INVESTIGATING INTERACTIONS BETWEEN MU AND DELTA OPIOID RECEPTORS USING BIFUNCTIONAL PEPTIDES

by

Lauren C. S. Purington

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Doctoral Committee:

Professor John R. Traynor, Chair
Professor Stephen K. Fisher
Professor Henry I. Mosberg
Professor James H.Woods

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1-Nal	3-(1-naphthylalanine)
2-Nal	3-(2-naphthylalanine)
[^{35}S]GTPγS	guanosine-5'-O-(3-[^{35}S]thio)triphosphate
AC	adenylyl cyclase
Aci	2-aminoindane-2-carboxylic acid
AcOH	acetic acid
ANOVA	analysis of variance
CTAP	D-Phe-[Cys-Tyr-D-Trp-Arg-Thr-Pen]-Thr-NH_2
DAMGO	[D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin
DIEA	diisopropylethylamine
DMEM	Dulbecco's Modified Eagle Medium
DMF	dimethylformamide
Dmt	2',6'-dimethyltyrosine
DPDPE	[D-Pen^{2,5}]-enkephalin
DPen	β,β, dimethyl Dcysteine
DPN	diprenorphine
EXL	extracellular loop
FBS	fetal bovine serum
Fmoc	9-fluorenylmetyloxycarboynyl
FSK	forskolin
GPCR	G protein-coupled receptor
HBTU	o-benzotriazole-N,N.N',N'-tetramethyl-uronium-hexafluoro phosphate
HOBT	1-hydroxybenzotriazole
IBMX	3-isobutyl-1-methylxanthine
MAPK	mitogen activated protein kinase
NTI	naltrindole
RP-HPLC	reverse phase-high performance liquid chromatography
PBS	phosphate buffered saline
SNC80	(+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide
Tic	1,2,3,4-tetrahydroisoquinoline, 3-carboxylic acid
TFA	trifluoroacetic acid
TM	transmembrane helix
U69,593	(5α,7α,8β)-(-)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4,5]dec-8-yl]benzene-acetamide

Abstract

Morphine and other opioids exert their effects by activation of opioid receptors, which belong to the G protein-coupled receptor (GPCR) superfamily. Clinically used opioids exert their effects through the mu (μ) opioid receptor although two other types of opioid receptors (delta (δ) and kappa (κ)) exist. Opioid receptors couple to $G\alpha_{i/o}$ proteins and agonists produce inhibition of adenylyl cyclase enzymes and Ca^{2+} channels with activation of K^+ channels. Chronic activation of μ receptors is known to produce adaptive side effects including tolerance and dependence, limiting the long-term utility of opioids as pain-relieving agents.

In this thesis, I examined the ability of the δ antagonist naltrindole to prevent acute antinociceptive tolerance after a single dose of the μ agonist morphine. I demonstrate reduced μ agonist-induced antinociceptive tolerance with δ antagonist administration *in vivo*. *Ex vivo*, morphine exposure produced a decrease in high-affinity μ receptors and decreased ability of subsequent μ agonist to stimulate G protein. These effects were reversed with δ antagonist administration.

Evidence for interactions between μ and δ receptors in the production of tolerance has furthered interest in novel drugs devoid of tolerance liability. Based on experiments in this thesis and previous reports, compounds displaying μ agonism for appropriate analgesia with δ antagonism to prevent tolerance development are desirable. Therefore, I synthesized bifunctional opioid peptide ligands displaying this mixed efficacy profile with equivalent binding affinities to both μ and δ receptors.

Novel opioid peptides were synthesized using both tetra- and pentapeptide scaffolds. Naphthylalanine-substituted pentapeptide ligands identified a novel μ/κ agonist, δ partial agonist/antagonist peptide. Development of tetrapeptide ligands led to the characterization of KSK-103, which demonstrated μ agonist efficacy on par with the

clinical standard morphine and δ antagonism at both the level of G protein stimulation and inhibition of adenylyl cyclase. *In silico* docking of peptides in computational models of the putative 'active' and 'inactive' conformations of the μ and δ receptors revealed steric hindrance in binding the δ 'active' conformation, potentially preventing δ agonist efficacy. These studies highlight the potential of bifunctional ligands displaying μ agonism and δ antagonism in producing analgesia without the limitations of tolerance development.

Chapter I

Introduction

Opioids and Opioid Receptors

Opioid drugs, including opium and its derivatives, have been primary treatments for acute and chronic pain conditions for thousands of years (Hamilton *et al.*, 2000; Trescot *et al.*, 2008). While these compounds are used successfully for the management of pain, the presence of deleterious side effects and neurochemical adaptations hinder their long-term utility. Specifically, acute use of opioids may lead to dangerous side effects including constipation and respiratory depression while prolonged exposure has been shown to produce long-lasting alterations in fundamental brain signaling pathways and may result in the development of both tolerance and dependence. Tolerance is an adaptation resulting in increasing doses of drug required to elicit the same desired effect (or decreasing effectiveness of the same dose of drug after repeated exposure). Tolerance develops to a number of opioid effects, including analgesia and euphoria. Dependence is characterized by an altered homeostasis whereby users and patients require the presence of drug to maintain normal function. Removal of drug from a dependent individual results in symptoms of withdrawal. Both tolerance and dependence are limiting factors in the treatment of pain conditions and dependence is one of the Diagnostic and Statistical Manual for Mental Disorders criteria for addiction (Ross *et al.*, 2009).

Morphine and other clinically used opioid drugs exert their analgesic and rewarding effects at three types of opioid receptors present in the brain and spinal cord. Opioid pharmacology was advanced in the early 1990s upon cloning of these receptors, with the delta (δ) opioid receptor (Evans *et al.*, 1992; Kieffer *et al.*, 1992) cloned first. This was followed rapidly by cloning and further characterization of the mu (μ) and

kappa (κ) opioid receptors (Chen *et al.*, 1993; Yasuda *et al.*, 1993). Opioid receptors belong to the broad superfamily of Class A G protein-coupled receptors (GPCR), having seven transmembrane-spanning domains, an extracellular N terminus, and an intracellular C terminus. This GPCR class also includes adrenergic receptors and rhodopsin. All GPCRs bind with heterotrimeric G proteins consisting of α, β, and γ subunits. Opioid receptors couple to adenylyl cyclase inhibitory G proteins, which include the α subunits $G\alpha_i$, $G\alpha_o$, and $G\alpha_z$. In addition, Gβγ subunits signal to specific downstream cellular effectors including Ca^{2+} and K^+ channels, and the MAP kinase pathway.

Opioid receptors are present at distinct locations within the central nervous system and peripherally, with μ receptors present primarily in the brainstem, thalamus, and cortex as well as the gastrointestinal tract. κ Receptors have been found in the limbic regions of the brain and spinal cord while δ receptors are highly expressed in the striatum and to a lesser extent throughout the brain (Mansour *et al.*, 1995; Trescot *et al.*, 2008).

The location of the μ receptor in brain regions described above contributes to the euphoric and rewarding effects of opioid drugs, potentially leading to their abuse. It is important to note that not all the effects occurring upon μ receptor activation develop tolerance or dependence after long-term exposure in the same manner as analgesia or euphoria. Deleterious side effects including respiratory depression and constipation do not decrease in severity with prolonged use of opioids at the same rate as analgesia or euphoria (although tolerance to these effects may appear after long-term use), thus chronic treatment must be carefully monitored to prevent occurrence of unwanted effects. As the euphoric and rewarding properties of abused μ agonist drugs including heroin and morphine do develop tolerance, abusers take increasing doses in order to achieve the same rewarding effects. This may lead to overdose *via* over-stimulation of specific opioid receptor cellular signaling pathways resulting in respiratory depression and other effects.

In contrast to μ receptors, compounds acting at the δ receptor are not used clinically, as the effects produced include mild analgesia (compared to morphine) and seizures. Research showing a potential antidepressant-like quality of δ agonists

(Jutkiewicz, 2006) as well as evidence that δ agonists are not self-administered in animals (Negus *et al.*, 1998) has generated proposals for a novel use of these compounds. Trafficking of δ receptors is the focus of several studies aiming to better understand how these receptors may be exploited in the search for novel pain therapeutics (see review in Cahill *et al.*, 2007). κ receptor agonists produce mild antinociception in animal models of pain, however they are also dysphoric, sedating, and may cause psychotomimetic effects (Schiller, 2010). Ligands displaying partial agonism at the κ receptor have been targeted as addiction therapeutics. Some drugs with mixed μ-κ agonist profiles (e.g. nalbuphine and pentazocine) are used clinically as analgesics for their μ properties (Trescot *et al.*, 2008).

Theories of tolerance development

The appearance of tolerance after several administrations of a μ agonist drug such as morphine (e.g. an increased number of doses administered or increased amount of drug given to a patient over time) greatly hinders long-term clinical use. While there are current protocols in place to switch a patient from an opioid drug to which they are tolerant to another opioid compound which does not develop complete cross-tolerance (e.g. transdermal fentanyl to oral methadone in patients with cancer; Benitez-Rosario *et al.*, 2004), this method is only partially effective. The cellular mechanism by which analgesic tolerance occurs is still unclear; however, several hypotheses have been made regarding changes in μ receptor expression and signaling capability. One hypothesis states that *in vitro* tolerance to μ agonists occurs after long-term application of drug due to internalization of the μ receptor, such that fewer receptors are available for subsequent agonist stimulation (von Zastrow *et al.*, 2003). It is known that opioid receptors undergo dynamic expression and internalization (von Zastrow, 2010). This hypothesis has been supported through the use of receptor binding studies which measure the change in expression of the μ receptor on the plasma membrane following treatment with μ agonists (Elliott *et al.*, 1997).

Internalization or desensitization of the μ receptor in tolerance development

Further evidence for internalized receptors has been the demonstration that chronic opioid agonist treatment *in vitro* results in a loss of opioid-mediated signal in tissue or cellular systems expressing the opioid receptors (Alt *et al.*, 2002; Alvarez *et al.*, 2002; Elliott *et al.*, 1997). Other studies have visualized the internalization of opioid receptors after binding with fluorescently-labeled opioid peptides *ex vivo* through confocal microscopy (Arttamangkul *et al.*, 2000).

The receptor internalization theory does not completely explain tolerance development to all clinically used opioids. It is well-established that many μ agonists (e.g. [D-Ala^2, N-Me-Phe^4, Gly-ol]-enkephalin (DAMGO) and fentanyl) produce robust internalization of the μ receptor with an associated loss in cell surface receptor expression and signaling capability (Alvarez *et al.*, 2002; von Zastrow *et al.*, 1993). However, the widely used opioid morphine does not produce internalization of the μ receptor in all circumstances (Keith *et al.*, 1996; Sternini *et al.*, 1996) and does not downregulate the μ receptor when given *in vivo*, yet has been demonstrated to decrease opioid receptor signaling and result in clinical analgesic tolerance. For morphine and other ligands which do not produce robust internalization, it is thought that application of these ligands results in receptor desensitization. Desensitization is an adaptation with loss of agonist ability to produce signaling endpoints through activation of the receptor (Rozenfeld *et al.*, 2007a; Sharma *et al.*, 1975), potentially through recruitment of kinases or arrestins (Zhang *et al.*, 1998; Zhang *et al.*, 1996). This produces a decrease in total receptor-effector activity and downstream signaling events, a precursor to tolerance development (Rozenfeld *et al.*, 2007a).

A decrease in the total amount of opioid receptor present on the plasma membrane following agonist exposure supports the thought that stimulation *via* endogenous or exogenous ligands can cause recruitment of intracellular molecules, 'marking' the receptor for internalization. Recent studies have examined the role of intracellular phosphatases or kinases in 'marking' tolerant receptors for either desensitization or internalization and the regulation behind these processes (von Zastrow *et al.*, 2003). Other work has analyzed the dynamic recycling opioid receptors undergo

and which signaling processes direct an internalized opioid receptor for recycling to the cell surface or degradation (Arttamangkul *et al.*, 2008) although no direct conclusions have yet been made.

One popular theory postulates that phosphorylation of Ser^{375} on the C-terminus of the μ receptor by G protein-coupled receptor kinases (GRKs) and subsequent recruitment of β-arrestin is required for proper internalization of the receptor (Bailey *et al.*, 2005; Christie, 2008; Williams *et al.*, 2001). Morphine is less effective at producing GRK-induced receptor phosphorylation (Schulz *et al.*, 2004) and β-arrestin recruitment (Bohn *et al.*, 2004) than other μ agonists such as DAMGO *in vitro*. In fact, a recently published article examining agonist efficacy and the ability of several μ agonists to recruit β-arrestin-3 reported that, while morphine was able to produce G protein activation to the same extent as DAMGO or Met-enkephalin, it was less able to recruit β-arrestin-3 to the μ receptor expressed in human embryonic kidney (HEK) cells (McPherson *et al.*, 2010). However, as morphine can develop analgesic tolerance to the same extent as other clinically used ligands, recruitment of β-arrestin-3 and phosphorylation of the μ receptor cannot be the sole molecular mechanism underlying this adaptive phenomenon. In fact, opioid receptors expressed in heterologous cellular systems and activated by selective agonists have shown roles for second messenger systems and kinases such as protein kinase A, protein kinase C, calcium/calmodulin-dependent kinase II (Ueda *et al.*, 2003), as well as phospholipase D_2 (Koch *et al.*, 2003) in desensitization of the μ receptor. Other research utilizing β-arrestin-2 knockout mouse models have demonstrated a role for this arrestin subtype in μ receptor antinociception, tolerance, and dependence (Bohn *et al.*, 2000; Bohn *et al.*, 1999). It is likely that there is a great deal of redundancy in opioid signaling and related systems, and a role for many cellular modulators in tolerance.

It is important to note that the trafficking mechanisms described above underlie *acute* regulation of opioid receptors. Long-term exposure to drug may alter signaling processes in completely different ways and change gross neuronal morphology. Cellular-based tolerance mechanisms may not completely explain the clinical manifestations of analgesic tolerance, which could in fact be due to more whole-system or synaptic plasticity mechanisms (Christie, 2008). Long-term (20 day) exposure to amphetamine, a

dopamine transporter substrate, has been shown to change neural structure and increase the number of dendritic spines expressed on medium spiny neurons (Jedynak *et al.*, 2007; Li *et al.*, 2003). Tolerance to some effects of amphetamine exposure (including anorectic effects (Carlton *et al.*, 1971) and responding for reinforcements (Schuster *et al.*, 1961)) has been shown to occur *in vivo*. While these studies were done utilizing different systems, it is logical to assume that similar long-lasting effects in neurons may occur after chronic opioid exposure as well, and these effects could play a significant role in the development and length of tolerance expression.

Role for the δ opioid receptor in modulating μ receptor-induced tolerance and dependence development

While it is well-established that analgesic effects and development of tolerance to clinically used opioid drugs derives from activation of the μ receptor, research in the late 1990s showed that the δ receptor may also play a role. Interactions between μ and δ receptors have been recognized for many years (Traynor *et al.*, 1993), and several studies showed activation of the δ receptor through addition of an agonist contributed to the acquisition of morphine tolerance in mice (Abdelhamid *et al.*, 1991; Miyamoto *et al.*, 1994; Miyamoto *et al.*, 1993). The analysis of these interactions or co-operativity by receptors in the development of tolerance led to several studies examining the effect of δ receptor blockade, *via* treatment with δ antagonists or through knockdown of the receptor with specific silencing RNA, on tolerance development. One study from the Kuhn lab (Hepburn *et al.*, 1997) demonstrated that long-term (5-7 days) pre- or co-administration of the δ antagonist naltrindole (NTI) with morphine produced a significant decrease in antinociceptive tolerance in rats. δ Receptor antagonism using NTI did not have an effect on acute morphine antinociception but also decreased the occurrence of withdrawal symptoms in these studies after chronic morphine administration. Earlier studies examining the properties of NTI documented a significant increase in the EC_{50} for morphine after long-term exposure, however co-administration of NTI returned the EC_{50} of morphine to baseline levels (Abdelhamid *et al.*, 1991; Figure 1.1). Additionally, development of δ receptor knockout mouse models allowed for an examination of the

link between μ and δ receptors in tolerance or dependence development. In several studies, the Pintar lab (Nitsche *et al.*, 2002; Zhu *et al.*, 1999) showed loss of morphine tolerance development in δ knockout mice when compared to wild type controls, along with a loss in physical withdrawal symptoms, indicating decreased dependence to morphine.

Mechanism for δ receptor activity in μ-induced tolerance

It is still unknown by what mechanism(s) δ receptors can modulate μ receptor activity and alter the appearance of the tolerance and dependence adaptations. The probability exists for an intracellular interaction between these receptors, as studies have reported both μ and δ receptors are expressed on the same neuron (Egan *et al.*, 1981; Fields *et al.*, 1980; Schoffelmeer *et al.*, 1990), although there are opposing views (see below, and Scherrer *et al.*, 2009). In conjunction with these data, a growing field of research is examining the potential for opioid receptors to form heterodimers, with different signaling properties than individual receptors (Daniels *et al.*, 2005; George *et al.*, 2000; Gomes *et al.*, 2004; Rozenfeld *et al.*, 2007a). However, the exact cellular location or mechanism by which dimerization occurs, especially in endogenous systems, is unknown.

The μ-δ heterodimer hypothesis suggests that blockade of the δ receptor *via* an antagonist compound will prevent the μ portion of the heterodimer from signaling appropriately to intracellular components, thus preventing *in vitro* opioid desensitization and/or internalization (Rozenfeld *et al.*, 2007a). Work by the Devi group has shown slower phosphorylation of extracellular signal-regulated kinases (ERK) by putative opioid receptor heterodimers (Rozenfeld *et al.*, 2007b) in heterologous expression systems using HEK cells when compared to singly expressed receptors. Alternatively, δ receptor occupation by an antagonist could alter the process by which intracellular internalization molecules (such as β-arrestins) bind the heterodimer and signal downstream components (Rozenfeld *et al.*, 2007b), allowing the dimer to maintain expression at the cell surface and decrease apparent tolerance.

A third potential heterodimer mechanism proposes that a δ antagonist may act as a pharmacological chaperone if cell-permeable (Cahill *et al.*, 2007), stabilizing a subset of the μ-δ heterodimers held in the endoplasmic reticulum and allowing trafficking of the dimer complex to the plasma membrane. Thus, the increase in available μ receptor binding sites on the cell surface allows increased μ receptor-specific signaling and would appear as decreased tolerance. A schematic of potential signaling mechanisms is shown below (Figure 1.2). In addition, several groups (Fuxe *et al.*, 2008; Jordan *et al.*, 1999) postulate that the difference in reported heterodimeric signaling from single receptor results may be attributed to opioid receptor subtypes (e.g. μ_1 and μ_2), explaining historical differences in subtype-specific signaling paradigms and actions by 'selective' agonists or antagonists at putative opioid receptor subtypes. Our lab and others have shown, without postulating heterodimerization, that different opioid receptors when endogenously expressed in the same cell will share access to G proteins and downstream signaling effectors (Alt *et al.*, 2002; Levitt *et al.*, 2011), which might provide an impetus for receptor heterodimerization and sharing of these signaling molecules. The three heterodimer mechanisms described above are postulated primarily for chronic μ agonist exposure; there is no consensus on measures to regulate acute μ receptor signaling *via* heterodimerization.

Additionally, activation of μ receptors can modulate the cell-surface expression and signaling capability of δ receptors *in vitro*. It is well-established that the majority of δ receptors are expressed inside the cell (Arvidsson *et al.*, 1995; Cahill *et al.*, 2001a) and are trafficked to the plasma membrane upon an as-yet unknown signal. In 2001, it was demonstrated that chronic (48 h) *in vitro* treatment of cortical neurons with morphine produced a significant increase in the number of δ receptors expressed on the cellular surface with a corresponding increase in signaling ability (Cahill *et al.*, 2001b). Shorter treatment times did not produce the same extent of δ mobilization. Multiple μ agonists could induce this trafficking phenomenon (Morinville *et al.*, 2003). Moreover, this increase in membrane-bound δ was transient; lasting no more than 12 h after μ agonist removal and indicated δ receptor expression at the plasma membrane is tightly regulated

via synthesis and endocytotic pathways. Other studies have shown cellular 'rescue' of intracellular δ receptors to the plasma membrane *via* treatment with δ agonists or antagonists (Petaja-Repo *et al.*, 2002), hypothesized to be due to stabilization of otherwise unstable conformations of the intracellular 'stored' δ receptor.

Increase in δ receptor expression at the cell surface was correlated with an increase in the potency of δ agonists to produce antinociception in multiple animal models of pain transmission (Cahill *et al.*, 2001b; Morinville *et al.*, 2003). The authors suggested that the δ receptor trafficking in response to chronic μ receptor stimulation may be a compensatory mechanism to maintain a constant level of enkephalin-type signaling in the brain by increasing the number of opioid-available receptors present at the cell surface.

While heterodimerization is feasible if receptors are co-expressed, an opposing field of research argues against the existence of opioid heterodimers (Scherrer *et al.*, 2009) as some studies have demonstrated μ and δ receptors exist in distinct populations in the brain (Rossi *et al.*, 1994; Scherrer *et al.*, 2009). This phenomenon therefore must be explained by separately expressed μ and δ receptors interacting through an intersecting neuronal pathway and utilizing an intercellular or physiological signaling mechanism. A feedback loop, while not requiring co-expression of receptors, is another possible explanation for other μ and δ receptor interactions, including the trafficking of δ receptors (Cahill *et al.*, 2001b; Morinville *et al.*, 2003). To date, there is no conclusive evidence that dimerization of opioid receptors or, indeed, other Class A GPCRs is necessary for signaling of the receptor. In fact, *single* rhodopsin (Whorton *et al.*, 2008), β_2-adrenergic (Whorton *et al.*, 2007), and μ opioid (Kuszak *et al.*, 2009) receptors have been shown to signal appropriately to downstream effectors. While there is a great deal of overlap in expression between μ and δ receptors in some regions of the brain, whether the interaction demonstrated between these receptors is produced by heterodimerization or another intra/extracellular interaction remains to be elucidated.

Interactions between μ and δ receptors in physical dependence and other effects of chronic opioid activation

The intensity of withdrawal symptoms in mice chronically treated with μ agonists was directly correlated with the number of δ binding sites expressed in brain membranes (Yukhananov *et al.*, 1994), lending more evidence to the hypothesis that μ–δ receptor interactions are important in tolerance and dependence development. The effect of μ–δ receptor interactions on μ agonist induced trafficking of δ receptors (Cahill *et al.*, 2001b; Morinville *et al.*, 2003) is a question posed in a recent review of δ receptor trafficking (Cahill *et al.*, 2007), but to date has not been studied.

Evidence also exists for interactions between μ and δ receptors in addition to analgesic tolerance or dependence development. Studies have shown that μ and δ receptors are linked using *in vivo* measures of opioid activation and rewarding effects (Chefer *et al.*, 2009; Shippenberg *et al.*, 2009). In these studies, δ antagonism could prevent tolerance development to the locomotor stimulating effects of morphine and conditioned place preference, a measure of the rewarding properties of a drug.

Development of multi-functional ligands as tools to investigate interactions between opioid receptors

Taken together, studies suggesting μ and δ receptor interactions in the development of adaptations after prolonged μ agonist treatment launched a new area of opioid research and compound development in the search for novel ligands displaying μ agonism (for appropriate analgesic effects) with concurrent δ antagonism (to lessen or altogether prevent development of tolerance and/or dependence) in treatment of long-term pain conditions. In addition, a drug with μ agonist and δ antagonist efficacies could decrease the appearance of adverse side effects such as respiratory depression or constipation when compared to morphine alone (Schiller, 2010), although this has yet to be demonstrated experimentally. The research approaches taken have been many and varied, with numerous studies reporting novel treatment modalities prepared from a mixture of μ agonists and δ antagonists or single mixed-efficacy compounds having both

desired efficacies in one compound. The mixed-efficacy compound literature is particularly broad. There are reports of peptide and non-peptide ligands, ligands displaying μ partial agonist efficacies with varying δ activities (Ananthan *et al.*, 1998; Ananthan *et al.*, 2004; Schiller *et al.*, 1999), and bivalent ligands (Daniels *et al.*, 2005; Yamamoto *et al.*, 2008; Figure 1.3). Small molecule bivalent ligands, such as those reported by the Portoghese lab at the University of Minnesota (Portoghese, 1989), consist of one opioid pharmacophore, based on structural elements known to be important in binding and activating μ receptors, and another opioid pharmacophore which displays δ receptor antagonism linked by a carbon chain spacer of varying lengths (Daniels *et al.*, 2005). These bivalent ligands have been proposed to bind μ-δ receptor heterodimers (Lenard *et al.*, 2007).

The breadth of research has helped further our understanding of the structural basis of efficacy for both the μ and δ receptors. A review published in 2010 nicely describes the potential for bi- or multi-functional opioid peptide drugs and highlights the major research findings in opioid ligands for the last decade (Schiller, 2010). Bifunctional ligands have been hypothesized to exert their effects in three unique ways. Bifunctional ligands may act *via* two separate, simultaneously acting pharmacophores connected directly or with a linker moiety (e.g. bivalent ligands, described above). Alternatively, a ligand displaying overlapping pharmacophores may interact at two distinct sites of action or highly integrated bifunctional pharmacophores (e.g. buprenorphine which acts with varied efficacy at the opioid and ORL-1 receptors) could produce effects in multiple receptor systems (Schiller, 2010).

Application of bi- or multi-functional ligands in clinical practice has been an idea growing in popularity over several years due to many promising factors. A single therapeutic agent able to modulate several targets simultaneously decreases the risk of drug-drug interactions and increases the probability of patient compliance in taking the medication (Morphy *et al.*, 2005; Morphy *et al.*, 2009). In addition, drug cocktails containing one or more components run an increased risk of adverse and unpredictable variability between patients. For ease of clinical use, a mixture of two ligands would need to display similar pharmacokinetic and pharmacodynamic profiles in order to ensure

proper and timely activation (or blockade) of target receptors to achieve the desired effects.

As there are few clinically used analgesic opioid having low μ efficacy and displaying the desired μ agonist/δ antagonist efficacy profile (e.g. buprenorphine, which also displays κ antagonist activity), many groups have focused on developing novel compounds to capitalize on the hypothesized interactions between these receptors in the development of tolerance and dependence. One such example is the peptide DIPP(Ψ)NH_2 (Dmt-Tic(Ψ)[CH_2NH_2]Phe-Phe-NH_2; (Schiller *et al.*, 1999), which was reported to display both μ agonism and δ antagonism and produced a low degree of antinociception in rats with a low propensity for tolerance development after chronic administration.

Investigation of the interactions between μ and δ receptors thus poses an area of research that could inform current clinical practice and lead to novel therapeutics or treatment strategies. While is it unclear at the present time the specific mechanism by which these interactions occur, regulation of the interactions by novel pharmaceuticals represents a potential strategy to improve pain management while simultaneously decreasing occurrence of unwanted side effects. Development of bifunctional ligands that take advantage of interactions between these receptors would represent a step forward in chronic pain management techniques.

Peptide ligands have been historically regarded as having low clinical relevance due to the inherent instability and low bioavailability (e.g. short half-life, fast cleavage *via* proteolysis, and poor oral bioavailability) after peripheral administration. Indeed, many centrally-acting peptide compounds must be studied *via* direct administration into the central nervous system, a route of administration impossible for routine clinical use. Still, peptide-based therapeutics has become a growing field of research due to significant progress being made to overcome these limiting factors. Recently, several peripherally acting peptide ligands such as exenatide (Byetta®) have been approved for treatment of Type-2 diabetes or autoimmune disorders or are in promising stages of development (Bellmann-Sickert *et al.*, 2010). Peptide ligands also represent a flexible field of research

as most peptide ligands may be more easily modified and offer a greater number of modification points than non-peptidic compounds (Schiller, 2010).

Hypothesis and Objectives

It is the goal of this body of research to better understand mechanisms underlying tolerance development and to further the development of novel, non-selective μ-δ peptide ligands which produce analgesia but are not hindered by tolerance or dependence liability. In order to better understand the mechanism behind the interaction between μ and δ receptors, a two-fold plan is proposed.

First, I will re-investigate interactions between μ and δ using behavioral and biochemical measures *in vivo* and *ex vivo*. While previous reports have analyzed the effect of δ antagonist co-administration on tolerance development after prolonged morphine exposure, my studies focus on the acute effects of agonist and antagonist administration and the immediate effects of receptor desensitization, a precursor to tolerance development. A second aim is the development of bifunctional peptide ligands displaying μ agonism and δ antagonism with equivalent binding affinities to each receptor. These bifunctional compounds would be useful to investigate effects of concurrent receptor activation on downstream signaling components. This will also allow verification of the hypothesized model of decreased tolerance liability as well as probe the structural requirements of opioid receptor binding.

Interactions between μ and δ receptors in vivo and in vitro

The first aim is to revisit effects of the δ receptor on μ agonist-induced tolerance on a behavioral and biochemical basis using both *in vivo* studies and *ex vivo* characterization. The studies performed for this thesis analyze the effect of concurrent δ antagonist treatment on the development of μ agonist-induced tolerance and dependence. These were to replicate previously published studies (Abdelhamid *et al.*, 1991; Hepburn *et al.*, 1997) using selective concentrations of both the μ agonists morphine and DAMGO and the δ antagonist NTI, determining whether the interactions between the receptors is at a cellular or more physiological level. The alteration in opioid receptor signaling on an

acute (e.g. single exposure) paradigm was performed and these results provide insight into mechanism(s) underlying the immediate opioid adaptive side effects. Determining what, if any, interactions between μ and δ receptors occur can inform current clinical practice and further development of novel ligands devoid of deleterious side effects tolerance and dependence.

Bifunctional Opioid Peptide Ligands

Synthesis of mixed-efficacy opioid peptides with affinity for both the μ and δ receptors requires a detailed understanding not only of proposed peptide structure but also the conformational and structural constraints inherent in the binding sites of each opioid receptor. The goals in design of these peptides were equivalent binding affinity to both μ and δ receptors (to make sure each receptor binding site is occupied similarly), and differential efficacies (μ agonism with concurrent δ antagonism). Through the use of conformationally constrained peptide ligands (Mosberg *et al.*, 1983) and computational modeling (Fowler *et al.*, 2004a; Fowler *et al.*, 2004b; Mosberg *et al.*, 2002; Pogozheva *et al.*, 1998; Pogozheva *et al.*, 1997; Pogozheva *et al.*, 2005), docking of a proposed ligand into putative 'active' and 'inactive' conformations of both μ and δ receptors was examined. Receptor-ligand interactions or steric hindrances were observed and these modeling experiments helped direct synthesis of novel peptide sequences that incorporate the required structural qualities. Conformationally constrained peptides were synthesized to mimic the modeling constraints imposed. The validation and utilization of the opioid receptor models is described in more detail in *Chapter 3* and *Chapter 4*.

The peptide ligands proposed in this thesis will be used primarily as tools to characterize interactions between μ and δ receptors and two approaches are examined in *Chapter 3* and *Chapter 4*. Development of ligands using both penta- and tetrapeptide scaffolds and displaying the proper bifunctional properties is the primary goal, followed by characterization of these ligands. These results allow greater understanding of the opioid receptor system and structural determinants to opioid receptor binding. Characterization of novel peptide ligands displaying the appropriate efficacies likewise represents a step forward in the development of pain therapeutics lacking tolerance and/or dependence liability.

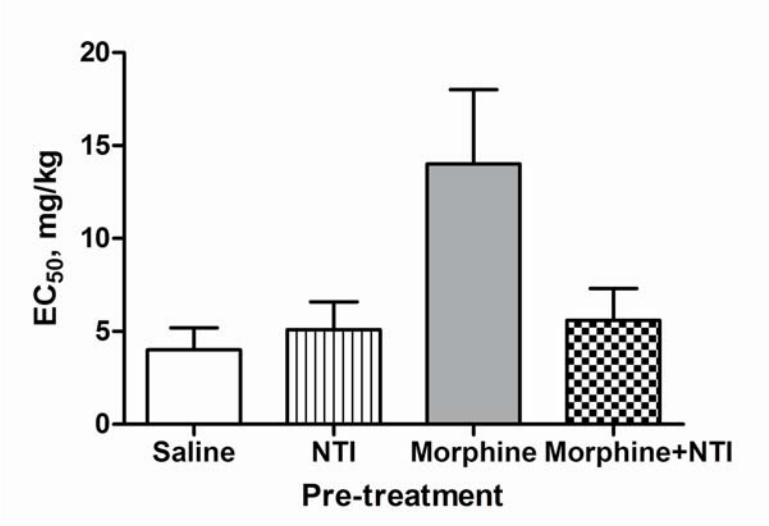

Figure 1.1 Long-term exposure of mice to the μ agonist morphine produces a significant increase in EC_{50} for the drug over saline-treated control animals. Co-administration of selective δ antagonist naltrindole (NTI) blocks this increase and decreases antinociceptive tolerance to baseline. NTI alone shows no effect on the antinociceptive effect of morphine. (Adapted from Abdelhamid *et al.*, 1991)).

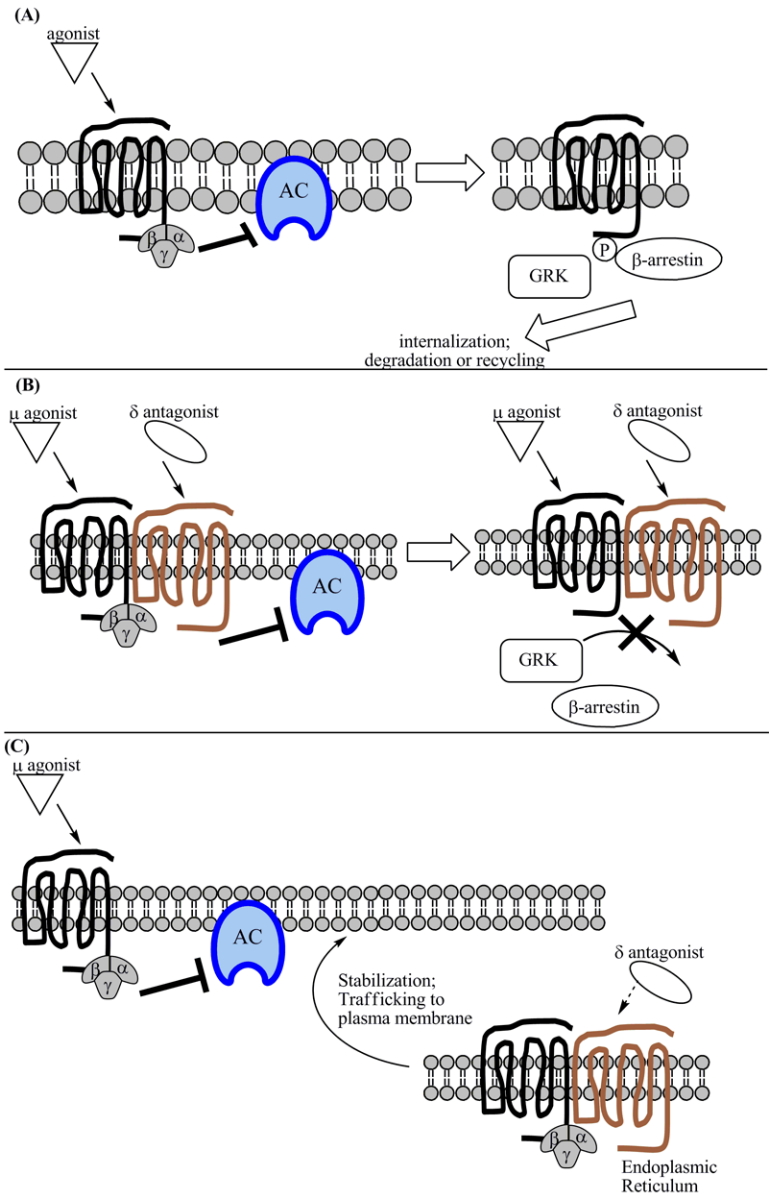

Figure 1.2 Potential mechanisms whereby heterodimerization can prevent μ agonist-induced tolerance. (A) While homomeric opioid receptors are internalized after agonist exposure, (B) heterodimerization may prevent receptor phosphorylation by GRK proteins and recruitment of β-arrestin, decreasing receptor internalization. (C) Application of a cell-permeable δ antagonist may stabilize heterodimers held in intracellular stores and traffic the heterodimer to the plasma membrane, providing additional μ receptor signaling potential.

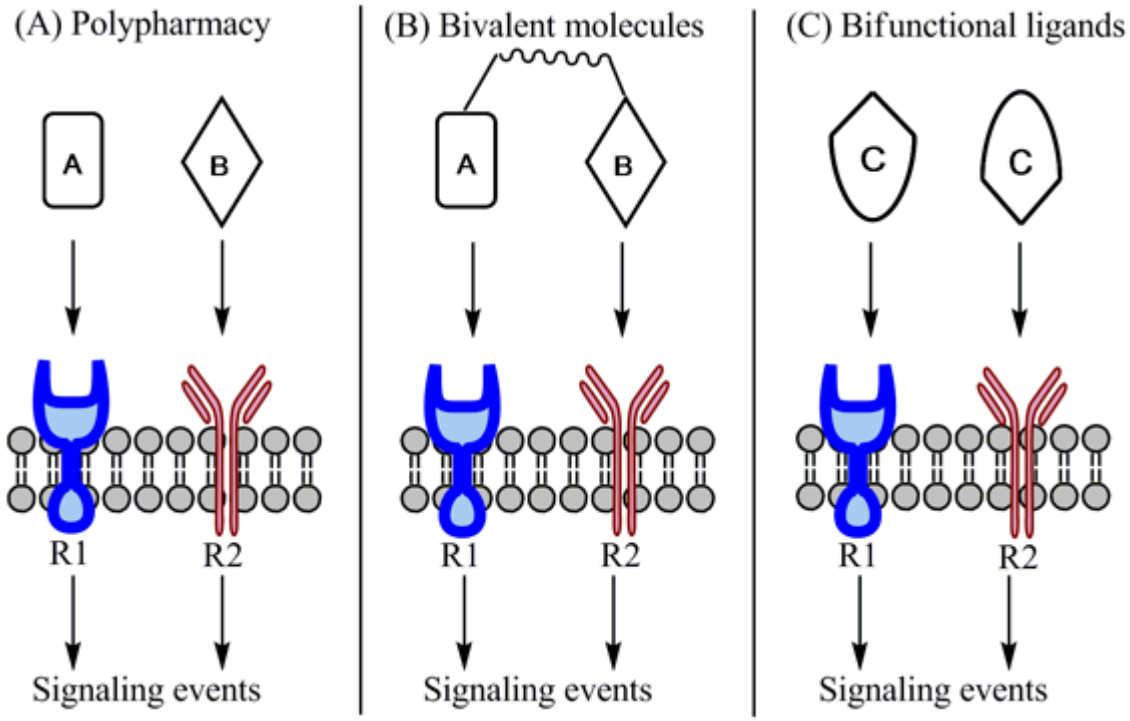

Figure 1.3 Schematic representation of different bifunctional ligands. (A) two co-administered ligands (polypharmacy); (B) a bivalent molecule linked by a spacer; and (C) a bifunctional ligand targeting both receptor 1 and 2 (R1 and R2).

Chapter II

Inhibition of acute morphine tolerance and dependence by the delta antagonist naltrindole

Summary

A positive role for the delta (δ) opioid receptor system in the production of adaptive effects to chronic morphine administration, including tolerance and dependence, has been suggested. This is based on experiments showing antagonism, knockdown, or knockout of the δ receptor slows the rate of development of morphine tolerance and/or dependence. Therefore, I hypothesized the effect of δ blockade might occur at an early stage in these adaptive processes. In this report, I show that tolerance and dependence can be observed after a single acute administration of morphine in mice, and show that these adaptations are prevented by co-administration of a single dose of the δ antagonist naltrindole (NTI). Thus, a single injection of 32 mg/kg morphine in 129S6 mice produced an antinociceptive tolerance to a subsequent morphine challenge that was fully blocked by co-administration of NTI at a dose that did not inhibit acute morphine antinociception in naïve mice. Acute tolerance was accompanied by a loss of high-affinity μ receptor binding sites; there was no change in the total number of opioid receptor binding sites. The morphine-induced loss of high-affinity binding was prevented by *in vivo* pretreatment with NTI. In addition, administration of naloxone 4 h after a single dose of morphine precipitated a number of typical opioid withdrawal signs. These signs were also reduced by pretreatment of NTI. The results provide further evidence for a physiological interaction between μ and δ receptors in the production of tolerance to and dependence on morphine and suggest this may occur at an early stage in the development of these adaptations.

Introduction

Long-term use of many illicit drugs including opiates produces neuronal adaptations (Fan *et al.*, 2003; Nestler, 2001) and can lead to both tolerance (decreased effectiveness of a drug) and dependence (requirement of drug to maintain behavioral and cellular homeostasis). Development of tolerance to the mu (μ) opioid agonist morphine has been shown to develop rapidly in animal models (Abdelhamid *et al.*, 1991; Cochin *et al.*, 1964) and lasts for a sustained period of time; (Kornetsky *et al.*, 1968). The vast majority of both clinical and illicit opioid agonists produce their effects *via* activation of the μ receptor. However, a number of studies (Abdelhamid *et al.*, 1991; Hepburn *et al.*, 1997; Kest *et al.*, 1996) have provided evidence of a role for the delta (δ) opioid receptor in modulating long-term adaptations to morphine, including tolerance and dependence.

The idea that opioid receptor interactions alter behavioral outcomes is not new (Egan *et al.*, 1981; Porreca *et al.*, 1992) and many studies have examined the role of δ receptors in μ receptor induced effects. Observations that certain outcomes are changed including μ agonist mediated antinociception (Cahill *et al.*, 2001b; Porreca *et al.*, 1992), tolerance (Chefer *et al.*, 2009; Kest *et al.*, 1996), and dependence (Fundytus et al., 1995) lead to the development of various hypotheses regarding interactions between μ and δ receptors. Behavioral studies supporting this idea showed that blockade of the δ receptor *via* antagonism (Abdelhamid *et al.*, 1991; Hepburn *et al.*, 1997) or genetic knockdown (Kest *et al.*, 1996) decreased the appearance of antinociceptive tolerance and/or dependence on morphine. Interactions between μ and δ receptors are not limited to antinociceptive effects, as further studies have demonstrated a link between these receptors in behavioral paradigms such as locomotor sensitization and conditioned place preference (Chefer *et al.*, 2009; Shippenberg *et al.*, 2009).

One hypothesis to explain the effect of δ antagonism on μ agonist-mediated behaviors is that δ receptors in a μ–δ heterodimeric complex modulate intracellular signaling produced by the μ receptor or that the μ–δ heterodimer uses alternative signaling pathways (Gomes *et al.*, 2004; Rozenfeld *et al.*, 2007a), such that development of adaptive side effects might be changed. Results from these and similar experiments

have founded an area of opioid ligand development aimed at the design of novel ligands that bind to both μ and δ receptors presenting with mixed efficacy at each receptor (e.g. μ agonism and δ antagonism; Purington et al., 2009; Schiller, 2010).

Advances have been made in understanding the cellular mechanism(s) underlying tolerance and dependence development after μ agonist treatment. Tolerance to μ agonists including morphine likely involves several redundant and compensatory mechanisms. Studies have shown μ agonist-specific responses to a number of cell signaling events, including G protein stimulation (McPherson *et al.*, 2010), receptor phosphorylation (Zhang *et al.*, 1998), desensitization (Alvarez *et al.*, 2002) and internalization (Arttamangkul et al., 2008). Most *in vitro* work has been performed in single (and often heterologous) cell systems after short treatment times (min-h), and with high concentrations of agonists, conditions inconsisten with behavioral studies in rodents published previously. In particular, studies examining the effect of δ antagonism on μ agonist-mediated antinociceptive tolerance or other behavioral effects were performed after prolonged exposure (5-7 days) to the μ agonist (Abdelhamid *et al.*, 1991; Hepburn *et al.*, 1997) and the effects of NTI were shown only to delay the progress of tolerance development. I therefore examined the early stages of the adaptive process.

Tolerance to the μ agonist morphine has been demonstrated in some behavioral measures to occur after a single exposure to the drug (Cochin *et al.*, 1964; Kornetsky *et al.*, 1968). I have utilized an acute tolerance paradigm in 129S6 mice to examine the effects of a single dose of morphine to bridge the gap between previously published *in vitro* and *in vivo* reports and also to look at effects on early time points. Analysis of the production of tolerance and dependence by both *in vivo* and *ex vivo* methods in the presence or absence of the selective δ antagonist naltrindole (NTI) (Portoghese *et al.*, 1988) was performed. NTI is the δ antagonist used most often to demonstrate interactions in opioid receptor systems (Abdelhamid et al., 1991; Hepburn et al., 1997; Shippenberg et al., 2009). The results show decreased acute antinociceptive tolerance and dependence to morphine in the presence of NTI and dampening of morphine-mediated changes at the level of receptor and G protein. The studies lend further evidence to the hypothesis that δ

receptor blockade may be useful in lessening the development of tolerance to, and dependence on, morphine.

Results

Acute tolerance to morphine

The ability of morphine to produce antinociception in male 129S6 mice in the hot plate assay was measured. The mice showed an antinociceptive response to morphine with an ED_{50} of 11.2 +/- 1.5 mg/kg, with a measured endpoint as forepaw licking or jumping (Lamberts et al., 2011). A 10 mg/kg dose of naltrindole (NTI, s.c.) administered 15 min before morphine did not have an effect on baseline latencies to paw lick, nor on the ability of morphine to produce antinociception in the hot plate assay (Figure 2.1A). Time-course studies of the effects of morphine indicated a loss in the antinociception 4 h after initial exposure (data not shown) and previous studies have shown 4 h to be sufficient time to visualize decreased effects of morphine (Kornetsky *et al.*, 1968). Therefore, acute tolerance was examined 4 h after a single morphine injection.

A challenge dose of 32 mg/kg morphine (i.p.) administered 4 h after the initial injection (Figure 2.1B) produced less antinociception than the initial morphine dose (reported as % Maximum Possible Effect (% MPE); % MPE (initial): 63.4 ± 7.3%; % MPE (challenge): 35.8 ± 5.4%; $p<0.05$). This effect was prevented by NTI pretreatment (% MPE (challenge): 60.2 ± 10.9%). NTI had no effect on the ability of morphine to produce antinociception in naïve animals (% MPE (initial): 72.0 ± 9.0%; $p>0.05$). Control groups of mice receiving saline or NTI pretreatment 4 h before a single dose of morphine (Figure 2.1B, inset) displayed similar antinociceptive effects (saline pretreatment, % MPE: 70.2 ± 9.9%; NTI pretreatment, % MPE: 77.7 ± 13.0%), indicating that NTI did not have direct off-target effects at the μ receptor and confirming the dose-effect data (Figure 2.1A). Statistical analysis by two-way ANOVA revealed a significant interaction between pretreatment and treatment ($F(3,43)=19.56$; $p<0.0001$). Additionally, there were significant main effects of pretreatment ($F(3,43)=5.076$; $p=0.0043$) and injection (either initial or challenge injections; $F(1,43)= 16.30$; $p=0.0002$).

In contrast to these data, mice treated seven days with morphine with or without NTI pretreatment did not develop antinociceptive tolerance, even with a higher morphine dose. There was no shift in the ED_{50} of the morphine dose-effect curve after chronic administration (ED_{50} initial: 16.0 ± 4.1 mg/kg; ED_{50} following treatment: 16.3 ± 4.0 mg/kg, $p>0.05$) and no decrease in maximum antinociception (data not shown). While this protocol has been used with success to develop tolerance (Bohn *et al.*, 2000), inability to produce tolerance in these studies and insensitivity to chronic morphine may be due to the species of mice used. Morphine antinociception and tolerance has been shown to be strain-specific and the 129S6 strain used in these studies does not develop antinociceptive tolerance as readily after chronic administration of morphine (Bryant *et al.*, 2006; Kest *et al.*, 2002).

Precipitated withdrawal following acute morphine treatment

To examine acute dependence, withdrawal was precipitated by a single dose of the non-selective opioid antagonist naltrexone (10 mg/kg, s.c.) Withdrawal symptoms observed included paw tremors, wet dog shakes, ptosis, body tremors, and teeth chattering (Divin *et al.*, 2008). Jumping and diarrhea, commonly observed symptoms of opioid withdrawal in mice (Maldonado *et al.*, 1996), were not seen after acute morphine exposure, which may indicate the withdrawal symptoms are differentially produced or develop under a prolonged time course (Kimes et al., 1993) and may not be observed after acute opioid exposure.

As seen in Figure 2.2, saline pretreated mice receiving 32 mg/kg morphine (i.p.) displayed 19.2 ± 2.0 counted withdrawal signs in the 20 min observation period, while mice receiving saline alone showed 5.8 ± 1.1 signs. As in the hot plate assay, pretreatment with 10 mg/kg NTI decreased the appearance of withdrawal signs measured in 20 min to 10.0 ± 1.5 ($p<0.05$ compared to saline-pretreated animals). NTI pretreatment had no effect on the number of baseline behavioral signs exhibited by saline treated mice (5.0 ± 1.0). Analysis by two-way ANOVA revealed significant main effects of both treatment and pretreatment (treatment: $F(1,20)=32.35$; $p<0.0001$, pretreatment: $F(1,20)=9.626$; $p<0.01$), and a significant interaction between the two ($F(1,20)=6.684$; $p<0.05$). Chronically treated mice did show naloxone-precipitated withdrawal symptoms

after 7 days, but the degree of withdrawal was no different from animals given a single dose of morphine (chronic morphine-treated mice: 20.5 ± 4.1 withdrawal signs in 20 min).

[^{35}S]GTPγS binding in brainstem membranes after morphine treatment

As an *ex vivo* measure of morphine antinociceptive tolerance, I examined the ability of μ agonist [D-Ala^2-N-Me-Phe^4-Gly^5-ol]enkephalin (DAMGO) to stimulate binding of the non-hydrolyzable GTP analog [^{35}S]GTPγS (Traynor *et al.*, 1995) in brainstem membranes (Bohn et al., 2000). Addition of increasing concentrations of DAMGO to brainstem membranes from saline treated animals stimulated [^{35}S]GTPγS binding to μ receptors, with an EC_{50} of 850 ± 220 nM and a maximum stimulation of 117 ± 11% over basal levels (Figure 2.3A). In contrast, 4 h after a single *in vivo* morphine treatment, there was a decreased the ability of DAMGO *ex vivo* to stimulate [^{35}S]GTPγS binding, (68 ± 8%, $p<0.05$), with a small but not significant increase in the DAMGO EC_{50} value (1040 ± 360 nM). NTI pretreatment *in vivo* rescued the loss of DAMGO-mediated [^{35}S]GTPγS stimulation in morphine treated animals (NTI-Morphine: 100 ± 20%; EC_{50}: 1290 ± 200 nM; $p>0.05$ compared to saline treated controls, Figure 2.3B). NTI pretreatment alone *in vivo* did not affect the ability of DAMGO to stimulate G protein in this assay (NTI-Sal: 100 ± 10% stimulation; EC_{50}: 410 ± 120 nM; data not shown).

Expression of μ receptor binding sites after morphine treatment

Measurement of high-affinity μ receptor binding sites in brainstem and striatal membrane preparations was examined following morphine treatment *in vivo*. I utilized radiolabeled [^{3}H]DAMGO to examine the number of μ receptor sites with or without morphine treatment. [^{3}H]DAMGO binding in brainstem membranes from saline-treated animals showed a Bmax of 108 ± 20 fmol binding sites/mg protein (Figure 2.4), while morphine-treated animals had a lower Bmax (Bmax: 57 ± 7 fmol/mg protein, $p<0.05$; Table 2.1). The binding affinity of [^{3}H]DAMGO (Kd) was unchanged between treatment groups (Kd (saline): 2.3 ± 0.7 nM; Kd (morphine): 2.4 ± 1.6 nM; $p>0.05$). [^{3}H]DAMGO binding in striatal membrane preparations displayed similar results. Saline treated mice showed a Bmax of 147 ± 12 fmol/mg protein with a Kd for [^{3}H]DAMGO of 3.3 ± 1.6

nM, while morphine treated animals had a non-significant decrease in μ receptor binding sites (86 ± 36 fmol/mg protein, $p>0.05$; Kd [^{3}H]DAMGO: 1.5 ± 0.8 nM).

Since DAMGO labels only high-affinity μ receptors, I examined the total number of opioid receptors and total μ receptor population in the brainstem and striatum, using the non-selective opioid antagonist [^{3}H]diprenorphine at a supramaximal concentration of 4 nM. As this was a single concentration radioligand binding assay, these results cannot be compared directly with the data from the [^{3}H]DAMGO binding assays. Total μ receptor population was defined by displacement of [^{3}H]diprenorphine with 300 nM of the μ-selective antagonist peptide D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP). In membranes from saline treated mice, [^{3}H]diprenorphine labeled 137 ± 12 fmol/mg protein of total opioid receptors in brainstem and 263 ± 43 fmol/mg protein in striatal membranes. The μ receptor-specific populations were 72 ± 12 fmol/mg protein and 93 ± 15 fmol/mg protein, respectively (Table 2.1). In membrane homogenates from morphine treated mice, [^{3}H]diprenorphine labeled 136 ± 12 fmol receptor/mg protein in the brainstem and 195 ± 60 fmol receptor/mg protein in the striatum; the total amount of μ receptors was determined to be 62 ± 13 fmol/mg in brainstem and 76 ± 13 fmol/mg protein in striatum (Table 2.1). A greater number of total opioid receptors was determined in striatal membranes over brainstem in saline-treated mice, in agreement with the differential expression of opioid receptors in the brain (Mansour *et al.*, 1995).

As no change in total μ receptor expression was seen between saline or morphine treated animals, our results suggest a lack of down-regulation of these receptors. The tolerance seen in these studies may therefore be due to the significant decrease in high-affinity binding sites following morphine treatment.

cAMP overshoot as a cellular measure of dependence

To examine the effect of acute morphine treatment *ex vivo*, I employed an adenylyl cyclase (AC) enzyme sensitization paradigm. AC sensitization (Watts *et al.*, 2005) is a cellular phenomenon that develops following morphine treatment and considered to be a withdrawal response. Administration of opioid drugs *in vivo* produces a re-setting of basal AC activity such that, upon removal of the agonist drug, the enzyme is ‘sensitized’ to subsequent stimulation. As the system requires presence of drug to

maintain homeostasis, this has become a model of cellular dependence and precipitated withdrawal (Divin *et al.*, 2009).

I examined the ability of morphine treatment *in vivo* to produce cAMP sensitization in striatal membrane preparations upon addition of the AC stimulator, forskolin (FSK). In striatal membrane preparations, the basal levels of cAMP were similar between membrane preparations from saline or morphine treated mice (data not shown). With addition of 5 μM FSK (Bohn *et al.*, 2000), there was an increase in cAMP accumulation but no difference was seen between morphine treated animals over saline treated controls.

Discussion

The studies reported here have examined *in vivo* and *ex vivo* measures of acute tolerance and dependence after a single administration of morphine. These lend evidence in support of the hypothesis that co-administration of morphine with a δ antagonist can prevent the development of μ agonist tolerance and dependence (measured by precipitated withdrawal) *in vivo*. The acute tolerance and dependence seen in 129S6 mice after a single administration of morphine may be due rapidly reversible changes at the receptor level (Gintzler *et al.*, 2008), compared to permanent structural changes and altered protein synthesis mechanisms after chronic administration, thus suggesting that the effects of δ antagonist treatment are dominant early in the process of tolerance development. I was able to determine acute tolerance and dependence *in vivo*; however, chronic administration of morphine at the doses employed did not produce tolerance. In contrast, long-term morphine exposure elicited dependence development as a number of precipitated withdrawal symptoms were measured following 7 day treatment.

Tolerance is known to arise *via* various mechanisms. Roles for β-arrestins (Bohn *et al.*, 2000), protein kinase C (Bailey et al., 2006), and other accessory proteins (Clark *et al.*, 2005) have all been shown in tolerance development after chronic morphine treatment. It is likely that complex compensatory mechanisms are activated upon chronic opioid agonist exposure and may be the underlying reason why no tolerance was observed in our system. Additionally, μ agonist exposure indirectly alters signaling

capabilities of non-opioid containing cells (Mao, 1999) to subsequent stimulation and interactions of the μ receptor with other inhibitory G protein-coupled receptors (including the α_2-adrenergic, cannabinoid, and orphanin (ORL-1) receptor) have been shown (Levitt *et al.*, 2011), providing another compensatory signaling mechanism to prevent observation of tolerance. In support of our findings in 129S6 mice, propensity to develop tolerance among several species of mice has been studied (Bryant *et al.*, 2006; Kest *et al.*, 2002), demonstrating that some inbred strains (including the 129S6 strain) are resistant to morphine tolerance and develop this adaptation less readily than other strains.

A reduction in the number of high-affinity μ receptor binding sites (without a loss in total μ receptor expression) and decrease in agonist ability to elicit receptor-G protein activation *ex vivo* may provide an explanation for the behavioral observations. I postulate the reduction in [^{3}H]DAMGO binding *ex vivo* in membrane preparations from morphine treated mice is due to a decrease in the high-affinity μ receptor state without a corresponding decrease in total μ receptor number. A reduced number of high-affinity states of the receptor is most likely due to receptor desensitization and uncoupling of G protein from the membrane-bound receptor (Zhang et al., 1996). This agrees with previous studies demonstrating morphine can produce receptor desensitization but not internalization (Kim et al., 2008; McPherson et al., 2010). The decrease in the number of high-affinity μ receptors available is not caused by the continued presence of morphine after membrane homogenization, as the membrane preparation involves several washing steps, there was no change in basal [^{35}S]GTPγS binding (data not shown) and no change in the Kd value for [^{3}H]DAMGO between treatment groups.

Acute exposure to morphine *in vivo*, while able to produce a significant number of withdrawal symptoms (Figure 2.2) may not produce large enough biochemical changes to see in the *ex vivo* procedure. This may be because of insufficient time to develop a high degree of sensitization of μ receptor-linked AC enzymes or because this effect may be short-lived and readily reversible (Watts *et al.*, 2005). AC isoform V has also been shown to play a role in AC sensitization (Kim et al., 2006) after long-term morphine exposure, but our results may indicate that striatal adenylyl cyclase isoforms IV and V (Kim et al., 2006) do not become sensitized after a single administration of μ agonist. It is possible

that AC sensitization may be visualized in other brain regions after a single dose of morphine (including AC isoforms I and VIII (Zachariou *et al.*, 2008) present in the cortex and other brain regions). On the other hand, this adaptation may be only visualized after chronic μ agonist administration (Bohn *et al.*, 2000; Kim *et al.*, 2006; Watts *et al.*, 2005; Zachariou *et al.*, 2008). Additionally, there is a possible loss of sensitized AC in the *ex vivo* assay; an acute exposure to morphine may not be sufficient to produce robust sensitization that would persist following our membrane preparation procedure.

The experiments in this report highlight the role of δ receptor blockade in decreasing production of adaptive side effects tolerance and/or dependence after brief exposure to morphine. Moreover, they strengthen clinical relevance of bifunctional ligands or multi-drug therapies simultaneously containing μ agonist and δ antagonist efficacies to effectively produce analgesia with reduced tolerance liabilities.

Materials and Methods

Drugs: All reagents, including [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) naloxone, and D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP) were from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated and of analytical grade. [^{35}S]-guanosine-5'-O-(3-thio)triphosphate ([^{35}S]-GTPγS; 1250Ci (46.2TBq)/mmol), [^{3}H]-DAMGO, and [^{3}H]-diprenorphine were purchased from Perkin Elmer (Boston, MA). Morphine sulfate was obtained from Research Triangle Institute (RTI, NC). All drugs were diluted in sterile water for both biochemical and behavioral assays.

Subjects: Male wild-type 129S6/SvEvTac mice (obtained from in-house breeding colony or Taconic (http://www.taconic.com)) were used for all behavioral studies. Mice were group-housed (4-6 animals per cage) in a temperature-controlled room maintained on a 12 hr light/dark cycle with lights on at 7:00 AM. For behavioral studies, subjects were between 8-12 weeks of age and weighed 20-30 g at the time of the studies. Groups of 6-8 mice were used in each of the behavioral experiments. All experimental procedures were approved by the University of Michigan Committee on Use and Care of Animals (UCUCA) and in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Behavioral Antinociceptive Testing: The hot plate test was used to evaluate supraspinal antinociception as described previously (Lamberts *et al.*, 2011). Mice were given two injections of saline (i.p.) to determine baseline latency, followed by three cumulative doses of morphine (i.p.) in 30-min intervals or a single 32 mg/kg morphine injection (i.p.). Mice were placed on a 52°C hot plate 30 min following each injection. The latency to lick forepaw(s) or jump was measured with a cutoff time of 60 s in order to prevent tissue damage. Agonist-stimulated antinociception is expressed as a percentage of maximum possible effect (% MPE), where % MPE = (post-drug latency-average baseline latency) / (cutoff latency-average baseline latency) x 100.

Acute Tolerance and Dependence: Acute tolerance to the antinociceptive effects of morphine (Kornetsky *et al.*, 1968) was induced by a single administration of 32 mg/kg morphine. Mice were assessed pre-drug exposure for baseline latency, given a pretreatment (s.c.) of saline or 10 mg/kg of the δ antagonist naltrindole (NTI), followed 15 min later by 32 mg/kg morphine (i.p.). 30 Min post-morphine, the latency to lick forepaws or jump from a hot plate as described above was determined. After 4 h incubation, the baseline latency was again determined (latencies were between 15-18 s) and mice given a challenge injection of 32 mg/kg morphine (i.p.). The post-challenge administration latency was measured after 30 min and all data are expressed as % MPE for groups containing 8 animals each.

Following determination of hot plate latency, mice were given a single injection of 10 mg/kg naloxone (s.c.) to precipitate withdrawal symptoms (Divin *et al.*, 2008). To assess withdrawal, each mouse was placed singly in a clear Plexiglass box (28 cm x 18 cm x 13 cm) and observed for 20 min. The total occurrence and frequency in 5 min intervals of the following behaviors was measured: wet dog shakes, paw tremors, ptosis, teeth chattering, and body tremors. Data are plotted as the total number of withdrawal symptoms (Maldonado *et al.*, 1996; Maldonado *et al.*, 1992) in a 20 min period and represent the average from groups of 6-8 mice.

Chronic morphine administration: Mice were treated for 7 days with morphine (Bohn *et al.*, 2000). A dose-effect curve for cumulative doses of morphine (10-100 mg/kg, i.p.) was produced on day 0, followed by twice daily administrations of 128

mg/kg morphine (i.p.) for 6 days. On each administration day, mice were weighed and baseline latency to lick forepaws on the hot plate examined. Mice were given an injection of saline or 128 mg/kg morphine (i.p.), followed 30 min later by analysis of morphine latency with a cutoff time of 60 sec as described above. For the evening injections, mice were weighed and administered drug only. On the 7th day, a second cumulative morphine schedule was employed (32-320 mg/kg, i.p.) and the dose-effect curve analyzed for changes from the initial data. Mice were assessed for physical dependence following the cumulative morphine dose schedule on day 7 as described above.

Brain Membrane Preparation: Mice were euthanized by cervical dislocation and brainstem or striatal tissue was removed, immediately frozen on dry ice and stored at -80 °C. Samples were used within one week of harvest. Brainstem or striatal membrane homogenates for radioligand or [^{35}S]GTPγS binding assays were prepared as previously described (Lester *et al.*, 2006). Briefly, the tissue was suspended in ice-cold 50 mM Tris-HCl buffer, pH 7.4 and homogenized with a Tissue Tearor (Biospec Products, Inc., Bartlesville, OK) for 20 s at setting 4. The homogenate was centrifuged at 20,000x*g* for 20 min at 4°C and the pellet re-homogenized in 50 mM Tris-HCl with a Tissue Tearor for 10 s at setting 2, followed by re-centrifugation. Final membrane pellets were resuspended in 50 mM Tris-HCl, pH 7.4 and used immediately. Protein concentration was determined using the BCA protein assay (Smith et al., 1985)Thermo Fisher Scientific, Rockford, IL) using bovine serum albumin as the standard.

Striatal membrane preparation used for the examination of cAMP sensitization. Striatum were thawed in 1 ml Tris-HCl buffer, pH 7.4 with 1 mM EDTA and 0.32 M sucrose in the presence of protease inhibitors and 1 mg/ml BSA. Samples were homogenized and centrifuged at 15,000 rpm for 30 min at 4 °C. The resulting pellet was resuspended in buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 100 mM NaCl and 5 mM $MgCl_2$. Protein content was analyzed as described above.

*Radioligand Binding Assays:*Total opioid receptor expression in brain membranes was determined in radioligand binding assays using [^{3}H]diprenorphine and brainstem or striatal membrane homogenates prepared as described above. The assay mixture, containing membrane suspensions (25-30 μg protein/tube) in 50 mM Tris-HCl buffer (pH

7.4) and 4 nM [^{3}H]diprenorphine was incubated at 25°C for 75 min with shaking to allow binding to reach equilibrium. The relative number of μ receptors present in the membrane preparations was determined by the displacement of 4 nM [^{3}H]diprenorphine by 300 nM of μ-selective antagonist peptide CTAP. This concentration was shown to be μ-selective as it was unable to produce a shift in the [^{35}S]GTPγS stimulation concentration-response curve for the δ agonist 4-[(*R*)-[(2*S*,5*R*)-4-allyl-2,5-dimethylpiperazin-1-yl](3-methoxyphenyl)methyl]-N,N-diethylbenzamide (SNC80) in SH-SY5Y cell membranes (data not shown).

Analysis of high-affinity μ receptor binding was determined *via* [^{3}H]DAMGO saturation binding. Membrane homogenate (50 μg protein/tube) was added to 50 mM Tris-HCl buffer, pH 7.4 with increasing concentrations of [^{3}H]DAMGO (0.5-25 nM) and the mixture incubated as described above. After incubation, samples were filtered rapidly through GF/C filters (Whatman, Middlesex, UK) using a Brandel harvester and washed three times with ice-cold 50 mM Tris-HCl buffer. The radioactivity retained on dried filters was determined by liquid scintillation counting after saturation with EcoLume liquid scintillation cocktail (MP Biomedicals, Solon, OH) in a Wallac 1450 MicroBeta (PerkinElmer, Waltham, MA). Non-specific binding was determined in all radioligand binding assays with 10 μM naloxone. Data were graphed using GraphPad Prism 5.01 software (GraphPad Software, La Jolla, CA). The results presented are the mean from at least three separate assays, each performed in triplicate.

[^{35}S]GTPγS Stimulation Assay: Binding of the non-hydrolyzable GTP analog [^{35}S]GTPγS was measured as a method to determine receptor activation as described previously (Traynor *et al.*, 1995). Prepared brainstem membranes (10 μg protein/tube) were incubated in GTPγS binding buffer (50 mM Tris-HCl, pH 7.4; 100 mM NaCl; and 5 mM $MgCl_2$) containing 0.1 nM [^{35}S]GTPγS, 100 μM GDP, 2 mM dithiothreitol, 0.4 U/ml adenosine deaminase, and varying concentrations (0.1–10,000 nM) of DAMGO for 90 min at 25°C. For all [^{35}S]GTPγS-binding assays, nonspecific binding was evaluated in the presence of 10 μM GTPγS. The reaction was terminated by rapidly filtering through GF/C filters and washing with 2 ml ice-cold GTPγS binding buffer. Retained

radioactivity was measured as described above. Experiments were performed at least three times in duplicate. EC_{50} values were determined by nonlinear regression analysis using GraphPad Prism 5.01 software.

Whole cell acute inhibition of adenylyl cyclase: Adenylyl cyclase activity (and cyclic AMP (cAMP) accumulation) was measured in striatal membrane preparations prepared as described above. Striatal membranes (10 μg/well) were incubated 5 min in 10 μl assay buffer containing 1 mM 3-isobutyl-1-methylxanthine, 10 μM GDP, 100 mM NaCl, 5 mM $MgCl_2$, 2 mM ATP, 100 mM phosphocreatine, and 1000 U/ml creatine phosphokinase with or without 5 μM forskolin (FSK) to stimulate cAMP accumulation at 37 °C (Bohn *et al.*, 2000). The assay was quenched with addition of 20 μl ice-cold 3% perchloric acid, neutralized with 5 μl 2.5M $KHCO_3$, and centrifuged 2 min at 10,000x*g*. cAMP accumulation in the presence or absence of FSK was measured from the supernatant using an ELISA kit from Cayman Chemical (Ann Arbor, MI), according to the manufacturer's instructions. Experiments were performed in triplicate and repeated a minimum of three times.

Statistical Analysis Data were analyzed using Student's two-tailed *t* test or two-way analysis of variance followed by Bonferroni's post-hoc test where appropriate using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA www.graphpad.com). *p* values less than 0.05 were considered to be significant.

Table 2.1. Loss in high-affinity μ receptor binding sites with morphine treatment, but no change in total opioid receptor binding sites in membrane preparations from brainstem and striatum

	Brainstem				Striatum			
Treatment	[^{3}H]DAMGO*		[^{3}H]Diprenorphine$^+$		[^{3}H]DAMGO		[^{3}H]Diprenorphine	
	Bmax (fmol/mg protein)	Kd (nM)	Total receptor (fmol/mg protein)	μ-specific (fmol/mg protein)	Bmax (fmol/mg protein)	Kd (nM)	Total receptor (fmol/mg protein)	μ-specific (fmol/mg protein)
Saline	108 ± 20	2.3 ± 0.7	137 ± 12	72 ± 12	147 ± 12	3.3 ± 1.6	263 ± 43	93 ± 15
Morphine	57 ± 7	2.4 ± 1.6	136 ± 12	62 ± 13	86 ± 36	1.5 ± 0.8	195 ± 60	76 ± 13

[^{3}H]DAMGO or [^{3}H]diprenorphine binding in brainstem or striatal membrane homogenates prepared 4 h after a single administration of saline or 32 mg/kg morphine (i.p.). *[^{3}H]DAMGO Bmax values determined from saturation binding curve. $^+$Total receptor expression in [^{3}H]diprenorphine assays estimated using a single supramaximal concentration (4 nM) of [^{3}H]diprenorphine. μ Receptor-specific binding sites measured *via* displacement of 4 nM [^{3}H]diprenorphine with 300 nM of the μ-selective antagonist D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2 (CTAP). There is a loss in agonist-bound, high-affinity [^{3}H]DAMGO binding sites in both brainstem and striatal membranes prepared after treatment with 32 mg/kg morphine (i.p.) with no change in the Kd value for DAMGO. There is no change in the total opioid receptor expression measured with antagonist [^{3}H]diprenorphine binding or in μ receptor-specific binding sites. In the striatum, there is a slight, but not significant, decrease in the number of [^{3}H]DAMGO binding sites after morphine treatment.

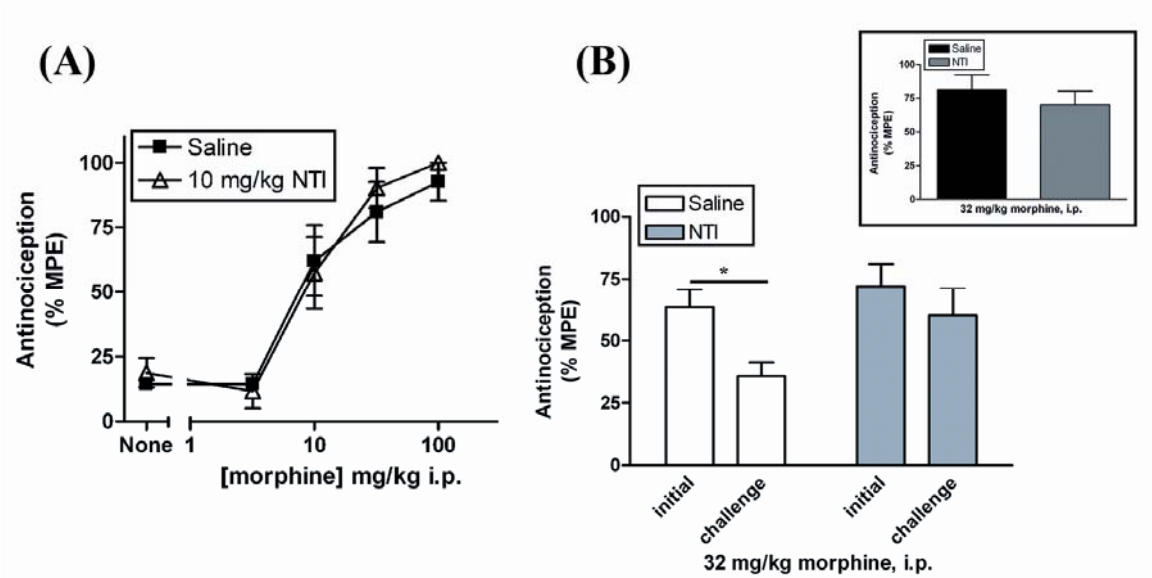

Figure 2.1: Morphine antinociception in the hot plate assay. Data are expressed as % maximum possible effect (% MPE), calculated as described in *Methods*. (A) 15 min pretreatment with 10 mg/kg NTI (s.c.) does not affect the antinociceptive properties of morphine in the hot plate assay and has no effect on the EC_{50} of morphine (i.p.) Baseline latencies were also unchanged after NTI pretreatment (latency (saline): 12.4 ± 2.0 sec; latency (NTI): 13.8 ± 0.7 sec; $p>0.05$). (B) Mice pretreated with saline or 10 mg/kg NTI (s.c.) exhibit the same degree of antinociception upon an initial dose of 32 mg/kg morphine (i.p.) (saline pretreated mice % MPE (initial): 63.4 ± 7.3%; NTI pretreated mice % MPE (initial): 72.0 ± 9.0%; $p>0.05$). A challenge dose of 32 mg/kg morphine (i.p.) administered after 4 h produced decreased antinociception in saline pretreated mice (% MPE (challenge): 35.8 ± 5.4%) but not NTI pretreated mice (% MPE (challenge): 60.2 ± 10.9%). NTI pretreatment did not have an effect on morphine antinociception 4 h after pretreatment (inset). Statistical analysis by two-way ANOVA revealed a significant interaction between pretreatment and treatment ($F(3,43)= 19.56$, $p<0.0001$) and significant main effects of pretreatment ($F(3,43)= 5.076$, $p=0.0043$) and injection (initial or challenge; $F(1,43)= 16.30$, $p=0.002$).

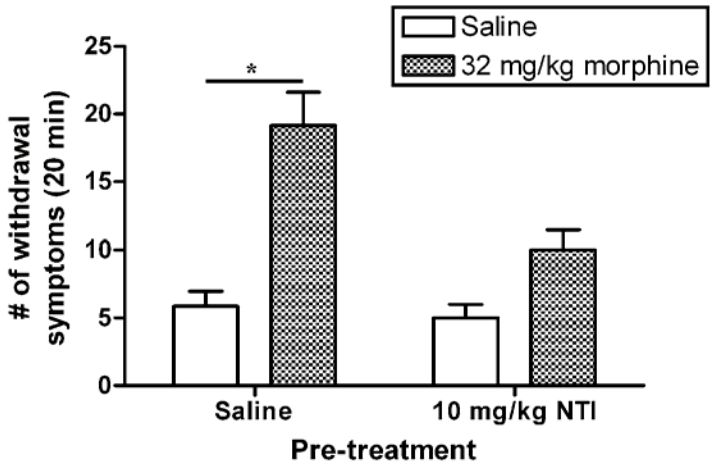

Figure 2.2. Withdrawal precipitated with 10 mg/kg naltrexone (s.c.) 4 h after morphine exposure. Mice given 32 mg/kg morphine (i.p.) followed by withdrawal precipitated by 10 mg/kg naltrexone (s.c.) exhibit an increase in the number of withdrawal symptoms in the 20 min observation period, while mice receiving a 10 mg/kg NTI (s.c.) pretreatment show no change in the number of withdrawal symptoms when compared to saline pretreated animals. Two-way ANOVA for these data demonstrated significant main effects of both treatment (saline or 32 mg/kg morphine (i.p.); $F(1,20)= 32.35$, $p<0.001$) and pretreatment (saline or 10 mg/kg NTI (s.c.); $F(1,20)= 9.626$, $p=0.0056$) and a significant interaction between pretreatment and treatment ($F(1,20)=6.684$, $p=0.0177$).

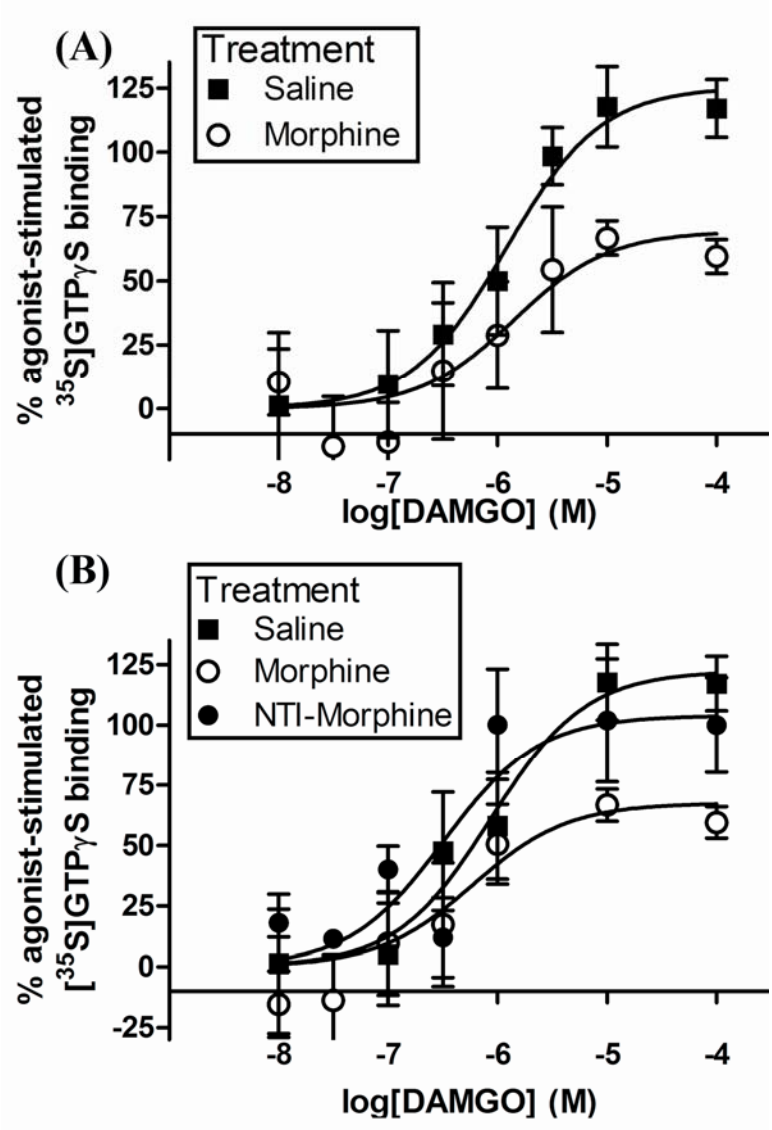

Figure 2.3. [^{35}S]GTPγS binding in mouse brainstem membranes *ex vivo* is decreased when measured in brainstem homogenates harvested 4 h after a single *in vivo* injection of 32 mg/kg morphine (i.p.) Mice were treated with saline or morphine as described above and brainstem membranes prepared as in *Methods*. (A) [^{35}S]GTPγS binding is stimulated by μ agonist DAMGO in brainstem membranes from mice treated *in vivo* with saline (i.p.), affording an EC_{50} value of 850 ± 220 nM. In contrast, there is a decrease in the total stimulation by DAMGO application (saline: 117 ± 11% stimulation over baseline; morphine: 68 ± 7.9%; $p<0.05$) and a non-significant 2.1-fold rightward shift in the concentration-response curve for DAMGO (morphine EC_{50}: 1040 ± 360) following *in vivo* treatment with 32 mg/kg morphine (i.p.). (B) *In vivo* NTI pretreatment of morphine treated animals improves stimulation by DAMGO *ex vivo* following morphine treatment ((NTI-morphine): 100 ± 20% stimulation over baseline, $p<0.05$ compared to saline-treated controls; EC_{50}: 1290 ± 200 nM).

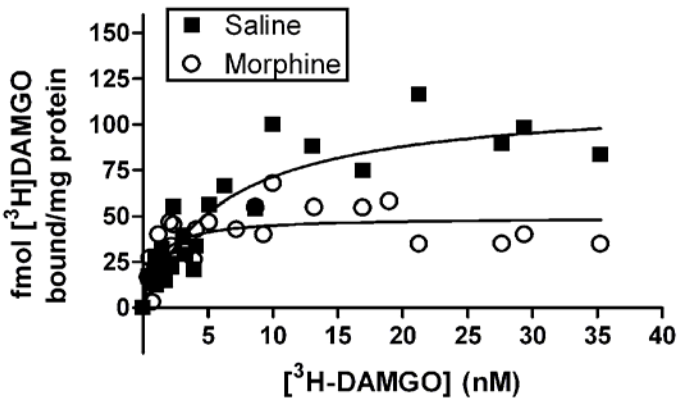

Figure 2.4. Loss in high-affinity μ receptor expression (labeled with [^{3}H]DAMGO) in brainstem membranes prepared 4 h after *in vivo* treatment of mice with a single injection of 32 mg/kg morphine (i.p.). Saline treated animals display a Bmax of 108 ± 20 fmol receptor/mg protein while morphine treated animals show a decrease in high-affinity μ receptors (Bmax: 57 ± 7 fmol/mg protein). There was no change in the Kd of DAMGO after morphine treatment (Kd (saline): 2.3 ± 0.7 nM, Kd (morphine treated): 2.4 ± 1.6 nM; $p>0.05$).

Chapter III

Pentapeptides displaying mu opioid receptor agonist and delta opioid receptor partial agonist/antagonist properties

Summary

Chronic use of μ agonists has been shown to cause neurochemical adaptations resulting in tolerance and dependence. While the analgesic effects of these drugs are mediated by the μ receptor, several studies have shown that antagonism or knockdown of δ receptors can lessen or prevent development of tolerance and dependence. Based on computational modeling of putative active and inactive conformations of both μ and δ receptors, I have synthesized a series of pentapeptides with the goal of developing a μ agonist/δ antagonist peptide with similar affinity at both receptors as a tool to probe functional opioid receptor interaction(s). The eight resulting naphthylalanine-substituted cyclic pentapeptides displayed variable mixed-efficacy profiles. The most promising peptide (**9**; Tyr-c(SCH_2S)[DCys-Phe-2-Nal-Cys]NH_2) displayed a μ agonist and δ partial agonist/antagonist profile and bound with equipotent affinity (Ki ~ 0.5 nM) to both receptors, but also showed κ receptor agonist activity.

Introduction

Mu (μ) opioid receptor agonists such as morphine are commonly used in the treatment of moderate to severe pain. However, use of such drugs is associated with side effects including the development of tolerance, limiting the usefulness of these

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compounds. It has been hypothesized that opioid compounds displaying μ agonism paired with a selective δ or κ receptor effects could lessen the severity of limiting side effects surrounding current μ agonist use (Schiller, 2009), including respiratory depression and constipation as well as tolerance. In particular, studies pointing to a role of the δ receptor in modulating the development of μ agonist-induced tolerance have led to the hypothesis that both μ and δ receptors play major roles in the development of tolerance after chronic morphine exposure. For example, work in δ receptor knockout rodent models (Kest *et al.*, 1996; Nitsche *et al.*, 2002; Zhu *et al.*, 1999) or using δ antagonists (Abdelhamid *et al.*, 1991; Daniels *et al.*, 2005; Fundytus *et al.*, 1995; Hepburn *et al.*, 1997) was shown to prevent or lessen the severity of tolerance development to chronic morphine exposure. More recent *in vivo* work also points to a role of the δ receptor in modulating morphine-induced behavioral sensitization and conditioned place preference in rodents (Chefer *et al.*, 2009; Shippenberg *et al.*, 2009; Timar *et al.*, 2005). It has been hypothesized that the formation of homo- or heterodimers of μ and δ receptors leads to changes in their pharmacological behaviors including alteration in tolerance or dependence development (Daniels *et al.*, 2005; George *et al.*, 2000; Gomes *et al.*, 2004; Rozenfeld *et al.*, 2007a).

The growing body of evidence implicating a role of the δ receptor in modulating μ agonist-induced tolerance suggests that opioid ligands with similar affinities at μ and δ, but displaying agonism at μ and antagonism at δ might be of great clinical potential, especially for the treatment of chronic pain conditions. Consequently, many groups have developed compounds with μ and δ receptor affinity, including peptidic (Balboni *et al.*, 2002a; Balboni *et al.*, 2002b; Salvadori *et al.*, 1999a; Schiller *et al.*, 1999; Yamamoto *et al.*, 2008) and non-peptidic (Ananthan *et al.*, 1998; Ananthan *et al.*, 1999; Ananthan *et al.*, 2004; Cheng *et al.*, 2007; Hiebel *et al.*, 2007) ligands displaying μ agonism and δ antagonism. However, many of these compounds, while displaying the desired efficacy profile, do not have equivalent binding affinities to both receptors, thus limiting their usefulness in probing μ-δ receptor interactions.

Our previous work led to the synthesis of peptide **1** (Tyr-c(SCH_2CH_2S)[DCys-Phe-Phe-Cys]NH_2 (Przydzial *et al.*, 2005b). Peptide **1** displayed a promising mixed-efficacy profile at μ and δ receptors, binding with high affinity to both μ and δ receptors while exhibiting full agonism at the μ and κ receptors, but only partial agonism at the δ receptor. I wished to improve peptide **1** by decreasing efficacy at the δ receptor while increasing affinity, retaining both efficacy and affinity at the μ receptor, and reducing affinity at the κ receptor. To pursue this aim, *in silico* docking of **1** into computational models of μ and δ receptors was performed. Based on modeling of putative active and inactive conformations of μ and δ receptors (Fowler *et al.*, 2004a; Fowler *et al.*, 2004b; Pogozheva *et al.*, 1998; Pogozheva *et al.*, 1997), and docking of **1** to these models, I focused on steric constraints surrounding the third and fourth Phe residues of **1.** I hypothesized that replacement of these Phe residues with bulkier side chains would decrease ligand affinity to the δ receptor active state, but not the inactive state and would not affect binding to the μ receptor, thus favoring the desired μ agonist/δ antagonist profile. Consequently, I designed and synthesized eight analogues of peptide **1** containing naphthylalanine in place of Phe^3 or Phe^4 to more fully explore the steric limits of the receptor binding pocket at either of these positions. The Mosber lab has previously used naphthylalanine substitution to add steric bulk in cyclic peptides (Heyl *et al.*, 1992) and this has been more recently applied to linear peptides (Fichna *et al.*, 2008). *In vitro*, our cyclic peptides displayed variable μ receptor efficacies and had decreased δ receptor efficacy. One compound (peptide **9**; Tyr-c(SCH_2S)[DCys-Phe-2-Nal-Cys]NH_2) displayed full agonism at the μ receptor (99% stimulation compared with the full μ agonist DAMGO) and was an antagonist at δ in the [^{35}S]GTPγS assay, but with partial agonist activity in the adenylyl cyclase inhibition assay. Although this compound retained κ efficacy and affinity, **9** bound with similar subnanomolar affinity to μ and δ receptors stably and independently expressed in C_6 rat glioma cells. Thus, incorporation of a substitution based on rational design and intended to highlight putative steric constraints resulted in a compound that had similar affinity for μ and δ receptors but

decreased δ efficacy without compromising μ agonism, an important step forward in the development of novel ligands presenting μ agonist and δ antagonist effects.

Results

Rationale for the design of pentapeptides displaying μ agonism and δ antagonism.

Previous work by our group led to the synthesis of the high-affinity, μ-selective cyclic pentapeptide **1** (Tyr-c(SCH_2S)[DCys-Phe-Phe-Cys]NH_2; Przydzial *et al.*, 2005b) which has picomolar affinity for the μ receptor (Ki = 0.016 nM) and nM affinities for the δ (Ki = 1.8 nM) and κ (Ki = 2.5 nM) receptors. When evaluated for efficacy at the three opioid receptors using the [^{35}S]GTPγS assay (Traynor *et al.*, 1995), **1** displayed full agonism at μ and κ receptors, but only partial agonism at the δ receptor (Table 3.1). In order to understand the molecular mechanism underlying the mixed-efficacy profile of **1**, computational models of μ and δ receptors were utilized. Peptide **1** was virtually docked in models of active and inactive conformations of the μ and δ receptors (Fowler *et al.*, 2004a; Fowler *et al.*, 2004b; Pogozheva *et al.*, 1998; Pogozheva *et al.*, 1997; Figure 3.1) in a similar manner as μ- and δ-selective tetrapeptides, JOM-6 and JOM-13, respectively, which were previously positioned in the μ and δ receptor models based on their structure-activity relationships and receptor mutagenesis data (Fowler *et al.*, 2004a; Fowler *et al.*, 2004b; Mosberg *et al.*, 2002; Pogozheva *et al.*, 2005). These receptor models have been designated as active and inactive receptor conformations of the μ and δ receptors based on the published crystal structure of the α_2-adrenergic receptor (Rasmussen *et al.*, 2007; as described in the *Materials and Methods* section). Though the new adrenoreceptor-based models of opioid receptors differ from our previously published rhodopsin-based models (Fowler *et al.*, 2004a; Mosberg *et al.*, 2002; Pogozheva *et al.*, 1998; Pogozheva *et al.*, 1997) by some helix shifts and an outward movement of extracellular loop (EXL) 2 (r.m.s.d. in the range 2.3-2.6 Å for all Cα-atoms, excluding EXL2), the ligand binding mode and receptor-ligand interactions with residues from TMs 3, 5, 6 and 7 of either the δ or μ receptor remained essentially the same. I found that the docking mode of peptide

ligands is more influenced by its interactions with helix residues than residues from EXL2, whose modeling is expected to be less accurate.

Examination of the position of pentapeptide **1** inside the binding pocket of the active and inactive μ receptor model (Figure 3.1A, B) allowed development of a low-energy conformation of **1** that did not have steric hindrances or other adverse interactions with residues in the receptor binding pocket in either receptor state. Peptide **1** docked in the μ receptor model showed favorable aromatic interactions between its Phe^3 side chain and the Trp^{318} side chain in transmembrane helix 7 (TM7), which were more pronounced in the active conformation of the μ receptor. These aromatic interactions may explain the preferential binding of **1** to μ, as compared to the δ or κ receptors, which have Leu^{300} or Tyr^{312} at the corresponding position. The same conformation of **1**, when fitted into the active δ receptor model (Figure 3.1C), demonstrated steric overlap between Phe^4 of the ligand and Trp^{284} from the TM6 of the δ receptor which was not observed in the inactive δ receptor model (Figure 3.1D). Docking of peptide **1** to both active and inactive conformations of the μ receptor and better compatibility with the inactive conformation of the δ receptor is consistent with the μ agonist/δ partial agonist profile of **1**.

Based on the above, I hypothesized that incorporation of a bulkier naphthylalanine side chain in either the third or fourth position of pentapeptide **1** would affect its binding and efficacy properties differentially at μ and δ receptors and could result in potent μ agonist/δ antagonist ligands. To test this hypothesis, I focused on replacement of Phe^3 and Phe^4 of **1** with the bulkier 3-(1-naphthyl)alanine or 3-(2-naphthyl)alanine (Figure 3.2) to provide eight new cyclic pentapeptides (**2-9**; Tables 3.1, 3.2). Peptides were cyclized *via* a disulfide bond (SS) or methylene dithioether (SCH_2S) linkage to allow for altered size and flexibility of the cycle.

Analysis of synthesized pentapeptides 2-9 for binding affinity and efficacy at opioid receptors.

Most naphthylalanine peptides demonstrated relatively high binding affinities to the μ, δ, and κ receptors as measured by competitive displacement of the radiolabeled non-selective opioid antagonist [^{3}H]diprenorphine. However, some loss in affinity to all

three opioid receptors was generally noted as compared to the highly potent pentapeptide **1**. At the μ receptor, the most significant loss of affinity occurred with peptide **6** ($p < 0.001$) while at the δ receptor four peptides had ≥ 10-fold decreased affinity (**2**, **3**, **4** $p < 0.001$, and **5** $p < 0.05$). At the κ receptor, peptides **4** and **5** had > 10-fold decreased binding affinity when compared to **1** ($p < 0.001$; Table 3.1). Of the eight peptides synthesized, only peptide **9** (Tyr-c(SCH_2S)[DCys-Phe-2-Nal-Cys]NH_2) showed similar low nanomolar binding affinity to the μ, δ, and κ receptors (Ki = 0.47 nM, 0.48 nM, and 1.3 nM, respectively).

Peptides **2-9** were also analyzed for efficacy at the μ, δ, and κ receptors as determined by maximal stimulation of [^{35}S]GTPγS binding (Traynor *et al.*, 1995) as a percentage of 10 μM opioid agonists DAMGO, SNC80, and U69,593 (Table 3.1). Peptides **3**, **6**, and **8** showed equivalent full agonism at the μ receptor as peptide **1** (~ 90% maximal stimulation), while peptides **2**, **4**, and **5** had decreased efficacy at the μ receptor (31-34% stimulation; $p < 0.001$). In contrast, peptides **7** and **9** showed greater stimulation of [^{35}S]GTPγS binding at the μ receptor than **1** ($p < 0.05$), displaying the same maximal stimulation. It is noteworthy that all peptides displaying decreased efficacy at the μ receptor (**2**, **4**, and **5**) were substituted at position 3 with naphthylalanine. In contrast, analogs with naphthylalanine substitution at position 4 (**6-9)** showed equal or greater efficacy at the μ receptor, indicating substitution at position 3 by residues with bulky side chains is deleterious for μ agonism. One exception was peptide **3**, which has naphthylalanine substitution at position 3 and displayed high efficacy at the μ receptor. Peptide **3** was cyclized *via* a methylene dithioether, possibly allowing greater flexibility of the peptide ring structure and leading to increased stimulation over the disulfide-cyclized counterpart **2**. At the δ receptor, peptides **2**, **4**, **8**, and **9** behaved essentially as antagonists, providing very little or no [^{35}S]GTPγS stimulation. Compounds **3**, **5**, **6**, and **7** displayed partial agonism with maximal stimulation varying from 15-23%. The eight peptides displayed varying efficacy profiles at the κ receptor, with most compounds behaving as partial to full agonists (maximal stimulation 35-100%).

Docking of peptide 9 to modeled μ and δ receptor active and inactive conformations.

The binding and efficacy studies described above identify peptide **9** as a candidate ligand displaying the desired μ agonist and δ antagonist profile. In order to better understand the mechanism of the decreased efficacy of **9** at the δ receptor, docking studies of this peptide in the modeled active and inactive conformations of μ and δ receptors similar to the docking of peptide **1** (Figure 3.3) was performed.

The lowest energy conformation of peptide **9** exhibited no hindrance in the binding pocket of either active or inactive conformations of the μ receptor (Figure 3.3A, B), nor in the inactive δ receptor conformation (Figure 3.3D). However, when compared to peptide **1** (Figure 3.2C), **9** shows greater overlap between its 2-Nal^4 and Trp^{284} in the active conformation of the δ receptor (Figure 3.3C). This reduced compatibility of **9** relative to **1** for the active state of the δ receptor supports the decreased agonism at δ. Modeling results also explain the high agonist efficacy of **9** at the μ receptor, because **9** fits both receptor states well and may promote a conformational shift toward the active conformation of the μ receptor where favorable aromatic interactions between Phe^3 and Trp^{318} of the μ receptor are more prominent.

Characterization of the functional properties of peptide 9 at opioid receptors.

In the [^{35}S]GTPγS binding assay, peptide **9** behaved as a full agonist at the μ receptor with EC_{50} of 1.2 ± 0.05 nM (Figure 3.4A). On the other hand, at 10 μM concentration **9** produced only 7% of SNC80-induced stimulation of δ receptor-mediated [^{35}S]GTPγS binding (Table 3.1).

The properties of **9** were further evaluated by measuring its ability to inhibit SNC80-stimulated binding of [^{35}S]GTPγS to G-proteins. Peptide **9** produced a 3.1-fold rightward shift in the dose-response curve of SNC80 in C_6-δ containing cells (Figure 3.4B); the EC_{50} for SNC80 was shifted from 75 ± 3.8 nM to 188 ± 31 nM in the presence of 100 nM **9** ($p = 0.02$). However, this shift and the calculated Ke value (48 ± 9.5 nM) for **9** was not consistent with its high binding affinity to the δ receptor (Ki = 2.1 nM), indicating **9** may have some partial agonist efficacy at the δ receptor which cannot be

fully observed using the high efficacy-requiring [^{35}S]GTPγS binding assay. To more fully assess the extent of this partial agonism, I measured the ability of **9** to inhibit cAMP accumulation as a measure of adenylyl cyclase activity (Clark *et al.*, 2004). Due to downstream signaling amplification, it is easier to visualize partial agonism using this system. Peptide **9** was shown to be more potent (EC_{50}: 36 ± 4.8 nM) than SNC80 (EC_{50}: 166 ± 43 nM; p = 0.01) and behaved as a partial agonist, able to produce 55% inhibition of that seen with SNC80 (Figure 3.4C). The δ-selective antagonist naltrindole (NTI) was without effect in this assay.

Discussion

The current studies were aimed toward the development of a potent compound with mixed μ agonist/δ antagonist properties, a profile that would be valuable to probe interactions of μ and δ receptors and that has considerable clinical promise. The eight newly synthesized cyclic pentapeptides represent modifications of our previously reported pentapeptide **1** (Tyr-c(SCH_2S)[DCys-Phe-Phe-Cys]NH_2; Przydzial *et al.*, 2005b), which was characterized by high affinity binding to all opioid receptors and a mixed efficacy profile. Based on our computational modeling of active and inactive conformations of μ and δ receptors and docking of **1** to these models (Figure 3.1), I focused on receptor-ligand interactions surrounding Phe^3 and Phe^4 residues of **1**. I designed and synthesized several derivative peptides containing naphthylalanine substitution to more fully explore the steric limits of the receptor binding pocket at either of these positions. All peptides were evaluated for their potential to interact at both μ and δ receptors *via* receptor binding and *in vitro* functional studies. The newly synthesized peptides demonstrated μ agonism with variable efficacies and had greatly decreased δ efficacy in the [^{35}S]GTPγS binding assay. One compound, peptide **9** (Tyr-c(SCH_2S)[DCys-Phe-2-Nal-Cys]NH_2), bound with similar subnanomolar affinity to μ and δ receptors stably expressed in rat glioma cells and was characterized as an agonist at the μ receptor and an antagonist or partial agonist at the δ receptor, depending on the

assay used. This latter difference highlights the importance of the choice of assay in efficacy determination (Nieland *et al.*, 2006).

The development of pentapeptide **9** represents a significant step forward in the development of a mixed-efficacy μ agonist/δ antagonist ligand. Previously reported mixed-efficacy ligands did not show the same equipotent affinity for both μ and δ receptors (Ananthan *et al.*, 1998; Ananthan *et al.*, 1999; Ananthan *et al.*, 2004; Balboni *et al.*, 2002a; Balboni *et al.*, 2002b; Cheng *et al.*, 2007; Hiebel *et al.*, 2007; Salvadori *et al.*, 1999a; Yamamoto *et al.*, 2008) or the same full μ agonist properties (Ananthan *et al.*, 1999; Ananthan *et al.*, 2004; Schiller *et al.*, 1999). These results also represent a validation of our receptor models and a novel demonstration of the use of differences in modeled active and inactive states to design ligands with prescribed properties. In this example, steric differences in the binding site of the active and inactive δ receptor models were exploited by incorporating bulkier naphthylalanine in place of phenylalanine in residues 3 and 4 of lead peptide **1** to generate ligands with the desired μ agonist/δ antagonist profile.

Although peptide **9** displays the desired μ agonist/δ antagonist mixed-efficacy profile, it also acts as a full agonist at the κ receptor in the [^{35}S]GTPγS binding assay with EC_{50} of 12 ± 0.1 nM although it is 10-fold selective in potency for μ receptors over κ (Figure 3.4A). This residual κ receptor activity is not surprising, as compound **1** was initially developed in a series aiming to improve κ receptor binding and efficacy (Przydzial *et al.*, 2005b). Using computational models of the κ receptor developed as described above for putative active and inactive conformation models of the μ and δ receptors, future studies will focus on development of analogs that are intended to exhibit reduced κ receptor affinity while retaining the desired μ agonist/δ antagonist profile. Such compounds will allow the characterization of μ and δ receptor interactions without the potential complication of concomitant κ receptor activation or antagonism.

Materials and Methods

Fmoc-protected amino acids were obtained from Advanced ChemTech (Louisville, KY) or Sigma-Aldrich (St. Louis, MO). Other reagents for peptide synthesis and characterization were from Sigma-Aldrich (St. Louis, MO) unless otherwise indicated. Fetal bovine serum, cell culture media and additives were purchased from Gibco Life Sciences (Grand Island, NY). [D-Ala^2, N-Me-Phe^4, Gly^5-ol]-enkephalin (DAMGO) and other biochemicals were obtained from Sigma-Aldrich and were of analytical grade. 4-[(*R*)-[(2*S*,5*R*)-4-allyl-2,5-dimethylpiperazin-1-yl](3-methoxyphenyl)methyl]-N,N-diethylbenzamide (SNC80) was obtained from the Narcotic Drug and Opioid Peptide Basic Research Center at the University of Michigan (Ann Arbor, MI). [^{35}S]-guanosine-5'-O-(3-thio)triphosphate ([^{35}S] - GTPγS; 1250Ci (46.2TBq)/mmol) and [^{3}H]-diprenorphine were from from Perkin Elmer (Boston, MA).

Structural modeling: Homology modeling of the inactive conformation of human δ (residues 45-338, UniProt accession code P41143) and mouse μ receptors (residues 64-354, UniProt accession code P42866) was performed as previously described (Fowler *et al.*, 2004a; Fowler *et al.*, 2004b; Przydzial *et al.*, 2005a) using the more recent structure of the β_2-adrenergic receptor fused with T4 lysozyme (2rh1 PDB code; Rasmussen *et al.*, 2007). Distance geometry calculations with DIANA (Guntert *et al.*, 1991) were used to provide helix shift and loop modeling. To model the active receptor conformation, several structural constraints between TM 3, 5, and 6 were included that have been shown to be compatible with active states of different GPCRs. (Fowler *et al.*, 2004b). Introduction of these constraints allowed reproduction of the significant movement of TM6 that has been suggested based on numerous experimental studies of different GPCRs, as well as on the comparison of the rhodopsin (1f88; Palczewski *et al.*, 2000a) and opsin (3dqb; Scheerer *et al.*, 2008) crystal structures.

3D structures of cyclic pentapeptides were generated by QUANTA (Accelrys Inc.) using residue substitution of previously modeled pentapeptides (Przydzial *et al.*, 2005b), followed by molecular mechanics computations using the CHARMm force field. Several conformations of disulfide or methylene dithioether bridges and different

rotamers of residues in the third and fourth positions of pentapeptides were tested during ligand docking. Several low energy conformations (within 2 kcal/mol) were manually positioned inside the receptor binding cavity similarly to tetrapeptides JOM-6 and JOM-13, whose docking in δ and μ receptors has been previously justified using conformational and mutagenesis analysis (Fowler *et al.*, 2004a; Fowler *et al.*, 2004b; Mosberg *et al.*, 2002; Pogozheva *et al.*, 2005). During ligand docking, low energy conformations were chosen that satisfied the following criteria: (1) provided interactions between Tyr^{1} and Phe^{3} and functionally important receptor residues (Asp^{147} from TM3, His^{297} from TM6 and Trp^{318} from TM7 of the μ receptor (Fowler *et al.*, 2004b) or corresponding Asp^{128}, His^{278}, and Leu^{300} in the δ receptor; (2) had minimal steric overlap; and (3) formed more hydrogen-bonds between receptor and ligand polar groups. The docking pose of each ligand was subsequently refined using the Solid Docking module of QUANTA. The opioid receptor models are available upon request.

Solid Phase Peptide Synthesis: All peptides were synthesized by solid phase methods (Stewart, 1984) on an ABI Model 431A solid phase peptide synthesizer (Applied Biosystems, Foster City, CA) as previously published (Przydzial *et al.*, 2005b). Rink resin (Advanced ChemTech) was used as the solid support for C-terminal carboxamide peptides. Peptide elongation on the peptide-resin involved treating resin with piperidine (Sigma-Aldrich) to cleave the Fmoc-protecting group, diisopropylethylamine (DIEA) activation, followed by coupling of the next amino acid with o-benzotriazol-1-yl-N,N,N',N'-tetramethyl uronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt; Applied Biosystems). These steps were repeated until the entire peptide was assembled. A solution of trifluoroacetic acid/H_2O/tri-isopropylsilane (9:0.5:0.5, v/v/v) was used to cleave the linear peptide from the resin and simultaneously remove the side chain-protecting groups. The peptide solution was filtered from the resin and subjected to semi-preparative reverse phase high-performance liquid chromatography (RP-HPLC) to afford the linear disulfhydryl-containing peptide (Table 3.2). Final product was obtained by high resolution mass spectroscopy (HRMS; Protein Structure Facility, University of Michigan, Ann Arbor MI).

General method for disulfide cyclization of peptides: To obtain disulfide-cyclized peptides, linear disulfhydryl-containing peptide was dissolved in a 1% (v/v) acetic acid (AcOH) in H_2O solution (saturated with N_2) at 5°C (1mg linear peptide/mL of aqueous AcOH solution). The pH of the peptide solution was raised to 8.5 using NH_4OH, followed by the addition of 4 equiv of $K_3Fe(CN)_6$. The reaction mixture was stirred for 2 min and quenched by adjusting the pH to 3.5 with AcOH. The mixture was then subjected to semi-preparative RP-HPLC to afford the disulfide-cyclized peptide.

General method for methylene dithioether cyclization of peptides: To form methylene dithioether-containing cyclic peptides, linear disulfhydryl peptide was added to dimethylformamide (DMF) and maintained at 5°C under a N_2 atmosphere (0.1mg linear peptide/mL DMF). Approximately 10 equiv of potassium *t*-butoxide was added to the peptide solution, followed by the addition of 10 equiv of dibromomethane. The reaction was quenched with 5 mL AcOH after 2 h and the solvent removed *in vacuo*. The residue was dissolved in water, filtered, and subjected to semi-preparative RP-HPLC to obtain the methylene dithioether cyclized peptide.

*Characterization of Peptides:*Final product peptides were >97% pure as assessed by analytical RP-HPLC on a Vydac 218TP C-18 column (The Nest Group, Southboro, MA) using two solvent systems; (A): 0.1% trifluoroacetic acid (TFA) in water (w/v)/0.1% TFA in acetonitrile with a gradient of 0–70% organic component in 70 min and (B): 0.1% TFA in water/0.1% TFA in methanol with a gradient of 20-70% organic component in 50 min, monitored at 230 nm. Peptides displayed the appropriate molecular weights as determined by HRMS (Table 3.2).

Cell Lines and Membrane Preparations: C_6-rat glioma cells stably transfected with a rat μ (C_6-μ) or rat δ (C_6-δ) opioid receptor (Lee *et al.*, 1999) and Chinese hamster ovary (CHO) cells stably expressing a human κ (CHO-κ) opioid receptor (Husbands *et al.*, 2005) were used. Cells were grown to confluence at 37°C in 5% CO_2 in either Dulbecco's Modified Eagle's Medium (DMEM; C_6 cells), or DMEM-F12 Medium (CHO-κ) containing 10% fetal bovine serum. To prepare membranes for biochemical assays (Clark *et al.*, 2003), confluent cells were washed twice with ice-cold phosphate-buffered saline (0.9% NaCl; 0.61mM Na_2HPO_4; and 0.38mM KH_2PO_4, pH 7.4), detached from the

plates by incubation in harvesting buffer (20mM HEPES, pH 7.4; 150mM NaCl; and 0.68mM EDTA) at room temperature, and pelleted by centrifugation at 200x*g* for 3 min. The cell pellet was suspended in ice-cold 50mM Tris-HCl buffer, pH 7.4 and homogenized with a Tissue Tearor (Biospec Products, Inc., Bartlesville, OK) for 20 s at setting 4. The homogenate was centrifuged at 20,000x*g* for 20 min at 4°C and the pellet re-homogenized in 50mM Tris-HCl with a Tissue Tearor for 10 s at setting 2, followed by re-centrifugation. The final pellet was re-suspended in 50mM Tris-HCl, to 0.5-1.0 mg/ml protein and frozen in aliquots at -80°C. Protein concentration was determined using the BCA protein assay(Smith *et al.*, 1985) (Thermo Fisher Scientific, Rockford, IL) using bovine serum albumin as the standard.

Radioligand Binding Assays: Opioid ligand-binding assays were based on the competitive displacement of [^{3}H]diprenorphine by the test compound from membrane preparations containing opioid receptors as described above. (Przydzial *et al.*, 2005b) The assay mixture, containing membrane suspension (10-20 μg protein/tube) in 50 mM Tris-HCl buffer (pH 7.4), [^{3}H]diprenorphine (0.2 nM), and increasing concentrations of test peptide, was incubated at 25°C for 1 h to allow binding to reach equilibrium. Subsequently, the samples were filtered rapidly through GF/C filters (Whatman, Middlesex, UK) using a Brandel harvester and washed three times with ice-cold 50 mM Tris-HCl buffer. The radioactivity retained on dried filters was determined by liquid scintillation counting after saturation with EcoLume liquid scintillation cocktail (MP Biomedicals, Solon, OH) in a Wallac 1450 MicroBeta (PerkinElmer, Waltham, MA). Non-specific binding was determined using 10 μM naloxone. Ki values were determined from nonlinear regression analysis to fit a logistic equation to the competition data using GraphPad Prism 5.01 software (GraphPad Software, La Jolla, CA). The results presented are the mean from at least three separate assays, each performed in duplicate where the SEM (standard error of the mean) was less than 10% of the mean value.

[^{35}S]GTPγS Binding Assay: Agonist stimulation of [^{35}S]GTPγS binding was measured as described previously (Traynor *et al.*, 1995). Membranes (20–30 μg of protein/tube) were incubated in GTPγS binding buffer (50 mM Tris-HCl, pH 7.4; 100 mM NaCl; and 5 mM $MgCl_2$) containing 0.1 nM [^{35}S]GTPγS, 100 μM GDP, and varying

concentrations (0.1–10,000 nM) or a maximum dose (10 μM) of opioid peptides, compared with standards DAMGO, SNC80, or U69,593 (10 μM) in a volume of 500 μl for 1 h at 25°C. The reaction was terminated by rapidly filtering through GF/C filters and washing four times with 2 ml ice-cold GTPγS binding buffer. Retained radioactivity was measured as described in radioligand binding assay methods. Experiments were performed at least three times in duplicate. EC_{50} values were determined by nonlinear regression analysis using GraphPad Prism 5.01 software as described above. To determine antagonism of **9**, [^{35}S]GTPγS binding was determined for SNC80 in the presence or absence of 100 nM **9**. The IC_{50} value in the presence of 100 nM **9** was divided by the IC_{50} value for SNC80 alone, and this ratio (DR) was employed to calculate the Ke value using the equation Ke = [antagonist]/(DR-1).

Whole cell acute inhibition of adenylyl cyclase: Inhibition of adenylyl cyclase by opioid standards or test peptides was measured in C_6-δ cells grown to confluence in 24-well plates (Clark *et al.*, 2004). Cells were washed in serum-free DMEM at least 30 min prior to the start of the assay and incubated with vehicle or various concentrations (0.1 – 1000 nM) of SNC80, naltrindole, or peptide **9** in serum-free media containing 5 μM forskolin (FSK) and 1 mM 3-isobutyl-1-methylxanthine for 10 min at 37°C. The assay was quenched by replacing media with 1 ml ice-cold 3% perchloric acid and 30 min incubation at 4°C. A 400 μl aliquot of sample was neutralized with 2.5M $KHCO_3$ and centrifuged 1 min at 11,000x*g*. Cyclic AMP (cAMP) was measured from the supernatant using a radioimmunoassay kit from GE Healthcare (Piscataway, NJ), according to the manufacturer's instructions. Inhibition of cAMP accumulation by **9** or standard opioid ligands was calculated as a percent of FSK-stimulated cAMP accumulation in vehicle-treated cells. EC_{50} values were calculated for each compound using GraphPad Prism 5.01 software. Experiments were performed in duplicate and repeated a minimum of three times.

Statistical Analysis. Data were analyzed using Student's two-tailed *t* test or a one-way analysis of variance followed by Bonferroni's post-hoc test using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA www.graphpad.com). *p* values less than 0.05 were considered to be significant.

Table 3.1. Binding affinities and efficacies of cyclic opioid pentapeptides 1-9 at μ, δ, and κ receptors.

Peptide	Sequence	Linker	Ki (nM) ± SEM			Efficacy (%) ± SEM		
			μ	δ	κ	μ	δ	κ
1	Tyr-c[DCys-Phe-Phe-Cys]NH_2	SCH_2S	0.016 ± 0.01	1.8 ± 0.8	2.5 ± 1.5	88 ± 1.1	45 ± 2.3	93 ± 3.4
2	Tyr-c[DCys-1-Nal[a]-Phe-Cys]NH_2	SS	0.08 ± 0.04	34 ± 4.5	21 ± 1.7	33 ± 1.6	0	59 ± 3.1
3	Tyr-c[DCys-1-Nal-Phe-Cys]NH_2	SCH_2S	0.33 ± 0.04	34 ± 0.7	5.8 ± 2.9	91 ± 4.5	23 ± 3.4	77 ± 6.3
4	Tyr-c[DCys-2-Nal-Phe-Cys]NH_2	SS	0.72 ± 0.05	55 ± 0.3	145 ± 11	34 ± 1.5	0.7 ± 1.0	7.5 ± 2.1
5	Tyr-c[DCys-2-Nal-Phe-Cys]NH_2	SCH_2S	0.47 ± 0.2	15 ± 1.7	69 ± 0.6	34 ± 3.8	23 ± 1.6	35 ± 4.0
6	Tyr-c[DCys-Phe-1-Nal-Cys]NH_2	SS	2.5 ± 0.9	7.1 ± 2.3	11 ± 1.8	88 ± 3.6	15 ± 1.5	100 ± 7.4
7	Tyr-c[DCys-Phe-1-Nal-Cys]NH_2	SCH_2S	0.61 ± 0.08	5.2 ± 0.4	6.0 ± 1.5	100 ± 3.8	22 ± 2.3	87 ± 2.4
8	Tyr-c[DCys-Phe-2-Nal-Cys]NH_2	SS	1.2 ± 0.3	11 ± 6.4	5.9 ± 0.8	90 ± 1.4	2.0 ± 1.0	49 ± 3.6
9	Tyr-c[DCys-Phe-2-Nal-Cys]NH_2	SCH_2S	0.47 ± 0.2	0.48 ± 0.2	1.3 ± 0.4	99 ± 1.8	7.0 ± 2.3	89 ± 3.6

[a]3-(1-naphthyl)alanine and 3-(2-naphthyl)alanine substitution abbreviated as 1-Nal and 2-Nal, respectively. Cyclization abbreviated as SS for disulfide linkage and SCH_2S for methylene dithioether linkage. Experiments were performed in C_6-μ, C_6-δ, or CHO-κ cells as described in *Methods*. Binding affinities (Ki) were obtained by competitive displacement of radiolabeled [^{3}H]diprenorphine. Efficacy of pentapeptides at the three opioid receptors was determined using the [^{35}S]GTPγS binding assay. Efficacy is presented as percent of the maximal level of [^{35}S]GTPγS binding obtained with standard agonists for μ, δ, and κ receptors (DAMGO, SNC80, or U69,593, respectively) at a 10 μM concentration.

Table 3.2. Analytical data of peptides 2-9.

Peptide	MW		HPLC (min; Rt)[b]	
	Calculated	Observed	System A	System B
2	728.2		35.23	31.97
3	742.3		35.13	31.60
4	728.2		35.71	32.14
5	742.3		35.81	32.18
6	728.2		35.88	33.22
7	742.3		35.79	32.71
8	728.2		35.85	32.84
9	742.3		35.92	33.09

[a] Molecular weights verified by high resolution mass spectrometry (HRMS).

[b] Retention time assessed by analytical high-performance liquid chromatography (HPLC) using two solvent systems; (A): 0.1% trifluoroacetic acid (TFA) in water (w/v)/0.1% TFA in acetonitrile with a gradient of 0–70% organic component in 70 min and (B): 0.1% TFA in water/0.1% TFA in methanol with a gradient of 20-70% organic component in 50 min, monitored at 230 nm, samples in H_2O with 0.1% TFA (elution column heated at 35º C). All peptides had > 97% purity determined by HPLC.

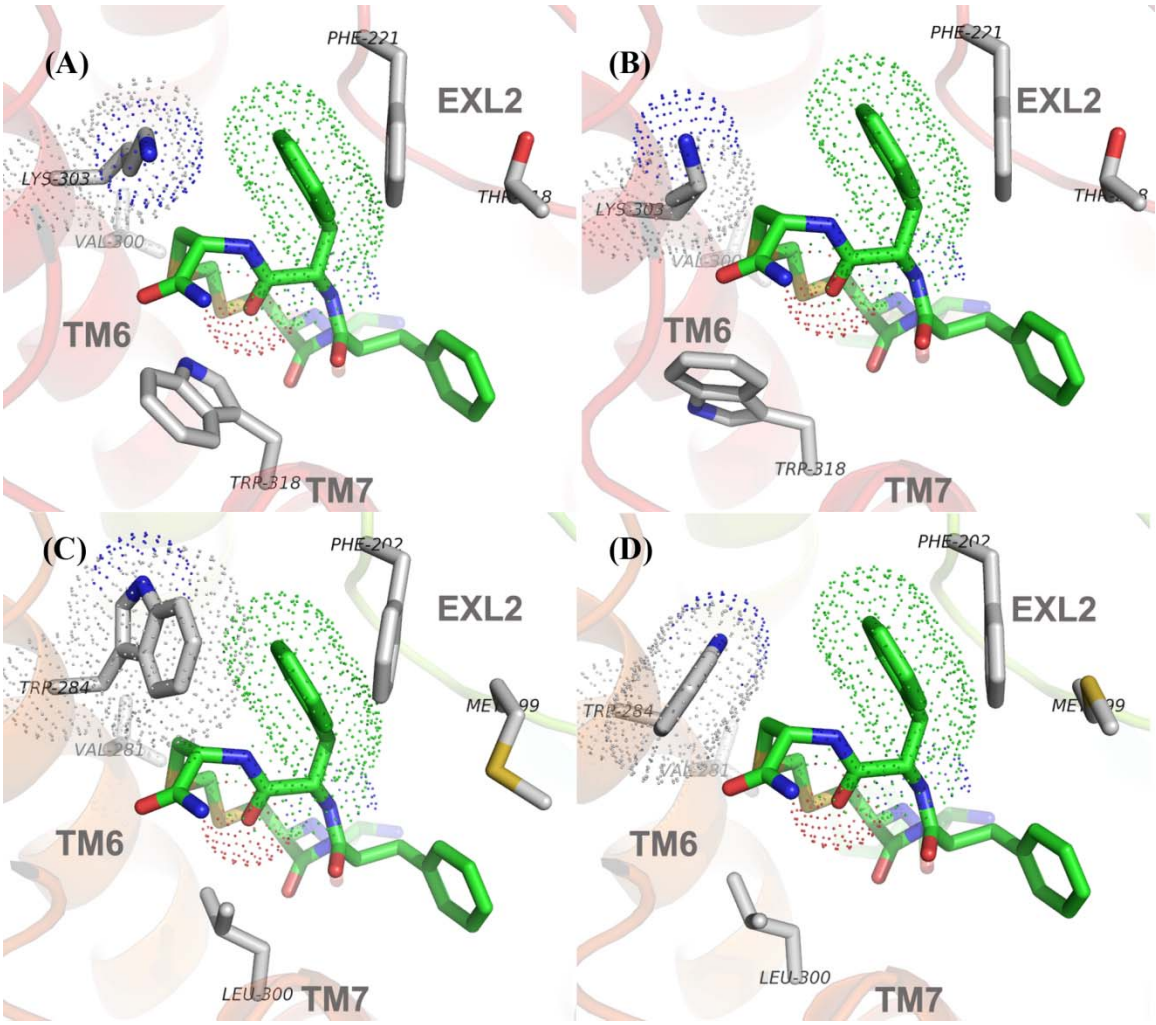

Figure 3.1: Modeling of peptide 1 in the binding pocket of putative active and inactive conformations of mouse μ and human δ opioid receptors. Peptide 1 docked in the putative active (A) and inactive (B) conformation of the μ receptor shows no noticeable unfavorable interactions between ligand side chains and residues from the receptor binding pocket. Peptide 1 docked in the active conformation of the δ receptor (C) shows a steric overlap of the peptide Phe4 side chain with the side chain of receptor Trp^{284} from transmembrane domain (TM) 6, while peptide 1 in the inactive conformation of the δ receptor (D) does not show a similar steric hindrance.

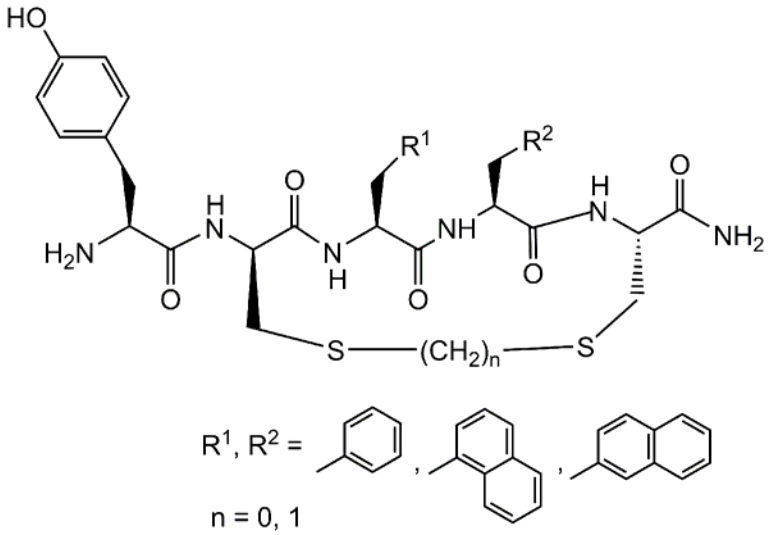

Figure 3.2: Naphthylalanine-containing analogues of peptide 1.

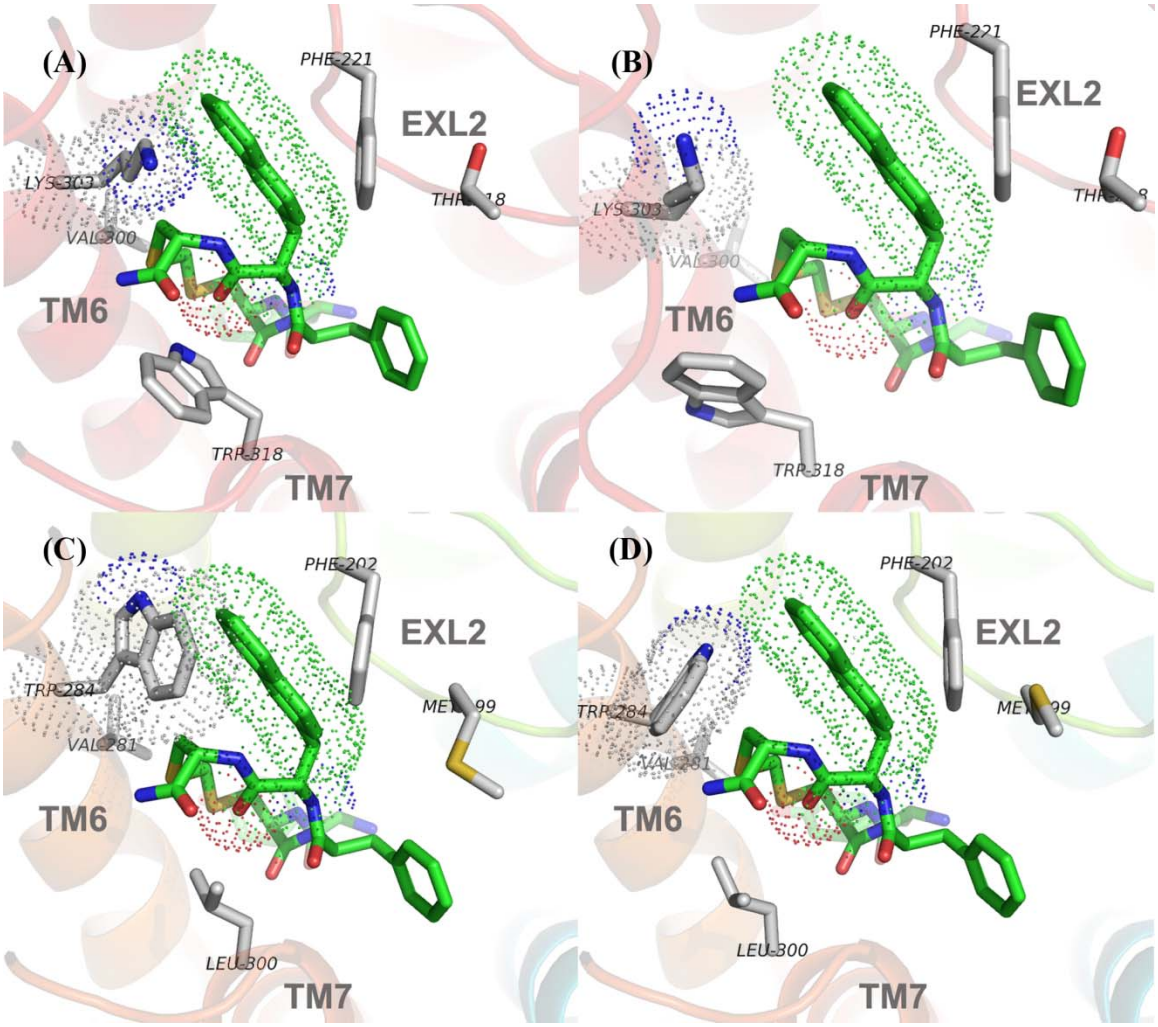

Figure 3.3: Pentapeptide 9 docked in the binding pocket of putative active and inactive conformations of mouse μ and human δ opioid receptors. The 2-naphthylalanine[4] side chain of peptide **9** shows minimal hindrance with receptor residue Lys^{303} in the (A) μ receptor active conformation but an increased steric overlap with Trp^{284} side chain in the (C) δ receptor active conformation. These hindrances are absent in the inactive conformations of both (B) μ and (D) δ receptor models.

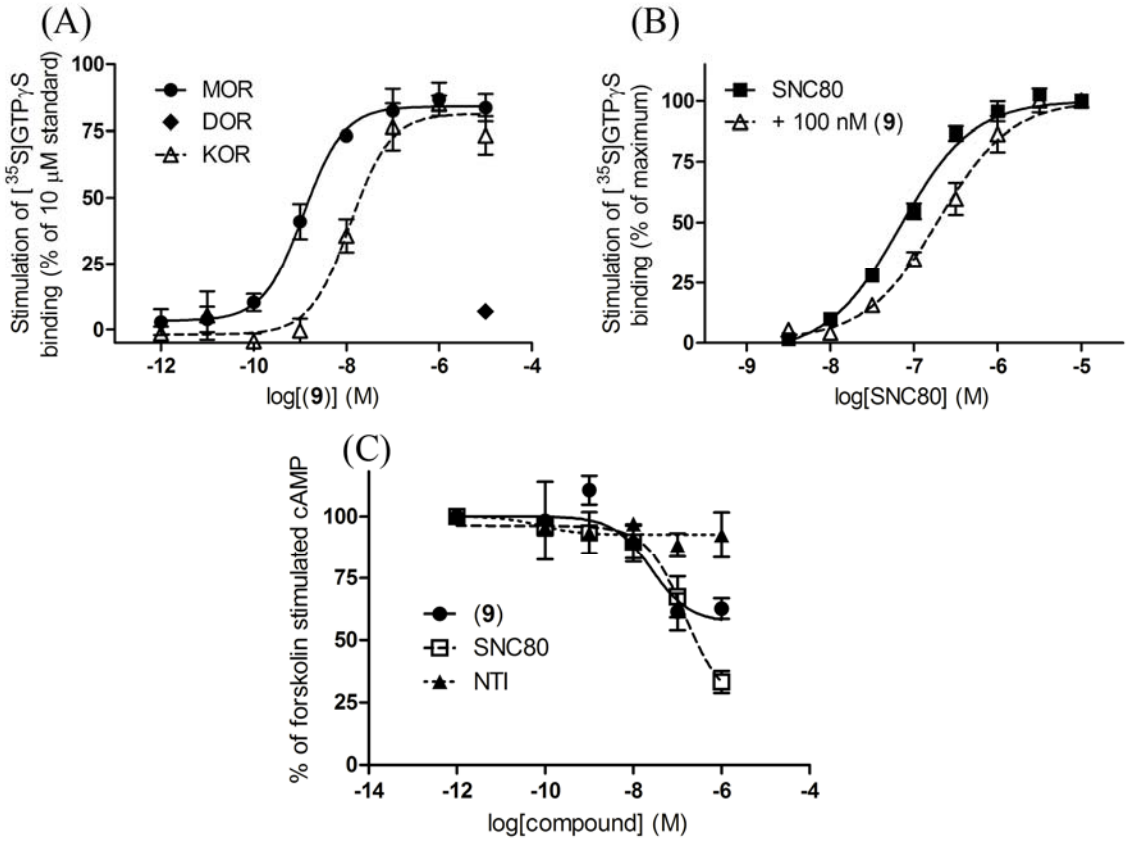

Figure 3.4: Pharmacological analysis of peptide 9. (A) Activity of peptide 9 in the [^{35}S]GTPγS binding assay at μ, δ, and κ receptors. Results are plotted as percent stimulation compared to a 10 μM concentration of opioid compounds (μagonist DAMGO, δ agonist SNC80, and κ agonist U69,593). Peptide **9** has 10-fold higher potency at μ receptors (EC_{50}: 1.2 ± 0.05 nM) than κ receptors (EC_{50}: 12 ± 0.1 nM) and 10 μM **9** produces only 6.9 ± 2.3% of SNC80-induced stimulation at the δ receptor. (B) δ Receptor antagonism of peptide **9** in the [^{35}S]GTPγS binding assay. Peptide **9** (100 nM) produces a 3.1-fold rightward shift in the SNC80 dose−response curve, indicating δ receptor antagonism. Calculated K_e = 48 ± 9.5 nM. Results are plotted as percentage of the maximum level of SNC80-stimulated [^{35}S]GTPγS binding. (C) Partial agonism of peptide **9** at the δ receptor as illustrated by inhibition of adenylyl cyclase. Results are shown as percent inhibition of 5 μM forskolin-stimulated adenylyl cyclase production in C_6-δ cells. SNC80 (1 μM) gave 67 ± 4.4% inhibition of adenylyl cyclase production with a calculated maximal effect of 76 ± 8.8%, while 1 μM peptide **9** produced 37 ± 4.2% inhibition with a calculated maximum of 43 ± 9.0% inhibition. Peptide **9** is more potent (EC_{50} = 36 ± 4.8 nM) than SNC80 (EC_{50} = 166 ± 43 nM) in this assay. The δ antagonist naltrindole (NTI) did not inhibit cAMP accumulation.

Chapter IV

Development and in vitro characterization of a novel bifunctional mu agonist/delta antagonist opioid tetrapeptide

Summary

The development of tolerance to and dependence on opioid analgesics greatly reduces their long-term usefulness. Previous studies have demonstrated that co-administration of a mu (μ) opioid receptor agonist and delta (δ) opioid receptor antagonist can decrease μ agonist-induced tolerance and dependence development after chronic exposure. Clinically, a single ligand displaying multiple efficacies (*e.g.* μ agonism concurrently with δ antagonism) would be of increased value over two drugs administered simultaneously. Guided by modeling of receptor-ligand complexes a series of potent non-selective opioid tetrapeptides that have differing efficacy at μ and δ receptors were developed. In particular, our lead peptide (KSK-103) binds with equal affinity to μ and δ receptors, acting as a μ agonist with similar efficacy but greater potency than morphine and a δ antagonist in cellular assays measuring both G protein stimulation and adenylyl cyclase inhibition.

Introduction

Opioid drugs such as morphine are the primary treatment for post-operative and chronic pain conditions through their actions at the mu (μ) opioid receptor. However, development of tolerance to and dependence on these drugs limits their usefulness. Thus, a novel ligand with analgesic properties but lacking tolerance and dependence liability would be of great value in clinical settings. Several published reports have documented that blockade of the delta (δ) opioid receptor, through either antagonism or knockdown of the receptor, leads to decreased development of tolerance or dependence in rodents

chronically treated with the μ agonist morphine (Hepburn *et al.*, 1997; Kest *et al.*, 1996; Nitsche *et al.*, 2002; Zhu *et al.*, 1999). Evidence for interactions between μ and δ receptors leading to altered signaling profiles has also been discussed in reference to locomotor sensitization (Chefer *et al.*, 2009; Shippenberg *et al.*, 2009) while other research has shown μ and δ cell-surface receptor expression levels to be linked (Cahill *et al.*, 2001b; Morinville *et al.*, 2003). The studies described above have highlighted roles for both μ and δ receptors in the development of morphine-provoked analgesic tolerance and/or dependence.

For pain relief, an optimal therapeutic would be a single drug containing two opioid receptor actions: μ agonism to promote analgesia along with δ antagonism to prevent μ agonist-induced tolerance and dependence development during chronic administration. Co-administration of two drugs acting separately at each individual receptor could be hampered by increased 'off-target' effects, differences in pharmacokinetic profiles, and user compliance (Morphy *et al.*, 2005; Morphy *et al.*, 2009). I focused therefore on the development and characterization of peptide ligands that simultaneously display μ agonism and δ antagonism while binding with equivalent affinity to each receptor.

The development of such bifunctional or mixed-efficacy ligands has become a topic of increasing interest in several therapeutic areas (Morphy *et al.*, 2009; Schiller, 2010). For example, bifunctional ligands have been proposed with δ-kappa (κ) opioid receptor efficacy (Daniels *et al.*, 2005) or μ-cholecystokinin receptor activities (Harikumar *et al.*, 2010), targeting tolerance liability (Lee *et al.*, 2006) or toward novel ligands for treatment of cocaine abuse (Maisonneuve *et al.*, 1994; Neumeyer *et al.*, 2003; Peng *et al.*, 2006). Similarly, early studies indicating that blockade of the δ receptor reduces the development of tolerance to μ agonists have stimulated several investigations into mixed μ agonist/δ antagonist compounds (Balboni *et al.*, 2002a; Purington *et al.*, 2009; Schiller *et al.*, 1999).

Small peptides and, in particular, receptor-specific ligands provide a means to determine structural or conformational requirements of binding to a particular membrane-

bound receptor. The Mosberg lab has previously used molecular modeling and conformationally restricted cyclic peptide ligands as tools to analyze determinants of ligand binding to μ, δ, and κ receptors. Through this methodology, the Mosberg lab developed peptides that bind selectively to different opioid receptors (McFadyen *et al.*, 2000b; Mosberg *et al.*, 1983; Mosberg *et al.*, 1988) and have expanded these concepts to develop peptides that bind non-selectively but with differing efficacy profiles. Recent work (Purington *et al.*, 2009) described the development of a non-selective opioid cyclic pentapeptide that displayed μ agonism, δ partial agonism, and κ agonism. The pentapeptides characterized in that study were designed to have decreased δ receptor efficacy compared with the parent ligand due to steric interactions inferred from ligand docking to a model of the active state of the δ receptor. Indeed, replacement of Phe residues in position 3 or 4 of the pentapeptide with bulkier 1-naphthylalanine (1-Nal) or 2-naphthylalanine (2-Nal) residues produced analogues with decreased δ efficacy, in agreement with modeling studies.

Current studies have extended this approach by re-examining previously synthesized, non-selective opioid peptides. As our earlier studies were aimed at development of selective opioid ligands for μ, δ, or κ receptors, resulting non-selective cyclic peptides were not evaluated beyond binding affinity. Re-evaluation of these previously synthesized ligands has led to the identification of potential leads with μ agonist/δ antagonist properties. Among these ligands were several analogs of the μ-selective tetrapeptide JOM-6 (Tyr-c(SCH_2CH_2S)[DCys-Phe-DPen]NH_2; McFadyen *et al.*, 2000a) and the δ-selective tetrapeptide JOM-13 (Tyr-c(SS)[DCys-Phe-DPen]OH; Mosberg *et al.*, 1994) where DPen is D-penicillamine (β, β–dimethyl-D-cysteine). Peptides were cyclized through the side chain sulfurs of DCys and DPen *via* an ethylene dithioether or a disulfide, respectively (denoted as c(SCH_2CH_2S) and c(SS), respectively). Modifications to these scaffolds included replacement of Phe^3 with a bulkier or more constrained aromatic residue that might be expected to bind differently to the active and inactive states of opioid receptors. Evaluation and further modification of the most promising candidates led to the two new analogs reported here, KSK-102 (Dmt-c(SCH_2CH_2S)[DCys-Aci-DPen]NH_2) and KSK-103 (Dmt-c(SCH_2CH_2S)[DCys-Aci-

DPen]OH), where Dmt is 2', 6'-dimethyltyrosine and Aci is 2'-aminoindane-2'-carboxylic acid (Figure 4.1). Of these peptides, KSK-103 displayed the desired bifunctional profile and behaved *in vitro* as a μ agonist with greater potency than the clinical standard morphine. KSK-103 was also found to be a δ antagonist at the level of receptor-G protein stimulation and at inhibition of the downstream effector enzyme adenylyl cyclase. By comparison, DIPP(Ψ)NH_2 (Dmt- Tic(Ψ)[CH_2NH_2]Phe-PheNH_2 (where Tic is tetrahydroisoquinoline-3-carboxylic acid; Schiller *et al.*, 1999) and UFP-505 (Dmt-Tic-GlyNH-benzyl; Balboni *et al.*, 2002a; Balboni *et al.*, 2010b), two previously described μ agonist/δ antagonist bifunctional peptides with reported decreased propensity to produce tolerance relative to morphine, displayed partial δ agonism in the adenylyl cyclase assay and had less desirable receptor binding properties.

Results

For development of the bifunctional peptides described here, I examined alterations to the tetrapeptide JOM-6 scaffold (McFadyen *et al.*, 2000b; Mosberg *et al.*, 1988) that included replacement of Tyr^1 with 2', 6'dimethyltyrosine (Dmt) and Phe^3 with the conformationally constrained 2-aminoindane-2-carboxylic acid (Aci). Additionally, C-terminal carboxamide (KSK-102) and carboxylic acid (KSK-103) containing analogs were compared. The computational docking of these peptides to the ligand binding pockets of models of active and inactive states of the μ and δ receptors, illustrated for KSK-103 in Figure 4.2, reveals a favorable interaction of Aci^3-containing peptides with the active and inactive states of the μ receptor (Figure 4.2A and 4.2B) and the inactive state of the δ receptor (Figure 4.2D), but a less favorable interaction with the δ receptor in the active state (Figure 4.2C). In particular, the conformationally constrained Aci^3 displays steric overlap with the bulky side chain of Met^{199} from extracellular loop 2 (EL2) of the active state δ receptor model. The corresponding residue in the μ receptor (Thr^{218}) has a smaller side chain, allowing favorable docking of Aci to the active μ receptor state.

Opioid Receptor Binding

The binding affinity of each peptide was determined at the μ, δ, and κ receptors from membrane preparations of C_6 rat glioma cells (μ or δ) or CHO cells (κ) (Table 4.1). As reported previously, JOM-6 displays 100-fold μ receptor selectivity in binding (Ki = 0.29 ± 0.04 nM affinity at the μ receptor and 25 ± 1.5 nM at the δ receptor; Table 4.1). Replacement of Tyr^1 with Dmt^1 and Phe^3 with Aci^3 while maintaining the same ring size with ethylene dithioether cyclization produced KSK-102. These alterations did not change the binding affinity at the μ receptor (0.6 ± 0.1 nM), but significantly increased affinity at the δ receptor (0.9 ± 0.2 nM) and at the κ receptor (9.8 ± 3.6 nM). Replacement of Tyr^1 with Dmt often results in decreased selectivity of the ligand by increasing the affinity at the less favored receptor (Balboni *et al.*, 2010a; Salvadori *et al.*, 1999b). Modification of the carboxamide by the carboxylic acid in KSK-103 also resulted in a slight decrease in binding affinity to both μ and δ receptors (2.4 ± 0.7 nM at the μ receptor and 2.3 ± 0.5 nM at the δ receptor) when compared to KSK-102.

DIPP(Ψ)NH_2 and UFP-505 were also analyzed for opioid receptor binding (Table 4.1). DIPP(Ψ)NH_2 binds equally well at μ and δ receptors with affinity values of 0.4 ± 0.1 nM at the μ receptor and 0.4 ± 0.04 nM at the δ receptor and has 10-fold selectivity for these receptors over the κ receptor (3.9 ± 0.2 nM). UFP-505, on the other hand, is δ receptor-selective, with a binding affinity of 0.2 ± 0.06 nM. UFP-505 binding affinity at the μ receptor is approximately 100-fold lower (26 ± 8 nM) and κ receptor affinity is reduced still further (128 ± 42 nM).

Stimulation of G protein.

The ability of the peptides to activate G protein at each receptor was assessed using the [^{35}S]GTPγS binding stimulation assay (Table 4.2). The [^{35}S]GTPγS binding results are reported as both maximal stimulation, employed as a read-out of efficacy as a percentage of the [^{35}S]GTPγS incorporation afforded by known opioid receptor agonist ([D-Ala^2-N-Me-Phe^4-Gly^5-ol]-enkephalin (DAMGO) at μ receptors, D-$Pen^{2,5}$-enkephalin (DPDPE) at δ receptors, and U69,593 at κ receptors) and potency as EC_{50} values.

KSK-102 and KSK-103 behaved as partial agonists at the μ receptor, giving maximal stimulation of 58 ± 8% and 59 ± 11%, respectively, compared to 10 μM DAMGO (Table 4.2, Figure 4.3A). By comparison, the clinically used analgesic morphine produced 57 ± 5% of DAMGO-induced stimulation and the endogenous μ receptor-selective peptide endomorphin-2 gave 49 ± 7% stimulation. Both KSK-102 and KSK-103 were far more potent at G protein stimulation than morphine (480- and 47-fold, respectively) or endomorphin-2 (310- and 31-fold, respectively). DIPP(Ψ)NH_2 displayed very low efficacy partial agonism at the μ receptor in this assay, giving a relative stimulations of 18 ± 1% compared with DAMGO (Table 4.2). UFP-505 displayed no significant efficacy at the μ receptor (maximal stimulation < 10%).

At the δ receptor, KSK-102 had the highest efficacy of all ligands tested, yielding 37 ± 4% stimulation compared to the δ peptide agonist DPDPE (Mosberg *et al.*, 1983), with an EC_{50} value of 1.4 ± 0.4 nM. In contrast, KSK-103 produced no significant stimulation of [^{35}S]GTPγS binding at the δ receptor (Table 4.2). In confirmation of this result, KSK-103 acted as an antagonist in this assay and produced a 26-fold rightward shift in the concentration-response curve for DPDPE (Figure 4.3B). The EC_{50} for DPDPE was shifted from 246 ± 45 nM to 6300 ± 1000 nM upon the addition of 100 nM KSK-103. The antagonist affinity constant (Ke) (Kosterlitz *et al.*, 1968) for KSK-103 calculated from this shift was 4.4 ± 1.4 nM in agreement with the binding affinity of KSK-103 for the δ receptor and suggesting neutral antagonism for this peptide.

Neither UFP-505 nor DIPP(Ψ)NH_2 caused detectable stimulation of [^{35}S]GTPγS binding at the δ receptor. At the κ receptor, none of the peptides produced significant stimulation of [^{35}S]GTPγS binding up to a concentration of 10 μM (Table 4.2).

Inhibition of Forskolin-Stimulated Adenylyl Cyclase.

The [^{35}S]GTPγS binding assay requires a compound to activate G protein under stringent conditions. As such, it is possible to misclassify a compound with downstream partial agonist activity as a pure antagonist in this assay (Purington *et al.*, 2009). Therefore, I examined the inhibition of adenylyl cyclase (measured as a decrease in forskolin-stimulated cAMP levels) by DPDPE in the presence or absence of KSK-103.

KSK-103 maintained the same δ antagonist profile at the downstream cellular effector adenylyl cyclase (Figure 4.4A). KSK-103 was unable to significantly reduce forskolin-stimulated cAMP accumulation even at a 10 μM concentration (91 ± 6% forskolin stimulation, or 9% inhibition) and at 100 nM KSK-103 produced a 9.5-fold rightward shift in the DPDPE concentration-response curve (EC_{50} DPDPE alone: 29 ± 7 nM; EC_{50} DPDPE + 100 nM KSK-103: 276 ± 69 nM), giving a calculated Ke value in the nM range (12 ± 3.3 nM), similar to results in the [^{35}S]GTPγS assay.

DIPP(Ψ)NH_2 and UFP-505 were also characterized further in the adenylyl cyclase inhibition assay. Both pseudopeptides were observed to have significant partial agonism at the δ receptor. DIPP(Ψ)NH_2 decreased forskolin stimulation of cAMP levels by 30 ± 4% with a potency (IC_{50}) of 2.2 ± 0.9 nM, while UFP-505 decreased cAMP levels by 28 ± 2% with an IC_{50} value of 0.7 ± 0.2 nM. (Figure 4.4B).

As both DIPP(Ψ)NH_2 and UFP-505 displayed very low μ partial agonist activity in the [^{35}S]GTPγS binding assay, they were further examined for the ability to inhibit adenylyl cyclase through activation of the μ receptor. Both DIPP(Ψ)NH_2 (48 ± 4% maximal inhibition, with an IC_{50} value of 5.1 ± 3.8 nM) and UFP-505 (35 ± 10% maximal inhibition with an IC_{50} value of 45 ± 13 nM) showed significant μ partial agonism in this assay (data not shown), underscoring the need to evaluate downstream signaling when stimulation of [^{35}S]GTPγS binding yields equivocal results.

Discussion

Development of bifunctional opioid peptides having distinct efficacy profiles at multiple receptors poses a challenge involving both understanding of receptor-ligand interactions and requirements for receptor-G protein activation. The desired profile for a μ-δ bifunctional ligand in these studies would combine high affinity binding to μ and δ receptors with much lower affinity for the κ receptor together with agonism at the μ receptor but antagonism at the δ receptor. I have synthesized and characterized a new cyclic tetrapeptide with the desired properties. Utilization of a conformationally constrained Aci^3, along with replacement of Tyr^1 by Dmt^1, and a C-terminal carboxylic

acid produced, in KSK-103, a ligand displaying the desired characteristics. This is in agreement with predictions from computational modeling of receptor-ligand complexes, providing further evidence for the value of receptor models for structure based drug design. Different interactions of KSK-103 with distinct functional states of the μ and δ receptors predict different efficacy of the ligand at both receptors: agonist action at the μ receptor and antagonist action at the δ receptor. These predictions were tested in *in vitro* assays evaluating receptor binding, G protein activation, and inhibition of cyclic adenosine monophosphate (cAMP) production by forskolin-stimulated adenylyl cyclase.

Additionally, I undertook development of the reported tetrapeptides with the goal of removing affinity to the κ receptor. Incorporation of a C-terminal carboxylic acid in KSK-103 in place of the carboxamide group of KSK-102 was designed to reduce κ receptor affinity, as a negative charge in this part of the ligand causes adverse electrostatic interactions at κ (Pogozheva *et al.*, 2005) and previous studies have shown a carboxamide to be beneficial in producing κ receptor affinity (Przydzial *et al.*, 2005a). In agreement, a C-terminal carboxylic acid motif for KSK-103 produced a 100-fold decrease in κ receptor affinity compared with KSK-102.

While it may be expected that KSK-102 and KSK-103 should behave similarly, having only one small change in the C-terminal sequence between them, KSK-102 behaved as a δ agonist in the [^{35}S]GTPγS binding assay. The δ agonist activity of KSK-102, which, like KSK-103, has the Aci^{3} residue and might be expected to bind poorly to the δ receptor active state, is likely attributed to its slightly deeper positioning in the binding pocket due to the ability of the KSK-102 C-terminal amide to form an H-bond with the backbone carbonyl of Leu^{200} (not shown). This slight readjustment of KSK-102 in the δ receptor binding pocket results in a small shift of Aci^{3} that relieves the steric interaction with the Met^{199} side chain in the active conformation of the δ receptor.

Analysis of previously reported pseudopeptides DIPP(Ψ)NH_2 and UFP-505 in both receptor binding and agonist assays determined alternate profiles from the published results. In contrast to KSK-103, neither DIPP(Ψ)NH_2 nor UFP-505 display the desired bifunctional profile (e.g. equivalent binding to the μ and δ receptors, much lower binding

affinity to κ receptors, and μ agonist/δ antagonist efficacies). However, our assays were performed using conditions slightly different from those under which these peptides were first reported (Balboni *et al.*, 2002a; Schiller *et al.*, 1999), and thus it is reasonable to expect our data to be dissimilar. DIPP(Ψ)NH_2 had equivalent binding affinities to the μ and δ receptors and was only 10-fold selective for these receptors over the κ receptor in our binding assays. UFP-505, on the other hand, was determined to be δ receptor-selective having a 130-fold or 640-fold lower affinity at μ and κ receptors, respectively. Moreover, both DIPP(Ψ)NH_2 and UFP-505 display partial agonism at the δ receptor, as indicated by their ability to inhibit adenylyl cyclase, rather than the desired δ receptor antagonism.

Thus, KSK-103 represents a step forward in the development of novel ligands potentially lacking tolerance and dependence liability. Future studies will investigate the *in vivo* actions of KSK-103 after both acute and chronic administration. These studies will determine tolerance liability of KSK-103 *versus* standard opioid ligands including DAMGO, morphine, and endomorphin-2. The propensity of KSK-103 to produce dependence using *in vitro* and *in vivo* models will also be used to further investigate hypothesized interactions between μ and δ receptors which result in adaptive side effects.

Materials and Methods

Materials. Reagents for peptide synthesis and characterization were from Sigma-Aldrich unless otherwise indicated. Fetal bovine serum, cell culture media and additives were purchased from Gibco Life Sciences. [D-Ala^2, NMePhe^4, Gly^5-ol]-enkephalin (DAMGO) and other biochemicals were obtained from Sigma-Aldrich. [^{35}S]-guanosine-5'-O-(3-thio)triphosphate ([^{35}S]GTPγS; 1250Ci (46.2TBq)/mmol) and [^{3}H]-diprenorphine were purchased from Perkin Elmer. DIPP(Ψ)NH_2 was a gift from the National Institute on Drug Abuse (NIDA) Drug Supply Program.

Solid Phase Peptide Synthesis and Cyclization of Peptides. KSK-102 and KSK-103 were synthesized in a sequential fashion by solid phase methods. Synthesis of the carboxylic acid terminal KSK-103 utilized chloromethylated polystyrene (Merrifield) resin crosslinked with 1% divinylbenzene (AdvancedChemTech), while synthesis of the

carboxamide terminal analog KSK-102 employed p-methylbenzhydrylamine resin (AdvancedChemTech). t-Butyloxycarbonyl (Boc) protection of the α-amino function was used throughout. The labile sulfhydryl groups of the D-Cys and D-Pen were protected with the p-methylbenzyl function. Both peptides were cleaved from the resin and deprotected by treating with 10 ml anhydrous HF in the presence of 0.5 g thiocresol and 0.5 g cresol. After stirring for 1 h at 0^{o}C, the solvent was removed under vacuum and the resin was washed several times with dry diethyl ether. The peptide was extracted from the resin with 5 ml washes of dimethylformamide (DMF)/80% acetic acid (9:1, v/v), diluted 20 fold with aqueous HPLC component, filtered and subjected directly to semipreparative HPLC (Waters Corporation). Purification of the resulting free sulfhydryl-containing linear peptides was effected by semi-preparative HPLC (Waters Corporation) on a Vydac Protein & Peptide C-18 column (2.2 cm x 25 cm) with the solvent system 0.1% trifluoroacetic acid (TFA) in H_2O/0.1% TFA in CH_3CN, using a 0-50% gradient of organic component. The identity and purity of the linear peptides were determined by electrospray ionization mass spectrometry (ES-MS; Agilent Technology, 6130 Quadrupole LC-MS) in positive mode. Dmt-Tic-GlyNH-Bzl (UFP-505) was synthesized according to the reported protocol (Balboni *et al.*, 2002a).

Dithioether cyclization of peptides A DMF solution of the linear peptide (15 mg/40 ml) containing 10 mol equivalents of 1,2-dibromoethane was added dropwise to a cooled round bottom flask containing 10 mol equivalents of potassium *tert*-butoxide in 100 ml anhydrous DMF. The stirring continued for 2 hrs and the reaction was quenched with 5 ml of acetic acid. Solvents were removed *in vacuo* and the residue purified using preparative RP-HPLC to afford the alkyl dithioether-cyclized peptide.

The purity of final peptides was determined using a Waters Alliance 2690 Analytical HPLC and molecular weight confirmed using ES-MS (KSK102 [M+1]=628.1; KSK103 [M+1]=629.1; Table 4.3).

Cell Lines and Membrane Preparations. C_6-rat glioma cells stably transfected with a rat μ (C_6-μ) or δ (C_6-δ) opioid receptor (Lee *et al.*, 1999) and Chinese hamster ovary (CHO) cells stably expressing a human κ (CHO-κ) opioid receptor (Husbands *et al.*, 2005) were used for all *in vitro* assays. Cells were grown to confluence at 37°C in 5%

CO_2 in either Dulbecco's Modified Eagle's Medium (DMEM; C_6 cells), or DMEM-F12 Medium (CHO cells) containing 10% fetal bovine serum and 5% penicillin-streptomycin. Membranes were prepared by washing confluent cells three times with phosphate-buffered saline (0.9% NaCl, 0.61 mM Na_2HPO_4, 0.38 mM KH_2PO_4, pH 7.4). Cells were detached from the plates by incubation in harvesting buffer (20 mM HEPES, 150 mM NaCl, 0.68 mM EDTA, pH 7.4) and pelleted by centrifugation at 200x*g* for 3 min. The cell pellet was suspended in ice-cold 50 mM Tris-HCl buffer, pH 7.4 and homogenized with a Tissue Tearor (Biospec Products, Inc.) for 20 s at setting 4. The homogenate was centrifuged at 20,000x*g* for 20 min at 4°C and the pellet re-homogenized in 50 mM Tris-HCl with a Tissue Tearor for 10 s at setting 2, followed by re-centrifugation. The final pellet was re-suspended in 50 mM Tris-HCl, to 0.5-1.0 mg/ml protein and frozen in aliquots at -80°C (Clark *et al.*, 2003). Protein concentration was determined using the BCA protein assay (Thermo Fisher Scientific) using bovine serum albumin as the standard.

Radioligand Binding Assays. Opioid ligand-binding assays (Przydzial *et al.*, 2005a) were performed using competitive displacement of 0.2 nM [^{3}H]diprenorphine by the test compound from membrane preparations containing opioid receptors. The assay mixture, containing membrane suspension (20-40 μg protein/tube) in 50 mM Tris-HCl buffer (pH 7.4), [^{3}H]diprenorphine, and various concentrations of peptide was incubated at 25°C for 1 h to allow binding to reach equilibrium. The samples were rapidly filtered through GF/C filters (Whatman) using a Brandel harvester and washed three times with 50 mM Tris-HCl buffer. The radioactivity retained on dried filters was determined by liquid scintillation counting after saturation with EcoLume liquid scintillation cocktail (MP Biomedicals) in a Wallac 1450 MicroBeta (Perkin Elmer). Non-specific binding was determined using 10 μM naloxone. Ki values were calculated using nonlinear regression analysis to fit a logistic equation to the competition data using GraphPad Prism version 5.01 for Windows. The results presented are the mean ± standard error from at least three separate assays performed in duplicate.

Stimulation of [^{35}S]GTPγS Binding. Agonist stimulation of [^{35}S]GTPγS binding was measured as described previously (Traynor *et al.*, 1995). Briefly, membranes (20–40

μg of protein/tube) were incubated 1 hr at 25°C in GTPγS buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4) containing 0.1 nM [^{35}S]GTPγS, 100 μM GDP, and varying concentrations (0.1–10,000 nM) of peptides. Peptide stimulation of [^{35}S]GTPγS was compared with 10 μM standard compounds DAMGO, D-Pen^{2,5}-enkephalin (DPDPE), or U69,593. The reaction was terminated by rapidly filtering through GF/C filters, washing three times with GTPγS buffer and retained radioactivity measured as described above. Experiments were performed at least three times in duplicate and EC_{50} values determined using nonlinear regression analysis with GraphPad Prism. To determine antagonist properties of peptides at the δ receptor, [^{35}S]GTPγS binding was determined for DPDPE in the presence or absence of a single concentration of peptide (Kosterlitz *et al.*, 1968). The EC_{50} value for DPDPE in the presence of peptide was divided by the EC_{50} value for DPDPE alone, and this ratio (DR) was employed to calculate the Ke value using the equation Ke = ([antagonist]/(DR-1)).

Whole Cell Acute Inhibition of Adenylyl Cyclase. Inhibition of adenylyl cyclase by opioid agonists or peptides was measured in C_6-δ or C_6-μ cells grown to confluence in 96-well plates. Cells were washed in serum-free DMEM at least 30 min prior to the start of the assay and incubated with various concentrations (1 – 1000 nM) of DPDPE or peptide in serum-free media containing 5 μM forskolin (FSK) and 0.25 mM 3-isobutyl-1-methylxanthine (IBMX) for 30 min at 37°C. The assay was quenched during a 30 min incubation at 4°C by replacing media with 0.1 ml lysis buffer (0.3% Tween-20, 5 μM HEPES in dH_2O, pH 7.4). Antagonism of DPDPE-mediated inhibition was measured by addition of a single concentration of KSK-103 in C_6-δ cells, as described above. Cyclic adenosine monophosphate (cAMP) was measured from samples in a 384-well plate with a BioTek MultiMode Microplate Reader using the AlphaScreen cAMP detection kit from Perkin Elmer according to manufacturer's instructions. Inhibition of cAMP accumulation was calculated as a percent of FSK-stimulated cAMP accumulation in vehicle-treated cells. EC_{50} values were calculated for each compound using GraphPad Prism. Experiments were performed in triplicate and repeated a minimum of three times.

Modeling. The homology models of human μ receptor (UniProt ID: P35372, residues 67-354), in the inactive and active states were developed using crystal structures

of bovine rhodopsin in the inactive (PDB ID: 1U19) and photoactivated (PDB ID:3DQB) conformations, respectively. The models were generated as previously described for gonadotropin-releasing hormone receptor modeling (Janovick *et al.*, 2011). The receptor models were refined using distance geometry calculations with structural restraints that involved receptor-specific H-bonds, natural and engineered disulfide bonds and metal-binding clusters described in our earlier publications (Fowler *et al.*, 2004a; Fowler *et al.*, 2004b), as well as contacts between receptor residues and the native μ agonist, endomorphin-1 (Tyr-Pro-Trp-PheNH_2) or synthetic μ antagonist, antanal-2 (Dmt-Pro-Phe-2-NalNH_2) (Fichna *et al.*, 2007) docked similarly to cyclic tetrapeptides (Pogozheva *et al.*, 2005; Przydzial *et al.*, 2005a). The models of human δ receptor (UniProt ID: P41143, residues 48-336) and human κ receptor (UniProt ID: P41145, residues 58-348) were developed from the μ receptor active and inactive state models by residue substitution followed by energy minimization with CHARMm potentials (QUANTA, Accelrys), dielectric constant, ε = 10, and the adopted basis Newton-Raphson minimization method (100 iterations). Coordinates of μ (active and inactive states) and δ (inactive state) receptor models with KSK-103 can be downloaded from our web site (http://mosberglab.phar.umich.edu/resources/).

Statistical Analysis. Data were analyzed using Student's two-tailed *t* test or analysis of variance followed by Bonferroni's post-hoc test using GraphPad Prism where appropriate. A *p* value less than 0.05 was used to determine significance.

Table 4.1: Opioid receptor binding affinity of peptides at μ, δ, and κ receptors [a]

Peptide	Sequence	Ki, nM (μ)	(δ)	(κ)
JOM-6	Tyr-c(SCH_2CH_2S)[DCys-Phe-DPen]NH_2	0.3 ± 0.04 [b]	25 ± 1.5[b]	9540 ± 620
KSK-102	Dmt[c]-c(SCH_2CH_2S)[DCys-Aci[c]-DPen]NH_2	0.6 ± 0.1	0.9 ± 0.2	9.8 ± 3.6
KSK-103	Dmt-c(SCH_2CH_2S)[DCys-Aci-DPen]OH	2.4 ± 0.7	2.3 ± 0.5	780 ± 150
DIPP(Ψ)NH_2	Dmt-Tic[c](Ψ)[CH_2NH_2]Phe-PheNH_2	0.4 ± 0.1	0.4 ± 0.04	3.9 ± 0.2
UFP-505	Dmt-Tic-GlyNH-Bzl	26 ± 8	0.2 ± 0.06	128 ± 42

[a]Opioid receptor binding studies in cell membrane preparations. Experiments were performed as described in *Methods* and affinity was determined by non-linear regression following displacement of 0.2 nM [^{3}H]diprenorphine from membrane preparations of opioid receptors individually expressed in C_6 rat glioma (μ and δ receptors) or Chinese hamster ovary cells (κ receptors). Results reported as mean Ki ± standard error from at least three experiments performed in duplicate. [b]JOM-6 affinity at μ and δ receptors taken from McFadyen, et. al. 2000. [c]Abbreviations include Dmt for 2', 6' dimethyltyrosine, Aci for 2-aminoindane- 2-carboxylic acid, and Tic for 1,2,3,4-tetrahydroisoquinoline, 3-carboxylic acid. Cyclization of peptides reported as (SCH_2CH_2S) for ethylene dithioether linkage.

Table 4.2: Efficacy and potency of cyclized peptides for stimulation of [^{35}S]GTPγS binding at opioid receptors

Peptide	Sequence	μ		δ		κ	
		% max.[a]	EC_{50}, nM[b]	% max.	EC_{50}	% max.	EC_{50}
KSK-102	Dmt-c(SCH_2CH_2S)[DCys-Aci-DPen]NH_2	58 ± 8	0.4 ± 0.02	37 ± 4	1.4 ± 0.4	n.s.[c]	---
KSK-103	Dmt-c(SCH_2CH_2S)[DCys-Aci-DPen]OH	59 ± 11	4.7 ± 0.7	n.s.	---	n.s.	---
DIPP(Ψ)NH_2	Dmt-Tic(Ψ)[CH_2NH_2]Phe-PheNH_2	18 ± 1	5.7 ± 3.3	n.s.	---	n.s.	---
UFP-505	Dmt-Tic-GlyNH-Bzl	n.s.	---	n.s.	---	n.s.	---
Morphine		57 ± 5	194 ± 21	n.t.[d]	n.t.	n.t.	n.t.
endomorphin-2		49 ± 7	125 ± 31	n.t.	n.t.	n.t.	n.t.

[a]Stimulation of [^{35}S]GTPγS binding in membrane preparations from cells stably expressing μ, δ, or κ receptors. % maximum (% max.) values represent percent of maximal [^{35}S]GTPγS binding obtained with 10 μM peptide compared to a 10 μM concentration of standard agonists DAMGO (μ), DPDPE (δ), and U69,593 (κ). [b]EC_{50} values determined from non-linear regression analysis of [^{35}S]GTPγS incorporation as described in *Methods*. Experiments were performed in duplicate at least three times and data reported is the mean ± standard error. [c]n.s. No significant stimulation (less than 10% of standard compound) , [d]n.t. - not tested. Abbreviations as in Table 4.1.

Table 4.3: Analytical data for KSK-102 and KSK-103

Peptide	Molecular Weight[a]		HPLC[b] (min; R_t)
	calculated (MW)	observed (MW +1)	
KSK-102	627.1	628.1	28.8
KSK-103	628.1	629.1	30.3

[a]Observed molecular weights determined by electrospray ionization mass spectrometry (ES-MS). [b]Retention times assessed by analytical high-performance liquid chromatography (HPLC) using the solvent system 0.1% trifluoroacetic acid (TFA) in water (w/v)/0.1% TFA in CH_3CN with a gradient of 0-50% organic component in 50 min, monitored at 230 nm with samples dissolved in a mixture of aqueous and organic HPLC components. The column was maintained at 35 °C. Both peptides were found to be 98-99% pure.

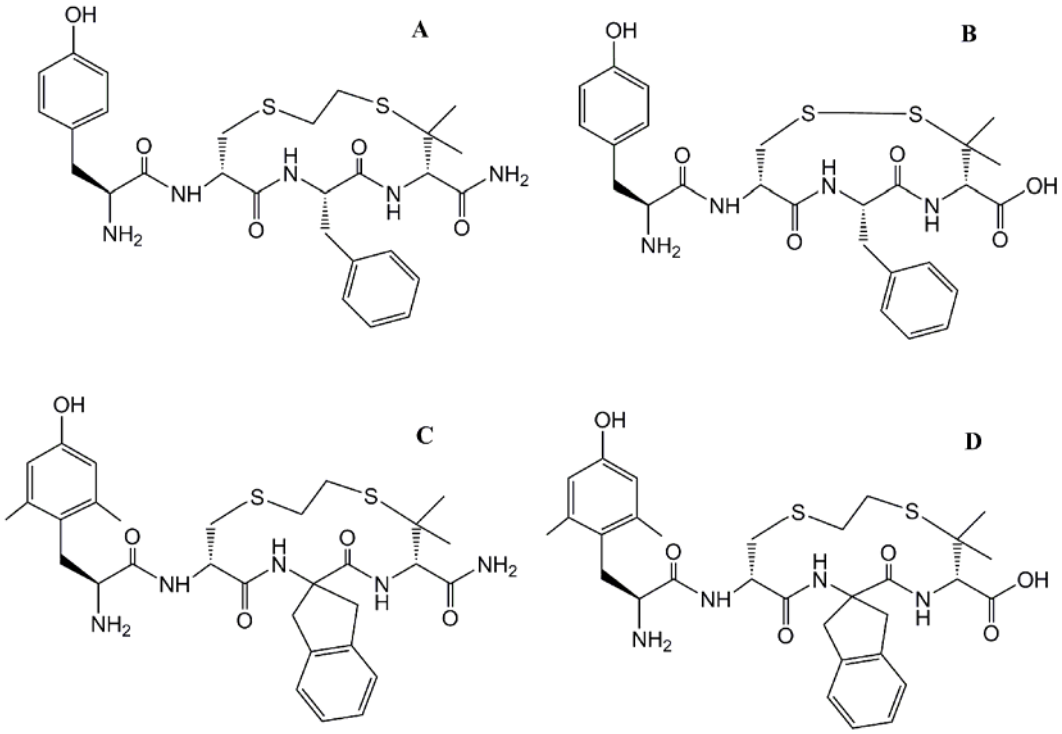

Figure 4.1: Structures of parent peptides (A) JOM-6 and (B) JOM-13 and new analogs (C) KSK-102 and (D) KSK-103.

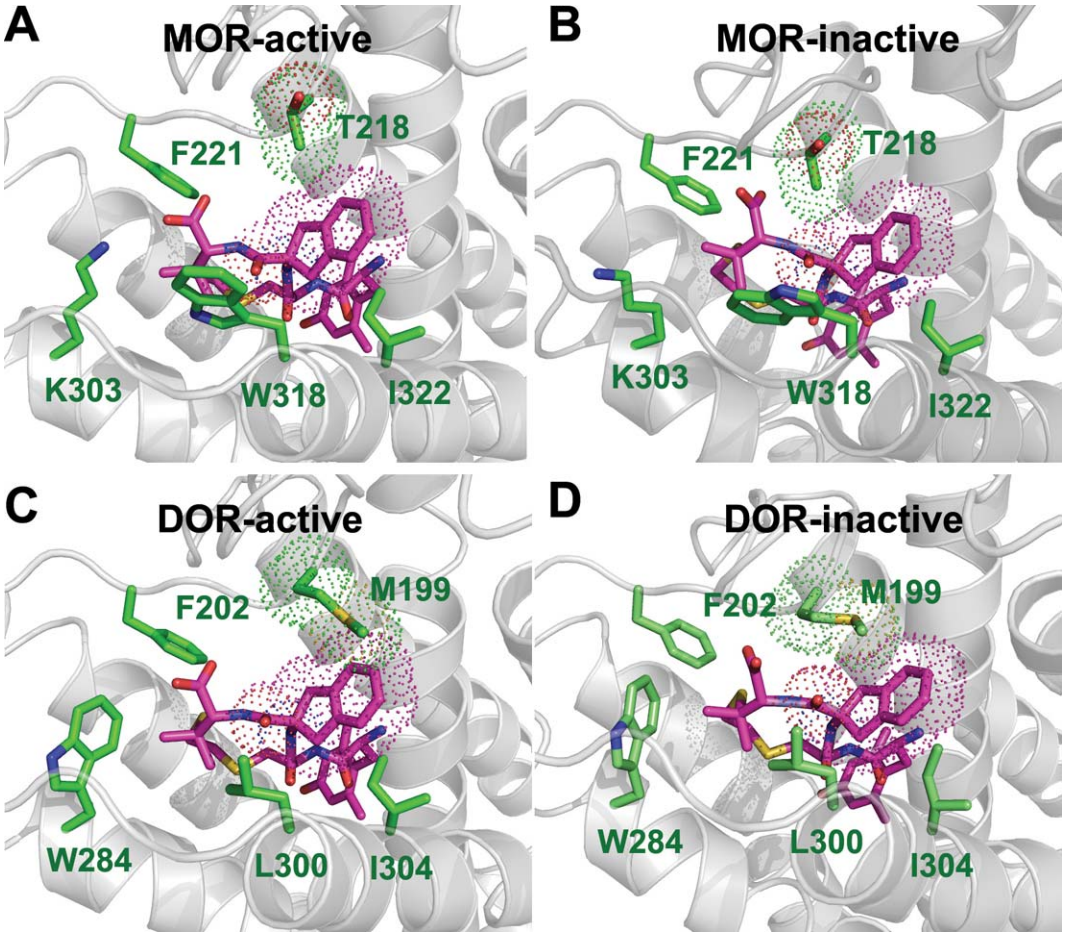

Figure 4.2: Computational modeling of KSK-103 in μ and δ receptor ligand binding pockets reveals structural determinants of ligand efficacy. KSK-103 can be docked without steric hindrances into the ligand binding pocket of the μ receptor models in the (A) active and (B) inactive conformations**,** but displays significant overlap between Aci^{3} of the ligand and Met^{199} of the receptor in the δ receptor (C) active conformation. This overlap is removed in the δ (D) inactive conformation, where Met^{199} is shifted away from the ligand binding pocket.

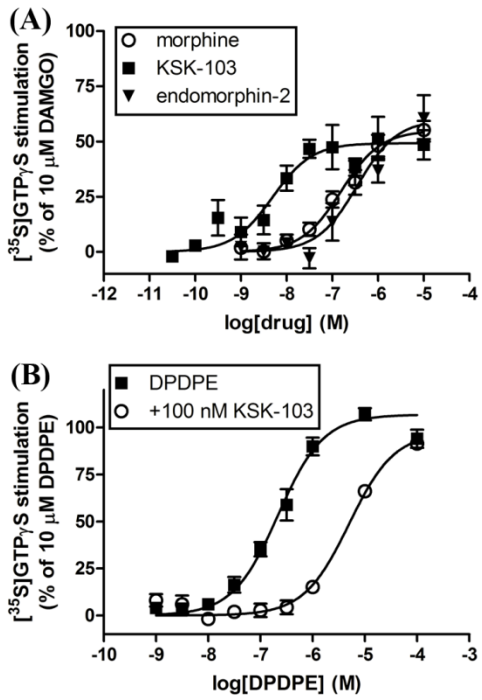

Figure 4.3: KSK-103 behaves as a μ agonist and δ antagonist in the [^{35}S]GTPγS stimulation assay. Incorporation of [^{35}S]GTPγS as a measure of G protein stimulation was analyzed in cell membrane preparations from C_6-rat glioma cells stably expressing either the μ or δ receptors. (A) At the μ receptor, KSK-103 behaved as a partial agonist, producing 59 ± 11% stimulation of G protein compared to μ agonist DAMGO (filled squares). Morphine (open circles) and endomorphin-2 (filled triangles) produced a similar percent stimulation; however, they were less potent than KSK-103. (B) Addition of 100 nM KSK-103 (open circles) produced a 26–fold rightward shift in the concentration-response curve for DPDPE at the δ receptor, affording a Ke value for the antagonist of 4.4 ± 1.4 nM.

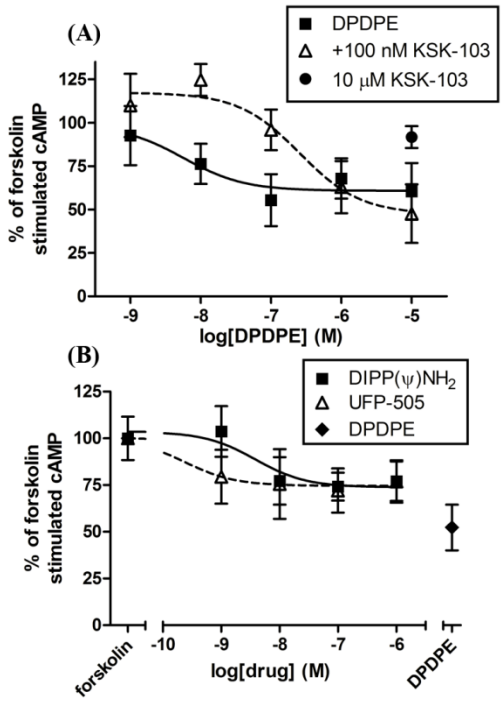

Figure 4.4: KSK-103 is a δ receptor antagonist in adenylyl cyclase inhibition while reference peptides DIPP(Ψ)NH_2 and UFP-505 display partial agonist efficacies. Inhibition of forskolin-stimulated cAMP production was measured utilizing a whole cell assay in C_6-rat glioma cells stably expressing the δ receptor. (A) In this assay, 10 μM KSK-103 (filled circle) was unable to significantly inhibit forskolin-stimulated cAMP levels (91 ± 6%) while addition of 100 nM KSK-103 (open triangles) produced a 9.5-fold rightward shift in the concentration-response curve for DPDPE. This afforded a Ke value for antagonism of 12 ± 3.3 nM. (B) Peptides DIPP(Ψ)NH_2 (filled squares) and UFP-505 (open triangles) behaved as partial agonists at the δ receptor in this assay, able to reduce forskolin-stimulated cyclase to 65 ± 10% and 72 ± 2%, of controls respectively. 1 μM δ peptide agonist DPDPE (filled diamond) inhibited forskolin stimulation to 52 ± 12%.

Chapter V

Discussion and Future Directions

Overview

Opioid agonists, while extremely useful in producing analgesia, are hampered by their propensity to develop tolerance and dependence. Previous studies, and research detailed in this thesis, have shown evidence for interactions between μ and δ receptors in the production of these unwanted effects. In particular, δ receptor blockade *via* antagonism reduces tolerance and dependence liability, measured both *in vivo* and *ex vivo* after a single administration of the μ agonist morphine. Previous studies that examined the role of the δ receptor in these phenomenon focused on tolerance or dependence appearing after chronic (5-7 day) exposure to morphine. My examination of behavioral and biochemical results after acute morphine exposure lends increased relevance to the hypothesis that δ antagonism produces alterations in subsequent μ receptor activity such that development of tolerance and dependence is lessened and that the interaction occurs early in the adaptive process. Although on a different time scale of morphine exposure, these results agree with the previously published data demonstrating in rodents (Abdelhamid *et al.*, 1991; Hepburn *et al.*, 1997) that δ antagonism is a viable target for reducing tolerance liability of opioid drugs. Therefore, a drug that contains both μ agonist and δ antagonist properties might be a viable analgesic with reduced tolerance and dependence liability.

As a start toward such a drug, development and characterization of bifunctional opioid peptides displaying μ agonism and δ antagonism simultaneously and utilizing two scaffolding systems (both penta- and tetrapeptides) was completed. These peptides are proposed first ligands in the development of novel improved clinical candidates for pain treatment while reducing the risk for tolerance and/or dependence development. The use

of constrained amino acid analogs and analysis of peptide binding to putative computational models of both the μ and δ receptor revealed important structural characteristics in these peptides. These results demonstrate that rational drug design is a useful tool to differentiate efficacy profiles between targets and can aid in designing peptides with dual actions at one or more receptors.

Whereas peptides themselves are not drug targets, modeling studies utilizing these constrained peptides can provide information on the structural components of efficacy. Indeed, studies described in this thesis have identified a region of the δ receptor that may be important in activation and production of downstream signaling. The results show the utility of structure-based design in the development of bifunctional ligands and provide strengthening evidence for the validity of the computational model receptor systems. Identification of favorable and unfavorable interactions between the proposed peptide ligands and the ligand binding domains of each receptor may be significant for the future development of more drug-like, non-peptidic ligands.

Role for the δ receptor in modulating μ receptor-induced tolerance: Interactions between receptors

My results examining acute tolerance *in vivo* demonstrated that co-administration of a δ antagonist compound can decrease both tolerance and dependence development, in agreement with previously published reports showing a role for δ receptor blockade in ameliorating effects of long-term treatment with morphine (Abdelhamid *et al.*, 1991; Hepburn *et al.*, 1997). The behavioral studies were complemented with *ex vivo* analysis of changes in receptor-G protein coupling and expression of high-affinity μ receptor binding sites to better understand cellular changes occurring upon agonist exposure. There was a loss of G protein stimulation on subsequent agonist administration following 4 h morphine exposure *in vivo*, as well as a loss of high-affinity, G protein bound μ receptors but not total opioid receptor expression.

Previous studies in our lab (Bradbury *et al.*, 2009; Levitt *et al.*, 2011) and others (Bailey *et al.*, 2005; Bohn *et al.*, 2000) have shown evidence for numerous, overlapping signaling pathways (e.g. GRK, β-arrestin, and protein kinase C) in tolerance mechanisms

at the single cell level after opioid agonist exposure. It is likely that the mechanism(s) producing tolerance utilize many signaling processes and cellular modulators, making it relatively difficult to differentially target and block the development of tolerance entirely. My results provide strong evidence for cross-talk between opioid receptors in the production of this adaptive effect. On the other hand, other research groups have found evidence for opioid agonists indirectly inducing non-opioid signaling mechanisms to overcome tolerance, including activation of N-methyl-D-aspartate (NMDA) or other glutamatergic signaling processes (Mao, 1999; Mao *et al.*, 1995). Conversely, inhibition of NMDA receptors *via* antagonism was demonstrated to decrease tolerance development (Trujillo *et al.*, 1991), showing a link between multiple receptors or signaling pathways in the production of adaptive effects.

The findings presented in this thesis agree with previous findings that development of receptor desensitization leads to reduced opioid receptor activation. In particular, it has been noted in several studies (McPherson *et al.*, 2010; von Zastrow *et al.*, 2003; Whistler *et al.*, 1999), that morphine is a μ agonist unable to induce receptor internalization, but still produces profound clinical analgesic tolerance. Results from studies in this dissertation show that the tolerance developed after an acute, single morphine exposure is due to an uncoupling of the μ receptor from G proteins and a decrease in subsequent agonist efficacy. This supports a theory that acute receptor desensitization is a precursor to complete tolerance development. While I have shown evidence for acute tolerance in the 129S6 mouse strain, the inability to visualize greater tolerance after chronic morphine administration studies points to the likelihood of more complex compensatory mechanisms (including glutamatergic activation or other cell signaling or gene expression modifications) in masking opioid tolerance in the particular mouse strain utilized for these studies. Further investigation of why this particular strain is resistant to tolerance, compared to other strains such as Swiss-Webster (Abdelhamid *et al.*, 1991) or C57BL6/129SvJ animals (Bohn *et al.*, 2000), previously shown to develop tolerance after chronic morphine exposure, may be very helpful in understanding mechanisms underlying tolerance. To further probe interactions between μ and δ receptors, it would be necessary to investigate more thoroughly endpoints beyond

antinociception, utilizing both *in vivo* behavioral studies, including conditioned place preference, sensitization paradigms, or respiratory depression experiments. *In vitro* model systems expressing both μ and δ receptors have been used to show interactions between these receptors (George *et al.*, 2000), however these studies were predominantly performed in heterologous systems, which may decrease the relevance of published results.

In conclusion, my results demonstrating interactions between μ and δ receptors in the development of both tolerance and dependence support suggestions that a mixed-efficacy approach would be beneficial in clinical applications.

Examination of interactions between μ and δ receptors using endogenous systems

An *in vitro* cellular system expressing both μ and δ receptors as a model to examine interactions between these receptors and to test the utility of the novel bifunctional opioid peptides described above would be advantageous. It is difficult to examine the long-term effects of the developed peptide ligands *in vivo* due to their instability and poor bioavailability and reported methodologies are few. An *in vitro* model system would allow fast and easy characterization of tolerance and dependence liability of novel peptidic ligands and also provides a method to study interactions between μ and δ receptors directly. This type of system would be a useful tool to determine whether the proposed interactions between μ and δ receptors are due to intracellular mechanisms based on access to common downstream signaling components (Levitt *et al.*, 2011), or intercellular mechanisms whereby both receptors activate their respective pathways and activation or blockade has a physiological effect at a distinct cellular location (Scherrer *et al.*, 2009).

There are several reasons to choose an endogenous cell system to study interactions between GPCRs. While a great deal of research has been published demonstrating the existence and alternate signaling of putative opioid (and other GPCR) heterodimers (George *et al.*, 2000; Gomes *et al.*, 2004; Rozenfeld *et al.*, 2007a), much of this research was performed using heterologously expressed cell systems. Over-expressed heterologous systems may lack crucial proteins or other components for proper

opioid signaling and desensitization (Bailey *et al.*, 2005). This is especially important when comparing to signaling in distinct brain regions. For example, neuronal cell membranes have increased cholesterol compared to other cells and this has been shown to be important for opioid receptor signaling (Levitt *et al.*, 2009). In addition, over-expression of the protein or receptor being studied may lead to non-specific interactions which would not normally occur *in vivo*. Using endogenous systems gives a more accurate indication of the necessary regulatory processes underlying adaptations after drug exposure (including tolerance and dependence) and endogenous systems provide the ability to view putative receptor interactions on a scale similar to that encountered *in vivo*. Such native systems may also be used to verify previously published results obtained in over-expression models. However, interactions between two receptors may be difficult to determine using this type of system due to the smaller number of receptors expressed. If interactions can be identified in an endogenous cell system or tissue preparation, previously reported results including the hypothesis that μ and δ receptor interactions led directly to decreased tolerance development (Hepburn *et al.*, 1997) might be further clarified.

Use of SH-SY5Y cells to examine cross-talk between μ and δ receptors

One such endogenous model system is the human SH-SY5Y neuroblastoma cell line. SH-SY5Y cells are second-generation subclone of SK-N-SH neuroblastoma cells (Biedler *et al.*, 1978). Our lab has used the SH-SY5Y cells extensively to better characterize opioid receptor signaling (Alt *et al.*, 2002; Elliott *et al.*, 1997; Elliott *et al.*, 1994; Levitt *et al.*, 2011; Wang *et al.*, 2009). The Traynor lab has shown that several GPCRs coupled to inhibitory G proteins ($G_{i/o}$) are endogenously expressed in these cells and compete for access to both G proteins and adenylyl cyclase enzymes (Alt *et al.*, 2002; Levitt *et al.*, 2011). In these SH-SY5Y cells, μ and δ receptors are expressed in a ratio of approximately 3 μ receptors:1 δ receptor.

Importantly, tolerance to μ agonists may be visualized in these cells (Alt *et al.*, 2002; Elliott *et al.*, 1997). Thus, pretreatment of SH-SY5Y cell monolayers with the μ agonist DAMGO resulted in a significant decrease in the amount of subsequent G protein

activation and a decrease in the number of μ receptors present on the plasma membrane (Elliott *et al.*, 1997). The apparent 'tolerant' effect in the treated cells was most likely due to desensitization and/or internalization of the μ receptor (Elliott *et al.*, 1997).

I have performed preliminary studies in the SH-SY5Y cells utilizing co-administration of the μ agonist DAMGO with or without the δ antagonist naltrindole (NTI). DAMGO treatment for 24 h produced a decrease in the level of μ cell-surface receptor expression measured by binding of the non-specific opioid antagonist [^{3}H]diprenorphine ([^{3}H]DPN; Figure 5.1A) without a loss of cell-surface δ receptors (Figure 5.1B) as measured by binding of the δ-selective antagonist [^{3}H]naltrindole ([^{3}H]NTI). This was coupled with a loss of DAMGO-induced G protein stimulation (Figure 5.1C). A similar decrease in [^{35}S]GTPγS binding stimulated by morphine (Figure 5.2A) was seen. Addition of a δ-selective NTI concentration (10 nM; Figure 5.2B) during the 24 h treatment was unable to prevent the loss of μ agonist-mediated [^{35}S]GTPγS binding (Figure 5.2C). Similar decreases in μ receptor cell-surface expression and loss in subsequent [^{35}S]GTPγS binding were also observed after 1 h of μ agonist DAMGO exposure, while application of the δ antagonist during this time was again unable to block these events (data not shown). In light of my *in vivo* results after a single administration of morphine, studies examining both the time-course of decreased [^{35}S]GTPγS binding after DAMGO exposure or with varying concentrations of DAMGO should be performed. Further studies in this system could analyze the effects of μ agonist treatment on the trafficking of δ receptors to the plasma membrane to examine correlates between this system and others previously reported (Cahill *et al.*, 2007; Cahill *et al.*, 2001b). Examination of δ receptor trafficking in response to μ agonist exposure (and vice versa) in the SH-SY5Y cells might provide further evidence for interactions between the receptors. Such studies could lend strength to the hypothesis for basal enkephalinergic tone which is maintained *via* trafficking of δ receptors in response to μ agonist-induced down-regulation (Cahill *et al.*, 2007). Effects with the δ receptor inverse agonist ICI 174864 in my preliminary studies which showed increased μ agonist-induced [^{35}S]GTPγS binding following ICI 174864 pretreatment point to such an interaction between μ and δ

receptors. However, it is unclear how these interactions could influence tolerance development in a single cell.

Mouse vas deferens as a model system for examining μ-δ receptor interactions

Another potential *in vitro* model for the analysis of μ and δ receptor interactions is the mouse vas deferens (MVD). The MVD was used as a system for the identification and analysis of novel opioid ligands until the cloning and over-expression of receptors in cellular systems became popular (Lee *et al.*, 1993). One advantage of the use of peripheral opioid systems including the MVD is that they respond rapidly to drug administration, do not need blood-brain barrier penetrating agents, and can be used to test a complete series of concentrations without loss of signal (Kosterlitz *et al.*, 1975). While the sensitivity of the system may be less than heterologous, over-expressed cell lines, the endogenous opioid receptor expression can provide insight into *in vivo* behavioral results on a mechanistic basis.

The MVD was shown to express all three opioid receptor subtypes (Lord *et al.*, 1977), but predominately expresses δ receptors (Wild *et al.*, 1993) and μ receptors over κ receptors. As an endogenous system, it is also suitable for examination of interactions between opioid receptors (Elliott *et al.*, 1995). μ and δ Receptors are not only present in this tissue, but have been shown to exist on the same neuron (Rogers *et al.*, 1990), although there is no evidence for a direct interaction between the two (Sheehan *et al.*, 1986). Additionally, the MVD was demonstrated to be an advantageous system in the study of tolerance development following morphine administration *in vivo,* as shown by several groups (Marshall *et al.*, 1981; Schulz *et al.*, 1984; Schulz *et al.*, 2004; Wuster *et al.*, 1982). Using this procedure, tolerance to either μ– or δ-selective agonists could be produced, but cross-tolerance between the two receptors was not seen (Herz *et al.*, 1982; Wuster *et al.*, 1983). These findings indicate the two receptors may not be intrinsically linked (e.g. no heterodimerization) and instead share cellular signaling proteins and downstream effectors. Interactions between μ and δ receptors in the development of tolerance have never been directly studied using this system.

The MVD tissue preparation provides a unique method to analyze signaling between μ and δ receptors outside of the central nervous system. While it is possible that the receptors contained in this tissue are different and signal in a differential manner than receptors expressed in the brain or spinal cord (Takemori *et al.*, 1989), similar G proteins and downstream effector molecules (including adenylyl cyclase; Bhoola *et al.*, 1986) have been identified in the MVD as in the brain. The endogenous expression of μ and δ receptors in this tissue and the reports that the two receptors may be expressed on the same neuron makes it a good candidate system for further studies of the research described in this thesis. Analysis of μ-specific tolerance development in the MVD with or without concurrent δ antagonism might further knowledge of reported interactions (Abdelhamid *et al.*, 1991; Hepburn *et al.*, 1997) between these two receptors.

Structural Components of Efficacy: Development of multi-functional ligands as tools to investigate interactions between opioid receptors

Bifunctional ligands having differential efficacy profiles at distinct sites of actions have numerous positive qualities. Firstly, bifunctional ligands are desirable in a clinical setting and would have improved relevance over a polypharmacy (either two drugs administered separately or a mixture of two different drugs administered simultaneously) technique (Morphy *et al.*, 2005). Secondly, a single bifunctional drug is a more desirable candidate over multiple drugs for patient compliance and would not have the complicated pharmacokinetic or pharmacodynamic profile of an administered mixture (Morphy *et al.*, 2009).

Use of both penta- and tetrapeptide scaffolds with incorporation of bulky, aromatic substituents and computational modeling studies identified several regions of interest in both the μ and δ receptor ligand binding pockets. Both peptide scaffolds incorporated bulky and constrained side groups to explore the limits of the δ receptor binding domain. While the hydrophobic residues incorporated into the 3rd and 4th positions of the tetra- and pentapeptide scaffolds did not appear to limit binding affinity or decrease agonist efficacy at the μ receptor, there was significant overlap between these residues and amino acids in the δ receptor binding pocket. The active and inactive

computational models of the μ and δ receptors were designed on homology modeling of the rhodopsin crystal structure in inactive (Palczewski *et al.*, 2000b) and photoactive (Salom *et al.*, 2006) states.

The pentapeptide scaffold led to development of lead peptide **9** *via* naphthylalanine incorporation, and identified a favorable interaction in the μ receptor 'active' state between the Tyr^{1} of peptide **9** and Trp^{318} of the receptor as described in *Chapter 3* (Figure 3.3C). While no favorable or unfavorable interactions were noted in the 'inactive' states of either μ or δ receptor with docked peptide **9**, analysis of the δ receptor active state identified a region of steric overlap between the 2-Nal^{4} of the peptide and Trp^{284} of the receptor. I hypothesized that hindrance in binding the δ active conformation (due to steric overlap) decreased the agonist efficacy of peptide **9** compared to the parent peptide. Peptide **9** was noted to be a partial agonist at the δ receptor when analyzed for ability to inhibit cAMP accumulation and also bound to the κ receptor with high affinity and was an agonist at this receptor.

Activity at the κ receptor is a complication in the design of bifunctional ligands as κ agonism produces dysphoria in humans. Thus, subsequent peptides were developed from leads which were devoid of κ receptor binding affinity. The tetrapeptide series described in *Chapter 4*, including KSK-103 (Figure 4.1), bound with very low affinity to the κ receptor, especially when compared to the μ and δ receptors. KSK-103 is a μ agonist and δ antagonist peptide (both at the level of G protein and adenylyl cyclase enzymes) and bound with equivalent affinity to both receptors. When analyzed for binding to the computational models of μ and δ receptors, KSK-103 was shown to have a favorable interaction with Lys^{303} in the μ receptor 'active' state, similar to the favorable interaction shown between peptide **9** and the μ receptor. This interaction was not sufficient to produce μ agonist efficacy on the same level as peptide **9**; however, KSK-103 had agonist ability on par with the standard drug morphine. Additionally, the conformationally constrained Phe analog 2-aminoindan, 2-carboxylic acid (Aci) moiety of KSK-103 showed a great deal of overlap with Met^{199} in the δ receptor 'active' conformation. This was not observed in the μ receptor, which has Thr^{218} in this position.

Again, this was hypothesized to prevent δ receptor-bound KSK-103 from producing a conformational shift in the receptor, activation of signaling and produced an antagonist profile. KSK-103 represents a promising lead in developing bifunctional μ agonist/δ antagonist therapies. Initial *in vivo* studies in mice with KSK-103 after central (i.c.v.) administration demonstrated a short-lived partial agonist antinociceptive profile of this peptide. Due to the instability of KSK-103 *in vivo*, *in vitro* screening methods will provide a faster and easier technique to assess tolerance liability of these bifunctional ligands.

The above discussion strengthens the hypothesis for modeling G protein-coupled receptor activation using a two-state receptor theory (Black *et al.*, 1983). This theory proposes agonist ligand incorporation in an 'inactive' state of the receptor, followed by a conformational shift to the 'active' state of receptor and subsequent downstream signaling or binding of ligand to a previously established active state. The 'inactive' state would allow equivalent binding of ligands behaving as either antagonists or agonists. However, the difference in efficacy between these ligands becomes apparent when the ability to produce a conformational change in the receptor to 'active' is measured, such that bound heterotrimeric G protein is activated. Agonist compounds elicit the conformational change in the receptor to activate downstream signaling processes (Leff, 1995), while antagonist ligands (such as KSK-103 at the δ receptor) are unable to produce this shift due to unfavorable interactions with the 'active' conformation of the receptor, therefore maintaining the receptor in an inactive, antagonist-bound position. As antagonist ligands can bind to the 'inactive' state (sometimes with very high affinity), they can prevent agonists of the receptor from binding and inducing the conformational shift necessary for agonist effects.

My studies examining the efficacy profiles of penta- and tetrapeptides revealed the importance of utilizing the correct assay to confirm antagonist efficacy (Nickolls *et al.*, 2011). It is not sufficient to hypothesize antagonism based on the absence of agonist actions at the level of G protein activation, as studies have found these assay conditions to be stringent and agonists with low partial agonist activity can be masked (Harrison *et al.*, 2003). Determination of antagonism can only be established *via* the ability to block a

known agonist's signaling processes (e.g. production of a rightward shift in the dose-response curve for G protein activation) as well as a lack of ability to alter downstream endpoints which take advantage of signal amplification (such as adenylyl cyclase inhibition). This was particularly noticeable in characterization of lead peptide **9** and reference peptides DIPP(Ψ)NH_2 and UFP-505, as all three demonstrated little to no [^{35}S]GTPγS binding at the δ receptor, but were able to inhibit cAMP accumulation showing they are partial δ agonists, not antagonists.

Future directions for development of bifunctional ligands

Further research should focus on more clinically viable therapeutics. While derivatives and analogs of endogenous neuropeptides are a logical drug category, little progress has been made in development of peptides as drugs due to their inherent instability and inactivity when administered peripherally. Upon the discovery of endogenous neuropeptides, including the β-endorphins, dynorphins, and enkephalins (Hughes *et al.*, 1975), it was hypothesized that novel neuropeptidic drugs could replace older drugs that had greater side effect and toxicity profiles (Polt *et al.*, 2005). However, the majority of synthesized opioid neuropeptides (including the μ agonist DAMGO) fail to cross the blood-brain barrier (BBB) and therefore cannot exert their actions after therapeutically viable (i.e. oral or parenteral) administration methods. An important goal in the development of novel opioid therapeutics is the synthesis of stable, potent ligands providing the desired analgesia with reduced side effect profiles. Whereas peptides provide a unique and rapid system for the analysis of structural components of efficacy, these are poor drug candidates. Thus, synthesis of more 'drug-like' peptide analogs has focused on improving peptide stability through a number of modifications.

Improving bioavailability by glycosylation of peptide ligands

Bioavailability of peptides can be improved in a number of ways that increase plasma half-life, stability in the brain, and entry *via* the BBB. Alteration of the peptide ligand through such processes as glycosylation has been shown to improve access of the synthesized peptide to the brain by increasing blood-brain barrier permeability (Egleton

et al., 2001). Other ligand alterations, including N-methylation (Biron *et al.*, 2008; Dechantsreiter *et al.*, 1999), have been used with success to improve BBB penetration for a variety of peptidic ligands (Biron *et al.*, 2008; Egleton *et al.*, 2005; Lowery *et al.*, 2007). In addition, modifications including polyethylene glycol (PEG)-ylation (Roberts *et al.*, 2002) or lipidization (Flinn *et al.*, 1996) have been demonstrated to improve both plasma bioavailability and brain penetration.

Glycosylation has been shown to improve the biodistribution of opioid ligands, including the δ agonist deltorphin (Tomatis *et al.*, 1997), met-enkephalin (Egleton *et al.*, 2000; Polt *et al.*, 1994), and leu-enkephalin (Bilsky *et al.*, 2000) and improved antinociceptive potencies upon peripheral administration. The hypothesis underlying these improvements focuses on the so-called 'biousian effect' (Dhanasekaran *et al.*, 2005). Glycosylation allows transport of the peptide through the BBB via a number of potential mechanisms, including the glucose transporter (Nomoto *et al.*, 1998), organic anion-transporting peptide transporter (Gao *et al.*, 2000), and/or *via* an adsorptive endocytotic mechanism (Banks *et al.*, 1997).

To glycosylate the bifunctional peptide KSK-103 would require incorporation of a C-terminal Ser or Thr residue, followed by attachment of the sugar moiety. It is critically important to re-evaluate the binding and efficacy characteristics of each iteration of KSK-103, as incorporation of any structural modifications may lead to differential changes in binding affinity or efficacy.

Peptidomimetic scaffolding for novel therapeutics

'Peptide mimic' drugs, or peptidomimetics, resemble a lead peptide but contain some synthetic, unnatural elements that help to reduce metabolism; these can be obtained through a variety of modifications (Pauletti *et al.*, 1997) and are useful to reduce the size and hydrophobicity of the compounds, aiding in absorption and bioavailability. The peptidomimetic approach to create more 'drug-like' compounds is not restricted to opioid chemistry, although many opioid peptidomimetics have been characterized (Ballet *et al.*, 2006; Birkas *et al.*, 2008; Liao *et al.*, 1998). Development of peptidomimetics in a number of fields including substance P antagonists (Tong *et al.*, 2000; Walpole *et al.*,

1998), bradykinin (Abe *et al.*, 1998), and endothelin analogs (Murugesan *et al.*, 1998) has resulted in orally available derivatives that are undergoing further clinical analysis, demonstrating the viability of this technique for furthering development of novel therapeutics. Synthetic alterations to create peptidomimetics focus on limiting peptide bond hydrolysis through modification of the peptide backbone in a number of ways. Firstly, addition of molecules designed to protect or hide the peptide bond from enzymes decreases metabolism, as does replacement of the peptide bond altogether using another linkage system (Patel *et al.*, 2009). Also, global changes to the peptide have been used to render the peptidomimetic unrecognizable to peptidases or other enzymes in order to prevent degradation (Plummer *et al.*, 1997). Examples of peptidomimetic alterations to improve stability and bioavailability include amide bond replacement (Shiosaki *et al.*, 1993; Shue *et al.*, 1993; Wang *et al.*, 1998) or cyclization (Li *et al.*, 2002) and have shown great success. Additionally, incorporation of unnatural amino acid derivatives to mimic transition state peptides has provided methods to bypass systemic degradation and promote receptor activation (Liao *et al.*, 2007). These alterations were noted to increase ligand selectivity, increase the plasma half-life, and are hypothesized to decrease apparent side effects, although there is limited data available to support such a hypothesis.

Utilization of an alternative scaffolding system taking advantage of identified key structural components represents another method by which to continue the development of bifunctional peptide ligands, such as KSK-103 described in this thesis into clinically relevant drugs. Our group has previously demonstrated the ability to produce these types of peptidomimetic drugs (Wang *et al.*, 1998) which behave similarly *in vitro* to their peptide counterparts but are able to be studied *in vivo* with less difficulty.

Overall summary and significance

The results presented in this thesis demonstrate a link between μ and δ receptors in the production of μ agonist-induced tolerance and dependence, after a single administration of μ agonist. Tolerance was developed both *in vivo* and *ex vivo*, while dependence was only seen *in vivo*. Due to the inability to establish dependence *ex vivo*, I

hypothesize that development of these adaptations is a short-term change in receptor signaling that does not persist following the cell membrane preparation process prior to *ex vivo* assays. The inability to produce tolerance after long-term morphine administration in the mice used in this study may be due in part to complex physiological mechanisms as well as complementary changes in gene expression. It is likely that production of cellular signaling processes (both acute and chronic) are very tightly regulated by many cellular modulators (e.g. G protein receptor kinases, β-arrestin, PKC, $G_{i/o}$ coupled receptors, etc.). The *in vivo*/*ex vivo* results demonstrated receptor desensitization occurs after acute morphine treatment, such that both antinociceptive and G protein tolerance is observed. Future work should analyze cellular dependence mechanisms after a different time-course of agonist exposure and *ex vivo* using endogenous systems to investigate interactions between μ and δ receptors, which may be due to distinct neuronal pathways.

Additionally, development of an *in vitro* model system with which to screen novel ligands for bifunctional efficacies is an important goal in furthering development of therapeutics devoid of tolerance and dependence liabilities. Future work should assess the effect δ antagonism has on μ agonist-induced cellular endpoints (including MAP kinase activation, inhibition of GIRK channels, and Ca^{2+} channel activity) to determine if there is a link between these cellular endpoints and tolerance development. Behavioral tests beyond antinociception and withdrawal should also be examined. Links have been demonstrated between μ and δ receptors in production of conditioned place preference to morphine (Chefer *et al.*, 2009; Shippenberg *et al.*, 2009; Timar *et al.*, 2005) and locomotor sensitization (Shippenberg *et al.*, 2009; Timar *et al.*, 2005) after long-term exposure. It would help to complete the picture of the role of the δ receptor by performing these assays after morphine exposure.

The studies performed in this thesis have added to knowledge of modulation of morphine tolerance and dependence by the δ receptor. Examining both behavioral and biochemical measures helped to frame the cellular changes occurring after μ agonist exposure. These results are significant in that they furthered the relevance of bifunctional drug therapy with reduced tolerance/dependence liability in the treatment of pain

conditions. In this regard, I have successfully developed and characterized two series of non-selective mixed μ/δ efficacy opioid peptide ligands. Studies with these ligands also highlighted rational structure-based drug design and the use of computational modeling to differentially create efficacies in a single ligand. The designed peptides identified important limits in the δ receptor binding pocket important for agonist efficacy.

In terms of clinical relevance, I have demonstrated that co-administration of μ agonist and δ antagonist ligands helps reduce tolerance development and argued that a ligand displaying this profile could be extremely useful in the treatment of chronic pain conditions by reducing the need to continuously increase dosage to achieve desired effects. Bifunctional ligands also display the advantages of simultaneous therapy over polypharmacy, highlighting important targets in future drug development. Future work should focus first on improving bioavailability and brain penetration of these ligands. Such compounds might then be used to demonstrate effective antinociception with reduced tolerance development after both acute and chronic exposure paradigms.

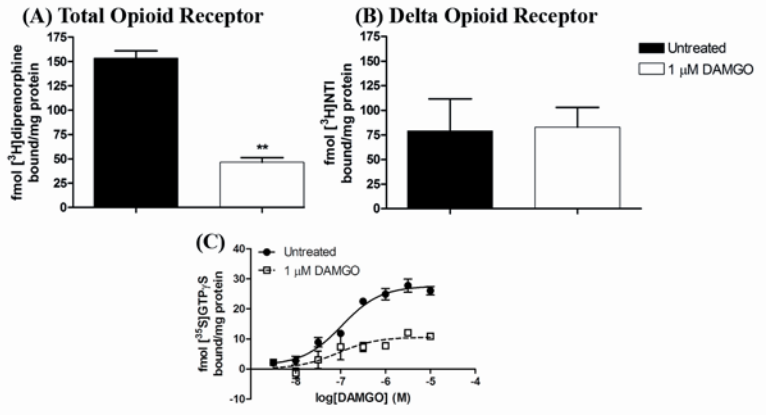

Figure 5.1 24 h treatment of SH-SY5Y cells with 1 μM μ agonist DAMGO. (A) There is a loss in [^{3}H]diprenorphine binding to SH-SY5Y cell membrane preparations after 24 h treatment with DAMGO indicating a decrease in cell-surface μ receptors but (B) no change in cell-surface δ receptors as binding with the δ-selective antagonist [^{3}H]naltrindole (NTI) is unchanged between treatment groups. (C) Application of μ agonist DAMGO in treated cell membrane preparations shows decreased ability to stimulate [^{35}S]GTPγS binding compared to untreated SH-SY5Y membranes.

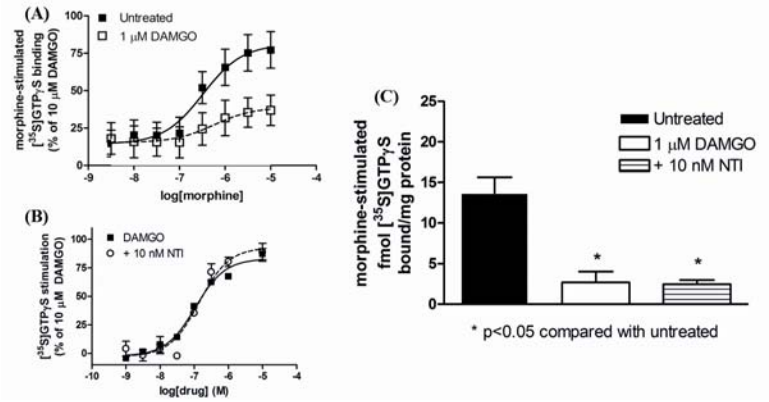

Figure 5.2 Co-administration of 1 μM μ agonist DAMGO and 10 nM δ antagonist naltrindole cannot prevent DAMGO-induced decreased [^{35}S]GTPγS binding by subsequent μ agonist application. (A) 24 h treatment of SH-SY5Y cells with 1 μM DAMGO produces less [^{35}S]GTPgS binding after application of the μ agonist morphine to cell membrane preparations compared to control cells. (B) A 10 nM concentration of the δ antagonist naltrindole (NTI) was determined to be a δ-selective concentration, as it was unable to produce any change in the dose-response curve for μ agonist DAMGO in untreated SH-SY5Y membrane preparations. (C) Co-administration of μ agonist DAMGO and δ antagonist NTI does not prevent the decreased [^{35}S]GTPγS binding by morphine in cell membrane preparations.

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