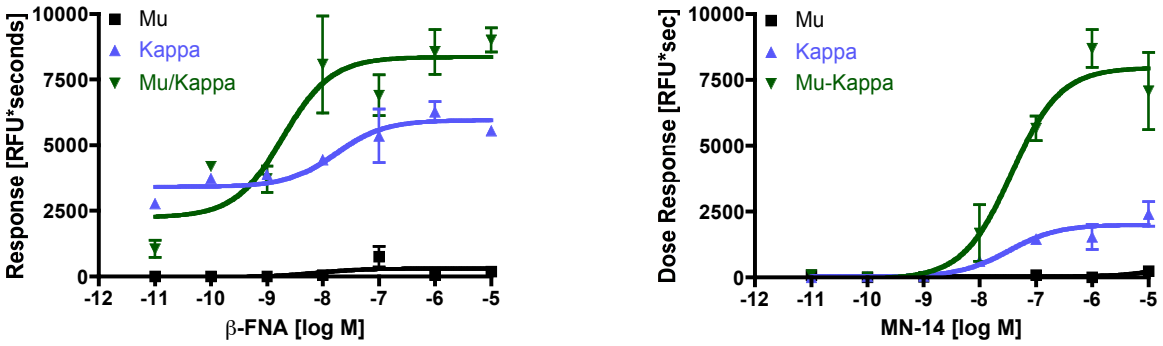



**Figure 5.7 β-FNA and the β-naltrexamine analog, MN-14, selectively activate mu-kappa opioid receptor heteromers.**

In the intracellular calcium release assay, both β-FNA and MN-14 produced strong calcium release in HEK293 cells stably expressing mu and kappa receptors

### 5.2.6. β-FNA binds irreversibly to both mu and mu-kappa receptors

**(Performed by Mike Powers)**

It is well established that β-FNA binds irreversibly to mu opioid receptors[185]. Due to the agonist activity in HEK293 cells containing coexpressed mu and kappa receptors, we evaluated the ability of β-FNA to

bind mu-kappa heteromers. HEK-293 cells expressing mu or kappa alone, or both mu and kappa together, were incubated with β-FNA or naloxone for 30 mins. These cells were then incubated with [$^3$H]Diprenorphine ([$^3$H]DIP) for another 30 mins and counted for radioactivity to determine their ability to inhibit diprenorphine binding. Another set of cells were incubated with β-FNA or naloxone for 30 mins, washed 3x with HEPES buffer and then incubated [$^3$H]DIP to determine if the inhibition was irreversible. We observed that β-FNA showed binding to kappa receptors that was reversed by washing. Singnificantly, β-FNA showed irreversible binding to both mu and mu-kappa receptors that was immune to washing with buffer (Fig 5.8). On the other hand, naloxone's binding was completely removed after washing, reiterating the well-known fact that naloxone is a reversible ligand for mu opioid receptors while β-FNA binds irreversible.

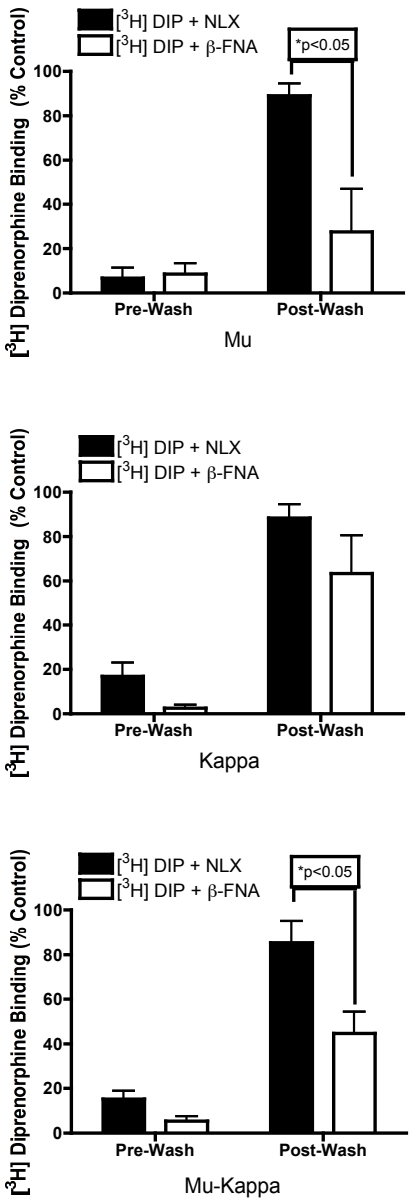



**Figure 5.8 β-FNA exhibits wash-resistant binding to mu and mu-kappa opioid receptors.**

To determine the irreversibility of binding, the ability of β-FNA and naloxone to bind mu, kappa and mu-kappa opioid receptors was tested before and after rigorous washing in HEK293 cells using the radioligand binding assay. β-FNA remained bound even after washing and inhibited [$^3$H]DIP binding to a greater extent in cells expressing mu and mu-kappa receptors.

## 5.3. Discussion

β-FNA[59, 185] and CTOP[62] were the first selective irreversible and reversible antagonists for mu opioid receptors, respectively. The evidence for their selectivity came from radioligand binding, ex vivo tissue, and in vivo antagonism studies and they have remained essential tools to study opioid receptors and ligands for decades. Liu-chen and coworkers[185] identified that the $Lys^{233}$ residue was necessary for the irreversible binding of β-FNA. Even though the existence of opioid receptor heteromers has been demonstrated for over a decade, there have been few systematic studies to evaluate the selectivity of ligands for the expanded field of receptor targets. We have performed experiments to determine the selectivity of both CTOP and β-FNA in vitro and in vivo, in light of the existence of opioid receptor heteromers in living cells.

To test the in vitro antagonist activity of CTOP, we evaluated its ability to antagonize DAMGO in HEK-293 cells stably expressing opioid receptors, singly or in pairs, using the intracellular calcium release assay. CTOP (1 μM) produced only ~ 25% antagonism of an $EC_{90}$ concentration of DAMGO (1 μM) in cells expressing mu receptors alone. Significantly, CTOP strongly attenuated DAMGO activity in cells coexpressing mu and delta opioid receptors but did not produce any antagonism in cells coexpressing mu and kappa receptors. These data provided the first indication for the requirement of mu-delta heteromers to be present for CTOP's antagonistic effects.

We then utilized the immunofluorescence labeling technique to determine the extent of receptor endocytosis induced by DAMGO in the presence and absence of antagonists. In HEK-293 cells expressing mu opioid receptors, DAMGO promoted strong internalization of mu receptors. Interestingly, DAMGO produced strong co-internalization of both mu and delta receptors in cells stably expressing both receptors. George and coworkers[130] have also previously shown that DAMGO can co-internalize mu and delta receptors when the receptors are coexpressed. CTOP did not inhibit DAMGO-induced mu receptor endocytosis. However, CTOP completely abolished mu-delta endocytosis in cells expressing both receptors. This is consistent with the significantly greater antagonism of calcium currents induced by DAMGO in cells expressing mu-delta heteromers when compared with cells expressing only mu receptors (Fig 5.2). On the other hand, β-FNA was able to attenuate receptor endocytosis in both mu and mu-delta cell lines. At this point, the data suggested that β-FNA can efficiently antagonize the mu receptor active site even if they are coupled with other opioid receptors, while CTOP needs the presence of the delta receptor to produce antagonism.

In an effort to elucidate the selectivity of CTOP in vivo, we conducted a series of antinociceptive studies in mice. First, we established dose response curves for DAMGO and the delta selective peptide, DPDPE, with and without CTOP pretreatment. Both DAMGO and DPDPE produced potent antinociception in the radiant heat tail-flick assay. Significantly, the antinociception of both ligands was reversed by CTOP to similar extents. To

determine the antagonist potency, $AD_{50}$s were determined. Even in this case, the $AD_{50}$ to antagonize DAMGO and DPDPE were equivalent, arguing for similar phenotypic receptors being targeted by CTOP. These data provide a qualitative correlation with the in vitro calcium and imaging results providing evidence for the selectivity of CTOP for mu-delta heteromers.

Given that both the in vitro and in vivo data indicated the preference of CTOP for mu-delta heteromers, we investigated its antagonistic ability in DOR-KO mice. These mice have been previously developed and lack functional delta receptors. In these mice, a WT control $ED_{90}$ dose of DAMGO produced similar antinociception when administered by itself. Surprisingly, CTOP did not produce any significant antagonism of DAMGO in these mice. This is very significant as these mice lack functional delta receptors, but do express functional mu receptors as evidenced by the antinociceptive activity of DAMGO. Even though CTOP has been long considered a mu selective antagonist, it was unable to antagonize the activity of DAMGO further corroborating the idea that CTOP is a selective mu-delta antagonist.

An interesting initial observation with β-FNA was that it produced short-acting, reversible agonism in the guinea pig ileum and in mice. However, its antagonist activity predominated which was long acting and irreversible. Prior studies had attributed the agonism to kappa receptors[59, 60]. In one of our pilot studies where we were looking at antagonist activity in cells expressing mu and kappa receptors, β-FNA produced strong calcium fluxes in calcium release experiments. This led us to study β-FNA in cells containing singly expressed

mu or kappa receptors, or coexpressed mu and kappa receptors using the intracellular calcium release assay. β-FNA produced potent agonism in both kappa and mu-kappa cell lines. While the $EC_{50}$s were similar, β-FNA was slightly more efficacious in cells coexpressing mu and kappa receptors, than kappa receptors alone. These data suggest that the agonism produced by β-FNA can be mediated via kappa or mu-kappa receptors.

It should be noted here that the agonistic effects of β-FNA were reversible, while the mu antagonism was wash-resistant. To further our understanding of the interaction between β-FNA and various opioid receptors, we conducted radioligand binding studies in HEK293 cells stably expressing mu, kappa, and mu-kappa receptors. As expected, β-FNA attenuated [$^3$H]DIP binding at mu opioid receptors even after washes, underscoring its wash-resistant nature at these receptors. Furthermore, β-FNA binding at kappa receptors was reversible as it blocked [$^3$H]DIP binding that was abolished upon washing. Surprisingly, the binding of β-FNA seemed to be irreversible, as the attenuated [$^3$H]DIP binding at mu-kappa receptors was not affected by washing. This data suggests that the presence of kappa receptors does not adequately affect the irreversible binding of β-FNA to the mu protomer within the mu-kappa heteromer.

In conclusion, we have presented data that clearly show that CTOP selectively inhibits the activation and internalization of mu-delta opioid receptor heteromers. The fact that CTOP inhibited the antinociceptive activity of both DAMGO and DPDPE at the same dose suggests that in vivo antagonism was

occurring via mu-delta heteromers. Indeed, CTOP was unable to antagonize DAMGO in DORKO mice strongly indicating that CTOP selectively antagonizes mu-delta heteromers, and not mu opioid receptors, as it is commonly believed, both in vitro and in vivo. On the other hand, β-FNA was able to antagonize both mu and mu-delta receptors. We also presented additional evidence to show that the short-term agonism observed with β-FNA can be mediated via kappa and/or mu-kappa receptors. All these data underscore the importance of incorporating heteromeric GPCRs into screening paradigms to get a better determination of ligand selectivity.

## 5.4. Methods

**Materials.** β-FNA and norBNI were synthesized as described previously[57, 66]. cDNAs encoding human kappa, mu and murine delta opioid receptors were inserted separately into the mammalian expression vector pcDNA3 (Invitrogen, Carlsbad, CA) and these were used to generate the singly expressing stable cell lines. HEK-293 cells stably co-expressing mu-kappa, kappa-delta and mu-delta receptors were obtained from Dr. Jennifer Whistler and their construction has been previously explained and verified[134, 159]. The chimeric G-protein, Δ6-$G_{\alpha qi4\text{-}myr}$, was graciously provided by Dr. Evi Kostenis and its construction has been previously described[160].

**Competition Binding.** Experiments were performed using HEK 293 cells genetically modified to produce wild-type human mu, kappa, or delta opioid receptors and coexpressing mu/kappa opioid receptors. Ten concentrations of

the tested compounds (50 μL) were added to test tubes containing 0.5 nM [$^3$H]diprenorphine (≈ 1.0 x $K_D$) (50 μL) or selective radioligands [$^3$H]DAMGO and [$^3$H]U69593 (both 2.0 nM) and whole cells (75 mm$^2$ plate, 80-90 % confluent) suspended in 12 ml HEPES buffer (25 mM, pH=7.4) (400 μL). Final volume was 500 μL. Non-specific binding was measured in the presence of 10 μM naloxone. Assays were incubated at room temperature for 90 min. and then filtered using a Brandel M-48 tissue harvester through Whatman GF/C filter paper that was pre-soaked in 0.25 % poly(ethyleniminie). Filters were washed three times with ice cold HEPES buffer (see above), and the radioactivity counted using a LS 6500 liquid scintillation counter (Beckman, Fullerton, CA). All measurements were performed in triplicate. $IC_{50}$ values were calculated using PRISM software (GraphPad, San Diego, CA) utilizing non-linear regression of the data normalized to fit a sigmoidal dose-response curve with a variable slope (100% defined at concentration = 0 (total binding) and 0% defined at the value of non-specific binding). $K_i$ values were determined from the Cheng-Prusoff equation assuming a single site binding model. Values reported are mean $K_i$ ± standard error of the mean (SEM) of three or more independent experiments.

**Immunocytochemistry:** Two-color immunofluorescence was employed to analyze co-expression of mu- and kappa-opioid receptors as previously described. Briefly, HEK-293 cells coexpressing HA-mu and FL-kappa were incubated with goat anti-HA (Abcam, Cat #ab9134) antibody and rabbit anti-FLAG (Abcam, Cat #1162) antibody at a final working concentration of 1:100

for 60 min at 4°C. After rinsing thrice with 50 mM PBS (pH 7.2), cells were fixed with 2% formaldehyde for 10 min at room temperature. Then cells were washed (3 x 15 min) with PBS and incubated at room temperature with the mixture of anti-goat NL-493 (Cat # NL003; R&D Systems, Inc.) and anti-rabbit NL-557 (Cat # NL004; R&D Systems, Inc.) fluorescent secondary antibodies (1:200 final dilutions). Cells were again rinsed with PBS (3 x 15 min) and mounted under coverslips with anti-fade mounting media iBright Plus (Cat # SF40000-10; Neuromics, Inc.) containing DAPI. Olympus FluoView1000 confocal microscope was used for image collection.

**Cell Culture and Intracellular $Ca^{2+}$ release assay.** HEK-293 cells were cultured at 37°C in Dulbelcco's modified Eagle's medium supplemented with 10% bovine calf serum, Pen/Strep antibiotics. For cells singly expressing opioid receptors, G418 was used as the selection antibiotic; G418 and Zeocin were used for selecting for cells coexpressing two opioid receptors. The protocol for the intracellular calcium release has been previously described in detail. Briefly, the stable opioid receptor cell lines were transiently transfected with 200 ng/20,000 cells of the chimeric G-protein, $\Delta$6-$G_{\alpha qi4\text{-}myr}$, using OptiMEM medium (Invitrogen) and Lipofectamine 2000 (Invitrogen, Carlsbad CA) reagent according to manufacturer's protocol (1:2 wt/vol ratio for DNA:Lipofectamine). The cells were seeded into 96 well plates (half-area; Corning) at 20,000 cells/well after 24 hours and assayed 48 hours after transfection using the FLIPR calcium kit (Molecular devices) in a Flexstation-III apparatus (Molecular

devices). The response was measured as area under the curve (Relative Fluorescence Units X seconds) and plotted using non-linear regression.

**[$^{35}$S]GTPγS assay.** The assay was setup as described previously[174]. Briefly, membranes from mu, kappa and mu/kappa cells were incubated with varying concentrations of NNTA and [$^{35}$S]GTPγS (Perkin-Elmer) in a 96 well plate. The incubation was performed at 37°C for an hour in membrane buffer (50mM Tris-HCl, 3mM $MgCl_2$, 0.2mM EGTA, 100mM NaCl and 0.5% BSA), filtered and counted for radioactivity. Three replications were performed for each treatment.

**Animals.** For studies performed at the University of Minnesota, male ICR-CD1 mice (18-25 g or 30-35 g; Harlan, Madison, WI), were housed in groups of 8 in a temperature and humidity controlled environment with unlimited access to food and water and maintained on a 12 hour light/dark cycle. The mu opioid receptor knockout mice (BALB/C X C57BL/6 MORKO) were a generous gift from Dr. Sabita Roy and have been described previously. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota.

Antinociceptive Studies.

All solutions were dissolved in distilled water. Controls with only distilled water showed no antinociception. All ligands were administered in a 5-µl volume in conscious mice according to the method Hylden and Wilcox[183] for i.t. and Haley and McCormick[184] for i.c.v. injections.

**%MPE method.** The tail flick assay was performed as described by D'Amour and Smith[52] and modified by Dewey et. al.[53]. For the measurement the latency of the tail-flick measurement, the mice were held gently in one hand with the tail positioned in the apparatus (Tail Flick Analgesia Meter, Columbus Instruments, Columbus, Ohio) for radiant heat stimulus. The tail-flick response was elicited by applying radiant heat to the dorsal side of the tail. The intensity of the heat was set at setting 8 so that the animal flicked its tail within 2 to 3 s. The test latency was measured once before drug treatment (control) and again after the drug treatment (test) at the peak time of the compound, a 10s maximum cut-off time was used to prevent damage to the tail. Antinociception was quantified according to the method of Harris and Pierson (54) as the percent maximal possible effect (%MPE) which is calculated as: %MPE = (Test - Control/10 – Control) x 100. At least three groups of eight to ten mice were used for each dose response curve, and each mouse was used only once. $ED_{50}$ values with 95% confidence intervals (C.I.) were computed with GraphPad Prism 4 by using nonlinear regression methods.

# CHAPTER 6

# 6. BIVALENT MU AGONIST/DELTA ANTAGONIST LIGAND (MDAN-21) BRIDGES MU-DELTA OPIOID HETEROMERS

## 6.1. Introduction

Opioid ligands, such as morphine, produce analgesia via Gi/Go protein coupled opioid receptors. However, they also produce side effects like respiratory depression, constipation, tolerance, physical dependence, etc that complicate their use in the clinic. There are three cloned opioid receptors, μ, κ and δ [2, 82] that are activated by opioid ligands. Several studies have suggested a major role for δ opioid receptors in participating in tolerance and physical dependence mediated by "μ" opioid ligands[136, 137, 161, 162]. Notably, it has been shown that co-administration of the δ antagonist, naltrindole[72] (NTI) attenuates morphine-induced tolerance and dependence[161]. These seminal observations, along with the discovery that mu and delta opioid receptors oligomerize to form heteromers[109, 115], led to the design of a series of MDAN bivalent ligands that contain mu agonist and delta antagonist pharmacophores tethered via spacers of varying length[138].

The rationale for the design of bivalent ligands is based on the concept that the two protomers in a heterodimer are physically associated, and that optimal bridging of the associated protomers takes place at a specific spacer length[139]. Several peptide[140-143] and opioid alkaloid[65, 144-148] bivalent ligands have now been characterized to show effects that are consistent with bridging

in vivo. However, to date, there has been limited direct evidence for bridging of protomers in a heteromeric complex. In an effort to obtain direct evidence for bridging, we have performed immunofluorescence and intracellular calcium release experiments utilizing the MDAN bivalent ligands that contain mu agonist and delta antagonist pharmacophores tethered together via a glycolic linker. In this series, MDAN-21 is the 21-atom spacer bivalent ligand that has been reported to produce potent antinociception devoid of tolerance, physical dependence, or place preference[138, 149]. Studies with this ligand, in comparison with the rest of the ligands in the series, provided the strongest possible correlation yet between bridging GPCR heteromers with the appropriate pharmacophores and therapeutic benefit[138, 149].

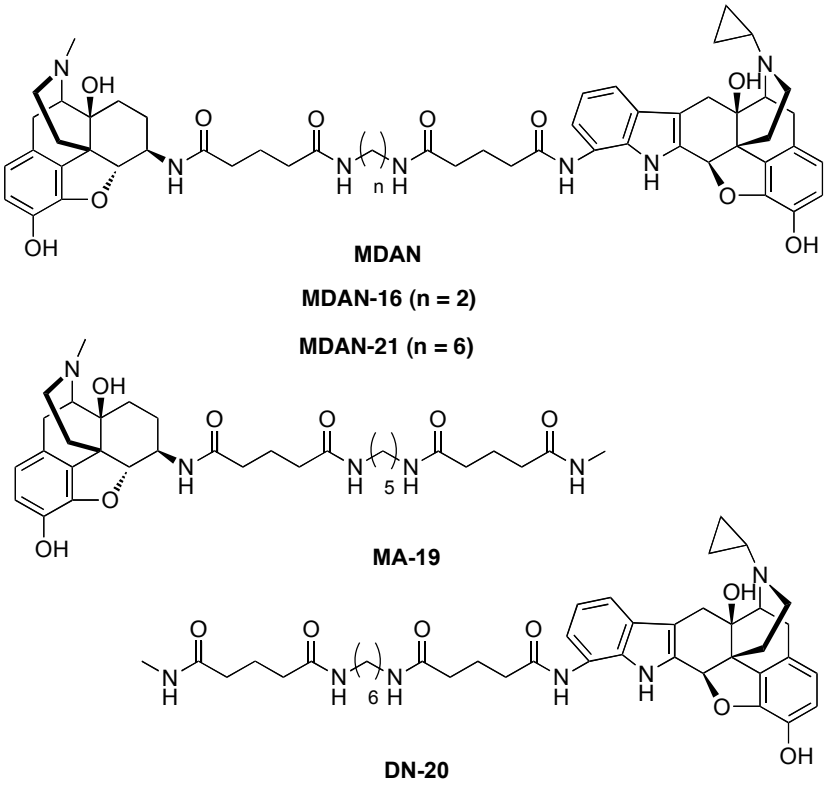



**Figure 6.1 Structures for bivalent and monovalent ligands in the MDAN series**

## 6.2. Results and Discussion

To investigate the possibility that MDAN-21 bridges mu-delta heteromers, an immunocytochemical approach was utilized. We hypothesized that the monovalent ligand MA-19 and the bivalent ligand, MDAN-16, both of which produce tolerance and physical dependence, do so through a univalent interaction. If this is the case, they would produce different effects on the endocytosis of mu-delta heteromers when compared with MDAN-21, whose

pharmacology suggested bridging of mu and delta protomers. HEK-293 cells containing FLAG tagged μ (FL-μ) and hemagglutinin tagged δ (HA-δ) were incubated with anti-FLAG and anti-HA primary antibodies (Abcam) for 2 hours on ice. The cells were then treated with the respective ligands for 30 minutes at 37°C, washed, and fixed with 4% paraformaldehyde for 10 minutes. Staining was performed using the corresponding fluorophore-tagged secondary antibodies.

Interestingly, MA-19 produced robust co-internalization of both mu and delta opioid receptors (Fig 6.2). When taken together with studies showing that mu and delta receptors are constitutively expressed as heteromers[124, 130], these data suggest that mu and delta receptors are physically coupled and trafficked together. Indeed, the mu agonist, DAMGO, also has been reported to co-internalize mu-delta receptors[130]. The fact that co-administration of the monovalent delta antagonist DN-20 with MA-19 did not block the co-internalization of mu-delta heteromers (Fig 6.2), suggests that occupancy of the delta opioid protomer by the delta antagonist pharmacophore was not negatively affecting the trafficking of the heteromer for this combination of ligands.

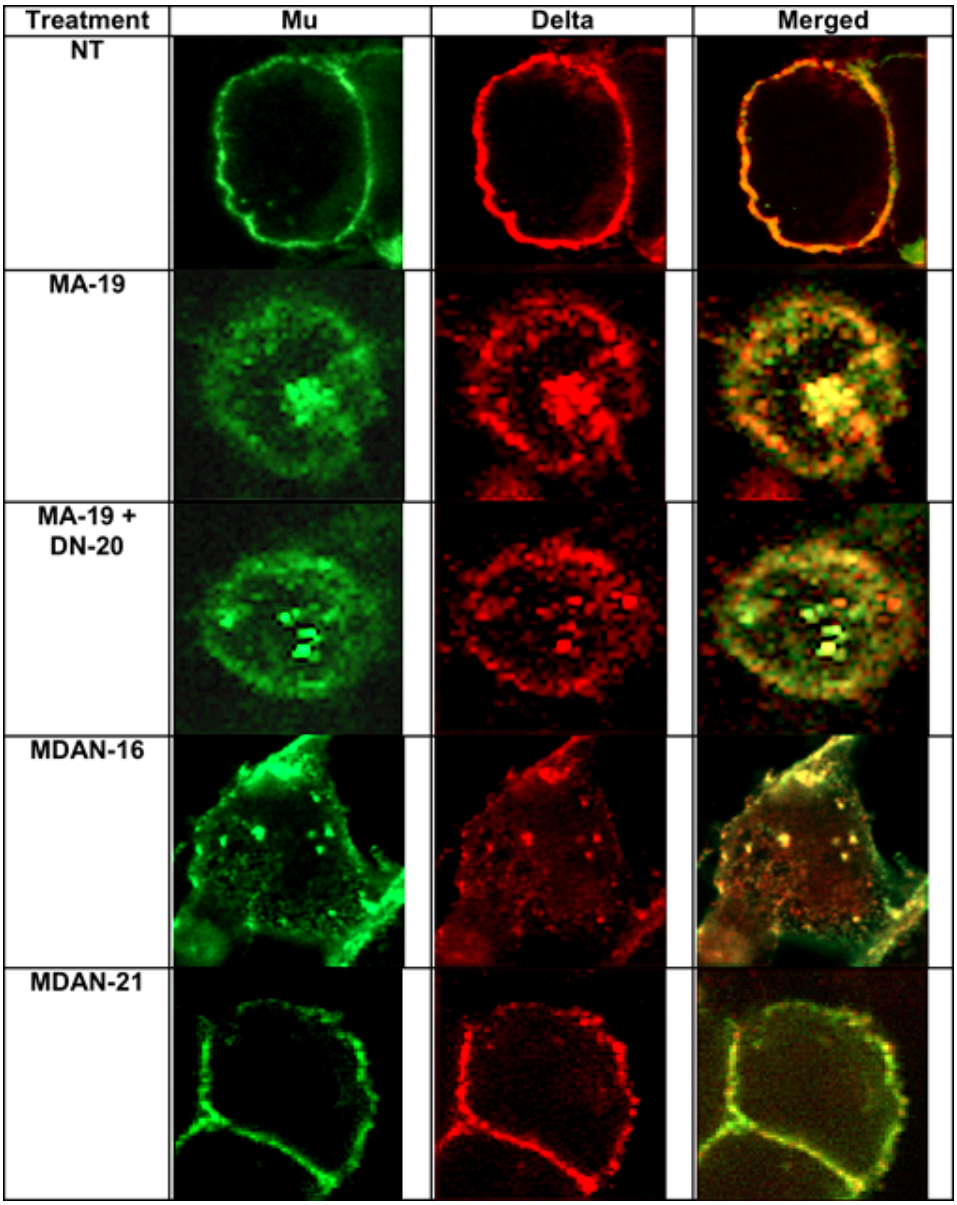



**Figure 6.2. MDAN-21 does not co-internalize mu-delta opioid receptor heteromers.**

The mu agonist monovalent (MA-19) produces strong internalization (row 2) that is not antagonized by the delta antagonist monovalent ligand (DN-20) when added together (row 4). However, the bivalent ligand MDAN-21 does not produce any internalization of either mu or delta receptor (row 5), providing evidence for the bivalent ligand bridging the mu-delta heteromers.

Significantly, MDAN-21 did not produce significant internalization of either mu or delta receptors in the mu-delta cell line (Fig 6.2). Since the bivalent ligand with the short spacer, MDAN-16, produced robust co-internalization of both mu and delta receptors, the data strongly suggest that MA-19 and MDAN-16 are involved in univalent interaction that leads to robust co-internalization of mu-delta heteromers. In contrast, the data suggest that MDAN-21 bridges adjacent mu and delta protomers, possibly stabilizing a receptor conformation that does not promote endocytosis of either receptor.

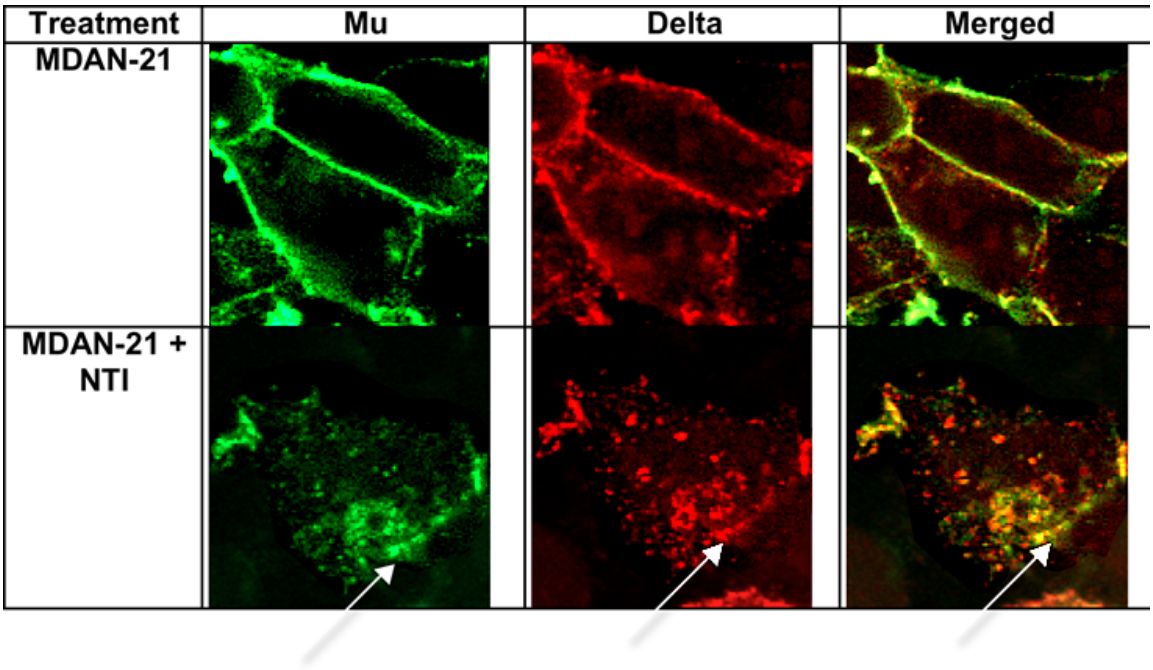



**Figure 6.3. Pretreatment with delta antagonist naltrindole (NTI) blocks bridging by MDAN-21.**

The bivalent ligand MDAN-21 does not produce any internalization of either mu or delta receptor (top row). However, after pretreatment with NTI for 10 mins MDAN-21 produces robust co-internalization of both mu and delta receptors (bottom row).

Further evidence for bridging of mu-delta protomers was obtained when cells were pretreated with the delta antagonist, NTI, 10 minutes before addition of MDAN-21. In this case, several punctae containing co-internalized mu-delta could be observed (Fig 6.3). This suggests that the bivalent interaction was disrupted due to displacement of the δ antagonist pharmacophore in MDAN-21 by NTI. Thus, in the presence of NTI, MDAN-21 functions univalently and promotes endocytosis as in the case of MA-19 and MDAN-16.

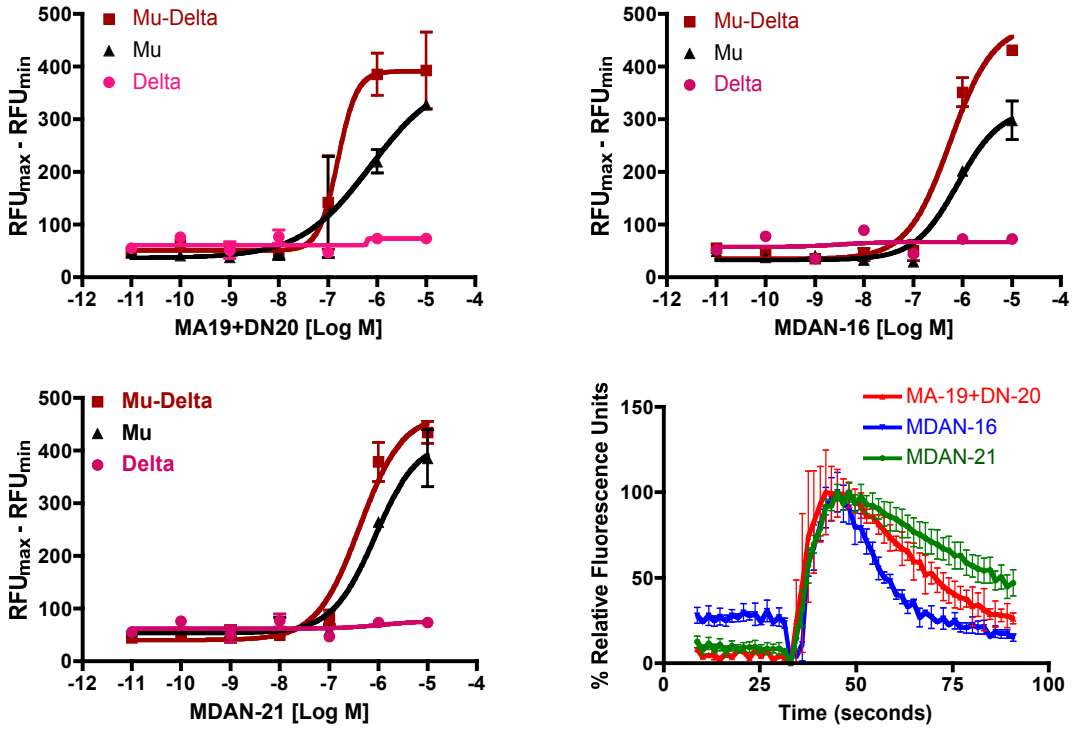



**Figure 6.4. MDAN-21 equivalently activates mu and mu-delta opioid receptors.**

Intracellular Ca2+ ion release mediated by increasing concentrations of mixed monovalents, MA-19+DN-20, and bivalent ligands MDAN-16, and MDAN-21, were measured in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric G-protein. Response was measured as Relative Fluorescence Units (RFU). The Y-axis plots the RFU values ± SEM (n = 8 - 12).

Given that bridging of μ-δ heteromers by MDAN-21 blocked endocytosis, we investigated whether or not the ability of MDAN-21 to activate the heteromers was also attenuated. We carried out intracellular calcium release experiments in HEK-293 cells that contain stably expressed mu, delta or mu-delta opioid receptors[134, 159, 174]. These cells were transiently transfected with Δ6-$G_{\alpha qi4\text{-}myr}$[160], a chimeric G protein that has been shown to couple opioid receptors to the calcium release mechanism[134, 174, 182]. We evaluated the bivalent ligands, MDAN-16 and MDAN-21, with MA-19 and DN-20 serving as monovalent controls. It should be noted here that we chose to co-administer the monovalent ligands, MA-19 and DN-20, rather than pretreating with DN-20 and then adding the agonist. This was done because in the bivalent ligands, both pharmacophores are tethered and would be able to interact with the respective protomers in a concerted manner. Hence, it would be required to co-administer the univalent ligands to serve as monovalent controls.

In HEK-293 cells, the monovalent agonist, MA-19, produced strong calcium release (~380 Relative Fluorescence Units (RFU)) that was unaffected by equimolar DN-20 in either mu or mu-delta cells (Fig 6.4). MDAN-16 produced slightly greater calcium release in mu-delta cells (EC50 = 565.7 nM, ΔRFU = 480 RFU) than in mu cells (EC50 = 796.6 nM, ΔRFU = 321 RFU). However, MDAN-21 was equivalently active in both mu-delta (EC50 = 411.6 nM, ΔRFU = 468 RFU) and mu cells (EC50 = 850.6 nM, ΔRFU = 420 RFU). Interestingly, all the three ligands had similar peak effect when added at a 10

μM concentration in cells containing mu-delta heteromers (Fig 6.4d). None of the ligands tested showed any significant activity in the delta opioid cells as all the ligands tested are antagonists at δ receptors. Significantly, the data demonstrate that MDAN-21 bridges the μ-δ heteromer and prevents endocytosis, while activating the heteromers to the same extent as homomeric mu receptors.

In addition to providing fresh evidence for bridging of heteromers, this study opens up the debate on the relationship between receptor endocytosis and the development of tolerance[186-188]. The regulation of opioid receptors by endocytosis has been suggested to play a significant role in the development of antinociceptive tolerance. Studies have indicated that the endocytosis of mu opioid receptors has an inverse relationship to tolerance[189, 190], whereas the endocytosis of delta receptors correlates with increased tolerance[191]. In this context, the trafficking properties of mu-delta heteromers are extremely relevant, as several studies have suggested a role for these heteromers in the tolerance produced by clinically used opioids[136, 137, 161, 162, 174], such as morphine. Since MDAN-21 has already been reported to not produce tolerance in mice[138], it is significant that MDAN-21 alone does not promote endocytosis of the mu-delta heteromers either.

In conclusion, immunohistochemistry-monitored trafficking of heteromeric mu-delta receptors has provided fresh evidence for bridging of mu-delta heteromers using MDAN-21, a bivalent ligand containing a spacer of 21-atoms. These data reveal that the lack of internalization of mu-delta heteromers in the

presence of MDAN-21 is correlated with the absence of antinociceptive tolerance and dependence reported for the MDAN series in mice. Further studies will be needed to determine if blocking endocytosis of mu-delta heteromers is a relevant strategy to attenuate opioid tolerance and dependence.

## 6.3. Methods

**Immunocytochemistry:** Two-color immunofluorescence was employed to analyze co-expression and internalization of mu- and delta-opioid receptors as previously described. Briefly, HEK-293 cells coexpressing HA-delta and FL-mu were incubated with goat anti-HA (Abcam, Cat #ab9134) antibody and rabbit anti-FLAG (Abcam, Cat #1162) antibody at a final working concentration of 1:100 for 60 min at 4°C. After rinsing thrice with 50 mM PBS (pH 7.2), cells were incubated with or without ligands for 30 mins at 37°C to identify ligands that promote internalization. In antagonism experiments the delta antagonist, NTI, was incubated with cells 10-min prior to the addition of agonists. The cells were then rinsed agained and were fixed with 4% formaldehyde for 10 min at room temperature. Then cells were washed (3 x 15 min) with PBS and incubated at room temperature with the mixture of anti-goat NL-493 (Cat # NL003; R&D Systems, Inc.) and anti-rabbit NL-557 (Cat # NL004; R&D Systems, Inc.) fluorescent secondary antibodies (1:200 final dilutions). Cells were again rinsed with PBS (3 x 15 min) and mounted under coverslips with anti-fade mounting media iBright Plus (Cat # SF40000-10; Neuromics, Inc.)

containing DAPI. Olympus FluoView1000 confocal microscope was used for image collection.

**Cells.** Human embryonic kidney cells (HEK-293) stably expressing single opioid receptors (human μ, κ and mouse δ) were generated. HEK-293 cells co-expressing human opioid receptors in pairs were procured from Dr. Jennifer Whistler (University of California, San Fransisco). Their construction and characterization has been described previously [134]. Briefly, HEK-293 cells were cotransfected with HA-μ and FLAG-δ (μ-δ), HA-δ and FLAG-κ (κ-δ), HA-μ and FLAG-κ (μ-κ) to make the different stable coexpression cell lines. The stably expressing cells were checked for the expression of μ-δ and κ-δ opioid receptor heteromers using co-immunoprecipitation [134, 159]. All the single and dual stable transfected cell lines were grown at 37°C and 10% $CO_2$ in Dulbelcco's modified medium (GIBCO) supplemented with 10% FBS and 1% Penicillin-streptomycin antibiotic solution. G418 alone was used for selecting cells expressing opioid receptors singly, while both G418 and Zeocin were used to select for dual coexpression cell lines.

**Intracellular $Ca^{2+}$ release assay.** HEK-293 cells stably expressing opioid receptors obtained from Dr. Jennifer Whistler [134] were transiently transfected with a chimeric $G_{\alpha}$-protein [160], $\Delta$6-$G_{qi4\text{-}myr}$ at a concentration of 200 ng/20,000 cells using Lipofectamine 2000 (Invitrogen, Carlsbad CA). The DNA for the chimeric G-protein was the only DNA that was transiently transfected. Cells were grown to a confluency of approximately 2 million cells in a petridish. The cells were then counted and DNA for the chimeric G-protein was added to a

ratio of 200 ng/ 20,000 cells. Lipofectamine 2000 at a ratio of 1:2 wt/vol (DNA:Lipofectamine) was used for the transfection. The cells were then seeded 24 hours later into half area black 96 well plates (Corning) at 20,000 cells per well. The FLIPR calcium kit (Molecular devices) was used for the assay. Cells were incubated with a $Ca^{2+}$ ion chelating dye from the kit, 48 hours after transfection, and incubated for an hour. The plates were then assayed in a Flexstation-III apparatus (Molecular Devices) using a range of concentrations of the opioid ligand. The response was measured as Relative Fluorescence Units (RFUs) and the time of the response was measured in seconds. A response window of 33 seconds after ligand addition was used to measure the response before calcium ion reuptake mechanisms caused a drop in fluorescence. $\Delta$RFU was computed for each concentration which was then plotted as a concentration response curve using non-linear regression. To incorporate well-well variability, four well replications were performed for each concentration of the ligand. Importantly, each ligand was tested in at least three independent replications where, each replicate experiment consists of cells transiently transfected with the chimeric G-protein on a separate day thus ensuring true biological replication. The representative curves, $EC_{50}$ and $RFU_{peak}$ values are all thus calculated from the four internal/dependent and three independent replications. Thus any variability due to transfection is contained in the error bars and has been accounted for.

# CHAPTER 7

# 7. NEW INSIGHTS INTO THE TRAFFICKING OF OPIOID RECEPTOR HETEROMERS.

## 7.1. Introduction

Coexpressed opioid receptors have been shown to form heteromers in cultured cells, and it has recently been reported that exogenous opioid ligands generally exhibit greater activation of such heteromeric receptors as compared to their corresponding homomers. As there are no reports on the activation of heteromeric and homomeric opioid receptors by endogenous opioid peptides, we have investigated the functional selectivity of [Met$^5$]enkephalin (M-enk), [Leu$^5$]enkephalin (L-enk), endomorphin-I (EM1), and endomorphin-II (EM2) in HEK-293 cells containing either coexpressed or singly expressed opioid receptors using an intracellular calcium release assay. The selectivity profiles of the opioid peptides at heteromeric opioid receptors differed significantly from those of homomers. In addition, while [Leu$^5$]enkephalin and endomorphin-I were selective agonists for mu-kappa heteromeric receptors, [Met$^5$]enkephalin and endomorphin-II were non-selective agonists at mu-containing receptors (mu, mu-kappa and mu-delta). We have also conducted immunofluorescent imaging studies that reveal distinct differences in trafficking between heteromers and homomers activated by the opioid peptides. Given the remarkable differences in ligand selectivity, potency, and activation-promoted trafficking between heteromeric and homomeric receptors, the results of the present study suggests that the functional roles of the endogenous ligands

require reevaluation, given that all published studies on the selectivity of these peptides were based on the assumption of their interaction with homomeric receptors.

**[Met$^5$]-enkephalin:** Tyr-Gly-Gly-Phe-Met

**[Leu$^5$]-enkephalin:** Tyr-Gly-Gly-Phe-Leu

**Endomorphin-1**: Tyr-Pro-Trp-Phe

**Endomorphin-2:** Tyr-Pro-Phe-Phe

**Figure 7.1 Amino acid sequences of endogenous enkephalins and endomorphins**

## 7.2. Results

### 7.2.1. Endomorphin-1 (EM1) selectively activates mu-kappa opioid heteromers, while Endomorphin-2 (EM2) is non-selective

We utilized the intracellular calcium release method to investigate the activity of endomorphin-1 (EM-1) and endomorphin-2 (EM-2) in HEK293 cells stably expressing opioid receptors (Fig 7.2). The coexpressed cell lines have been shown to contain heteromers and $Ca^{2+}$ release method has been established previously[134, 159, 174, 182]. EM-1 showed partial agonist properties in cells containing mu ($EC_{50}$ = 2.4 nM, $RFU_{max}$ = 485.2 RFU), and mu-delta ($EC_{50}$ = 0.56 nM, RFUmax = 488.2 RFU) receptors. However, it was most active in cells that coexpressed mu and kappa receptors ($EC_{50}$ = 24.4 nM, RFUmax = 1422 RFU). Interestingly, while EM1 did not show significant activation at either kappa or delta receptors, it promoted calcium release in cells coexpressing kappa and delta receptors ($EC_{50}$ = 64.3 nM, RFUmax = 785.2 RFU).

On the other hand, EM2 was equivalently active at mu ($EC_{50}$ = 2.3 nM, RFUmax = 553.7 RFU), mu-kappa ($EC_{50}$ = 4.0 nM, RFUmax = 762.6 RFU), and mu-delta receptors ($EC_{50}$ = 0.53 nM, RFUmax = 650.2 RFU). There was little to no activity in cells expressing kappa and delta receptors singly, or together.

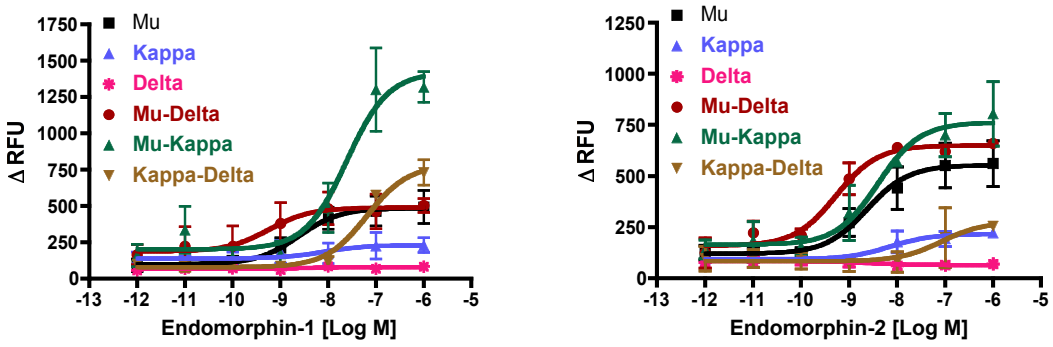



**Figure 7.2. Endomorphin-1 (EM1) selectively activates mu/kappa heteromers while endomorphin-2 (EM2) equivalently activates mu, mu/kappa and mu/delta opioid receptors.**

Intracellular $Ca^{2+}$ ion release mediated by increasing concentrations of EM1 or EM2 were measured in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric $G_{qi}$ protein. Response was measured as Relative Fluorescence Units (RFU) and time was measured in seconds. The Y-axis plots the RFU values ± SEM (n = 12-16).

### 7.2.2. Kappa receptors block mu endocytosis induced by endomorphins

Given that the EM1 selectively activated mu-kappa receptors, while EM2 was non-selective, we hypothesized that they differentially regulate the trafficking of mu-kappa heteromers. To test this we utilized the

immunofluorescence imaging technique in HEK293 cells that stably expressed epitope tagged opioid receptors. The cDNA for mu receptors was tagged with hemagglutinin (HA) while kappa receptors were tagged with FLAG epitope. Cells were grown in 8-well chamber slides and incubated with anti-HA (goat) and anti-FLAG (rabbit) primary antibodies for 1 hr at 4C. Cells were then washed with PBS and incubated with either EM1 or EM2 (both 1 μM) for 30 mins at 37C. They were then rinsed and stained with fluorophore-tagged secondary antibodies for goat and rabbit for 30 mins at RT and mounted using anti-fade mounting medium (Neuromics). In cells expressing mu opioid receptors, both EM1 and EM2 promoted significant internalization of mu receptors (Fig 7.3). However, the internalization was completely abolished in cells that contained coexpressed mu and kappa receptors (Fig 7.3).

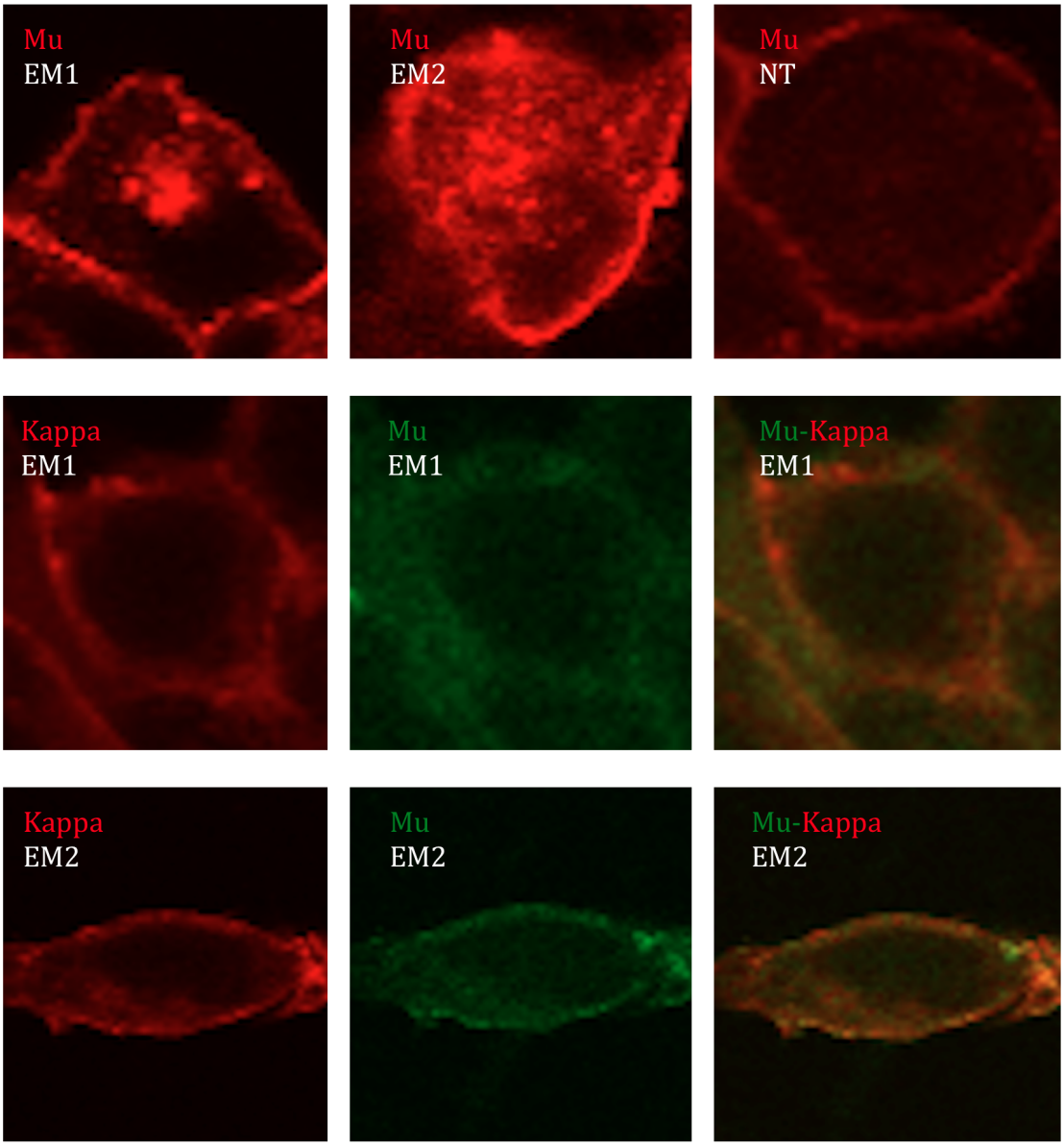



**Figure 7.3. Kappa receptors block mu internalization induced by endomorphins.**

In immunocytochemical studies, endomorphin-1 (EM1, 1 μM) and endomorphin-2 (EM2, 1 μM) strongly internalize mu opioid receptors (top row) in HEK-293 cells. However, in cells stably expressing both mu and kappa receptors, both EM1 (middle row) and EM2 (bottom row) do not produce any internalization. All the experiments have been replicated thrice and shown above are representative cells for the respective treatments. (NT = no treatment)

### 7.2.3. Selectivity of enkephalins

We then investigated the selectivity of enkephalin peptides, Leu-enkephalin (L-enk) and Met-enkephalin (M-enk) in HEK293 cells stably expressing opioid receptors using the intracellular calcium release assay (Fig 7.4). L-enk selectively activated cells containing both mu and kappa receptors ($EC_{50}$ = 1.4 nM, RFUmax = 1074 RFU). It showed some activity in cells expressing mu, mu-delta and kappa-delta receptors. There was no significant activity in cells expressing kappa or delta receptors. In contrast, M-enk equivalently activated mu ($EC_{50}$ = 0.9 nM, RFUmax = 394.9 RFU), mu-delta ($EC_{50}$ = 2.6 nM, RFUmax = 541.4 RFU) and mu-kappa receptors ($EC_{50}$ = 3.5 nM, RFUmax = 635.4 RFU). There was limited calcium release from cells containing kappa and delta receptors expressed alone or together.

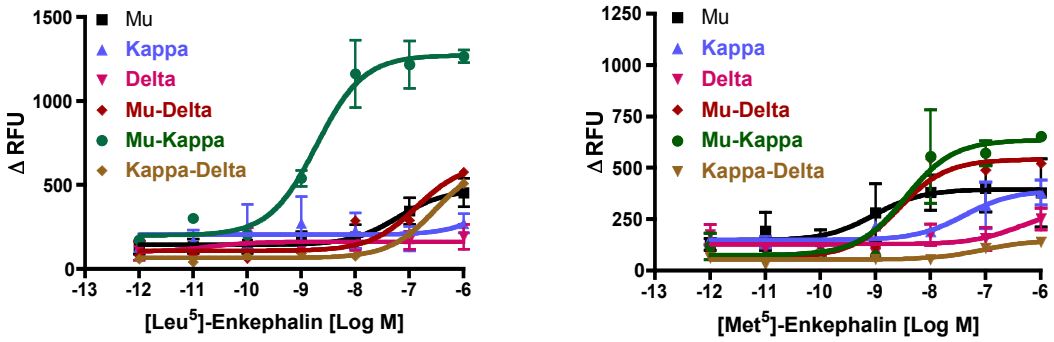



**Figure 7.4. [Leu$^5$]-enkephalin selectively activates mu/kappa heteromers while [Met$^5$]-enkephalin non-selectively activates mu, kappa, mu/kappa and mu/delta opioid receptors.**

Intracellular $Ca^{2+}$ ion release mediated by increasing concentrations of enkephalin ligands were measured in HEK-293 cells stably expressing opioid receptors and transiently transfected with the chimeric G-protein. Response was measured as Relative Fluorescence Units (RFU) and time was measured in seconds. The Y-axis plots the RFU values ± SEM (n = 12-16).

### 7.2.4. Kappa receptors block mu endocytosis promoted by [Leu$^5$]-enkephalin (L-enk), but not [Met$^5$]-enkephalin (M-enk)

To investigate the trafficking patterns induced by the enkephalin peptides, we used the immunofluorescence technique in cells containing mu or mu-kappa receptors. Similar to studies with endomorphins, cells were incubated with the respective primary antibodies, treated with L-enk or M-enk (both 1 μM), and then stained using fluorophore-tagged secondary antibodies. Interestingly, both L-enk and M-enk promoted significant endocytosis of mu opioid receptors in cells stably expressing just the mu receptors (Fig 7.5). However, in cells

coexpressing both mu and kappa receptors, L-enk did not produce any internalization of either mu or kappa receptors. On the other hand, M-enk promoted strong internalization of mu receptors even in the mu-kappa cell line.

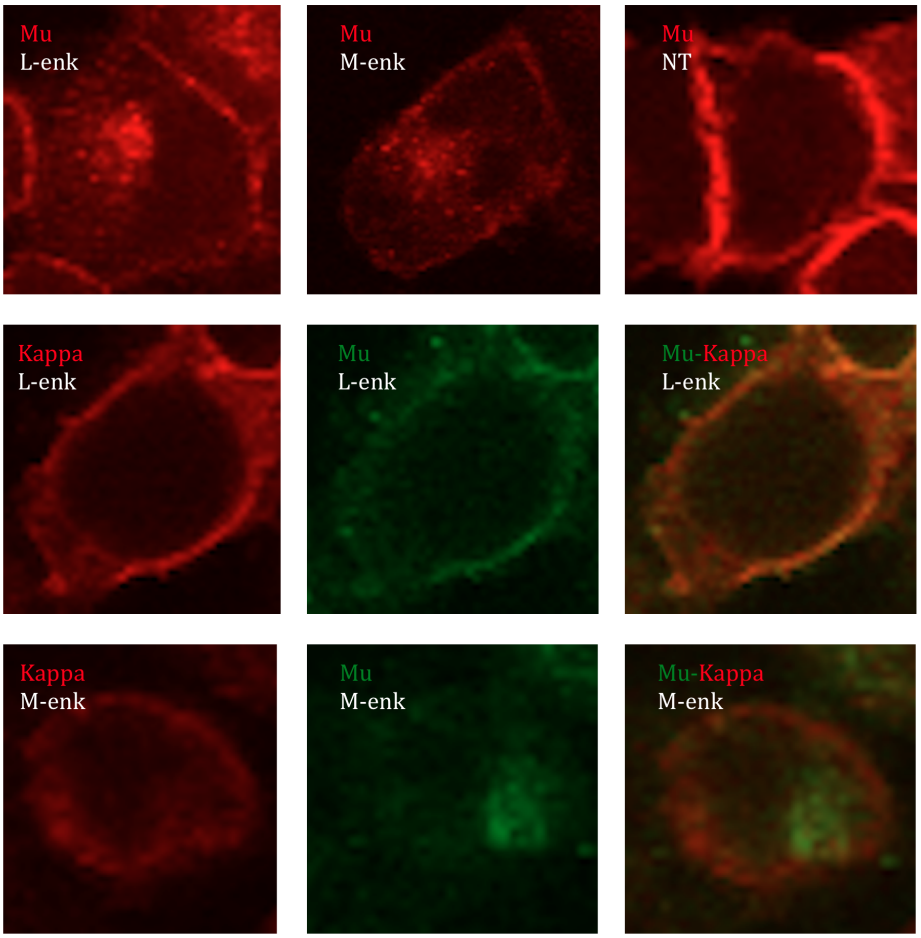



**Figure 7.5. Kappa protomers block mu internalization induced by [Leu$^5$]-enkephalin but not [Met$^5$]-enkephalin.**

In immunocytochemical studies, [Leu$^5$]-enkephalin (L-enk, 1 μM) and [Met$^5$]-enkephalin (M-enk, 1 μM) strongly internalize mu opioid receptors (***top row***) in HEK-293 cells. However, in cells stably expressing both mu and kappa receptors, both L-enk (***middle row***) does not produce any mu or kappa internalization while M-enk-induced internalization (***bottom row***) of mu opioid receptors remains unchanged. All the experiments have been replicated thrice and shown above are representative cells for the respective treatments. (NT = no treatment)

### 7.2.5. Delta receptors are translocated to the cell membrane by enkephalins

Due to several reports[2, 17] that enkephalins selectively target delta opioid receptors we conducted immunofluorescence experiments to determine their ability to traffic delta receptors. In HEK293 cells expressing delta receptors, the cells were stained with the receptor selective $DOR_{3\text{-}17}$ primary antibodies (Neuromics Inc.) with or without a 30 min treatment with L-enk or M-enk (1 μM). Significantly, most of the receptor staining was cytoplasmic in the no-treatment control experiments. However, treatment with both L-enk and M-enk resulted in increased surface localization of delta receptors (Fig 7.6).

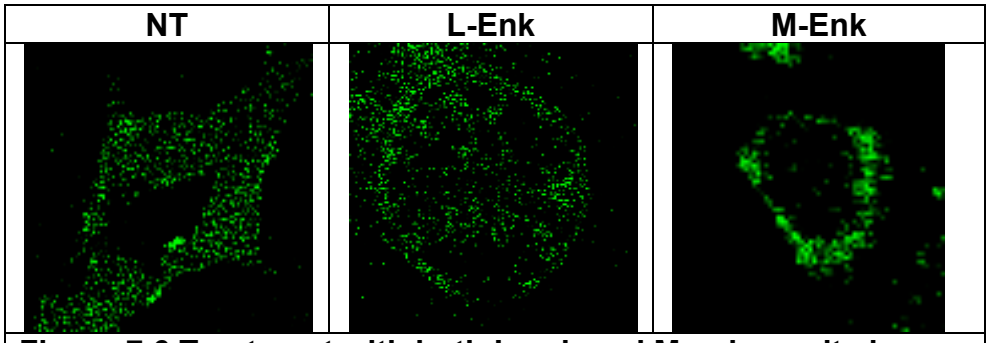



**Figure 7.6 Treatment with both L-enk and M-enk results in delta receptor translocation onto the cell surface of HEK293 cells.**

### 7.2.6. Pretreatment with DPDPE increases the efficacy of enkephalins in HEK-293 cells expressing delta opioid receptors

Since delta ligands were able to increase delta receptor surface expression, we tested enkephalin-induced calcium release after a 10 min pretreatment with the

delta-selective agonist, DPDPE (1 nM). A low dose of DPDPE was used to reduce extent of background calcium release due to the agonist. Enkephalins (1 μM) did not show much activation in delta cells without DPDPE. However, after DPDPE pretreatment both L-enk and M-enk promoted significant calcium release (Fig 7.7).

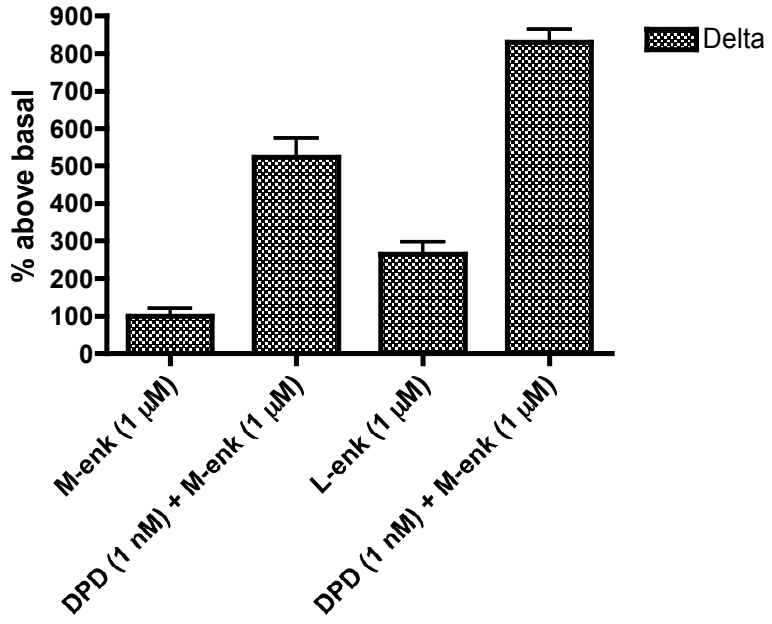



**Figure 7.7 In HEK-293 cells pretreatment with the delta agonist, DPDPE (1 nM), for 10 minutes leads to increased calcium release due to increased expression of delta opioid receptors on the cell surface.**

### 7.2.7. Mu opioid receptor expression promotes the surface translocation of delta opioid receptors

To determine the effect of mu opioid receptor expression on delta trafficking, we utilized the immunofluorescence imagining assay. cDNA vectors containing mu opioid receptors (200 ng/ 20,000 cells) were transfected into HEK293 cells stably

expressing delta opioid receptors. The delta receptors were predominantly expressed in the cytoplasm of HEK293 cells (Fig 7.6). However, the receptors were significantly surface localized upon transient expression of mu opioid receptors (Fig 7.8). Immunofluorescent imaging of HEK293 cells stably expressing both FLAG-mu and HA-delta receptors also showed that most of the staining for delta receptors was surface localized (Fig 7.8).

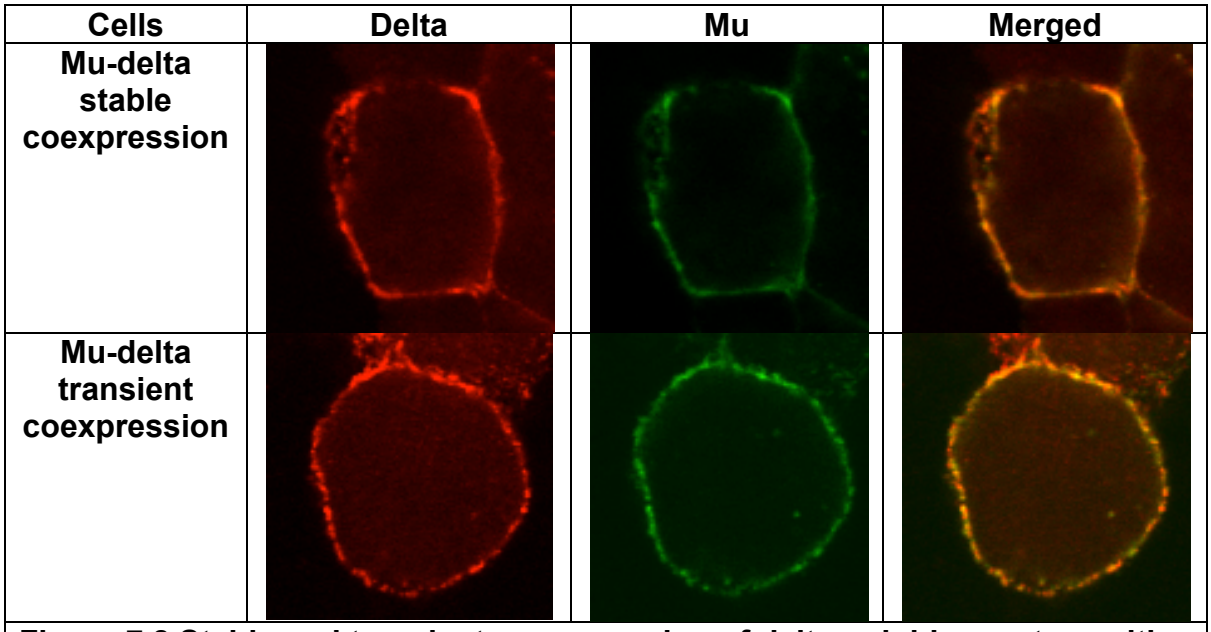



**Figure 7.8 Stable and transient co-expression of delta opioid receptors with mu receptors leads to increased delta receptors on the cell surface of HEK293 cells.**

### 7.2.8. Co-internalization of mu-delta receptors by DAMGO, morphine and SNC80

To determine the effects of DAMGO, morphine and SNC80 on the trafficking of mu-delta heteromers, we used again used the immunofluorescence assay with

HEK293 cells expressing just mu receptors, or mu and delta together. Morphine (1 μM) did not promote any internalization of mu receptors, while DAMGO (1 μM) promotes strong internalization of mu receptors when they are expressed alone (Fig 7.9). However, both morphine and DAMGO promote significant endocytosis of both mu and delta receptors in cells where they are coexpressed (Fig 7.10).

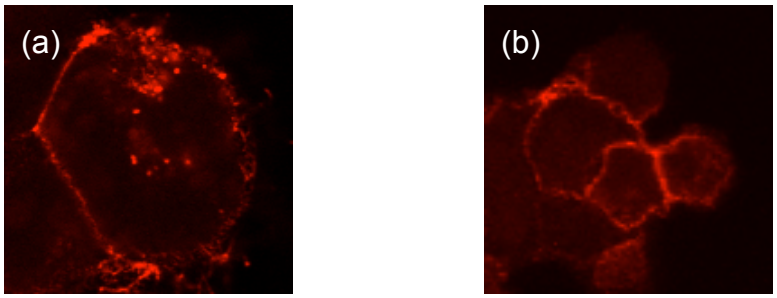



**Figure 7.9 (a) DAMGO produces strong internalization of mu opioid receptors while (b) morphine does not promote any internalization of mu receptors in HEK293 cells.**

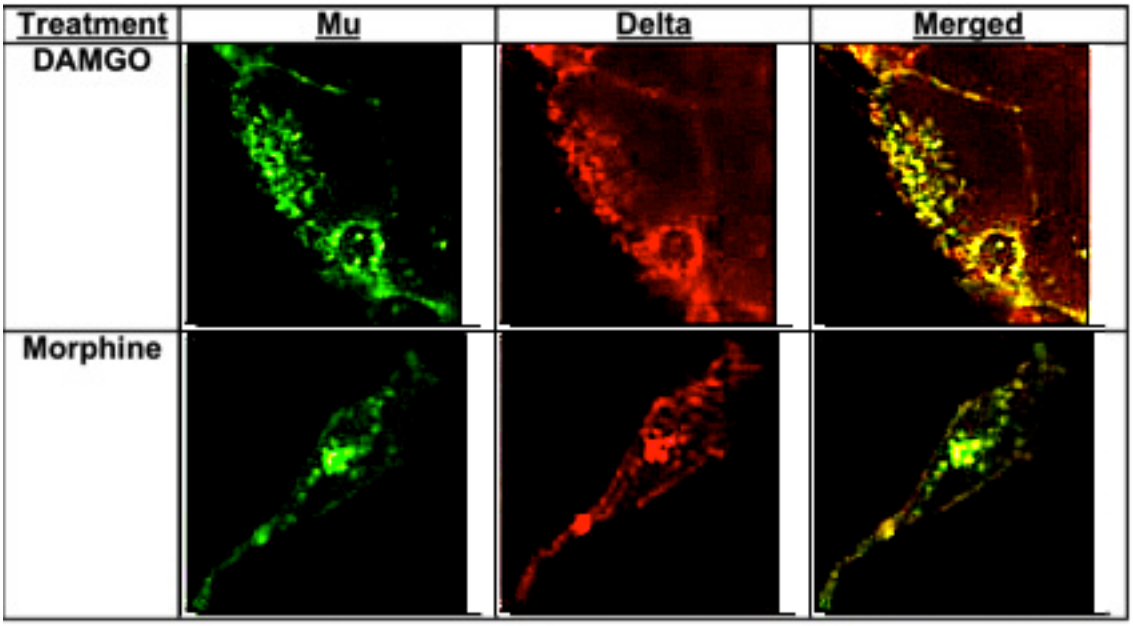



**Figure 7.10 DAMGO and morphine promote strong co-internalization of mu and delta receptors in HEK293 cells.**

The delta selective agonist, SNC80 (1 μM), also promoted strong co-internalization of both mu and delta receptors (Fig 7.11). This co-internalization was abolished when cells were pre-incubated with the delta antagonist, NTI (1 μM). Interestingly, pre-incubation with the mu antagonist, CTAP (1 μM), only blocked mu internalization while delta receptors were still internalized by SNC80 (Fig 7.11).

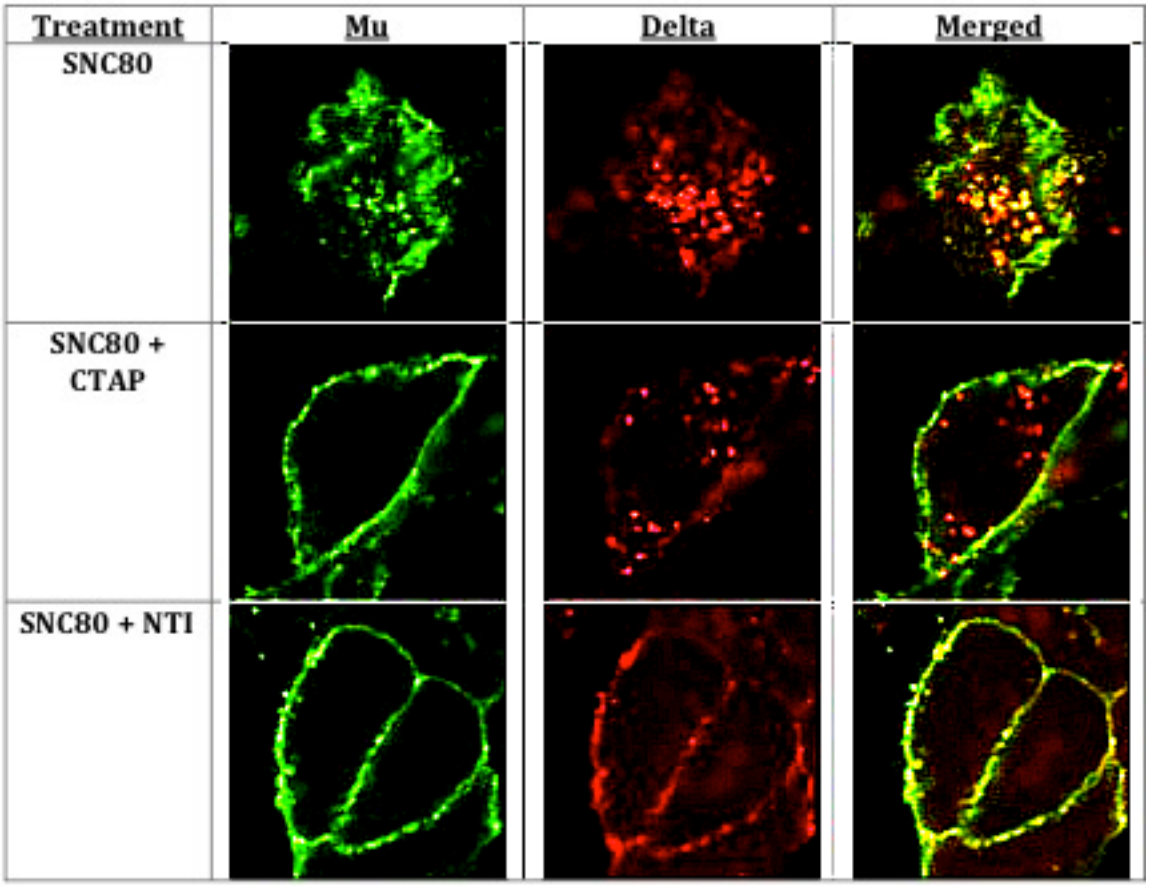



**Figure 7.11 SNC80 promotes strong co-internalization of mu-delta heteromers by targeting the delta protomers.**

Pretreatment with the (a) mu antagonist CTAP only blocked the internalization of mu receptors while (b) the delta antagonist, NTI, completely abolished the internalization of both mu and delta receptors due to SNC80.

## 7.3. Discussion

Even though there has been burgeoning evidence for the existence opioid receptor heteromers over the past decade, their role in mediating physiological effects has not been clarified. An important step in this process would be the delineation of ligands that selective target heteromers. We have previously

shown that several commonly used standard opioids and clinical analgesics selectively activate different opioid receptor heteromers in HEK-293 cells (See chapters 2-4). However, the selectivity of the endogenous opioid peptides has not been evaluated in the context of homomeric and heteromeric receptors. Therefore, we have conducted intracellular calcium release experiments to determine the selectivity of endogenous ligands and applied them to study the patterns of trafficking of opioid receptor heteromers.

Endomorphin-1 (Tyr-Pro-Trp-Phe-$NH_2$) and endomorphin-2 (Tyr-Pro-Phe-Phe-$NH_2$) are tetrapeptides that were originally described to have high affinity and selectivity for mu opioid receptors[192, 193]. However, it has long been suspected that they may also target distinct phenotypic receptors as several studies have pointed out the divergent activities of the two peptides[201-207]. In this regard, even though in vitro studies showed that both EM1 and EM2 are partial agonists of mu receptors[194-196], and their activity is attenuated in MORKO mice[197-201], antinociceptive studies have suggested that their pharmacological actions are mediated via different phenotypic receptors[201-203]. Moreover, mice pretreated i.t. with EM1 developed some antinociceptive cross-tolerance to EM2, while mice pretreated with EM2 did not develop any cross-tolerance to EM1[204]. Immunofluorescence studies revealed that EM1 was expressed to a greater extent supraspinally, while EM2 was more prevalent in the spinal cord suggesting that they may act tissue-specifically[205-207]. These studies led us to determine the selectivity of the peptides at homomeric and heteromeric opioid receptors. In intracellular calcium release experiments, EM1

selectively activated mu-kappa receptors, while being a partial agonist at mu and mu-delta receptors. In contrast, EM2 was a non-selective agonist at mu, mu-delta and mu-kappa receptors (Fig 7.2).

Both EM1 and EM2 have been previously shown to induce the internalization of mu opioid receptors[208]. In view of our studies showing that EM1 selectively activates mu-kappa receptors, we investigated the trafficking of mu and mu-kappa receptors using the immunofluorescence technique. Our studies revealed that EM1 and EM2 promoted strong endocytosis of mu opioid receptors (Fig 7.3). However, neither ligand produced any internalization in cells coexpressing mu and kappa receptors (Fig 7.3). The data suggested that in certain cases, kappa protomers inhibit the ligand-induced internalization of mu protomers when expressed together. Kappa receptors have been previously shown to have similar effects on delta and adrenergic receptors[154].

Enkephalins were the first endogenous opioid ligands discovered by Hughes and coworkers[12] in 1975. Initially, the two peptides were postulated to be delta selective[18] but later studies have shown that target both mu and delta receptors equivalently[17]. To elucidate the selectivity of the enkephalins, we again used the intracellular calcium release assay in HEK-293 cells expressing opioid receptors (Fig 7.4). Interestingly, [Leu$^5$]enkephalin (L-enk) was selective for mu-kappa receptors while [Met$^5$]enkephalin (M-enk) was non-selective for mu, mu-delta and mu-kappa receptors.

The unexpected results suggesting that L-enk selectively activates mu-kappa receptors, while M-enk is a non-selective mu agonist seemed to parallel

our findings with the endomorphins. Hence, we investigated the trafficking of mu-kappa receptors promoted by enkephalins (Fig 7.5). The enkephalins promoted profound internalization of mu receptors in HEK293 cells expressing only mu receptors as has been observed previously[209]. In a result similar to EM1, L-enk also failed to promote any endocytosis in cells expressing both mu and kappa receptors. However, M-enk promoted strong endocytosis of mu receptors in the mu-kappa cell line (Fig 7.5). This suggests that the trafficking of the individual protomers within a mu-kappa heteromer is ligand dependent.

Even though the enkephalins have been shown to target delta opioid receptors by various investigators, they did not produce any calcium release in HEK293 cells expressing delta receptors (Fig 7.4). To investigate this puzzling result, we conducted immunocytochemical studies to determine the cellular localization of delta receptors. Our results showed that the native delta receptors are distributed in the cytoplasm of HEK293 cells (Fig 7.6). Prior studies in PC12 cells and DRG neurons have also revealed a similar distribution pattern for delta receptors[151, 210]. Since the enkephalins are membrane impermeable peptides, this would explain why there was little activation observed in these cell lines.

An interesting facet of delta receptor trafficking is the fact that delta ligands have been shown to “externalize” cytoplasmic receptors onto the cell surface of DRG neurons. To determine if a similar phenomenon occurred in HEK293 cells, we conducted immunofluorescence imaging experiments using delta-selective and anti-FLAG primary antibodies, with and without agonist

treatment. Both L-enk and M-enk promoted strong surface expression of delta receptors after a 10-min incubation (Fig 7.7).

Additionally, we observed that delta receptors were predominantly located on the cell surface in HEK293 cells stably coexpressing mu and delta receptors (Fig 7.8). Given that delta receptors when expressed alone were localized in the cytoplasm, we hypothesized that mu opioid receptors modulate the trafficking of delta receptors onto the cell surface. To test this, we transiently expressed cDNA for mu opioid receptors in the delta cell line. Mu receptor expression again led to predominantly surface localized delta receptors suggesting that the coexpression of mu receptors has a profound effect on the trafficking of delta receptors (Fig 7.8). Moreover, our study provides more proof-of-principle for a previous study that suggested that mu-delta heteromers are coupled in the ER itself and are secreted onto the cell surface as constitutive heteromers[130].

We have previously reported that morphine and DAMGO activate mu opioid receptors, but are selective for mu-delta heteromers[174]. It is widely known that morphine does not promote endocytosis of mu receptors, while DAMGO produces profound internalization (Fig 7.9). Since we observed that mu receptors modulate delta receptor trafficking, we evaluated the possibility of some reciprocal effects on mu receptor trafficking. First, we observed that DAMGO produced strong co-internalization of both mu and delta receptors when expressed together (Fig 7.10). Significantly, morphine also produced significant co-internalization of both mu and delta receptors in HEK293 cells

(Fig 7.10). These data strongly suggest that both DAMGO and morphine target mu-delta heteromers leading to unique downstream signaling events.

This co-internalization was not limited to the “mu” agonists alone, as the delta-selective agonist, SNC80, promoted strong co-internalization of mu and delta receptors (Fig 7.11). The co-internalization was completely abolished in cells that were pre-incubated with NTI. However, when cells were pre-incubated with the mu antagonist, CTAP, only the trafficking of mu receptors was inhibited (Fig 7.11). Delta receptors were internalized by SNC80 under this condition.

These data suggest that SNC80 predominantly produces its effects via the delta protomers that can be abolished completely by NTI, as it would prevent SNC80 from binding. Moreover, the fact that CTAP does not block delta trafficking from within the mu-delta heteromer suggests that CTAP cannot allosterically inhibit the delta receptors.

In conclusion, we have shown results that suggest that the endomorphins and enkephalins target heteromeric opioid receptors, in addition to homomeric receptors. Kappa opioid receptors inhibit the trafficking of mu receptors when they are expressed together possibly as a consequence of being organized as heteromers. However, such effects are ligand dependent and M-enk is the only ligand, of those tested, which can still promote mu endocytosis in the mu-kappa cell line. We also showed that mu opioid receptors directly modulate the trafficking of delta opioid receptors by promoting cell surface localization. Ligands targeting either protomer can also induce co-internalization of both mu

and delta receptors. Even though morphine does not produce mu endocytosis in HEK293 cells, it can produce significant co-internalization of coexpressed mu and delta receptors. All these data provide strong support to the hypothesis that the individual protomers within opioid receptor heteromers modulate each other, possibly by physical association as heteromers, and lead to unique outcomes upon ligand activation. These conclusions have profound ramifications in understanding the role of ligands and receptors in vivo.

## 7.4. Methods

**Immunocytochemistry:** Two-color immunofluorescence was employed to analyze co-expression and internalization of mu- and delta-opioid receptors as previously described[174]. Briefly, HEK-293 cells coexpressing HA-delta and FL-mu were incubated with goat anti-HA (Abcam, Cat #ab9134) antibody and rabbit anti-FLAG (Abcam, Cat #1162) antibody at a final working concentration of 1:100 for 60 min at 4°C. After rinsing thrice with 50 mM PBS (pH 7.2), cells were incubated with or without ligands for 30 mins at 37°C to identify ligands that promote internalization. In antagonism experiments the delta antagonist, NTI, was incubated with cells 10-min prior to the addition of agonists. The cells were then rinsed agained and were fixed with 4% formaldehyde for 10 min at room temperature. Then cells were washed (3 x 15 min) with PBS and incubated at room temperature with the mixture of anti-goat NL-493 (Cat # NL003; R&D Systems, Inc.) and anti-rabbit NL-557 (Cat # NL004; R&D Systems, Inc.) fluorescent secondary antibodies (1:200 final dilutions). Cells

were again rinsed with PBS (3 x 15 min) and mounted under coverslips with anti-fade mounting media iBright Plus (Cat # SF40000-10; Neuromics, Inc.) containing DAPI. Olympus FluoView1000 confocal microscope was used for image collection.

**Cells.** Human embryonic kidney cells (HEK-293) stably expressing single opioid receptors (human μ, κ and mouse δ) were generated. HEK-293 cells co-expressing human opioid receptors in pairs were procured from Dr. Jennifer Whistler (University of California, San Fransisco). Their construction and characterization has been described previously [134]. Briefly, HEK-293 cells were cotransfected with HA-μ and FLAG-δ (μ-δ), HA-δ and FLAG-κ (κ-δ), HA-μ and FLAG-κ (μ-κ) to make the different stable coexpression cell lines. The stably expressing cells were checked for the expression of μ-δ and κ-δ opioid receptor heteromers using co-immunoprecipitation [134, 159]. All the single and dual stable transfected cell lines were grown at 37°C and 10% $CO_2$ in Dulbelcco's modified medium (GIBCO) supplemented with 10% FBS and 1% Penicillin-streptomycin antibiotic solution. G418 alone was used for selecting cells expressing opioid receptors singly, while both G418 and Zeocin were used to select for dual coexpression cell lines.

**Intracellular $Ca^{2+}$ release assay.** HEK-293 cells stably expressing opioid receptors obtained from Dr. Jennifer Whistler [134] were transiently transfected with a chimeric $G_{\alpha}$-protein [160], $\Delta$6-$G_{qi4\text{-}myr}$ at a concentration of 200 ng/20,000 cells using Lipofectamine 2000 (Invitrogen, Carlsbad CA). The DNA for the chimeric G-protein was the only DNA that was transiently transfected. Cells

were grown to a confluency of approximately 2 million cells in a petridish. The cells were then counted and DNA for the chimeric G-protein was added to a ratio of 200 ng/ 20,000 cells. Lipofectamine 2000 at a ratio of 1:2 wt/vol (DNA:Lipofectamine) was used for the transfection. The cells were then seeded 24 hours later into half area black 96 well plates (Corning) at 20,000 cells per well. The FLIPR calcium kit (Molecular devices) was used for the assay. Cells were incubated with a $Ca^{2+}$ ion chelating dye from the kit, 48 hours after transfection, and incubated for an hour. The plates were then assayed in a Flexstation-III apparatus (Molecular Devices) using a range of concentrations of the opioid ligand. The response was measured as Relative Fluorescence Units (RFUs) and the time of the response was measured in seconds. A response window of 33 seconds after ligand addition was used to measure the response before calcium ion reuptake mechanisms caused a drop in fluorescence. $\Delta$RFU was computed for each concentration, which was then plotted as a concentration response curve using non-linear regression. To incorporate well-well variability, four well replications were performed for each concentration of the ligand. Importantly, each ligand was tested in at least three independent replications where, each replicate experiment consists of cells transiently transfected with the chimeric G-protein on a separate day thus ensuring true biological replication. The representative curves, $EC_{50}$ and $RFU_{peak}$ values are all thus calculated from the four internal/dependent and three independent replications. Thus any variability due to transfection is contained in the error bars and has been accounted for.

# CHAPTER 8

# 8. PRELIMINARY EVIDENCE FOR ALLOSTERIC COUPLING BETWEEN OPIOID AND NON-OPIOID RECEPTORS

## 8.1. Introduction

One of the hallmarks of GPCR heteromers is the possibility of allosteric modulation between the protomers. Key evidence for such allosterism comes from studies with obligate heteromers such as $GABA_B$. $GABA_B$ heteromers are constituted by $GABA_{B1}$ and $GABA_{B2}$ receptors. Expressing either of these receptors alone did not show much affinity or activity due to GABA stimulation[211, 212]. However, when both receptors are coexpressed, enhancement in ligand binding and activity are observed[213-215]. Indeed, it was later determined that $GABA_{B1}$ predominantly binds to GABA, while disruption in the C-term tail of $GABA_{B2}$ is enough to prevent G protein activation[216].

Even though there are few examples of such obligate heteromers, several cases of allosteric modulation have been observed for various heteromers[216-218]. In the case of opioid receptor heteromers, allosteric modulation has been best studied with mu-delta receptors. Radioligand binding studies have shown that delta selective ligands ($TIPP_\Psi$ and DELT II) enhance the binding affinity for mu ligands (DAMGO) at mu-delta heteromers[115]. This enhancement is reciprocal, in that DAMGO pretreatment also increased the binding affinity of DELT II. In a follow up study[217], pretreatment with low concentrations of delta agonists and antagonists were shown to increase morphine induced

[$^{35}$S]GTPγS binding to membranes expressing both mu and delta receptors, but not in cells expressing only mu receptors.

While studies with altered binding provide hints for allosteric coupling between two receptors, an important feature of the allostery is trans-activation where a ligand for one receptor causes signal activation of the other. Such trans-activation can lead to altered signaling and novel physiological consequences. However, efforts to determine such effects are complicated if both receptor partners in a heteromer activate similar G proteins. For instance, in the case of a $CXCR_2$-delta opioid heteromer, both delta opioid receptors and $CXCR_2$ activate Gi/o proteins and reduce cAMP levels. To circumvent this, researchers have used a technique called functional reconstitution[102, 218]. $CXCR_2$ that carries mutations in the second extracellular loop rendering it incapable of ligand activation was fused with a pertussis toxin-insensitive G protein and coexpressed with a fusion construct of delta receptor with a non-functional Gi protein. In this system, deltorphin II produced Gi protein activation, which was non-existent in cells expressing only the delta and non-functional Gi fusion construct, clearly demonstrating transactivation between the receptors.

A major flaw with such reconstitution studies is that the receptors carry mutations that may not adequately reflect their native conformations. It would then be ideal if the receptor protomers within a heteromer are coupled to different signal effectors where unique endpoints can be monitored using native receptors. The recent discovery of mu-mGluR5 and mu-CCR5 heteromers[120, 127] presents such a novel opportunity. mGluR5 and CCR5 promote intracellular

calcium mobilization by coupling with Gq protein which would promote calcium release, while mu receptors are coupled with Gi/o and inhibit cAMP synthesis. The demonstration of mu-mGluR5 and mu-CCR5 heteromers also has significant implications in opioid therapy. Studies have shown that concomitant administration of opioid analgesics with either an mGluR5 or CCR5 antagonist results in attenuated analgesic tolerance and withdrawal[219-222]. In addition, both mu and CCR5 agonists have also been shown to cross-desensitize each other[120, 223, 224]. Another study showed that co-administration of the mGluR5 antagonist, MPEP, along with DAMGO reduced mu phosphorylation, internalization and desensitization in HEK293 cells coexpressing mu and mGluR5[127]. Given the therapeutic relevance and difference in coupling to signaling effectors, we have utilized calcium mobilization as an end point to study allosteric modulation of mu-mGluR5 and mu-CCR5 heteromers.

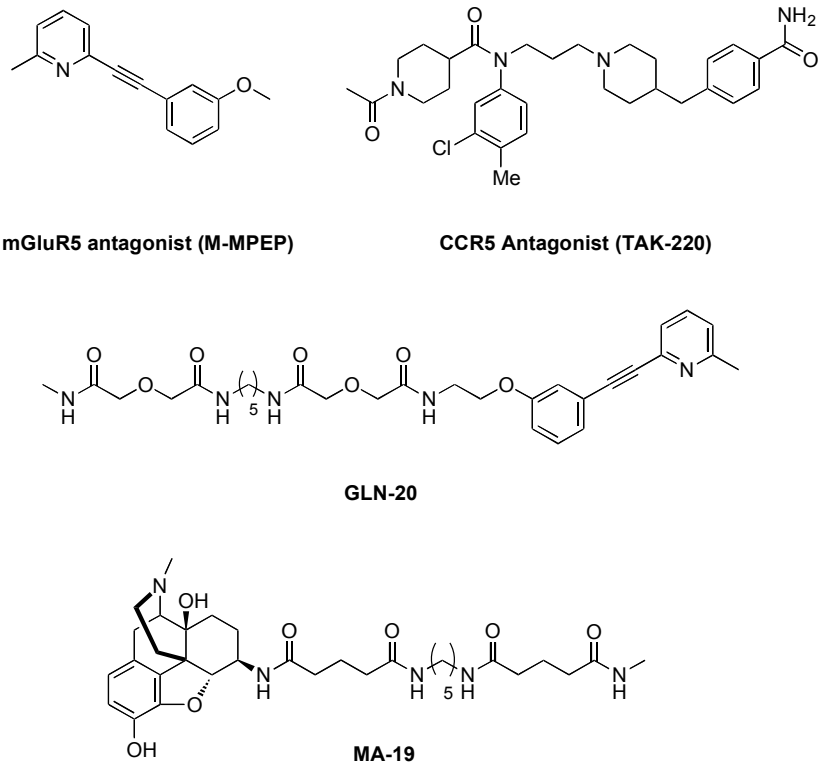



**Figure 8.1 Structures for ligands used to study allosterism of mu-mGluR5 and mu-CCR5 heteromers (synthesized by Dr. Irfan Javed)**

## 8.2. Results

### 8.2.1. Mu agonists promote calcium release when mu and mGluR5 are coexpressed in HEK293 cells

As mentioned previously, mu opioid receptors inhibit cAMP levels via activation of Gi/o proteins. Hence, when mu opioid receptors are expressed in HEK293 cells ligand-mediated receptor activation does not lead to any significant intracellular calcium ion release from the ER. However, in cells containing coexpressed mu and mGluR5, DAMGO (1 μM) induced strong calcium fluxes. Additionally, we also tested a oxymorphamine analog tethered

to a 19-atom spacer, MA-19 (1 μM), that also promoted strong calcium release (Fig 8.2).

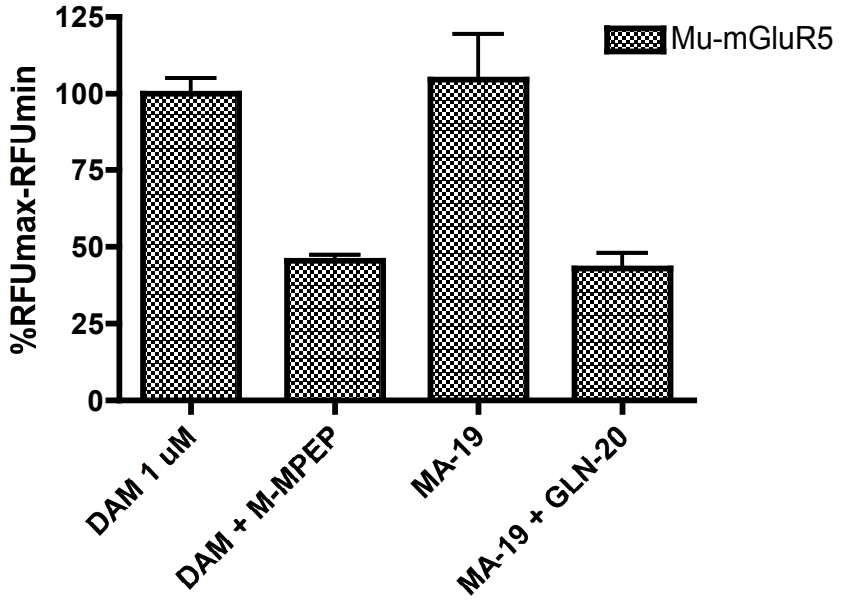



**Figure 8.2 DAMGO and MA-19 stimulate calcium release in HEK293 cells containing both mu and mGluR5 by allosterically modulating mGluR5 via mu receptors. The data are reported as mean +/- SEM for at least three biological replications.**

### 8.2.2. Effect of mGluR5 and opioid antagonists

In an effort to discern the extent of allosteric coupling between mu and mGluR5, we pretreated HEK-293 cells transiently expressing both receptors with an mGluR5 antagonist, MPEP. The calcium release induced by DAMGO (1 μM) was significantly inhibited by an equimolar concentration of M-MPEP. Since the monovalent mu agonist, MA-19[138], also produced calcium release, a corresponding monovalent M-MPEP analog, GLN-20 (1 μM), was administered

to cells prior to addition of MA-19 (1 μM). GLN-20 also inhibited calcium fluxes induced by MA-19.

Since antagonists at mGluR5 were capable of inhibiting the calcium release mediated by mu agonists, we tested the possibility of reciprocal antagonism of mGluR5 agonists by narcotic antagonist, naltrexone (1 μM). L-glutamate (100 μM) produced strong calcium currents that was significantly inhibited by naltrexone (Fig 8.3). Control experiments were performed to show that NTX or M-MPEP cannot antagonize mGluR5 or mu receptors expressed singly in HEK-293 cells (Fig 8.4)

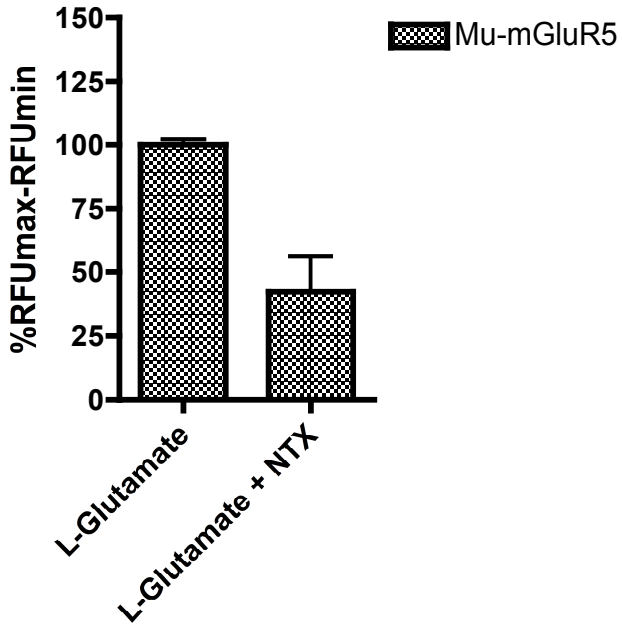



**Figure 8.3 Naltrexone significantly inhibits the calcium release stimulated by L-glutamate in HEK293 cells containing both mu and mGluR5 by allosterically antagonizing mGluR5 via mu receptors. The data are reported as mean +/- SEM for at least three biological replications.**

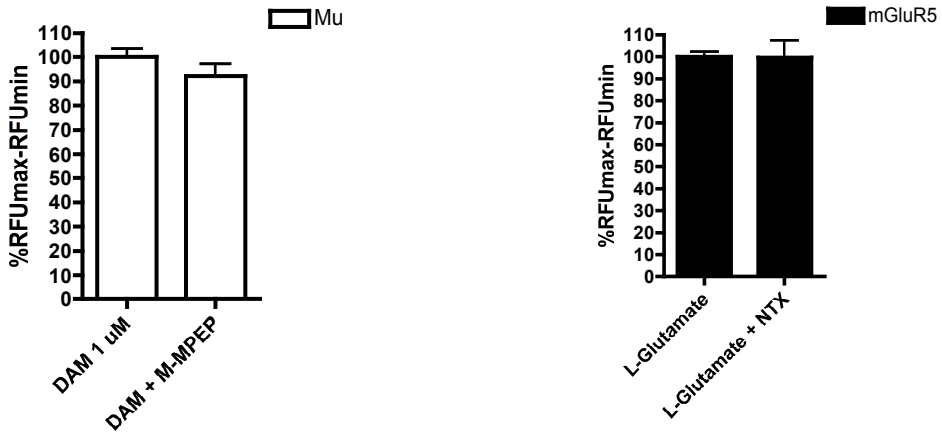



**Figure 8.4 Control experiments show that NTX and M-MPEP do not antagonize mGluR5 or mu receptors respectively, when expressed singly in HEK-293 cells.**

### 8.2.3. Mu agonists promote calcium release when mu and CCR5 are coexpressed in HEK-293 cells

Since CCR5 is also coupled to calcium release via Gq proteins[225, 226], we tested the ability of mu agonists in allosterically activating calcium release via CCR5 in HEK293 cells expressing both mu and CCR5. Both DAMGO (1 μM) and MA-19 (1 μM) produced strong calcium release in these cells.

### 8.2.4. Effect of CCR5 and opioid antagonists when mu and CCR5 are coexpressed in HEK-293 cells

To test if inhibition at CCR5 was able to block the calcium release mediated by the mu agonists, we employed the small molecule CCR5 antagonist, TAK-220. HEK293 cells containing coexpressed mu and CCR5 were pretreated with TAK220 for 10 mins prior to the administration of agonists. The calcium release

elicited by both DAMGO and MA-19 (1 μM) was significantly inhibited by TAK-220 (1 μM) (Fig 8.5).

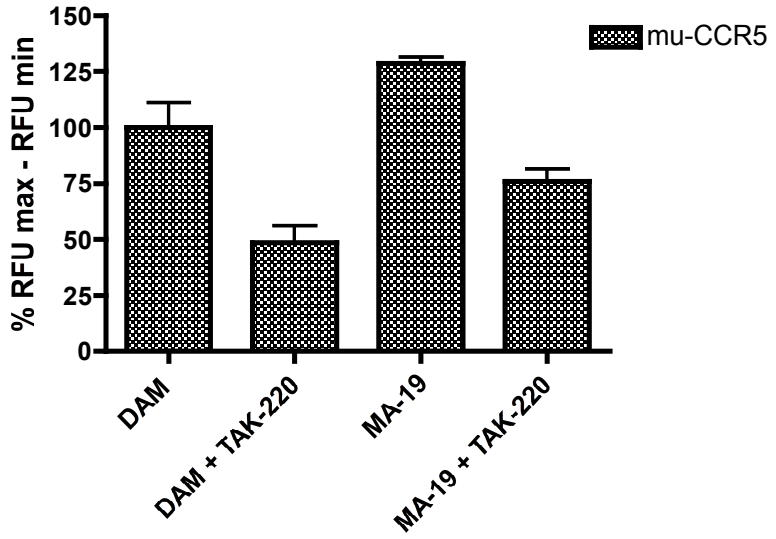



**Figure 8.5 DAMGO- and MA-19-induced activation of mu-CCR5 is inhibited by the CCR5 antagonist, TAK-220. The data are reported as mean +/- SEM for at least three biological replications.**

In a related experiment, we employed naltrexone to determine if inhibiting the mu receptors could also block the CCR5 agonist, RANTES. Cells that were pre-incubated with naltrexone (1μM) showed significantly lesser calcium release upon RANTES stimulation (1 μM) when compared with cells treated only with RANTES (Fig 8.6). Control experiments were performed to show that neither NTX, nor TAK-220, can antagonize CCR5 or mu receptors respectively, when expressed singly in HEK-293 cells.

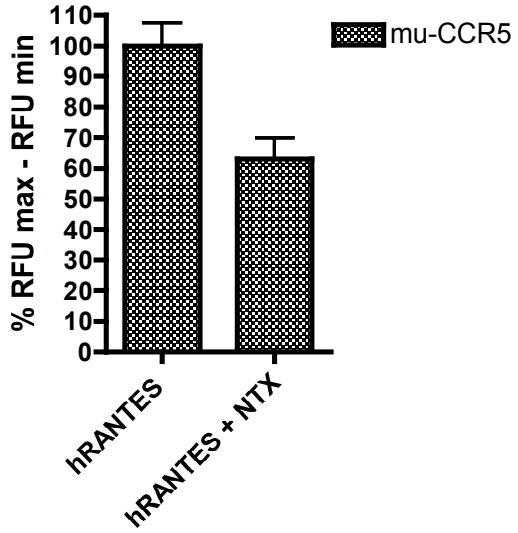
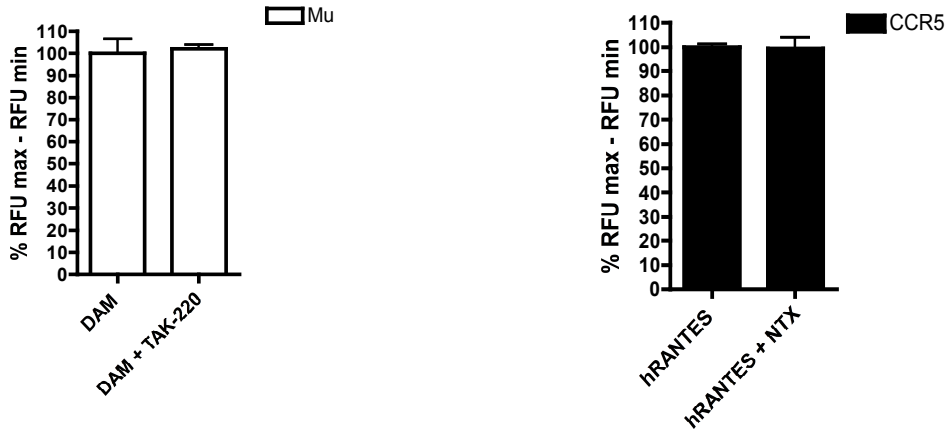



**Figure 8.6 Naltrexone significantly inhibits the calcium release stimulated by hRANTES in HEK293 cells containing both mu and CCR5 by allosterically antagonizing CCR5 via mu receptors. The data are reported as mean +/- SEM for at least three biological replications.**




**Firgure 8.7 Control experiments showing that neither NTX, nor TAK-220, can antagonize singly expressed CCR5 or mu receoptors respectively, in HEK-293 cells**

## 8.3. Discussion

The recent demonstration of mu-mGluR5[127] and mu-CCR5[120] heteromers has profound implications in developing pain therapeutics. It has been reported in the literature that co-administration of opioid agonists with antagonists for either mGluR5 or CCR5 can attenuate narcotic tolerance and/or dependence[219-222]. It is thus possible that the putative heteromers may play a role in mediating some of these effects. To gain a mechanistic understanding of the allosterism between these receptors, we have conducted a series of calcium mobilization experiments in HEK293 cells expressing mu opioid receptors along with mGluR5 or CCR5.

An important premise for the design of these experiments comes from the G proteins that are predominantly activated by the three receptors. Opioid receptors inhibit cAMP synthesis via Gi/o proteins[2], whereas mGluR5 receptors are coupled to intracellular calcium release via Gq proteins[227]. CCR5 receptors are a more complex system as they can inhibit biosynthesis of cAMP (via Gi) and promote calcium release (via Gq)[225, 226]. When HEK293 cells containing mu receptors are treated with mu agonists, there is no observable accompanying calcium release. However, since both mGluR5 and CCR5 can be stimulated to release intracellular calcium, the coexpression of mu-mGluR5 and mu-CCR5 provides an ideal platform to test the potential of allosteric modulation between the receptor pairs.

Prior studies have suggested that mu opioid receptor effects may be allosterically modulated by mGluR5. For instance, pre-incubation of HEK293

cells with the mGluR5 antagonist, MPEP resulted in attenuated phosphorylation, internalization and desensitization of mu opioid receptors[127]. However, the study did not directly measure trans-activation between mu and mGluR5. Therefore, we first tested the ability of mu agonist, DAMGO, in stimulating calcium release in HEK293 cells transiently coexpressing mu and mGluR5. While DAMGO (1 μM) did not produce any calcium release in cells expressing either mu or mGluR5, it stimulated strong calcium fluxes in cells containing both mu and mGluR5 suggesting that it was allosterically activating (or trans-activating) mGluR5 via mu receptors. To ensure that this effect was not limited to DAMGO, we employed a monovalent oxymorphone analog, MA-19. The oxymorphone pharmacophore has been used as a mu agonist in several bivalent ligands synthesized in our lab[132, 138]. To discern the effects of the glycolic linker connecting the two pharmacophores, we designed MA-19 which has a 19-atom spacer conjugated to the mu agonist pharmacophore. This ligand serves as a unique pharmacological scaffold and the data can be used as controls to design mu agonist-mGluR5 antagonist bivalent ligands in the future. Similar to DAMGO, even MA-19 (1 μM) promoted significant intracellular calcium release in cells coexpressing both mu and mGluR5.

Since the agonistic activity is possibly being mediated by allosteric activation of Gq via mGluR5, it could be argued that administering an antagonist for mGluR5 would mitigate the activity. To test this we pre-incubated cells with M-MPEP, which is a selective mGluR5 antagonist, and its monovalent analog, GLN-20. M-MPEP and GLN-20 significantly antagonized

the activity of DAMGO and MA-19, respectively, suggesting that the allosteric changes modulated by the mu agonists are attenuated in the presence of the mGluR5 antagonists. These data suggest that the attenuated calcium release may mediate the inhibition of mu receptor phosphorylation and internalization by mGluR5 antagonists, as the ability of mu to inhibit cAMP is not affected by MPEP.

To test the reciprocity of this allosteric coupling, cells expressing mu and mGluR5 were first pre-incubated with the opioid antagonist, naltrexone, prior to the administration of L-glutamate, the endogenous agonist for mGluR5. Surprisingly, the occupancy of the mu protomer by naltrexone significantly inhibited the extent of calcium release due to L-glutamate. This strongly suggests that the active sites of both receptors can be modulated by each other suggesting reciprocal allosteric modulation within the mu-mGluR5 heteromer.

At this juncture an important discrepancy needs to be addressed. Koch and coworkers showed that MPEP administration did not affect the ability of mu receptors to promote GTP$\gamma$S binding or inhibit the levels of cAMP[127]. Hence, the authors suggested that functional coupling to G proteins is not allosterically modulated within the mu-mGluR5 heteromer. However, our data with intracellular calcium release as an end point seems to contradict that hypothesis. If we assume that the individual protomers within the mu-mGluR5 heteromer may be coupled to the G$\alpha$ proteins that they are usually associated with, Gi and Gq, respectively, the simplest explanation for the discrepancy may be that the mGluR5 receptor is more susceptible to allosteric modulation when

compared with the mu receptor. Therefore, ligands at either protomer would affect conformational changes modulating Gq activation to a greater extent than Gi.

Additionally, there has been considerable debate on the stoichiometry of homomers vs heteromers in a heterologous expression system. Since Koch and coworkers used cAMP as an endpoint[127], the inhibition of cAMP due to a mu agonist can be mediated by both mu homomers and mu-mGluR5 heteromers. The extent of inhibition due to MPEP will depend on the relative numbers of homomers vs heteromers and thus complicate interpretation. A similar argument can be made for the GTPγS experiments with an additional caveat arising from the fact that the assay measures GTPγS incorporation into all Gα subtypes. Since the mu-mGluR5 heteromers can couple with both Gi and Gs proteins, even if MPEP reduced GTP binding onto Gs, it would represent a fraction of the GTP bound to Gi coupled with mu homomers and mu-mGluR5 heteromers, and may not show up as a significant difference in that assay. In our system, all the above scenarios are avoided as we are measuring an endpoint that can only be achieved due to allosteric coupling within a mu-mGluR5 heteromer that allows for a clearer interpretation.

In the original report describing the mu-CCR5 heteromer, agonists at both receptors were shown to cross-desensitize each other[120]. Recently, another study suggested that PKC may mediate the cross-desensitization downstream of the receptors[228]. However, neither study explored the potential for allosteric modulation between these receptors. We, therefore, conducted experiments

with the mu agonists, DAMGO and MA-19, to determine if these ligands could allosterically stimulate intracellular calcium release via CCR5. Both DAMGO and MA-19 (1 μM) promoted strong calcium release in HEK293 cells expressing both mu and CCR5.

We then determined the effect of antagonists in the allosteric modulation between mu and CCR5. The CCR5 antagonist, TAK-220, significantly antagonized both DAMGO and MA-19. Interestingly, naltrexone (1 μM) was also able to inhibit calcium fluxes due to the CCR5 agonist, RANTES showing that the antagonists at mu receptors can also inhibit CCR5 agonists. The reciprocal inhibition by antagonists at either protomer suggests that, just like for the mu-mGluR5 heteromers, the calcium release mechanism is quite sensitive to allosteric conformational changes in mu-CCR5 heteromers.

### 8.4. Future directions

The preliminary studies described above suggest that a novel and sensitive allosteric regulation is possible within the mu-mGluR5 and mu-CCR5 heteromers. Future studies can be conducted to study the trafficking and phosphorylation patterns with agonist and antagonist treatments. The data can then be correlated with the calcium release studies to understand how these all these mechanisms are modulated in the context of heteromers. Additionally, bivalent ligands containing mu agonist (oxymorphone) and either mGluR5 antagonist (M-MPEP) or CCR5 antagonist (TAK-220) are being synthesized in the lab. The distance between the pharmacophores will be varied similar to the

MDAN series of ligands to study the effect of bridging the mu-mGluR5 and mu-CCR5 heteromers. Calcium release, immunofluorescence imaging and in vivo experiments are being conducted to determine the activity of the ligands.

## 8.5. Methods

**Cells.** Human embryonic kidney cells (HEK-293) stably expressing single opioid receptors (human μ, κ and mouse δ) were generated. HEK-293 cells co-expressing human opioid receptors in pairs were procured from Dr. Jennifer Whistler (University of California, San Fransisco). Their construction and characterization has been described previously [134]. Briefly, HEK-293 cells were cotransfected with HA-μ and FLAG-δ (μ-δ), HA-δ and FLAG-κ (κ-δ), HA-μ and FLAG-κ (μ-κ) to make the different stable coexpression cell lines. The stably expressing cells were checked for the expression of μ-δ and κ-δ opioid receptor heteromers using co-immunoprecipitation [134, 159]. All the single and dual stable transfected cell lines were grown at 37°C and 10% $CO_2$ in Dulbelcco's modified medium (GIBCO) supplemented with 10% FBS and 1% Penicillin-streptomycin antibiotic solution. G418 alone was used for selecting cells expressing opioid receptors singly, while both G418 and Zeocin were used to select for dual coexpression cell lines.

**Intracellular $Ca^{2+}$ release assay.** HEK-293 cells were transiently transfected with cDNA vectors for mu opioid receptors together with mGluR5 or CCR5 at a concentration of 200 ng/20,000 cells each using Lipofectamine 2000 (Invitrogen, Carlsbad CA). Cells were grown to a confluency of approximately 2

million cells in a petridish. The cells were then counted and DNA for the chimeric G-protein was added to a ratio of 200 ng/ 20,000 cells. Lipofectamine 2000 at a ratio of 1:2 wt/vol (DNA:Lipofectamine) was used for the transfection. The cells were then seeded 24 hours later into half area black 96 well plates (Corning) at 20,000 cells per well. The FLIPR calcium kit (Molecular devices) was used for the assay. Cells were incubated with a $Ca^{2+}$ ion chelating dye from the kit, 48 hours after transfection, and incubated for an hour. The plates were then assayed in a Flexstation-III apparatus (Molecular Devices) using a range of concentrations of the opioid ligand. The response was measured as Relative Fluorescence Units (RFUs) and the time of the response was measured in seconds. A response window of 33 seconds after ligand addition was used to measure the response before calcium ion reuptake mechanisms caused a drop in fluorescence. $\Delta$RFU was computed for each concentration which was then plotted as a concentration response curve using non-linear regression. To incorporate well-well variability, four well replications were performed for each concentration of the ligand. Importantly, each ligand was tested in at least three independent replications where, each replicate experiment consists of cells transiently transfected with the chimeric G-protein on a separate day thus ensuring true biological replication. The representative curves, $EC_{50}$ and $RFU_{peak}$ values are all thus calculated from the four internal/dependent and three independent replications thus accounting for any variability.

# CHAPTER 9

# 9. NALOXONE PRESENTS A TWO-PRONGED APPROACH TO DEVELOP THERAPEUTICS FOR INFLAMMATORY PAIN AND NEUROAIDS

## 9.1. Introduction

Pain is perceived via nerve endings commonly known as nociceptors. Inflammation is mediated and maintained by various cellular cytokines (TNF$\alpha$, IL-6, etc.) and chemokines (CCL5, SDF-1, etc.), and pain due to inflammation is one of its cardinal signs [229]. In inflammatory disease conditions such as, arthritis or sickle cell anemia, noxious stimuli produce enhanced nociception that is termed hyperalgesia [230]. Glutamate is a major excitatory neurotransmitter that targets both ionotropic [231] and metabotropic glutamate receptors [232] and has been implicated in mediating inflammation-induced hyperalgesia [231, 233, 234]. It has also been shown that inflammation causes changes in central and peripheral expression of glutamate receptors, [235] and glutamate antagonists can attenuate hyperalgesia associated with inflammation [236].

Opioid analgesics are commonly used in the clinic for treatment of chronic pain due to inflammatory diseases such as, arthritis and sickle cell anemia. However, deleterious side effects like tolerance, physical dependence, respiratory depression, and constipation complicate their use [2]. Loperamide, a peripherally active opioid has been shown to be effective against experimental arthritis that may help circumvent some of the central side effects [237]. However, newer approaches to treat inflammatory pain are desirable.

We have previously shown that the opioid antagonist, naloxone, paradoxically produces antinociception in transgenic mouse models of sickle cell anemia [238]. This phenomenon could be replicated in wild-type mice pretreated with the pro-inflammatory CC chemokine ligand 5 [CCL5, (RANTES)], suggesting that the antinociception was related to the inflammatory state and not limited to sickle cell anemia. Significantly, we had then suggested that naloxone interacts with a "naloxone receptor" that may be heteromerized with CCR5 [238]. Here we describe the results that show that naloxone can directly interact with mGluR5 and induce strong internalization of mGluR5 and CCR5 without evoking $Ca^{2+}$ release. Naloxone also produces strong co-internalization of both mGluR5 and CCR5 receptors in BV-2 murine microglia and HEK-293 cells containing both receptors, but not CCR5 receptors when expressed alone, suggesting that mGluR5 and CCR5 receptors may exist as physically coupled heteromers.

## 9.2. Results and Discussion

In our previous study, we had shown that naloxone produced antinociception in control mice treated with CCL5 that was unaffected in mu opioid receptor knockout mice [238]. However, there existed the possibility that delta or kappa opioid receptors could play a role, as naloxone is a non-selective opioid ligand. To examine this, we employed opioid receptor triple knockout (OPRKO) mice [239]. We pretreated the OPRKO (n = 10) and wild type control (n = 10) mice i.c.v with 20 ng/mouse of CCL5 for 20 mins before

administration of naloxone (25 pmol/mouse, i.c.v.) so that both ligands peak at the same time. Under these conditions, naloxone produced similar antinociception in both control (70%) and OPRKO mice (80%) in the tail-flick assay (Fig 9.1) indicating that naloxone was producing antinocicpetion independent of opioid receptors.

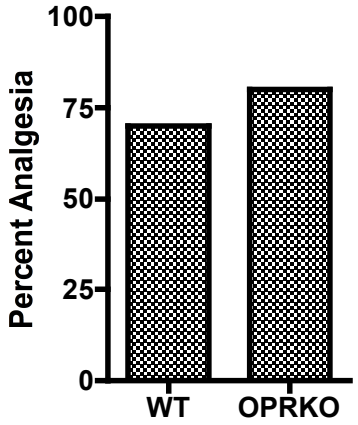



**Figure 9.1 Naloxone produces equivalent antinociception in both WT and opioid receptor triple KO (OPRKO) mice. (performed by Mary Lunzer)**

Since opioid receptors are not involved in the antinociceptive action of naloxone, we reanalyzed the microarray data produced in the previous study to identify potential receptor targets [238]. We found that mGluR5 receptors were significantly down regulated after naloxone treatment in the NY1DD sickle cell mice. mGluR5 receptors have been previously implicated in various nociceptive modalities, including inflammatory pain [240, 241]. Moreover, increased chemokine levels in turn cause a concomitant increase in glutamate levels that lead to

hyperalgesia and pain [242-244]. To test if mGluR5 receptors were involved in producing antinociception after CCL5 pretreatment, we employed the mGluR5 antagonist, MPEP, using a similar CCL5 pretreatment protocol [238]. ICR-CD1 mice were pretreated i.c.v with various doses of CCL5 for 10 mins before administration of MPEP (50 nmol/mouse, i.c.v). MPEP by itself did not produce any significant antinociception, but showed potent antinociception with increasing doses of CCL5 (Fig 9.2). These data suggest that mGluR5 can modulate the hyperalgesia induced by CCL5.

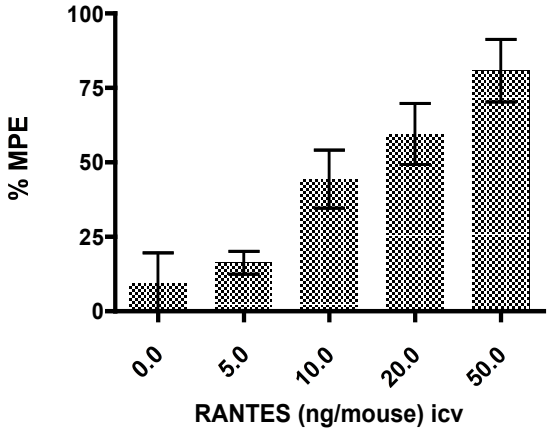



**Figure 9.2 MPEP (50 nmol/mouse) produces antinociception after pre-treatment with increasing doses of RANTES in ICR-CD1 mice. (performed by Mary Lunzer)**

Given MPEP's antinociceptive activity, we studied the possibility that naloxone may antagonize mGluR5. To determine the functional activity of naloxone, we employed the intracellular calcium release assay [134, 174] with HEK-293 cells. These cells were transfected with cDNA for mGluR5 and assayed 48

hours later in a FLIPR apparatus. Naloxone did not produce any $Ca^{2+}$ release in the cells even at the 100 μM concentration (Fig 9.3a). Remarkably, naloxone (100 nM) significantly antagonized calcium currents induced by L-glutamate (10 μM) showing that naloxone was able to inhibit calcium signaling by mGluR5 (Fig 9.3b). This data may explain the paradoxical antinociception produced by naloxone after CCL5 pretreatment. It has been shown that CCL5 (RANTES) can directly modulate glutamate release via CCR1 and CCR5 receptors in the human neocortex [242] and this may be true for other brain regions. The increased glutamate levels in the brain would lead to hyperalgesia which, if blocked by an antagonist (MPEP or naloxone), would provide alleviate pain due to the inflammation.

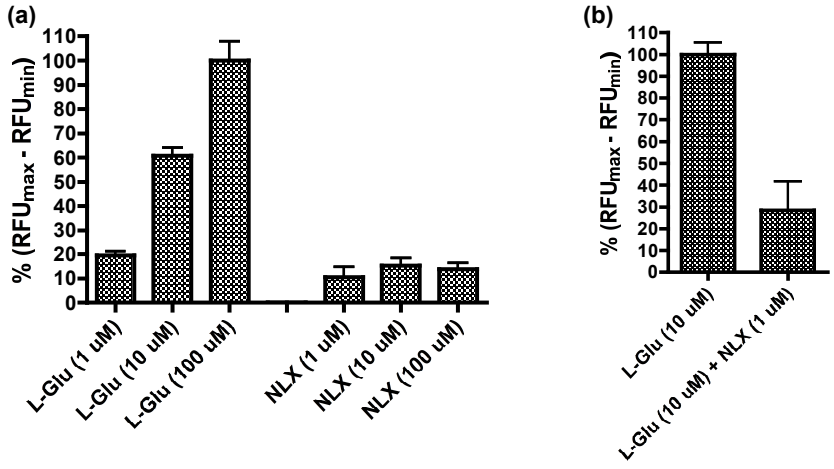



**Figure 9.3 Naloxone inhibits calcium currents induced by L-glutamate in HEK293 cells expressing mGluR5.**

(a) L-glutamate produces calcium release with increasing concentrations while naloxone (at even 100 μM) is unable to produce any significant calcium release. (b) Naloxone (1 μM) significantly inhibits the calcium release promoted by L-glutamate (10 μM).

To determine the effects of naloxone on the trafficking of mGluR5 and CCR5, we utilized the immunofluorescence technique [174] in HEK-293 cells transiently expressing the receptors. HEK-293 cells were transfected with cDNA for rat mGluR5a or human CCR5 (16 μg/ 2 million cells) and seeded 24 hours later into 8-well chamber slides. After allowing for a further 24 hours for the cells to settle and equilibrate, they were incubated with various ligands (1 μM) for 30 mins. The cells were then washed, fixed with 4% paraformaldehyde and incubated with the respective selective primary antibodies (R&D Systems) overnight at 4C. The cells were then washed the next day and stained with respective fluorescent secondary antibodies (red for mGluR5, green for CCR5). Naloxone produced strong internalization of mGluR5 receptors but not CCR5 receptors (Fig 9.4). In a control experiment using similar conditions, L-glutamate internalized mGluR5 receptors, and CCL5 produced internalization of CCR5 receptors (Fig 9.4).

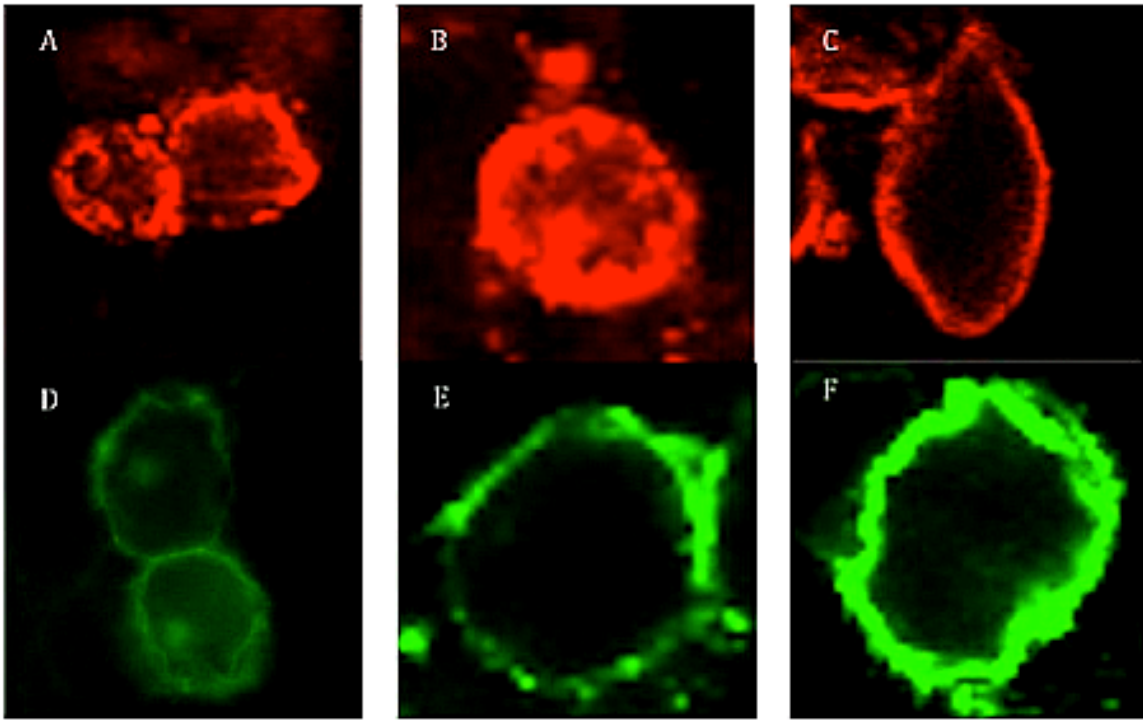



**Figure 9.4 Naloxone induces the internalization of $mGluR_5$ (red), but not $CCR_5$ (green) in HEK-293 cells when expressed alone .**

(**A - C**) L-glutamate (1 uM) and naloxone (1 uM) produce strong internalization of $mGluR_5$ (red) but remain on the surface without any treatment. (**D - F**) CCL5 produces internalization of $CCR_5$ while the receptors remain on the surface after naloxone (1 uM) or without any treatment.

Since naloxone produced internalization of mGluR5, we studied the effects of naloxone in HEK293 cells that expressed both mGluR5 and CCR5. Significantly, naloxone produced strong co-internalization of mGluR5 and CCR5 in these cells (Fig 9.5). These data, taken together with the fact that naloxone internalized mGluR5, but not CCR5 when expressed alone, suggests that mGluR5 and CCR5 may be physically associated as heteromers when coexpressed. However, such heteromers may or may not be relevant to the

antinociception produced by naloxone, as CCL5 also produces strong co-internalization of both receptors (Fig 9.6). Hypothetically, since mice are pretreated with CCL5, 20 mins before administration of naloxone, the putative mGluR5-CCR5 heteromers may be internalized, leaving homomeric mGluR5 as the predominant receptor target for naloxone.

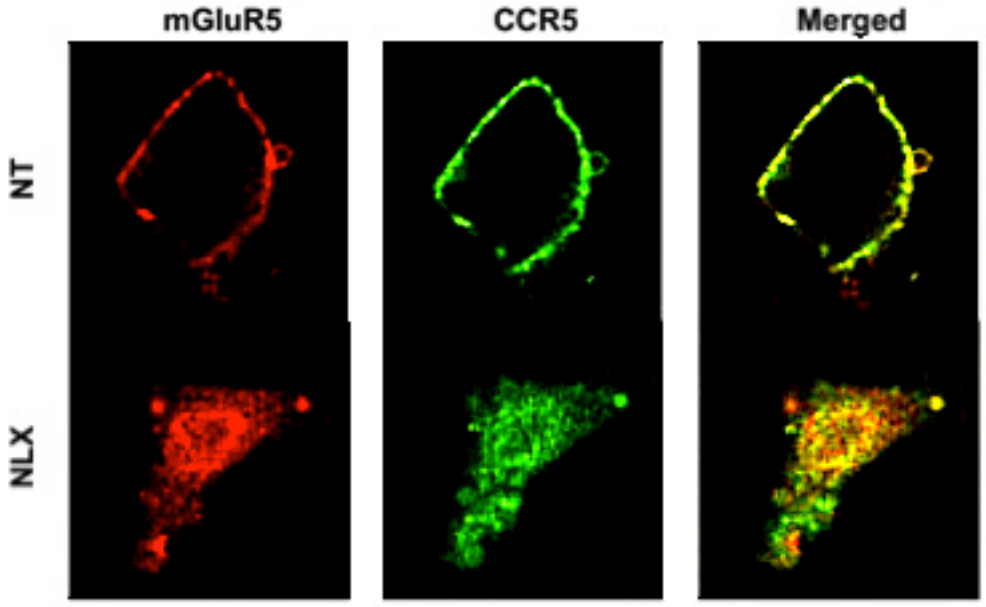



**Figure 9.5 $mGluR_5$ and $CCR_5$ receptors are co-internalized by naloxone in HEK-293 cells.**

(***Top row***) $mGluR_5$ (red) and $CCR_5$ (green) remain on the surface without any treatment. (***Bottom row***) $mGluR_5$ (red) and $CCR_5$ (green) are strongly co-internalized (yellow punctae in merged panel) by naloxone (1 uM) in HEK-293 cells coexpressing both receptors.

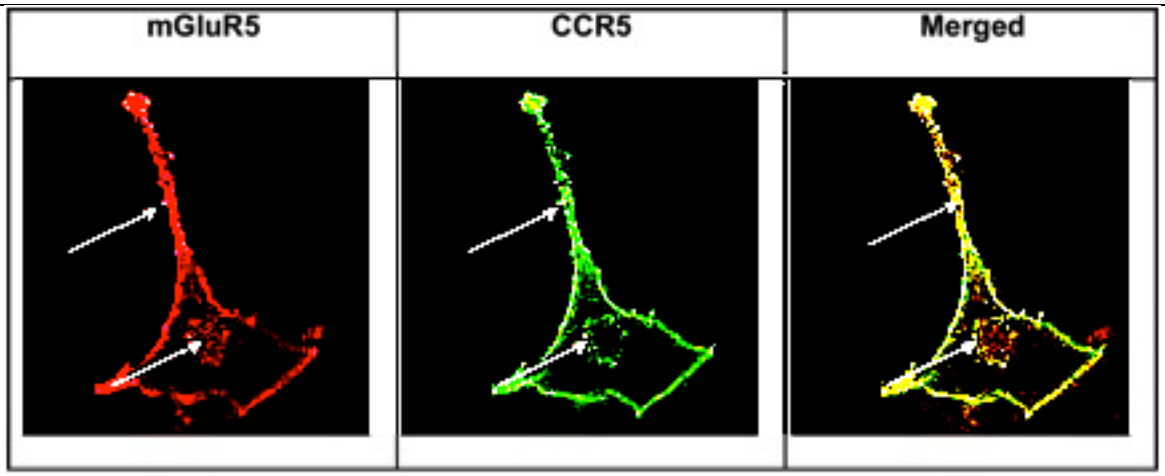



**Figure 9.6 RANTES cointernlizes mGluR5-CCR5.**

$mGluR_5$ (red) and $CCR_5$ (green) are strongly co-internalized (yellow punctae in merged panel) by RANTES (1 μM) in HEK-293 cells coexpressing both receptors.

To determine the effects of naloxone in a neuronal cell line, we utilized the BV2 murine microglial cells that endogenously co-express both mGluR5 and CCR5 (Fig 9.7). We observed strong surface and cytoplasmic staining for both receptors in these cells. Interestingly, naloxone induced co-clustering of both mGluR5 and CCR5 in the microglia (Fig 9.7). Surface plots show extensive relocation of both receptors at the cell surface. Such clustering has been suggested to represent a higher order interaction between GPCRs that allows for superior signal integration in the cluster [245]. The exact outcome of such a cluster would depend on the receptor guiding the cluster formation. Moreover, CCR5 receptors have been shown to cluster towards chemotactic stimuli [246, 247], and the subsequent internalization inhibits HIV-1 uptake into cells [248, 249]. Given that microglia are the primary target for HIV in neuroAIDS [248], the co-clustering may provide a therapeutic option against neuroAIDS.

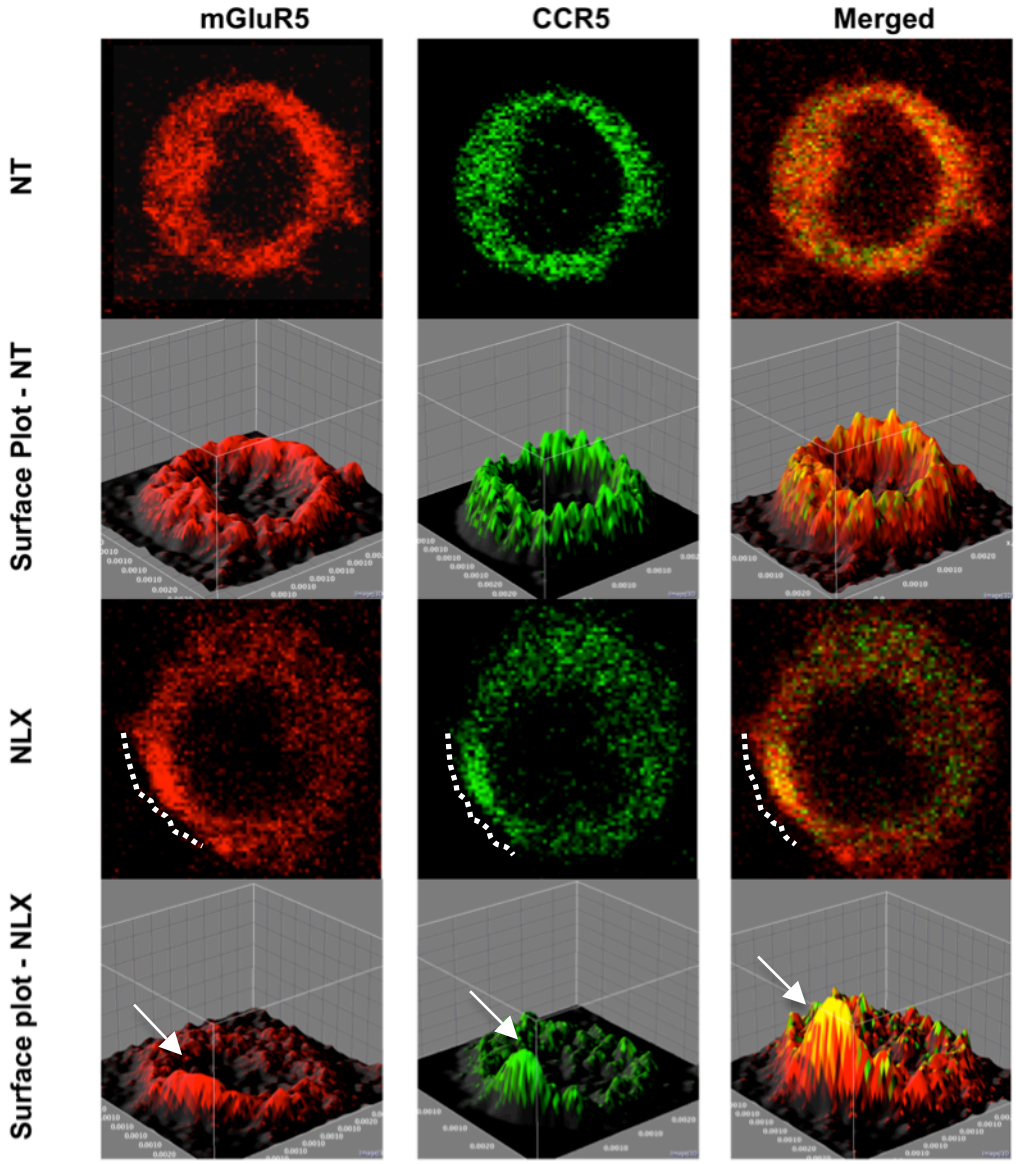



**Fig 9.7 $mGluR_5$ and $CCR_5$ are co-clustered by naloxone in BV-2 murine microglia.**

(***Row 1 and 2***) $mGluR_5$ (red) and $CCR_5$ (green) receptors are distributed evenly throughout the surface and cytoplasm without any treatment as indicated by the images and surface plots. (***Row 3 and 4***) $mGluR_5$ (red) and $CCR_5$ (green) are strongly co-clustered (yellow punctae near dotted line in merged panel) by naloxone (1 uM) in BV-2 microglial cells.

A striking feature of the antinociception produced by naloxone was the fact that none of the other opioid antagonists produced a similar effect [238]. Given naloxone's unique activity, we evaluated naltrexone in view of its close structural relationship. We also evaluated oxymorphone in the immunofluorescence assay, as it is also a close structural analog of naloxone (Fig 9.8). Interestingly, neither naltrexone nor oxymorphone produced any internalization in cells expressing mGluR5. This data underscores the uniqueness of naloxone in mediating these effects. Given that these molecules only differ in the *N*-substitution on the piperidine ring, our data suggests that the allyl group in naloxone is necessary for its antinociceptive activity. Additionally the data from our study indicate that naloxone can inhibit mGluR5 receptors also produce internalization at the same time. This puts naloxone in the same group with other such ligands in the literature for various GPCRs, including $5HT_{2A}$, neuropeptide YY1 and CCK receptors [250-252].

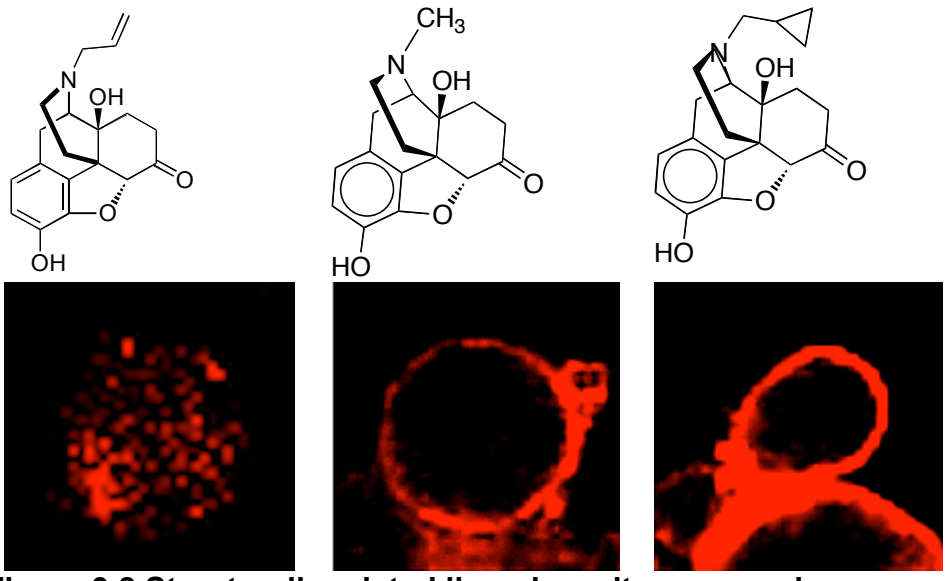



**Figure 9.8 Structurally related ligands, naltrexone and oxymorphone do not internalize mGluR5 suggesting that the *N*-allyl moiety in naloxone may be contributing to the ability of naloxone to internalize mGluR5.**

In conclusion, we have shown that naloxone produces robust antinociception in control mice pretreated with CCL5 and in opioid receptor triple KO mice, indicating that a non-opioid mechanism is involved. We show for the first time that naloxone can directly interact with mGluR5 receptors without activation and antagonizes L-glutamate with concomitant internalization. This novel mechanism of action of naloxone in inflammatory disease conditions suggests a new pharmacotherapic approach to treat inflammatory pain, as naloxone is an FDA-approved drug with limited toxic side effects. Significantly, naloxone does not produce any internalization of CCR5 receptors when expressed alone, but produces robust co-internalization of both mGluR5 and CCR5 receptors when coexpressed in HEK-293 cells. This

suggests that mGluR5 and CCR5 receptors may form heteromers. Moreover, naloxone promotes co-clustering of mGluR5 and CCR5 in murine microglia. Since CCR5 is a coreceptor for HIV-1 uptake into cells, studies are underway to utilize this effect to inhibit neuroAIDS. In effect, the present study provides new insights to develop a two-pronged approach to treat inflammatory pain and neuroAIDS.

## 9.3. Methods

**Animals.** For studies performed at the University of Minnesota, male ICR-CD1 mice (18-25 g or 30-35 g; Harlan, Madison, WI), were housed in groups of 8 in a temperature and humidity controlled environment with unlimited access to food and water and maintained on a 12 hour light/dark cycle. The mu opioid receptor knockout mice (BALB/C X C57BL/6 MORKO) were a generous gift from Dr. Sabita Roy and have been described previously[253, 254]. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota.

**Antinociceptive Studies.** Recombinant murine RANTES (CCL5) and MPEP were procured from Peprotech Inc. (Rocky Hill, NJ) and Tocris Biosciences, respectively. Naloxone HCl was supplied by Mallinkrodt (St. Louis, MO). All solutions were dissolved in distilled water. Controls with only distilled water showed no antinociception. All ligands were administered in a 5-µl volume in conscious mice according to the method of Haley and McCormick [184] for i.c.v. injections.

**Naloxone antinociception in WT and OPRKO mice.** For measuring the antinociception of naloxone, the drugs were administered so that the ligand effects would peak simultaneously. Antinociception was measured using the modified radiant heat tail-flick test as described previously [238]. Briefly, a radiant heat source was applied to the dorsal side of the tail, and the latency to flick away from the heat source was recorded. The data were made quantal by designating a positive antinociceptive response of an animal as those that increased their latency to tail flick (after drug treatment) by at least three standard deviations above the mean of the baseline latency of the whole group (50). The light source was manually turned off if the mouse did not flick its tail after the three standard deviation criteria for a positive response. At least three groups of 8-10 mice were used for each drug paradigm, and each mouse was used only once.

**MPEP antinociceptive activity.** The tail flick assay was performed as described by D'Amour and Smith[52] and modified by Dewey et. al.[53] . For the measurement the latency of the tail-flick measurement, the mice were held gently in one hand with the tail positioned in the apparatus (Tail Flick Analgesia Meter, Columbus Instruments, Columbus, Ohio) for radiant heat stimulus. The tail-flick response was elicited by applying radiant heat to the dorsal side of the tail. The intensity of the heat was set at setting 8 so that the animal flicked its tail within 2 to 3 s. The test latency was measured once before drug treatment (control) and again after the drug treatment (test) at the peak time of the compound, a 10s maximum cut-off time was used to prevent damage to the

tail. Antinociception was quantified according to the method of Harris and Pierson[54] as the percent maximal possible effect (%MPE) which is calculated as: %MPE = (Test - Control/10 – Control) x 100. At least three groups of eight to ten mice were used for each dose response curve, and each mouse was used only once. All calculations were computed with GraphPad Prism 4 by using nonlinear regression methods.

**Materials for *in vitro* assays.** cDNA for rat mGluR5 receptors was a generous gift from Dr. Jean-Philippe Pin (Montpellier, France), while the hCCR5 cDNA was procured from Missouri S&T cDNA resource center (cDNA.org). Naloxone, naltrexone and oxymorphone HCl were supplied by Mallinkrodt (St. Louis, MO) and L-Glutamate was procured from Tocris Biosciences.

**Cell Culture and Intracellular $Ca^{2+}$ release assay.** The assay was performed as described previously [182]. Briefly, HEK-293 cells were cultured at 37°C in Dulbelcco's modified Eagle's medium supplemented with 10% bovine calf serum, Pen/Strep antibiotics. These cells were transiently transfected with 200 ng/20,000 cells of the mGluR5 cDNA using OptiMEM medium (Invitrogen) and Lipofectamine 2000 (Invitrogen, Carlsbad CA) reagent according to manufacturer's protocol (1:2 wt/vol ratio for DNA:Lipofectamine). The cells were seeded into 96 well plates (half-area; Corning) at 20,000 cells/well after 24 hours and assayed 48 hours after transfection using the FLIPR calcium kit (Molecular devices) in a Flexstation-III apparatus (Molecular devices). For inhibition studies, cells were pretreated with naloxone (1 μM) for 10 min before

addition of L-glutamate. The response was measured as peak Relative Fluorescence Units ($RFU_{max}$ – $RFU_{min}$) and plotted using Prism 4 (Graphpad Inc).

**Immunocytochemistry:** Two-color immunofluorescence was employed to analyze co-expression of mGluR5 and CCR5 using a previously described method [182]. Briefly, HEK-293 cells containing mGluR5 or CCR5, singly or together, and BV-2 microglia were incubated with ligands (1 μM) for 30 min at 37C and were then fixed with 4% formaldehyde for 10 min at room temperature. After rinsing thrice with 50 mM PBS (pH 7.2), cells were incubated with sheep anti-mGluR5 (R&D Systems, Cat #af4514) antibody and mouse anti-CCR5 (R&D Systems, Cat # mab181) antibody at a final working concentration of 1:100 overnight at 4°C. Then cells were washed (3 x 15 min) with PBS and incubated at room temperature with the mixture of anti-goat NL-557 (Cat # NL003; R&D Systems, Inc.) and anti-mouse NL-493 (Cat # NL004; R&D Systems, Inc.) fluorescent secondary antibodies (1:200 final dilutions). Cells were again rinsed with PBS (3 x 15 min) and mounted under coverslips with anti-fade mounting media iBright Plus (Cat # SF40000-10; Neuromics, Inc.) containing DAPI. Olympus FluoView1000 confocal microscope was used for image collection.

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