Uncovering the Functional Implications of µ- and δ-Opioid Receptor Heteromerization in the Brain

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Graduate Department of Pharmacology and Toxicology

University of Toronto

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ABSTRACT

Opioid Receptors (ORs) are involved in the pathophysiology of several neuropsychiatric conditions yet remain an untapped therapeutic resource. Although only μ-, δ-, and κ-OR types have been cloned, additional subtypes result from complexes generated by direct receptor-receptor interactions. μ- and δ-ORs form a heteromeric receptor complex with unique pharmacological and signalling properties distinct from those of μ- and δ-OR homomers. In these studies, we sought to characterize the ligand binding pocket and agonist-induced internalization profile of the μ-δ heteromer, to investigate μ-δ heteromer-specific signalling in brain, and to interrogate the contribution of this receptor complex to opioid-mediated behavioural effects.

In competition radioligand binding studies, δ-agonists displaced high affinity μ-agonist binding from the μ-δ heteromer but not the μOR homomer, suggestive of δ-agonists occupying or allosterically modulating the μOR ligand binding pocket within the heteromer. δ-agonists induced internalization of the μ-δ heteromer in a dose-dependent, pertussis toxin resistant, and μOR- and δOR-dependent manner from the cell surface via the clathrin and dynamin endocytic machinery. Agonist-induced internalization of the μ-δOR heteromer
persisted following chronic morphine treatment conditions which desensitized the µOR homomer.

Using Go-specific GTPγS binding assays, we demonstrated that µ-δ heteromer signalling previously characterized in cell lines was present in the striatum and hippocampus, and did not desensitize following prolonged morphine treatment conditions which desensitized µOR homomer-mediated signalling.

Since δ-agonists which also target the µ-δ heteromer possess antidepressant-like and anxiolytic-like properties, we investigated the role of this receptor complex in mood regulation. We devised a strategy to selectively analyze the effects of the µ-δ heteromer by dissociating it using a specific interfering peptide aimed at a sequence implicated in µ-δ heteromerization. The interfering peptide abolished the unique pharmacological and trafficking properties of δ-agonists at the µ-δ heteromer and dissociated this receptor complex in vitro. Intra-accumbens administration of the interfering peptide disrupted the µ-δ interaction in vivo and allowed for isolation of the µ-δ heteromer contribution to the mood-regulatory effects of a δ-agonist with activity at the heteromer. Activation of the µ-δ heteromer in the nucleus accumbens produced antidepressant-like and anxiolytic-like actions in animal models of depression and anxiety.
LIST OF PUBLICATIONS

Kabli, N., Nguyen, T., O’Dowd, B.F., and S.R. George. The \( \mu-\delta \) opioid receptor heteromer mediates the anti-depressant-like effects of \( \delta \)-agonists in the nucleus accumbens. Manuscript in preparation.


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DEDICATION

To my parents
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<td>5'-NTII</td>
<td>naltrindole 5'-isothiocyanate</td>
</tr>
<tr>
<td>β-FNA</td>
<td>β-funaltrexamine</td>
</tr>
<tr>
<td>μOR</td>
<td>mu opioid receptor</td>
</tr>
<tr>
<td>δOR</td>
<td>delta opioid receptor</td>
</tr>
<tr>
<td>κOR</td>
<td>kappa opioid receptor</td>
</tr>
<tr>
<td>%</td>
<td>percent</td>
</tr>
<tr>
<td>±</td>
<td>plus or minus</td>
</tr>
<tr>
<td>°C</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>BL</td>
<td>baseline</td>
</tr>
<tr>
<td>BNTX</td>
<td>7-benzylidenenaltrexone</td>
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<tr>
<td>BRET</td>
<td>bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>C</td>
<td>carboxyl</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>calcium</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CB</td>
<td>cannabinoid</td>
</tr>
<tr>
<td>CGRP</td>
<td>calcitonin gene related peptide</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>con A</td>
<td>concanavalin A</td>
</tr>
<tr>
<td>CTOP</td>
<td>D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH₂</td>
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<td>CTRL</td>
<td>control</td>
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<td>DADLE</td>
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<td>DAMGO</td>
<td>[D-Ala², NmePhe⁴, Gly-ol⁵]enkephalin</td>
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<tr>
<td>Deltorphin II</td>
<td>[D-Ala², Glu⁴]deltorphin II, H-Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH₂</td>
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<tr>
<td>DPDPE</td>
<td>[D-Pen⁵, D-Pen⁵]enkephalin, where pen is penicillamine</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglia</td>
</tr>
<tr>
<td>DSLET</td>
<td>[D-Ser²,Leu⁵,Thr⁶]enkephalin</td>
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<tr>
<td>DYN K44A</td>
<td>dynamin dominant negative mutant</td>
</tr>
<tr>
<td>DZP</td>
<td>diazepam</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPM</td>
<td>elevated plus maze</td>
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<tr>
<td>Endomorphin-1</td>
<td>Tyr-Pro-Trp-Phe-NH₂</td>
</tr>
<tr>
<td>Endomorphin-2</td>
<td>Tyr-Pro-Phe-Phe-NH₂</td>
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<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FST</td>
<td>forced swim test</td>
</tr>
<tr>
<td>g</td>
<td>gram(s)</td>
</tr>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<tr>
<td>GRK</td>
<td>G-protein receptor kinase</td>
</tr>
<tr>
<td>GTPγS</td>
<td>guanosine 5'-γ-thiotriphosphate</td>
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**Abbreviations**

HA = hemagglutinin  
%H A = percent high affinity  
HDL = high density lipoprotein  
HEK = human embryonic kidney  
hr = hour(s)  
IMI = imipramine  
i.p. = intraperitoneal  
ISH = In-situ hybridization  
K+ = potassium  
kDa = kilo Dalton  
KAI = high affinity binding constant  
KL = low affinity binding constant  
LDCV = large dense core vesicle  
Leu-enkephalin = H-Tyr-Gly-Gly-Phe-Leu-OH  
LHR = leutenizing hormone receptor  
MAPK = mitogen-activated protein kinase  
MEM = minimum essential medium  
Met-enkephalin = H-Tyr-Gly-Gly-Phe-Met-OH  
min = minute(s)  
mRFP = monovalent red fluorescent protein  
mRNA = messenger ribonucleic acid  
MS = morphine sulfate  
N = amino  
NAC = nucleus accumbens  
NAKT = naltrindole  
NIH = novelty-induced hypophagia  
OR = opioid receptor  
ORL-1 = opioid receptor-like-1  
PDYN = prodynorphin  
PEND = proendormorphin  
PENK = proenkephalin  
PEP = peptide  
PKC = protein kinase C  
PNS = peripheral nervous system  
POMC = proopiomelanocortin  
PTX = pertussis toxin  
R = receptor  
RT-PCR = reverse-transcriptase polymerase chain reaction  
s = second(s)  
s.c. = subcutaneous  
SDS = sodium dodecyl sulfate  
SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis  
SEM = standard error of the mean  
SNC80 = (+)-4-[(αR)-α-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide  
SNSR = sensory neuron specific G protein-coupled receptor
<table>
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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>SP</td>
<td>substance P</td>
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<tr>
<td>UFP-512</td>
<td>H-Dmt-Tic-NH-CH(CH₂-COOH)-Bid</td>
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<td>VGCC</td>
<td>voltage-gated calcium channels</td>
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1. INTRODUCTION

1.1 A brief history of opioids

Opium, a powerful tincture extracted from the poppy seeds of *Papaver somniferum*, has been used and abused for several centuries for its potent analgesic and euphoric effects. Following its isolation in 1803 as the active ingredient, morphine rapidly gained status as the gold standard analgesic. A few decades later, heroin was synthesized by deacetylating morphine and was touted as a non-habit-forming opiate. Opioids were used to treat pain, cough, diarrhoea and in some instances melancholia. However, with their widespread use, it became clear that prolonged exposure to opioids leads to the development of tolerance and dependence, greatly limiting their therapeutic utility. The addictive properties of opiates could not be ignored and prompted the prohibition of opium and the morphine derivative heroin in the early 1900s. One hundred years later and despite its adverse effects, morphine remains the most widely used analgesic in the clinic and one of the key indispensable weapons in the physician’s arsenal against moderate to severe pain. Today, the abuse of prescription opioid narcotics has become a growing problem far surpassing heroin abuse and affecting a wider tranche of society. Thus, exploring the opioid system is necessary to conceive strategies aimed at leveraging its therapeutic analgesic and mood-elevating effects while minimizing unwanted side effects. Research into the pharmacology of the opioid system suggests that this seemingly lofty goal may be attainable.

1.2 Opioid binding sites

Opioid binding sites were first proposed as early as the 1950s (Beckett and Casy 1954) and models for ligand interactions with the “analgesic-receptor” were beginning to be
elucidated as early as the 1960s (Portoghese 1965). However it was not until 1973 that three independent research groups used tritiated agonist and antagonist radioligand binding to demonstrate that opioids exert their effects by binding to membrane receptors in mammalian brain and myenteric plexus (Pert and Snyder 1973; Simon 1973; Terenius 1973). A number of opioid receptor (OR) types was postulated based on competition radioligand binding and autoradiographic studies using various opioid agonist and antagonist combinations. The distinct binding patterns of various opioid ligands suggested the existence of several OR subtypes or several forms of the ORs. Table 1.1 catalogues a list of selective OR ligands. By the late 1970s and early 1980s, µ, κ, σ, and ε OR types had been postulated to exist based on radioligand binding using morphine, ketocyclazocine, SKF10047, and β-endorphin, respectively (Waldhoer et al., 2004). The δ receptor was characterized in studies using vas deferens preparations (Zaki et al., 1996). However, subsequent studies demonstrated that σ was in fact a single transmembrane (TM) protein and the ε receptor simply reflected β-endorphin binding to both µ- and δ-ORs. Thus, by the end of the 1980s, the overall consensus was that there were only three types of ORs: µ, δ, and κ.

1.3 More than three types of opioid receptors?

The multiplicity of ligand binding profiles and differential antagonism in behavioural and molecular studies led to the division of the three OR types into eight subtypes µ₁,₂,₃, δ₁,₂, and κ₁,₃ (Dietis et al., 2011). Of these, δOR subtypes have been the most widely studied and characterized owing in part to the clear and largely reproducible differences in their pharmacology and to the variety of available pharmacological and radiolabelled ligands
<table>
<thead>
<tr>
<th></th>
<th>µOR</th>
<th>δOR</th>
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<td>UFP-512</td>
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<td>DADLE</td>
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<td>Ethylketocyclazocine (EKC)</td>
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<td><strong>Endogenous peptide agonists</strong></td>
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<td>Endomorphin-1</td>
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<td>β-endorphin</td>
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<td><strong>Antagonists</strong></td>
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<td>Naltrindole</td>
<td>Nor-binaltrophimine (nor-BNI)</td>
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<tr>
<td>β-Funaltrexamine</td>
<td>Naltrindole 5' isothiocyanate (NTII)</td>
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<td>BNTX</td>
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<td>Naloxone</td>
<td>ICI-174,864</td>
<td>Naloxone</td>
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Compiled from (Gulya et al., 1988; Portoghese et al., 1988; Erspamer et al., 1989; Sofuoglu et al., 1991; Zadina et al., 1997; Quock et al., 1999; Waldhoer et al., 2004; Alexander et al., 2011).
targeting this receptor (Zaki et al., 1996). A number of δ-acting ligands had been developed based on the backbone of the endogenous δ-preferring agonist enkephalin, each grouped by their actions at two pharmacologically-distinct forms of the δOR. In behavioural and molecular studies, the effects of peptide δ-agonists DPDPE, DADLE, and DALCE were selectively antagonized by BNTX and those of DSLET and deltorphin II were selectively antagonized by naltrindole 5'-isothiocyanate and naltriben (Vaughn et al., 1990; Negri et al., 1991; Sofuoglu et al., 1991). The DPDPE- and DSLET-sensitive binding sites were termed δ₁ and δ₂, respectively (Zaki et al., 1996). Further, little cross-tolerance develops between DSLET and DPDPE or DPDPE and deltorphin II and autoradiographic binding patterns of ³H-DPDPE and ³H-DSLET are overlapping but not identical (Quock et al., 1999). However, structurally dissimilar alkaloid δ-ligands SNC80, BW373U86 and TAN-67 do not fit into a δ₁/δ₂ subcategorization, and the non-selective δ-antagonist naltrindole is used to block the effects of all δ-agonists. Despite this extensive in vivo evidence, radioligand binding and functional assays do not distinguish between δ₁ and δ₂ subtypes in heterologous expression systems and cell lines endogenously expressing the δOR (Toll et al., 1997; Parkhill and Bidlack 2002).

Until now, the molecular basis for OR subtypes has remained elusive. However, it is believed that this pharmacology, which is observed in tissues, may be explained by OR interactions with other receptors in vivo. Investigating the molecular basis of OR subtypes has become a goal in the opioid field since it is hoped that targeting pharmacologically-distinct OR subtypes may isolate specific therapeutic outcomes while avoiding unwanted side effects.
1.4 Endogenous opioid ligands

Endogenous opioid peptides are a family of neuropeptides derived from the three precursor genes proopiromelanocortin (POMC), preproenkephalin (PENK) and preprodynorphin (PDYN). POMC, PENK, and PDYN give rise to the gene products β-endorphin (µOR-preferring), Met- and Leu-enkephalin (δOR-preferring), and dynorphin (κOR-preferring). Although each peptide displays a slightly higher affinity at its cognate receptor, µ- and δ-acting peptides have relatively equal affinities for both µ- and δ-ORs. This may stem from the fact that the peptides are all structurally similar and share the hallmark amino terminal Tyr-Gly-Gly-Phe sequence which interacts with ORs (Kieffer 2009). Further, β-endorphin — the longest peptide with a 31 amino acid sequence - encompasses the enkephalin sequence. The most recent additions to the opioid peptide family are the highly µ-selective tetrapeptides endomorphin-1 and endomorphin-2 which are structurally different and have yet unidentified precursor genes (Zadina et al., 1997).

1.5 The cloning of opioid receptors

The advent of molecular biological techniques catalyzed the cloning of three OR types: µ (“mu” named after its ligand morphine), δ (“delta” derived from vas deferens where it was first identified) and κ (“kappa” named after its ligand ketocyclazocine) (Evans et al., 1992; Kieffer et al., 1992; Chen et al., 1993; Meng et al., 1993; Yasuda et al., 1993). Although, the isolation of three OR genes did not solve the mystery of OR subtypes, it did succeed in facilitating the development of genetic tools to manipulate and study the OR
system in vivo. Despite the isolation of only three OR genes, the pharmacology of opioid ligands observed in vivo suggested the existence of more OR subtypes.

1.5.1 Opioid receptor splice variants

The cloning of ORs also paved the way for the detection of a number of splice variants of the µOR in brain and spinal cord, each differing in their carboxyl tail sequence (Abbadie et al., 2000; Abbadie et al., 2001). Although µOR variants have overlapping but distinctive localization patterns in brain, their pharmacology is not sufficiently different so as to account for the pharmacological division of OR subtypes. In contrast, δ- and κ-OR splice variants have not been studied as extensively as their µOR counterparts. A δOR splice variant lacking the third intracellular loop was detected in the SHSY-5Y neuroblastoma cell line but not in normal human or rat brain or human monocytes. Another variant containing an insertion between the first and second exons was isolated in mouse brain (Gaveriaux-Ruff et al., 1997; Mayer et al., 2000). A κOR variant with a deletion and insertion producing a truncated final product was isolated in monocytes and T lymphocytes (Gaveriaux-Ruff et al., 1997). The regulation, function, or physiological relevance of the δ- and κ-OR variants remains unknown.

1.6 Localization of opioid receptors in the CNS and PNS

Prior to the cloning of ORs, knowledge about OR localization was deduced from autoradiography and radioligand binding studies which are limited by their resolution and inability to distinguish cell types. In situ hybridization and immunohistochemical techniques have been instrumental in shedding light on the cellular and subcellular localization of ORs.
ORs are expressed in several tissues and are widely distributed throughout the peripheral and central nervous systems (CNS and PNS) in rodents. Their widespread distribution in several regions of the CNS and PNS supports their involvement in modulating several biological processes. The concentration of ORs in regions implicated in the processing of nociceptive and emotional stimuli underscores their important role in modulating these behaviours in animals.

1.6.1 Cellular distribution of ORs in the CNS

1.6.1.1 µ- and δ-opioid receptor distribution in the CNS

Immunohistochemical, in situ hybridization and autoradiographic studies demonstrate that µ- and δ-ORs have distinct but overlapping expression profiles and colocalize to several regions including the cortex, striatum, nucleus accumbens, caudate putamen, amygdala, hippocampus, olfactory bulb, globus pallidus, ventral pallidum, hypothalamus, thalamus, colliculi, periaqueductal gray, ventral tegmental area, locus coeruleus, parabrachial nucleus and the spinal cord (Sharif and Hughes 1989; Mansour et al., 1993; George et al., 1994; Mansour et al., 1994; Arvidsson et al., 1995a; Arvidsson et al., 1995b; Cahill et al., 2001a; Goody et al., 2002).

µ- and δ-ORs are co-localize to the same neurons in several brain regions including the ventral tegmental area, nucleus accumbens, caudate putamen, hypothalamus, medial forebrain bundle, hippocampus, substantia nigra, ventral pallidum, medial thalamus, globus pallidus, bed nucleus of the stria terminalis, periaqueductal gray, and nucleus raphe magnus (Wang and Pickel 2001; Stumm et al., 2004; Gray et al., 2006). An extensive
characterization of the cell types co-expressing μ- and δ-ORs in all brain regions has not been undertaken. In the hippocampus, both μ- and δ-OR transcripts are present in GABAergic parvalbumin-containing basket cells and somatostatinergic oriens layer neurons in the hippocampal formation and low levels of transcript are expressed in vesicular glutamate transporter-1-containing granule cells (Stumm et al., 2004). In the caudate putamen nucleus, μ- and δ-ORs are expressed both individually and together in the terminal, axon, spine and dendritic neuronal compartments (Wang and Pickel 2001).

Neurons expressing μ- or δ-ORs alone also exist but are morphologically similar – with some exceptions – to those co-expressing both receptors in most brain regions (Stumm et al., 2004; Gray et al., 2006). In the hippocampus, calreticulin-containing GABAergic neurons express μOR but not δOR (Stumm et al., 2004).

1.6.1.2 κOR distribution in the CNS

Gray and colleagues (2006) undertook an extensive immunohistochemical study to generate a co-localization map of ORs in rodent brain. While the degree of μ- and δ-OR co-localization was extensive, κORs were observed in morphologically-distinct neuronal populations that did not express μORs but were physically apposed to μOR-expressing cells. These immunohistological findings were in line with behavioural studies demonstrating synergy and co-operativity between μ- and δ-ORs and antagonism between μ- and κ-ORs (Schmidt et al., 2002b). κORs will not be examined in detail in this dissertation.
1.6.2 Subcellular localization of μ- and δ-ORs in the CNS

At the subcellular level, μORs are expressed mainly at the neuronal plasma membrane with minimal localization to intracellular vesicles whereas δORs are localized to intracellular compartments with minimal cell surface expression (Arvidsson et al., 1995a; Arvidsson et al., 1995b; Svingos et al., 1997; Trafton et al., 2000; Wang and Pickel, 2001; Hack et al., 2005; Lucido et al., 2005; Ma et al., 2006). Thus, μ- and δ-ORs would be expected to co-localize both at the level of the plasma membrane and within intra-cellular compartments. For their part, δORs are mobilized to the cell surface following prolonged treatment with morphine in several brain regions including the nucleus accumbens, dorsal striatum, periaqueductal grey and nucleus raphe magnus, and in the spinal cord (Cahill et al., 2001b; Cahill et al., 2003; Hack et al., 2005; Lucido et al., 2005; Ma et al., 2006). This subcellular localization has pronounced ramifications for the behavioural and biochemical effects of opioid ligands which can interact readily with ORs targeted to the plasma membrane.

The distribution of the δOR was examined using two different commercially available antibodies, one targeting an epitope at the amino (N) terminal domain and the other targeting the carboxyl (C) terminus of the receptor (Cahill et al., 2001a). At the regional level, these antibodies produced similar localization maps in the CNS. However, the N-terminus antibody reacted more strongly with a higher molecular weight species of the δOR whereas the C-terminus antibody reacted against the lower molecular weight species, suggesting that antibodies stain distinct immunogenic forms of the δOR (Cahill et al., 2001a). The N terminal antibody produced greater than three-fold more intense δOR
staining, which suggests differential expression of the various immunogenic forms of the receptor, assuming that the two antibodies interact with the same affinity towards their respective epitopes. The most interesting differences arose when examining the subcellular distribution of δOR in neurons. The immunoreactive species of δOR detected by the N-terminus antibody was mainly localized to intracellular stores, and a greater proportion of this form was expressed in axon terminals. In contrast, a higher proportion of the immunoreactive δOR species detected by the C-terminus antibody was localized to the neuronal plasma membrane. However, absolute levels of plasma membrane expression of the former immunogenic form of the δOR were likely greater since N-terminus antibody staining was more intense, assuming similar antibody-epitope affinities. Both antibodies labelled nerve cell bodies but the δOR immunogenic species detected by the N terminal antibody was expressed to a greater extent in the nerve terminals. Not only do these findings demonstrate that antibodies interact with differential preference to various molecular species of the δOR, they also suggest that different molecular forms of the receptor may be targeted to different neuronal compartments.

While there is consensus about the regional localization of ORs, there is much debate about the subcellular localization of δORs. Using a knock-in mouse expressing GFP-tagged δORs (δOR<sub>GFP</sub>), Scherrer and colleagues contested the intracellular localization of δORs and demonstrated that these receptors are present at the cell surface in various brain regions including the cortex and the caudate putamen (Scherrer et al., 2006; Scherrer et al., 2009). However, other groups advance that the GFP moiety altered the subcellular localization of δOR in the knock-in mouse as this tag has been shown to shift localization of tagged proteins
to the plasma membrane (Wang et al., 2008), thus limiting the validity of findings from the δOR<sub>eGFP</sub> knock-in mouse model.

### 1.6.3 Cellular distribution of μ- and δ-ORs in the PNS

In the peripheral nervous system, μ- and δ-ORs are co-expressed in primary sensory afferents of the dorsal root ganglia (DRG) (Ji et al., 1995; Zhang et al., 1998b; Wang and Wessendorf 2001; Mennicken et al., 2003; Walwyn et al., 2009; Wang et al., 2010). Although μORs have been shown to be expressed in most neuron profiles of the DRG, they are most highly expressed in small-diameter unmyelinated peptidergic neurons which convey painful sensory information from the periphery (Ji et al., 1995; Wang et al., 2010). δORs are expressed in unmyelinated peptidergic and non-peptidergic small-diameter, myelinated medium-diameter, and myelinated large-diameter neurons which transmit painful (small and medium neurons) and mechanical touch sensations (large neurons), respectively. However, δORs are more highly expressed in large neurons, a localization that is thought to confer them with greater efficacy at inhibiting painful responses to mechanical stimuli (Scherrer et al., 2009). Since μORs predominate in small neurons, μ- and δ-ORs exhibit greater co-localization in this neuronal population (Wang et al., 2010).

Using the δOR<sub>eGFP</sub> knock-in mouse model, Basbaum and colleagues contested the co-localization of μ- and δ-ORs to the same DRG neurons (Scherrer et al., 2009). They reported that δORs are almost exclusively localized to large-diameter myelinated neurons and that they selectively modulate mechanical pain stimuli. μORs had a complementary distribution, being expressed mainly in small-diameter neurons and selectively modulating thermal pain.
In their double-labelling immunohistological experiments, this group reported minimal co-localization between δOR_{eGFP} and μORs. Further, these authors advanced that the δOR antibodies that had been used to characterize the expression of this receptor produced immunostaining in δOR gene-deleted animals. Basbaum and colleagues suggested that δ-agonists do not inhibit thermal pain when administered peripherally and that the positive effects reported in the literature were due to the use of high drug doses and were mediated by μORs instead (Scherrer et al., 2009). However, the utility of the δOR_{eGFP} knock-in mouse model has been questioned due to the enhanced levels of δOR expression and function in the CNS and PNS of these transgenic animals (Scherrer et al., 2006; Scherrer et al., 2009). Increased δOR expression has been shown to reduce the maturity of μORs, which may mask μ- and δ-OR co-localization (Decaillot et al., 2008).

The Basbaum study prompted a lively debate and a rapid response from groups who had previously reported on the expression of δORs in small-sized peptidergic neurons. Using single-cell PCR, Wang and colleagues demonstrated that μ- and δ-OR transcripts are present in the same neuronal populations of the DRG (Wang et al., 2010). While δORs are more highly expressed in myelinated large-diameter fibers, they nonetheless co-exist with μORs in small and large (to a lesser extent) fibers. Further, Wang and colleagues (2010) reasserted the selectivity of the δOR antibodies by demonstrating that staining is only observed in δOR gene-deleted animals at low dilutions of the antibody. In addition, Walwyn and colleagues used electrophysiological techniques to demonstrate that both μ- and δ-agonists inhibit Ca^{2+} currents in cultured small-diameter DRG cells, thus confirming the co-existence of these receptors in the same neurons (Walwyn et al., 2009).
1.6.4 Subcellular localization of µ- and δ-ORs in the PNS

While it is generally agreed that µORs mainly localize to the plasma membrane, the subcellular localization of δORs has been debated in the PNS as well (Cahill et al., 2001a; Bao et al., 2003; Walwyn et al., 2009). It had been generally accepted that δORs are mainly intracellular with limited cell surface expression (Cahill et al., 2001a; Bao et al., 2003; Gendron et al., 2006). However, while Cahill and colleagues (2001a) report that δORs are associated with RER, golgi bodies and membranes of small and large clear vesicles in unmyelinated axons of the spinal cord, which have their cell bodies in small-diameter neurons of the DRG, Bao and colleagues (2003) report that δORs are mainly associated with Calcitonin Gene-Related Peptide (CGRP)-containing large dense core vesicles (LDCV) which are present in these small neurons. Interaction with protachykinin (the precursor for the nociceptive molecule substance P which is expressed in small-diameter peptidergic neurons), this group argues, is essential for the sorting of δORs into LCDVs (Guan et al., 2005). Accordingly, they demonstrate that δORs are not present in this subset of neurons in preprotachikinin gene-deleted animals (Guan et al., 2005).

Using the δOR<sub>αGFP</sub> knock-in mouse model, Scherrer and colleagues (2009) contested the widely-held view that δORs are mainly intracellular and demonstrated that these receptors are present on the cell surface of large-diameter myelinated neurons of the DRG under basal conditions. The requirement of protachykinin for the proper sorting of δORs into peptidergic neurons was also disputed as the authors reported no alterations in the distribution of δORs in tachykinin gene-deleted animals (Scherrer et al., 2009).
Irrespective of the exact localization of δORs, both Cahill and Bao concede that δORs are targeted to the plasma membrane following a variety of exogenous stimuli (Cahill et al., 2001b; Bao et al., 2003). Cahill and colleagues (2001b) demonstrate that prolonged morphine treatment results in enhanced targeting of δORs to the plasma membrane of primary sensory neurons. Further, cell-permeable antagonists such as naloxone act as chaperones, stimulating the trafficking of δORs to the cell surface. Alternatively, Bao and colleagues (2003) advance that short duration treatment with the δ-agonist deltorphin I or nerve depolarization result in exocytosis of CGRP-containing vesicles (which express δOR) and insertion of δORs in the plasma membrane in a calcium-dependent manner. According to the latter paradigm, δOR stimulation would produce pain as it would induce release of the pro-nociceptive peptide CGRP. These observations were used to rationalize the use of δ-antagonists for the treatment of pain conditions. However this notion is not supported by behavioural findings which demonstrate that δ-agonists are extremely effective therapeutic interventions in animal models of chronic and inflammatory pain (Cahill et al., 2003; Morinville et al., 2004; Kabli and Cahill 2007; Shinoda et al., 2007; Pradhan et al., 2011; Saloman et al., 2011). Further, Bao and colleagues (2003) investigated δOR processing in a subset of small-diameter DRG neurons, and thus these mechanisms of δOR regulation do not apply in phenotypically-different sensory fibers. On the other hand, prolonged morphine treatment and chronic pain-induced regulation of the δOR appears to be a general phenomenon that occurs in many types of neurons and regions of the nervous system (Cahill et al., 2001b; Hack et al., 2005; Lucido et al., 2005; Gendron et al., 2006; Ma et al., 2006).
1.7 Opioid receptor function

1.7.1 Opioid receptor gene-deleted animals: insight into opioid receptor function

The generation of mice with targeted deletions in OR genes has provided a tremendous contribution to our understanding of the function of the opioid system. Animals in which all three OR genes have been disrupted are viable and have been instrumental in verifying non-opioid mechanisms of action (Simonin et al., 2001; Contet et al., 2004b).

1.7.2 The µOR: a potent analgesic receptor with limitations

Studies in µOR gene-deleted animals indicate that this receptor mediates both the therapeutic and adverse effects of morphine and other µ-acting agonists. Morphine-induced analgesia, reward, respiratory depression, inhibition of gastric motility, dependence and self-administration are abolished in µOR gene-deleted mice (Matthes et al., 1996; Sora et al., 1997; Matthes et al., 1998; Roy et al., 1998; Sora et al., 2001). Although µ-agonists possess potent antinociceptive properties, they have the highest abuse liability (Kieffer 1999; Contet et al., 2004a).

1.7.3 The δOR: a modest analgesic receptor with a special role in modulating mood

On the other hand, the δOR is implicated not only in analgesia but it also plays a significant role in regulating emotional responses. Animals in which the δOR gene is disrupted display depressive and anxiogenic behaviour in various behavioural assays including the forced swim test and elevated plus maze (Filliol et al., 2000; Kieffer and Gaveriaux-Ruff 2002; Pradhan et al., 2011). δ-Agonists elicit well-documented, potent antidepressant-like and anxiolytic-like properties in several animal models (Kastin et al.,
1978; Baamonde et al., 1992; Broom et al., 2002a; Broom et al., 2002b; Jutkiewicz 2006; Perrine et al., 2006). Preproenkephalin gene-deleted animals display depressive and anxiogenic phenotype similar to their δOR counterparts (Filliol et al., 2000; Ragnauth et al., 2001), confirming a parallel role of both the endogenous peptide and receptor in modulating basal emotional tone. Although δ-agonists are less potent analgesics, they have reduced addictive potential and less adverse effects (Cowan et al., 1988; Rapaka and Porreca 1991; Lazarus et al., 1999; Pradhan et al., 2011).

1.7.4 The κOR: a peripherally-restricted analgesic receptor with limited therapeutic utility

κORs mediate analgesia and dysphoria (Simonin et al., 1998). Thus, the therapeutic utility of κ-acting agonists is mainly restricted to their effects in the periphery, although some studies indicate that κOR antagonists may have antidepressant effects (Carr et al., 2010). κORs have in some instances been described as an anti-opioid system. For example, κOR activation antagonizes μOR- and δOR-mediated analgesia in the nucleus accumbens (Schmidt et al., 2002b).

1.8 Opioid receptors are G-Protein Coupled Receptors

Opioid receptors belong to the 7-transmembrane (7-TM) or heptahelical domain superfamily of membrane receptors, also named G-protein coupled receptors (GPCRs). Originally named because they transduce signals by activating heterotrimeric guanine nucleotide binding proteins (G-protein), GPCRs are alternatively referred to using the 7-TM domain receptor nomenclature following reports of G-protein-independent signalling (Pierce
et al., 2002). GPCRs represent the largest gene family of membrane proteins and the third largest gene family in the human genome, with > 1200 genes identified, 800 of which have been de-orphanized. This family of receptor represents half of current drug targets (George et al., 2002; Pierce et al., 2002; Fotiadis et al., 2006).

1.8.1 Opioid receptor structure

Opioid receptors belong to the class A rhodopsin family of GPCRs and have the signature 7-TM helical domain topology with an extracellular amino terminus, three extracellular and three intracellular loop domains, and an intracellular carboxyl terminus (Minami and Satoh 1995). The three ORs display ~ 60% overall sequence homology with the least variance in the TM core region which displays greater than 70% homology among the three receptors and contains residues forming the ligand binding cavity (Waldhoer et al., 2004; Granier et al., 2012; Manglik et al., 2012; Wu et al., 2012). The amino terminal domain and extracellular loops vary greatly between ORs and play a role in ligand selectivity (Kane et al., 2006). Select sequences within the intracellular loops and proximal carboxyl terminus are implicated in the interaction with G-proteins, receptor kinases and the complement of intracellular signalling machinery (Merkouris et al., 1996; Law et al., 2000; Xie et al., 2007). Similar to other class A GPCRs, ORs have the conserved D/ERY (Asp/Glu-Arg-Tyr) motif at the intracellular portion of TM III, which forms a salt bridge between the Arginine and Aspartate/Glutamate of TM VI. This interaction acts as an ionic lock which stabilizes the receptor in an inactive conformation. Further, ORs also share the NPXXY (Asn-Pro-X-X-Tyr) motif in the intracellular portion of TM VII which is thought to play a role in the transition from inactive to active states of the receptor as well as high-
affinity agonist binding, G-protein coupling and postreceptor trafficking (Wess et al., 1993; Barak et al., 1995; Fritze et al., 2003).

1.9 The regulation of opioid receptors

1.9.1 Opioid receptor stimulation, agonist binding and G-protein activation

Agonist binding to ORs induces conformational changes which modify the helical packing and induce rearrangements of TM helices III, VI, and VII (Kobilka 2007). The activated receptor conveys the extracellular signal by activating the G-protein α subunit which acts as a guanine exchange factor promoting the dissociation of GDP and activation of GTP. The activated G-protein heterotrimer dissociates into α subunit and βγ dimer. However, increasing evidence suggests that the dissociation of α and βγ is “functional” rather than “physical” (Rasmussen et al., 2011; Westfield et al., 2011). The current thinking is that the Gα and Gβγ subunits are pulled apart from one another while remaining closely associated with the receptor. This, a paradigm shift from the traditional model that saw the subunits dissociating and moving away from the receptor, is in line with an energy conservation framework where the signalling components are kept in close proximity and poised to participate in another fast receptor activation cycle.

1.9.2 Opioid receptor G-protein coupling and effector activation

ORs regulate many signalling pathways by activating multiple types of G-proteins. ORs couple mainly to the pertussis toxin-sensitive Gαi/o family of inhibitory G-proteins, which includes Gαi1, Gαi2, Gαi3, Gαz, Gαo1, and Gαo2 to initiate a multitude of downstream signalling events (Jordan and Devi 1998; Law et al., 2000; Albert and Robillard 2002; Tso
and Wong 2003). Opioid receptors also couple to calcium mobilization through the $G_{\alpha_q}$ and phospholipase C and $G_{\alpha_i/o}$ pathways in vitro and in vivo (Murthy and Makhlouf 1996; Rhim et al., 1996; Ho et al., 2001; Narita et al., 2003). In vitro, most opioid-induced signals can be blocked by pertussis toxin, suggesting predominant coupling to the PTX-sensitive $G_{\alpha_i/o}$ proteins (Hsia et al., 1984; Rhim et al., 1996; Clark et al., 1997; Pak et al., 1999; Tso and Wong 2003).

Opioid receptor activation leads to the inhibition of adenylyl cyclase resulting in a decrease in cAMP levels, activation of inward-rectifying $K^+$ channels and inhibition of voltage-gated calcium channels (VGCC) in a variety of cell line and neuronal preparations (Childers 1991; Jordan and Devi 1998; Law et al., 2000). Opioid-induced inhibition of VGCC is thought to occur via coupling to PTX-sensitive G-proteins (Rhim et al., 1996). Overall, the effects of OR activation are inhibitory, as agonists inhibit neurotransmitter release from the pre-synaptic cleft and hyperpolarize post-synaptic neurons (Waldhoer et al., 2004). In addition to G-protein-mediated signalling, ORs similar to other GPCRs also signal through G-protein-independent mechanisms through $\beta$-arrestin2 scaffolding to the mitogen-activated protein kinase (MAPK) and extracellular signal regulated kinase (ERK) signalling cascades (Zheng et al., 2008). $\beta$-arrestin2 recruits mediators of kinase signalling to the receptors both at the plasma membrane and after receptor sequestration and endocytosis.

1.9.3 **Dampening of opioid receptor signalling and receptor endocytosis**

Regulators of G-protein Signalling (RGS) proteins act as a break mechanism, accelerating GTP hydrolysis to GDP and leading to the reassociation of the G-protein
heterotrimer and termination of the activation cycle. The activated OR with its changed conformation recruits G-protein receptor kinases (GRKs) and/or tyrosine kinases to phosphorylate Serine/Threonine and/or Tyrosine residues, respectively, at its carboxyl tail and third intracellular loop (Chavkin et al., 2001; Qiu et al., 2003). β-arrestins get recruited to the phosphorylated receptor to uncouple it from its associated G-protein. So named because they are said to “arrest” signalling, β-arrestins recruit the endocytic machinery to initiate a process of receptor sequestration and endocytosis through clathrin-coated pits at the plasma membrane. This trafficking of the receptor toward intracellular compartments, also termed internalization is an adaptive response that promotes both the desensitization (as it removes the receptor from being accessible to ligand at the cell surface) and resensitization of the signal (as it promotes agonist dissociation of the receptor and its recycling towards the cell surface) (Ferguson, 2001; von Zastrow 2003).

Agonist-induced internalization of µORs and δORs has been visualized in cell lines and in neural tissues from the brain, spinal cord and dorsal root ganglia (Arden et al., 1995; Trafton et al., 2000; Scherrer et al., 2006). In addition to their role in binding to and recruiting the endocytic machinery, β-arrestins have a dual role as promoters of signalling through the MAPK cascade, thus allowing the receptor to continue signalling long after it has internalized (Zheng et al., 2008).

1.9.4 Post-endocytic sorting of opioid receptors

Overall ORs undergo a similar sequence of events until they are endocytosed and sequestered in vesicles. While µORs are rapidly recycled (Arden et al., 1995; Finn and
Whistler 2001), δORs undergo lysosomal or proteasomal degradation (Trapaidze et al., 1996; Tsao and von Zastrow 2000). Whereas μORs possess the LENLEAE sorting signal in their carboxyl tail which directs their recycling to the cell surface (Tanowitz and von Zastrow 2003), δORs interact with the G-protein coupled associated sorting protein (GASP) at their carboxyl terminus which transports them to the lysosomes for degradation (Whistler et al., 2002).

1.9.5 GPCR conformations and ligand-directed signalling: the chicken or the egg?

The phenomenon of ligand-directed signalling has been documented for many GPCRs (Audet et al., 2008; Evans et al., 2010; Baker et al., 2011). Different selective ligands are thought to stabilize their corresponding receptor in unique conformations that recruit a specific set of signalling molecules to the intracellular portion of the receptor, resulting in activation of different effectors. For example, the β2-adrenergic receptor is differentially targeted towards β-arrestin-mediated signalling versus G-protein-mediated signalling following activation by different ligands (Evans et al., 2010; Baker et al., 2011). The μOR also undergoes ligand-directed signalling. Whereas μ-agonists morphine and methadone activate ERK in a G-protein-dependent manner whereby activated ERK is cytosolic and activates the transcription factor cAMP response element binding protein, fentanyl and etorphine activate ERK in a β-arrestin-dependent manner leading to the nuclear translocation of activated ERK which in turn activates the transcription factor Elk-1 (Zheng et al., 2008). In this manner, ligands are said to dictate the signalling cascade to which the receptor couples.
However, a growing number of studies is demonstrating that receptors actually pre-couple with G-protein subunits and a complement of signalling molecules in the endoplasmic reticulum and are transported as one complex, or a signalosome, to the plasma membrane where the receptor is available for activation by ligands (Dupre et al., 2006; Dupre et al., 2007; Audet et al., 2008). According to this model, the ligand plays a limited role in “directing” the signalling of the receptor. However, these two models may not be mutually exclusive. Studies examining ligand affinities in heterologous cells over-expressing various G-protein α subunits relative to the receptor or expressing OR-G-protein fusion constructs demonstrate that agonists have slightly varying affinities for differentially G-protein-coupled receptors (Snook et al., 2008; Yan et al., 2008). Using the substituted cysteine accessibility method, Roth and colleagues demonstrated that κ-agonists have 2-3 fold changes in affinity for κOR coupled to Ga_{i2} versus Ga_{16} and that these small changes in affinity actually translated to conformational changes within the receptor (Yan et al., 2008). Thus, agonists may in fact “direct” signalling by binding to different signalosomes with different affinities.

1.9.6 Receptor acrobatics: the many conformations of the δOR

The δOR has been the subject of intense study as peptide and alkaloid ligands have been shown to differentially regulate this receptor in heterologous cells endogenously and exogenously expressing δOR (Quock et al., 1999; Varga et al., 2004). However, the implications of these observations were not immediately evident. Recently, Pineyro and colleagues used Bioluminescence Resonance Energy Transfer (BRET) proximity assays to probe the conformation of δORs when bound to different ligands (Audet et al., 2008). They tagged the receptor and G-protein α or βγ subunits with fluorophores and assessed ligand-
induced conformational shifts in the receptor. Confirming previous biochemical observations of differential δ-agonist effects on internalization and signalling, biophysical approaches demonstrated that δORs adopt distinct ligand-dependent conformations which are propagated downstream to components in the signalling cascade. The ligand-induced conformational changes are encoded within the conformation of the receptor, thus creating a unique “barcode” for communication with intracellular signalling components. By asserting that receptors have multiple ligand-stabilized active states, this framework challenges the traditional and simplistic two-state model of receptor activation that postulates that receptors interconvert between a ligand-bound active and inactive state and, and thus cannot adequately explain the differential efficacies of ligands in activating different effectors. The physiological relevance of these observations has recently been demonstrated. Using SNC80 and ARM390, two δ-agonists with similar analgesic efficacy but different internalization (and subsequent receptor degradation) potencies, it was demonstrated that prolonged administration of SNC80 induced tolerance to its analgesic, locomotor and anxiolytic-like effects whereas ARM390 selectively induced tolerance to analgesic responses with no desensitization in locomotion or anxiolysis (Pradhan et al., 2010). This effectively demonstrated that the differential regulation of the δOR actually affects the function of the receptor and differentially affects tolerance to different behavioural effects of δ-agonists.

More recently, Audet and colleagues demonstrated that agonist-specific recycling patterns of δOR and differential receptor interactions with β-arrestin2 lead to varying degrees of functional desensitization and acute analgesic tolerance (Audet et al., 2012). In contrast with SNC80, DPDPE induced δOR recycling and a different conformational interaction with β-arrestin2 in vitro, which correlated with reduced cAMP desensitization and an absence of
acute analgesic tolerance in vivo. Thus, different conformations of receptors have different potencies in activating/initiating the mechanisms of tolerance.

1.9.7 μOR regulation: morphine refuses to play by the rules

The short term regulation of μOR in response to various agonists is thought to correlate with their propensity to produce tolerance in animals (Whistler et al., 1999). In contrast with other μ-agonists, morphine is a poor recruiter of protein kinases and does not induce appreciable phosphorylation of the μOR, and thus morphine-bound μORs do not adopt the conformation conducive to β-arrestin2 recruitment (Arden et al., 1995; Keith et al., 1996; Sternini et al., 1996; Segredo et al., 1997; Burford et al., 1998; Keith et al., 1998; Zhang et al., 1998a; Whistler et al., 1999; He et al., 2002). Morphine-bound μORs do not internalize and do not undergo recycling and re-insertion into the plasma membrane since they undergo kinase-mediated phosphorylation to a lesser extent compared to other μ-agonists (Arden et al., 1995; Whistler et al., 1999).

Morphine’s lack of internalization was thought to be associated with its propensity to produce tolerance and dependence. This, termed the RAVE hypothesis advanced that high efficacy ligands such as DAMGO, methadone, fentanyl and etorphine which induce phosphorylation of the receptor, β-arrestin2 recruitment and subsequent internalization were shown to produce less tolerance than morphine in cellular and animal models (Whistler et al., 1999; Finn and Whistler 2001; He et al., 2002).
If the lack of β-arrestin2 recruitment accounted for morphine’s inefficiency in internalization and high tolerance-inducing profile, then one would expect that animals lacking the gene for β-arrestin2 would have even more pronounced morphine tolerance. Paradoxically, this is not the case. β-arrestin 2 gene-deleted animals display enhanced morphine-mediated analgesia and do not develop morphine tolerance, suggesting β-arrestin2 is essential for the regulation of morphine-stimulated µORs and suggesting that β-arrestin2 does interact with morphine-stimulated µORs to induce their internalization and desensitization (Bohn et al., 2000).

This notion that β-arrestin2 is essential for the G-protein uncoupling and post-receptor regulation of µORs is further supported by the finding that β-arrestin2 gene-deleted mutants have increased µOR G-protein coupling in brain and spinal cord (Bohn et al., 2004). Further, spinal and supraspinal morphine analgesia is increased in these animals, suggesting that β-arrestin2 is essential for the desensitization of µOR following their activation by morphine (Bohn et al., 2000; Bohn et al., 2002). What these studies suggest is a framework whereby morphine-bound µORs do interact with β-arrestin2, albeit weakly, and these receptors may remain desensitized at the plasma membrane (Whistler et al., 1999).

Thus, although their interaction with β-arrestin2 may not influence the short-term regulation of morphine-bound µORs, it does become significant under conditions of more prolonged morphine treatment. Indeed, animals treated with various chronic morphine treatment regimens display decreased responsiveness to other µ-acting agonists as manifested by desensitization in G-protein coupling (Selley et al., 1997; Bohn et al., 2000;
Sim-Selley et al., 2007) and adenylyl cyclase inhibition (Noble and Cox 1996). In cell lines, overexpression of the receptor kinase GRK2 results in robust β-arrestin2 translocation to μORs at the plasma membrane (Zhang et al., 1998a; Bohn et al., 2004). Thus exogenous addition of a kinase to ensure phosphorylation of morphine-bound μORs is sufficient. Indeed, chronic morphine treatment has been shown to increase GRK expression in brain, and this would be expected to lead to the phosphorylation of μOR upon stimulation with morphine, arrestin recruitment, internalization and desensitization (Terwilliger et al., 1994; Ozaita et al., 1998; Hurle 2001). Additionally the loss of tolerance to morphine analgesia in β-arrestin2 gene-deleted animals can be explained by recent evidence that β-arrestin2 can contribute to cellular morphine tolerance by inhibiting the resensitization of μORs and preventing their return to the cell surface (Dang et al., 2011).

1.9.8 Morphine-induced μOR internalization: a neuronal compartment-specific phenomenon

In 2003, the notion that morphine does not promote μOR trafficking was dispelled by von Zastrow and colleagues (Haberstock-Debic et al., 2003). In this landmark study, animals were treated with morphine systemically and the subcellular localization of μORs was examined using immunoelectron microscopy in the nucleus accumbens. Morphine did not induce μOR internalization in the cell body but stimulated a robust intracellular trafficking of the receptor in dendritic processes (Haberstock-Debic et al., 2003). Similar results were observed in a μOR virally transfected nucleus accumbens neuron preparation. This suggests that agonists may have different endocytic properties in different cellular compartments. Indeed, several proteins are expressed in a compartment-specific manner in neurons (Abul-
Husn et al., 2009). The complement of proteins in a micro-environment likely influences the endocytic fate of the receptor after its stimulation.

1.10 Interactions between opioid receptors: synergy and co-operativity between $\mu$OR and $\delta$OR

1.10.1 Behavioural evidence of $\mu$- and $\delta$-opioid receptor interactions

Early evidence of $\mu$- and $\delta$-OR interaction came mainly from studies demonstrating the cross-potentiation of behavioural effects of $\mu$- and $\delta$-agonists. $\mu$- and $\delta$-OR ligands potentiate one another’s anti-nociceptive effects both spinally and supra-spinally (Gomes et al., 2004; Malmberg and Yaksh 1992; Porreca et al.; 1990; Porecca et al., 1992). Intrathecal administration of subanalgesic doses of the endogenous $\delta$-preferring agonist Leu-enkephalin potentiated morphine-induced analgesia in the tail immersion assay, thus providing evidence of interactions at the level of the pain circuits in the spinal cord and primary sensory afferents of the dorsal root ganglia (Larson et al., 1980). Similarly, Leu-enkephalin has been documented to potentiate morphine-induced analgesia, ileal muscle contraction and abdominal constriction among other behavioural effects (Vaught and Takemori 1978; 1979). $\delta$-agonists also potentiate the analgesic effects of $\mu$-agonists in chronic pain models (Zhang et al., 1998a; Schramm and Honda 2010). Further, the irreversible $\mu$-antagonist $\beta$-funaltrexamine has been shown to antagonize the effects the $\delta$-antagonist ICI174864 on $\delta$-agonist-induced anti-convulsant effects and striatal cAMP modulation (Tortella et al., 1985; Holaday et al., 1986).
1.10.2 Molecular evidence of µ- and δ-opioid receptor interactions

µ- and δ-ORs have also been shown to interact and modulate each other’s activity in a number of molecular functional assays. δOR ligands increase µOR binding sites, µOR-mediated G-protein activation, and µOR-mediated MAPK activation (Gomes et al., 2004). Prolonged µOR stimulation has been shown to enhance the targeting of intracellularly stored δORs to the plasma membrane in the brain, spinal cord and dorsal root ganglia (Cahill et al., 2001b; Hack et al., 2005; Lucido et al., 2005; Gendron et al., 2006; Ma et al., 2006). This plasmalemmal targeting of the δOR was accompanied by increased antinociceptive potency and G-protein activation of the δ-agonist deltorphin II and was not observed in µOR gene-deleted animals (Morinville et al., 2003; Morinville et al., 2004).

Indeed, studies in OR gene-deleted animals support the existence of functional interactions between µ- and δ-ORs in specific central neuronal circuits (Kieffer and Gaveriaux-Ruff 2002). Targeted gene deletion of ORs in mice has provided a significant leap not only in our understanding of the function of each OR type but has been instrumental in confirming the interactions that occur between ORs that were previously postulated to exist based on radioligand binding and behavioural studies. Although some studies reported that the analgesic effects of δ-agonists were maintained in µOR gene-deleted mutants (Loh et al., 1998), a more weighty body of evidence suggests otherwise (Matthes et al., 1998; Morinville et al., 2004; Scherrer et al., 2004; Gendron et al., 2007). The spinal and supraspinal analgesic effects of the δ-agonist DPDPE are abolished or decreased in µOR gene-deleted mice (Scherrer et al., 2004). Similarly, deltorphin II-induced thermal antinociceptive effects are decreased in these animals (Matthes et al., 1998). The use of
different pain testing paradigms probing various pain modalities likely accounted for slightly differing findings between studies, as ORs differentially modulate various pain circuits. Further, δOR-mediated G-protein activation is reduced or abolished in brains of mice lacking the μOR gene (Hosohata et al., 2000). Another demonstration of the interdependence of μOR and δOR function came from a study showing that μOR-mediated inhibition of calcium currents in the dorsal root ganglia was attenuated in DRG neurons from δOR gene-deleted animals (Walwyn et al., 2009). Further, cell surface expression of μOR in DRG neurons was also reduced, as has been reported in brain (Goody et al., 2002), which suggests that δORs may be required for cell surface expression of μOR (Walwyn et al., 2009).

The role of the δOR in the cellular mechanisms of morphine tolerance is still disputed with some studies demonstrating that δOR gene-deleted animals do not develop morphine tolerance (Zhu et al., 1999) and others asserting that morphine tolerance is intact in these animals (Scherrer et al., 2009).

Overall, these studies suggest that μ- and δ-ORs interact in vivo and that these interactions are necessary for the full therapeutic responses to some opioid agonists.

### 1.11 GPCR Oligomerization

Many GPCRs have been shown to form homo-oligomers (homomers) and hetero-oligomers (heteromers) with similar or different subtypes or classes of receptors, respectively. GPCR oligomers have been demonstrated using a variety of techniques including co-immunoprecipitation, radioligand binding, bioluminescence and fluorescence
resonance energy transfer, functional complementation of inactive receptor pairs and co-trafficking of receptor partners by stimulating one of the subunits (George et al., 2002). Homo-oligomerization may be a universal phenomenon for GPCRs and has implications for receptor trafficking after synthesis in the endoplasmic reticulum (George et al., 2002). For example, homodimerization of the β2 adrenergic receptor has been shown to be a prerequisite for cell surface receptor expression and adenylyl cyclase function (Hebert et al., 1996). The functional roles for hetero-oligomerization of GPCRs range from chaperone-like aiding of cell surface localization to novel pharmacology and signal transduction properties, selective activation of signalling cascades, generation of signalling diversity, and pharmacological tissue specificity depending on the interacting receptors (George et al., 2002; Pierce et al., 2002; Rozenfeld et al., 2012).

For many types of GPCRs, formation of a heteromeric signalling complex is a prerequisite to signalling (Terrillon et al., 2003). The GABA_B-R is an example of an obligate heterodimer (Galvez et al., 2001). The GABA_B-R1 subunit contains the ligand binding cavity, but does not reach the cell surface when expressed alone. On the other hand, the GABA_B-R2 subunit is trafficked to the plasma membrane but cannot bind the putative ligand GABA. When co-expressed, GABA_B-R1 and GABA_B-R2 interact via a coiled-coil at their carboxyl termini and reconstitute a functional GABA_B-R that is expressed at the cell surface and can bind ligand to transduce signals intracellularly.

Recently, Huhtaniemi and colleagues provided an elegant demonstration of GPCR homomers (Rivero-Muller et al., 2010). The authors achieved functional complementation
of a ligand binding- and signalling-defective mutant leutnenizing hormone receptor (LHR) pair in vitro. By mating two LHR gene-deleted mouse lines each expressing one of the LHR mutants, the group demonstrated a near-complete reversal of the hypogonadism phenotype and full complementation in the transgenic mice expressing two non-functional LHRs (Rivero-Muller et al., 2010). Not only do these data demonstrate LHR oligomerization in vivo, they suggest that ligand binding to one protomer is sufficient to induce activation of signal transduction by the neighbouring protomer.

1.11.1 Can a monomeric GPCR function on its own?

With the exception of obligate GPCR heteromers such as the GABA<sub>B</sub>-R, it is not definitively known whether homo- and hetero-oligomerization is crucial for GPCR function (Gurevich and Gurevich 2008). The question of whether GPCRs can function as monomers pre-occupied researchers in the GPCR community and led to the conception of a method whereby a single receptor molecule is reconstituted phospholipid bilayer, allowing for the dissection of its signalling properties (Whorton et al., 2007; Kuszak et al., 2009). A single purified fluorophore tagged µOR in a high density lipoprotein (HDL) molecule receptor bound one single molecule of fluorescently-labelled ligand and activated exogenously-added Ga<sub>12</sub> proteins (Kuzak et al., 2009). While these findings demonstrated that oligomerization is not a prerequisite for µOR function, the HDL molecule remains a synthetic medium where receptors are forced to exist as monomers. It is not known whether monomeric µORs exist in vivo or whether they can recruit downstream signalling molecules efficiently and undergo endocytosis. Thus, these findings do not preclude the possibility that µOR exist as oligomers in physiological tissues. Accordingly, structural modelling studies indicate that although a
single GPCR may interact with G-proteins and β-arrestins, two molecules of receptor produce the best fit and conformation (Liang et al., 2003; Filipek et al., 2004; Fotiadis et al., 2006; Modzelewska et al., 2006).

1.12 Anatomy of an opioid receptor

Structural insight into the organization of OR and their mode of ligand binding have been historically derived from structure-function studies utilizing site-directed mutagenesis, receptor chimeras, and truncation mutants. The early part of our decade saw the unveiling of the crystal structures of class A receptors rhodopsin and subsequently that of the β2-adrenergic receptor which have been used as template in homology studies elucidating modes of ligand binding and receptor structure (Palczewski et al., 2000; Rasmussen et al., 2007). The recent unveiling of the crystal structures of all three ORs has provided a significant leap in our understanding of the structure of these receptors (Granier et al., 2012; Manglik et al., 2012; Wu et al., 2012).

1.12.1 Crystal structure of the µOR

The µOR was crystallized in association with the irreversible µ-antagonist β-funaltrexamine (β-FNA) (Manglik et al., 2012). µORs are arranged as parallel dimers and associate with one another through helices TM5 and TM6. Interdimeric contacts occur between TM1, TM2 and helix 8, suggesting the formation of higher-order complexes composed of dimers as a basic unit. While dimers may have formed as an artefact of the crystallization conditions, the interaction interfaces are mostly in agreement with those predicted by computational modeling studies (Filizola and Weinstein 2002). The µOR
ligand binding pocket is exposed to the extracellular surface, which may explain the rapid
dissociation kinetics of opioid agonists. The antagonist β-FNA is within close proximity to
14 residues and interacts directly with 9 of these, making contact with TM3, TM5, TM6 and
TM7 domains of the µOR. The peptide agonist DAMGO is hypothesized to occupy an
overlapping but larger ligand binding pocket. Overall, this structure was in general
agreement with previous mutagenesis studies implicating certain key µOR residues in ligand
binding to β-FNA although binding of structurally-dissimilar ligands may vary slightly as
they may induce a different µOR conformation.

1.12.2 Crystal structure of the κOR

The crystal structure of the κOR bound to the highly selective κ-antagonist JDTic
reveals that these receptors, too, are arranged in parallel dimers and contact one another
through TM helices I, II and VIII similar to the µOR (Wu et al., 2012). The κOR is
characterized by an expansive ligand binding pocket compared to other members of the same
class such as the chemokine CXCR-4 receptor. JDTic binds deeply within the ligand pocket
interacting with the receptor via ionic, polar and hydrophobic bonds.

1.12.3 Crystal structure of the δOR

Two months following the unveiling of the µOR and κOR crystal structures, the
crystal structure of the murine δOR bound to the antagonist naltrindole was solved (Granier
et al., 2012). In contrast to the µORs, δORs were arranged in an anti-parallel fashion likely
as an artefact of the crystallization process but these receptors, too, are also characterized by
an exposed solvent-accessible binding pocket. This structure provides even greater insight
into the ligand binding mode of ORs and provides structural credence to the message-address concept advanced by Portoghese and colleagues as early as the 1960s (Portoghese et al., 1965). Indeed, the crystal structure of the δOR confirms that the inner aspect of the ligand binding pocket that is housed within the deeper aspects of the TM core is conserved among the three ORs and binds the “message” portion of the ligand which is conserved among morphinan OR ligands. The more superficial portion is composed of divergent amino acid residues which confer ligand selectivity and binds to the “address” component of the ligand that endows it with receptor selectivity (Granier et al., 2012).

1.12.4 Key lessons from opioid receptor crystallography studies: limitations and future directions

These OR crystal structures have provided great insight into the conformation of ORs and the potential domains that may be implicated in oligomerization, and validated previous biochemical determinations of the structural basis of ligand binding. Despite the immense insight gained from these crystal structures, they remain a snapshot of the receptor in the most thermodynamically-favourable conformation imposed by the crystallization conditions. In the case of the OR structures, conditions were biased toward generating structural data about the mode of ligand binding to receptors stabilized in an inactive state bound to classical morphinan antagonists. Greater insight into structural determinants of ligand binding to peptide agonists and other ligands would be achieved from solving the crystal structures of ORs in their agonist-bound active conformations. The general consensus within the opioid field is that ligands stabilize different receptor conformations (Whistler et al., 1999; Varga et al., 2004; Audet et al., 2008). Morphine, the most frequently used analgesic
in the clinic for moderate to severe pain, is thought to stabilize a conformation of the \( \mu \)OR that does not appreciably recruit \( \beta \)-arrestin2 and the complement of endocytic machinery required for receptor internalization, and this, in turn, is thought to contribute to morphine’s propensity to cause tolerance (Whistler et al., 1999; He and Whistler 2002). Comparing the conformation of morphine-bound \( \mu \)ORs to fentanyl- or methadone-bound receptors which are potent recruiters of the endocytic machinery would provide a structural basis for these molecular and behavioural features of morphine. Similarly, it is generally accepted that the \( \delta \)OR is conformationally plastic, taking on various agonist-stabilized conformations that lead to varying degrees and manifestations of analgesic tolerance (Pradhan et al., 2010). Providing a structural basis for this conformational diversity and its relation to therapeutic and unwanted effects of opioid ligands would be a significant advance towards designing improved opioid therapeutics.

1.13 Opioid receptors form heteromers with many GPCRs

1.13.1 Opioid-opioid receptor heteromers

In addition to forming homo-oligomers, ORs associate into heteromeric receptor complexes with one another (Levac et al., 2002). \( \delta \)ORs interact with \( \kappa \)ORs to generate \( \delta \)-\( \kappa \) OR heteromers with novel pharmacological characteristics (Jordan and Devi 1999). The \( \delta \)-\( \kappa \) OR heteromer interacts with non-selective opioid ligands such as naloxone and diprenophine with greater affinity than \( \delta \)- and \( \kappa \)- agonists and antagonists (Jordan and Devi 1999). More recently, this receptor heteromer was isolated from sensory neurons where it accounted for allosteric interactions between \( \delta \)- and \( \kappa \)-selective ligands (Berg et al., 2012). The \( \kappa \)OR antagonist norbinaltorphimine enhanced the potency of DPDPE - but not other \( \delta \)-selective
agonists - at inhibiting adenyl cyclase activity in sensory neurons and reversing prostaglandin E2-induced allodynia in an animal model of thermal pain (Berg et al., 2012). Interestingly, the κOR antagonist 5’-guanidinonaltrindole (5’-GNTI) attenuated the molecular and behavioural effects of DPDPE, suggesting that different ligands induce specific conformations in the protomers of a heteromeric receptor complex. The bivalent ligand GDNII selectively targeted the δ-κ OR heteromer and produced analgesia only peripherally following intrathecal administration (Waldhoer et al., 2005). Co-immunoprecipitation studies confirmed that δ- and κ-ORs only associated in a complex in spinal cord but not in brain. This study demonstrated the feasibility of bivalent ligands and demonstrated that OR heteromers although co-expressed in many tissue, can have a tissue-specific distribution.

μ-κ OR heteromers have been isolated in spinal cord tissue where their activation produces antinociception (Chakrabarti et al., 2010). This receptor complex was expressed in a sexually dimorphic manner with an enhanced expression in female rodents, and may account for the recruitment of κOR to produce morphine antinociception in female rodents.

μ- and δ-ORs interact to form a heteromer with unique signalling properties and great therapeutic potential in neuropsychiatric disorders (George et al., 2000; George et al., 2002; Levac et al., 2002; Gomes et al., 2004; Costantino et al., 2012). The μ-δ OR heteromer will be the focus of this dissertation and is discussed in more detail in Section 1.14.
1.13.2 Opioid-adrenergic receptor heteromers

Both δ- and κ-ORs form heteromers with β2-adrenergic receptors resulting in profound changes in receptor trafficking (Jordan et al., 2001). Whereas δ-receptors in β2-δ heteromers undergo endocytosis in response to β2-agonist stimulation and vice versa, κ-β2 receptors do not internalize following β2-adrenergic receptor or κOR stimulation. Further, stimulation of either protomer of the β2-δ heteromer leads to MAPK activation whereas β2-κ heteromers induce significantly less activation of this functional response (Jordan et al., 2001).

Both δ- and µ-ORs interact with α2-adrenergic receptors which may account for the opioid-adrenergic interactions observed in vivo (Jordan et al., 2003). Animals expressing a mutated form of the α2-adrenergic receptor displayed attenuated morphine-induced spinal analgesia (Stone et al., 1997). µ-α2 heteromers were isolated from cultured sensory neurons and cell lines co-expressing the receptor pair. For their part, µ-α2 heteromers are more potently activated by agonist targeting one member of the protomer. Application of µ- and α2-selective ligands results in a reduction in signalling in various functional assays including the MAPK and G-protein activation (Jordan et al., 2003).

1.13.3 Other opioid-GPCR heteromers

δORs interact with the Sensory Neuron Specific Receptor-4 to form a heteromer that is activated by either δ- or SNSR-4-selective agonists (Breit et al., 2006). In contrast, co-activation of both protomers using BAM-22 or co-application of protomer-selective ligands only activated the SNSR-4 specific responses with no effect on δORs (Breit et al., 2006).
More recently, cannabinoid-1 (CB1) receptors have been added to the growing list of δOR interacting partners (Rozenfeld et al., 2012). δ-CB1 receptor heteromers isolated from neuronal cell lines display interesting properties whereby δ-CB1 receptor heteromerization leads to a reduction in CB1-mediated signalling and enhanced recruitment of β-arrestin-2 and subsequent CB1 receptor desensitization. Most notably, CB1 agonist stimulation of δ-CB1 heteromers leads to activation of a novel neuronal survival signalling pathway that results in reduced pro-apoptotic enzymes and enhanced cell survival (Rozenfeld et al., 2012).

μORs and CB1 also form heteromers in cell lines with interesting consequences on signal transduction. Interestingly, using receptor-G-protein fusion constructs, Hojo and colleagues (2008) demonstrated that μ- and CB1-selective agonists can activate their corresponding receptor or the other interacting partner.

In addition to these pairs, μORs have been shown to form heteromers with somatostatin (Pfeiffer et al., 2002), dopamine D1 (Juhasz et al., 2008), opioid-receptor-like-1 (Wang et al., 2005b) and the substance P neurokinin-1 receptor (Pfeiffer et al., 2003). δORs have been shown to heteromerize with chemokine 2 receptors (Parenty et al., 2008). κORs have been shown to interact with the human apelin receptor (Li et al., 2012).

All of these interactions generate novel functional properties for the interacting partners, expand the signalling mechanism ascribed to each receptor, and illustrate that ORs interact with GPCRs with seemingly different functions. Thus, OR heteromerization is a
more widespread phenomenon and would be dictated by several factors including tissue- and cell compartment-specific distribution of the interacting partners.

1.14 The µ-δ OR heteromer: µOR and δOR together are a force to be reckoned with!

Literature on the synergistic interactions between µ- and δ-ORs and their extensive colocalization along with the pharmacological evidence of OR subtypes that remain unaccounted for by cloning studies suggested that µ- and δ-ORs may interact physically to form a pharmacologically-distinct signalling unit. Indeed, a µ-δ OR receptor complex was hypothesized to exist as early as 1980s based on a series of radioligand binding studies (Rothman and Westfall 1982). In their initial analyses Rothman and colleagues discovered that the µ-agonist morphine reduced binding of tritiated endogenous δ-agonist Leu-enkephalin in competition radioligand binding assays in whole brain homogenate and isolated striatal and cortical tissues (Rothman and Westfall 1982). This finding was initially interpreted as morphine allosterically modulating the enkephalin receptors, which would necessitate that the “morphine” and “enkephalin” receptors be in close physical proximity and in a receptor complex. Importantly, Rothman and Westfall (1982) predicted that different brain regions would express different proportions of µOR and δOR, which would affect the number and/or stoichiometries of µ-δOR heteromers formed and would affect ligand pharmacology at the receptor.

1.14.1 Altered pharmacology of the µ-δ heteromer

It was not until the year 2000 that George and colleagues provided the first physical evidence of µ- and δ-OR association (George et al., 2000). Co-immunoprecipitation studies
revealed that μ- and δ-ORs form heteromeric and homomeric receptor complexes (George et al., 2000). In the absence of a definitive demonstration of the exact stoichiometry of the μ-δ OR complex, it is referred to throughout this thesis as a μ-δ OR heteromer or heterooligomer. The μ-δ OR heteromer displayed novel ligand binding and signalling properties distinct from those of its constituent μ- and δ-ORs (George et al., 2000). Competition radioligand binding using a variety of μ-selective and δ-selective agonists revealed a different agonist potency rank order at the μ-δ heteromer, suggestive of the formation of a novel ligand binding pocket (George et al., 2000). For example, the μ-agonist DAMGO and δ-agonist DPDPE bound co-expressed μ- and δ-ORs with equal affinities that were ten-fold lower than the affinities at their corresponding receptors, and produced bi-phasic competition radioligand binding curves with both high and low affinity sites (George et al., 2000). Furthermore, the guanine nucleotide analog GTPγS which uncouples μ- and δ-OR homomers from the Gα protein and results in a loss of agonist-detected HA binding does not abolish agonist-detected high affinity antagonist binding to the μ-δ heteromer (George et al., 2000). Thus, in the case of the μ-δ heteromer, the HA state may be independent of G-protein coupling (George et al., 2000).

Subsequent to the report from our laboratory, an independent research group also used co-immunoprecipitation and biophysical BRET techniques to demonstrate the presence of the μ-δ heteromer in cell lines (Gomes et al., 2000). Further, they demonstrated that μ- and δ-agonists modulated one another’s binding with δ-agonists or δ-antagonists increasing μOR binding sites in heterologous cells co-expressing the receptors (Gomes et al., 2000;
Gomes et al., 2004). Devi and colleagues subsequently demonstrated that the \( \mu-\delta \) heteromer may represent the basis for such interactions between \( \mu- \) and \( \delta \)-ligands (Gupta et al., 2010).

**1.14.1.1 Bivalent opioid ligands**

Early reports of \( \mu- \) and \( \delta \)-OR interactions and the possibility that these receptors may form a complex prompted the development of bivalent opioid ligands. The MDANs (\( \mu \)-agonist \( \delta \)-antagonist) ligands possess two pharmacophores that are thought to selectively target both protomers of a \( \mu-\delta \) OR heterodimer (Daniels et al., 2005). MDAN bivalent ligands were designed on the premise that inactivating the \( \delta \)OR while activating the \( \mu \)OR would prevent the development of morphine tolerance as observed in \( \delta \)OR gene-deleted animals (Zhu et al., 1999) although the latter view has recently been challenged (Scherrer et al., 2009).

The therapeutic utility and potency of MDAN ligands in various behavioural paradigms requires further study as \( \delta \)-agonists have anti-nociceptive properties in animal models of pain (Cahill et al., 2003; Morinville et al., 2004; Kabli and Cahill 2007; Shinoda et al., 2007; Pradhan et al., 2011; Saloman et al., 2011). Further, studies in gene-deleted animals demonstrate that endogenous \( \delta \)ORs have positive modulatory effects on mood (Filliol et al., 2000), another desired effect which would be reversed by inactivating \( \delta \)ORs. \( \delta \)-agonists have also been shown to potentiate the analgesic effects of \( \mu \)-activating ligands (Larson et al., 1980; Schramm and Honda 2010; Yekkirala et al., 2012) and \( \delta \)-antagonists attenuate \( \mu \)OR-mediated analgesia and dopamine release (Hirose et al., 2005). By activating \( \mu \)ORs and antagonizing \( \delta \)ORs, MDAN ligands may only produce modest analgesic effects.
Nonetheless, MDAN ligands constitute an important and valuable tool in probing the activity of μ-δ heterodimers.

### 1.14.2 Affinity of opioid receptor protomers for each other

Wang and colleagues (2005a) used quantitative BRET techniques to investigate the interaction between pairs of μ-, δ- and κ-ORs differentially tagged with energy donor renilla luciferase and energy acceptor green fluorescent protein. The BRET\textsubscript{50} measurement was employed to elucidate the affinity of OR pairs for one another. BRET\textsubscript{50} represents the ratio of acceptor to donor protein at which 50% of the maximum BRET signal occurs and has been validated to represent the affinity of the donor for the acceptor (Mercier et al., 2002). Accordingly, μ-, δ- and κ-ORs interact with the same or a different OR with similar – but not equal – affinity (Wang et al., 2005a). For example, μORs form homo-oligomers (BRET\textsubscript{50} = 2.7 ± 0.5 nM) with a slightly greater propensity than μ-δ OR heteromers (BRET\textsubscript{50} = 3.7 ± 0.2 or 3.2 ± 0.5 depending on tag location) (Wang et al., 2005a). Similarly, δORs form homo-oligomers (BRET\textsubscript{50} = 1.8 ± 0.1) with a slightly greater propensity than μ-δ OR heteromers (BRET\textsubscript{50} = 3.2 ± 0.5) (Wang et al., 2005a). Further, this group confirmed that OR oligomers form early in the biosynthetic pathway and traffic as oligomers to the plasma membrane to become available for ligand binding and signal transduction (Wang et al., 2005a). Around the same time, an independent research group showed that μ-δ OR heteromers can also form and dissociate at the cell surface (Law et al., 2005). Overall, these observations suggest that the interaction between μ- and δ-ORs is not covalent, which concurs with co-immunoprecipitation and gel electrophoresis studies demonstrating that the
interaction between this receptor pair is detergent-sensitive and likely less structurally robust than the interaction between ORs in a homomer (George et al., 2000; Gomes et al., 2000).

1.14.3 A switch in signalling time course

μ-δ OR heteromerization has been shown to result in a switch in the time course of ERK signalling (Rozenfeld et al., 2007). Activation of μ- or the δ-ORs expressed alone resulted in transient ERK phosphorylation peaking at 3-5 minutes whereas stimulation of the μ-δ heteromer using either the μ-agonist DAMGO or δ-agonist deltorphin II resulted in a sustained ERK response peaking at 5 min and remaining elevated for 30 minutes (Rozenfeld et al., 2007). Further, phosphoERK (pERK) produced downstream of μOR activation localized to the nucleus in contrast to the pERK downstream of μ-δ heteromer activation which localized to the cytoplasm. Further, this study showed that μ-δ OR heteromers are constitutively associated with β-arrestin2, which suggests that they may internalize readily following agonist stimulation. In contrast to their previous reports where the μ-δ heteromer is activated using combinations of μ-agonist and δ-antagonist or δ-agonist and μ-antagonist, in this study, Devi and colleagues report that these ligand combinations destabilize the μ-δ heteromer and revert the signalling patterns to those of μOR expressed alone (Rozenfeld et al., 2007). These findings fall in line with a body of behavioural and molecular studies demonstrating cross-antagonism of μ- and δ-OR functions (Tortella et al., 1985; Holaday et al., 1986; Scherrer et al., 2004; Hirose et al., 2005; Yekkirala et al., 2012).
1.14.4 Role in immune function?

Recently, a group from Rutgers University demonstrated that the µ-δ heteromer is expressed in immune natural killer cells and splenocytes isolated from rats (Sarkar et al., 2012; Yekkirala et al., 2012). Combinations of a µ-agonist and δ-antagonist or a δ-agonist and µ-antagonist resulted in a reduction µ-δ heteromers levels and led to increased formation of cytolytic factors which promote immune function. Further, the use of a combination of the δ-agonist DPDPE and the µ-antagonist naltrexone resulted in decreased tumour incidence, number and volume as well as a decreased malignancy rate in an animal model of mammary adenocarcinoma (Sarkar et al., 2012). Extra-neuronal expression of the µ-δ OR heteromer suggests that its roles extend beyond the CNS and encourage further characterization of µ-δ OR heteromer function and regulation in immune tissues.

1.14.5 Structural determinants of µ- and δ-opioid receptor heteromerization

In a quest to identify the receptor domains involved in forming the µ-δ heteromer, George and colleagues generated µOR and δOR mutants with a truncation in the distal carboxyl tail domain. Co-expressing wild type µOR with a δOR distal 15 a.a. carboxyl tail truncation mutant greatly attenuated the co-immunoprecipitation of µ- and δ-ORs into the same physical complex and abolished the µ-δ heteromer-specific pharmacology, suggesting that the terminal portion of the δOR is implicated in µ-δ OR heteromer formation (Fan et al., 2005). Walwyn and colleagues (2009) corroborated these findings using electrophysiological approaches. Opioid agonist-induced inhibition of voltage-gated calcium channel currents in DRG neurons required functional µ- and δ-ORs and was not fully restored in neurons expressing the δOR distal 15 amino acid truncation mutant
(Walwyn et al., 2009). More recently, the last three glycine residues of the δOR carboxyl tail have been shown to play a critical role in μ-δ heteromer formation (O'Dowd et al., 2012).

1.14.6 Altered G-protein coupling

Pertussis toxin (PTX), a compound which inactivates G-protein αi/o subunits through ADP-ribosylation, did not abolish high affinity ligand binding or agonist-induced cAMP inhibition in cells co-expressing μ- and δ-ORs (George et al., 2000). In contrast, PTX abolished high affinity binding of μ- and δ-selective agonists to their corresponding receptors as well as cAMP inhibition when μ- and δ-ORs were expressed individually (George et al., 2000). PTX sensitivity of μ- and δ-OR signalling had long been established and PTX had been used routinely to block OR function in molecular and biochemical assays (Hsia et al., 1984; Clark et al., 1997; Pak et al., 1999; Tso and Wong 2003). Thus, the finding that OR signalling subsisted in the presence of PTX only in cells co-expressing μ- and δ-ORs suggested coupling of the μ-δ heteromer to functional responses through a PTX-resistant inhibitory G-protein.

Subsequent to their initial report, George and colleagues used a Ga-specific [35S]-GTPγS assay to demonstrate that the δ-agonist deltorphin II activates the μ-δ OR heteromer and links it to the activation of the PTX-resistant Gaζ G-protein (Fan et al., 2005). In contrast, deltorphin II and other μ- and δ-selective agonists activated the PTX-sensitive Gaζ3 G-protein in cells expressing δOR or μOR individually (Fan et al., 2005). Using BRET techniques, the μ-δ OR heteromer was shown to interact more favourably with Gaζ than Gaζ3 (Hasbi et al., 2007). Using sucrose density centrifugation followed by BRET, Gaζ was
found to associate with the µ-δ OR heteromer early in the biosynthetic pathway in the endoplasmic reticulum (Hasbi et al., 2007). The Ga\(_z\)-bound µ-δ heteromer trafficked to the plasma membrane as a pre-assembled signalling complex. Altogether, these studies demonstrate that deltorphin II-induced Ga\(_z\) only occurs in cells expressing the µ-δ heteromer and thus is thought to represent a fingerprint µ-δ heteromer signalling.

1.14.6.1 \(Ga_z\): a G-protein with interesting properties

\(Ga_z\) is a member of the \(Ga_{i/o}\) family of G protein \(\alpha\) subunits. Although structurally homologous to its family members, \(Ga_z\) has distinct biochemical properties beginning with an amino acid modification which renders it resistant to PTX inactivation (Premont et al., 1989; Albert and Robillard 2002). \(Ga_z\) has been shown to couple to several downstream effectors including adenylyl cyclase (Wong et al., 1992), mitogen-activated protein kinase, phospholipase C, and potassium channels (Jeong and Ikeda 1998).

1.14.6.1.1 Localization of \(Ga_z\)

Unlike its family members, \(Ga_z\) has a largely neuronal localization but is also expressed in the spleen and heart (Fong et al., 1988; Hinton et al., 1990; Hendry et al., 2000). \(Ga_z\) co-localizes extensively with µ- and δ-ORs in the CNS (Table 1.2, Delfs et al., 1994; George et al., 1994; Mansour et al., 1994; Wittert et al., 1996; Table 1.2, Friberg et al., 1998; Kelleher et al., 1998; Hendry et al., 2000; Leck et al., 2004). However, an extensive characterization of the cell types co-expressing \(Ga_z\), µOR and δOR in the brain has not been conducted.
1.14.6.1.2 The role of $\Gamma_{\alpha z}$ in morphine tolerance

$\Gamma_{\alpha z}$ gene-deleted animals exhibit a faster onset and a greater degree of morphine tolerance as evidenced by a faster and greater right-ward shift in the morphine dose response curve in the hot and cold plate pain tests (Hendry et al., 2000; Leck et al., 2004). A greater shift was mainly observed in supra-spinal pain circuits, implicating $\Gamma_{\alpha z}$ in supra-spinal mechanisms of analgesic morphine tolerance. The enhanced tolerance to morphine analgesia was gene dose-dependent and was not the result of pharmacokinetic or behavioural tolerance and the absence of $\Gamma_{\alpha z}$ had no effect on the affinity of morphine for its receptor (Leck et al., 2004). Although not tested directly, the exogenous expression of $\Gamma_{\alpha z}$ in cell lines resulted in a decrease in adenylyl cyclase superactivation, a measure frequently used to assess morphine tolerance in cellular models (Ozawa et al., 1999). Thus, $\Gamma_{\alpha z}$ likely plays an important role in modulating morphine tolerance.
Table 1.2  A survey of mRNA expression levels of µ- and δ- opioid receptors and $G_{\alpha_z}$ in rodent brain.

<table>
<thead>
<tr>
<th>CELL GROUPS</th>
<th>µOR</th>
<th>δOR</th>
<th>$G_{\alpha_z}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Striatum</td>
<td>*</td>
<td>**</td>
<td>*</td>
</tr>
<tr>
<td>Nucleus accumbens</td>
<td>**</td>
<td>**</td>
<td>*</td>
</tr>
<tr>
<td>Globus pallidus</td>
<td>**</td>
<td>*</td>
<td>1/2</td>
</tr>
<tr>
<td>Ventral pallidum</td>
<td>***</td>
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<td>*</td>
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<tr>
<td>Subthalamic nucleus</td>
<td>** 1/2</td>
<td>*</td>
<td>**</td>
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<tr>
<td>Substantia nigra - pars compacta</td>
<td>**</td>
<td>*</td>
<td>**</td>
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<tr>
<td>Substantia nigra - pars reticulata</td>
<td>*</td>
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<tr>
<td>Hippocampus - CA1</td>
<td>* 1/2</td>
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<td>** 1/2</td>
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<tr>
<td>Hippocampus - CA3</td>
<td>**</td>
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<tr>
<td>Hippocampus - dentate gyrus</td>
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<td>Cerebral cortex</td>
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</tr>
<tr>
<td>Cerebellum</td>
<td>**</td>
<td>1/2</td>
<td>* 1/2</td>
</tr>
</tbody>
</table>

Asterisks correlate with levels of mRNA expression. Results compiled from (Delfs et al., 1994; George et al., 1994; Mansour et al., 1994; Wittert et al., 1996; Friberg et al., 1998; Kelleher et al., 1998).
1.15 STATEMENT OF PROBLEM

While the sum of anatomical, behavioural, molecular, electrophysiological and genetic studies provides indisputable evidence of µ- and δ-OR interactions and the reports of µ-δ heteromers are steadily increasing in the literature, physical evidence for the existence of µ-δ OR complexes in native tissues has been difficult to obtain owing to a number reasons. Firstly, there are no selective opioid receptor antibodies that can be employed for co-immunoprecipitation studies. Some of the commercially available reagents target opioid receptor epitopes which are implicated in the µ-δ OR heteromeric interaction. Secondly, there are no selective pharmacological ligands exclusively targeting the µ-δ heteromer, rendering it challenging to isolate the contribution of this specific opioid receptor complex to opioid-mediated effects. Furthermore, our understanding of the agonist-induced regulation of the µ-δ heteromer in cell lines is rudimentary but is a crucial stepping stone towards understanding the properties of this receptor complex. Thus, characterizing the unique signalling mediated by the µ-δ heteromer is a prerequisite to delineating its therapeutic utility.

In light of the current state of knowledge and available experimental tools, the aims and hypotheses that this thesis research endeavoured to accomplish and to test, respectively, were three-fold:
1.16 STUDY I: The agonist-induced regulation profile of the µ-δ OR heteromer in a cell line

Objective I: To investigate the agonist-induced regulation of the µ-δ OR heteromer in a mammalian cell line

Hypothesis I: The µ-δOR heteromer has a unique agonist-induced regulation profile that differs from that of the µOR homomer

Rationale for Study I:

The ligand binding profile and the affinities of several endogenous and exogenous opioid ligands for co-expressed µ- and δ-ORs have been investigated by us and others (George et al., 2000; Gomes et al., 2000; Gomes et al., 2004; Fan et al., 2005; Law et al., 2005; Wang et al., 2005a). On the other hand, the nature of the ligand binding pocket and mechanism of action of agonists and their regulation of the µ-δOR heteromer have not been explored extensively.

GPCR regulation via internalization is an important determinant of drug responsiveness in vivo (Trafton et al., 2000; Scherrer et al., 2009). Internalization plays a key role in regulating receptor number at the cell surface and is an important mechanism by which receptor function is tightly regulated (Ferguson et al., 1998). Further, the adaptations in OR trafficking that occur following prolonged morphine treatment are of clinical significance. While internalization of the individual µ- and δ-OR is impaired in a cellular model of morphine tolerance (Eisinger et al., 2002), the regulation of the µ-δOR heteromer in this paradigm is not known. The intracellular trafficking of the µ-δ complex has tremendous implications for its potential suitability as a drug target, and requires further investigation.
1.17 STUDY II: The presence of μ-δ OR heteromer-specific signalling in the brain

Objective II: To demonstrate that the μ-δ OR heteromer-specific signalling observed in cells occurs in brain regions under normal physiological conditions and after prolonged morphine treatment

Hypothesis II: μ- and δ-ORs form functional hetero-oligomeric receptor complexes in brain which are regulated by prolonged morphine exposure

Rationale for Study II:

Autoradiographic, electrophysiological, immunohistochemical, in situ hybridization, and single-cell PCR studies converge on the extensive co-localization of μ- and δ-ORs in the same neurons in several neuronal structures including the striatum (nucleus accumbens and caudate putamen), hippocampus, midbrain, spinal cord and DRG (Sharif and Hughes 1989; Mansour et al., 1993; George et al., 1994; Mansour et al., 1994; Arvidsson et al., 1995a; Arvidsson et al., 1995b; Cahill et al., 2001a; Wang and Pickel 2001; Goody et al., 2002; Stumm et al., 2004; Gray et al., 2006; Wang et al., 2010). Thus, these receptors are poised for functional interactions in specific neuronal circuits.

In cell lines, μ-δ heteromer signalling is PTX-resistant and occurs via Ga\textsubscript{z} in contrast to μOR and δOR homomer-mediated signalling which is PTX-sensitive and is transduced by Ga\textsubscript{i3} (George et al., 2000; Fan et al., 2005). In vivo evidence pointing to the existence of μ-δ heteromer signalling, namely deltorphin II-induced Ga\textsubscript{z} activation, is provided by several studies. Firstly, deltorphin II’s analgesic effects are not entirely abolished by PTX following intracerebroventricular or intrathecal administration, suggesting that activation of PTX-resistant Ga proteins is involved in transducing the spinal and supraspinal antinociceptive
effects of deltorphin II (Tseng and Collins, 1996). Secondly, Ga_z, the G protein to which the μ-δOR heteromer couples selectively, has a predominant neuronal localization and co-localizes with μ- and δ-ORs (Delfs et al., 1994; George et al., 1994; Mansour et al., 1994; Wittert et al., 1996; Friberg et al., 1998; Kelleher et al., 1998). Further, deltophin II-induced analgesia is reduced in μOR gene-deleted animals, further supporting the involvement of both μOR and δOR in the actions of deltorphin II (Matthes et al., 1998).

Several lines of evidence suggest that the μ-δOR heteromer may be intimately involved in the cellular mechanism of morphine tolerance. Firstly, prolonged morphine treatment has been shown to induce the targeting of δOR from intracellular stores to the plasma membrane in various brain regions such as the striatum and in the spinal cord (Cahill et al., 2001b; Morinville et al., 2003; Morinville et al., 2004; Hack et al., 2005; Lucido et al., 2005; Ma et al., 2006). This δOR targeting required the μOR, was absent in animals lacking the μOR gene and manifested as a potentiation of deltorphin II-induced antinociceptive effects (Cahill et al., 2001b; Morinville et al., 2004). Secondly, ablation of the gene for Ga_z leads to accentuated morphine tolerance in rodents, which suggests that signalling through Ga_z may be protective in conditions of prolonged morphine treatment (Hendry et al., 2000; Leck et al., 2004). Thus, these independent reports converge on a possible modulatory effect of prolonged morphine treatment on the μ-δ heteromer.
1.18 STUDY III: Functional consequences of disrupting the μ-δ OR heteromeric interaction in a cell line and in animal systems

Objective III: To explore the function of the μ-δ heteromer by determining the functional consequences of disrupting the μ-δ OR hetero-oligomeric interaction in a mammalian cell line and in animal systems

Hypothesis III: The μ-δ OR heteromer plays a role in the physiological effects of opioid agonists

Rationale for Study III:

δ-agonists have a more complex pharmacology than previously thought and are also agonists at the μ-δ heteromer as revealed by their ability to modulate μ-agonist binding to the heteromer and intracellular trafficking of this receptor complex (Kabli et al., 2010; Milan-Lobo and Whistler 2011). A variety of peptidic and non-peptidic δ-agonists were shown to displace high affinity μ-agonist binding to the μ-δ heteromer and to induce internalization of this receptor complex by occupying both μ- and δ-OR binding pockets and sorting the heteromer to the clathrin-dynamin endocytic pathway (Kabli et al., 2010). The reports of unique actions of δ-agonists at the μ-δ heteromer in vitro, the partial PTX sensitivity of δ-agonist behavioural effects in vivo (Tseng and Collins 1996) and their reversal by μOR antagonists in vivo (Scherrer et al., 2004) as well as the requirement of the μOR for δ-agonist-mediated behavioural and molecular effects (Cahill et al., 2001b; Gendron et al., 2007), indicate that the μ-δ heteromer likely plays a role in the cellular and behavioural effects of these ligands.
An emerging body of evidence converges on the critical role of the δOR system in regulating mood. δ-Agonists elicit well-documented, potent antidepressant-like and anxiolytic-like properties in several animal models (Kastin et al., 1978; Baamonde et al., 1992; Filliol et al., 2000; Broom et al., 2002a; Broom et al., 2002b; Saitoh et al., 2005; Jutkiewicz 2006; Perrine et al., 2006). Further, δ-receptor gene-deleted animals display depressive and anxiogenic behaviour (Filliol et al., 2000; for review see Gaveriaux-Ruff and Kieffer 2002). Studies on the involvement of the µOR demonstrate that the function of this receptor in regulating mood is less well-defined, but indicate a role for it as well (Filliol et al., 2000; Nyhuis et al., 2008; Zarrindast et al., 2008; Ide et al., 2010; Berrocoso et al., 2012). Interestingly, Ga2-gene deleted animals also display pro-depressive and anxiogenic behaviour (Oleskevich et al., 2005).

Using a µ-δ heteromer-selective antibody, it has recently been confirmed that the heteromer is expressed in several brain regions including the nucleus accumbens (Gupta et al., 2010), a region of demonstrated importance in animal and human studies of depression and anxiety (Sturm et al., 2003; Epstein et al., 2006; Nestler and Carlezon 2006; Zarrindast et al., 2008; Alexander et al., 2010; Kitamura et al., 2010). These findings taken together with the fact that δ-agonists – which possess potent mood-elevating properties – act as agonists at the µ-δ heteromer point to a possible role of the µ-δ heteromer in regulating mood.

Our laboratory has shown that the distal portion of the δOR carboxyl terminus plays an important role in generating the heteromeric interaction and novel pharmacology of the µ-δOR heteromer (Fan et al., 2005). Truncation of the terminal amino acids of the δOR
diminished the ability of μ- and δ-ORs to hetero-oligomerize, abolished the unique μ-δ heteromer pharmacology, and compromised the full functional response to μ-opioid agonists in cultured neurons (Fan et al., 2005; Walwyn et al., 2009). Peptides derived from the sequence of one of the interacting protein partners have been successfully used to inhibit oligomerization (George et al., 1998; Aarts et al., 2002). Thus, an interfering peptide targeting the key contact points between μ- and δ-ORs would interfere with the heteromeric interaction and would be a useful strategy to assess the physiological importance of μ-δ hetero-oligomerization in vivo.
2. MATERIALS AND METHODS

2.1 STUDY I METHODS

2.1.1 Drugs and chemical reagents: Deltorphin II, DAMGO, DPDPE, naltrindole hydrochloride, SNC80, Leu-enkephalin, Met-enkephalin, endomorphin-1, β-funaltrexamine, and naloxone hydrochloride were purchased from Sigma (Saint Louis, MO, USA). CTOP was purchased from Tocris (Ellisville, MO, USA). Morphine sulfate (MS) was purchased from BDH Chemicals (Toronto, ON, Canada). UFP-512 was synthesized by Balboni et al., (2002). Concanavalin A (con A) was purchased from Calbiochem (Los Angeles, CA, USA).

2.1.2 cDNA Constructs: The Transformer site-directed mutagenesis kit (Clontech, Mountain View, CA) was used to insert epitopes into μ- and δ-OR as previously described (George et al., 2000). To generate cMyc-μOR and FLAG-δOR, the c-Myc (EQKLISEEDL) or FLAG (DYKDDDDK) epitopes were inserted after the N-terminal start methionine of rat μ- or δ-OR cDNAs, respectively. cDNAs were then inserted together or separately into the mammalian expression vector pBudCE4.1 (Invitrogen, Burlington, ON, Canada). To generate green fluorescent protein-tagged μOR (μOR GFP) and monovalent red fluorescent protein (mRFP)-tagged δOR (δORmRFP) in pEGF-N1 (Invitrogen, Burlington, ON, Canada), the GFP and mRFP sequences were inserted at the carboxy termini of the μOR or δOR, respectively, as described previously. Sequencing ensured the correct orientation of the polymerase chain reaction products in the expression vector and absence of sequence errors. cDNA encoding rat G protein αz subunit (Gαz) was subcloned into the mammalian expression vector pcDNA3.1 (Invitrogen, Burlington, ON, Canada), and sequenced to ensure accuracy and correct orientation. The dominant negative dynamin mutant DYN K44A
construct in pCB1 vector was a kind gift from Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA, USA) and was generated as described (Zhang et al., 1996). Cells were transfected with 2 μg of Gαz or DYN K44A cDNA using LipofectAMINE (Invitrogen, Burlington, ON, Canada) according to manufacturer’s protocols. Cells were used 48 hours post-transfection. Gαz expression and its abundance relative to Gαi3 was determined using Western blotting analysis.

2.1.3 Expression in Mammalian Cells: HEK 293T human embryonic kidney cells (American Type Culture Collection, Manassas, VA, USA) were maintained as a monolayer at 37°C with 5% CO2 saturation in advanced minimal essential medium supplemented with 6% fetal bovine serum and antibiotics (Invitrogen, Burlington, ON, Canada). Cells were stably transfected with the pBudCE4.1 expression vector containing μ- and/or δ-OR using LipofectAMINE reagent (Invitrogen, Burlington, ON, Canada). Clones expressing each of the receptors at a density of 150 – 175 fmol/mg were used (150 – 350 fmol/mg total receptor protein).

2.1.4 Cell Membrane Preparation: Cells were rinsed with phosphate-buffered saline, suspended, pelleted and lysed by polytron homogenisation in a 5mM Tris-HCl and 2mM EDTA solution containing a protease inhibitor cocktail (5 μg/ml leupeptin, 10 μg/ml benzamidine and 5 μg/ml soybean trypsin inhibitor) as previously described (George et al., 2000). Unbroken cells and nuclei were pelleted by centrifugation at 100g. The supernatant was centrifuged at 40,000g for 20 min at 4°C to prepare the crude membrane fraction (P2).
Membrane protein content was determined using the Bradford assay (Bio-Rad, Hercules, CA, Canada) as per manufacturer’s protocol.

2.1.5 Competition Radioligand Binding: Experiments were performed in duplicate on cell membrane preparations with increasing concentrations of competing ligand (10^{-12} to 10^{-4} M). Both [{\textsuperscript{3}}H]-diprenorphine (50 Ci/mmol) and [{\textsuperscript{3}}H]-DAMGO (36.8 Ci/mmol) were purchased from Perkin Elmer Life Sciences (Waltham, MA USA). The concentration of radioligand used approximated its $K_D$ (1 nM [{\textsuperscript{3}}H]-diprenorphine, and 2 nM [{\textsuperscript{3}}H]-DAMGO). Bound ligand was isolated by rapid filtration through a 48-well cell harvester (Brandel, Gaithersburg, MD, USA) using GF/C filters (Whatman, Florham Park, NJ, USA). Filters were washed with cold 50 mM Tris-HCl buffer (pH 7.4), placed in vials containing scintillation fluid and counted for tritium.

2.1.6 Pertussis Toxin Treatment: Cells were pre-treated with 100 ng/ml pertussis toxin (PTX) (Sigma, Saint Louis, MO, USA) for 24 hours prior to radioligand binding experiments. This concentration of PTX has been shown to abolish OR signalling (Clark et al., 1997).

2.1.7 SDS – Polyacrylamide Gel Electrophoresis and Immunoblotting: Membrane proteins (20 μg protein/lane) were resolved on a 10 % Tris-Glycine precast gel (Novex, San Diego, CA, USA) under denaturing conditions by SDS-PAGE and then electroblotted onto a polyvinylidene difluoride membrane as described previously (Fan et al., 2005). Immunoreactivity was revealed by incubating in $\alpha_z$ or $\alpha_{i3}$ antibody diluted 1: 250 (Sc-388...
or Sc-262, respectively, Santa Cruz Biotechnology, Santa Cruz, CA, USA), HRP-conjugated goat-anti-rabbit secondary antibody diluted 1:1000 (Bio-Rad, Hercules, CA, USA), and enhanced chemiluminescent reagents (KPL, Gaithersburg, MD, USA), and then exposing to film. Immunoreactive bands are expected at 41kDa for $G_\alpha_z$ (Premont et al., 1989) and $G_\alpha_{i3}$ (Holz et al., 1989) as reported in the literature. Blots were stripped using $\beta$-mercaptoethanol buffer and reprobed with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody diluted 1:10,000 (Abcam, Cambridge, MA, USA), which served as the loading control.

2.1.8 Immunoprecipitation Studies: The membrane fraction obtained from cells co-expressing $\mu$- and $\delta$-ORs in the absence or presence of transfected $G_\alpha_z$ was re-suspended and stirred with protease inhibitors and the homogenate centrifuged. The solubilized portion was isolated and incubated with 5 µg anti-$G_\alpha_z$ (Sc-388, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or 5 µg anti-$G_\alpha_{i3}$ (Sc-262, Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody and then protein-G-agarose beads. The immunoprecipitate was washed and solubilized in SDS sample for gel electrophoresis.

2.1.9 Chronic Morphine Treatment: Cells were treated with 10 µM morphine sulfate in Advanced-MEM for 48 hours. Cells were washed three times in PBS pH 7.4 prior to incubation in medium with or without opioid agonists.

2.1.10 Intact Cell Internalization Assay: $\mu$OR binding was performed on intact cells stably expressing opioid receptors using $[^3H]$-DAMGO at its $K_D$ (2 nM). $[^3H]$-DAMGO would only label cell surface receptors because it is a hydrophilic peptide ligand and so does not
penetrate the plasma membrane (Blake et al., 1997; Koch et al., 1998). Non-specific binding was assessed using 10 μM naloxone. Cells were pretreated with agonist for 1 hr at 37°C, rinsed, treated with [³H]-DAMGO for 4 hrs at 4°C, then rinsed. Cell surface radioactivity was measured using the liquid scintillation counter. The disappearance of [³H]-DAMGO binding sites on the cell surface served as an indicator of μOR internalization. In separate experiments δOR internalization was assessed using 1nM [³H]-diprenorphine following the same protocol as above.

2.1.11 Cell Surface μOR Immunofluorescence: Cells stably expressing cMyc-μOR and FLAG-δOR were seeded onto a 96-well plate at a density of 50,000 cells/well. Cells in the same plate were pretreated with vehicle (saline) or 10 μM SNC80 (δ-agonist) for 1 hr at 37°C, rinsed, and then fixed using 4% paraformaldehyde prior to incubation with blocking solution (4% bovine serum albumin) to prevent binding of antibody to non-specific sites. To label cell surface μORs, cells were then incubated with mouse monoclonal anti-cMyc 4A6 antibody (05-724, Upstate, Billerica, MA, USA) for 2 hours at room temperature, then rinsed and incubated with Alexa Fluor® 488 goat anti-mouse IgG antibody (A11001, Molecular Probes, Eugene, OR, USA) for 2 hours at room temperature. Cells were rinsed again and signals were collected using a plate reader spectrofluorometer (Victor®, Perkin Elmer Instruments, Waltham, MA, USA). The decrease in cell surface receptor immunofluorescence in δ-agonist-treated compared to vehicle-treated cells served as an indicator of μOR internalization.
2.1.12 **Receptor Visualization using Confocal Microscopy:** Cells were transfected with cDNAs encoding \( \mu \text{OR}_{\text{GFP}} \) and \( \delta \text{OR}_{\text{mRFP}} \) and split onto 60 mm plates 24 hours post-transfection. Forty-eight hours post-transfection, living cells were examined on the LSM 510 Zeiss confocal microscope (Carl Zeiss, Toronto, ON, Canada) prior to (basal) and following administration of 100 nM UFP-512 (\( \delta \)-agonist). Cells were visualized using 40X Archoplan wet objective lens. Images were assembled using the Zeiss LSM Image Browser software (Carl Zeiss, Toronto, ON, Canada).

2.1.13 **Data Analysis:** Statistical analyses and graph generation were performed using GraphPad Prism software 3.01 (San Diego, CA, USA). The results were presented as means ± standard error of the mean (SEM). Data from competition radioligand binding experiments were analyzed by nonlinear least-squares regression. An F-test was used to compare the coefficients of the goodness-of-fit and to determine whether a two- or a one-site was a statistically significant better fit. The unpaired Student’s t-test was used to assess statistical significance in receptor internalization experiments. \( P < 0.05 \) was deemed significant.
2.2 STUDY II METHODS

2.2.1 Animals: Adult male Sprague Dawley rats weighing 250-350 g (Charles River, Québec, Canada) were housed in pairs in cages in a temperature-controlled room with corn chip bedding and free access to standard rodent chow and water. Rats were maintained under a standard 12-h/12-h light/dark cycle (lights on at 07:00 AM). Testing was performed during the light cycle. Animal protocols were approved by the University of Toronto Animal Care Committee and were in accordance with the guidelines set by the Canadian Council on Animal Care.

2.2.2 Drugs: Morphine sulfate (MS) was purchased from BDH Chemicals (Toronto, ON, Canada). Deltorphin II, DAMGO, and DPDPE were purchased from Sigma (Saint Louis, MO).

2.2.3 Prolonged Morphine Treatment: Rats were injected subcutaneously (s.c.) with ascending doses of morphine sulfate (5, 8, 10, and 15 mg/kg, s.c. every 12 h) for 48 hours and sacrificed 8 – 12 hours following the last morphine injection according to the protocol of Cahill and colleagues (2001b). Control rats were injected with equal volumes of saline.

2.2.4 Tail Immersion Antinociception Assay: To verify that morphine sulfate doses administered produced analgesia, rats were tested in the tail immersion thermal assay after receiving the 5 mg/kg and 10 mg/kg doses of morphine sulfate. Saline animals acted as controls. Rats were restrained gently and the distal 5 cm of their tail dipped in a bath of water maintained at 52°C. The latency to tail withdrawal from water was measured. Three pre-
drug baseline (BL) measures were obtained and averaged. A cut-off of 4 x BL latency was imposed to minimize tissue damage. Rats were habituated to the tail immersion assay before testing. % Maximum Possible Effect was calculated according to the equation: % Maximum Possible Effect = (Latency - BL latency) / (Cut-off Latency – BL latency) x 100.

2.2.5 Brain Membrane Preparation: Rats were decapitated using a guillotine. The brain was removed and the striatum (nucleus accumbens and caudate putamen), hippocampus, and spinal cord were isolated, placed in buffer (50 mM Tris, 250 mM sucrose, 25 mM KCl, 5 mM MgCl₂, pH 7.4), homogenized and centrifuged at 100g. The supernatant was centrifuged at 40,000g for 20 min at 4°C to isolate the crude membrane fraction.

2.2.6 Total and Go-specific [³⁵S]-GTPγS Binding: Membranes (200µg / tube) from rat striatum, hippocampus, and spinal cord were pre-incubated at 30°C for 5 min, then treated with vehicle or 10 µM agonist in the presence of 2 nM [³⁵S]-GTPγS (1250 Ci/mmol, Perkin Elmer Life Sciences, Waltham, MA) and 10 µM GDP for 1 min. Binding was terminated by the addition of 1 ml ice-cold assay buffer (10 mM HEPES, 100 mM NaCl, 10 mM MgCl₂, pH 7.4) and centrifugation at 20,000g for 10 min at 4°C. The pellet obtained was dissolved in 100 µl of ice-cold solubilization buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA, 1.25 % (v/v) Igepal 630/NP-40, pH 7.4) and 0.18% SDS for 1 hour (hr) at 4°C. Unsolubilized debris was pelleted at 20,000g for 20 min. To detect total G protein activation, the pellet obtained after the first centrifugation step was washed three times with assay buffer by centrifugation at 2,500 rpm for 3 min each time. Scintillation cocktail (Ready Safe™, Beckman Coulter, Mississauga, ON) was added and radioactivity was counted (Beckman LS
6500 Counter, Beckman Coulter, Mississauga, ON). In order to detect agonist-induced activation of specific G proteins, the supernatant was incubated with 5 µg of Gαz or Gαi3 antibody (Sc-388 or Sc-262, respectively, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. Protein G agarose 50:50 was added and the tubes rotated at 4°C for 4 hours. Agarose beads were washed 4 times with solubilization buffer by centrifugation at 2,500 rpm for 3 min each time. Beads were then suspended in scintillation cocktail and radioactivity was counted.

2.2.7 Data Analysis: Statistical analysis and graph generation were performed using GraphPad Prism software 3.01 (San Diego, CA). The results are presented as a percentage over unstimulated (basal) controls, which were designated as 0%. The results were presented as means ± standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Dunnett’s post hoc tests were utilized to analyze the statistical significance of agonist-induced effects on G-protein activation in comparison to unstimulated controls. The unpaired Student’s t-test was used to analyze the statistical significance of the difference in agonist-induced G-protein activation in brain samples from animals treated with repeated morphine or saline control injections. P < 0.05 was deemed significant.
2.3 STUDY III METHODS

2.3.1 Drugs and Chemical Reagents: Naltrindole hydrochloride, deltorphin II, DAMGO, DPDPE and imipramine were purchased from Sigma (Saint Louis, MO, USA). CTOP was purchased from Tocris (Ellisville, MO, USA). UFP-512 was synthesized by Balboni et al., (2002).

2.3.2 Expression in Mammalian Cells: HEK 293T human embryonic kidney cells (American Type Culture Collection, Manassas, VA, USA) were maintained as a monolayer at 37°C with 5% CO₂ saturation in advanced minimal essential medium supplemented with 6% fetal bovine serum and antibiotics (Invitrogen, Burlington, ON, Canada). HEK 293T stably expressing μ- and/or δ-ORs were generated as described previously (Kabli et al., 2010). Briefly, cells were stably transfected with the pBudCE4.1 expression vector containing μ- and/or δ-OR using LipofectAMINE reagent (Invitrogen, Burlington, ON, Canada). Cell lines expressing each of the receptors at a density of 150 – 175 fmol/mg were used (150 – 350 fmol/mg total receptor protein).

2.3.3 Minigene Constructs: cDNA fragments expressing either the distal 16 or 22 amino acids (a.a.) of the δOR carboxyl tail were subcloned into pcDNA3.1. The Transformer site-directed mutagenesis kit (Clontech, Mountain View, CA) was used to insert the hemaglutinin (HA) epitope (YPYDVPDYA) at the N-terminal of the δOR carboxyl tail peptide using the same approach previously described (Fan et al., 2005). Constructs were sequenced to ensure accuracy and correct orientation. Cells were transfected using LipofectAMINE (Invitrogen,
Burlington, ON, Canada) according to manufacturer’s protocols. Cells were used 48 hours post-transfection.

2.3.4 Cell Membrane Preparation: Cells were rinsed with phosphate-buffered saline, suspended, pelleted and lysed by polytron homogenization in a 5mM Tris-HCl and 2mM EDTA solution containing a protease inhibitor cocktail (5 \( \mu \)g/ml leupeptin, 10 \( \mu \)g/ml benzamidine and 5 \( \mu \)g/ml soybean trypsin inhibitor) as previously described (George et al., 2000). Unbroken cells and nuclei were pelleted by centrifugation at 100g. The supernatant was centrifuged at 40,000g for 20 min at 4°C to prepare the crude membrane fraction (P2). Membrane protein content was determined using the Bradford assay (Bio-Rad, Hercules, CA, Canada) as per manufacturer’s protocol.

2.3.5 Competition Radioligand Binding: Experiments were performed in duplicate on cell membrane preparations with increasing concentrations of competing ligand (10\(^{-12}\) to 10\(^{-4}\) M). \[^3\text{H}]-\text{DAMGO} (36.8 \text{ Ci/mmol}) and \[^3\text{H}]-\text{diprenorphine} (50.0 \text{ Ci/mmol}) were purchased from Perkin Elmer Life Sciences (Waltham, MA USA). The concentration of radioligand used approximated its K\(_D\) (2 nM \[^3\text{H}]-\text{DAMGO} and 1 nM \[^3\text{H}]-\text{diprenorphine}). Bound ligand was isolated by rapid filtration through a 48-well cell harvester (Brandel, Gaithersburg, MD, USA) using GF/C filters (Whatman, Florham Park, NJ, USA). Filters were washed with cold 50 mM Tris-HCl buffer (pH 7.4), placed in vials containing scintillation fluid (Ready Safe™, Beckman Coulter, Mississauga, ON) and counted for tritium using a liquid scintillation counter (Beckman LS 6500 Counter, Beckman Coulter, Mississauga, ON).
2.3.6 Dot Blotting Analysis: Cells were rinsed with phosphate-buffered saline, pelleted then lysed in a 5mM Tris-HCl and 2mM EDTA solution containing a protease inhibitor cocktail (5 μg/ml leupeptin, 10 μg/ml benzamidine and 5 μg/ml soybean trypsin inhibitor). The lysate was incubated on ice with frequent vortexing and subsequently centrifuged at 13,000 rpm for 30 min to obtain the supernatant. Five μl of cell lysate was spotted onto nitrocellulose membrane and then incubated with anti-hemaglutinin (HA) antibody diluted 1:2000 (AB 3254, Chemicon, Temecula, CA, USA). Immunoreactivity was revealed by incubating with HRP-conjugated anti-chicken secondary antibody diluted 1:1000 (Bio-Rad, Hercules, CA, USA) followed by enhanced chemiluminescent reagents (KPL, Gaithersburg, MD, USA), and then exposing to film. Background immunoreactivity was determined in cell lysates not expressing the HA-tagged peptide. Immunoreactivity was not quantified and assay variance was no greater than ten percent.

2.3.7 Immunoprecipitation Studies: The membrane fraction obtained from cells co-expressing μ- and δ-ORs in the absence or presence of δOR distal carboxyl tail interfering or control peptide was re-suspended and stirred with protease inhibitors and the homogenate centrifuged. The solubilized portion was isolated and incubated with 10 μg anti-cMyc (Millipore, Billerica, MA, USA) antibody and then protein-G-agarose beads. The immunoprecipitate was washed and solubilized in SDS sample for gel electrophoresis.

2.3.8 SDS – Polyacrylamide Gel Electrophoresis and Immunoblotting: Membrane proteins were resolved on a 10 % Tris-Glycine precast gel (Novex, San Diego, CA, USA)
under denaturing conditions by SDS-PAGE and then electroblotted onto a polyvinylidene difluoride membrane as described previously (Fan et al., 2005). Immunoreactivity was revealed by incubating in FLAG antibody diluted 1:5000 (Sigma, Saint Louis, MO, USA) or cMyc antibody diluted 1:1000 (Millipore, Billerica, MA, USA), HRP-conjugated goat-anti-mouse secondary antibody diluted 1:2000 (Bio-Rad, Hercules, CA, USA), and enhanced chemiluminescent reagents (KPL, Gaithersburg, MD, USA), and then exposing to film. No cMyc immunoreactivity was observed in cells expressing FLAG-tagged δORs and not cMyc-tagged μORs, confirming specificity of the antibody.

2.3.9 Intact Cell Internalization Assay: μOR binding was performed on intact cells stably co-expressing μ- and δ-opioid receptors using [³H]-DAMGO at its Kᵟ (2 nM). [³H]-DAMGO would only label cell surface receptors because it is a hydrophilic peptide ligand and so does not penetrate the plasma membrane (Blake et al., 1997; Koch et al., 1998). Non-specific binding was defined using 10 μM naloxone. Cells were pretreated with agonist for 1 hr at 37°C, rinsed, treated with [³H]-DAMGO for 4 hrs at 4°C (to eliminate any potential for radiolabel internalization), then rinsed. Cell surface radioactivity was measured using the liquid scintillation counter. The disappearance of [³H]-DAMGO binding sites on the cell surface served as an indicator of μOR internalization.

2.3.10 Animals: Adult male Sprague Dawley rats weighing 250-400 g (Charles River, Québec, Canada) were housed in pairs (and singly-housed following surgery) in a temperature-controlled room with corn chip bedding and free access to standard rodent chow and water. Rats were maintained under a standard 12-h/12-h light/dark cycle (lights on at
07:00 AM). Testing was performed during the light cycle. Animal protocols were approved by the University of Toronto Animal Care Committee and were in accordance with the guidelines set by the Canadian Council on Animal Care.

2.3.11 Stereotaxic Surgery: Rats were mounted on a stereotaxic frame under isoflurane anesthesia. Stainless steel guide cannulae were implanted bilaterally into the NAc (AP +1.6, ML +3.0, DV -6.9) at a 10° angle from the midline or into the lateral cerebral ventricle (AP -0.8, ML +1.4, DV -3.6) at 90° angle to the skull using co-ordinates from Paxinos and Watson (1998). Guide cannulae were positioned 1 mm anterior to the NAc to prevent tissue damage at the target site. Cannulae were secured onto the skull using stainless steel screws and dental cement. Dummy stylets were inserted into guide cannulae to maintain patency. Rats were allowed to recover and behavioural testing was conducted 7 days following surgery. Drugs and peptides were administered using an internal cannula by Hamilton syringe in a total volume of 1.0 µl over 120 sec. The internal cannula was kept in place for 60 sec after micro-injection. Placement of guide cannulae was verified by brain sectioning and histological examination at the end of the experiment.

2.3.12 TAT Fusion Peptides: The peptide fragment comprising the distal portion of the δOR carboxyl tail (VTACTPSDGPGGAAAA) implicated in the interaction between μ- and δ-ORs (Fan et al., 2005) was conjugated to the HIV protein transduction domain TAT (YGRKKRRQRRR) to create a TAT fusion peptide for transduction into neurons (Schwarze et al., 1999). A scrambled sequence of the same 16 amino acids was used as control. Dansyl chloride was conjugated to the TAT peptide to allow for easy detection of the fusion peptide
in vivo. Interfering or scrambled fusion peptides (15 pmol / side) were injected bilaterally into the NAc.

### 2.3.13 Forced Swim Test:

The forced swim test (FST) was performed as described by Porsolt and colleagues (1978) with the following modifications. Briefly, rats were placed in a cylindrical container filled to a depth of 30 cm with water (at 25 ± 1 °C). During the initial session, rats were placed in the container and allowed to swim without behavioural scoring for 15 min. This priming session serves to induce immobility on test day. On the next day, animals underwent a test session and their predominant behaviour was recorded at 5 sec intervals for 5 minutes using the time sampling method validated by Lucki and colleagues (1994). Rat behaviour was scored as climbing (directed movements with forepaws against walls of container in an effort to climb out), swimming (active moving around cylindrical container) or immobility (floating in water and making only the necessary movements to maintain head above the water). Total duration of each behaviour was calculated. Following the swim sessions, rats were dried and placed under a heat lamp and monitored until recovery.

### 2.3.14 Elevated Plus Maze Test:

The elevated plus maze (EPM) apparatus (Colbourn Instruments, Whitehall, PA, USA) consisted of a black plexiglas maze with four arms extending out in a cross pattern from a central zone. Arms measured 45 cm in length and 10 cm in width. Two arms were open and two arms were covered by vertical black plexiglas walls measuring 30 cm in height. The maze was elevated 55 cm above ground. At the beginning of testing, rats were placed in the central zone and allowed to move freely around
the maze. The 5 min session was recorded by video and the amount of time spent in the open arms was assessed. Maze arms were cleaned after each animal.

2.3.15 Novelty-induced Hypophagia Test: Novelty-induced hypophagia (NIH) was assessed in singly-housed rats. Animals were trained to drink a sweetened milk solution in their home cage for three days. Only animals that consumed milk solution were included. On the fourth day, the latency to drink the milk solution and the amount of milk consumed were assessed in the home cage. On the fifth and final day, rats were placed in a novel cage without corn cob bedding under bright light conditions and the latency to drink the milk solution and amount of milk consumed were assessed in this novel and stressful environment.

2.3.16 Locomotor Activity: Locomotor activity was assessed using automated activity monitors equipped with motion sensors (Accuscan Instruments, Columbus, OH, USA). Rats were placed in activity monitor chambers (45 cm x 45 cm) and their movement monitored for 60 min. Total horizontal distance travelled was used as an index of locomotor activity.

2.3.17 Tail Immersion Antinociception Assay: Rats were restrained gently and the distal 5 cm of their tail dipped in a bath of water maintained at 52°C. The latency to tail withdrawal from water was measured. Three pre-drug baseline (BL) measures were obtained and averaged. A cut-off of 4 x BL latency was imposed to minimize tissue damage. Rats were habituated to the tail immersion assay before testing. % Maximum Possible Effect was
calculated according to the equation: % Maximum Possible Effect = (Latency - BL latency) / 
(Cut-off Latency – BL latency) x 100.

2.3.18 Verification of Cannula Placement: Rats were perfused transcardially with saline 
followed by 10% formalin fixative under brief isoflurane anesthesia. The brain was 
harvested and post-fixed in fixative solution overnight. Brain samples were then transferred 
to 30% sucrose in 0.1 M phosphate buffer (pH 7.4) for 48 hours and stored at 4°C. Brain 
samples were subsequently frozen at -70°C and sectioned into 20 µm slices using a cryostat. 
Sections were collected onto gelatin-coated slides and allowed to dry. Slides were viewed on 
the LSM 510 Zeiss confocal microscope (Carl Zeiss, Toronto, ON, Canada) using 20X Plan-
Apochromat objective lens. Images were assembled using the Zeiss LSM Image Browser 
software (Carl Zeiss, Toronto, ON, Canada).

2.3.19 Data Analysis: Statistical analyses and graph generation were performed using 
GraphPad Prism software 3.01 (San Diego, CA, USA). The results were presented as means 
± standard error of the mean (SEM). Data from competition radioligand binding experiments 
were analyzed by nonlinear least-squares regression. An F-test was used to compare the 
coefficients of the goodness-of-fit and to determine whether a two- or a one-site analysis was 
a statistically significant better fit for the radioligand competition binding curves. One-way 
ANOVA followed by Dunnett’s post hoc analysis was used to assess statistical significance 
of cell surface radioligand binding, forced swim test, elevated plus maze, novelty-induced 
hypoglycemia and locomotor activity data. P < 0.05 was deemed significant.
3. STUDY I RESULTS AND DISCUSSION

The agonist-induced regulation profile of the $\mu$-$\delta$ OR heteromer in a cell line

Findings have been published in the British Journal of Pharmacology (Kabli, N., Martin, N., Fan, T., Nguyen, T., Hasbi, A., Balboni, G., O’Dowd, B.F., and S.R. George. Agonists at the $\delta$-Opioid Receptor Modify the Binding of $\mu$-Agonists to the $\mu$-$\delta$ Opioid Receptor Hetero-oligomer. British Journal of Pharmacology 2010 (161): 1122-1136) and are reproduced by permission from John Wiley and Sons and the British Journal of Pharmacology.
3.1 Modulatory role of $\Gamma z$ on peptidic and non-peptidic agonist affinities for the $\mu$-$\delta$OR heteromer.

G protein coupling plays an important role in modulating the conformation of ORs (Yan et al., 2008). Since we showed that the $\mu$-$\delta$ heteromer preferentially coupled to $\Gamma z$ (Fan et al., 2005; Hasbi et al., 2007), agonist affinities at the $\Gamma z$-coupled receptor complex were investigated. The affinities of peptidic (endogenous and synthetic) and non-peptidic $\mu$- and $\delta$-opioid agonists were compared in cells co-expressing $\mu$- and $\delta$-ORs in the absence or presence of transfected $\Gamma z$ using competition of antagonist $[^3H]$-diprenorphine (non-selective $\mu/\delta$ antagonist) binding. Whereas $\Gamma i$ proteins are ubiquitously expressed in HEK cells, $\Gamma z$ is not abundantly expressed (Figure 3.1A; Fan et al., 2005). Immunoblotting with $\Gamma z$ antibody revealed an immunoreactive band with an approximate weight of 41 kDa as reported previously (Casey et al., 1990; Hinton et al., 1990; Hendry et al., 2000; Fan et al., 2005). Similarly, immunoblotting with $\Gamma i3$ antibody revealed an immunoreactive band with an approximate weight of 41 kDa as reported previously (Codina et al., 1983; Codina et al., 1988; Gerhardt and Neubig 1991). Blots were stripped and reprobed with GAPDH as the loading control. Transfection resulted in an approximately three-fold increase in $\Gamma z$ expression over endogenous levels, as determined by densitometry analysis (ImageJ software, NIH, Bethesda, MD, USA). The specificity of the $\Gamma z$ and $\Gamma i3$ antibodies was ascertained by immunoprecipitating $\Gamma z$ or $\Gamma i3$ and probing with $\Gamma z$ or $\Gamma i3$ antiserum (Figure 3.1B). Whereas the $\Gamma z$ antiserum detected immunoprecipitated $\Gamma z$ protein, the latter was not detected by the $\Gamma i3$ antiserum, and vice versa. Thus, each antibody reacted selectively with its corresponding antigen.
Ga<sub>z</sub> co-expression significantly increased high affinity binding (K<sub>H</sub>) of the δ-agonist DPDPE by 10-fold, the endogenous agonist Leu-enkephalin by 3-fold and the μ-agonist DAMGO by 10-fold for the μ-δ OR heterooligomer (Table 3.1). Whereas Ga<sub>z</sub> has no effect on binding affinities of μ- or δ-selective agonists at their individual receptors consistent with preferential coupling of these homomers to the PTX-sensitive Ga<sub>i</sub> proteins (Fan et al., 2005), Ga<sub>z</sub> co-expression resulted in an overall leftward shift of the competition curves resulting in higher affinities both at the high and low affinity sites. High affinity binding of the synthetic δ-agonists UFP-512 and SNC80 and the endogenous μ-agonist endomorphin-1 were unaltered by Ga<sub>z</sub> co-expression (Table 3.1). Overall, enhanced affinities for select agonists were demonstrated at the μ-δ-Ga<sub>z</sub> complex, an effect that was agonist-specific and did not depend on ligand chemical structure. The percentage of receptors in the agonist-detected high affinity state (% HA) was not significantly different following Ga<sub>z</sub> co-expression, except in the case of Leu-enkephalin and UFP-512 where it was reduced. In this case, the change in % HA did not seem to relate to the effect of Ga<sub>z</sub> coupling on agonist affinities, since the affinity of Leu-enkephalin was enhanced upon Ga<sub>z</sub> co-expression whereas that of UFP-512 was not altered. According to the ternary complex model of ligand binding to GPCRs, the high and low affinity states are thought to reflect agonist binding to the G-protein-coupled and -uncoupled receptors, respectively (DeLean and Lefkowitz 1980). Thus, increased expression of Ga proteins would be expected to increase the proportion of receptors in the HA state. However, in the case of the μ-δ heteromer, the HA state seems to be independent of G-protein coupling (George et al., 2000). The guanine nucleotide analog GTPγS, which uncouples μ- and δ-OR homomers from Ga proteins and results in a loss of agonist-detected HA binding, does not abolish agonist-detected high affinity binding to the
μ-δ heteromer (George et al., 2000). Thus, the shift in high and low affinity states we observed cannot be explained by the ternary complex model of ligand binding to GPCRs. The latter is a simplistic interpretation of the results and does not account entirely for the multiple affinity states detected by radioligand binding studies which can result from multiple factors such as G protein coupling, ligand co-operativity, and different oligomeric states of the GPCR (Chidiac et al., 1997; Green et al., 1997; Christopoulos and Kenakin 2002; Kara et al., 2009). For example, in studies of radioligand antagonist competition by a μ-opioid agonist in cells co-expressing μ- and δ-ORs, the HA site may represent an average affinity describing the interaction of the agonist with the μ-δ heteromer as well as the G-protein-coupled μOR. On the other hand, the low affinity site may be representative of agonist interactions with the μ-δ heteromer (low affinity species), the G-protein-uncoupled μOR and the G-protein-uncoupled δOR. Similarly, if a δ-agonist is used as the competing ligand, then the HA site may represent an average affinity describing the interaction of this agonist with the μ-δ heteromer as well as the G-protein-coupled δOR and the low affinity site may be representative of agonist interactions with the μ-δ heteromer (low affinity species), the G-protein-uncoupled δOR and the G-protein-uncoupled μOR.

In order to assess true agonist affinity at the Gαz-coupled μ-δ OR complex, PTX (100 ng/ml overnight) was used to inactivate Gαi/o proteins and to minimize the contribution of Gαi/o-coupled receptors to the high affinity binding detected. PTX treatment resulted in the emergence of a high affinity binding site for the δ-agonist SNC80 with a 160-fold increase in affinity, whereas Leu-enkephalin-detected high affinity states were not significantly enhanced (Table 3.1). Whereas PTX treatment is known to abolish high affinity agonist
binding to µ- and δ-OR homomers, it was necessary to reveal the affinity of SNC80 at the µ-δ OR complex. In the absence of PTX, multiple binding sites could not be resolved on a multi-phasic competition curve likely accounting for the monophasic SNC80-[³H]-diprenorphine competition curve. PTX, by inactivating Gαi proteins and removing the contribution of any Gαi-coupled receptors to the binding detected by [³H]-diprenorphine, unmasked the true affinity of SNC80 at the µ-δ OR heterooligomer.

Our finding that the associated G protein enhanced the affinity of select ligands for ORs has also been documented for the κOR and OR-G protein fusion constructs (Snook et al., 2008; Yan et al., 2008). In the case of κOR, use of the substituted cysteine accessibility method showed that 2- to 3-fold changes in agonist affinity at the differentially coupled receptor were due to conformational changes induced by G protein binding to the receptor (Yan et al., 2008). Since changes in agonist affinity reflect receptor conformational changes, the Gαz-coupled µ-δ heterooligomer likely had an altered conformation.

3.2 Comparison the δ-agonist-detected ligand binding pocket in the µ-δ OR heterooligomer and the µOR homomer.

To examine δ-agonist displacement of µ-agonist binding from the µ-δ OR heteromer and the µOR homomer, competition radioligand binding was performed using [³H]-DAMGO in cells co-expressing µ- and δ-ORs or singly expressing µOR. DAMGO is a highly selective µ-agonist having approximately 1600-fold higher affinity for µOR than δOR (George et al., 2000). Furthermore, [³H]-DAMGO binding was inhibited by the selective µ-antagonist naltrexone with similar affinity both in cells co-expressing µ- and δ-ORs (K₁ =
0.51 ± 0.05 nM; n = 3) or singly expressing μOR (Kᵢ = 0.49 ± 0.13 nM; n = 3). At the concentration used, DAMGO did not detect δOR as confirmed by the lack of binding in cells expressing δOR alone (Figure 3.2C). All δ-agonists tested detected a single low affinity binding site in cells expressing μOR as indicated by monophasic competition of [³H]-DAMGO binding (Figure 3.2; Table 3.2). In contrast, a high-affinity binding site emerged when δ-agonists displaced [³H]-DAMGO binding in cells co-expressing μ- and δ-ORs (Figure 3.2; Table 3.2). All of SNC80, UFP-512, Deltorphin II and DPDPE displaced [³H]-DAMGO binding with statistically significantly higher affinities (380-, 165-, 1800-, and 200-fold, respectively) in cells co-expressing μ- and δ-ORs compared to cells expressing μOR alone (Table 3.2), and this was evidenced by a left-ward shift in the agonist competition curve (Figure 3.2A and B). The percentage of receptors in the δ-agonist-detected high affinity state was approximately 20-30%.

To confirm that the binding pocket detected by [³H]-DAMGO was composed of μOR, we pre-treated cells with 50 nM of the irreversible μ-receptor antagonist β-funaltrexamine (β-FNA). This concentration was selected since it resulted in approximately 80 % inhibition of [³H]-DAMGO binding in cells expressing μOR and only 30 % inhibition of [³H]-diprenorphine binding in cells expressing δOR (Figure 3.3). In membranes from β-funaltrexamine-treated cells co-expressing μ- and δ-ORs, specific [³H]-DAMGO binding was greatly attenuated and SNC80 could not displace [³H]-DAMGO (Figure 3.2C), indicating that δ-agonists did indeed compete for binding within a ligand binding pocket comprising μOR.
In separate experiments, SNC80 competition for \[^{3}H\]-DAMGO binding was assessed in cells transfected with additional \(G_{\alpha}z\) and pre-treated with PTX. SNC80 displaced \[^{3}H\]-DAMGO binding with high nanomolar affinity under all these conditions (Figure 3.2B), suggesting that the binding pocket detected is unique to the \(\mu-\delta\) heterooligomer as it was PTX-resistant.

\(\delta\)-opioid agonist displacement of the selective \(\mu\)-agonist \[^{3}H\]\-DAMGO from its binding site with high affinity in cells co-expressing \(\mu\)- and \(\delta\)-ORs but not in those expressing \(\mu\)OR individually suggests that the \(\mu-\delta\) heteromer may contain a novel \(\delta\)-agonist-detected ligand binding pocket in the \(\mu\)OR. The binding site labeled by \[^{3}H\]\-DAMGO was comprised of \(\mu\)OR since binding of this radioligand was abolished following pre-treatment with an irreversible \(\mu\)OR antagonist at a concentration that selectively inhibited \(\mu\)OR. Secondly, this selective \(\mu\)-agonist radioligand did not detect \(\delta\)OR at the concentration used, as confirmed by the lack of binding in cells that expressed \(\delta\)OR alone. It follows that \[^{3}H\]\-DAMGO selectively labels \(\mu\)OR-containing receptor complexes. Thus, the high affinity \(\mu\)OR binding site detected may represent the interaction of the \(\delta\)-agonist with the \(\mu-\delta\) heteromer whereas the low affinity site may be representative of the interaction with the \(\mu\)OR homomer. Our observations in these radioligand binding studies could be explained by a competitive binding interaction whereby \(\delta\)-agonists compete with \(\mu\)-agonists for the same binding site and occupy the \(\mu\)OR ligand binding pocket or an allosteric interaction whereby \(\delta\)-agonists, by binding to the \(\delta\)OR, modify the conformation of the \(\mu\)OR protomer thereby modulating \(\mu\)-agonist binding. The emergence of a \(\delta\)-agonist-detected high affinity \(\mu\)OR binding site within the \(\mu-\delta\) heteromer suggests that the \(\delta\)-agonist-targeted allosteric site is
conformationally-coupled to the high affinity μOR ligand binding pocket. In any case, these binding interactions could only occur if the two receptors were physically interacting, as in a receptor heterooligomer.

Our findings indicate that δ-agonists may not only be binding to their putative δOR, but also occupying or modulating a high affinity μOR binding pocket within the μ-δ heteromer. It follows that the behavioural effects of δ-agonists in vivo may not result solely from the activation of δOR, but also from their ability to interact with the μ-δ heteromer. Overall, these findings suggest that δ-agonists have previously unrecognized high affinity for the μ-δ heterooligomer and provide a possible explanation for the attenuation of δ-agonist-mediated effects in μOR gene-deleted animals.

3.3 Opioid agonist-induced internalization of the μ-δ OR heteromer.

To determine how opioid ligands modulated the density of cell surface μ-δ heteromers, an internalization assay was performed to track the disappearance of cell surface [³H]-DAMGO labelling as an index of μOR internalization in intact cells co-expressing μ- and δ-ORs. δ-agonist-induced internalization of μOR in this expression background would serve as an index of μ-δ heteromer internalization. All endogenous and synthetic μ- and δ-opioid agonists tested induced internalization of μOR following agonist (10 μM for 1 hr) exposure (Figure 3.4). As expected, μ-agonists induced significantly greater internalization of μOR than δ-agonists, since they may be expected to induce internalization of both μ-δ heteromers and μOR homomers present in the cells. The endogenous agonists endomorphin-
1, and Met- and Leu-enkephalin resulted in $52 \pm 4.7$, $62 \pm 3.5$, and $50 \pm 2.9\%$ internalization, respectively (Figure 3.4 bars 2-3). The $\mu$-agonist DAMGO induced $72 \pm 3.3\%$ attenuation of cell surface $\mu$OR (Figure 3.4 bar 1). $\delta$-agonists resulted in loss of cell surface $\mu$OR, with UFP-512, SNC80, deltorphin II and DPDPE each resulting in internalization of $48 \pm 3.3$, $30 \pm 3.5$, $23 \pm 2.5$ and $22 \pm 3.4\%$ cell surface receptors, respectively (Figure 3.4 bars 4-9). Vehicle-treated cells, where no receptor internalization occurred, served as controls. Agonist-induced internalization was dose-dependent (Figure 3.5).

Internalization plays a key role in regulating receptor number at the cell surface and is an important mechanism by which receptor function is tightly regulated (Ferguson et al., 1998; Whistler et al., 1999). Reduction of cell surface binding of the $\mu$-agonist $[^3H]$-DAMGO was used as a quantitative measure of $\mu$OR endocytosis. $[^3H]$-DAMGO would only label cell surface receptors because it is a hydrophilic peptide ligand and so does not penetrate the plasma membrane (Blake et al., 1997; Koch et al., 1998). In addition, incubation with $[^3H]$-DAMGO was conducted at $4^\circ$C to prevent any possible internalization of $[^3H]$-DAMGO bound to the $\mu$OR and to prevent recycling of receptors to the cell surface (Blake et al., 1997; Segredo et al., 1997; Koch et al., 1998; Toews 2000). This protocol has been validated as a sensitive and reliable approach by several other groups (Segredo et al., 1997; Koch et al., 1998).

$\delta$-agonist-induced internalization of $\mu$OR was also analyzed using epitope-tagged eMyc$\mu$OR and FLAG$\delta$OR in a cell surface receptor immunofluorescence assay (Figure 3.6).
The decrease in cell surface cMyc immunofluorescence in agonist-treated versus vehicle-treated control cells served as an indicator of μOR internalization. SNC80 treatment (10 μM for 1 hr) induced a 20% decrease in cell surface μOR, which was comparable to the internalization by SNC80 measured using radioligand binding (Figures 3.5 and 3.6). δ-agonist-induced disappearance of cell surface μOR labelling in cells co-expressing μ- and δ-ORs suggested that the receptor population lost from the cell surface were likely μOR hetero-oligomerized with δOR. These findings confirm that the μ-δ heteromer was expressed on the cell surface and that its function was regulated by both endogenous and synthetic opioid agonists. Further, they confirm that decreased cell surface [³H]-DAMGO binding measured in whole cell binding assays represented μOR endocytosis and was not merely a result of loss of affinity for the radioligand or persistent receptor occupancy by δ-agonists.

δ-agonist-induced mobilization of μOR was visualized by confocal microscopy. Cells co-expressing δORₘRFP and μOR₆FP were treated with 100 nM UFP-512 and observed in real-time. Under basal conditions prior to agonist treatment, μ- and δ-ORs fluorescence was present mostly on the cell surface (Figure 3.7 A-C). Following agonist treatment, μ- and δ-ORs were observed as yellow puncta inside the cell (Figure 3.7 D-F, arrows). These observations support our findings from the intact cell internalization assay, indicating that this δ-agonist induced mobilization of both μ- and δ-ORs. In separate experiments, UFP-512 induced a dose-dependent internalization of δOR expressed alone, but had no effect (at concentrations between 0.1 and 10 μM) on μOR expressed individually (Figure 3.8). The
lower drug dose and experimental temperature (20-22 °C) likely account for the seemingly reduced extent of internalization observed by confocal microscopy.

The finding that the µ-δ heteromer readily internalized in response to a wide array of agonists may be explained by evidence that the µ-δ heteromer is constitutively associated with β-arrestin 2, a key component of the endocytic pathway (Rozenfeld et al., 2007). Thus, not only did δ-agonists displace µ-agonist binding with high affinity, they also induced internalization of the µ-δ OR complex.

### 3.4 Role of δOR and µOR in δ-agonist-induced internalization of the µ-δ OR heteromer.

To probe the involvement of δOR and µOR in δ-agonist-induced internalization of µOR in cells co-expressing µ- and δ-ORs, cells were pretreated with the highly selective δ-antagonist naltrindole (Portoghese et al., 1988) or the µ-antagonist CTOP (Gulya et al., 1988) (Figure 3.9A). Naltrindole abolished SNC80- and deltorphin II-induced internalization (Figure 3.9A; 2.0 ± 5.4 and -2.3 ± 11 % internalization, respectively). Blocking the µOR with CTOP abolished UFP-512, SNC80- and deltorphin II-induced internalization (Figure 3.9A; 1.1 ± 5.2, 0.0 ± 2.1, and -3.5 ± 6.6 % internalization, respectively), providing evidence that δ-agonist-induced internalization of the µOR required the occupancy of both µ- and δ-OR binding pockets within the heterooligomer. DAMGO-induced internalization was significantly attenuated by naltrindole and abolished by CTOP (Figure 3.9A; 72 ± 3.3 versus 37.2 ± 3.3 and -3.2 ± 7.8 % internalization in control versus naltrindole- or CTOP-treated cells, respectively), suggesting that both δ- and µ-ORs may be required for DAMGO-
induced internalization of the $\mu$-$\delta$ receptor complex. On the other hand, endomorphin-1-induced internalization was unaffected by $\delta$OR blockade but was abolished by blocking the $\mu$OR (52 ± 4.7 versus 48.3 ± 6.4 and -2.0 ± 8.7 % internalization in control versus naltrindole- or CTOP-treated cells, respectively), suggesting that this endogenous $\mu$-agonist may not occupy the $\delta$OR binding pocket within the $\mu$-$\delta$ heterooligomer, or that it may induce internalization of the heteromer by a different mechanism. Given that $\delta$-agonist-induced $\mu$OR internalization required occupancy of both $\delta$- and $\mu$-ORs and that these agonists displaced $\mu$-agonist binding with high affinity from the $\mu$-$\delta$ heteromer, the receptors internalized were likely $\mu$OR that were complexed with $\delta$OR.

3.5 Opioid agonist-induced internalization profile of the $\mu$OR homomer.

To examine $\delta$-agonist effects at the $\mu$OR homomer, internalization was investigated in intact cells singly expressing this receptor by tracking the disappearance of cell surface $[^3H]$-DAMGO binding (Figure 3.9B). The $\delta$-agonists UFP-512, SNC80, deltorphin II and DPDPE did not induce $\mu$OR internalization (Figure 3.9B; 6.5 ± 3.5, 0.7 ± 0.6, 1.9 ± 2.0 and 3.4 ± 7.0 % internalization, respectively). Met-enkephalin, endomorphin-1 and DAMGO induced 45 ± 1.3, 54 ± 1.9 and 48 ± 1.1 % internalization of cell surface $\mu$OR, respectively.

3.6 Effect of PTX treatment or $G\alpha_z$ transfection on agonist-induced internalization of the $\mu$-$\delta$ OR heteromer.

To determine whether internalization of the $\mu$-$\delta$ heteromer was PTX-sensitive and to investigate the effect of $G\alpha_{i/o}$ inactivation on internalization, cells were pre-treated with PTX
for 24 hrs prior to the internalization assay (Figure 3.10A). PTX treatment significantly attenuated Met-enkephalin- and DAMGO-induced internalization but had no effect on endomorphin-1 (41 ± 2.1, 31 ± 1.2 and 50 ± 8.3 compared to 62 ± 3.5, 72 ± 3.3 and 52 ± 4.7 % internalization respectively, in non-PTX-treated controls). PTX attenuation of DAMGO-induced internalization is consistent with the notion that a proportion of µOR internalized in cells co-expressing µ- and δ-ORs are homomeric. The lack of PTX effect on endomorphin-1-induced internalization suggests that a large proportion of endomorphin-1- internalized µOR in cells co-expressing µ- and δ-ORs may be within a heteromeric complex. SNC80-, deltorphin II- and DPDPE-induced internalization of the µ-δ heteromer was not affected by PTX treatment (32 ± 6.6, 24 ± 4.3 and 24 ± 8.2, compared to 30 ± 3.5, 23 ± 2.5 and 22 ± 3.4 % internalization, respectively, in controls not treated with PTX). Agonist-induced internalization of individually-expressed µOR by selective agonists is PTX-sensitive, whereas δOR internalization is PTX-resistant (Remmers et al., 1998; Zaki et al., 2000; Bradbury et al., 2009). Here, both µ- and δ- agonist-induced internalization of the µ-δ heteromer was PTX-resistant.

Over-expression of G-proteins has been used as a method to elucidate the effects of receptor coupling to specific G-proteins on ligand binding and receptor function (Snook et al., 2008; Yan et al., 2008). To determine the effect of Gαz on internalization of the µ-δ heteromer, agonist-induced endocytosis was assessed in the presence and absence of transfected Gαz (Figure 3.10B). Western blotting confirmed the enhanced expression of Gαz relative to endogenous levels and its comparable abundance with endogenously-expressed Gαi3 proteins (Figure 3.1). Further, the modulatory effect of Gαz on agonist affinities
suggests that transfected Ga\(\alpha\)z interacted with receptor and had a similar disposition as its endogenous counterpart. Ga\(\alpha\)z had no additional effect \(\mu\)- or \(\delta\)-agonist-induced internalization of the \(\mu\)-\(\delta\) heteromer. Thus, inactivation of Ga\(\alpha_{i/o}\) or transfection of additional Ga\(\alpha\)z did not affect \(\delta\)-agonist-induced internalization of the \(\mu\)-\(\delta\) heteromer, suggesting that endogenous levels of Ga\(\alpha\)z may be sufficient or that this internalization process may not involve G proteins.

3.7 Effect of disruption of the clathrin-dynamin endocytic machinery on \(\delta\)-agonist-induced internalization of the \(\mu\)-\(\delta\) OR heteromer.

To investigate the pathway by which \(\delta\)-agonists induced internalization of the \(\mu\)-\(\delta\) heterooligomer, cells were either co-treated with the clathrin endocytosis disrupter concanavalin A or transfected with the dynamin dominant negative construct DYN K44A. Both concanavalin A and DYN K44A abolished SNC80- and deltorphin II-induced internalization (Figure 3.11; con A: 3.0 \(\pm\) 5.6 and 3.7 \(\pm\) 0.8 %; DYN K44A: 4.4 \(\pm\) 3.7 and 4.1 \(\pm\) 3.2 % internalization, respectively), suggesting that the \(\mu\)-\(\delta\) heteromer utilized the clathrin and dynamin endocytic machinery for internalization. In separate control experiments, transfection of empty vector had no effect on \(\delta\)-agonist-induced internalization. Thus, the \(\mu\)-\(\delta\) heteromer undergoes endocytosis via similar mechanisms as \(\mu\)- and \(\delta\)-OR homomers.

3.8 Effect of chronic morphine exposure on agonist-induced internalization of the \(\mu\)-\(\delta\) OR heteromer.

To investigate the internalization profile of the \(\mu\)-\(\delta\) heteromer in a cellular model of morphine tolerance, cells were pre-treated with 10 \(\mu\)M morphine sulfate for 48 hrs prior to
the internalization assay (Figure 3.12A). SNC80-, deltorphin II- and DPDPE-induced internalization was not altered by chronic morphine exposure (31 ± 5.0, 22 ± 7.0 and 19 ± 5.1 compared to 23 ± 3.5, 23 ± 2.5 and 22 ± 3.4 % internalization, respectively, in cells not pre-treated with morphine). DAMGO-induced internalization was significantly attenuated whereas endomorphin-1’s effects remained intact following morphine exposure (44 ± 2.1 and 52 ± 4.7 compared to 72 ± 3.3 and 44 ± 3.5 % internalization respectively, in cells not pre-treated with morphine). In contrast, this morphine regimen almost abolished DAMGO- and endomorphin-1-induced internalization in cells expressing μOR individually (Figure 3.12B).

The adaptations in OR trafficking that occur following chronic morphine treatment are of clinical significance. However, the regulation of the μ-δ heteromer under these conditions is not known. In this study, the prolonged morphine exposure paradigm used produces a cellular correlate of morphine tolerance (Sharma et al., 1975; Avidor-Reiss et al., 1995; Blake et al., 1997; Finn and Whislter, 2001). Whereas chronic morphine exposure has been shown to impair internalization of the individual μ- and δ-ORs by their selective agonists (Eisinger et al., 2002), we found that δ-agonist-induced internalization of the μ-δ heteromer still occurred without impairment. Chronic morphine exposure resulted in a reduction in - but not a loss of - DAMGO-induced internalization. Consistent with the possibility that endomorphin-1-internalized receptors in cells co-expressing μ- and δ-ORs are mostly heteromeric, endocytosis induced by this agonist was not impaired by chronic morphine exposure. Since internalization is a measure of function, our findings suggest that μ-δ heteromer function was not impaired in a paradigm of morphine tolerance. Since OR desensitization following chronic morphine exposure is thought to be a contributory
mechanism to the loss of opioid analgesic efficacy, the lack of desensitization in \( \mu-\delta \) heteromer function implies that targeting this receptor complex may be a better therapeutic strategy in treating neuropsychiatric conditions requiring prolonged exposure to morphine.

3.9 Conclusions

In conclusion, this study provides novel insight into the nature of the ligand binding pocket of the \( \mu-\delta \) heteromer and its regulation following exposure to a range of opioid agonists. The novel conformation of \( \mu- \) and \( \delta- \) ORs induced by their heterooligomerization resulted in preferential association with \( G\alpha_z \) and the enhanced affinities of select agonists at the \( \mu-\delta \) heteromer. \( \delta \)-agonists displacement of \( \mu \)-agonist binding with high affinity from the \( \mu-\delta \) heteromer but not the \( \mu \)-OR homomer suggested that the \( \mu-\delta \) hetero-oligomer may contain a novel \( \delta \)-agonist-detected \( \mu \)-OR ligand binding pocket or an allosteric site conformationally coupled to a \( \mu \)-OR high affinity binding site. The \( \mu-\delta \) receptor complex was expressed on the cell surface and internalized in response to endogenous and synthetic agonists via the clathrin and dynamin endocytic machinery. \( \mu-\delta \) heteromer internalization by \( \delta \)-agonists required occupancy of both \( \mu \)- and \( \delta \)-OR binding pockets. In contrast to the \( \mu \)-OR homomer, \( \mu-\delta \) heteromer internalization occurred after chronic morphine exposure without evidence of attenuation. Our finding that \( \delta \)-agonists target the \( \mu-\delta \) heteromer in addition to the \( \delta \)OR suggests that these agonists may have unique pharmacological specificity for the heteromer. This receptor complex represents a novel opioid signalling complex, and understanding its pharmacology and regulation will provide further insight into the functions of the opioid
system and the possible identification of selective compounds targeting the µ-δ heteromer exclusively.
Figure 3.1  Expression of $\Gamma\alpha_z$ and $\Gamma\alpha_i$ in HEK 293T cells and antibody specificity verification.  A. Membrane preparations (20 μg protein/lane) from cells were denatured and electrophoresed on 10% Tris-Glycine gels. Proteins were electroblotted onto a polyvinylidene difluoride membrane and immunoblotted using $\Gamma\alpha_z$ or $\Gamma\alpha_{i3}$ antiserum diluted 1:250. Cells co-expressing $\mu$- and $\delta$-ORs were (lanes 1 and 3) or were not (lane 2) transfected with additional $\Gamma\alpha_z$. Immunoblotting with $\Gamma\alpha_z$ antibody revealed an immunoreactive band with an approximate weight of 41 kDa as reported previously (Premont et al., 1989). Similarly, immunoblotting with $\Gamma\alpha_{i3}$ antibody revealed an immunoreactive band with an approximate weight of 41 kDa as reported previously (Holz et al., 1989). Blots were stripped and reprobed with GAPDH antibody diluted 1:10000 to serve as the loading control.  B. Cells co-expressing $\mu$- and $\delta$-ORs were transfected with additional $\Gamma\alpha_z$, immunoprecipitated with $\Gamma\alpha_z$ or $\Gamma\alpha_{i3}$ antiserum, and then probed with either $\Gamma\alpha_z$ or $\Gamma\alpha_{i3}$ antibody. Molecular weights were estimated by comparing immunoreactive band migration with calibrated protein ladder.
Table 3.1  Effect of $G_\alpha_z$ on peptidic and non-peptidic agonist affinities for the $\mu$-$\delta$ OR heteromer.

<table>
<thead>
<tr>
<th></th>
<th>$\mu$-OR</th>
<th>$\mu$-OR + $G_\alpha_z$</th>
<th>$\mu$-OR + $G_\alpha_z$ + PTX</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>$K_H$ (nM)</td>
<td>$K_L$ (nM)</td>
<td>% HA</td>
</tr>
<tr>
<td>DPDPE</td>
<td>32 ± 11</td>
<td>16,161 ± 1662</td>
<td>48 ± 4.8</td>
</tr>
<tr>
<td>Leu-Enkephalin</td>
<td>5.9 ± 1.2</td>
<td>201 ± 42</td>
<td>62 ± 5.2</td>
</tr>
<tr>
<td>DAMGO</td>
<td>28 ± 6.0</td>
<td>3774 ± 312</td>
<td>32 ± 3.8</td>
</tr>
<tr>
<td>Endomorphin-1</td>
<td>148 ± 37</td>
<td>7305 ± 702</td>
<td>21 ± 2.4</td>
</tr>
<tr>
<td>UFP-512</td>
<td>1.7 ± 0.1</td>
<td>1165 ± 101</td>
<td>58 ± 3.4</td>
</tr>
<tr>
<td>SNC80</td>
<td>n/a</td>
<td>298 ± 10</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Values shown represent mean ± S.E.M. of n = 3 - 6 experiments performed in duplicate. $K_H$ = agonist-detected high affinity site binding constant, $K_L$ = agonist-detected low affinity site binding constant, % HA = percentage of receptors in the agonist-detected high affinity state. Competition radioligand binding was performed using the non-selective $\mu$/δ antagonist [3H]-diprenorphine. An F-test was used to determine whether a two-site or a one-site binding curve provided a statistically significant better fit for agonist competition for [3H]-diprenorphine binding. Nd = not determined; n/a = not applicable; PTX = pertussis toxin. The unpaired Student’s t-test was used to compare $K_H$ and % HA values. * p < 0.05, *** p < 0.001 relative to cells expressing $\mu$- and $\delta$-ORs.
**Figure 3.2  δ-agonist detection of the μ ligand binding pocket in cells co-expressing μ- and δ-ORs or singly expressing μOR or δOR.** Competition of [³H]-DAMGO binding in membranes from HEK 293T cells expressing μOR or μ- and δ-ORs by UFP-512 (A), cells expressing μOR or μ- and δ-ORs in the presence/absence of PTX pre-treatment by SNC80 (B), or cells expressing δOR or μ- and δ-ORs in the presence/absence of β-FNA (β-funaltrexamine) pre-treatment by SNC80 (C). Grey and black arrowheads and arrows point to the high and low ligand binding affinities in μOR-, μ-δOR-, or PTX pre-treated μ-δOR-expressing cells, respectively. Cells expressed 150 – 350 fmol/mg of total receptor protein, whereby each receptor was expressed at a density of 150 – 175 fmol/mg. Results shown are mean ± S.E.M. and curves are representative of n = 3-6 experiments performed in duplicate.
Table 3.2  δ-agonist affinities for the μ-ligand binding pocket in cells co-expressing μ- and δ-ORs compared to cells expressing μOR alone.

<table>
<thead>
<tr>
<th></th>
<th>μOR</th>
<th>μ-δOR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>K_i (nM)</td>
<td>K_H (nM)</td>
</tr>
<tr>
<td>SNC80</td>
<td>9220 ± 1358</td>
<td>24 ± 7.0 **</td>
</tr>
<tr>
<td>UFP-512</td>
<td>83 ± 9.0</td>
<td>0.5 ± 0.1 ***</td>
</tr>
<tr>
<td>DPDPE</td>
<td>3120 ± 490</td>
<td>15 ± 4.0 **</td>
</tr>
<tr>
<td>Deltorphin II</td>
<td>6230 ± 1098</td>
<td>3.5 ± 1.2 **</td>
</tr>
</tbody>
</table>

Competition radioligand binding was performed using [3H]-DAMGO. Values shown represent mean ± S.E.M. of n = 3 – 6 experiments performed in duplicate. K_i = agonist-detected affinity site binding constant, K_H = agonist-detected high affinity site binding constant, K_L = agonist-detected low affinity site binding constant, % HA = percentage of receptors in the agonist-detected high affinity state. Cells expressed 150 – 350 fmol/mg of total receptor protein. ** p < 0.01, *** p < 0.001 relative to cells expressing μOR alone. An F-test was used to determine whether a two-site or a one-site binding curve provided a statistically significant better fit for δ-agonist binding to the μ-ligand binding pocket in cells co-expressing μ- and δ-ORs (SNC80: p<0.0001; UFP-512, p<0.0001; Deltorphin II, p<0.001; DPDPE, p<0.05).
Figure 3.3  Determination of β-funaltrexamine concentration required to block binding to μOR with the least effect on δOR. Competition of [3H]-DAMGO (A) or [3H]-diprenorphine (B) binding to membranes from HEK 293T cells expressing μOR (shaded circles) or δOR (open circles) by β-funaltrexamine.
Figures 3.4 Agonist-induced internalization of cell surface μOR detected by [3H]-DAMGO in intact HEK 293T cells co-expressing μ- and δ-OR. Cells were treated with 10 μM agonist for 1 hr at 37°C. Data shown represent drug-induced loss of cell surface receptors as a percentage of cell surface receptors in vehicle-treated control cells (where no internalization occurred), and are expressed as mean ± S.E.M. for n = 3-5 experiments performed in triplicate.
Figure 3.5  **Dose-dependence of agonist-induced internalization.** Cell surface μOR were detected by [³H]-DAMGO in intact HEK 293T cells co-expressing μ- and δ-OR. Cells were treated with 1 or 10 μM agonist for 1 hr at 37°C. Data shown represent drug-induced loss of cell surface receptors as a percentage of cell surface receptors in vehicle-treated control cells (where no internalization occurred), and are expressed as mean ± S.E.M. for n = 3-5 experiments performed in triplicate [* p < 0.05, *** p < 0.001 relative to cells treated with 1 μM agonist concentration].
Figures 3.6  SNC80-induced internalization of cell surface μOR detected by immunofluorescence in cells co-expressing cMyc-tagged μOR and FLAG-tagged δOR. Cells were treated with 10 μM agonist for 1 hr at 37°C and then processed for immunofluorescence. Data shown represent drug-induced loss of cell surface receptors as a percentage of cell surface receptors in vehicle-treated control cells (where no internalization occurred), and are expressed as mean ± S.E.M. for n = 3 experiments performed in triplicate.
Figure 3.7  Agonist-induced internalization of mRFP-tagged δOR and GFP-tagged μOR in a living HEK 293T cell followed in real time. The cell was treated with the δ-agonist UFP-512 (100 nM) and observed under the Zeiss LSM 510 confocal microscope. Panels A-C and D-F show the cell prior to drug treatment and following UFP-512 treatment for 90 min at room temperature, respectively. Arrows point to μ- and δ-OR, or δOR fluorescence inside the cell. Red and green represent δOR and μOR fluorescence, respectively. This is a representative example of n = 16 cells. Overlay is shown in yellow. Scale bar = 10 μm.
Figure 3.8  UFP-512-induced internalization of cell surface μOR or δOR detected by [³H]-DAMGO or [³H]-diprenorphine, respectively, in intact HEK 293T cells expressing μ- or δ-ORs individually. Cells were treated with 10, 1 or 0.1 μM agonist for 1 hr at 37°C. Data shown represent drug-induced loss of cell surface receptors as a percentage of cell surface receptors in vehicle-treated control cells (where no internalization occurred), and are expressed as mean ± S.E.M. for n = 3 experiments performed in triplicate.
Figure 3.9  Agonist-induced internalization of cell surface μOR detected by [³H]-DAMGO in intact HEK 293T cells co-expressing μ- and δ-ORs and treated with 100 nM naltrindole or 1 μM CTOP (A) or singly expressing μOR (B). Cells co-expressing μ- and δ-ORs were pre-treated with 100 nM naltrindole or 1 μM CTOP for 30 min at 37°C, rinsed, then treated with 10 μM agonist for 1 hr at 37°C (A). Cells singly expressing μOR were treated with 10 μM agonist for 1 hr at 37°C (B). Data shown represent drug-induced loss of cell surface receptors as a percentage of cell surface receptors in vehicle-treated control cells and are expressed as mean ± S.E.M. for n = 3-5 experiments performed in triplicate. Nd = not determined. Statistical significance in (A) was determined using an unpaired Student’s t-test [* p < 0.05, ** p < 0.01, *** p < 0.001 relative to cells not pre-treated with naltrindole or CTOP].
Figure 3.10  Effect of pertussis toxin treatment or $\text{G}_\alpha_z$ transfection on agonist-induced internalization of cell surface $\mu$OR detected by $[^3\text{H}]$-DAMGO in intact HEK 293 cells co-expressing $\mu$- and $\delta$-OR. Cells were pre-treated with 100 ng/ml PTX for 24 hrs and rinsed (A) or transfected with $\text{G}_\alpha_z$ 48 hours in advance (B) prior to 10 $\mu$M agonist treatment for 1 hr at 37°C. Data shown represent a percentage of the vehicle-treated control cells and are expressed as mean ± S.E. for n = 3 experiments performed in triplicate. Statistical significance was determined using an unpaired Student’s t-test [* $p < 0.05$, *** $p < 0.001$ relative to cells not pre-treated with PTX or not transfected with additional $\text{G}_\alpha_z$].
Figure 3.11 Effect of concanavalin A (con A) treatment or co-expression of the dynamin dominant negative mutant DYN K44A on agonist-induced internalization of cell surface μOR detected by [3H]-DAMGO in intact HEK 293T cells co-expressing μ- and δ-ORs. Cells were co-treated with 275 μg/ml con A and 10 μM agonist for 1 hr at 37°C or were transfected with the DYN K44A construct 48 hours prior to agonist treatment. Data are expressed as a percentage of the receptor density in vehicle-treated control cells and as mean ± S.E.M. for n = 3 experiments performed in triplicate. Statistical significance was determined using an unpaired Student’s t-test [** p < 0.01 relative to cells not co-treated with con A or not expressing DYN K44A].
Figure 3.12 Effect of chronic morphine exposure on agonist-induced internalization of cell surface μOR detected by \[^{3}H\]-DAMGO in intact HEK 293T cells co-expressing μ- and δ-ORs (A) or singly expressing μOR (B). Cells were pre-treated with 10 μM morphine sulfate (MS) for 48 hrs, washed, then incubated with medium with or without 10 μM agonist for 1 hr at 37°C. Data shown represent drug-induced loss of cell surface receptors as a percentage of cell surface receptors in vehicle-treated control cells, and are expressed as mean ± S.E.M. for n = 3-4 experiments performed in triplicate. Statistical significance was determined using an unpaired Student’s t-test [*** p < 0.001 relative to cells not pre-treated with morphine].
4. STUDY II RESULTS AND DISCUSSION

The presence of $\mu$-$\delta$ OR heteromer-specific signalling in the brain

Findings from Study II are outlined in the following manuscript in preparation:

Kabli, N., Fan, T., O’Dowd, B.F., and S.R. George. $\mu$-$\delta$ opioid receptor heteromer-specific signalling is present in the striatum and hippocampus.
4.1 Agonist-induced Gαző activation in rodent striatum and hippocampus under basal conditions.

In order to interrogate the presence of the µ-δ OR heteromer in the striatum and hippocampus, we used the \[^{35}\text{S}]\text{-GTPγS}\) incorporation assay followed by selective immunoprecipitation using specific antibodies to assess agonist-induced activation of Gαző. Deltorphin II significantly enhanced \[^{35}\text{S}]\text{-GTPγS}\) incorporation into Gα зло over unstimulated levels (which were designated as 0% activation) in the striatum and hippocampus (Figure 4.1A and 4.1B; hatched bars). Neither DAMGO (µ-agonist) nor DPDPE (δ-agonist) induced significant \[^{35}\text{S}]\text{-GTPγS}\) incorporation into Gα зло over unstimulated levels in these regions (Figure 4.1A and 4.1B; hatched bars). In separate experiments, brains were sectioned to obtain tissue from the nucleus accumbens and periaqueductal gray. However, the protein yield from these regions was extremely low. Conducting the \[^{35}\text{S}]\text{-GTPγS}\) assay would have necessitated the pooling of tissue from several animals in order to perform a sufficient number of samples and replicates, which precluded us from analyzing these regions.

Deltorphin II-induced activation of Gα зло only occurs in cells co-expressing µ- and δ-ORs and is used as an index of the presence of functional µ-δ heteromers (Fan et al., 2005). Here, deltorphin II activated Gα зло in brain regions that strongly co-express µOR, δOR and Gα зло (Delfs et al., 1994; George et al., 1994; Mansour et al., 1994; Wittert et al., 1996; Friberg et al., 1998; Kelleher et al., 1998; Wang and Pickel, 2001; Stumm et al., 2004; Gray et al., 2006), suggesting that the µ-δOR heteromer is expressed in the striatum and hippocampus. In contrast, the µ-agonist DAMGO and δ-agonist DPDPE did not activate Gα зло. Our data is in accordance with a study by Garzon and colleagues who demonstrated that
administration of antisense oligodeoxynucleotides targeting $\mathrm{G}\alpha_{\delta/z}$ into the periaqueductal gray attenuated the analgesic effects of deltorphin II, but not those of DPDPE (Garzon et al., 1997). We observed slight but non-significant activation of $\mathrm{G}\alpha_z$ by DAMGO, which suggests that DAMGO may also activate the $\mathrm{G}\alpha_z$-coupled $\mu$-$\delta$ heteromer, although not as efficaciously as deltorphin II. In Garzon’s study, DAMGO’s analgesic effects were also attenuated by reducing the expression of $\mathrm{G}\alpha_{\delta/z}$ in the periaqueductal gray, however, a more potent inhibition of DAMGO’s effects was obtained by inactivation of PTX-sensitive $\mathrm{G}\alpha$ proteins (Garzon et al., 1997).

4.2 The effect of repeated morphine treatment on agonist-induced $\mathrm{G}\alpha_z$ activation in rodent striatum and hippocampus.

To determine the effect of repeated morphine treatment on opioid agonist-induced $\mathrm{G}\alpha_z$ activation, animals were treated with ascending doses of morphine sulfate s.c. for 48 hours (every 12 hours) and their brain tissue harvested 8-12 hours following the last injection for use in the $[^{35}\mathrm{S}]$-GTP$\gamma$S assay. Deltorphin II significantly enhanced $[^{35}\mathrm{S}]$-GTP$\gamma$S incorporation into $\mathrm{G}\alpha_z$ over unstimulated levels in the striatum and hippocampus under conditions of repeated morphine treatment (Figure 4.1A and 4.1B; filled bars). Neither DAMGO nor DPDPE significantly activated $\mathrm{G}\alpha_z$ in these regions (Figure 4.1A and 4.1B; filled bars). Thus, the $\mu$-$\delta$OR heteromer did not desensitize following repeated morphine treatment.
4.3 Agonist-induced $\alpha_i$ activation in rodent striatum and hippocampus under basal conditions.

In order to confirm the presence of $\mu$- and $\delta$-OR homomers in the striatum and hippocampus, we assessed whether the same $\mu$- and $\delta$-agonists activated $\alpha_{i3}$ in these regions. In cell lines expressing $\mu$- or $\delta$-ORs alone, selective agonists for each receptor activate the PTX-sensitive $\alpha_{i3}$ G-protein (Fan et al., 2005). All opioid agonists tested significantly enhanced $[^{35}\text{S}]-\text{GTP}_\gamma\text{S}$ incorporation into $\alpha_{i3}$ over unstimulated levels in the striatum and hippocampus and induced comparable activation of this G-protein (Figure 4.2A and 4.2B; hatched bars). As G protein activation is a proximal step in the GPCR regulatory pathway and serves as a functional measure of receptor activity, these findings also confirm the presence of functional $\mu$- and $\delta$-OR homomers in these brain regions.

4.4 The effect of repeated morphine treatment on agonist-induced $\alpha_i$ activation in rodent striatum and hippocampus.

To determine the effect of repeated morphine treatment on opioid agonist-induced $\alpha_{i3}$ activation, animals were subjected to the 48-hour repeated morphine treatment regimen and their brain tissue harvested for analysis in the $[^{35}\text{S}]-\text{GTP}_\gamma\text{S}$ assay. $\alpha_{i3}$ activation over basal unstimulated levels by DAMGO, deltorphin II, or DPDPE was reduced significantly in the striatum and hippocampus of animals receiving repeated morphine injections compared with saline-treated controls (Figure 4.2A and 4.2B; filled bars). Thus, repeated morphine treatment led to the desensitization of $\mu$- and $\delta$-OR homomer-induced $\alpha_{i3}$ activation.
4.5 **Opioid agonist-induced activation of all Gα-protein subtypes in rodent brain and spinal cord.**

In order to verify that all opioid agonists activated G-proteins in rodent brain, total G protein activation was assessed using the $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ incorporation assay without immunoprecipitation of specific Gα proteins. All of DAMGO, deltorphin II and DPDPE induced comparable and significant $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ incorporation into Gα proteins over unstimulated levels in the striatum (Figure 4.3A hatched bars), hippocampus (Figure 4.3B, hatched bars), and spinal cord (Figure 4.3C, hatched bars). These data confirm the presence of functional ORs in regions assayed and verify that the lack of Gα$_z$ activation by DAMGO and DPDPE was due to the lack of coupling to this particular subtype of G protein, and not due to an inability to activate Gα proteins in this assay.

4.6 **The effect of repeated morphine treatment on opioid agonist-induced activation of all G-protein subtypes in rodent brain and spinal cord.**

To determine the effect of repeated morphine treatment on overall opioid agonist-induced activation of G-proteins, animals were subjected to the 48-hour repeated morphine treatment regimen and their brain and spinal cord tissue harvested for use in the $[^{35}\text{S}]-\text{GTP}_{\gamma}\text{S}$ activation assay. Repeated morphine treatment significantly reduced G-protein activation by DAMGO, deltorphin II, or DPDPE in all neural tissues tested (Figure 4.1.3A - D; filled bars). Thus, repeated morphine treatment leads to the desensitization of OR G-protein activation which may be due to the uncoupling of these receptors from select populations of G-proteins.
4.7 Differential effect of prolonged morphine exposure on agonist-induced opioid receptor homomer and heteromer G-protein activation.

Prolonged morphine treatment leads cellular adaptations which act to dampen OR signalling. Reduction in G-protein coupling is one of the desensitization mechanisms which limit the clinical utility of the current clinically-used μ-agonists (Tso and Wong 2003; Christie 2008; Dang and Christie 2012). Until now, the effect of repeated morphine treatment on agonist-induced activation of specific Ga proteins had not been explored in vivo. In this study, coupling of μ- and δ-agonists to Ga proteins - and specifically the PTX-sensitive Ga$_{13}$ - was reduced suggestive of μOR and δOR homomers uncoupling from G-proteins following chronic morphine treatment. The observed reductions are in accordance with previous studies demonstrating marked attenuation in opioid receptor G-protein coupling following various chronic morphine regimens in cell lines and in brain regions such as the locus coeruleus (Klee and Streaty 1974; Brady et al., 1989). Receptor density is unlikely to account for this effect as reductions in μOR number are not consistently observed following chronic morphine treatment (Tao et al., 1993; Selley et al., 1997; Sim-Selley et al., 2007). Rather, this reduction in G-protein activation is one of the mechanisms of drug tolerance and is thought to reflect the uncoupling from G-proteins (Kabli et al., 2010).

In contrast, deltorphin II-induced activation of the PTX-resistant Ga$_{z}$ protein was unaffected in striata and hippocampi from animals pre-treated repeatedly with morphine, suggesting that μ-δ heteromer signalling did not desensitize. Prolonged morphine exposure has been shown to induce δOR targeting to the plasma membrane in a μOR-dependent manner (Cahill et al., 2001b; Morinville et al., 2004). Thus, it is likely that a proportion of
the δORs undergoing plasmalemmal targeting may be complexed with μORs in a μ-δOR heteromer. Recent studies using a newly-synthesized μ-δ heteromer antibody revealed that chronic morphine treatment resulted in an upregulation of the μ-δ receptor complex in several brain regions including the striatum, nucleus accumbens and hippocampus (Gupta et al., 2010). These observations fall in line with our findings from functional GTPγS binding assays. This unique regulation of μ-δ heteromer signalling by morphine exposure has interesting therapeutic implications since the μ-δ heteromer remains functional under prolonged morphine exposure conditions which desensitize μOR function contributing to the reduced efficacy of μ-opioid analgesics and their limited clinical utility.

4.8 Verification of the behavioural effects of morphine in the tail immersion nociceptive assay.

To confirm the efficacy of morphine doses used in the repeated morphine treatment regimen, rats underwent behavioural testing in the tail immersion thermal nociception assay. Rats were tested following the 5 mg/kg, s.c. and 10 mg/kg, s.c. doses. Morphine injected induced sustained analgesia peaking from 30-45 min (5 mg/kg, s.c.), and from 30-150 min (10 mg/kg, s.c.) following drug administration (Figure 4.4). No analgesia was observed in saline-treated control animals (Figure 4.4). Since morphine produced analgesia, these data confirm that any lack of desensitization in G-protein activation following repeated morphine treatment is not due to morphine not reaching the nervous system.
4.9 Conclusion

In conclusion, this study demonstrates that $\mu$-$\delta$ heteromer-specific signalling is present in the striatum and the hippocampus and that this signalling did not desensitize following conditions of repeated morphine treatment which desensitized $\mu$OR and $\delta$OR homomers. The $\mu$-$\delta$ heteromer is regulated differently following morphine treatment and represents a therapeutic target for treating neurologic and neuropsychiatric disorders.
Figure 4.1  Quantification of agonist-induced [35S]-GTPγS incorporation into immunoprecipitated Gaζ in the striatum and hippocampus of rats treated with saline or repeated morphine injections. Rats were injected subcutaneously (s.c.) with saline or increasing doses of morphine sulfate (MS; 5, 8, 10, and 15 mg/kg, s.c. every 12 hr) and sacrificed 8 – 12 hours following the last injection. Membrane preparations from striatum (A) or hippocampus (B) were treated with 10 μM DAMGO, deltorphin II, or DPDPE. Data are presented as % activation over basal (designated as 0%) and are shown as means ± S.E.M. for n = 3-4 separate experiments.

DAMGO-, DPDPE- and deltorphin II-induced Gaζ activation relative to basal in each group was assessed for statistical significance using one-way ANOVA followed by Dunnett’s post hoc analysis. [*** p < 0.001, ** p < 0.01 relative to unstimulated basal]. Differences in % Gaζ activation induced by the same drug in striata and hippocampi from saline-treated control versus morphine-treated animals were assessed for statistical significance using the unpaired Student’s t-test. [p > 0.05 relative to control saline-injected animals].
Figure 4.2  Quantification of agonist-induced [$^{35}$S]-GTPγS incorporation into immunoprecipitated Gαi3 in the striatum and hippocampus of rats treated with saline or repeated morphine injections. Rats were injected subcutaneously (s.c.) with saline or increasing doses of morphine sulfate (MS; 5, 8, 10, and 15 mg/kg, s.c. every 12 hr) and sacrificed 8–12 hours following the last injection. Membrane preparations from striatum (A) or hippocampus (B) were treated with 10 μM DAMGO, deltorphin II, or DPDPE. Data are presented as % activation over basal (designated as 0%) and are shown as means ± S.E.M. for n = 3 separate experiments. Differences in % Gαi3 activation induced by the same drug in striata and hippocampi from saline-treated control versus morphine-treated animals were assessed for statistical significance using the unpaired Student’s t-test. [* p < 0.05 relative to control saline-injected animals].
Figure 4.3 Quantification of agonist-induced \( ^{35}S \)-GTP\( \gamma \)S incorporation into all G\( \alpha \) G-protein subtypes in the striatum, hippocampus, or spinal cord of rats treated with saline or repeated morphine injections. Rats were injected subcutaneously (s.c.) with saline or increasing doses of morphine sulfate (MS; 5, 8, 10, and 15 mg/kg, s.c. every 12 hr) and sacrificed 8 – 12 hours following the last injection. Membrane preparations from striatum (A), hippocampus (B), or spinal cord (C) were treated with 10 \( \mu \)M DAMGO, deltorphin II, or DPDPE. Data are presented as % activation over basal (designated as 0%) and are shown as means ± S.E.M. for n = 3-6 separate experiments. Differences in % G\( \alpha \) activation induced by the same drug in striata and hippocampi from saline-treated control versus morphine-treated animals were assessed for statistical significance using the unpaired Student’s t-test. [* p < 0.05; ** p < 0.01; *** p < 0.001 relative to control saline-injected animals].
Figure 4.4 The analgesic effects of morphine in the tail immersion nociceptive assay. Rats were injected subcutaneously (s.c.) with saline or morphine sulfate (MS 5 mg/kg or 10 mg/kg). The latency to tail withdrawal from warm water (52°C) was measured at baseline (BL) and following saline or MS injection. The arrow denotes the time of the morphine or saline injection. A cut-off of 4 x BL latency was imposed to minimize tissue damage. % Maximum Possible Effect was calculated according to the equation: % Maximum Possible Effect = (Latency - BL latency) / (Cut-off Latency – BL latency) x 100.
5. STUDY III RESULTS AND DISCUSSION

Functional consequences of disrupting
the μ-δ OR heteromeric interaction in a cell line
and in animal systems

Findings from Study III are outlined in the following manuscript in preparation:
5.1 Effect of the interfering δOR carboxyl tail peptide on the δ-agonist-detected ligand binding pocket in the μ-δ OR heteromer and the μOR homomer.

To examine whether a minigene expressing the δOR distal carboxyl tail peptide interfered with the μ-δ heteromeric interaction and dissociated the μ-δ heteromer, δ-agonist displacement of μ-agonist binding from the μ-δ heteromer was assessed. We previously demonstrated that δ-agonists detect a novel high affinity μOR ligand binding site only when the μOR is complexed with δOR (George et al., 2000). Competition radioligand binding was conducted using [3H]-DAMGO in cells stably co-expressing μ- and δ-ORs in the presence or absence of a short (16 a.a.) or longer (22 a.a.) peptide derived from the distal carboxyl tail of the δOR. The δ-agonists UFP-512 and SNC80 displaced high affinity [3H]-DAMGO binding in cells co-expressing μ- and δ-ORs but not in those expressing μOR alone (Figure 5.1A and 5.1C), which is suggestive of δ-agonist occupancy or allosteric modulation of the μOR ligand binding pocket. The latter two scenarios could only occur if μ- and δ-OR were physically associated in a receptor complex. Co-expression of the distal 16 a.a. carboxyl tail peptide of the δOR (interfering peptide) abolished UFP-512- and SNC80-induced displacement of high affinity μ-agonist binding within the μ-δ heteromer, and resulted in a right-ward shift to a monophasic low affinity competition curve identical to the agonist binding to the μOR expressed alone (Figure 5.1B and 5.1D). The distal 22 a.a. carboxyl tail peptide of the δOR had no effect on high affinity δ-agonist modulation of the μOR binding site within the μ-δ heteromer, and will be designated as the control peptide in the remaining in vitro studies (Figure 5.1E and 5.1F). It is not clear why the 22 a.a. peptide had no effect; this longer peptide may adopt a conformation that is not efficient at disrupting the μ-δ heteromer.
The interfering peptide had no effect on UFP-512 (Figure 5.2A) or DAMGO (Figure 5.2C) displacement of μ-agonist binding to the μOR homomer or UFP-512 binding to the δ receptor pocket within the δOR homomer (Figure 5.2B). The highly selective μ-agonist DAMGO has approximately 1600-fold higher affinity for μOR than δOR (Kabli et al., 2010) and at the 2 nM concentration used, DAMGO would not detect δOR (Kabli et al., 2010). The interfering peptide-induced selective loss of δ-agonist modulation of μ-agonist binding to the μ-δ heteromer suggests dissociation of the μ-δ receptor complex since this competitive or allosteric ligand binding interaction could only occur when μ- and δ-ORS are physically associated.

5.2 Effect of the interfering δOR carboxyl tail peptide on the co-immunoprecipitation of μ- and δ-ORS.

Dot blotting analysis confirmed the expression of the interfering and control peptides encoded by the minigenes in transfected cells (Fig. 5.3A). In cells co-expressing μ- and δ-ORS, immunoprecipitation of μOR using a cMyc antibody and subsequent blotting using a FLAG antibody targeting the δOR revealed an immunoreactive band at ~ 65 – 70 kDA consistent with immunoprecipitation of the δOR and co-immunoprecipitation of μ- and δ-ORS (Fig. 5.3B, lane 1). Expression of the interfering δOR carboxyl tail peptide resulted in a loss of the immunoreactive band at ~ 65 – 70 kDa and prevented co-immunoprecipitation of the δOR with the μOR, suggesting a dissociation of μ- and δ-ORS in the μ-δ heteromer (Fig. 5.3B, lane 2). The control peptide had no effect on μ- and δ-OR co-immunoprecipitation (Fig. 5.3B, lane 3). Immunoblots depict the expression of μOR in lysates from cells expressing μ- and δ-ORS alone (Fig. 5.3B, lane 4) or in the presence of expressed interfering
(Fig. 5.3B, lane 5) or control peptide (Fig. 5.3B, lane 6). The specificity of the cMyc antibody is demonstrated by absence of an immunoreactive signal in membranes from cells expressing FLAG-tagged δOR (Fig. 5.3B, lane 7). The expression of δOR is demonstrated in immunoblots of lysates from cells expressing μ- and δ-OR alone (Fig. 5.3B, lane 8) or in the presence of expressed interfering (Fig. 5.3B, lane 9) or control peptide (Fig. 5.3B, lane 10). The specificity of the FLAG antibody is demonstrated by absence of an immunoreactive signal in membranes from cells expressing cMyc-tagged μOR (Fig. 5.3B, lane 11).

These experiments confirm that the interfering peptide resulted in a disruption of the μ-δ heteromer.

5.3 Effect of the interfering δOR carboxyl tail peptide on δ-agonist-induced internalization of the μ-δ OR heteromer.

To determine the effect of dissociating the μ-δ heteromer on δ-agonist-induced internalization of the μ-δ heteromer, receptor endocytosis in intact cells stably expressing μ- and δ-ORs in the presence and absence of the interfering δOR carboxyl tail peptide was assessed. The reduction of cell surface [3H]-DAMGO labelling following δ-agonist treatment served as an index of μ-δ heteromer internalization (Lucki 1997; Cryan et al., 2002; Nestler et al., 2002b). The δ-agonists UFP-512 and SNC80 (10 μM for 1 hr) induced internalization of μOR by 49% and 30%, respectively, in cells co-expressing μ- and δ-ORs (Figure 5.4, left panel). Co-expression of the interfering peptide completely abolished δ-agonist-induced endocytosis of μOR in these cells (Figure 5.4, left panel). Neither UFP-512 nor SNC80 induced internalization of μOR expressed alone (Figure 5.4, right panel). Vehicle-treated cells, where no receptor internalization occurred, served as controls. The interfering δOR carboxyl tail peptide disrupted and dissociated the μ-δ heteromer since it
abolished δ-agonist-induced internalization of μOR - an effect seen only when μ- and δ-ORs are complexed in a heteromer.

In vitro, the peptide minigene disruption strategy was successful in dissociating the μ-δ heteromer and selectively abolishing the previously reported actions of δ-agonists the μ-δ heteromer – namely, δ-agonist modulation of high affinity μ agonist binding to the μ-δ heteromer and δ-agonist-induced internalization of the μ-δ heteromer and co-trafficking of μ- and δ-ORs. The interfering peptide had no effect on isolated μ- or δ-OR homomer pharmacology. These results suggest that targeting the points of contact between μ- and δ-ORs results in the successful dissociation of the μ-δ heteromer and can be employed as a key tool for dissecting the physiological role of this receptor complex in vivo.

5.4 Effect of intra-accumbens micro-injection of the TAT-conjugated δOR carboxyl tail interfering peptide on δ-agonist-induced antidepressant-like action in the forced swim test.

Since all available drugs targeting homomeric ORs may also have effects at the μ-δ heteromer we sought to devise a strategy to selectively analyze the effects of the μ-δ receptor complex in vivo. To this end, we used a TAT peptide fusion construct expressing the distal carboxyl tail peptide of the δOR to disrupt the μ-δ interaction and isolate the contribution of the μ-δ heteromer in mediating antidepressant-like and anxiolytic-like activity in animal paradigms modelling features of depression and anxiety. In order to determine the contribution of μ-δ heteromers in the nucleus accumbens (NAc) to the antidepressant-like action of the δ-agonist UFP-512, rats were micro-injected with interfering peptide or a
scrambled control peptide prior to UFP-512 and examined in the forced swim test (FST). The FST behavioural paradigm models learned helplessness behaviour with immobility signifying a state of despair (Lucki 1997; Cryan et al., 2002; Nestler et al., 2002b). Pharmacological interventions which decrease an animal’s immobility in this paradigm modify depressive-like behaviour in humans, giving the FST great reliability and predictive validity (Vergura et al., 2008). Further, the FST is also sensitive to both depressive-like and anti-depressive-like behaviour.

Cannula placement in the NAc is illustrated (Figure 5.5A). Micro-injection of UFP-512 (1.5 µg) bilaterally into the NAc resulted in a significant reduction in the duration of immobility in the FST at 30 min following drug administration, suggestive of antidepressant-like action (Figure 5.6A). Pre-treatment with TAT-conjugated interfering peptide (30 pmol) followed in 1 hour by UFP-512 reversed the UFP-512-induced reduction in immobility whereas the scrambled peptide control had no effect (Figure 5.6A). Neither interfering nor scrambled peptides affected basal responding in the FST when administered alone (Figure 5.6A). TAT peptide delivery in the NAc is represented by dansyl chloride fluorescence (Figure 5.5B). We elected to use the δ-agonist UFP-512 as it is devoid of convulsive activity (Vergura et al., 2008), has antidepressant-like and anxiolytic-like properties in animals (Kabli et al., 2010), is devoid of locomotor effects (Vergura et al., 2008), recruits the μOR for its actions, and activates the μ-δ heteromer with the highest affinity in vitro (Baamonde et al., 1992; Broom et al., 2002a; Broom et al., 2002b; Jutkiewicz 2006; Perrine et al., 2006). Our data is in accordance with extensive literature documenting the mood-elevating properties of systemically-administered δ-agonists in rodents in several animal models of
depression and anxiety (Dulawa and Hen 2005; Duman 2010). We extend on the literature by demonstrating that the \( \mu-\delta \) heteromer is a receptor target and the NAc a neural substrate for the actions of the \( \delta \)-agonist UFP-512.

The tricyclic antidepressant imipramine administered intraperitoneally (20 mg/kg, i.p.) served as a positive control showing a significant reduction in immobility (Figure 5.6B). UFP-512’s effects in the FST were comparable to those of imipramine. To determine whether \( \mu-\delta \) heteromers in the NAc play a role in imipramine’s effects and to assess the selectivity of the interfering peptide, rats were micro-injected with the interfering peptide bilaterally into the NAc prior to imipramine administration and testing. Dissociating \( \mu-\delta \) OR heteromers in the NAc had no effect on imipramine’s antidepressant effects in the FST (Figure 5.6B). Thus, the interfering \( \delta \)OR carboxyl tail peptide selectively antagonized the effects of agonists acting at the \( \mu-\delta \) heteromer and this receptor complex plays a role in the antidepressant-like effects of \( \delta \)-agonists in the NAc.

5.5 Role of \( \delta \)OR and \( \mu \)OR in \( \delta \)-agonist-induced antidepressant-like effects in the forced swim test.

To investigate the contribution of \( \delta \)OR and \( \mu \)OR in the UFP-512-mediated antidepressant-like action in the NAc, rats were micro-injected bilaterally into the NAc with the highly selective \( \delta \)-antagonist naltrindole or the \( \mu \)-antagonist CTOP prior to UFP-512 administration and behavioural testing in the FST. Both naltrindole (1 \( \mu \)g) and CTOP (1 \( \mu \)g) administered into the NAc reversed the decrease in immobility induced by UFP-512 (Figure 5.6C). Thus, both \( \mu \)- and \( \delta \)-ORs were required for this \( \delta \)-agonist’s antidepressant-like action.
in the NAc. These findings complement reports of µOR requirement for δ-agonist behavioural effects (Kieffer and Gaveriaux 2002; Scherrer et al., 2004; Gendron et al., 2007) and support the role of the µ-δ OR heteromer in mediating UFP-512’s behavioural effects in the FST.

5.6 Effect of intra-accumbens micro-injection of the TAT-conjugated δOR carboxyl tail interfering peptide on δ-agonist-induced antidepressant-like and anxiolytic-like action in the novelty-induced hypophagia test.

In order to determine the contribution of µ-δ heteromers in the NAc to the antidepressant-like and anxiolytic-like effects of UFP-512 in an additional model of anxiety and anhedonia, rats were micro-injected bilaterally into the NAc with interfering peptide prior to UFP-512 and examined in the novelty-induced hypophagia (NIH) test. The rat NIH paradigm was adopted as it models features of anxiety and anhedonia (Dulawa and Hen 2005; Duman 2010). The latter is defined as decreased responsiveness to pleasurable stimuli and is a predominant feature in depression (Dulawa and Hen 2005; Duman 2010). In NIH, the stress of the novel environment induces a state of anhedonia demonstrable as increased latency to consume a palatable milk solution, and this is reversed by a variety of antidepressants and anxiolytics (Pellow et al., 1985). Intra-accumbens injection of UFP-512 (1.5 µg) resulted in a significant reduction in the latency to drink milk in a novel environment 30 min following drug administration, suggestive of antidepressant-like action (Figure 5.7A). Pre-treatment with TAT-conjugated interfering peptide (30 pmol) followed in 1 hour by UFP-512 reversed the UFP-512-induced reduction in latency to drink milk whereas the scrambled peptide control had no effect (Figure 5.7A). Neither interfering nor
scrambled peptides affected basal responding in the NIH paradigm when administered alone (Figure 5.7A). Thus, the intact \( \mu-\delta \) heteromer reduced the latency to drink in a novel stressful environment in the NIH paradigm, suggesting a role for this receptor complex in modulating anxiety and anhedonia.

5.7 Role of \( \delta \text{OR} \) and \( \mu \text{OR} \) in \( \delta \)-agonist-induced antidepressant-like and anxiolytic-like action in the novelty-induced hypophagia test.

To investigate the contribution of \( \delta \text{OR} \) and \( \mu \text{OR} \) in the centrally-mediated antidepressant-like and anxiolytic-like actions of UFP-512, rats received intracerebroventricular (i.cv.) micro-injections of the highly selective \( \delta \)-antagonist naltrindole or the \( \mu \)-antagonist CTOP prior to UFP-512 administration and behavioural testing in the NIH paradigm. Both naltrindole (10 nmol) and CTOP (10 nmol) reversed the UFP-512-induced (80 nmol) decrease in latency to drink milk (Figure 5.7B). Neither antagonist produced an effect when administered alone (Figure 5.7B). Thus, both \( \mu \)- and \( \delta \)-ORs were required for this \( \delta \)-agonist’s centrally-mediated antidepressant-like and anxiolytic-like action in the NIH paradigm.

5.8 Effect of intra-accumbens micro-injection of the TAT-conjugated \( \delta \text{OR} \) carboxyl tail interfering peptide on \( \delta \)-agonist-induced anxiolytic-like action in the elevated plus maze.

In order to determine the contribution of accumbens \( \mu-\delta \) heteromers to the anxiolytic-like effects of UFP-512, rats were micro-injected with interfering peptide prior to UFP-512 and examined in the elevated plus maze (EPM) test. The EPM paradigm has great face,
construct and predictive validity for modeling fear and anxiety (Filliol et al., 2000; Ragnauth et al., 2001). Intra-accumbens injection of UFP-512 (1.5 µg) resulted in a significant increase in the time spent in the open arms of the EPM at 30 min following drug administration, suggestive of anxiolytic-like action. Pre-treatment with TAT-conjugated interfering peptide (30 pmol) followed 1 hour later by UFP-512 reversed the UFP-512-induced increase in time spent in open arms whereas the scrambled peptide control had no effect (Figure 5.8A). Neither interfering nor scrambled peptides affected basal responding in the EPM when administered alone (Figure 5.8A). The benzodiazepine diazepam (1 mg/kg, i.p.) served as a positive control (Figure 5.8B). Therefore, positive modulatory effects of accumbens µ-δ heteromer activation are not limited to the FST, but improve features of despair and anhedonia as well as anxiety.

5.9 Role of δOR and μOR in δ-agonist-induced anxiolytic-like action in the elevated plus maze.

To investigate the contribution of δOR and μOR in the centrally-mediated anxiolytic-like actions of UFP-512, rats received i.cv. micro-injections of the highly selective δ-antagonist naltrindole or the μ-antagonist CTOP prior to UFP-512 administration and behavioural testing in the EPM. Both naltrindole (10 nmol) and CTOP (10 nmol) reversed the UFP-512-induced (80 nmol) reduction in the time spent in open arms of the maze (Figure 5.8C). Neither naltrindole nor CTOP produced an effect when administered alone (Figure 5.8C). These findings demonstrate that both μ- and δ-ORs were required for this δ-agonist’s centrally-mediated anxiolytic-like action in the EPM test.
5.10 Role of the µ-δ heteromer in modulating basal depressive-like and anxiogenic behaviour

In this study, neither disruption of the µ-δ heteromer nor blockade of µ- or δ-receptors in the NAc affected basal responding in any of the behavioural paradigms used, suggesting that the µ-δ heteromer in NAc may not modulate basal emotional affect. Congruent with our findings, δOR inactivation by antagonist in the amygdala has no effect on basal anxiety levels (Alexander et al., 2010). However, our findings do not preclude the possibility that a selective knockdown of µ-δ heteromers in a discrete cluster of functionally-linked brain regions or neuronal ensembles could affect basal mood.

5.11 Involvement of the opioid system in mood regulation

The effect of activating a heteromeric complex composed of both δ- and µ-ORs on depressive and anxiolytic-like behaviour was not known. Although the contribution of the δOR system to mood regulation has been cemented (Filliol et al., 2000; Ide et al., 2010; Berrocoso et al., 2012), the role of µOR has been somewhat controversial owing to dissimilar findings in µOR gene-deleted animals (Extein et al., 1981; Emrich et al., 1982; Bodkin et al., 1995; Nyhuis et al., 2008; Berrocoso et al., 2009). Evidence clearly demonstrates that the µOR has positive effects on mood, with µOR-acting agonists such as buprenorphine, morphine and methadone being used in the clinic for the successful treatment of patients with major refractory depression (Zarrindast et al., 2008). In animals, micro-injection of µ-agonists into the NAc reduces anxiety in the elevated plus maze (Darko et al., 1992; Zubieta et al., 2003; Kennedy et al., 2006). Furthermore, human studies demonstrate a dysregulation of the endogenous β-endorphin system and the µOR in depressed patients (Mansour et al.,
In our study, we demonstrated that both µ- and δ-ORs are required and involved in UFP-512’s NAc- and centrally-mediated antidepressant-like and anxiolytic-like effects. Disrupting the µ-δ heteromer in the NAc had no effect on the systemic antidepressant effects of imipramine, a modulator of monoamine neurotransmitter reuptake, demonstrating the specificity of the interfering TAT fusion peptide used for µ-δ heteromer involvement only.

5.12 Involvement of nucleus accumbens in mood regulation

Our data solidify the case for opioidergic mechanisms in the NAc having a role in modulating depressive and anxiety-related symptoms. We focused our line of investigation on the function of µ-δ heteromers in the NAc for several reasons. µ- and δ-ORs co-localize extensively in the NAc and are present in the same cells (Gupta et al., 2010), and the expression of µ-δ heteromers in this locus has been recently confirmed (Hirose et al., 2005; Hipolito et al., 2008). This area where opioids act directly in the NAc core sub-region to induce dopamine release and modulate the excitability of the NAc (Nestler et al., 2002a; Epstein et al., 2006; Zarrindast et al., 2008; Alexander et al., 2010; Kitamura et al., 2010; Morales-Mulia et al., 2012), has been implicated as a major player in modulating depressive-like behaviour and anxiety in humans and animals (Kitamura et al., 2010). Intra-accumbens administration of bupropion produces potent antidepressant actions in the FST (Sturm et al., 2003; Bewernick et al., 2011; Grubert et al., 2011) while intra-accumbens administration of µ-opioid agonists elicits anxiolytic-like actions in the EPM in animals (Zarrindast et al., 2008). To this end, NAc has been targeted in deep brain stimulation with promise in reversing depressive symptoms such as anhedonia in patients with major depression and
anxiety and is gaining attention and interest for its role in mood modulation (Hirose et al., 2005). The mechanism by which NAc μ-δ heteromers participate in mood regulation can be surmised by the positive regulatory effects of opioid agonists on dopamine release in this region. Dopamine release in the NAc occurs within minutes following infusion of δ-agonists into the same region (Willner 1997). Indeed, dopamine release is thought to account for the more rapid onset of antidepressant effects in faster-acting antidepressant agents (Hirose et al., 2005) and likely plays a role in the observed antidepressant-like effects of μ-δ heteromer activation in the NAc. As we and others observed, the antidepressant-like actions of UFP-512 occur rapidly following administration, supporting dopamine release as a likely mechanism of action. Further, at doses similar to those used in our study, δ-agonist-induced dopamine release was reversed by intra-accumbens administration of μ- or δ-antagonists (Schmidt et al., 2002a; Schmidt et al., 2002b; 2003; Gear and Levine 2011). The latter study confirms the requirement of both μ- and δ-ORs in the actions of δ-agonists in the NAc and suggests the involvement of the μ-δ receptor complex in the dopamine modulatory effects. Overall, these studies provide a likely explanation for the faster onset of antidepressant effects following μ-δ heteromer activation compared to classical antidepressants and substantiate our behavioural data that both receptors of the μ-δ heteromer are recruited to mediate mood-regulatory actions of δ-agonists in the NAc.

5.13 Effect of intra-accumbens micro-injections of μ- and δ-opioid agonists on analgesia in the tail immersion nociceptive assay.

A limited number of studies have reported on opioidergic NAc-mediated analgesia (Schmidt et al., 2002a; Schmidt et al., 2002b; 2003; Gear and Levine 2011). Thus, we
sought to determine whether the \(\mu-\delta\) heteromer in the NAc would play a role in analgesia. To assess possible analgesic effects of opioid agonists in our behavioural paradigm, rats were micro-injected with saline, UFP-512 (1.5 \(\mu\)g), UFP-512 (1.5 \(\mu\)g) and DAMGO (1.5 \(\mu\)g), or morphine (10 \(\mu\)g) bilaterally into the NAc and their nociceptive thresholds measured in the tail immersion nociceptive assay. Neither morphine, nor UFP-512 alone or in combination with DAMGO altered nociceptive thresholds at any time point tested (Figure 5.9). Thus, at the doses used, UFP512 selectively produced anti-depressant-like and anxiolytic-like effects – but not analgesia – through the NAc. These findings eliminate a role for the \(\mu-\delta\) heteromer in NAc-mediated analgesia in the tail immersion nociceptive assay.

5.14 Effect of intra-accumbens micro-injection of TAT-conjugated peptides and UFP-512 on locomotor activity.

In order to determine whether locomotor effects played a role in the behavioural effects of TAT-conjugated peptides and UFP-512 in the FST, NIH and EPM, rats were micro-injected with saline, TAT peptides, or UFP-512 bilaterally into the NAc and their locomotor activity quantified using activity monitoring chambers. The total distance traveled by animals treated with saline, interfering or scrambled peptides and UFP-512 was not significantly different (Figure 5.10A), and neither were their patterns of locomotor activity (Figure 5.10B). Thus the behavioural effects of the TAT peptides and the reduction in immobility, increased time spent in open arms, and reduced latency to drink milk induced by UFP-512 in the FST, EPM, and NIH paradigms, respectively, were not due to non-specific drug effects on locomotion.
5.15 Conclusions

In conclusion, our data demonstrate that µ-δ OR heteromers in the NAc are functional and play a critical role in regulating mood and affect. Activation of the µ-δ heteromer in the NAc of rodents by a δ-agonist produced potent antidepressant-like and anxiolytic-like effects similar in magnitude to the effects of clinically-relevant antidepressants and anxiolytics. Interfering minigene and TAT fusion peptide constructs derived from the distal δOR carboxyl tail sequence implicated in mediating µ-δ OR heteromerization abolished the unique pharmacological and trafficking properties of δ-agonists at the heteromer in vitro and their behavioural effects in vivo, indicating dissociation of the µ-δ heteromer. Thus, the peptide disruption strategy allowed us to confirm the involvement of the µ-δ heteromer in novel δ-agonist pharmacology and activity in cells and neural tissue co-expressing µ- and δ-ORs. Depression and anxiety disorders affect millions of individuals worldwide. Available treatment strategies focusing on serotonergic and monoaminergic neurotransmitter mechanisms only work for a proportion of patients and only begin to exert their effects weeks following their administration. Thus, other therapeutic measures are needed and ought to be targeted. We propose that the µ-δ heteromer warrants further exploration as a therapeutic target for treatment-resistant major depression.
Figure 5.1  Effect of the interfering δOR distal carboxyl tail peptide on δ-agonist binding to the μ- receptor ligand binding pocket in the μ-δ OR heteromer. δ-agonist detection of the μ ligand binding pocket in cells co-expressing μ- and δ-ORs in the absence and presence of the interfering inhibitory (PEP) or control peptide (CTRL) minigene, or in cells expressing μOR alone. Competition of [3H]-DAMGO (μ-agonist) binding to membranes from HEK 293T cells by δ-agonists UFP-512 (A,B,E) or SNC80 (C,D,F). Grey and black arrowheads point to the high and low ligand binding affinities where applicable. An F-test was used to determine whether a two-site or a one-site binding curve provided a statistically significant better fit. Cells expressed 150 – 350 fmol/mg of total μ- and δ-OR protein, whereby each receptor was expressed at a density of 150 – 175 fmol/mg. Results shown are mean ± S.E.M. and curves are representative of n = 3-6 experiments performed in duplicate.
**Figure 5.2** Effect of the interfering δOR distal carboxyl tail peptide on μ- and δ-agonist binding to the μ-receptor in the μOR homomer or on δ-agonist binding to the δ-receptor ligand pocket within the δOR homomer. Agonist detection of the μ or δ ligand binding pocket in μOR- or δOR-expressing cells, respectively, in the absence or presence of the interfering inhibitory peptide minigene. Competition of [3H]-DAMGO μOR binding to membranes from HEK 293T cells by (A) UFP-512 (δ-agonist) or (C) DAMGO (μ-agonist) or [3H]-diprenorphine δOR binding to membranes from HEK 293T cells by (B) UFP-512. Grey and black arrowheads point to the high and low ligand binding affinities where applicable. An F-test was used to determine whether a two-site or a one-site binding curve provided a statistically significant better fit. Opioid receptor density was approximately 150 – 175 fmol/mg. Results shown are mean ± S.E.M. and curves are representative of n = 3 experiments performed in duplicate.
Figure 5.3 Expression of the hemaglutinin-tagged interfering δOR distal carboxyl tail peptide in HEK 293T cells co-expressing μ- and δ-ORs and its effect on co-immunoprecipitation of cMyc-tagged μORs and FLAG-tagged δORs. A. Cell lysate preparations from cells co-expressing μ- and δ-ORs in the absence or presence of the HA-tagged δOR distal carboxyl tail interfering (PEP) or control minigene (CTRL PEP) were dotted onto nitrocellulose membranes and blotted using HA antiserum diluted 1:1000. B. Immunoprecipitation of μOR using a cMyc antibody and subsequent blotting using a FLAG antibody targeting the δOR in lysates from cells expressing μ- and δ-ORs alone (lane 1) or in the presence of interfering (lane 2) or control peptide (lane 3). In cells co-expressing μ- and δ-ORs, an immunoreactive band was revealed at approximately 65 - 70 kDa which is consistent immunoprecipitation of the δOR (lane 1); this band was abolished in cells expressing the interfering peptide (lane 2) but remained intact in cells expressing the control peptide (lane 3). Expression of μOR in immunoblots of lysates from cells expressing μ- and δ-ORs alone (lane 4) or in the presence of interfering (lane 5) or control peptide (lane 6), or in cells expressing δOR alone (lane 7). Expression of δOR in immunoblots of lysates from cells expressing μ- and δ-OR alone (lane 8) or in the presence of expressed interfering (lane 9) or control peptide (lane 10), or in cells expressing μOR alone (lane 11). Molecular weights were estimated by comparing immunoreactive band migration with calibrated protein ladder. Immunoreactivity in A and B was revealed following exposure to secondary antibodies and film.
Figure 5.4  Effect of the interfering δOR distal carboxyl tail peptide on δ-agonist-induced internalization of the μOR within the μ-δ OR heteromer. δ-agonist-induced internalization of cell surface μOR in cells co-expressing μ- and δ-ORs in the absence and presence of the interfering peptide minigene (PEP), or in cells expressing μOR alone. Agonist-induced reduction in [3H]-DAMGO labelling in intact HEK 293T served as an index of μOR internalization. Cells were treated with 10 μM agonist for 1 hr at 37°C. Data shown represent drug-induced loss of cell surface receptors as a percentage of cell surface receptors in vehicle-treated control cells, and are expressed as mean ± S.E.M. for n = 3-4 experiments performed in triplicate. The unpaired Student’s t-test was used to assess statistical significance [** p < 0.01, *** p < 0.001 relative to cells not treated with the interfering peptide].
Figure 5.5  Cannula placement in the rat nucleus accumbens core.  A. Schematic representation of micro-injection sites into the nucleus accumbens (NAc) core (filled circles). Each dot represents a cannula placement in the NAc; one dot is used despite overlap in the location of the cannulae.  B. Photomicrograph of dansyl chloride fluorescence in the NAc demonstrating the presence of TAT-conjugated interfering peptide in cells of the NAc. Scale bar = 20 µm. Co-ordinates are relative to Bregma according to the Atlas of Paxinos and Watson (1998).
Figure 5.6 Effect of dissociating μ- and δ-ORs or antagonizing μ- or δ-ORs on the antidepressant-like effects of the δ-agonist UFP-512 in the forced swim test. A. Rats were micro-injected bilaterally into the NAc with either saline or UFP-512, TAT-conjugated interfering (PEP) or scrambled (PEP SCR) δOR distal carboxyl tail peptide followed 60 minutes later by UFP-512, or TAT-conjugated interfering peptide (PEP) or scrambled peptide (PEP SCR) followed 60 minutes later by saline. B. Rats were injected intraperitoneally (i.p.) with saline, imipramine (10 mg/kg, i.p.) or imipramine (20 mg/kg, i.p.). A separate group of animals was micro-injected bilaterally into the NAc with TAT-conjugated interfering peptide (PEP) followed 60 minutes later by imipramine (20 mg/kg, i.p.). C. Rats were micro-injected bilaterally into the NAc with either the δOR antagonist naltrindole or the μOR antagonist CTOP followed 30 minutes later by saline or UFP-512. Depressive-like behaviour was assessed using the forced swim test 30 minutes following the last injection. Rat behaviour was scored at 5 sec intervals for 5 minutes, whereby the predominant behaviour for each interval was recorded according to the Lucki (1994) method. Immobility represented no activity except that which is required to keep the rat’s head above water. Statistical significance was determined using one-way ANOVA followed by Dunnett post-hoc analysis [*** p<0.001 relative to saline-treated rats; n = 6 – 12 animals per group].
Figure 5.7 Effect of dissociating μ- and δ-ORs or antagonizing μ- or δ-ORs on the anxiolytic-like and anti-depressant-like effects of the δ-agonist UFP-512 in the novelty-induced hypophagia paradigm. A. Rats received bilateral intra-accumbens micro-injections of saline or UFP-512 or micro-injections of TAT-conjugated interfering peptide (PEP) or scrambled peptide (PEP SCR) followed 60 minutes later by UFP-512 or saline. B. A separate group of rats received intracerebroventricular (i.cv) micro-injections of saline or UFP-512 or injections of the δ-antagonist naltrindole (NALT) or μ-antagonist CTOP followed by UFP-512 or saline. Anhedonia was assessed 30 minutes following the last injection. The latency to drink from palatable milk solution in a novel and stressful environment was used as an index of anxiolytic-like and anti-depressant-like action. Statistical significance was determined using one-way ANOVA followed by Dunnett post-hoc analysis [** p<0.01, *** p<0.001 relative to saline-treated rats; n = 4-9 animals per group].
Figure 5.8 Effect of dissociating μ- and δ-ORs or antagonizing μ- or δ-ORs on the anxiolytic-like effects of the δ-agonist UFP-512 in the elevated plus maze. A. Rats received bilateral intra-accumbens micro-injections of saline or UFP-512 or micro-injections of TAT-conjugated interfering peptide (PEP) or scrambled peptide (PEP SCR) followed 60
minutes later by UFP-512 or saline. B. A separate group of rats received intraperitoneal (i.p.) injections of saline or the anxiolytic diazepam (1 mg/kg, i.p.). C. A separate group of rats received intracerebroventricular micro-injections of saline or UFP-512 or micro-injections of the δ-antagonist naltrindole (NALT) or μ-antagonist CTOP followed by UFP-512 or saline. Anxiety was assessed using the elevated plus maze 30 minutes following the last injection. Rat behaviour was scored for 5 min whereby total time spent in open arms was calculated. Statistical significance was determined using one-way ANOVA followed by Dunnett post-hoc analysis [* p < 0.05, ** p<0.01, *** p<0.001 relative to saline-treated rats; n = 4-9 animals per group].
Figure 5.9  Effect of morphine or the δ-agonist UFP-512 administered alone or in conjunction with the µ-agonist DAMGO on analgesia in the tail immersion nociceptive assay. Rats received bilateral intra-accumbens micro-injections of saline, UFP-512, UFP-512 and DAMGO, or morphine. The latency to tail withdrawal from warm water (52°C) was measured at baseline (BL) and following saline or drug injection. The arrow denotes the time of the saline or drug micro-injection. A cut-off of 4 x BL latency was imposed to minimize tissue damage. % Maximum Possible Effect was calculated according to the equation: % Maximum Possible Effect = (Latency - BL latency) / (Cut-off Latency – BL latency) x 100. Statistical significance was determined using one-way ANOVA for n = 5 – 6 animals per group.
Figure 5.10 Effect of the δ-agonist UFP-512 or TAT-conjugated peptides on locomotor activity. Rats received bilateral intra-accumbens micro-injections of saline, UFP-512, TAT-conjugated interfering δOR distal carboxyl tail peptide (PEP) or scrambled peptide (PEP SCR). Locomotor behaviour was assessed for 60 minutes using automated activity monitors equipped with motion sensors. The total horizontal distance traveled was used as an index of locomotor activity. Statistical significance was determined using one-way ANOVA for n = 5 – 9 animals per group.
6. GENERAL DISCUSSION

6.1 Localization of the µ-δ OR heteromer in the nervous system

Constructing a thorough anatomical map of the localization of the µ-δ OR heteromer within the central and peripheral nervous systems is a critical prerequisite to elucidating its physiological relevance in neuronal circuits. Findings from Study II describe the presence of µ-δ heteromer-specific signalling in the striatum and hippocampus using modified Gα-specific GTPγS techniques. Findings from Study III illustrate the presence and functional role of the µ-δ heteromer in the nucleus accumbens using a disruptive interfering peptide targeting the interaction sites within this receptor complex. The lack of available µOR and δOR antibodies for co-immunoprecipitation studies coupled with the lack of selective ligands exclusively targeting the µ-δ heteromer necessitated our adoption of indirect approaches to study this receptor complex in vivo.

6.1.1 Inferences about the localization of the µ-δ heteromer from the literature

Autoradiographic, in situ hybridization, immunohistological, and single-cell PCR studies converge on the co-localization of µ- and δ-ORs to the same neurons in the CNS and PNS (Sharif and Hughes 1989; Mansour et al., 1993; Delfs et al., 1994; George et al., 1994; Mansour et al., 1994; Arvidsson et al., 1995a; Arvidsson et al., 1995b; Cahill et al., 2001a; Goody et al., 2002; Gray et al., 2006). Co-localization, although prerequisite, does not equate with hetero-oligomerization. Examining the immunohistological literature that emerged following the cloning of the ORs in the context of the δOR domains implicated in µ-δ heteromerization provides clues into the possible anatomical localization of the µ-δ OR complex.
Since the distal portion of the δOR carboxyl tail is implicated in forming the µ-δ OR heteromer, antibodies targeting the δOR amino terminal domain epitope are likely to interact with the µ-δ heteromeric receptor species in addition to δOR homomers. The conformation of the δOR carboxyl tail is likely to be altered when in a complex with µOR as suggested by the alterations in G-protein specificity. Thus, antibodies recognizing the δOR C-terminus epitope are not likely to react with the immunogenic form of the δOR that is complexed with µOR. The majority of commercially available µOR antibodies also target the carboxyl terminus of this receptor with the exception of one N-terminus antibody, however their comparative immunohistochemical map has not been investigated.

In their characterization of commercially available δOR antibodies, Cahill and colleagues (2001a) noted that the N-terminus δOR antibody reacted more strongly with a higher molecular weight species of the δOR in contrast with the antibody directed against the C-terminus of the receptor. The N-terminus antibody also produced greater than three-fold more intense immunoreactive staining and displayed a slightly differing intra-neuronal distribution (Cahill et al., 2001a). While it has not been definitively shown that the N-terminus antibody reacts with the µ-δ heteromer, it is noteworthy that this antibody reacts with a distinct immunogenic form of the δOR which is theoretically poised to interact with µORs. It is noteworthy that this same immunogenic form of the δOR is targeted to the plasma membrane following prolonged morphine treatment in a µOR-dependent manner in brain regions including the nucleus accumbens and periaqueductal gray and in the spinal cord (Petaja-Repo et al., 2002).
6.1.2 Mapping the μ-δ heteromer in the nervous system

Recently an antibody directed against the μ-δ OR heteromer was synthesized by subtractive immunization (Gupta et al., 2010). The clone that reacted with cells co-expressing μ- and δ-ORs but not μOR or δOR alone or tissue from animals lacking μOR or δOR was used in enzyme-linked immunosorbant assays (ELISA) to assess the regional expression of the μ-δ heteromer in the brain. The μ-δ heteromer was found to be expressed in the nucleus accumbens, hippocampus, rostral ventral medulla, ventral tegmental area, medial nucleus of the trapezoid body, cortex, pons, hypothalamus, prefrontal cortex, and dorsal root ganglia (Gupta et al., 2010). This expression pattern is consistent with a role for the μ-δ heteromer in modulating neuronal circuits involved in pain, mood, and reward. This group’s findings corroborate our own and confirm the expression of the μ-δ heteromer in the brain regions that we investigated. Furthermore, chronic morphine treatment resulted in an increase in μ-δ heteromer abundance in the above regions (Gupta et al., 2010), which is in line with our own findings of the persistence of μ-δ heteromer G-protein activation and signalling following prolonged morphine treatment. Interestingly, prolonged treatment with the δ-antagonist naltriben also induced an increase in μ-δ heteromer abundance through an unknown mechanism (Gupta et al., 2010). In separate studies, naltriben has been shown to act as a chaperone, increasing the cell surface trafficking of δORs (Tso et al., 2000; Sanchez-Blazquez et al., 2009). Thus, it is likely that a proportion of δORs targeted to the cell surface by the chaperone are associated with μORs in regions where μ- and δ-ORs are co-expressed.

Future studies should be aimed at characterizing the immunogenic forms of the μ-δ heteromer that may be expressed in a tissue and neuronal compartment-specific manner. A
more detailed characterization of the phenotype(s) of neurons expressing the µ-δ heteromer would shed more light on the modality of neuronal communications that this receptor complex modulates. In the hippocampus, double in-situ hybridization histochemical studies demonstrated that parvalbumin-containing and somatostatinergic GABAergic neurons expressed µ- and δ-ORs, which suggests that the µ-δ heteromer is likely to be present in these neuronal populations of the hippocampal formation (Stumm et al., 2004). The latter scenario would render the µ-δ heteromer poised to modulate GABAergic innervation of the perisomatic region and distal dendrites of principal cells, thus possibly facilitating the excitation of principal cells in an indirect manner.

Overall, our findings converge with the literature and underscore the need to delineate the signalling cascades downstream of the µ-δ heteromer in specific brain regions and neuronal compartments. The wide distribution of the µ-δ heteromer suggests extensive involvement and a possible role in modulating signalling by the µOR and δOR homomers. The balance of signalling between the opioid receptor homomers and heteromers is likely to play a role in fine-tuning a neuron’s responsiveness to stimuli. Moreover, prolonged morphine treatment which desensitizes µOR and δOR and uncouples these receptors from G-proteins, upregulates µ-δ heteromers and these receptors remain functional and coupled to Gαz. The exact mechanism of the altered regulation of the µ-δ heteromer under conditions of prolonged morphine treatment is not known but likely involves intact coupling to Gαz in the brain.
6.2 **Regulation of the µ-δ OR heteromer**

6.2.1 **Ga\(_z\) and µ-δ heteromer signalling**

Characterization of the downstream signalling cascade to which Ga\(_z\) couples the µ-δ heteromer would shed more light onto the function of this receptor complex. Exactly which of the known signalling cascades modulated by Ga\(_z\) - adenylyl cyclase, mitogen-activated protein kinase pathways, phospholipase C, ERK1/2, CREB, tyrosine kinases or K\(^+\) channels (Zachariou et al., 2003) - is downstream of µ-δ heteromer activation under basal conditions and following prolonged morphine treatment would shed light on the unique regulation of this receptor complex.

Desensitization of opioid signalling by Regulators of G-protein Signalling (RGS) proteins is also thought to be involved in the mechanism of morphine tolerance (Glick et al., 1998; Wang et al., 1998; Mao et al., 2004). Ga\(_z\) preferentially interacts with the RGS-Rz family of RGS proteins which includes RGSZ1 and RGSZ2 (Glick et al., 1998; Wang et al., 1998; Mao et al., 2004). Similar to Ga\(_z\), RGSZ1 and 2 are primarily expressed in the brain (Lounsbury et al., 1991; Glick et al., 1998). Additionally Ga\(_z\) undergoes protein kinase C-dependent phosphorylation which renders it less sensitive to RGS-induced dampening (Metcalf et al., 2012). Altered interactions between Ga\(_z\) and its regulators may be one of the mechanisms underlying the persistent and intact µ-δ OR heteromer signalling under prolonged morphine treatment and warrants further exploration.
6.2.2 Ligand binding properties of the μ-δ OR heteromer

Overall, our findings are consistent with the μ-δ heteromer as a distinct signalling entity with properties different from those of μ- or δ-ORs. Recently, Portoghese and colleagues (He et al., 2011; Milan-Lobo and Whistler 2011) also corroborated our finding that δ-agonists can activate the μ-δ heteromer. Our findings of δ-agonist-induced co-internalization of μ- and δ-ORs have also been corroborated by two separate laboratories (He et al., 2011; Milan-Lobo and Whistler 2011). Congruent with our data, antagonizing either the μ- or δ-OR protomer abolished opioid-induced co-internalization of μ- and δ-ORs (Filizola and Weinstein 2002; Manglik et al., 2012).

Alterations in ligand binding properties in μ- and δ-ORs upon heteromerization were also identified by other groups (Gomes et al., 2000; Gomes et al., 2004; Law et al., 2005; Metcalf et al., 2012). Consistent with our radioligand binding studies, the μ-δ heteromer has a novel μ receptor ligand binding pocket or an allosteric site to which δ-agonists bind. Pretreatment of μ-δ co-expressing cells with δ-agonists or antagonists results in an increase in μOR binding sites by up to three-fold, and similarly, μ-antagonist pre-treatment increases δOR binding sites (Gomes et al., 2004). In the case of the μ-δ heteromer, ligand binding to one of the protomers would alter the conformation of the associated protomer because conformational changes are transferred to interacting receptors. We observed δ-agonist displacement of high affinity [$^3$H] μ-agonist binding only in cells expressing the μ-δ heteromer. The latter observations could be explained by a competitive binding interaction whereby δ-agonists compete with μ-agonists for the same binding site and occupy the μOR ligand binding pocket. One of the approaches to interrogate the latter possibility would
entail a re-examination of $[^3H] \mu$-agonist binding in the presence of $\delta$-agonist in cells expressing $\delta$OR with mutations in key residues within the $\delta$OR ligand binding pocket. Alternatively, $\delta$-agonists, by binding to the $\delta$OR, could act as allosteric modulators to modify the conformation of the $\mu$OR protomer thereby modulating $\mu$-agonist binding. In our experiments, the emergence of a $\delta$-agonist-detected high affinity $\mu$OR binding site within the $\mu$-$\delta$ heteromer suggests that the $\delta$-agonist-targeted allosteric site is conformationally-coupled to the high affinity $\mu$OR ligand binding pocket. Interrogating this possibility would involve equilibrium and non-equilibrium (kinetic) radioligand binding experiments in addition to receptor function assays (Christopoulos and Kenakin 2002). The endeavour is further complicated by the fact that multiple allosteric sites can exist on the surface of a GPCR and that allosteric interactions are unique for each ligand pair (Christopoulos and Kenakin 2002).

In the case of the $\mu$-$\delta$ OR heteromer, it is not known whether $\delta$-agonists activate only the $\delta$OR protomer because they also displace high affinity $\mu$-agonist binding, which is suggestive of modulation of both $\mu$- and $\delta$-OR ligand binding pockets (Kabli et al., 2010). Recent work undertaken by He and colleagues demonstrates that $\mu$-agonists and $\delta$-agonists only induce phosphorylation of their corresponding $\mu$- or $\delta$-OR protomer (He et al., 2011). Phosphorylation of the $\delta$OR is required for co-internalization of both $\mu$- and $\delta$-ORs (He et al., 2011). On the other hand, the post-endocytic fate of the $\mu$OR is not definitively known. While He and colleagues (2011) advanced that $\delta$-agonists but not $\mu$-agonists lead to $\mu$-$\delta$ heteromer degradation, Milan-Lobo et al., (2011) demonstrated $\mu$-agonist-induced degradation of the $\mu$-$\delta$ heteromer. In contrast, using their $\mu$-$\delta$ heteromer antibody, Gupta et
al., (2010) demonstrated that treatment with several μ- or δ-agonists had no effect on μ-δ heteromer receptor density.

He and colleagues (2011) advanced that administration of a peptide derived from TM domain I of the μOR abolished the interaction between μ- and δ-ORs in the spinal cord. Further, this group demonstrated that the TM I peptide abolished morphine analgesic tolerance which led the authors to conclude that disrupting the μ-δ heteromer would be an effective therapeutic strategy to prevent tolerance. However, TM I of the μOR has been postulated to be the homo-oligomerization interface between μORs both by modeling studies and by the recently-unveiled crystal structure of the μOR (Dupre et al., 2006; Dupre et al., 2007; Hasbi et al., 2007). In addition, the inhibitory peptide had no effect on the analgesic effects of a δ-agonist which had been shown to target the μ-δ heteromer by the same group (He et al., 2011). Thus, future studies are required to elucidate the differential effect of the TM I inhibitory peptide on ligands acting at the μ-δ heteromer.

Future efforts should be focused on designing selective μ-δ heteromer ligands. These pharmacological tools would aid in dissecting the function and signalling downstream of μ-δ heteromer activation. MDAN ligands consisting of a μ-agonist-δ-antagonist pharmacophore (Daniels et al., 2005) were designed on the premise that the μ-δ heteromer is a 1:1 heterodimer. The exact stoichiometric composition of the μ-δ heteromer is not yet definitely known. Further, the μ-δ heteromer could pre-couple with various signalling molecules and get trafficked to the plasma membrane as signalosomes, each of which may interact differently with ligand. Indeed, the μ-δ heteromer and several GPCRs including β2-
adrenergic receptors pre-assemble into signalling complexes prior to plasma membrane targeting (Fotiadis et al., 2006; Modzelewksa et al., 2006; Han et al., 2009; Zylbergold and Hebert 2009). In addition to our own observations of constitutive association with $G\alpha_z$, the $\mu$-$\delta$ receptor complex has also been shown to be physically associated with $\beta$-arrestin2, thus expanding its signalling repertoire and conformations (Hasbi et al., 2007; Rozenfeld et al., 2007). Our observations in radioligand binding thus generate an average affinity which encompasses an agonist’s binding to several different populations of receptors and signalosomes. Ligand-directed signalling is likely to play a role in the agonist-induced regulation of the $\mu$-$\delta$ heteromer and thus different classes of ligands should be investigated for their signalling effects at this receptor complex.

6.2.3 $\mu$-$\delta$ OR heteromer: G-protein stoichiometry

Exploring the stoichiometry of the $\mu$-$\delta$ heteromer to G-protein interaction is also imperative. Current modeling studies suggest a 2:1 ratio of receptor to G-protein with an asymmetric arrangement that sees the $G\alpha$ subunit interacting more closely with one protomer and the $G\beta\gamma$ with the other member of the pair (Han et al., 2009; Zylbergold and Hebert 2009). Elegant experiments using a receptor-G-protein fusion construct and functionally-mutant receptors showed that a receptor homodimer is asymmetrically coupled to the G-protein heterotrimer such that only one protomer interacts with the $G\alpha$ subunit (Johnson et al., 2006). While this puzzle is beginning to be solved for homomers, the $\mu$-$\delta$ heteromer represents a challenge since both $\mu$OR and $\delta$OR couple to inhibitory G proteins and can signal effectively through receptor-G-protein fusion constructs (Snook et al., 2008).
It is not yet clear which of the $\mu$-$\delta$ heteromer protomers interact with $G\alpha_z$. It remains to be determined whether occupancy of the $\mu$- or $\delta$- OR protomers leads to transactivation of the $G\alpha$ protein on the other protomer, such that ligand binding to one protomer can modify the state of the neighbouring subunit into a conformation that activates $G\alpha$ without the requirement for direct ligand binding. Despite any asymmetric interactions of signalling molecules with the receptor complex, it is conceivable that binding to one protomer would still have a modulatory effect on its interacting partner—irrespective of its magnitude—because conformational changes in the ligand-bound protomer would likely transfer to the other partner. However, whether these conformational rearrangements are sufficient to activate G-proteins remains to be investigated.

6.2.4 Desensitization of the $\mu$-$\delta$ OR heteromer

A more rigorous examination of the desensitization mechanisms of the $\mu$-$\delta$ OR heteromer is a crucial stepping stone towards evaluating its therapeutic utility. As is the case for $\mu$OR and $\delta$OR, desensitization mechanisms of the $\mu$-$\delta$ OR heteromer are likely agonist-dependent (Sanchez-Blazquez et al., 2003; Georgoussi et al., 2006; Xie et al., 2007). This is consistent with the fact that different ligands stabilize different receptor conformations that in turn result in exposure of distinct residues at the intracellular domains of the receptor or the induction of specific conformations which differentially recruit signalling molecules (He and Whistler 2002; Johnson et al., 2006; Xie et al., 2007).

While RGS proteins 1, 2, 4, 6, 9, 10, 11, 12, 19, and 20 down-regulate $\mu$OR-mediated signalling (Xie et al., 2007; Wang et al., 2009), $\delta$OR signalling is modulated by RGS 4, 9
RGS-induced desensitization of G-protein signalling is likely to be different for the µ-δ heteromer and may involve the RGS-Rz family which selectively interacts with Gaζ. Phosphorylation by protein kinases or tyrosine kinases is another key step in the desensitization cascade. In the case of the µOR, DAMGO-induced desensitization is GRK2-dependent and GRK6- and protein kinase C (PKC)-independent, whereas morphine-bound µORs desensitize via PKC and not GRK2 or GRK6 (Johnson et al., 2006). A different complement of signalling molecules is likely to be involved in regulating the µ-δ heteromer.

Ultimately, the goal of thoroughly characterizing the various interactions of ligands with the µ-δ heteromer is to relate different agonist-stabilized conformations or µ-δ heteromer signalosomes to the therapeutic versus unwanted effects of opioids in order to design improved therapeutic agents capable of selectively targeting discrete signalling cascades.

6.3 Function of the µ-δ OR heteromer

6.3.1 Mood regulation

Anatomical localization, G-protein coupling, and functional assays provide clues about the possible functions of the µ-δ OR heteromer. However, by disrupting the µ-δ heteromer in vivo and assessing which functional properties were lost, we determined that this receptor complex is intimately involved in modulating the antidepressant-like and anxiolytic-like effects of the δ-agonist UFP-512. Interestingly, Gaζ-gene deleted animals also display pro-depressive and anxiogenic behaviour (Tortella et al., 1985; Holaday et al.,
1986; Matthes et al., 1998; Scherrer et al., 2004; Hirose et al., 2005), further implicating μ-δ heteromer signalling in mood modulation. Our observations that δ-agonists have unique actions at the μ-δ heteromer in vitro are congruent with behavioural and functional evidence demonstrating that they require the μOR for their effects in vivo (Hirose et al., 2005; Hipolito et al., 2008). Further, we corroborated our colleagues’ findings of μ-δ heteromer expression in the nucleus accumbens (Gupta et al., 2010) and expanded the field by demonstrating the functional role of these complexes. The μ-δ heteromer represents a potential therapeutic target for treatment-resistant refractory depression.

The mechanisms of μ-δ heteromer-mediated mood regulation remain to be investigated but may involve modulation of dopamine release. Micro-dialysis studies demonstrate that μ- and δ-agonists stimulate dopamine release in the nucleus accumbens (Hirose et al., 2005). Interestingly, μ-agonist-mediated dopamine release is reversed by δ-antagonists and vice versa (Willner 1997), implicating both μ- and δ-ORs in modulating dopamine release in response to μ- and δ-agonists in this nucleus. The time-course of opioid-induced dopamine release in the NAc correlates with the onset of behavioural effects in our experiments. Further, dopamine release contributes to the actions of faster-acting rapid-onset antidepressant agents (Kitamura et al., 2010). The anti-depressant effects of intra-accumbens buproprion are abolished by dopamine receptor blockade, demonstrating that the mechanism of action of this atypical antidepressant at the level of the NAc involves dopamine modulation (Larson et al., 1980; Scherrer et al., 2004; Gendron et al., 2007; Schramm and Honda 2010). Future studies should investigate the mechanism of μ-δ heteromer-mediated antidepressant and anxiolytic-like actions and the effect of disrupting the
μ-δ heteromer on opioid agonist-induced dopamine release. In addition, μ- and δ-ligand combinations remain to be assayed in paradigms of depression and anxiety.

### 6.3.2 Analgesia

Spinal μ-δ OR heteromers have been implicated in analgesia (Gomes et al., 2004). Activation of the μ-δ OR heteromer using a combination of morphine and a δ-antagonist such as TIPPψ potentiated morphine’s effects and attenuated pain behaviour in the tail immersion assay (Gomes et al., 2004). This pharmacological approach to μ-δ heteromer activation was based on studies in cell lines where δ-agonists and antagonists increased μOR binding sites and μ-antagonists increased δOR binding sites (Gomes et al., 2000). Future studies exploring the mechanism of TIPPψ potentiation of morphine analgesia in vivo would shed light on the role of μ-δ heteromerization in this process. Several studies document cross-antagonism of μ- and δ-agonist effects and a synergistic effects between μ- and δ-agonists in vitro and in paradigms of analgesia in vivo. Thus, the possibility that different μ-δ heteromer signalosomes may be activated more potently by different combinations of ligands and expressed in distinct neuronal circuits cannot be ruled out. Exploring μ-δ heteromer function in the nociceptive pathway where μ- and δ-OR interactions are well-documented would likely yield novel therapeutics for treating pain syndromes.

### 6.3.3 Immune function

A more recent study suggests a role for the μ-δ heteromer in modulating immune function (Sarkar et al., 2012). In contrast with Gomes and colleagues who utilize combinations of μ-agonist/δ-antagonist to activate the μ-δ heteromer (Gomes et al., 2000;
Gomes et al., 2004), this group advances that combinations of a \( \mu \)-agonist and \( \delta \)-antagonist or a \( \delta \)-agonist and \( \mu \)-antagonist lead to a reduction in the levels \( \mu-\delta \) heteromers as detected by co-immunoprecipitation and Western blotting analyses (Sarkar et al., 2012). The authors proposed that using a \( \delta \)-agonist and \( \mu \)-antagonist resulted in a reduction in tumour growth and thus linked the improved outcomes in an animal model of adenocarcinoma to a reduction in \( \mu-\delta \) heteromer expression (Sarkar et al., 2012). However, the effects of the \( \delta \)-agonist or \( \mu \)-antagonist alone were not evaluated in control experiments. Further, the reduction in \( \mu-\delta \) heteromer expression in cell lines could not be definitively linked to improved indices of cytolytic activity in cultured splenocytes. Nonetheless, these findings about the role of the \( \mu-\delta \) heteromer in immune function encourage future studies in this line of investigation.

### 6.4 Conclusion

The \( \mu-\delta \) heteromer represents a unique physiological entity and pharmacological target with immense therapeutic promise in mood disorders, chronic pain, and other neurologic conditions involving opioidergic mechanisms. The availability of selective \( \mu-\delta \) heteromer ligands, antibodies and specific inhibitory peptides as well as the advent of more sophisticated molecular tools would allow for the functional mapping of the \( \mu-\delta \) heteromer in several tissues and cell types. Such experimental tools would ultimately accelerate the assessment of the clinical utility of the \( \mu-\delta \) OR heteromer at a time when there are inadequate treatments for refractory major depression and pain, two debilitating disorders which affects millions of individuals worldwide and have wide-ranging personal, societal and economic ramifications.
7. REFERENCES


Breit A, Gagnidze K, Devi LA, Lagace M, Bouvier M. Simultaneous activation of the delta opioid receptor (deltaOR)/sensory neuron-specific receptor-4 (SNSR-4) hetero-oligomer by the mixed bivalent agonist bovine adrenal medulla peptide 22 activates SNSR-4 but inhibits deltaOR signaling. Mol Pharmacol 2006;70(2):686-696.


Ho MK, Yung LY, Chan JS, Chan JH, Wong CS, Wong YH. Galpha(14) links a variety of G(i)- and G(s)-coupled receptors to the stimulation of phospholipase C. Br J Pharmacol 2001;132(7):1431-1440.


Johnson EA, Oldfield S, Braksator E, Gonzalez-Cuello A, Couch D, Hall KJ, Mundell SJ, Bailey CP, Kelly E, Henderson G. Agonist-selective mechanisms of mu-opioid receptor...


Lazarus LH, Bryant SD, Cooper PS, Salvadori S. What peptides these deltorphins be. Prog Neurobiol 1999;57(4):377-420.


Lucki I. The forced swimming test as a model for core and component behavioral effects of antidepressant drugs. Behav Pharmacol 1997;8(6-7):523-532.


Scherrer G, Befort K, Contet C, Becker J, Matifás A, Kieffer BL. The delta agonists DPDPE and deltorphin II recruit predominantly mu receptors to produce thermal analgesia: a


Wu H, Wacker D, Mileni M, Katritch V, Han GW, Vardy E, Liu W, Thompson AA, Huang XP, Carroll FI, Mascarella SW, Westkaemper RB, Mosier PD, Roth BL, Cherezov V,


Zarrindast MR, Babapoor-Farrokhran S, Babapoor-Farrokhran S, Rezayof A. Involvement of opioidergic system of the ventral hippocampus, the nucleus accumbens or the central amygdala in anxiety-related behavior. Life Sci 2008;82(23-24):1175-1181.


