Unraveling the determinants of G protein activation as a measure of relative opioid drug efficacy

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Abstract

Opioids are powerful and effective drugs used for pain management, but their therapeutic usage is limited due to their side effects. Therefore, obtaining an extensive understanding of the pharmacological properties that underlie the actions of these drugs is much needed. Efficacy is the extent to which an agonist can stimulate the activity of the receptor it interacts with, and many studies have claimed to determine the efficacy of a wide range of opioid agonists. However, these opioids reportedly appear to be full agonists in some studies but seem to be partial agonists in others. Discrepancies from previous findings hamper the determination of accurate measurements of the efficacy of these drugs. As such, several assays focus on different aspects of opioid receptor signaling to deduce how efficacious these drugs may be. In this study, we focus on the μ-opioid receptor (MOR) as agonists that act on it represent the majority of clinically used opioids. We take advantage of a unique cellular model that captures the differential activation of each Gαi/o/z protein on top of measuring the relative efficacies of each tested opioid agonist. Using various cell-based assays, we demonstrate that these can be tools used to directly look at the interaction between the receptor and its effectors through the coupling of inhibitory heterotrimeric G proteins. The distinctions between each functional readout reveal insights about the nature of each established system, highlighting their advantages as well as their limitations. Key details about the mechanistic basis of inhibitory G protein activation are also uncovered. Precise determination of the efficacy of opioids could ultimately impact the understanding of opioid-mediated neuromodulation, as further links can be made between this important pharmacological parameter and the extent in which it induces analgesia and limits the side effects typically associated with opioid intake.

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List of Abbreviations

AC	adenylyl cyclase
AT1R	angiotensin II type 1 receptor
BCS	bovine calf serum
BRET	bioluminescence resonance energy transfer
$CaCl_2$	calcium chloride
cAMP	cyclic adenosine monophosphate
DAG	diacylglycerol
DAMGO	(D-Ala(2)-mephe(4)-gly-ol(5))enkephalin
DMEM	Dulbecco's Modified Eagle's Medium
DOR	delta/δ-opioid receptor
ECL	extracellular loop
FBS	fetal bovine serum
FRET	fluorescence resonance energy transfer
FSK	forskolin
G protein	guanine nucleotide-binding protein
GDP	guanosine diphosphate
GIRK	G protein-coupled inwardly rectifying potassium channels
GPCR	G protein-coupled receptor
GRK	G protein receptor kinase
GTP	guanosine triphosphate
Gαi/o/z	inhibitory Gα proteins
HBSS	Hank's balanced salt solution

HEK	human embryonic kidney
ICL	intracellular loop
IP3	inositol triphosphate
ISO	isoproterenol
IUPHAR	International Union of Pharmacology
KCl	potassium chloride
KH_2PO_4	potassium phosphate monobasic
KOR	kappa/κ-opioid receptor
M2R	M2 muscarinic receptor
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulfate
MOR	mu/μ-opioid receptor
N/OFQ	nociceptin/orphanin FQ
Na_2HPO_4	sodium phosphate dibasic
NaCl	sodium chloride
NanoBiT	nanoluc binary technology
NOR	nociceptin opioid receptor
ORL1	opioid receptor-like 1
OUD	opioid use disorder
PAG	periaqueductal grey
PKC	protein kinase C
PBS	phosphate-buffered saline
PDYN	preprodynorphin

PEI	polyethylenimine
PENK	preproenkephalin
PLL	poly-L-lysine
PNOC	prepronociceptin
POMC	proopiomelanocortin
RGS	regulators of G protein signaling
PTX	pertussis toxin
TM	transmembrane
YFP	yellow fluorescent protein
β2AR	β2-adrenergic receptor

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CHAPTER I: INTRODUCTION

1.1 General Overview: Pain, Opioids, and the Opioid Crisis

Pain is a vital function of the nervous system since it is a common sensory and emotional experience necessary for warning of injury or disease to the body (1). However, it is also perceived as an unpleasant and distressing sensation often described as burning, stabbing, aching, and throbbing, which can restrict individuals from performing daily activities and even reduce their quality of life (2). Accompanied by a wide and diverse spectrum of diseases, chronic pain, especially, has certainly been a prevalent concern worldwide as it is estimated that 1 in 10 adults are diagnosed with the condition each year (3, 4). Regardless of the demographics of the population, pain affects everyone at least once in their lives, even if the degree at which the severity of pain is perceived varies per individual. As an integral part in the practice of healthcare professionals, pain management is continuously regarded as a public health challenge. However, it remains a global health priority as it can even be considered a fundamental human right (4). In order to reduce the burden of suffering from pain, there have been several proposed and applied medications as well as therapies that have been deemed effective in doing so (5). However, there is one particular class of drugs that has stood the test of time – opioids.

For thousands of years, opioids, which are derived from the opium poppy, *Papaver somniferum*, have been commonly prescribed for the treatment of moderate to severe pain (6). Although considered to be very powerful in relieving pain, regularly taking these medications also poses a risk to their users due to major side effects such as respiratory depression, nausea, and severe constipation (7). Other significant clinical concerns of opioid administration

include physical dependence, tolerance, and addiction, which often lead to opioid-related overdoses and deaths (8). With the likes of morphine and fentanyl, potent opioid analgesics have found their way into the hands of many. The misuse of these drugs has thus sparked a serious public health concern known as the opioid crisis (**Figure 1.1**). Some key factors, such as increased opioid prescriptions, low cost, and availability of illicitly manufactured opioids, have driven the crisis to new heights (9). What makes matters worse is that people who have opioid use disorder (OUD) are likely to simultaneously use other substances as well in order to enhance the euphoric feeling typically associated with opioid intake (10).

The magnitude of the opioid crisis has led to the rise of morbidity and mortality rates which have already been in an upward trend over the last few decades. In the United States alone, the number of reported opioid-related overdose deaths has increased by around 4-fold since 1999 (11). Without any strategic interventions, over a million people in North America are also projected to die due to opioid overdoses by the end of the decade (12). As the opioid crisis continues to intensify because of its complex and evolving nature, the devastating impact on communities has increasingly drawn attention to the matter (12, 13). The international recognition gained from the long-standing opioid epidemic in North America has even allowed other countries to take notes to prevent the crisis from emerging in their own nations (14–16). Hence, there is a pressing need for a multifaceted approach to combat the war against opioid-related overdoses and deaths. From enforcing revised public health policies through government intervention to spreading more awareness about the risks of opioid use, the strategies on how to face the problematic crisis can vary tremendously. However, our contribution towards this collaborative effort stems from a pharmacological point of view towards opioids, the root of it all.

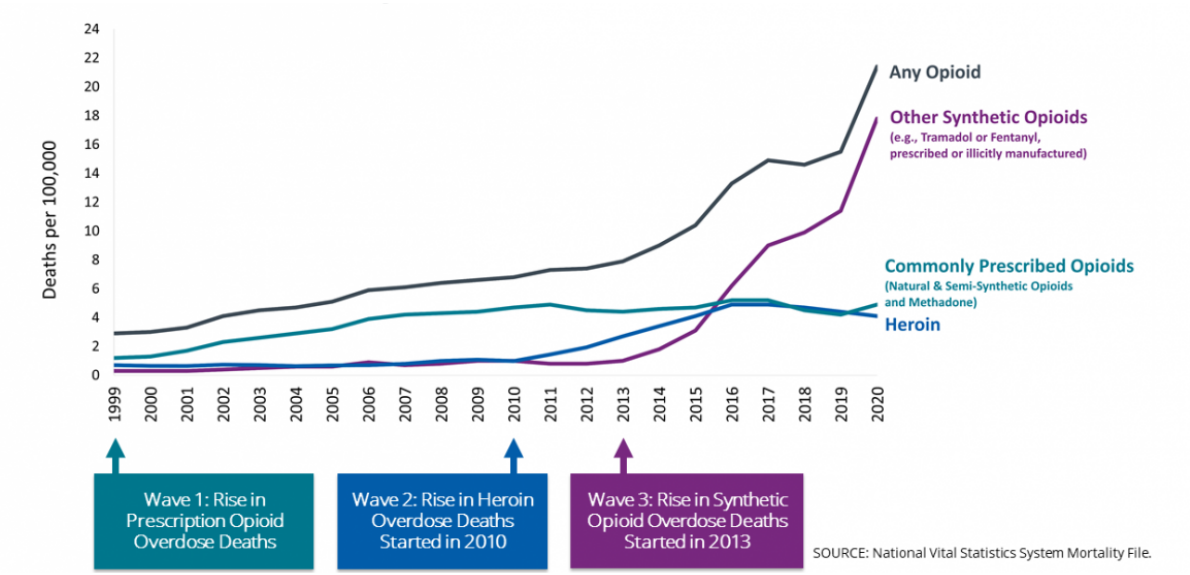

Deaths per 100,000
24
22
20
18
16
14
12
10
8
6
4
2
0
1999
2000
2001
2002
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
2014
2015
2016
2017
2018
2019
2020
Any Opioid
Other Synthetic Opioids
(e.g., Tramadol or Fentanyl, prescribed or illicitly manufactured)
Commonly Prescribed Opioids
(Natural & Semi-Synthetic Opioids and Methadone)
Heroin
Wave 1: Rise in Prescription Opioid Overdose Deaths
Wave 2: Rise in Heroin Overdose Deaths Started in 2010
Wave 3: Rise in Synthetic Opioid Overdose Deaths Started in 2013
SOURCE: National Vital Statistics System Mortality File.

Figure 1.1 The State of the Opioid Crisis. Since 1999, the number of opioid-related deaths have been on the rise in North America. Coming in waves due to different driving factors, the opioid crisis has become one of the worst public health crises that has ever been seen. Currently, synthetic opioids have been the main driver of overdose deaths, especially as it coincides with the increased production of illicitly manufactured drugs that are laced with fentanyl. Image is taken from the Centers of Disease and Control Prevention.

1.2 The Opioid System

1.2.1 Classification of Opioid Receptors

Opioids primarily produce their physiological effects by activating opioid receptors, wherein the main subtypes include the mu (μ), delta (δ), kappa (κ) receptors. The way in which they are named is derived from the following: the μ-opioid receptor (MOR) from morphine, the most common exogenous ligand that acts on it; the δ-opioid receptor (DOR) from mouse vas deferens, where it was first identified; and the κ-opioid receptor (KOR) from ketocyclazocine, the first ligand discovered to act on the receptor (17). Other terminologies to designate each receptor have been previously suggested as well. The International Union of Pharmacology (IUPHAR) regarded the DOR as OP1, KOR as OP2, and MOR as OP3 based on the order in which the receptors were cloned (18). In addition, the genes that encode for the MOR, DOR and KOR are OPRM1, OPRK1 and OPRD1, respectively (19). They been cloned from humans, and other vertebrates such as rats and mice (20). Multiple putative opioid receptor subtypes have also been proposed (μ1, μ2, μ3; δ1, δ2; κ1, κ2, κ3) as a suggestion to explain the variations in receptor responses based on different ligands. However, these subclassifications remain elusive due to the lack of supporting evidence and likely represent different signaling complexes rather than different receptors. (21).

More recently, a non-classical member has been added to the opioid receptor family called the nociceptin receptor (NOR), which is encoded by the OPRL1 gene. When it was first identified in 1994, the receptor was named opioid receptor-like 1 (ORL1). Although it is highly homologous to the other three known opioid receptors, the NOR is distinct since its pharmacological profile differs from them in terms of being unable to respond to naloxone, a classical opioid antagonist (17). Furthermore, there have been additional non-GPCR receptors

that have been introduced such as the sigma (σ), epsilon (ε), lamda (λ) and zeta (ζ) receptors. However, they are poorly characterized and there is still insufficient evidence to support their classification as part of the opioid receptor family (22).

1.2.2 Distribution of Opioid Receptors

In terms of the distribution of opioid receptors, they are primarily found to be widely present throughout the nervous system, mainly in the cerebral cortex, limbic system, and respiratory control centers that include the brainstem. The MOR is abundantly expressed in the amygdala, thalamus, and brainstem; the DOR is mainly found in the neocortex, olfactory system, striatum, and limbic cortex, while the KOR is especially present in the claustrum, striatum, and hypothalamus (**Figure 1.2**) (23). Within the descending pain pathways, opioid receptors, especially the MOR, are concentrated in regions such as the periaqueductal grey (PAG) and locus coeruleus in the brain, as well as the rostral ventromedial medulla (RVM) and the dorsal horn of the spinal cord (24). However, they are also expressed in the gastrointestinal tract and reportedly even in some immune cells (25). Although their primary role is to produce analgesia, each receptor appears to have distinct expression patterns in various locations that influence the differences in their range of effects. In general, these may include but are not limited to, reward, depression, anxiety, and addiction. The side effects typically associated with opioids are associated with those that occur upon the stimulation of the MOR. Other than analgesia, it can lead to respiratory depression, nausea, and a reduction in gastrointestinal motility. The DOR has been linked to anxiolysis, while the KOR has been shown to mediate diuresis and dysphoria (26, 27).

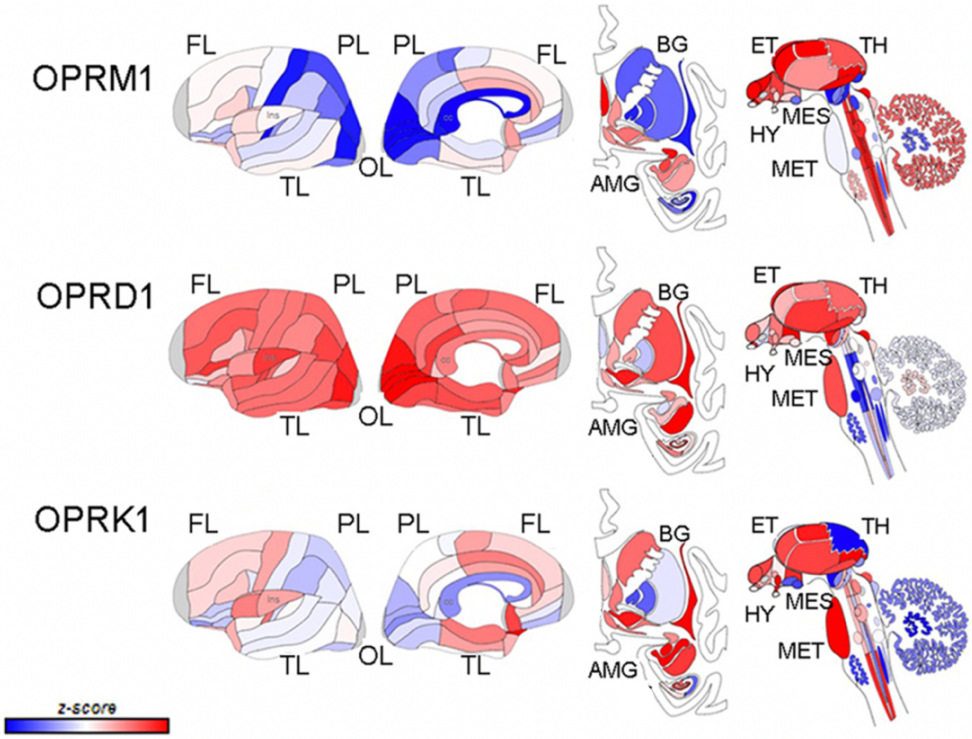

OPRM1
FL
PL
PL
FL
TL
OL
TL
BG
AMG
ET
TH
MES
HY
MET
OPRD1
FL
PL
PL
FL
TL
OL
TL
BG
AMG
ET
TH
MES
HY
MET
OPRK1
FL
PL
PL
FL
TL
OL
TL
BG
AMG
ET
TH
MES
HY
MET
z-score

Figure 1.2 Distribution of Opioid Receptors in the Human Brain. The data is from a 55-year-old, Caucasian male. OPRM1, OPRD1 and OPRK1 are the genes that encode for the MOR, DOR and KOR, respectively. The outer and inner surfaces of the left hemisphere of the brain are shown on the first two columns. Subcortical structures are also displayed from the frontal view along with brainstem structures from the side view. The color bar represents the expression values using z-score normalization. FL: frontal lobe; PL: parietal lobe; TL: temporal lobe; OL: occipital lobe; BG: basal ganglia; AMG: amygdala; ET: epithalamus; TH: thalamus; MES: mesencephalon; HY: hypothalamus; MET: medulla oblongata. The image was modified from Valentino et al. (28), with permission from https://creativecommons.org/licenses/by/4.0/.

1.2.3 Endogenous and Exogenous Opioid Ligands

The range of opioid ligands can be categorized as either endogenous or exogenous in nature. Endogenous opioids for each opioid receptor are involved in the modulation of reward mechanisms, mood, and stress. They also have been discovered to have selective affinities for each opioid receptor subtype: β-endorphins (29) for the MOR, enkephalins (30) for the DOR and dynorphins (31) for the KOR. In addition, nociceptin/orphanin FQ (N/OFQ) was found to be the natural ligand for the NOR and it is for which the receptor was named after. These ligands are derived from large proteins that are broken down by proteolytic cleavage. In particular, proopiomelanocortin (POMC), preproenkephalin (PENK), preprodynorphin (PDYN) and prepronociceptin (PNOC) are known to be the polypeptide precursors of β-endorphins, enkephalins, dynorphins and nociceptin/orphanin FQ, respectively (23, 32). Moreover, endomorphin-1 and -2 were identified in 1997 as additional opioid peptides that specifically act at the MOR (33). However, the search for their precursor proteins to understand the biosynthesis of these endogenous opioids continues as previous efforts have yielded inconclusive results (34).

Exogenous opioids can be categorized as either naturally occurring opioid alkaloids, semi-synthetic, or synthetic compounds. Naturally occurring compounds include morphine, codeine, thebaine and papaverine. Later on, many semi-synthetic compounds were created as a result of chemical modifications on opioid alkaloids. These include heroin, buprenorphine, naloxone, and oxycodone. Fully synthetic opioids have been identified more recently and are grouped according to their chemical structures. There are morphinan derivatives, diphenylheptane derivatives, benzomorphan derivatives and phenylpiperidine derivatives (17). Other than the prototypical opioid receptor ligands, novel ones have also been proposed to

bind to more than one type of opioid receptor with the objective of producing additive analgesic effects from their synergistic actions (35). Moreover, exogenous opioids may be classified according to their relative strengths as well. Weak opioids include codeine and tramadol, while strong opioids include morphine and methadone (36). As a key driver of the opioid crisis, one of the strongest synthetic compounds, fentanyl, has been shown to be up to 100 times more potent than morphine and it can penetrate the blood-brain barrier much faster (37), making it extremely powerful and dangerous to use if it has fallen into the wrong hands.

In terms of their effects on opioid receptors, opioids can be described as full agonists, partial agonists, or antagonists (**Figure 1.3**). Full or complete agonists produce a maximal biological response after activating the receptor similar to that of endogenous ligands, while partial agonists bind to the receptor, but only induce submaximal activation even at saturating conditions. On the other hand, antagonists do not elicit any functional response upon binding to the receptor (38). To make matters more complex, some opioids have mixed receptor action such as buprenorphine, which has agonist-antagonist activity depending on the subtype of opioid receptor it interacts with (39).

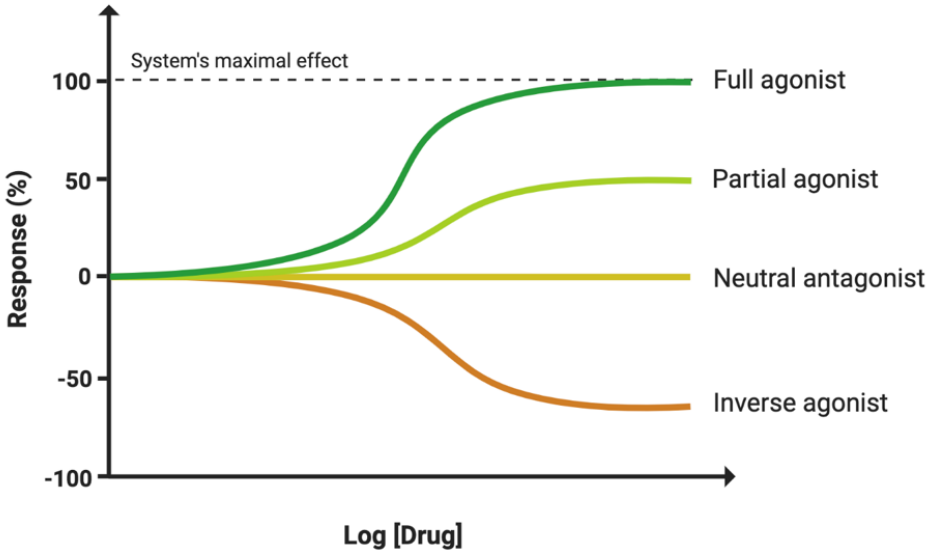

System's maximal effect
100
50
0
-50
-100
Response (%)
Log [Drug]
Full agonist
Partial agonist
Neutral antagonist
Inverse agonist

Figure 1.3. Classification of Drugs Based on their Effects upon Receptor Activation. Dose-response curves indicate the different effects of drugs on physiological systems. Full agonists reach the system's maximal effect. Partial agonists produce submaximal effects. Neutral antagonists bind to the receptor but do not alter its constitutive activity. Inverse agonists inhibit the constitutive activity of the receptor.

1.3 The μ-Opioid receptor: A G Protein-Coupled Receptor

1.3.1 GPCR Superfamily

The MOR belongs to the superfamily of G protein-coupled receptors (GPCRs). Having over 800 different genes in humans, they comprise the largest and most diverse family of membrane proteins in the human genome. They are subdivided according to their evolutionary homology and functional similarities. The most recognized way in which GPCRs are categorized divides them into six classes. These include rhodopsin-like, secretin, metabotropic glutamate, fungal mating pheromone, cyclic AMP and frizzled receptors, which are designated as classes A to F, respectively (**Figure 1.4**) (40). Another classification system called GRAFS is based on phylogenetic studies that group GPCRs present in vertebrates (41, 42). Various hormones, neurotransmitters, drugs and other stimuli interact with GPCRs to elicit their cellular and physiological responses. Among the different classes, class A or the rhodopsin-like family is by far the largest, and opioid receptors fall under this category. Because of the ubiquitous distribution and abundance of these receptors throughout the human body, as well as their involvement in cellular communication, GPCRs as therapeutic targets for drug discovery have become very popular. In fact, they currently represent one of the major targets of pharmaceutical research as approximately one-third of the United States Food and Drug Administration (FDA)-approved drugs on the market act on them (43).

Among the different subtypes of opioid receptors, the MOR predominantly mediates the physiological actions of clinically used opioids (44). Unfortunately, DOR and KOR do not mediate analgesia to the same extent as the MOR. Present in key points in pain pathways such as the dorsal horn of the spinal cord, activation of the MOR prevents the onset of action potentials that results in the depression of neural firing. Opioids acting on the MOR also

regulate both pre- and postsynaptic neurons as they work synergistically to reduce the perception of pain (22). At the same time, the central dopamine reward pathways that enhance euphoria are activated. As a prototypical MOR agonist, morphine remains to be part of the list of essential medicines by the World Health Organization because of its effectiveness in pain management (45). Nevertheless, the MOR has continued to represent the target of opioid agonists as they are considered as the gold standard for treating pain (46).

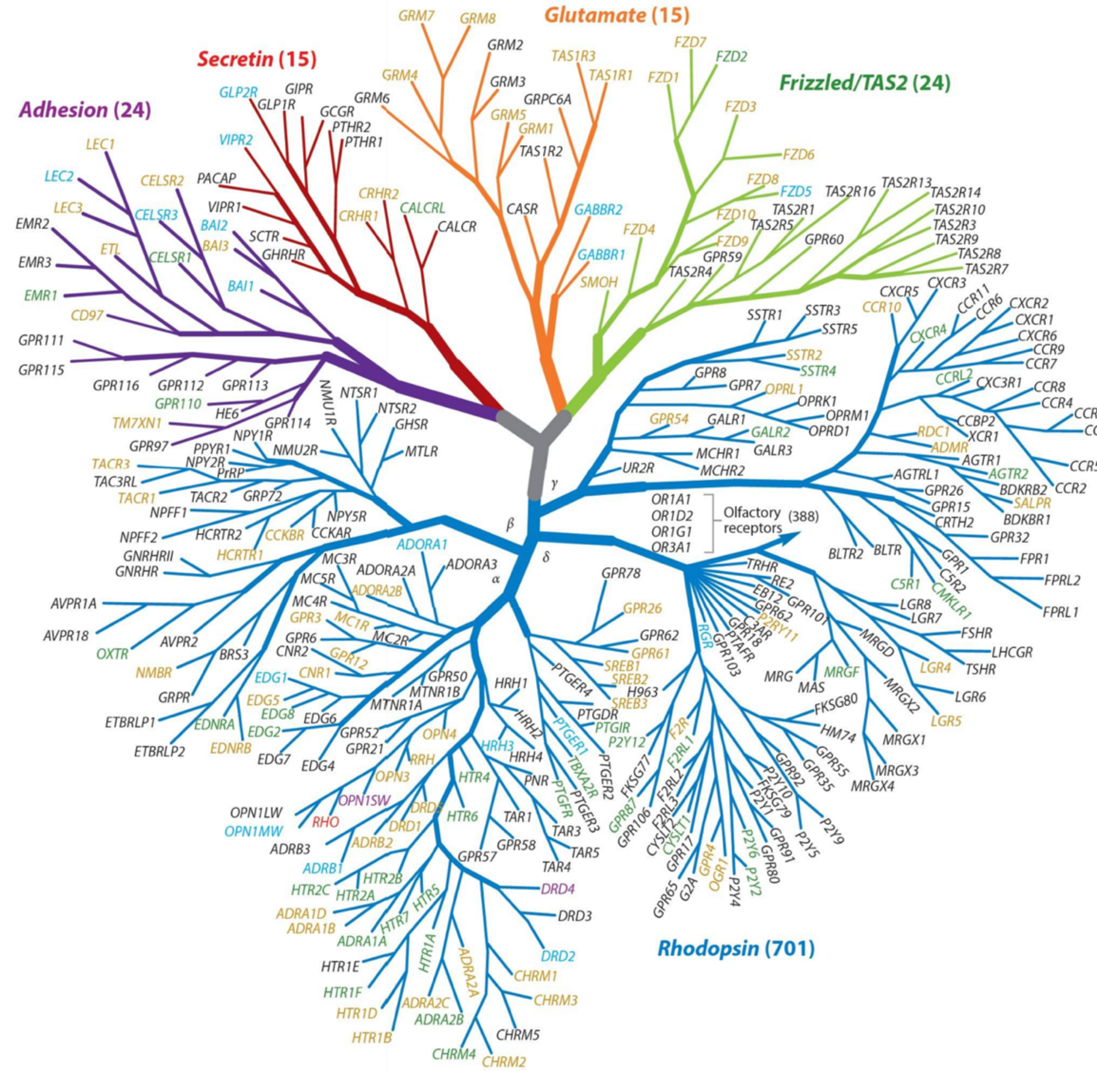

Glutamate (15)
Secretin (15)
Adhesion (24)
Frizzled/TAS2 (24)
Rhodopsin (701)
Olfactory receptors (388)
OR1A1
OR1D2
OR1G1
OR3A1
α
β
γ
δ
GRM7
GRM8
GRM2
GRM3
GRM4
GRM6
GRM5
GRM1
GRPC6A
TAS1R3
TAS1R1
TAS1R2
CASR
GABBR2
GABBR1
SMOH
FZD7
FZD2
FZD1
FZD3
FZD6
FZD8
FZD5
FZD10
FZD9
FZD4
TAS2R16
TAS2R13
TAS2R14
TAS2R10
TAS2R3
TAS2R9
TAS2R8
TAS2R7
TAS2R1
TAS2R5
TAS2R4
GPR60
GPR59
GLP2R
GIPR
GLP1R
GCGR
PTHR2
PTHR1
VIPR2
PACAP
VIPR1
SCTR
GHRHR
CRHR2
CRHR1
CALCRL
CALCR
LEC1
LEC2
LEC3
CELSR2
CELSR3
CELSR1
BAI2
BAI3
BAI1
EMR2
EMR3
EMR1
ETL
CD97
GPR111
GPR115
GPR116
GPR112
GPR113
GPR110
HE6
TM7XN1
GPR114
GPR97
SSTR1
SSTR3
SSTR5
SSTR2
SSTR4
GPR8
GPR7
OPRL1
OPRK1
OPRM1
OPRD1
GPR54
GALR1
GALR2
GALR3
MCHR1
MCHR2
UR2R
CXCR5
CXCR3
CCR10
CCR11
CCR6
CXCR2
CXCR1
CXCR6
CCR9
CCR7
CXCR4
CCRL2
CX3CR1
CCR8
CCR4
CCR1
CCR3
CCBP2
XCR1
RDC1
ADMR
AGTR1
CCR5
CCR2
AGTRL1
AGTR2
BDKRB2
GPR26
GPR15
SALPR
CRTH2
BDKBR1
GPR32
BLTR2
BLTR
GPR1
C5R2
FPR1
FPRL2
FPRL1
C5R1
CMKLR1
LGR8
LGR7
FSHR
LHCGR
TSHR
LGR4
LGR6
LGR5
MRGD
MRG
MAS
MRGF
MRGX2
FKSG80
HM74
MRGX1
MRGX3
MRGX4
GPR55
GPR35
P2Y9
P2Y5
GPR91
GPR80
P2Y2
P2Y4
P2Y6
OGR1
GPR4
G2A
GPR65
GPR17
TRHR
RE2
EB12
GPR101
GPR62
P2RY11
RGR
GPR103
PTAFR
GPR78
GPR26
GPR62
GPR61
SREB1
SREB2
SREB3
H963
PTGER4
PTGDR
PTGIR
P2Y12
F2R
F2RL1
F2RL2
FKSG77
GPR87
GPR106
CYSLT1
P2Y1
GPR92
P2Y10
FKSG79
NTSR1
NMU1R
NTSR2
GHSR
MTLR
NMU2R
NPY1R
PPYR1
NPY2R
PrRP
TACR3
TAC3RL
TACR1
TACR2
GRP72
NPFF1
NPY5R
NPFF2
HCRTR2
CCKBR
CCKAR
HCRTR1
GNRHRII
GNRHR
ADORA1
ADORA2A
ADORA3
ADORA2B
MC3R
MC5R
MC4R
MC1R
MC2R
GPR3
GPR6
CNR2
GPR12
CNR1
AVPR1A
AVPR18
OXTR
AVPR2
BRS3
NMBR
GRPR
EDG1
EDG5
EDG8
EDG6
EDG2
EDNRA
EDNRB
ETBRLP1
ETBRLP2
EDG7
EDG4
GPR50
MTNR1B
MTNR1A
GPR52
GPR21
OPN4
RRH
OPN3
OPN1SW
OPN1LW
OPN1MW
RHO
DRD5
DRD1
ADRB2
ADRB3
ADRB1
HTR2C
HTR2B
HTR2A
ADRA1D
ADRA1B
ADRA1A
HTR7
HTR5
HTR1E
HTR1A
HTR1F
HTR1D
HTR1B
ADRA2C
ADRA2B
ADRA2A
CHRM4
CHRM2
CHRM5
CHRM3
CHRM1
DRD2
DRD3
DRD4
GPR57
GPR58
TAR1
TAR3
TAR4
TAR5
PNR
HTR4
HTR6
HRH1
HRH2
HRH3
HRH4
PTGER1
TBXA2R
PTGFR
PTGER3
PTGER2

Figure 1.4 Phylogenetic Tree of the GPCR Superfamily. Having over 800 types found in humans, the family of GPCRs is very abundant and diverse. The opioid receptors (OPRM1: μ-opioid receptor, OPRD1: δ-opioid receptor, OPRK1: κ-opioid receptor) are part of the largest subfamily of GPCRs, the Rhodopsin family. Other major branches such as Adhesion, Secretin, Glutamate and Frizzed receptors are also labeled. The classifications are made based on sequence similarity. The image was modified from Chen et al. (47) with permission from Annual Reviews via the Copyright Clearance Centre.

1.3.2 Structural Insights of GPCRs

Obtaining the structure of GPCRs has been nothing short of a challenge. If not for the generation of two-dimensional crystals of rhodopsin (48), the first insights into the general architecture of GPCRs would not have been revealed. After some time, more information regarding their specific structural details came to light when three-dimensional crystal structures were resolved. Some of the GPCRs crystallized include opioid receptors (**Figure 1.5**) (49–52), β2-adrenergic receptor (β2AR) (53, 54), M2 muscarinic receptor (M2R) (55, 56), and angiotensin II type 1 receptor (AT1R) (57). Because of the dynamic nature of GPCRs, the ability to monitor between the different receptor states has presented some difficulties. However, progress has been made in developing tools to aid in stabilizing receptors in a certain conformational state. One such method is reportedly the use of nanobodies. As single-chain antibodies, nanobodies are very small and can be optimized to display high affinity for the receptor. For example, Nb6 and Nb39 stabilize inactive and active-like states of the KOR, respectively (51, 58). There has also been a report of the use of an antibody fragment derived from a monoclonal antibody that aids with receptor stabilization as well (59).

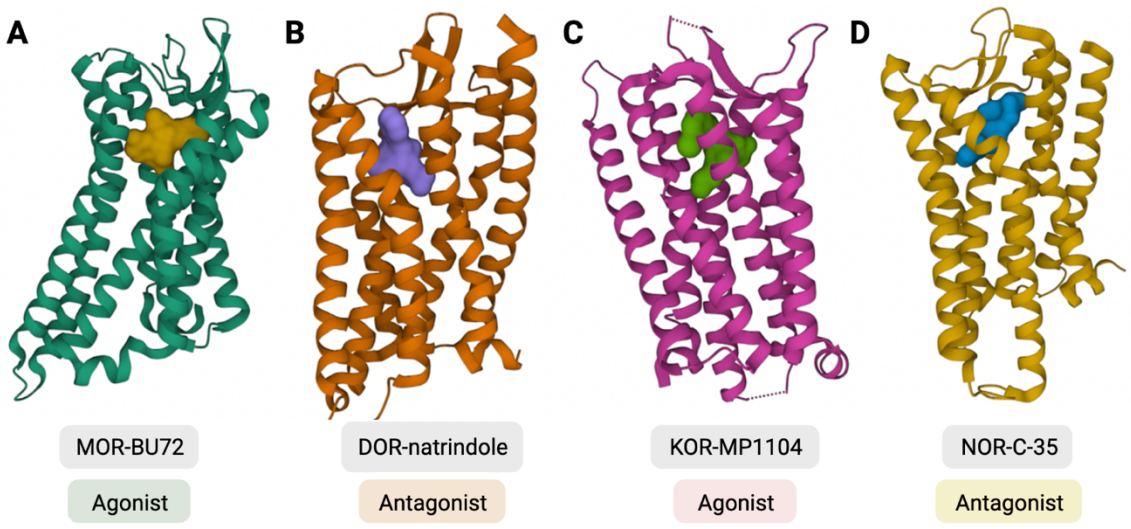

A
B
C
D
MOR-BU72
Agonist
DOR-natrindole
Antagonist
KOR-MP1104
Agonist
NOR-C-35
Antagonist

Figure 1.5 Resolved Crystal Structures of Opioid Receptors. The different opioid receptors, including their bound ligands, are shown. (A) MOR-BU72 (PDB ID: 5C1M); (B) DOR-natrindole (PDB ID: 4EJ4); (C) KOR-MP1104 (PDB ID: 6B73); (D) NOR-C-35 (PDB ID: 5DHG). The cartoon representation of the different receptors is shown while the molecular surface of ligands is displayed. Nanobodies were used to stabilize some of the structures but were omitted in this figure.

From extensive efforts to resolve their structure, it is now well known that GPCRs are seven-transmembrane-spanning receptors that comprise of seven hydrophobic transmembrane helical domains (TM1-TM7), connected by three intracellular loops (ICL1-ICL3) and three extracellular loops (ECL1-ECL3), along with an extracellular amino (N) terminus, and an intracellular carboxyl (C) terminus (60) (**Figure 1.6**). When it comes to the classical opioid receptors, the MOR shares similar structural features to them and are highly homologous (60%) in terms of their amino acid compositions. Their most identical regions are in their transmembrane regions, while they differ greatly in their extracellular loops, as well as their N- and C-terminal regions (61). These receptors are mainly known to transduce signals from extracellular ligands to intracellular effectors to regulate numerous cellular and physiological responses.

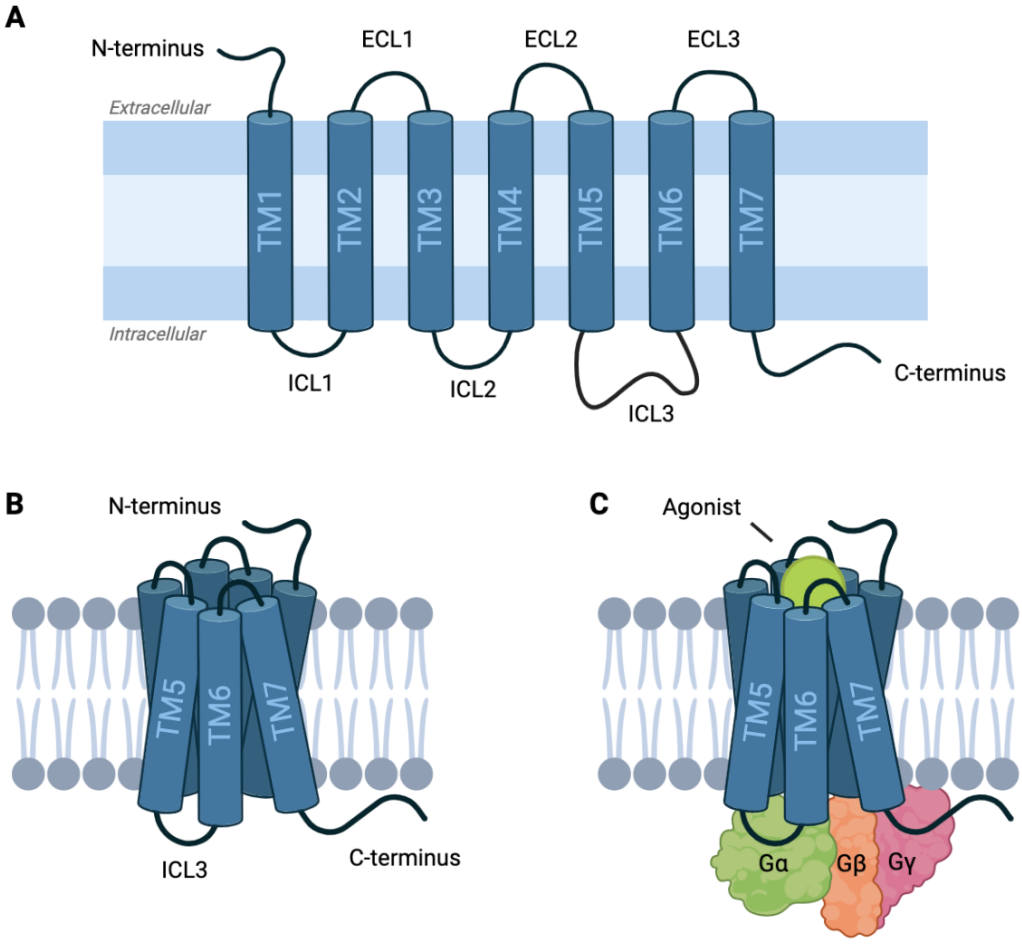

A
N-terminus
ECL1
ECL2
ECL3
Extracellular
Intracellular
TM1
TM2
TM3
TM4
TM5
TM6
TM7
ICL1
ICL2
ICL3
C-terminus
B
N-terminus
TM5
TM6
TM7
ICL3
C-terminus
C
Agonist
TM5
TM6
TM7
Gα
Gβ
Gγ

Figure 1.6 Schematic Representation of the General Structure of GPCRs. (A) GPCRs are membrane proteins that consist of seven transmembrane (TM) helices connected by three extracellular (ECL1-3) and three intracellular loops (ICL1-3). Their N- and C-terminus are found in the extracellular and intracellular regions, respectively. (B) The assembly of a typical GPCR is done in a counter-clockwise manner to form a functional receptor. (C) Upon agonist binding, the receptor is activated and recruits the heterotrimeric G proteins. These proteins bind to the ICL3 and C-terminus of the receptor.

1.3.3 GPCR Signaling

GPCRs are named after their ability to bind to guanine nucleotide-binding proteins, also known as G proteins. In terms of their primary function, these receptors mediate intracellular signaling either through a G protein-dependent or a G protein-independent manner that involves G protein activation or β-arrestin recruitment (62) (**Figure 1.7**). Opioid-induced analgesia suppresses neuronal excitability and promotes neuronal hyperpolarization through the classical G protein pathway (63). A general understanding of how these receptors signal through G proteins is that once a ligand binds to the receptor, a conformational change facilitates its coupling to heterotrimeric G proteins, composed of the Gα, Gβ and Gγ subunits. Thus, binding to the receptor causes their activation, which involves GTP binding to the Gα subunit in exchange for guanosine diphosphate (GDP), which is then released. As a result, the guanosine triphosphate (GTP)-bound Gα subunit, along with the Gβγ dimers, dissociate from each other and propagate signals to a multitude of downstream effector proteins. Some of these effector proteins that translate various signals upon GPCR activation are adenylyl cyclase (AC) and ion channels such as voltage-gated calcium channels (VGCC) and G protein-coupled inwardly rectifying potassium (GIRK) channels. Signaling is terminated when the intrinsic GTPase activity of the Gα subunit promotes the hydrolysis of GTP into GDP for the heterotrimeric G protein to return to its basal state. Another important mechanism of the regulation of GPCR signaling is the recruitment of the adaptor proteins called β-arrestins. They are known to promote the decoupling of the receptor and G proteins, regulate desensitization and facilitate receptor internalization (63). In the classical view of this pathway, G protein receptor kinases (GRK) mediate the phosphorylation of the receptor, which is subsequently

recognized by β-arrestin. When bound to the receptor, β-arrestin sterically hinders G protein coupling, leading to the downregulation of G protein-dependent signaling events (64).

Along with GPCR signaling pathways are several mechanisms that tightly regulate them, such as desensitization and internalization. Post-translational modifications govern some of these regulatory processes, which include phosphorylation, glycosylation, palmitoylation and ubiquitination (65). When it comes to receptor phosphorylation, several proteins take part in doing so, including protein kinase C (PKC) and GRK proteins. As previously mentioned for GRK proteins, receptor phosphorylation regulates its activity through desensitization and internalization. Glycosylation and palmitoylation occur during receptor maturation in the endoplasmic reticulum and Golgi apparatus, after which, regulate receptor localization to the plasma membrane. On the other hand, ubiquitination regulates receptor degradation. In addition to these post-translational modifications, there are other families of proteins that play a role in the modulation of G protein signaling (66). These include regulators of G protein signaling (RGS). These proteins act to enhance the GTPase activities of the GTP-bound Gα subunits which in turn, increase the rate of GTP hydrolysis into GDP, preventing further G protein signaling (46).

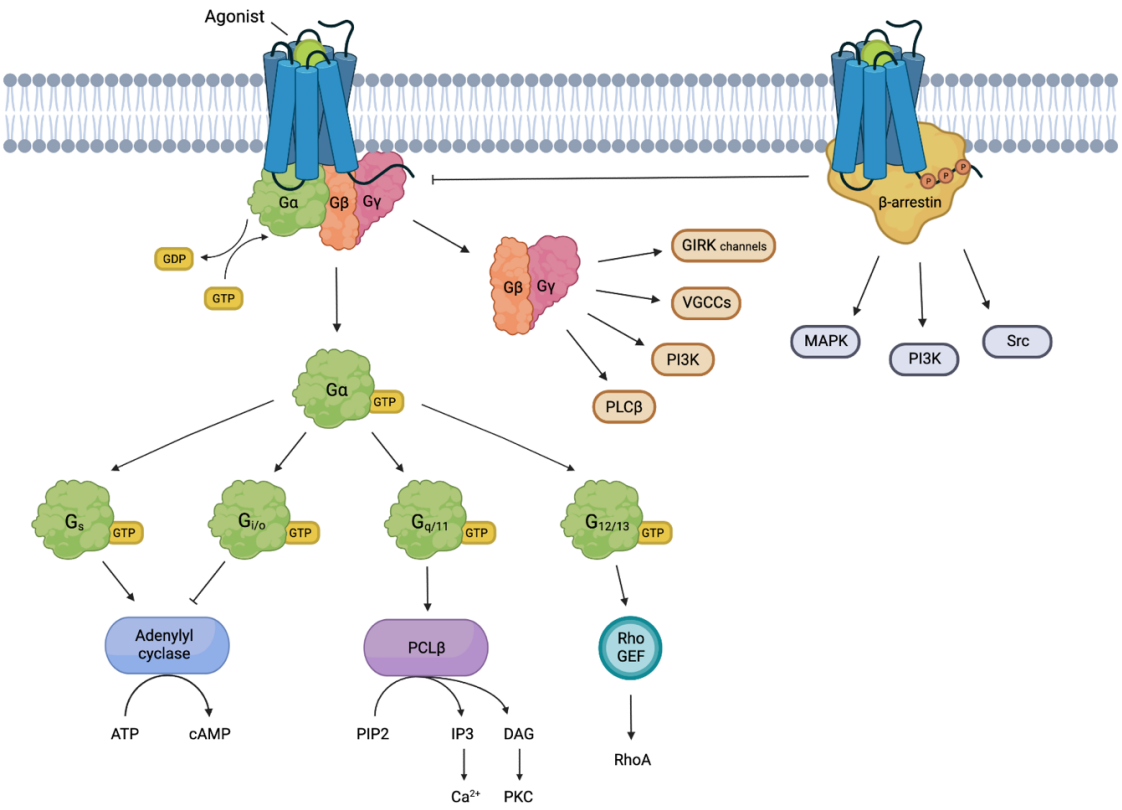

Agonist
Gα
Gβ
Gγ
GDP
GTP
Gβ
Gγ
GIRK channels
VGCCs
PI3K
PLCβ
β-arrestin
MAPK
PI3K
Src
Gα
GTP
Gs
GTP
Gi/o
GTP
Gq/11
GTP
G12/13
GTP
Adenylyl cyclase
ATP
cAMP
PCLβ
PIP2
IP3
DAG
Ca2+
PKC
Rho GEF
RhoA

Figure 1.7 Pathways Involved in GPCR Signaling. The binding of an agonist to a GPCR activates G-protein-dependent and -independent pathways that modulate cellular signaling. The dissociation of the heterotrimeric G protein and the recruitment of β-arrestin allows for the activation of a variety of downstream effectors and messengers. G protein signaling is terminated through the recruitment of β-arrestin as well. The signaling of GPCRs involves a highly-regulated network and only some examples of the pathways involved are highlighted.

1.3.4 Heterotrimeric G proteins

Heterotrimeric G proteins are classified into four groups according to their Gα subunit: Gs, Gi/o/z, Gq/11 and G12/13. Generally, stimulatory Gα proteins (Gs) enhance the activity of AC which leads to an increase in intracellular levels of cyclic adenosine monophosphate (cAMP). Conversely, inhibitory Gα proteins (Gi/o/z) inhibit certain AC isoforms which leads to reduced intracellular levels of cAMP. However, prolonged exposure to an agonist leads to an elevation of cAMP levels. This is a reflection of a neuroadaptive mechanism that takes place known as heterologous sensitization that increases the activity of AC (67). In the case of the MOR, elevated levels of cAMP have been found to be a hallmark of opioid dependence in mice (68). In the case of Gq/11 proteins, they stimulate another pathway that involves stimulating phospholipase C, eventually producing secondary messengers, diacylglycerol (DAG) and inositol triphosphate (IP3) which leads to the activation of calcium signaling. On the other hand, G12/13 proteins activate pathways that involve remodeling the cytoskeleton which underlies the regulation of cell shape and motility (69). Most studies identify G protein heterotrimers by the Gα subunit; however, it is also important to note that there are also several Gβγ subunit combinations possible that can interact with each Gα subunit. More specifically, there are 5 Gβ and 12 Gγ genes in human and mouse genomes (70). Although the interaction of both the Gα and Gβγ subunits to different cellular effectors is already well-established, the heterogeneity of the Gβγ isoform combinations with regards to their *in vitro* biochemical activities generally do not significantly differ (71). Different receptors under the GPCR superfamily also appear to preferentially couple to different types of G proteins. However, due to the high sequence similarity of G protein isoforms within the same family, receptors can

often interact with more than one of them (72). With regards to the MOR, it preferentially interacts with the inhibitory G-protein class, Gi/o/z, upon its activation by opioid ligands.

1.3.5 The Inhibitory G Protein Class

Inhibitory Gα proteins, collectively known as Gi/o/z, are the largest and most diverse family of Gα subunits as they are expressed ubiquitously throughout the human body (73). As the name implies, they are generally known to suppress the actions of their main effectors. The family includes six main subtypes: Gi1, Gi2, Gi3, GoA, GoB and Gz. Their sensitivity to pertussis-toxin (PTX) has been used as a classification scheme for these proteins whereby Gi1, Gi2, Gi3, GoA and GoB proteins are sensitive to it, while Gz is not. The general agreement when it comes to the G protein-dependent MOR signaling is that its activation causes the inhibition of AC that reduces the production of cAMP while the stimulation of GIRK channels hyperpolarizes the membrane and subsequently inhibits voltage-gated sodium and calcium channels (74).

The Gαi family includes the subtypes Gi1, Gi2 and Gi3, and are designated with an ‘i’ because of their inhibitory effect on AC. They are highly homologous and are found in many different tissues. Along with the crystal structures of the MOR with various opioid ligands (75), the structure of the MOR with an inhibitory G protein, Gi, has provided an opportunity to understand the biomechanics of G protein signaling (76, 77).

On the other hand, Gαo proteins include GoA and GoB, which are sometimes referred to in the literature as Go1 and Go2, respectively. They are designated with an ‘o’ which stands for ‘other’ as these subtypes were discovered during the purification of Gi proteins from the membranes of the bovine brain (78). Nonetheless, Gαo is the most highly expressed protein

in the mammalian central nervous system, making up approximately 1% of membrane protein in the brain. Not only is it found in the nervous system, but also in the heart and endocrine system. It is highly conserved throughout evolution, as well as across different species, which indicates its importance in signal transduction. Although the abundance of these proteins is apparent, there is still much to be unraveled concerning the mechanistic basis of Go signaling. In fact, not many studies report that Gαo has two splice variants, GαoA and GαoB, that are encoded by GNAO1. The two isoforms in humans are 94% homologous and differ by only 20 amino acids at the C-terminal end of the protein (79). In terms of their role in the nervous system, Gαo has been reported to be a requirement for the regulation of motor control (80) since mutations within the GNAO1 gene have been found to cause epileptic encephalopathy, which is characterized by severe impairments of motor control that include hyperkinetic involuntary spasms and tremors (81, 82). Further mouse studies have highlighted these notable neurological deficiencies as well. A knock-in mouse model, carrying one of the most common mutations found in clinical variants, has also been shown to replicate the development of behavioral phenotypes observed with patients who have de novo mutations in the GNAO1 gene (83). Mice that were deficient in Gαo have also been observed to display hyperalgesia, hyperactivity, and an inability to display coordinated movements (84).

Although Gαo is believed to not directly modulate adenylyl cyclase activity (85), it still influences the production of cAMP (86). Although it is clear that all members of the Gi/o family couple to the MOR, we have previously found that unlike all other inhibitory G proteins, including GαoB, only GαoA does not lead to a decrease in cAMP. Consistent with a previous study (87), among all the Gi/o/z members, GαoA lacks an inhibitory effect on AC activity *in vitro*. GαoA has also been found to have a lower affinity for Gβγ compared to other G proteins

(88). Unfortunately, understanding the discrepancy between both Gαo isoforms has not progressed much since these findings. In fact, studies that distinguish both isoforms from each other are very limited. Even though it does not apply to opioid receptors, a difference between the two has been discovered as GαoA, but not GαoB, was found to be specifically required for transmission of signals in retinal bipolar cells that are important for processing visual information (89). This just highlights the fact that more work needs to be done to uncover the molecular mechanisms that underlie the observations found regarding the roles of GαoA and GαoB, especially in neurotransmission.

1.4 Functional Selectivity

Functional selectivity, also known as biased signaling, is the ability of certain drugs to preferentially activate one intracellular pathway over another (90) (**Figure 1.8**). Not only has this concept been previously shown to occur with the MOR (91), but also for 5-HT2 serotonin, dopamine, β-adrenergic and V2 vasopressin receptors (92), which are also part of the family of GPCRs as well. Upon the activation of the same receptor by different ligands, the receptors are found to adopt multiple conformations due to their highly dynamic nature and structural plasticity. However, biased ligands are believed to stabilize the receptor in a distinct conformation. In turn, the subsequent intracellular events that occur are distinguishable from each other due to the engagement of different effectors. Currently, it remains to be a recognized and accepted concept within the field of GPCR research as it has been deemed to pave the way for improved therapeutics. Many studies have integrated the idea of functional selectivity into the basis of their work, with the promising idea of designing and finding biased ligands (93, 94).

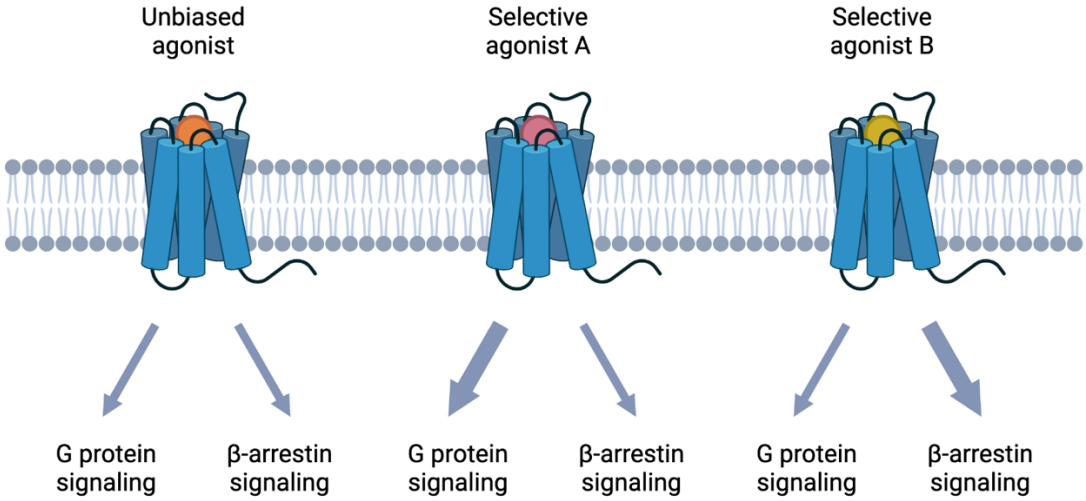

Unbiased agonist
Selective agonist A
Selective agonist B
G protein signaling
β-arrestin signaling
G protein signaling
β-arrestin signaling
G protein signaling
β-arrestin signaling

Figure 1.8 Functional Selectivity at GPCRs. Depending on the type of ligand that act on the same receptor, different signaling pathways can be preferentially activated. Unbiased agonists activate both G protein and β-arrestin signaling pathways while G protein-biased and β-arrestin-biased agonists selectively activate specific pathways that lead to distinct cellular responses.

In the last decade, ligands that selectively activate G protein signaling through the MOR have been discovered based on the premise that the recruitment of β-arrestin could be linked to the undesirable effects of opioid use, particularly respiratory depression (68). One of the first biased ligands to be introduced was oliceridine (TRV130), a compound generated by Trevena, Inc. (95) Another biased MOR ligand worth noting is PZM21, which was generated through virtual screening and molecular docking simulations (94). There have even been recent reports of novel biased MOR agonists that are analogues of already existing ones (96). Compared to morphine, an unbiased MOR ligand, both TRV130 and PZM21 have been found to be more effective in eliciting robust, longer-lasting analgesia while significantly minimizing respiratory depression and constipation typically associated with opioid administration (63). However, these were observations found in preclinical studies that unfortunately did not translate to those found in clinical trials, at least for TRV130. After submission of further data to support the drug, TRV130 was approved by the FDA in 2020 as it was proven to provide rapid onset of analgesia while having a more favourable side effect profile compared to that of morphine (97). Other than morphine, DAMGO, a highly selective MOR agonist, has been the most commonly used reference ligand that activates G protein-dependent and G protein-independent signaling to a similar extent (98). Ultimately, the discovery of G protein biased ligands may improve the therapeutic outcome upon opioid receptor activation by solely narrowing in on the analgesic effects of G protein signaling and dissociating from the adverse events following β-arrestin recruitment. In fact, G protein selectivity has been shown to correlate to broader therapeutic windows that allows for a better separation between opioid-induced nociception and its associated side effects (99).

In the past, several approaches have also been taken to potentially differentiate each Gi/o/z family member. Some of these strategies include using G protein subtype-selective antisense oligonucleotides (100), as well as subtype-selective immunoprecipitation of G proteins that are coupled to the receptor (101). Although these studies are insightful, the only concern with them is that the systems established may not be conducive to study further aspects of G protein signaling. Thereby, the gaps in knowledge with regards to the activation of specific G proteins by GPCRs need to be filled. However, since the most commonly used cell lines routinely express multiple Gi/o/z family members, methods to directly unravel the molecular identity of the G proteins responsible for the intracellular events that occur upon the activation of the receptor have been lacking. Fortunately, Grundmann et al. developed HEK293 cells with no functional G proteins by knocking out all four inhibitory Gα protein families using CRISPR/Cas9 (102, 103). Using this unique cellular model, mechanistic links with regards to G protein signaling can be revealed.

Monitoring G protein activation is certainly an area of interest in the field, and a number of approaches to explore this phenomenon have been introduced over the years. Bioluminescence resonance energy transfer (BRET)- and fluorescence resonance energy transfer (FRET)-based assays have been developed for different pathways that underlie G protein signaling (104). For example, a BRET-based biosensor assay was used to explore the differential activation of inhibitory G proteins at the dopamine D2 receptor (105). In addition, the same approach was used in a recent study which found that among the different Gi/o/z subtypes, GαoB is selectively activated by an agonist for adenosine A1 receptors (106). Although there are studies such as those aforementioned that extensively dissected the individual functional roles of each Gi/o/z protein, there are also studies such that of Saidak et

al., which limit their scope to simply using Gi1 and GoA as a representative of the Gi and Go subfamilies (107).

1.5 Key Pharmacological Concepts

Understanding opioids and how they interact with the MOR also raises questions regarding the properties of each drug. Not only does the toxicity and safety of drugs in physiological systems matter, but also characterizing the drugs themselves is important to build a clearer picture of their pharmacological profiles. The mechanism in which these drugs produce functional responses has long been a fundamental question in pharmacology as well. Nevertheless, there are two inherent properties of drugs that have been established: affinity and efficacy.

The affinity of a drug is its capacity to bind to a receptor as it is determined by the strength of the attraction between the two. It is one of the factors that determine the potency of drugs, which is a measure of the quantity of drugs that can produce an effect at a given magnitude. On the other hand, efficacy is a pharmacological concept that determines the effect of a drug on the activity of the receptor it binds to (108) (**Figure 1.9**). In the case of this study, it would be from the point of view of measuring a response relative to that caused by a reference ligand. However, since there have been different interpretations of how efficacy is defined, how it is truly measured *in vitro* remains to be poorly understood. Furthermore, as a purely drug-dependent property, efficacy, like affinity, is theoretically useful for the characterization of drugs and receptors, but difficult to assess directly compared to affinity. Thus, the efficacy of a drug in relation to the pathway and the system can be generalized as relative efficacy.

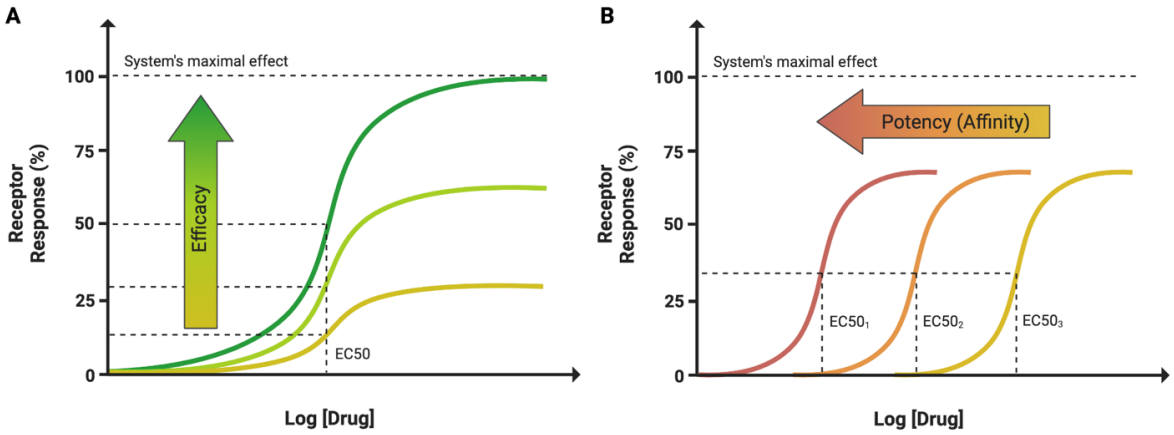

A
System's maximal effect
100
75
50
25
0
Receptor Response (%)
Efficacy
EC50
Log [Drug]
B
System's maximal effect
Potency (Affinity)
100
75
50
25
0
Receptor Response (%)
EC50$_1$
EC50$_2$
EC50$_3$
Log [Drug]

Figure 1.9 Relationship Between Drug Potency, Affinity and Efficacy. A series of agonists acting on the same receptor can result in differences of responses from each dose-response curve. (A) The maximal responses from each agonist indicate the efficacy of each one. In this case, the different agonists vary in efficacy, but have the same affinity for the receptor. (B) Potency is a measure of the amount of drug required to reach half of the maximal response (EC50), which reflects the differences in the affinity of the drug to the receptor.

An early understanding of efficacy is that it was simply a drug-dependent property defined as the capacity of an agonist to induce a conformational change and thus, activate a single population of receptors. However, based on this particular definition, efficacy can only be measured indirectly. Further research has prompted the interpretation of efficacy to be re-evaluated since models have been proposed to further explain the term (108). It has been shown that for GPCRs, which include the MOR, cellular responses can be recorded even in the absence of a ligand, indicating that some receptors are already in an active state prior to the introduction of an agonist. This basal level of receptor signaling activity, also known as constitutive activity, has been exhibited with the MOR in SH-SY5Y cells (109) and transfected HEK293 cells as well (110). Therefore, the concept of receptors existing in an equilibrium between a variety of conformational states was introduced, where some may be present in an active (R*) or inactive (R) form at a given time. There has also been some evidence that the efficacies of drugs that act on the same receptor can vary depending on the response measured. In this case, it does not necessarily mean that if an agonist appears to be a full agonist in one system, it can be assumed that it will also be one in another (111). For example, PZM21 and TRV130 have been described as partial agonists by some studies (112, 113). In contrast, Cong et al. and Ehrlich et al. have found that these same opioid ligands behaved as full agonists instead (91, 114).

When it comes to assessing the relative efficacy of drugs, many studies continue to rely on the functional responses that occur upon receptor activation to measure this important property. Although widely accepted to be sufficient proof, taking into account the characteristics of the system in which the drugs are tested has not been emphasized enough (108). The environment in which the receptors are exposed to has a role in their conformational

states, which can lead to a ligand inducing differential signaling profiles. Additionally, the expression level of the cellular components required for signaling may also influence the functional readout, which, in turn, is used to represent the relative efficacy of the tested drugs. In addition, the level of overexpression as well as the presence of a high receptor reserve may also influence the discrepancies observed from various functional assays (111). The receptor reserve (spare receptors) refers to the condition whereby the agonist activates only a small fraction of the existing receptor population to produce the maximal system response. This implies that a drug can have partial agonism in an equilibrated system (no receptor reserve), whereas it can display full agonism in a system with a receptor reserve. As the receptor reserve serves as a limiting factor, the apparent measurements taken do not reflect drug efficacy, but rather are a result of signaling components involved in the process (115). To be physiologically relevant, the measured drug efficacy should reflect its relative efficacy at a pathway and not the efficacy in a specific *in vitro* system. While efficacy will always be system-dependent, a partial agonist should be partial in all *in vitro* assays used.

Finding suitable tools for quantification is important in order to obtain the most accurate assessment of the true relative efficacy of drugs. The results can then be used to translate the values more easily from *in vitro* systems to animal models and human disease to further study MOR-mediated behaviours, and better reflect the therapeutic potential of each tested drug. Especially for the MOR, developing safer and more effective ligands that act on this GPCR will require unraveling all the important pharmacological parameters that make each one unique.

As already mentioned, one of the holy grails of research in the realm of opioid pharmacology is to improve the side effect profile of opioid ligands. Although much progress

has been made in terms of characterizing opioids and the mechanism behind their actions, there are still concerns with regards to explaining how an improved side effect profile can be achieved. Because of this, many studies have been intensively focused on the idea of functional selectivity since its conception. However, it is worth considering whether the clinical efficacy and reduction of adverse effects observed from novel MOR-biased agonists are due to G protein bias or low relative efficacy (116). After all, one common feature between known MOR-biased agonists such as TRV130 and PZM21 is that they are partial agonists in most *in vitro* assays. Gillis et al. provided some evidence suggesting that improving the side effect profile of opioid ligands concerns their low efficacy instead of their signaling bias (112). For example, a correlation between low efficacy opioid analgesics such as PZM21 and reduced respiratory depression has been observed in mice (94). However, the same observation was found in humans when buprenorphine, a partial agonist at the MOR, reduced the risk of respiratory depression, deeming it safer to use than fentanyl, a full MOR agonist (117). However, Stahl and Bohn are challenging this hypothesis and argue that G protein signaling bias does play a role in reducing the unwanted side effects associated with opioid intake (118). Nevertheless, the connection between low efficacy ligands and the provision of effective analgesia with reduced adverse effects raises the possibility to further investigate not just biased, but also unbiased opioid agonists at the MOR (116).

1.6 Rationale and Study Objectives

Several studies have tackled measuring relative drug efficacy through different methods. However, the relevance of their findings must be put into perspective. A limiting factor in previous studies is that the responses measured to determine the relative efficacy of

drugs may not necessarily reveal their true nature. Recognizing the limitations of each technique should reveal the most appropriate one to accurately interpret experimental results. With that, the main hypothesis of the project is that relative drug efficacy can only be accurately assessed by using a direct measurement of the interaction or activation between the receptor and its effectors without an intermediary component. Instead of looking at more downstream functional responses of opioid ligands acting at the MOR, we shift our focus closer towards the immediate actions that take place once the ligand is bound to the receptor. Through G protein signaling, we could expect to not only gain more insight with regards to the efficacies of each tested drug, but also reveal distinct characteristics of each inhibitory G protein.

To this end, we have concentrated on the following specific objectives:

1) To establish tools to study the direct inhibitory G protein-coupling with the MOR or any GPCR (NanoBiT)
2) To measure and compare various drug efficacies using different G protein-based assays (Glosensor, BRET, NanoBiT with mini-G and full-length G proteins)

The results generated from the study could ultimately establish the primary framework in which the relative efficacy of other opioid ligands can be measured. Obtaining more clarification with regards to this important drug property is needed to further understand how opioids facilitate their analgesic effects. We would also be able to interpret signaling data with caution and analyze them with more accuracy so that we can correlate them to *in vivo* outcomes and prevent high attrition rates in drug development. Collectively, the tools used to study the

interaction between the MOR and AC will further shed light into the molecular basis of G protein signaling. The ultimate goal is to contribute to advancing the field so that we could take one step closer to developing a new generation of effective opioid receptor drug candidates with improved therapeutic potential, and devoid of the side effects associated with currently available opioids.

CHAPTER II: MATERIALS AND METHODS

2.1 Materials

2.1.1 Cell Culture and Transfection

Human embryonic kidney (HEK) 293 cells knockout for all Gα proteins were provided by Dr. Evi Kostenis' laboratory (University of Bonn, Germany) (102). Culturex rat collagen I was purchased from R&D systems. Fetal bovine serum (FBS), 100X antibiotic, antimycotic solution, 0.25% trypsin-EDTA (IX) and Opti-MEM were purchased from Gibco. Dulbecco's Modified Eagle's medium (DMEM) was from Corning. Bovine calf serum (BCS) was from Millipore Sigma, while polyethylenimine (PEI) was from Polysciences, Inc. Phosphate-buffered saline (PBS) was prepared with 1.1 mM potassium phosphate monobasic (KH_2PO_4), 155 mM sodium chloride (NaCl) and 3 mM sodium phosphate dibasic (Na_2HPO_4). Poly-L-Lysine (PLL) was from Sigma-Aldrich. Tissue culture dishes and multi-well plates were from different sources, including Thermo Fisher Scientific and VWR.

2.1.2 Cell-Based Functional Assays

Plasmid constructs of the TRUPATH biosensors were purchased from Addgene, while the MOR-SmBiT and LgBiT-mGα proteins were previously cloned in the laboratory. Hank's balanced salt solution (1X HBSS) was prepared with 1.26 mM calcium chloride ($CaCl_2$) (anhydrous), 0.49 mM magnesium chloride ($MgCl_2$), 0.41 mM magnesium sulfate ($MgSO_4$), 5.3 mM potassium chloride (KCl), 0.44 mM potassium phosphate monobasic (KH_2PO_4), 137.9 mM sodium chloride (NaCl), 0.34 mM sodium phosphate monobasic (Na_2HPO_4) and 0.56 mM D-glucose (dextrose). Coelenterazine 400a (DeepBlueC™) was from NanoLight

Technologies. Furimazine was obtained from ChemShuttle. D-Luciferin was purchased from Cayman Chemical, while forskolin (FSK) was from LC Laboratories.

2.1.3. Opioid Ligands

DAMGO and fentanyl were obtained from Cayman Chemical. Morphine was purchased from Toronto Research Chemicals. Loperamide was from Tocris Bioscience. TRV130 was from Adooq Bioscience, while PZM21 was from Biovision.

2.2 Methods

2.2.1 Cell Culture

In a humidified atmosphere at 37°C with 5% CO2, human embryonic kidney (HEK) 293 cells knockout for all Gα proteins (4GKO) (102) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 5% bovine calf serum (BCS) and 1% antibiotic-antimycotic (anti-anti). They were also passaged and transfected using the aforementioned medium. To simplify the name of this cell line, we have dubbed it as 'G Less' cells and this term will be used henceforth. On a weekly basis, G Less cells were passaged twice as they were maintained at approximately 90% confluency. These cells were generally passaged for at most 20 times before thawing frozen cells. To keep cells in stock, they were resuspended in freezing media (70% DMEM, 20% FBS and 10% DMSO) before being frozen at -80°C using the CoolCell LX Freezing Container (Corning). Prior to their use, cell culture dishes and plates were coated with 50µg/mL rat tail collagen I to aid with cell adherence. For every assay mentioned hereafter, the way in which the cells are first prepared is identical. Depending on the size of the cell culture dish used, the

density of plated cells varies. For each well a 6-well plate, 700,000 cells were plated while half this number was used for a 12-well plate. The cells were then allowed to grow overnight before subjecting them to transfection.

2.2.2 Transient Transfection

Once the cells are determined to have reached around 60% confluency after being grown overnight, transient transfection was performed using polyethylenimine (PEI). Since the functional assays require the incorporation of multiple plasmid DNA constructs in each tested condition, every component is added to the DNA mix in an equal ratio. In total, 3 μg of DNA in Opti-MEM was prepared for each well in a 6-well plate. An addition of PEI was followed at a ratio of 3:1 of PEI to DNA. The prepared mixes were vortexed, then incubated for 30 minutes at room temperature before being added dropwise to the cells. In addition, a construct that contains a gene that encodes for yellow fluorescent protein (YFP) was transfected alongside each set of conditions to get a gauge of the transfection efficiency, which is determined 24h later.

2.2.3 GloSensor Assay

Originally developed by Fan et al. (119), but now tied to the Promega Corporation, the GloSensor assay allows for the quantitative detection of cAMP in live cells as it is used as a measurement of G protein activation. It is based on a genetically modified firefly luciferase enzyme that emits light in the presence of its substrate (D-Luciferin) (120) (**Figure 2.1**). Modifications have been made to the original protocol, but the overarching principle of this assay is still maintained. HEK293 cells knocked out for all Gα proteins were co-transfected

with different constructs after verifying that there was an appropriate number of cells in the cell culture dish. These constructs include the human MOR (hMOR), each of the inhibitory Gα proteins, and a plasmid containing a gene that encodes for a split luciferase-based cAMP biosensor (Glosensor). After 24h, the cells were harvested with 0.05% trypsin-EDTA, followed by centrifugation to collect the cells. The cells were resuspended in starvation media (DMEM, 1% FBS, 1% anti-anti) before an appropriate cell number was seeded in each well of a 384-well, flat, clear-bottom, white plate pre-coated with rat tail collagen I. Following incubation overnight at 37°C with 5% CO2, the media was removed from the plate. Immediately after, 20 µL of 1 mg/mL D-Luciferin in the assay buffer (1X HBSS, 20 mM HEPES, pH 7.4) was added to each well. The cells were then incubated in the dark for 30 minutes before they were treated with opioid agonists at 3X of the desired final concentrations. After 15 minutes at room temperature, forskolin (FSK) at a final concentration of 2 µM was added to the cells and incubated for another 10 minutes. Since FSK is a direct activator of AC, its addition causes cAMP levels to increase. However, the main purpose of FSK is to simply increase the level of detection of cAMP reduction, which in turn, is used as a reflection of the inhibitory effect of Gαi/o/z protein signaling. Reading the subsequent luminescence signals post-incubation was completed using the HiDex Sense microplate reader. As a comparative measure, the same assay was completed using HEK293T cells that stably expressed hMOR and Glosensor. The cells were then seeded in plates pre-coated with PLL before running the experiment with the panel of 6 opioid ligands.

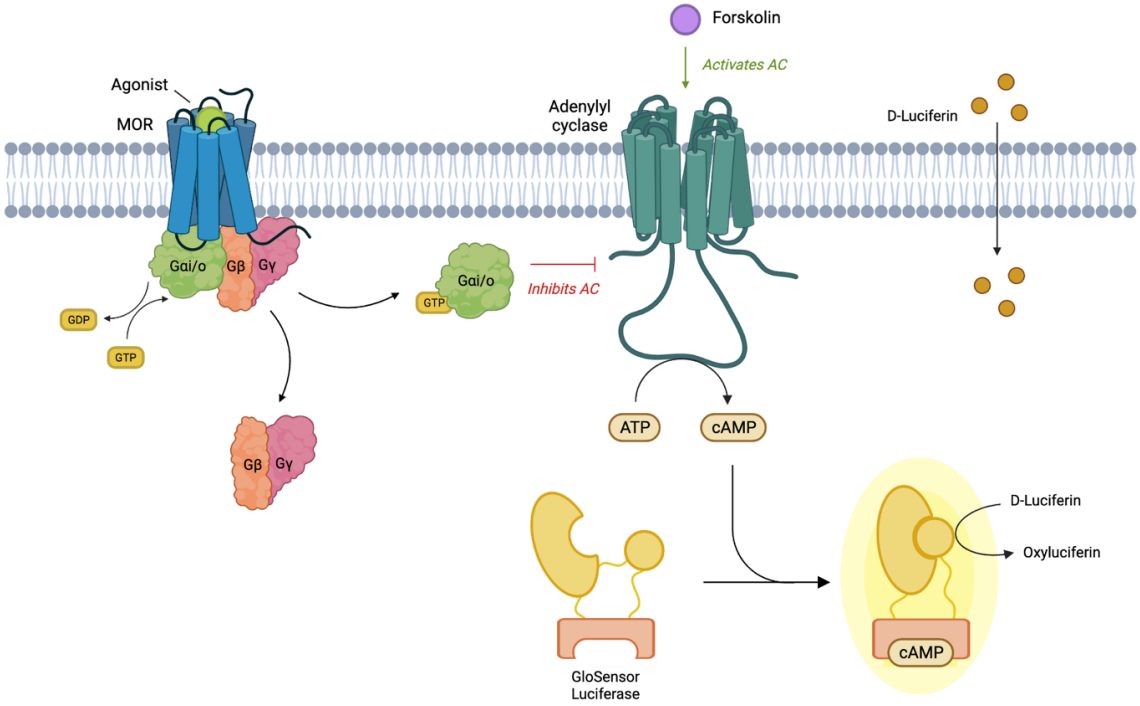

Forskolin
Activates AC
Agonist
MOR
Adenylyl cyclase
D-Luciferin
Gαi/o
Gβ
Gγ
GDP
GTP
Gαi/o
GTP
Inhibits AC
Gβ
Gγ
ATP
cAMP
D-Luciferin
Oxyluciferin
cAMP
GloSensor Luciferase

Figure 2.1 Schematic Representation of the GloSensor Assay. Forskolin directly activates AC to increase the production of cAMP. As an agonist binds to the MOR, the dissociation of the heterotrimeric G protein allows the Gαi/o/z subunit to inhibit AC activity, thus reducing the amount of cAMP produced. The binding of cAMP to GloSensor Luciferase causes a conformational shift that allows for the oxidation of the D-Luciferin substrate. As a result, light is produced and the measured light intensity corresponds to the amount of cAMP generated, reflecting the extent in which AC is activated.

2.2.4 TRUPATH BRET2 Assay

The BRET2 assay system developed by Olsen et al. was adopted to directly measure G protein dissociation upon the activation of the MOR using TRUPATH Gαβγ biosensors (121) (**Figure 2.2**). Although the original protocol identified and suggested the most optimal Gβ/Gγ-GFP2 biosensors to use for each Gα-RLuc8, we have decided to move forward with the same set of Gβ/Gγ-GFP2 for the purpose of this study. HEK293 cells knocked out for all Gα proteins were plated at a density of 700,000 cells per well in a 6-well plate. They were grown overnight in DMEM supplemented with 10% FBS/BCS and 1% anti-anti. Cells were then co-transfected with 4 plasmids encoding the human MOR, Gα-RLuc8, Gβ3, and Gγ9-GFP2 at a 1:1:1:1 ratio (750 ng/construct). Transfection was completed using PEI at a ratio of 3:1 of PEI to DNA in Opti-MEM. After 24h, cells were washed with PBS before being detached with 0.05% trypsin-EDTA and plated with starvation media (DMEM, 1% FBS, 1% anti-anti) in a 96-well clear bottom, white plate pre-coated with rat tail collagen I at a density of 60,000 cells/well. The next day, the starvation media was carefully aspirated and replaced with 60 µL of assay buffer (1X HBSS, 20 mM HEPES, pH 7.4). In each well, 10 µL of 50 µM coelenterazine 400a was added before incubating for 5 minutes at room temperature. The addition of an increasing concentration of opioid agonists was done right after at 30 µL each. After an additional 5 minutes at room temperature, the plates were read serially at least 4 times using the BioTek Synergy Neo2 Multi-Mode Reader with 395 nm and 510 nm emission filters. BRET2 ratios were immediately calculated by the reader as a ratio of GFP2 to RLuc8 emissions. A reduction in the BRET2 signal was expected as the activation of the MOR promotes the dissociation of the heterotrimeric G protein (Gαβγ) complex.

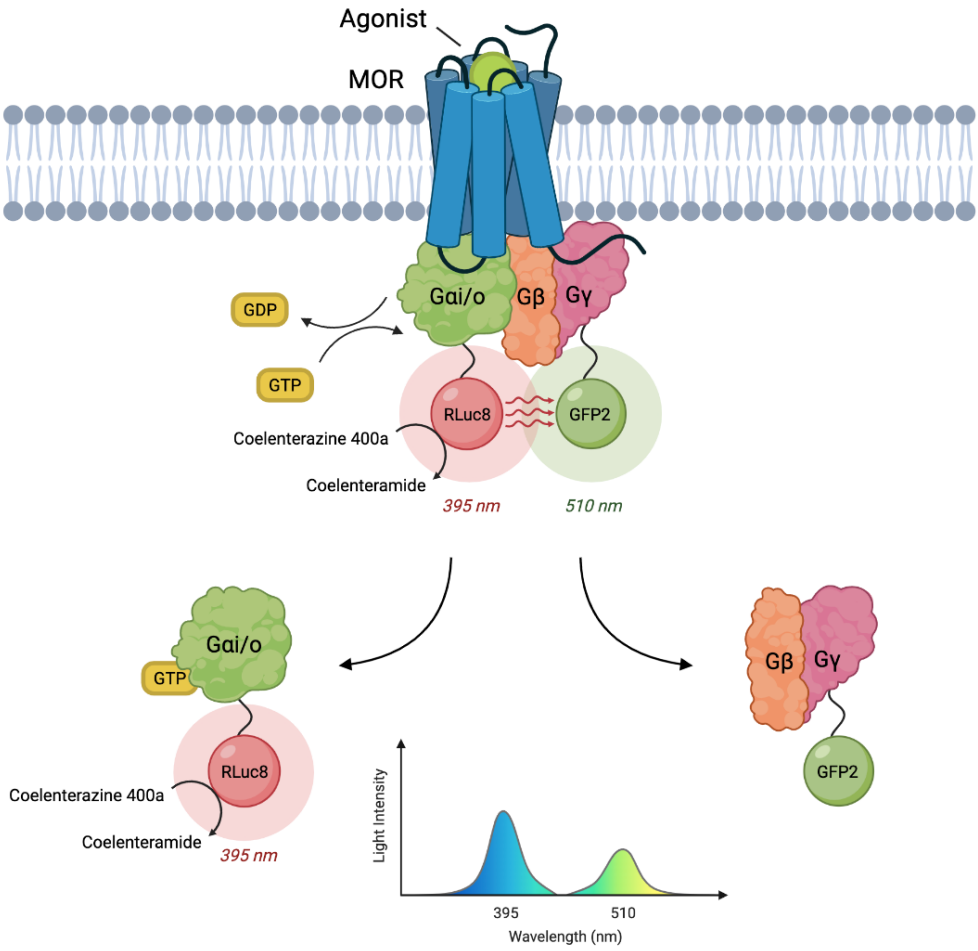

Agonist
MOR
Gαi/o
Gβ
Gγ
GDP
GTP
RLuc8
GFP2
Coelenterazine 400a
Coelenteramide
395 nm
510 nm
Gαi/o
GTP
RLuc8
Coelenterazine 400a
Coelenteramide
395 nm
Gβ
Gγ
GFP2
Light Intensity
395
510
Wavelength (nm)

Figure 2.2 Principle of the TRUPATH BRET2 Assay. The dissociation of the heterotrimeric G protein upon receptor activation is monitored by tagging Gα with RLuc8 and Gγ with GFP2. To facilitate resonance energy transfer, RLuc8 acts as the luciferase donor, while GFP2 is the fluorophore acceptor. These advanced biosensors were developed in order to study protein-protein interactions based on proximity. In the presence of the luciferase substrate, coelenterazine 400a, RLuc8 emits light at a wavelength of approximately 395 nm, which subsequently excites GFP2 to emit a fluorescent signal at 510 nm to indicate the close interaction between the two proteins of interest. The BRET signal is quantified by the ratio of the emissions of GFP2 to those of RLuc8.

2.2.5 NanoBiT Assay

NanoLuc Binary Technology (NanoBiT) has been developed as a structural complementation assay to study protein-protein interactions. The novel reporter used in this assay is known as NanoLuc luciferase. Its hallmark feature is that it is divided into two subunits wherein one is designated as Large BiT (LgBiT; 18 kDa) and the other as Small BiT (SmBiT; 1.3 kDa). The principle of the assay is that when these are fused to proteins of interest, the interaction of the tagged target proteins allows for the LgBiT and the SmBiT to form an active enzyme. As a result, it generates a bright, luminescent signal in the presence of its substrate, furimazine, a coelenterazine analogue (**Figure 2.3**). In the case of this study, we looked at full-length Gαi/o/z proteins to mini-Gαi/o/z proteins fused to LgBiT in how they compare in terms of being recruited to the MOR fused to SmBiT. As described by Wan et al. (122), the difference between the two sets of proteins fused to LgBiT is that the mini-G proteins have several modifications that include a truncated N terminus that prevents it from anchoring to the membrane and binding to the Gβγ dimer; a deleted α-helical domain; several mutations to enhance its stability *in vitro*; and a mutation in its C-terminal region that stabilizes the way in which they make a complex with the receptor in the presence of guanine nucleotides.

As for the workflow of preparing the assay, it starts with co-transfecting several constructs in G Less cells that include the MOR-SmBiT and each inhibitory G protein family member fused to LgBiT. Upon successful transfection, the cells are harvested with 0.05% trypsin-EDTA. They are resuspended in starvation media (DMEM, 1% FBS, 1% anti-anti), and subsequently seeded in a 384-well, flat, clear-bottom, white plate pre-coated with rat tail collagen. On the day of running the assay, the media is removed before 20 µL of 5 µM

furimazine with assay buffer (1X HBSS, 20 mM HEPES, pH 7.4) is added to the cells. The plate is incubated for 10 minutes at room temperature before luminescence readings were collected using the Fluorescent Imaging Plate Reader (FLIPR) Tetra system (Molecular Devices). Prior to any additions, baseline luminescence levels were measured. To stimulate MOR-SmBiT, 10 μL of drugs at 3X of the final concentrations were added into each well. The subsequent changes in relative luminescence signals were recorded over time and were used as a reflection of Gαi/o/z protein recruitment.

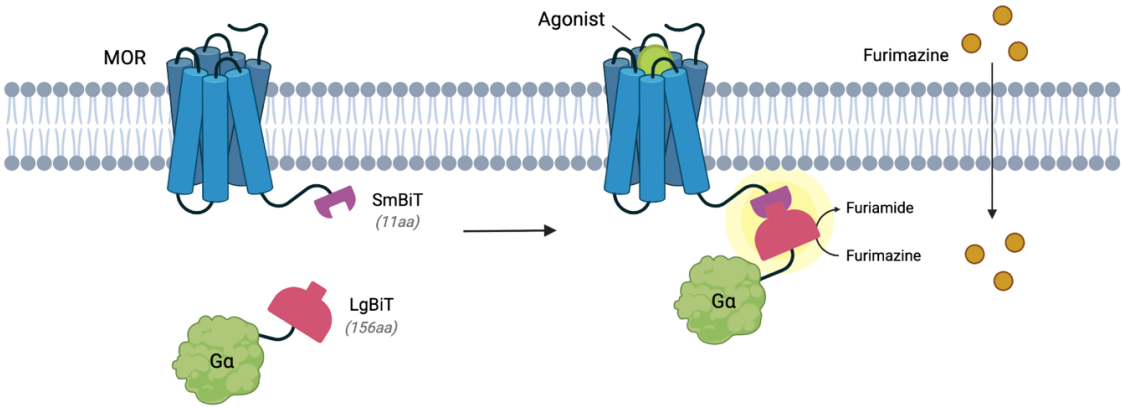

MOR
SmBiT
(11aa)
LgBiT
(156aa)
Gα
Agonist
Furimazine
Furiamide
Furimazine
Gα

Figure 2.3 Monitoring G Protein Coupling to the MOR via the NanoBiT Assay. To monitor the real-time interaction of the Gα subunit to the MOR, both the receptor and the Gα protein were tagged with SmBiT and LgBiT, respectively. The structural complementation of SmBiT and LgBiT is the basis of the NanoLuc Binary Technology (NanoBiT) system. Upon receptor stimulation by an agonist, the Gα protein is recruited to the receptor that facilitates the interaction of SmBiT and LgBiT, forming a complete NanoLuc luciferase enzyme. A luminescent signal in the presence of the furimazine substrate follows.

CHAPTER III: RESULTS

3.1 Profiling the relative efficacies of opioid ligands through G protein activation using cell-based assays

In this study, we focus on the MOR as the most effective opioid agonists target this receptor to mediate their analgesic effects to combat acute and chronic pain (123). We intended to profile the relative efficacies of a panel of opioid agonists against the MOR with respect to how the receptor discriminates between the six members of the Gi/o/z family. To do so, we used cell-based assays that take advantage of advanced biosensors. More specifically, we use the GloSensor, TRUPATH BRET2, and NanoBiT assays to measure the inhibition of forskolin-stimulated levels of cAMP, dissociation of the G protein heterotrimer, and the interaction between the MOR and each Gα protein, respectively. By using the three distinct experimental systems, the aim was to obtain a clearer picture of what occurs proximally upon receptor activation according to the tested opioid ligands, not to mention the role of the individual Gα subunit.

In all cases, the same cell line was employed as a system to investigate the activation of G proteins. Although there is a wide range of available opioid ligands that act on the MOR, only a selected few were chosen to be part of this study. These ligands include DAMGO, morphine, fentanyl, TRV130, PZM21 and loperamide. All drugs were tested simultaneously to efficiently compare their relative potencies and efficacies. In terms of the generation of dose-response curves, fitting the data points with nonlinear regression, correcting the baseline of these curves, determining the EC50 values for each one and extracting kinetic parameters were performed using GraphPad Prism 9.

3.1.1 Assessing G protein activation via cAMP reduction using the GloSensor assay

As a means to look at the capacity of each drug to activate the MOR with regards to each Gα protein, we measured the inhibition of forskolin-stimulated levels of cAMP using the GloSensor assay in G Less cells (**Figure 3.1A**). Having DAMGO as the reference ligand, fentanyl appears to be the most potent out of the panel of ligands tested, while TRV130 and PZM21 display the lowest efficacies among them. Each drug that activated Gαi1, Gαi2 and Gαi3 are the most similar in terms of their potencies and efficacies, while morphine appears to be generally less potent than the rest of the ligands in all conditions. The most notable differences, however, are observed with GαoA and GαoB. As clearly seen, when GαoA is involved, the drugs only inhibit the production of cAMP to a maximum of 60% unlike all other inhibitory Gα proteins. Not only do their efficacy profiles differ, but each drug seems to be less potent as well. On the other hand, GαoB displays properties that are more comparable to the majority of the Gα proteins. However, the appearance of TRV130 and PZM21 as partial agonists compared to the full agonist, DAMGO, is most evidently seen with GαoB. With both Gαo proteins, morphine seems to be a partial agonist, which contrasts with its full agonist activity shown with Gαi and Gαz proteins.

Since we took advantage of using G Less cells for the succeeding experiments, we ran the GloSensor assay using a stable HEK293T cell line that expresses the human MOR and GloSensor to consolidate the benefits of using G Less cells over its regular HEK293 counterpart for the purpose of this study (**Figure 3.1B**). Among the 6 opioid ligands tested, all appear to be full agonists with morphine and loperamide being less potent than the rest. The results in terms of efficacies and potencies are also similar to that of Gαi in G Less cells, with PZM21 having a different profile as it is less potent than it appears to be in HEK293T cells.

Since HEK293T cells endogenously express multiple members of the Gαi/o/z family, it appears that Gαi proteins are the most predominantly activated G proteins to cause a reduction in cAMP levels upon the activation of the MOR. In terms of the discrepancy observed in the potency of PZM21 in G Less and HEK293T cells, there may be unknown factors that played a role in the affinity between the drug and the receptor.

Furthermore, we noticed that there was a pattern of forskolin-stimulated levels of cAMP that was followed by each Gα protein in G Less cells when the receptor was not exposed to any ligands. As summarized in **Figure 3.2**, the increase in the basal levels of cAMP is not quite significant with GαoA and Gαz, as well as that of Gαi2 to some degree. The difference between the two Gαo isoforms is also highlighted wherein the basal cAMP level of GαoB is almost three times as high as that of GαoA. Although there are variations with these measurements, it is worth noting again that all Gα isoforms can exhibit a complete inhibition of cAMP except for GαoA.

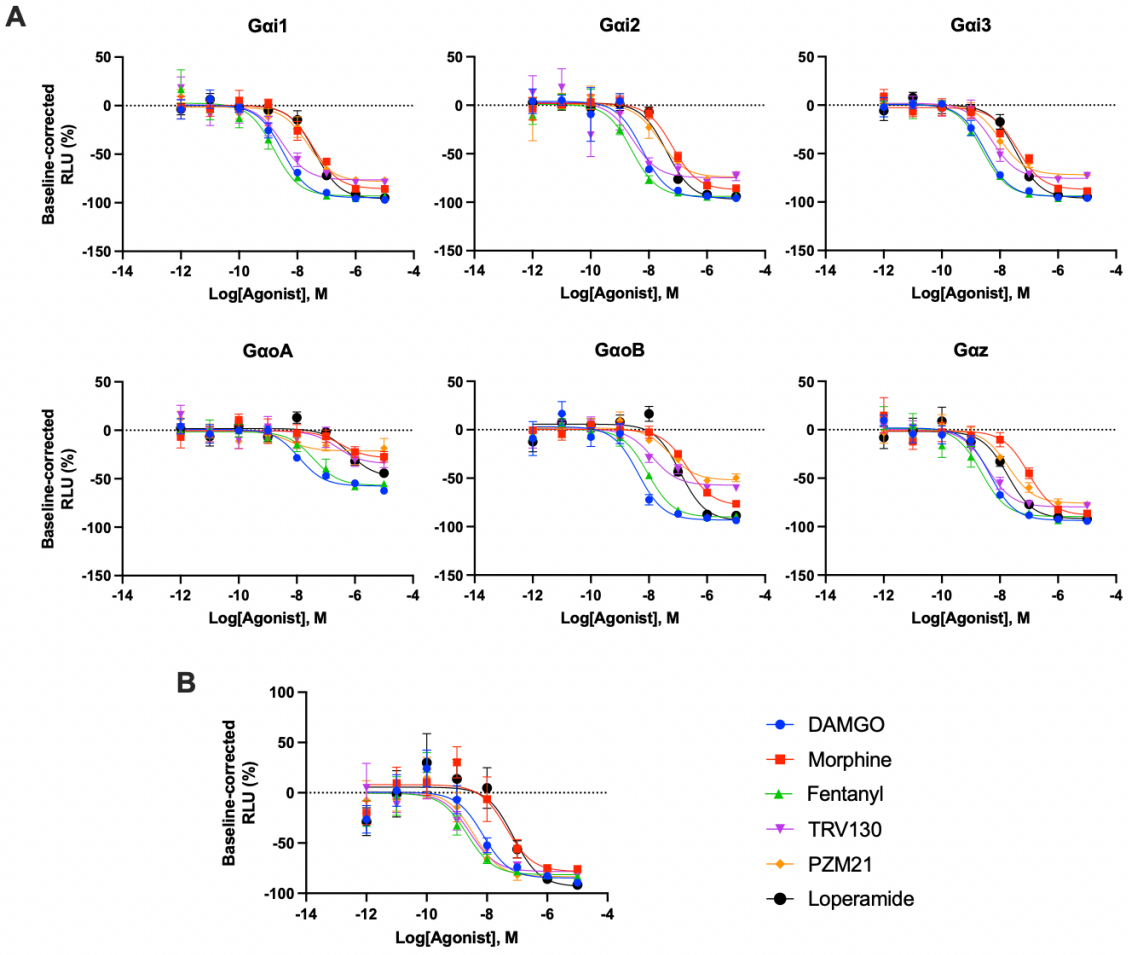

A
Gαi1
Gαi2
Gαi3
GαoA
GαoB
Gαz
Baseline-corrected RLU (%)
Log[Agonist], M
B
DAMGO
Morphine
Fentanyl
TRV130
PZM21
Loperamide

Figure 3.1 The reduction of cAMP as a measure of G protein activation. (A) G Less cells and (B) HEK293T cells stably expressing hMOR and GloSensor were treated with 2 μM FSK and increasing concentrations of a panel of opioid ligands. These drugs were tested against the MOR in relation to each Gα protein in G Less cells. With the baseline being FSK with no agonist, the data is represented as baseline-corrected dose-response curves which show the percentage of luminescence, reflecting the extent in which cAMP was inhibited. Each data point is a representation of the average of three replicate luminescence measurements along with their respective standard errors.

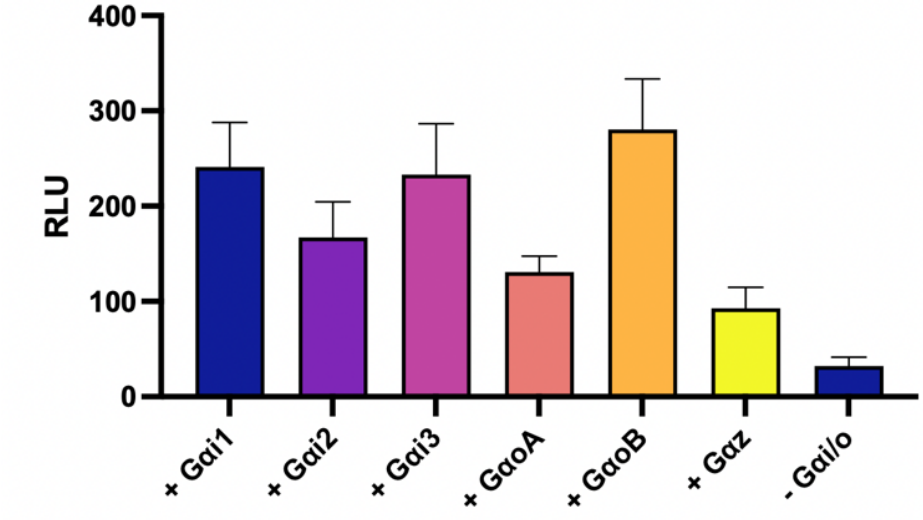

400
300
200
100
0
RLU
+ Gαi1
+ Gαi2
+ Gαi3
+ GαoA
+ GαoB
+ Gαz
- Gαi/o

Figure 3.2 Forskolin-stimulated levels of cAMP without receptor stimulation. In the presence of the MOR and each inhibitory Gα isoform, the increase of the basal levels of cAMP was measured after the addition of 2 μM FSK in G Less cells. Since the total amount of DNA transiently transfected was kept constant, an empty pcDNA3.1(+) vector was used to make up for the lack of Gα proteins and serve as a control. Having been observed in multiple experiments, the data included at least six replicate luminescence measurements.

3.1.2 Assessing G protein activation using BRET2-based biosensors

To detect the dissociation of the Gαβγ heterotrimer upon the activation of the MOR by a variety of ligands, we took advantage of BRET-based biosensors from the TRUPATH platform (121). Although the original report recommends the use of a particular combination of βγ-GFP2 chimeric acceptors for each Gα-RLuc8 chimeric donor, we have decided to use Gβ3 and Gγ9-GFP2. The reason behind this choice is to limit the number of variables that could possibly deter from a focal point of the study, which is to determine the differences between each inhibitory Gα protein. Olsen et al. also observed that Gβ3γ9 is the most optimal construct for most Gα subunits, with our own optimization efforts confirming that these do provide the most robust G protein activation among the possible combinations of dimer constructs.

As shown in **Figure 3.3**, the overall maximum response recorded in the system, regardless of the ligand, is approximately the same for each inhibitory Gα protein except for Gαi1, which displays around half the maximum of the others. Although not shown, the signals obtained for Gαi1 only stabilized after around 15 minutes post-stimulation, whereas all others more or less displayed the same results from 5 minutes onwards. In general, the data shows drug efficacies and potencies distinct from those observed from the GloSensor assay. One example of such observation is with regards to Gαi3 as all ligands appear to be less potent than those from the GloSensor assay. In addition, all ligands, except for morphine, have weaker efficacies relative to that of DAMGO. When it comes to GαoA, a striking difference is seen as it appears to be well-activated using this assay that measures the dissociation of the G protein heterotrimer as opposed to its ability to inhibit cAMP.

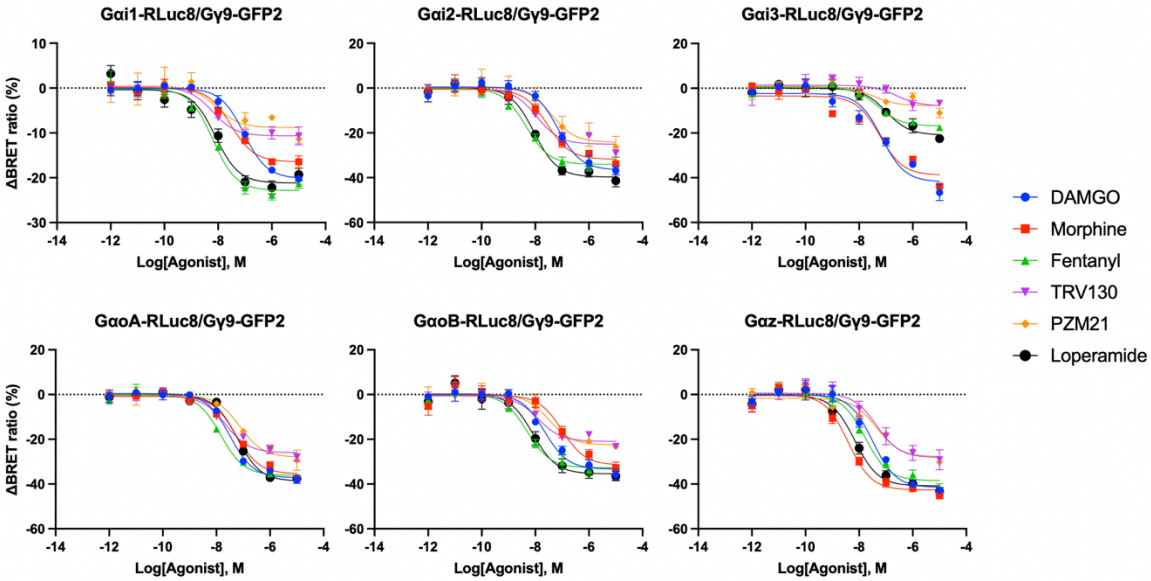

Gαi1-RLuc8/Gγ9-GFP2
Gαi2-RLuc8/Gγ9-GFP2
Gαi3-RLuc8/Gγ9-GFP2
GαoA-RLuc8/Gγ9-GFP2
GαoB-RLuc8/Gγ9-GFP2
Gαz-RLuc8/Gγ9-GFP2
ΔBRET ratio (%)
Log[Agonist], M
DAMGO
Morphine
Fentanyl
TRV130
PZM21
Loperamide

Figure 3.3 BRET-based responses in G Less cells upon stimulation with MOR agonists. TRUPATH biosensors for the heterotrimeric G proteins were used, specifically using each Gα with RLuc8, Gβ3 and Gγ9-GFP2. The BRET ratios, calculated as fluorescence over luminescence signals, reflect the dissociation of the heterotrimeric G protein complex upon receptor activation. The data is a record of responses obtained at 25 minutes after the stimulation of the receptor. Baseline-corrected dose-response curves are placed above the data points shown as the mean and standard errors from experiments performed in duplicate.

3.1.3 Assessing G protein activation by the recruitment of mini-G and full-length G proteins to the receptor using the NanoBiT assay

GPCRs are conformationally dynamic and reach a stasis between the ligand-free inactive and active conformation. Measuring the dynamic interaction between a GPCR and G protein is difficult, given the transient nature of this interaction. Recently, our collaborator, Dr. Nevin A. Lambert, elegantly reported the dynamic studies of the interaction between the 5HT7 receptor and Gαs using mini-G proteins (124). We, therefore, chose to study the dynamic interaction between the MOR and G protein using mini-G proteins (mGα). These engineered Gα subunits were designed as an *in vitro* tool to stabilize the active conformation of GPCRs (122) and thus provide a good tool to study live conformational transitions when coupled with the NanoBiT complementation assay as previously described (122). In this case, we fused the SmBit peptide to the C-terminus of the MOR (hOPRM1 isoform) and synthesized codon-optimized mini-G proteins, which were then co-transfected in G Less cells. When the G protein is recruited to the receptor upon its activation, a functional NanoLuc luciferase enzyme is formed, allowing for agonist-concentration dependent luminescence signals to be obtained in the presence of the substrate, furimazine.

As presented in **Figure 3.4**, the recruitment profile of each mini-G protein slightly varies. In terms of their maximal fold change, mGαi1, mGαoA and mGαoB display the highest among all mini-G proteins with around a 60-fold increase, with this increase referring to the change in RLU between the apparent measurements and their respective basal levels. In decreasing order, mGαz, mGαi3 and mGαi2 follow. When it comes to the potencies of the drugs, they seem to fall under a narrow range that does not significantly change between each mini-G protein. From the panel of opioid ligands, DAMGO seems to generally show the

highest efficacy, with fentanyl and loperamide next. Morphine, TRV130 and PZM21 appear to have partial agonist activity that is at least half of the maximum efficacy in each condition. It even has an efficacy lower than that of TRV130 and PZM21 for mGαi2. Another observation is that the G protein-biased agonists, TRV130 and PZM21, have a much lower apparent efficacy than those found in previous assays. We explain this discrepancy because GloSensor and BRET2 are amplification systems, wherein an active receptor will catalytically activate many G proteins, which in turn, can modulate the enzymatic activity of AC. However, the NanoBiT assay involves a linear measurement. With the mini-G protein complementation approach, a receptor can only complement one mini-G protein resulting in a linear correlation (no amplification). It is important to mention that we are referring to apparent coupling efficacy as mini-G proteins might not reflect the coupling mechanism of full-length G proteins as they lack all proper conformational dynamics of full-length G proteins and most likely do not interact with G protein regulators.

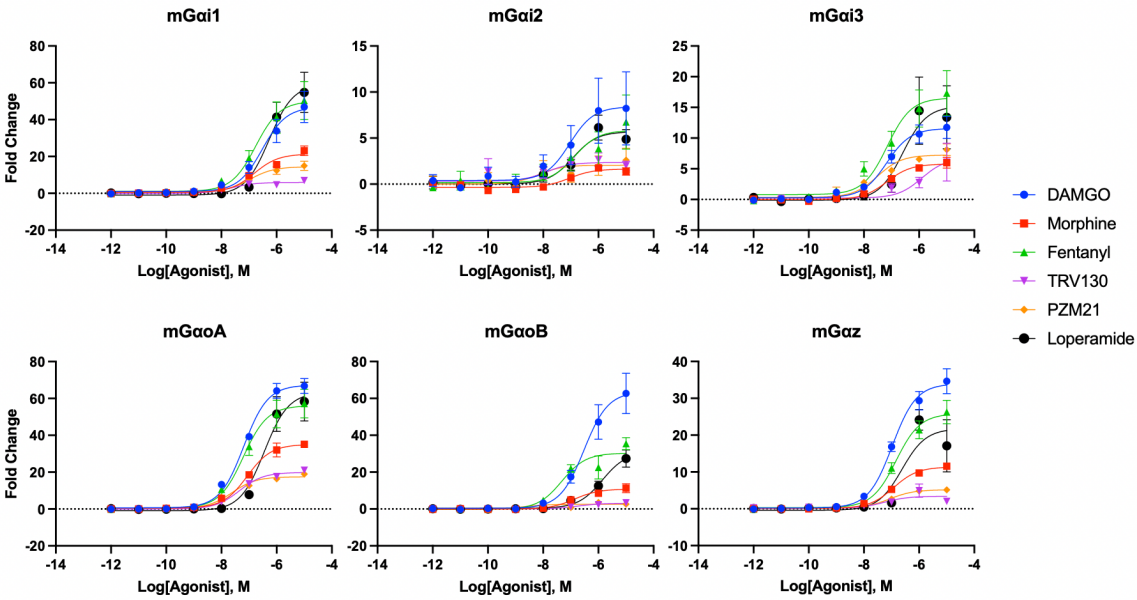

mGαi1
mGαi2
mGαi3
mGαoA
mGαoB
mGαz
Fold Change
Log[Agonist], M
DAMGO
Morphine
Fentanyl
TRV130
PZM21
Loperamide

Figure 3.4 Dose-curve responses based on mini-G protein recruitment to the MOR. Taking advantage of the NanoBiT assay, MOR-SmBiT and LgBiT-mGαi/o/z proteins were transiently co-transfected in G Less cells. The stimulation of the MOR by a series of opioid agonists recruited each inhibitory Gα subunit to the receptor, which is reflected by the corresponding luminescence values measured. Data are shown as mean ± SEM of baseline-corrected maximum luminescence readings from experiments performed in triplicate.

3.2 Identifying factors that affect the differential activation of G proteins at the level of the receptor

The GloSensor, BRET2 and NanoBiT assays allowed us to assess the relative efficacies and potencies of a series of opioid ligands at a specific time point. The design of the assays has also provided different perspectives on how inhibitory G proteins are activated as well. Although the differential activation of G proteins is not a new concept, previous studies fall short of extensively examining the entire family. For this reason, we made sure to include all inhibitory G protein isoforms that are known to couple to the MOR. However, when it comes to GPCR signaling kinetics, they do not have a linear correlation or even reach an equilibrium. GPCRs are large allosteric machines where signaling is tightly controlled by many intrinsic and extrinsic factors. Moreover, the signaling kinetics may be different for each signaling pathway of interest.

For these reasons, the timing of each measurement could be an important factor to consider. For most of these assays, multiple readings are taken at different times and the reading with the highest Emax value is selected. However, the time at which a full agonist reaches its Emax may differ from that of a partial agonist, leading to an underestimation of its efficacy and thus, its potency as well.

Having the tools to look at the proximal aspects of G protein signaling gave us the opportunity to further define what factors play a role in their distinct activities because these essentially affect the measured apparent efficacies of ligands that act on the receptor. In the case of this study, we used the NanoBiT assay to do so since it is a readily available tool that we had which allowed us to examine the real-time kinetics between the receptor and each G protein.

3.2.1 Kinetics of G protein recruitment and dissociation upon receptor activation

The NanoBiT assay used in this study is designed to detect the live, real-time, reversible action of the two fragments, SmBiT and LgBiT, which are associated with the MOR and mGαi/o/z proteins, respectively. Although the initial purpose of running this assay was to profile a panel of opioid ligands in terms of mini-G protein recruitment to the receptor, the manner at which the data was collected already revealed the kinetics of the interaction as relative luminescence measurements were captured over time. Not only does the assay provide information regarding the efficacies of each drug, but also uncovers more details regarding the mechanisms that determine these important parameters. In addition, since mini-G proteins lack the helical domain and switch regions, they do not interact with guanine nucleotides, thereby do not change conformation after receptor activation as well. Therefore, there is no allosteric cross-regulation between the activated receptor and each G-protein. We expected mini-G proteins would only interact with ligand-activated receptors considering their initial design.

As illustrated in **Figure 3.5**, the kinetic profile of mGαoA and mGαoB is different from that of mGαi1, mGαi2, mGαi3 and mGαz regardless of the type of ligand the receptor was stimulated with. After the addition of the different opioid agonists, the responses associated with mGαoA and mGαoB displayed an initial rapid increase, reaching a peak before it declined to a level above the baseline. On the other hand, the rest of the responses also started with a rapid increase but reached a point wherein it relatively remained constant over time. By comparing the overall kinetics, both mGαo isoforms display a biphasic response called rise-and-fall to steady-state curve while the others show a monophasic association exponential curve. These terms were used by Hoare et. al to effectively describe some of the time-course profiles typically associated with GPCR signaling (125, 126). For mGαi1, mGαi2, mGαi3 and

mGαz, it seems that their recruitment to the MOR upon agonist stimulation subsequently led to the formation of a stable complex between the two for the duration of the assay. However, for both mGαo isoforms, their recruitment to the MOR was almost immediately met with their dissociation from the receptor. These results suggest that the way in which the mini-G proteins interact with the receptor upon its activation differs. Especially for the characteristic kinetic profiles of mGαoA and mGαoB, the involvement of varying types of regulatory processes may have likely played a role as well.

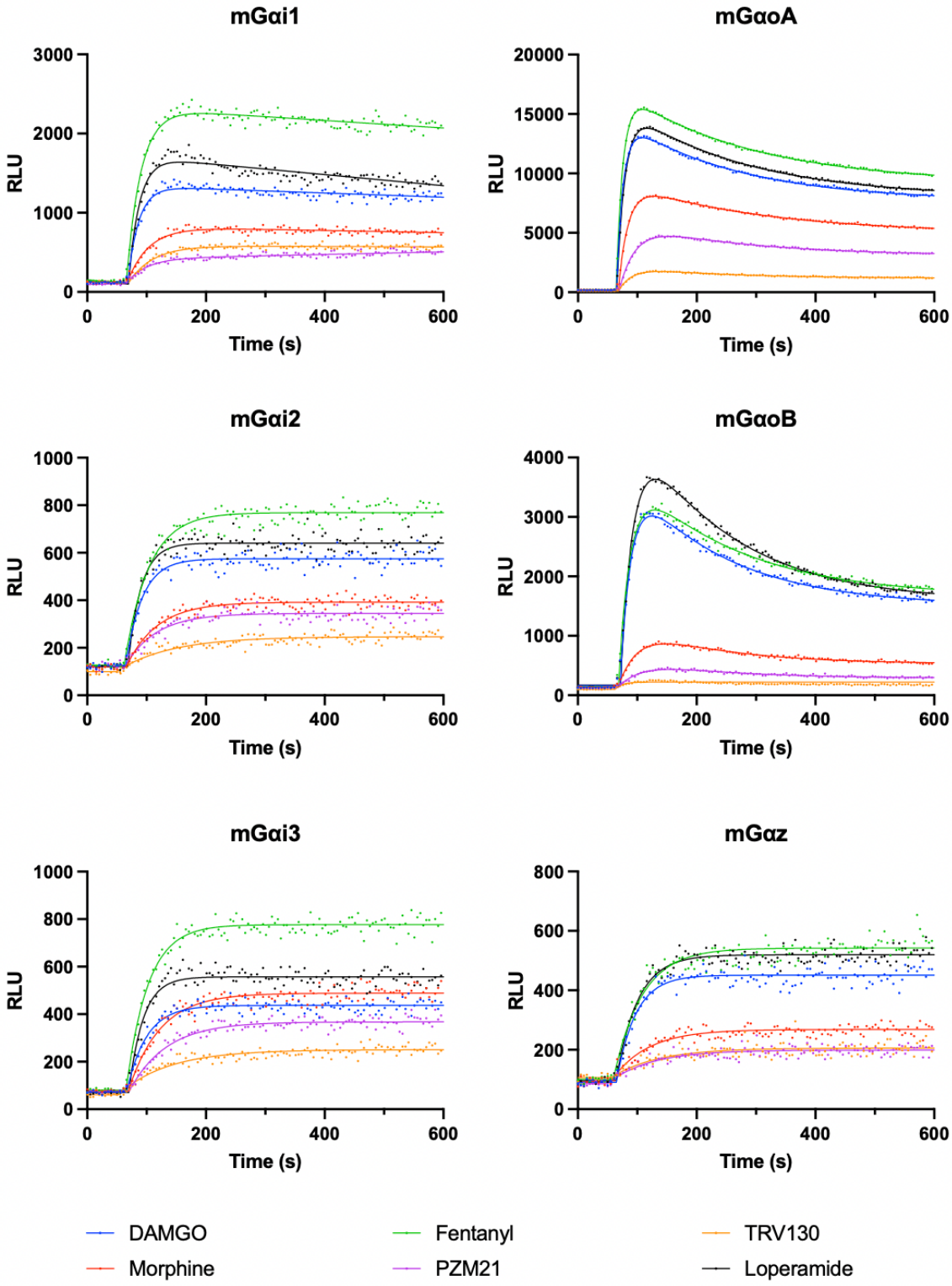

mGαi1
mGαoA
mGαi2
mGαoB
mGαi3
mGαz
RLU
Time (s)
DAMGO
Morphine
Fentanyl
PZM21
TRV130
Loperamide

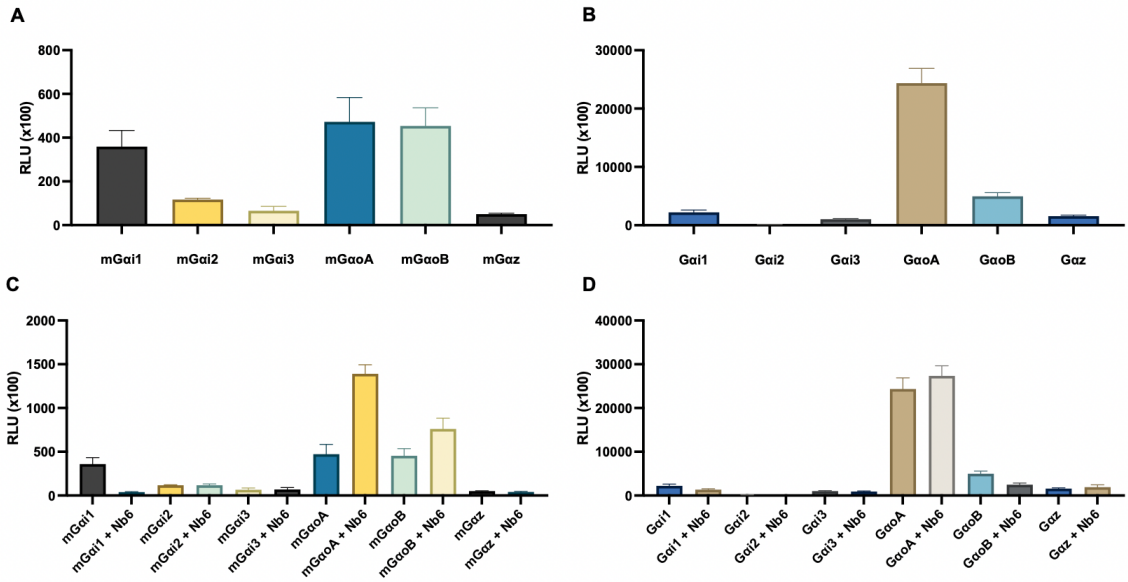
Figure 3.5 Monitoring the kinetics of mini-Gαi/o recruitment to the MOR. The NanoBiT assay was used to follow the dynamics between the proteins of interest in G Less cells since the interaction between SmBiT and LgBiT is reversible. In particular, agonist-stimulated recruitment of mGαi/o/z-LgBiT to MOR-SmBiT was monitored and reflected through relative luminescence measurements over time. In all cases, 1 μM of each ligand was simultaneously added by the FLIPR Tetra system approximately 1 minute after reading the basal signals. The data represent the means from experiments performed in quadruplicate. Using GraphPad Prism 9, the corresponding curves that overlie the data points were fitted into the "plateau followed by one phase association" equation for all mGαi/o/z proteins except for mGαoA and mGαoB, where the curves were fitted based on the "baseline then rise-and-fall to steady state time course" equation defined by Hoare et. al (125, 126)

3.2.2 Heterotrimeric Gαi/o/z pre-coupling with the MOR

While monitoring the kinetics of G protein recruitment and dissociation, we noticed that the basal levels of mGαoA, mGαoB and mGαi1 were higher than the others at steady state before the addition of an agonist (**Figure 3.6A**). When it came to the full-length Gαi/o/z subunits, GαoA showed a significantly stronger basal luminescence signal which is almost 5 times higher than that of GαoB, and even more so for the others (**Figure 3.6B**). Looking back at the principle of the NanoBiT assay, it requires the NanoLuc luciferase-based fragments, SmBiT and LgBiT to come together and form a functional enzyme that emits luminescence in the presence of the substrate, furimazine. In this case, we have the MOR and each Gα subunit tagged with SmBiT and LgBiT, respectively. This suggests that the luminescence detected prior to the addition of opioid ligands was from the Gα proteins pre-coupled with the receptor. Since the classical understanding of the G protein activation pathway involves the Gαβγ heterotrimer being recruited to the receptor once it is stimulated by an agonist, these findings were certainly intriguing.

The mechanistic scheme behind agonist binding to the receptor is most commonly described as a trigger that causes a shift in the equilibrium between its inactive and active states, with this shift being in favour of the active state. Since the state of the MOR transfected in G Less cells was not initially controlled in this study, we utilized nanobodies (51) to stabilize them. As part of the collected data from running the NanoBiT assay, we compared the basal levels of luminescence with and without the presence of Nb6, which stabilizes the inactive state of the receptor. When it comes to mini-Gα proteins, it appears that Nb6 reduces the interaction between mGαi1 and the receptor. However, it boosts the extent at which both mGαo proteins are recruited to the receptor prior to agonist, remarkably so for mGαoA with

an almost 3-fold increase in addition to a 1.5-fold increase observed with mGαoB (**Figure 3.6C, E**). This same observation cannot be said for other mini-Gα proteins as they do not seem to prefer being at the receptor prior to its activation (**Figure 3.6E**). In comparison to full-length Gα proteins, the presence of Nb6 does not significantly alter the basal levels of recruitment of GαoA to the receptor, having it only slightly increase (**Figure 3.6D**). However, unlike what was observed with mGαoB, Nb6 reduces the interaction between full-length GαoB and mGαi1 and the receptor as highlighted in **Figure 3.6F**. In this case, the difference between GαoA and GαoB is further showcased. Although there are differences observed between mini-G and full-length G proteins in terms of their basal luminescence levels from the NanoBiT assay, the results have provided important insights regarding the receptor state dependency of each Gαi/o/z isoform. Overall, our results suggest that only GαoA can pre-couple with the inactive receptor. Given that GαoA differs from GαoB by only 20 residues within the GTPase domain, this opens the door for further structure-activity-relationship to reveal the molecular determinants behind the observed difference.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RLU (x100)
800
600
400
200
0
mGαi1
mGαi2
mGαi3
mGαoA
mGαoB
mGαz
B
RLU (x100)
30000
20000
10000
0
Gαi1
Gαi2
Gαi3
GαoA
GαoB
Gαz
C
RLU (x100)
2000
1500
1000
500
0
mGαi1
mGαi1 + Nb6
mGαi2
mGαi2 + Nb6
mGαi3
mGαi3 + Nb6
mGαoA
mGαoA + Nb6
mGαoB
mGαoB + Nb6
mGαz
mGαz + Nb6
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Gαi1 + Nb6
Gαi2
Gαi2 + Nb6
Gαi3
Gαi3 + Nb6
GαoA
GαoA + Nb6
GαoB
GαoB + Nb6
Gαz
Gαz + Nb6

Figure 3.6 Basal luminescence levels with mini-G and full-length G proteins ± Nb6. In G Less cells, MOR-SmBiT was co-transfected with (A) mGαi/o/z-LgBiT and (B) full-length Gαi/o/z-LgBiT. At steady state and prior to MOR stimulation by 1μM DAMGO, luminescence signals were recorded to detect the recruitment of each Gα subunit to the receptor. To stabilize the inactive state of the receptor, Nb6 was additionally co-transfected alongside the (C) mGαi/o/z-LgBiT and (D) full-length Gαi/o/z-LgBiT proteins. Data are expressed as mean ± SEM from experiments performed in quadruplicate.

3.2.3 Receptor state-dependent activation of Gαi/o/z proteins

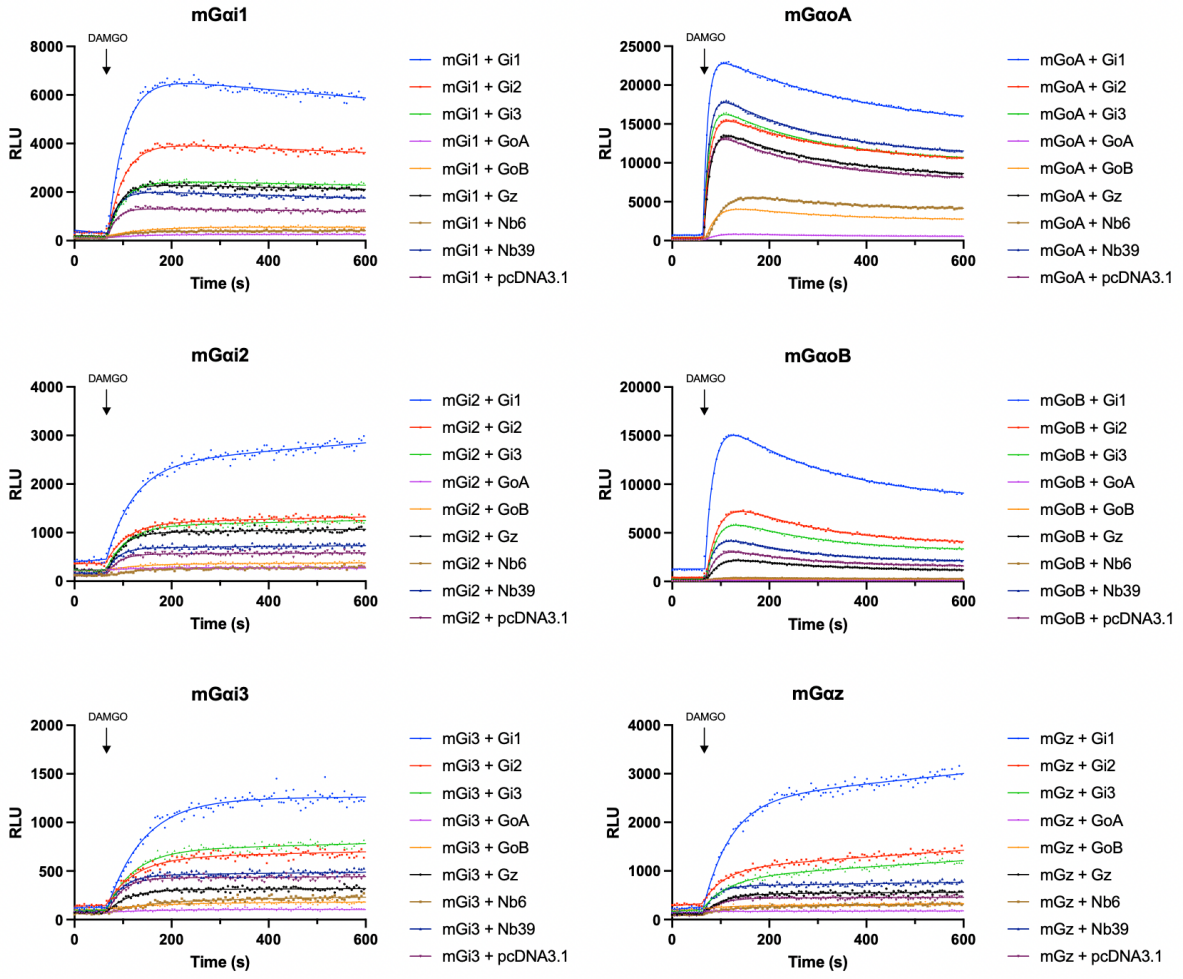
Performing the NanoBiT assay with mini-Gα proteins has certainly provided information regarding their relationship with the receptor in terms of their basal levels and kinetics. However, the modifications made to create mini-Gα proteins have their limitations since they do not entirely reflect the natural process that takes place upon receptor activation. As mentioned earlier, mini-Gα proteins have been modified to interact and stabilize the active receptor and are not allosterically controlled by guanine nucleotides. For these reasons, it was very surprising to observe such differences especially for mini-GαoA which seems to interact with the inactive receptor. Given the fast turnover of the interaction between full-length G proteins and the active receptor, it is unfortunately extremely challenging to perform kinetics studies with them. This is still under investigation in the laboratory while we are trying to build tools for this specific purpose.

To further understand this state selectivity, we incorporated wild-type full-length Gαi/o/z proteins in our study but as competitors of the mini-G proteins. As each one is set up against every mGαi/o/z protein, we presumed that it would provide more clarity regarding their differential activation profiles, especially since agonist-induced responses should reflect the activity of the receptor in its active conformation. The rational was that if a full-length G protein favourizes a specific state, we might see a differential competition behavior.

As illustrated in **Figure 3.7**, the kinetics of mGαi/o/z recruitment to the MOR follows a similar profile with mGαi1, mGαi2, mGαi3 and mGαz, but differs with mGαoA and mGαoB. As expected, the degree at which each mGαi/o/z is recruited to the MOR is lower with Nb6, which stabilizes the inactive state of the receptor, compared to that with Nb39, which stabilizes the active state. With these as reference curves, it appears that full-length Gαi1 markedly raises

the relative luminescence for all mGαi/o/z proteins. Other than that, full-length Gαi2, Gαi3 and Gαz also generally have the same effect as Gαi1, but to a lesser extent. It must be noted that Nb39 interacts with the G protein binding site on the receptor and thus competes with them which probably explains the modest effect. However, the presence of Nb39 seems to increase the number of active receptors as we can still detect an increase of the interaction between the mini-G proteins and the receptor.

Another notable feature found in all cases is that full-length GαoA and GαoB cause a significant reduction in the recruitment of all mGαi/o/z proteins, regardless of the subtype tested. The reduction is also observed to be at a level similar to that caused by Nb6. This suggests that both Gαo isoforms have a much higher affinity for the active state of the receptor, in addition to coupling with the receptor in its inactive state as clearly shown for GαoA in **Figure 3.6**. Conversely, Gαi1 is highly selective for the active receptor state but has a much lower affinity for the receptor as it competes less than any other G protein. One possible explanation is that Gαi1 has a much faster GTP exchange rate as it is released from the receptor quicker than any other G protein. This is still under investigation in the laboratory while we are trying to build tools for this specific purpose. Although the recorded responses shown in **Figure 3.7** correspond to receptor activation by DAMGO, similar profiles were observed upon their activation by the other opioid ligands previously tested.


mGαi1
DAMGO
RLU
Time (s)
mGi1 + Gi1
mGi1 + Gi2
mGi1 + Gi3
mGi1 + GoA
mGi1 + GoB
mGi1 + Gz
mGi1 + Nb6
mGi1 + Nb39
mGi1 + pcDNA3.1
mGαoA
mGoA + Gi1
mGoA + Gi2
mGoA + Gi3
mGoA + GoA
mGoA + GoB
mGoA + Gz
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mGi2 + Gi2
mGi2 + Gi3
mGi2 + GoA
mGi2 + GoB
mGi2 + Gz
mGi2 + Nb6
mGi2 + Nb39
mGi2 + pcDNA3.1
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mGoB + Gi2
mGoB + Gi3
mGoB + GoA
mGoB + GoB
mGoB + Gz
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mGαz
mGz + Gi1
mGz + Gi2
mGz + Gi3
mGz + GoA
mGz + GoB
mGz + Gz
mGz + Nb6
mGz + Nb39
mGz + pcDNA3.1

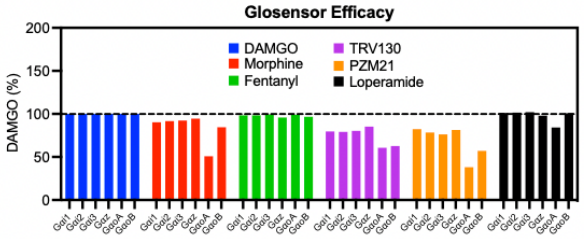
Figure 3.7 Competition between mini-G and full-length G proteins in terms of their kinetic profiles of recruitment to the MOR. In G Less cells, MOR-SmBiT, each LgBiT-mGαi/o/z and each full-length Gα protein were co-transfected. The inclusion of Nb39 and Nb6 was intended to compare the kinetics of mGαi/o/z recruitment as it relates to the stabilization of the active and inactive states of the receptor, respectively. In all cases, the MOR was stimulated by its well-known selective agonist, DAMGO. The data points shown are raw relative luminescence measurements taken in quadruplicate from an independent experiment. Using GraphPad Prism 9, the corresponding curves that overlie the data points were fitted into the "plateau followed by one phase association" equation for all mGαi/o/z proteins except for mGαoA and mGαoB, where the curves were fitted based on the "baseline then rise-and-fall to steady state time course" equation defined by Hoare et. al (125, 126).

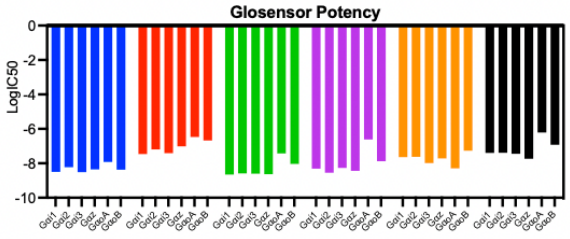
CHAPTER IV: DISCUSSION

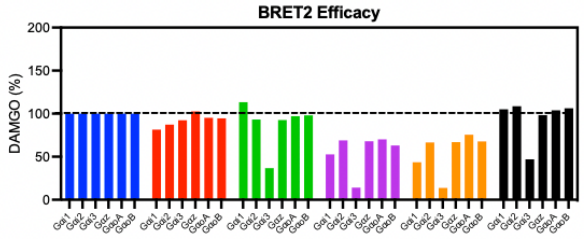
One of the most important aspects to understanding the pharmacology of drugs is by determining their inherent properties, such as their relative efficacies. Among the multitude of drug classes known, opioids have been in the limelight in recent years, especially with the rise of opioid use in North America (12). Although they are very effective in inhibiting nociceptive pain transmission, opioids are associated with numerous side effects (8). Therefore, it is crucial to gather as much information about this class of drugs, particularly the molecular signaling mechanisms that underlie their actions. In the case of this study, we target the μ-opioid receptor (MOR) since agonists that act on it provide very effective pain relief (123). Although there are numerous drugs available that target the MOR, a panel of 6 opioid ligands has been chosen for the purpose of this study. These ligands include (D-Ala(2)-mephe(4)-gly-ol(5))enkephalin (DAMGO), morphine, fentanyl, oliceridine (TRV130), PZM21 and loperamide. DAMGO is often used as a reference ligand because of its ability to display full agonist activity and high MOR specificity (98). Morphine is the archetypal opioid in which the MOR is named after, while fentanyl is 100X stronger than morphine in terms of its potency and rapid onset because of faster brain penetration (17). TRV130 and PZM21 are more recently discovered MOR ligands that are biased towards G protein signaling (94, 95). As a unique MOR agonist, loperamide is used as an antidiarrheal agent as it mainly acts to slow down gastrointestinal motility rather than provide pain relief through the central nervous system (127).

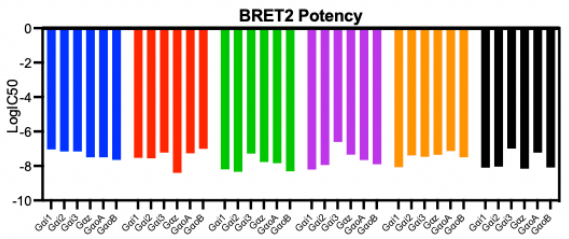
Many studies that take advantage of *in vitro* test systems have attempted to measure the efficacy of opioid ligands by looking at their signaling capacities upon the activation of the receptor. Typically, concentration-response curves are generated when analyzing data as it provides a measurement of activity at various drug concentrations. They also provide

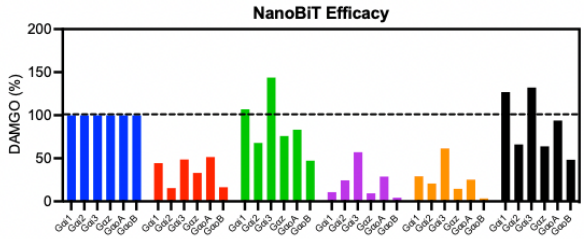
important drug parameters such as potency (EC50) and efficacy (Emax), which can be indicators of drug effects *in vivo*. However, most methods used to determine these parameters have been confounded by the system as it is highly dependent on the assays used. As a result, conflicting reports of efficacies by different groups have prompted us to re-evaluate these parameters for the selected opioid ligands briefly described earlier. We intended to do so by profiling their activities against the MOR using advanced biosensors in terms of the activation of each member of the inhibitory G protein family using G Less cells. We particularly utilized three different cell-based assays (GloSensor, TRUPATH BRET2, NanoBiT) to obtain a clear gauge of how the relative efficacies fare between them as they vary in terms of the measured responses, reflecting the *in vitro* inhibition of forskolin-stimulated cAMP, dissociation of the Gαβγ heterotrimer, and the recruitment of inhibitory G proteins to the MOR. At the same time, we provided a kinetic analysis of the NanoBiT assay that provides supportive data to back up the findings from the typical process of analysis with regard to measuring drug efficacy.

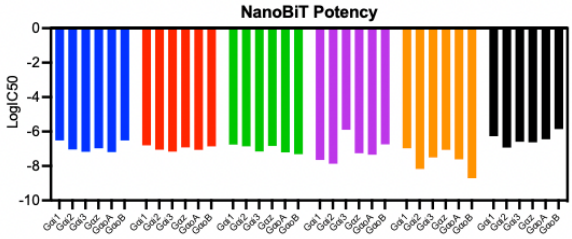

Glosensor Efficacy
DAMGO (%)
DAMGO
Morphine
Fentanyl
TRV130
PZM21
Loperamide


Glosensor Potency
LogIC50


BRET2 Efficacy
DAMGO (%)


BRET2 Potency
LogIC50


NanoBiT Efficacy
DAMGO (%)


NanoBiT Potency
LogIC50

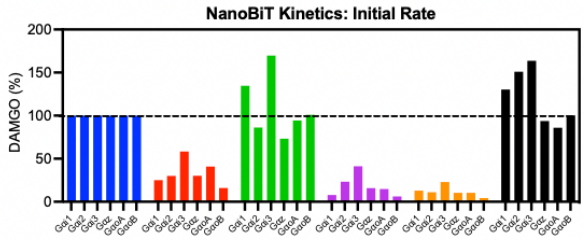

NanoBiT Kinetics: Initial Rate
DAMGO (%)

Figure 3.8 Comparison of the observed logistic parameters between the different assays. The observed Emax (efficacy) from the Glosensor, BRET2 and NanoBiT assays performed in G Less cells were extracted and normalized toward DAMGO, while the potencies for the same assays were extracted as shown and plotted as LogIC50. For the kinetic measurements using the NanoBiT assay with mini-Gαi/o/z proteins, a saturating concentration of the ligands (1μM) was used as the maximal initial rate (IRmax = $k\tau$). To obtain these values, mGαi1, mGαi2, mGαi3 and mGαz were fitted using the rise to steady-state model with robust regression while mGαoA and mGαoB were fitted using the rise-and-fall to steady-state model with least square regression as described by Hoare et. al (125, 126).

After assessing the efficacies of the selected opioid ligands by the activation of each Gαi/o/z protein, common observations and details can be deduced across the different assays as summarized in **Figure 3.8**. In general, DAMGO, fentanyl and loperamide appear to be full agonists, with morphine slightly being ambiguous since it seems to display partial agonist activity at times. It is not surprising to see morphine display variable efficacy profiles than the others as it has previously been reported to be dependent on the assays performed (111). When it comes to TRV130 and PZM21, their efficacies have not always been clear in the literature as they have once been claimed to be similar to that of morphine (94, 128). However, both can be called low efficacy partial agonists with certainty as they exhibited the profile consistently regardless of whether their relative efficacies were a measure of G protein activation by its recruitment to the receptor, dissociation with the βγ dimer or ability to reduce cAMP. The only difference lies in the extent at which these drugs elicit the responses. Another notable observation that has been consistent throughout the study is that GαoA has distinct characteristics that are unlike those observed with all other Gα proteins, especially GαoB. As previously mentioned, these proteins are isoforms of Gαo that only differ by a few amino acids. As a result, it is easy to assume that both exert near-identical activities because of how alike they are in terms of their amino acid composition. However, it is intriguing how dissimilar they are in terms of their activity profiles upon receptor activation. The GloSensor assay revealed that GαoA does not efficiently inhibit cAMP, complementing previous findings that Gαo does not regulate AC activity (85) but still affects it (86). Although previous findings did not distinguish the specific isoform of Gαo, it is assumed that they refer to GαoA. Moreover, the NanoBiT assay showed that GαoA does indeed differ compared to all other inhibitory G proteins as its full-length version was the only functional one in terms of being strongly

recruited to the MOR. The exact properties that determine why GαoA is set apart from all others remain unclear. However, the observations collected from the different assays have built a case for it that requires further exploration.

The observed efficacies of highly efficient and potent opioid ligands such as fentanyl, DAMGO and loperamide, have generally displayed quite similar profiles between the three experimental systems. However, partial agonists such as morphine, PZM21 and TRV130 showed a higher level of variability, indicating the challenge to determine the most accurate measurements of their drug properties. For example, in terms of its ability to activate Gαi3, fentanyl appears to be a full agonist with the GloSensor and NanoBiT assays, but a partial agonist in the BRET2 assay. The same observation in terms of their efficacies can be generally applied to the rest of the opioid ligands tested. As seen in **Figure 3.8**, the net BRET ratio is lowest with Gαi3 regardless of the drug that activated the receptor. Although the reasoning behind this may not be clear, one possible explanation is that there may be a low level of pre-formed heterotrimers with Gαi3 at the steady state. The BRET2 configuration may not be optimal for this particular G protein as well, resulting in efficacy measurements that are less than ideal. In terms of potencies, the recorded measurements appear to be very similar between the different assays. This observation clearly indicates that potency is not an important parameter that could explain the divergent physiological responses of the panel of selected opioid ligands. Additionally, regardless of the different configurations involved in the assays, affinity was not highly affected, which would have been reflected in the recorded drug potencies. Nevertheless, the deviations from the current understanding of the efficacies of each drug may simply be a reflection of the distinct characteristics of certain Gαi/o/z protein isoforms. However, it may also be observed due to the design and nature of each assay as it

has been previously shown to affect the efficacy measurements of other ligands. Therefore, we turn the spotlight towards critically assessing the limitations of the GloSensor, TRUPATH BRET2, and NanoBiT assays in terms of accurately determining the relative efficacies of the panel of opioid ligands tested.

The GloSensor assay measures the intracellular levels of cAMP, which is a second messenger produced from the catalytic action of AC as it is regulated by Gαs or Gαi/o/z proteins. In the case of this study, we look at the effect of each Gαi/o/z on cAMP production upon the activation of the MOR. The measurement of cAMP levels through luminescence readings is an indirect means to determine relative drug efficacy and may be a suboptimal way to determine this important property due to several factors that need to be taken into consideration. One limitation could be the endogenous expression of the AC that may have led to the variations observed from running the assay more than once under the same conditions. In addition, there are also multiple known transmembrane isoforms of AC (129) that may be differentially expressed in the cellular system. It is possible that some isoforms may be more receptive to the regulation of each Gαi/o/z subunit than others. For example, it has been shown that Gαo proteins might have effects only on AC1 (130). With that, we have yet to verify which AC isoforms are predominantly present in G Less cells. The presence of endogenous regulators of the cAMP signaling pathway, such as phosphodiesterases (PDE) (131), may also have affected the observed results.

The TRUPATH BRET2 assay relies on the resonance energy transfer between donor and acceptor proteins that are in close proximity to each other. In this study, the BRET-based biosensors, Gα-RLuc8, Gβ3, and Gγ9-GFP2, are used to measure the dissociation of the heterotrimer as a measure of G protein activation. However, the original design of these

biosensors by Olsen et al. was optimized to obtain certain BRET probe pairs (121). Although Gβ3 was used consistently, each Gαi/o/z isoform was mainly paired with a different Gγ isoform. To put the size of the family of Gβ and Gγ subunits into perspective, there are 4 Gβ and 12 Gγ isoforms (70). Because of the sheer diversity of Gβ and Gγ subunits, it may be possible that the responses measured may be due to the efficiency of resonance energy transfer between the BRET probe pairs rather than from the distinct identity of each Gαi/o/z protein. Another limitation of this assay is the substrate, coelenterazine 400a, as it seems to not be very stable at room temperature. In addition, the luminescence and fluorescence measurements may have been affected by the conformational dynamics of the G protein heterotrimer as orientations of the two proteins fused with the RLuc8 and GFP2 may change upon their dissociation from each other.

On the other hand, the NanoBiT assay is another method in which the proximity of two proteins of interest is measured but requires a physical interaction between two subunits. Specifically, it takes advantage of the SmBiT and LgBiT fragments of NanoLuc luciferase fused to the MOR and mini-Gαi/o/z proteins, respectively. After stimulation of the receptor by an agonist, the resulting recruitment of each mini-Gαi/o/z protein to the MOR is measured. Although the assay effectively gives an idea of this step in activating the G protein pathway, the MOR and Gα proteins involved are recombinant proteins wherein the fused NanoLuc luciferase fragments need to be in an optimal spatial orientation in order to become a functional enzyme that produces luminescence. Furthermore, the mini-Gαi/o/z proteins contain modifications, the most significant of which is a mutation in its C-terminal α5 helix that prevents the release of nucleotides, resulting in the stabilization of the receptor-mG protein complex (122). Although these proteins are modified, they are essential in better

understanding the recruitment profile of G proteins since we observed that most full-length Gα proteins did not appear to be functional in this assay, and it may likely be due to their GTPase activities. This observation also confirms the purpose of why the mini-G proteins were generated in the first place, which is to stabilize the normally transient interaction of the MOR-G protein complex so that we can more easily capture the dynamics between the two.

Among the different assays used in the study, the NanoBiT assay is by far the one that considers the closest relationship to the receptor as it directly measures the coupling capacity between the receptor and each Gα protein. Compared to GloSensor and BRET2, the NanoBiT assay is clearly the most efficient in determining partial agonism (**Figure 3.8**). Given that there are variations in drug efficacy measurements between each assay, the nature of the NanoBiT assay being a non-amplification assay with results that have a linear correlation makes it an optimal method to reflect differences in drug efficacy. In other words, it seems that focusing on the direct relationship between the receptor and each G subunit, rather than other downstream events, reveals most about this important drug property. One reason behind this is that looking elsewhere apart from the level of the receptor may open up the possibility of other endogenous proteins and processes affecting the observed results. The recruitment of Gαi/o/z proteins to the MOR is also directly affected by the conformational changes that occur upon binding of an agonist to the receptor. The accurate determination of the relative efficacies of opioid ligands is much needed and seemingly depends on a specific aspect of the mechanism that underlies receptor activation. Without the use of mini-G proteins, the detection of the direct coupling of G proteins to GPCRs would be more complicated as other techniques have been used to do so. For example, radiolabeled GTP analogs have been widely used for this purpose, but lack selectivity between G protein subunits, making it difficult to study the

dynamics between each one and the receptor. It is also limited to membrane preparations or cell homogenates in addition to nucleotide exchange and hydrolysis being rate-limiting steps that may affect the kinetics obtained for each G protein. Since looking at the interaction between the receptor and each G protein is technically challenging, most assays have been developed to assess G protein activation rather than GPCR-G protein complex interactions.

Moreover, we took a step further in understanding the dynamics between the MOR and each Gαi/o/z protein. We already had the data from the NanoBiT assay at our disposal, and because of the default nature of the assay, real-time kinetic analysis of the recruitment of mGαi/o/z proteins to the MOR was possible as it was characterized over a period of time before and after receptor stimulation. Although kinetic measurements using BRET are feasible, there are some challenges associated with this assay. First, the stability of the substrates used is a limitation since it affects the luminescence intensities generated over time by *Renilla Luciferase* (RLuc8). In the case of BRET2, coelenterazine 400a is stable for approximately 10 minutes while coelenterazine h, the substrate for BRET1, is much less stable (< 30 seconds). Another limitation of measuring kinetics using BRET is the device used to measure the RLuc8 and GFP2 emissions. For this study, we used a conventional microplate reader which takes approximately 5 minutes to read a full microplate on both emission wavelengths. Based on the kinetics obtained from the NanoBiT assay, Emax is reached at 75-100 seconds and a steady state at 200 seconds. Lastly, if an injector was used to add the ligands, only one well can be monitored at a time, which makes it tedious to look at multiple drugs at once. Hence, we have proceeded with solely looking at the kinetics obtained from the NanoBiT assay which is performed using a high-throughput cellular kinetic screening system.

In general, evaluating the kinetics of underlying mechanisms of GPCR signaling is typically set aside when it comes to determining the efficacies of ligands as it is common practice to do so using dose-response curves. However, it may provide additional important information or even simply confirm the efficacies obtained from the classical route of using dose-response curves as observed in the case of this study. Using mathematical models, quantification of kinetic parameters that reflect the efficacy of drugs acting on GPCRs was made possible by Hoare et al (125, 126), and this method was the basis of our analysis. As seen in **Figure 3.8**, we found that the maximal initial rate (IRmax) of the kinetic analysis of the NanoBiT assay run at a saturating concentration of each drug correlated quite well with the efficacies extracted from the dose-response curve of the same assay. This rate constant indicates the rate at which the response is generated by the agonist-occupied receptor and a signal precursor, reflecting the likes of the initial rate constant obtained with enzyme kinetics. The initial linear increase of the recorded response of a time course would thus be beneficial in determining efficacy as the signal would not be subject to regulatory mechanisms yet, which are reflected in the latter part of the time course. Moreover, the highly correlated efficacies quantified using the Emax of the dose-response curve and the IRmax of the kinetic analysis are interesting as it opens the door for further investigations using this approach to determine drug efficacy by a single dose through a live assay. An example of a future investigation could be studying the effect of various allosteric modulators on the efficacy of drugs. In addition, one striking revelation from our kinetic analysis of mGαi/o/z recruitment to the MOR is that the profiles of mGαoA and mGαoB are remarkably different from that of the other mGαi/o/z proteins regardless of the opioid ligand used to stimulate the receptor. The difference in time-course profiles is not new in the field of GPCRs as it has been observed with other aspects of

GPCR signaling. However, the rise-and-fall to steady state recruitment profile of mGαoA and mGαoB is known to involve more complex mechanisms than the association exponential profile of mGαi1, mGαi2, mGαi3 and mGαz (126). Nevertheless, the mechanistic details to explain each profile in terms of G protein activation are subject to further investigation. Although there is still more work that needs to be done in terms of correlating these observations with full-length G proteins as well, we are confident that the NanoBiT assay represents a sensitive and indicative approach that can discriminate between the relative efficacies of drugs. After all, measuring the direct interaction between the MOR and each Gαi/o/z protein may just be the key determinant of relative drug efficacy.

Furthermore, while investigating the kinetics of inhibitory G protein activation, we also noticed that the basal luminescence readings varied in that both mGαo isoforms and full-length GαoA exhibited higher values than others. Since the luminescent signals are only produced when SmBiT and LgBiT come together, this observation could imply that these mGαo proteins have already been pre-coupled to the receptor even before it was exposed to an agonist. The concept of G proteins pre-coupled to the receptor before its activation has been previously explored (132, 133) as BRET and FRET studies have elegantly set the basis of this new pre-coupled paradigm. Moreover, this finding goes against the classical understanding of GPCR signaling. As it applies to the MOR, GPCR signaling is typically known to involve the stimulation of the inactive receptor with an agonist, shifting it in favour of its active conformation. This conformational change then drives the heterotrimeric G proteins to be recruited to the receptor where GDP is exchanged for GTP at the Gα subunit. Subsequently, the GTP-bound Gα and Gβγ subunits dissociate from each other to trigger a variety of cellular responses through different effectors.

Since the pre-formation of a receptor-G protein complex seems to be the case for some Gαi/o/z proteins, Nb6 and Nb39 were used to stabilize the inactive and active states of the MOR, respectively. Although they have been shown to be effective in pushing the receptor to a specific state, it is also important to note that these nanobodies bind allosterically to the receptor and do not seem to interfere with G protein binding (51). Finding that Nb6 significantly increases the basal luminescence levels of mGαoA, as well as mGαoB to a lesser extent, confirms the previous assumption that pre-coupling to the receptor may be the most likely explanation for the observation. Understandably, the number of cells, transfection efficacy, and resulting expression levels of each G protein need to be accounted for in this case. As for not observing the same significant increase with the full-length GαoA and Nb6, it may likely be due to the innate ability of GαoA to strongly stabilize the receptor even without the presence of the nanobody. It even suggests that GαoA has a high affinity towards the inactive state of the MOR, unlike other inhibitory Gα proteins. In addition, the competition assay between mGαi/o/z and full-length Gαi/o/z proteins revealed that GαoA displays high affinity towards the active state of the receptor as well. These findings have considerably uncovered the receptor-state dependency of each member of the Gαi/o/z family.

CHAPTER V: CONCLUSION

Being one of the leading causes of overdose-related deaths in North America, opioids have become well known not only for their ability to effectively provide pain relief, but also for their side effects, including dependence and addiction. These effects have inevitably led to the rise of the opioid crisis as it continues to claim lives. Thus, there is a pressing need to face the matter before it reaches insurmountable heights. To contribute to the growing demand of obtaining information about this class of drugs, we focused on finding ways to accurately assess the relative efficacies of a series of opioid ligands that act on the MOR since there have been previous reports that show some discrepancies. We take advantage of three cell-based assays that have different functional readouts, targeting various aspects of agonist-mediated MOR signaling in order to evaluate their differences in terms of inhibitory G protein activation. Due to the possibility of external factors affecting the measurements from each assay, we deemed that focusing on the direct interaction between the MOR and Gαi/o/z proteins using the NanoBiT assay was the optimal method to accurately determine the relative efficacies of drugs. Along the way, we also discovered that GαoA displays characteristics that set it apart from other Gαi/o/z proteins, considering that it does not considerably differ with its splice isoform, GαoB, in terms of their amino acid compositions. Some unique features of GαoA include (i) its inability to modulate AC, (ii) its rise-and-fall to steady state kinetic recruitment profile and (iii) its capacity to strongly bind to both inactive and active states of the receptor.

Further studies could focus on correlating the data from mini-G proteins to full-length G proteins once an effective approach to study this interaction is established. We could also look into the factors in which Gα proteins, especially GαoA, are dependent on. Since the observations between each Gα isoform have certainly been intriguing, the exact functional

role of each as well as the mechanisms that control their differential functional profiles are of particular interest. One example could be looking at possible regulatory factors that distinguish between GαoA and GαoB, following up on evaluating the potential role of their GTPase activities. Another could be from the perspective of the effect of these isoforms on effectors aside from AC, such as calcium channels and inwardly rectifying potassium channels. The specific interactions between the ligand, receptor and G proteins can also be put under a microscope to closely understand what drives the efficacy of drugs. Moreover, this study was solely focused on MOR activation. However, the methods in which the findings have been determined can be applied to other GPCRs, especially since our cellular system is a conducive environment to study the activation of other subfamilies of G proteins. As mentioned earlier, determining drug efficacy not only by using the classical dose-response curves, but doing so using kinetic terms may also be used for further investigations.

All in all, not only has the goal of the study been met, but more has come out from it. Our findings lay the groundwork to explore the interesting possibility of GαoA playing a unique role in the physiological effects of opioid use as it interplays with other pathways involved in the functional interactome of the MOR. It also provides insights into the complex mechanisms that underlie central nervous system disorders, such as epileptic encephalopathies, that stem from changes in neural functioning. Mapping the activation mechanisms of each G protein by different ligands has also yielded important aspects to the determination of relative drug efficacy. Once done, the design of a new generation of opioid ligands can ultimately be fine-tuned in order to improve the side effects typically associated with these drugs.

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