A battle against the opioid crisis: Deciphering the molecular control of opioid receptors in an effort to design safer analgesics

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A thesis submitted to
the Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements
for the Master's degree in Biochemistry

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ABSTRACT

Opioid receptors are central to the development of tools that can be used to manage and fight against the opioid crisis that is prevalent in North America. They are part of a large protein family called G-protein-Coupled Receptors (GPCRs), which are the most therapeutically targeted receptors within the human body. Once activated, the receptors lead to the activation of multiple different signaling pathways such the β-arrestin and G-protein signaling pathways. The β-Arrestin pathway is usually associated with the side effects of opioid analgesics. An allosteric site that binds to sodium was identified within the delta-opioid receptor (DOR). Previous studies have found that the sodium cavity can regulate the activation of different signaling pathways and thus act at the functional selectivity level. Our lab has identified a subset of small molecules targeting this cavity. This finding supports the druggability of this site and thus opens the door for the development of a novel pharmacological entity to control opioid receptor activities. This thesis focuses on the characterization of the sodium cavity by performing structure activity relationship (SAR) studies on the delta opioid receptor with three allosteric modulators: MIA, HMA and zoniporide. We report that, through site-directed mutagenesis and functional studies, mutations in the allosteric sodium site has an impact on the receptor functionalities including ligand recognition, efficacy and also allosterism by small molecules; however, the mutations do not prevent the binding of the allosteric modulators to the receptor. We also report the development of a novel biomedical tool that can be used to study the recruitment of the G-protein subtypes as well as the arrestin subtypes. Our data suggest it is possible to design drugs that will target the sodium pocket and this site has a major role within DOR and could be used to design novel modulators with unique pharmacological properties. My work will serve as a platform to study other members of the opioid receptor family and for the future rational design of novel modulators.

Acknowledgements

First and foremost, I would like to express my sincere gratitude to my supervisor Dr. Patrick Giguère for his continued support and encouragement throughout my graduate studies. His wisdom and guidance for the past couple of years and his helpful insight throughout the writing process have been instrumental to the completion of this Master's degree. In other words, *merci pour tout patron.* I would also like to thank my Thesis Advisory Committee (TAC) members and thesis evaluators, Dr. Mario Tiberi, Dr. Jean-François Couture and Dr. Eric Lee for all of their helpful feedback and the time they invested in the corrections of my TAC reports and thesis.

I would like to also thank the members of the Giguère lab for their endless help for the past couple of years and for making my entire experiences unforgettable. Special thanks to our research associate, Dr. Geneviève Laroche, for her guidance and patience. I would also like to thank the graduate students, Nuria Blgacim, Stephanie Farhat and Manel Zeghal as well as the undergraduate students, Homa Sadeghi-Emamchaie, Rebecca Wang, and Nibras Ghanmi for the support throughout the years.

Last but not least, a very special thanks to my family and friends who have stood by me and for giving me invaluable support and encouragement throughout the many challenges that I had to endure in my graduate studies. I will be forever indebted to my extremely wonderful family.

Abdulhamid Mohamud

Ottawa, August 2019

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List of Abbreviations

(A_{2A}-AR): Adenosine receptor 2A

μ-OR/MOR: Mu-opioid receptor

7TM: Seven transmembrane helices

Ago-PAM: Agonist-Positive allosteric modulator

Arg/R: Arginine

ASIC3: Acid-sensing ion channel 3

Asn/N: Asparagine

Asp/D: Aspartic acid

ATP: Adenosine triphosphate

BCS: Bovine calf serum

cAMP: Cyclic adenosine monophosphate

CXCR4: C-X-C chemokine receptor type 4

Cys /C: Cysteine

D3: Dopamine receptor

DADLE: [D-Ala_2, D-Leu_5] enkephalin

DAG: Diacylglycerol

DMEM: Dulbecco's modified Eagle's medium

DOR/δ-OR: Delta-opioid receptor

ECL1, ECL2, and ECL3: Extracellular loops 1, 2 and 3

ENaCs: Epithelial sodium channels

FBS: Fetal Bovine Serum

$G\alpha_{i/o}$: inhibitory G alpha protein family

$G\alpha_s$: excitatory G alpha protein family

GABA: Gamma-Aminobutyric acid

GDP: Guanosine diphosphate

GIRKs: G-protein-coupled inwardly-rectifying potassium channels

GPCRs: G-protein-coupled receptors

GRKs: G-protein-coupled receptors kinases

GTP: Guanosine triphosphate

HEK293T: Human Embryonic Kidney 293T cell line

ICL1, ICL2, and ICL3: Intracellular loops 1, 2 and 3

IP_3: Inositol -1,4,5-trisphosphate

KOR/κ-OR: (kappa) for *K*etocyclazocine receptor

Leu/L: Leucine

LgBit: Large fragment of Nanoluc

M2: Muscarinic receptor 2

NAM: Negative allosteric modulator

NHE1: Na_+-H_+ exporter

NOP/NOR: Nociception/orphanin FQ peptide receptor

PAM: positive allosteric modulator

PAR1: Protease-activated receptor 1

PIP_2: 4,5-biphosphate

PKA: Protein kinase A

PKC: Protein kinase C

R*: Active state of the receptor

R: Inactive state of the receptor

RLU: Relative Luminescence Unit

SAR: Structure-activity-relationship

SmBit: 11-amino acid Nanoluc fragment

TM: Transmembrane domains or transmembrane region

Trp/W: Tryptophan

YFP: Yellow fluorescent protein

α1: Alpha-1 adrenergic receptor

α2: Alpha-2 adrenergic receptor

β-AR: β-adrenergic receptor

β-arr1: β-arrestin1

β-arr2: β-arrestin2

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1.0 General Introduction

1.1. Introduction

Opioids are widely used for the treatment of chronic pain and addictive disorders and they represent some of the most prescribed and abused medications in the developed world. While opioid agonists are addictive themselves, opioid antagonists and partial agonists are used for the treatment of addictive disorders. Over-prescription of opioids for chronic pain is accompanied by an increase in prescription opioid overdose, abuse and addiction. It's being considered an epidemic problem by health-care institutions (1,2). This highlights the need for better and safer narcotic analgesics. To address this major issue, this study focuses on the molecular targets of opioids in the human body: the opioid receptors.

Endogenous opioids lead to the activation of the three classical opioid receptors (μ, κ, δ-OR) to elicit their pain-relieving effects (3-5). Opioid receptors belong to the large G-protein-coupled receptors (GPCRs) family, and their expression is enriched within the Central Nervous System (CNS), especially the cortex spinal cord, periaqueductal gray and the thalamus (6). The main function of opioid receptors is the control of pain perception and management (7). The expression of opioid receptors outside the nervous system argues in favour of other important physiological roles as suggested for the respiratory and gastrointestinal tract modulation by opioids (6-8). Morphine-related drugs are the oldest and best characterized opiates used at the clinical level. However, their weak selectivity and the divergent pharmacological behaviour toward each member of the classical opioid receptor family (μ, κ, δ- OR) lead to numerous side effects, which limits their use (9).

1.2 GPCR Superfamily

G-protein-coupled receptors represent the largest family of proteins in the human genome (10), and they are molecular targets for more than half of the therapeutic drugs that are currently on the market (11). There are more than 800 receptors in this superfamily, which about 450 are part of the druggable genome, whereas most of which have not yet being exploited at this time. The potential for discovering new drug targets in this rather large family is enormous. This makes them the most pursued molecular drugs targets in the human proteome. Some of the various endogenous ligands that can activate GPCRs include amines, ions, organic odorants, photons, peptides, proteins, lipids as well as nucleotides (12). These ligands interact with the extracellular side and transmembrane bundle of G-protein-coupled receptors to induce an intracellular response. The opioid receptors are part of this GPCR family; therefore, this section will briefly discuss the classification, activation, signalling as well as the conserved structural motifs of these receptors.

1.2.1 Classification

The human proteome contains more 800 G-protein-coupled receptors, which are classified in five major families: Rhodopsin (Class A), Secretin (Class B), Glutamate (Class C), Adhesion, and Frizzled/smoothened receptors (Class F). The rhodopsin family is the largest of them all with more than 700 members. Receptors in this family have a small N-terminus and their ligand binding site is in the middle of the transmembrane domain. There are a variety of receptors in this family, which include light, neurotransmitter, peptides and hormone receptors. This large family is further subclassified into groups: the α-, β-, γ- and δ-groups based on sequence homology. The groups are further subclassified into clusters. For instance, the opioid receptors in part of the SOG cluster (Somatostatin, Opioid, Galanin cluster) of the γ-group in the rhodopsin family (12,13).

The second family of GPCRs is the Secretin receptor family (Class B), which consists of 16 members. They have a large binding site that can accommodate large peptides with similar sequence identity. They also have a large N-terminus that is about 60-80 amino acids long with a conserved Cysteine residue that is important for the binding of peptides. The third family of GPCRs is the Adhesion receptor family, which contains 33 receptors. This receptor family has an even larger N-terminus than the Secretin receptor family with about 200 to 2800 amino acids in length. Their N-terminus contains residues that might be involved in cell adhesion, hence their name as the Adhesion receptor family (14).

The fourth family of GPCRs is the Glutamate receptor family (Class C). This family contains 22 receptors including metabotropic glutamate, GABA, calcium-sensing and taste (TAS1) receptors. Similar to the receptors in Class B, this family of receptors has relatively large N-terminus with about 200 to 580 amino acids in length. The large N-terminus contains the ligand binding domain as well as a dimerization determinants (14). The fifth and the last family of GPCRs are the Frizzled/TAS2 receptor family (Class F). There are 24 members in this family, which is divided into two clusters: Frizzled receptors and TAS2 receptors. Although these two are in the same family, the length of their N-termini is different. The Frizzled receptors have large N-terminus with about 200 amino acids in length while the TAS2 receptors have a rather short N-terminus (14).

Despite knowing the existence of this vast majority of GPCRs in the human proteome, some of the endogenous ligands of these receptors are not currently known and they are referred to as “orphan GPCRs”. These receptors can serve as potential drug targets but only once their endogenous ligands are identified or they have been annotated by small molecule ligands. There is a lot of research being done in deorphanizing these receptors at the moment (15).

Figure 1.1.

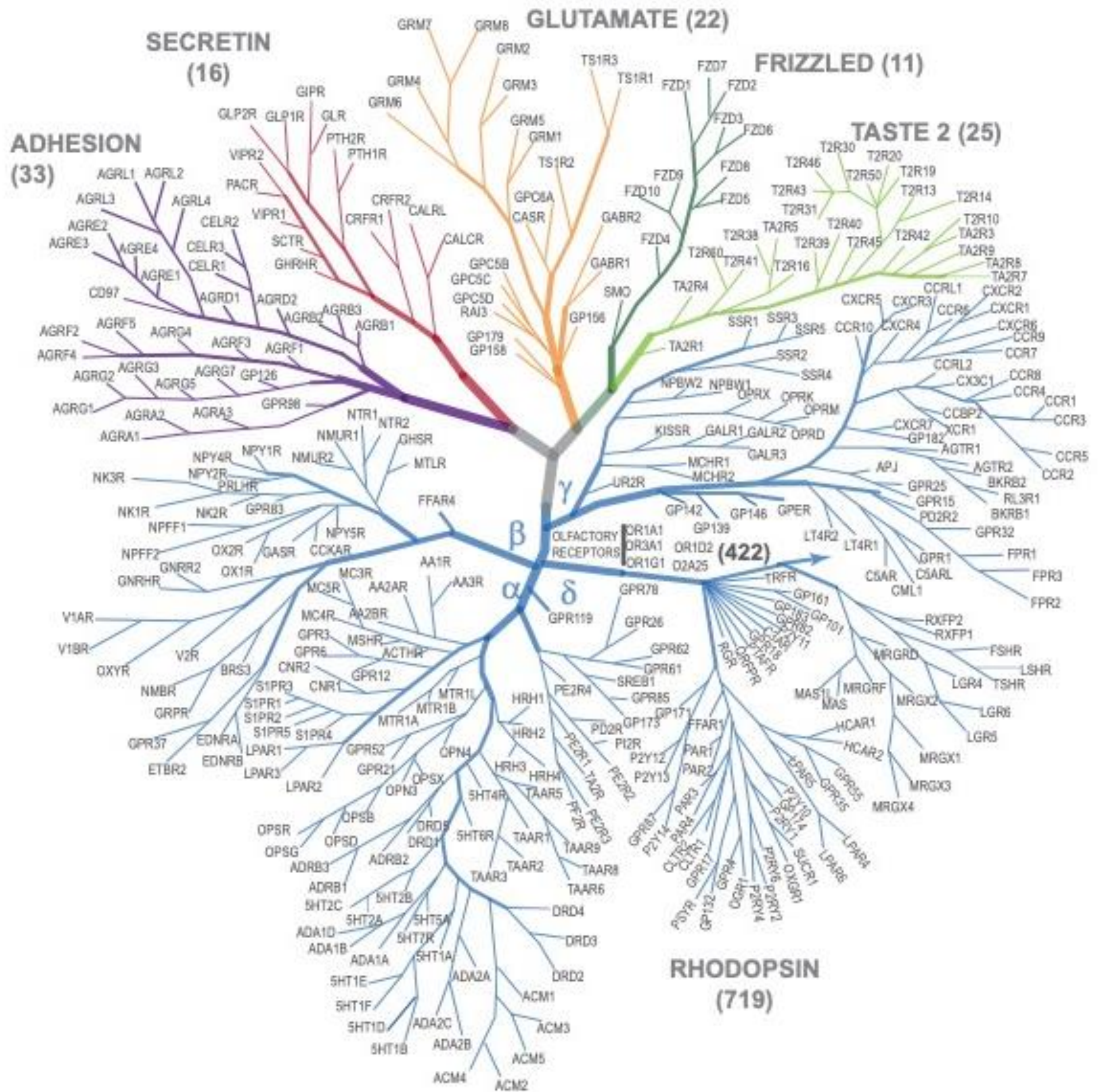


Figure 1.1. Classification of GPCRs Superfamily. This dendrogram shows the different classes of GPCRs, which are grouped together based on their sequence identity. As shown in the figure, the opioid receptors are part of the γ-branch of the Rhodopsin family of GPCRs, modified from (13).

1.2.2. Structural features of G-protein-coupled receptors

Due to advances in structural biology in recent years, there has been an increase in solving and determining the structures of G-protein-coupled receptors. These structures identify key similarities and differences between different classes of GPCRs as well as individual receptors in terms of ligand recognition and signal transduction. This new fast growing field of information allows researchers in discovering new drugs that are specific to each GPCR and its associated disease (16). The first crystal structure of the rhodopsin was solved in 2000 (17), followed by the structure of the β2-adrenergic receptor, which was solved seven years later (18). The structures of the β2-adrenergic and rhodopsin receptors were solved in their inactive conformations; with the β2-adrenergic bound to an antagonist and rhodopsin in the dark. Since then, several other GPCRs were crystallized; for instance, the β1-adrenergic receptor (19) and A2A-adenosine receptor (20), both were solved in 2008. By 2012, the chemokine receptor CXCR4(21), dopamine D3 receptor (22), histamine H1 receptor (23), M2 muscarinic receptor(24), kappa opioid receptor (25), and mu-opioid receptor (26) were solved with antagonist or inverse agonist ligands. Various techniques were used to stabilize these receptors such as stabilizing mutations, truncations, ligand-binding and binding of fab fragments and protein fusions (27).

Despite the similar overall structure of GPCRs, these recently determined GPCR structures allude to subtle similarities and differences between the four classes of this protein superfamily. Each GPCR has a seven transmembrane domains (7TM), connected to each other by three extracellular loops (ECLs) and three intracellular loops (ICLs) with an extracellular amino (N-terminus) and intracellular Carboxyl (C-terminus) termini (28). The length of the N-terminus, which accounts for majority of differences between GPCRs, widely varies and can range from 10s to 1000s of amino acids in length. The Adhesion family or class B has by far the largest N-terminus

of any GPCR and it is 1000s of amino acid residues in length. The large N-terminus in this class of GPCRs is said to be important for receptor activation and ligand binding. In comparison to the Adhesion family, the glutamate family of receptors, class C, have a medium sized N-terminus, which is in the 100s (350-600) of amino acids in length. The N-terminus in this class C forms a bilobed structure (flytrap) that is uniquely characteristic of this family and also serves as their ligand-binding region. The monoamine and peptide receptors of rhodopsin family or class A has the smallest N-terminus of GPCRs, and it is in the 10s (10-50) of amino acids in length. These receptors have their ligand-binding site in the upper part of the transmembrane region (29). The transmembrane region (TM) contains seven α-helices ranking from TM1 to TM7, and it has been known as the most conserved component of GPCRs (30). This latter region binds and transfers the information from the external part of the cell to its intracellular region, and it is implicated in forming the different ligand binding sites (16). Finally, the intracellular part consists of three intracellular loops coined as ICL1, ICL2, and ICL3 and the Carboxyl group (C-terminus). The C-terminus domain seems to be important in transducing the downstream signaling pathways by binding to diverse effectors, for instance, β-arrestins, G-protein-coupled receptor kinases, and others (31,32).

In spite of the similarities between G-protein-coupled receptors, they interact with variety of extracellular signals that are as small as photons and as large as peptides. It is intriguing how these structures of a similar overall fold can interact with signals as diverse and translate them into intracellular responses. These various ligands binds to different areas of these receptors; the larger ligands such as the proteins and peptides interact with the N-terminus and the upper region of the transmembrane (TM) domain, whereas small ligands are small enough to go deeper into the receptors and bind to the TM region (33).

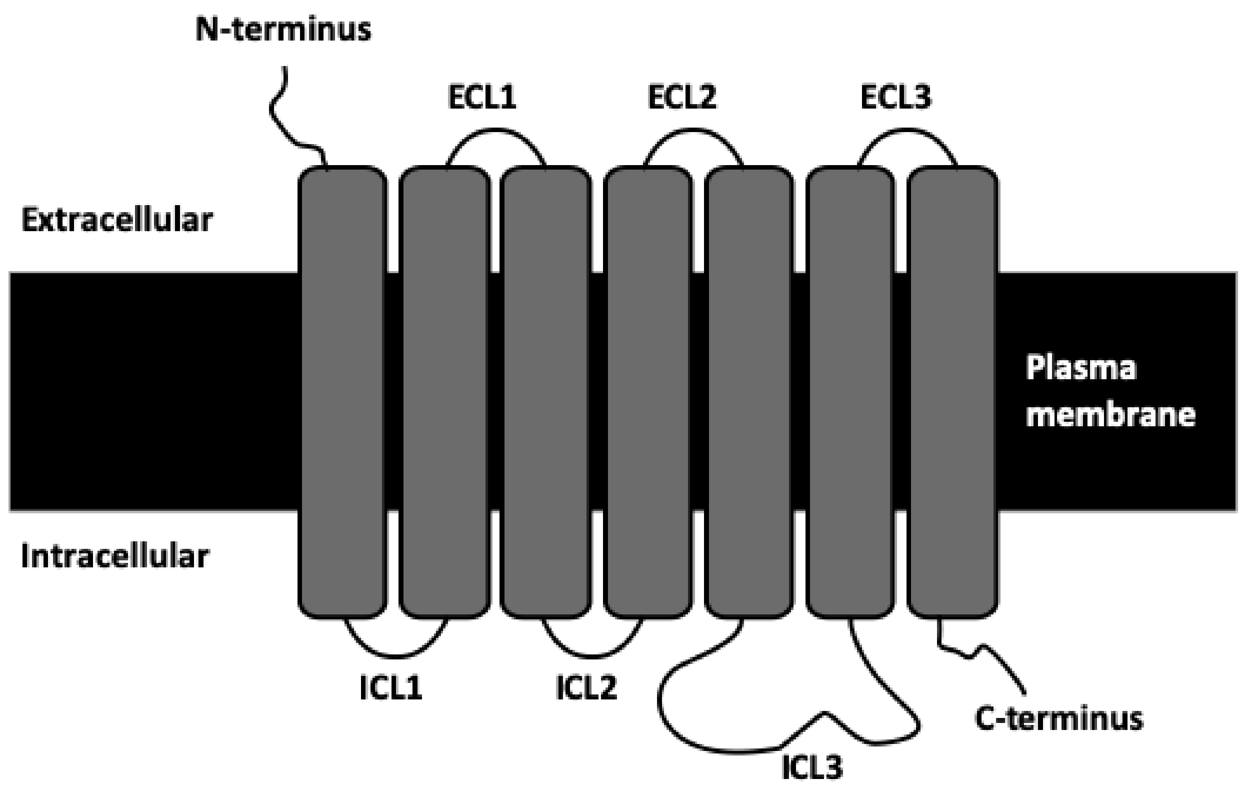

N-terminus
ECL1
ECL2
ECL3
Extracellular
Plasma
membrane
Intracellular
ICL1
ICL2
ICL3
C-terminus

Figure 1.2. Cartoon representation of GPCR. This figure highlights the overall structure of a GPCR, which has 7-transmembrane (7TM) domains imbedded in the plasma membrane, 3 extracellular loops and 3 intracellular loops with extracellular N-terminus and intracellular C-terminus.

Biochemical and structural studies identified four motifs in class A GPCRs that are important for receptor activation upon binding by agonists. The most conserved motif is; $D[E]R_{3.50}$ Y (the superscript number which is above the arginine residue represents numbering using the Ballesteros Weinstein nomenclature (34)) is located in helix III. In term of its function, it is a part of ''ionic lock'' by forming a salt bridge with $D/E_{6.30}$ in helix VI. This ionic lock is noticeable in an inactive state of Rhodopsin family receptors blocking the G-protein binding at this intracellular region (35). The second most conserved motif is $CW_{6.48}xP$ located in helix VI. The Trp residue of CWxP motif undergoes a conformation transition that constraints the receptor in a specific conformation. It is also known as a microswitch or toggle switch with a distinct orientation between the active and inactive state of the receptor. In addition to the above two conserved motifs, the interaction of the asparagine of the $NP_{7.50}xxY$ motif in helix VII with either residues located in helix VI as well as the aromatic-aromatic interaction between the Tyr of $NP_{7.50}xxY$ with an aromatic residue within the intracellular helix VIII also contributed to constraint the receptor in a specific state(36). The final conserved motif in G-protein-coupled receptors is the disulfide bridge, which forms between the two highly conserved cysteine residues: $Cys_{3.25}$ at the top of helix III and the one in the extracellular loop 2 (37).

1.2.3. Activation of G-protein-Coupled Receptors

The dynamic nature of G-protein coupled receptors allows them to transit between different conformational states ranging from the inactive states (R state) to the active (R* state). Agonists stabilize the active (R*) conformation of the receptor, which leads to the recruitment and the activation of intracellular partners. Antagonist and inverse agonists, however, stabilize the inactive (R) conformation of the receptors. At steady state, receptors are in equilibrium between the two functional state resulting in basal activation in absence of ligand (also called constitutive activation) (38). This basal level of activation is unique for each receptor with some receptors mostly in the inactive conformation whereas others adopt various active conformations. The efficacy, which is defined as the effect of the ligand on the coupling and functional properties of the receptor, is responsible for pushing the equilibrium towards either the active or the inactive state (39). The full agonist shifts the equilibrium towards the active state and stabilizes the R* while the inverse agonist binds to and shifts the equilibrium towards the inactive state R. The inverse agonist leads to a decrease in the level of basal activity. Partial agonist seems to have an affinity for both active R* and inactive state R. Its partial efficacy will thus depend on its preference for the active R* state when compared to a full agonist that solely binds to the R* state. The neutral antagonist does not affect the basal equilibrium. Therefore, it has no efficacy because it binds to both active and inactive conformations. The main role of the neutral antagonist is to compete with other ligands that bind to the same site (40).

It was thought that the GPCR activation mechanism was a simple bimodal system where there are only two conformations of the receptor: active and inactive conformations. Recent evidence showed that the activation mechanism of GPCRs is not an on/off switch but rather that the receptors can adopt multiple different conformations between the inactive to active state with

unlimited intermediate states (41). Depending on the ligand that is bound to the GPCRs, a specific conformation of the receptor can be stabilized, which in turn tune the efficacy of the recruitment of the GPCR's intracellular partners (42).

There are few structural changes in the receptor that occur upon the binding of ligand to a GPCR. In the transmembrane region, there are important residues such as $Pro_{5.50}$ and $Phe_{6.44}$ that induce distortions and rotations of TM5 and TM6, which leads to an outward movement at their cytoplasmic region (43,44). The outward movements of TM5 and TM6 lead to an opening in the cytoplasmic region of the receptor that will accommodate the binding of the G-protein. There is also an "ionic lock" that is characteristic to GPCR activation. It is formed in the cytoplasmic region TM3 in a conserved motif referred to as the DRY motif. The $Arginine_{3.50}$ of this motif forms an ionic bond with $Glu_{6.30}$ and this "ionic lock" is a hallmark of receptor activation because it is usually broken by most agonists (38).

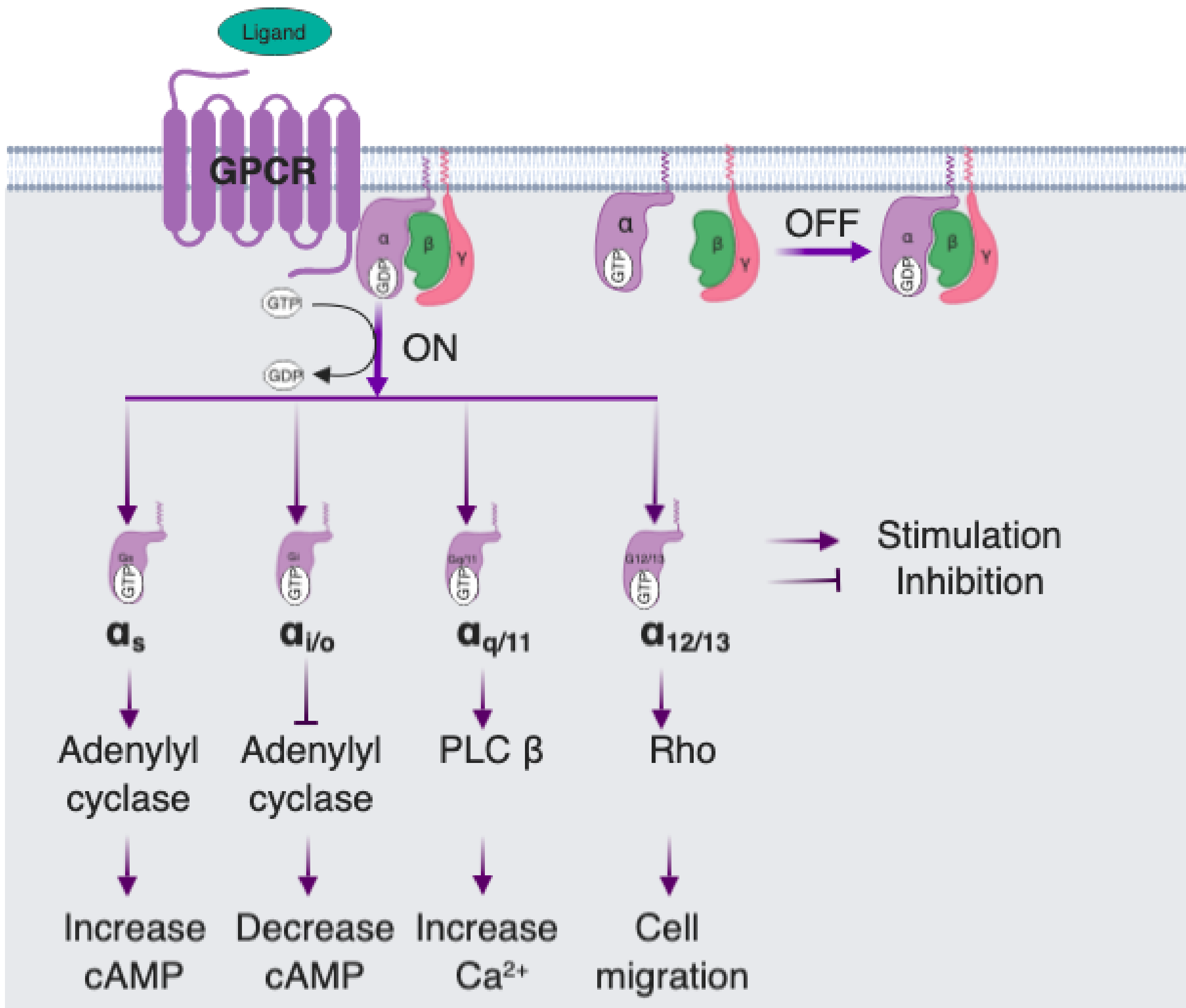
1.2.4. Signaling of GPCRs

GPCRs share a common signal transduction mechanism that involves a change in the conformation of the receptor upon the binding of an agonist, followed by the allosteric activation of the bound alpha subunit of a heterotrimeric G-protein (12,45). Signaling through the G-protein-coupled receptors occurs by the activation of at least one member of the guanine nucleotide binding protein alpha (Gα) family, hence their name G-protein-coupled receptors. The Gα subunit is part of a heterotrimeric G-protein with α, β, γ subunits that are primarily responsible for the signal transduction of GPCRs. The heterotrimeric G-protein subunits were first purified by Martin Rodbell in 1971 (46). Following the binding of the agonist, the receptor undergoes conformational changes, allowing GDP to be exchanged by GTP on the Gα subunit, and having the receptor act

as a guanine nucleotide exchange factor. Then, the activated Gα dissociates from the tightly bound Gβγ dimer (47,48). The activated G-protein subunits propagate downstream signaling by activating effector proteins. There are 16 Gα subunits that are grouped into 4 families depending on their function: $G\alpha_s$ family, which stimulate AC and thus increases cAMP levels inside the cell, $G\alpha_{i/o}$ family that have the opposite effects of $G\alpha_s$ and inhibit AC, $G\alpha_{q/11}$ family that are responsible for the activation of the phospholipase Cβ (PLC_β), which is responsible of cleaving phosphatidylinositol 4,5-biphosphate (PIP_2) into two second messengers, diacylglycerol (DAG) and inositol -1,4,5-trisphosphate (IP_3) and lastly, the $G\alpha_{12/13}$ family that leads to the activation of Rho Guanine Nucleotide Exchange Factors (GEFs). The increase of cAMP by the $G\alpha_s$ family leads to activation of cAMP-dependent pathways such as the activation of the protein kinase A (PKA), which is responsible for the phosphorylation of several downstream effector proteins. The $G\alpha_{i/o}$ family tends to have the opposite effect and inhibit cAMP dependent pathways. The $G\alpha_{q/11}$ family is responsible for the activation of protein kinases and release of calcium ions within the cell. The protein kinases phosphorylate downstream effector proteins, whereas the calcium ions serve as cofactors for various proteins and enzymes. The $G\alpha_{12/13}$ family activate cellular signaling pathways that regulate the actin cytoskeleton and, therefore, are important for cell migration (49). The other two subunits of the heterotrimer, the Gβγ, are primarily responsible for stabilizing and keeping the Gα subunits in its in GDP-bound inactive form acting as Guanine Dissociation Inhibitors (GDIs). In addition to this, they also activate many downstream effector proteins including, but no limited to, G-protein-coupled inwardly-rectifying potassium channels (GIRKs), lipid kinases (PI3K) and phospholipase Cβ (PLCβ) (14). The Gα subunit possesses an intrinsic GTPase activity that leads to the hydrolysis of the bound GTP into a GDP. The hydrolysis of the

GTP into GDP marks the inactivation of the G-proteins and its reassociation with the Gβγ subunits (50).

A wide range of biochemical, biophysical and pharmacological studies have provided evidence of ligand-dependent dynamicity between different functional states of GPCRs (13). This novel concept in GPCR pharmacology, which is termed "functional selectivity" or "biased signaling", has altered our concept of linear ligand-GPCR activation complex signaling, in which one ligand activates one receptor, leading to activation of signaling pathways with the same efficacy and potency (balanced signaling) (51). The design and discovery of functionally selective drugs can be very important in reducing the side effects that are associated with some of the drugs that on the market, which target GPCRs. This new area can only be exploited if the underlying mechanisms are thoroughly researched and understood. Currently, there is a little that is known about the molecular determinants that lead to biased signaling upon the binding of a ligand (52).



Ligand
GPCR
α
β
γ
GDP
GTP
ON
OFF
Stimulation
Inhibition
α_s
$\alpha_{i/o}$
$\alpha_{q/11}$
$\alpha_{12/13}$
Adenylyl cyclase
Adenylyl cyclase
PLC β
Rho
Increase cAMP
Decrease cAMP
Increase Ca^{2+}
Cell migration

Figure 1.3. Gα protein subtypes and their downstream signaling partners. The figure highlights the propagation of GPCR signaling through the 16 Gα subunits. They are grouped into 4 families: $G\alpha_s$, which stimulate adenylyl cyclase (AC); $G\alpha_{i/o}$, which inhibit AC; $G\alpha_{q/11}$, which activate PLC β; and $G\alpha_{12/13}$, which activate the Rho pathway. The Gβγ re-associates with the Gα subunits to complete the cycle of G-protein signaling.

1.2.5. Regulation of GPCRs signaling

There are a number of different ways that the signaling of G-protein-coupled receptors can be attenuated inside the cell through processes known as desensitization, internalization and downregulation. The desensitization involves the prevention of G-protein binding to the receptor through the recruitment of intracellular partners known as arrestins. These proteins are named arrestins because they “arrest” the signaling of G-protein-coupled receptors. In response to an overstimulation of the receptor, β-arrestin is recruited to the receptor in order to prevent the re-coupling of G-proteins. It’s not quite clear whether or not the β-arrestin competes with G-proteins in order to block the signaling. The overexpression of β-arrestin has little to no effect on G protein signaling. This argued in favor of different receptor pools instead of a sequential activation pathway at the same receptor. Signaling is attenuated by desensitization or downregulation of the receptor. If the activation of the receptor persists, the cell takes a more radical approach that involves the internalization of the whole receptors, eventually degrading the receptor.

The β-arrestins are recruited to the activated and phosphorylated G-protein-coupled receptors to either desensitize the receptor or internalize it all together. As mentioned earlier, the recruitment of the β-arrestins leads to the desensitization of the receptor by blocking G-protein interaction/recruitment. The phosphorylation of the receptor is achieved by different Ser/Thr kinases such as the G-protein-coupled Receptor Kinases (GRKs), protein kinases A or C (PKA/PKC), which phosphorylate the receptor at serine and threonine residues in either the C-terminus or ICL3 in some cases. When β-arrestin is recruited to the phosphorylated receptor, it becomes activated and releases its C terminus which leads to the recruitment at the clathrin internalization machinery. Following the recruitment to the clathrin-coated pits, the whole receptor in internalized (53). There are two outcomes for the internalized receptors; 1- it can be recycled

back to the plasma membrane or 2- they can traffic to the lysosome where they get degraded. The degradation of the receptor is what is referred to as down-regulation, where some of the receptor are completely removed from the plasma membrane.

There are four known arrestins that are found in humans. Two of them are widely distributed in all tissues, namely β-arrestin-1 and β-arrestin-2, whereas the expression of the other two arrestins are restricted to the retina, cone arrestin (arrestin 1) and visual arrestin (arrestin 2). (54).

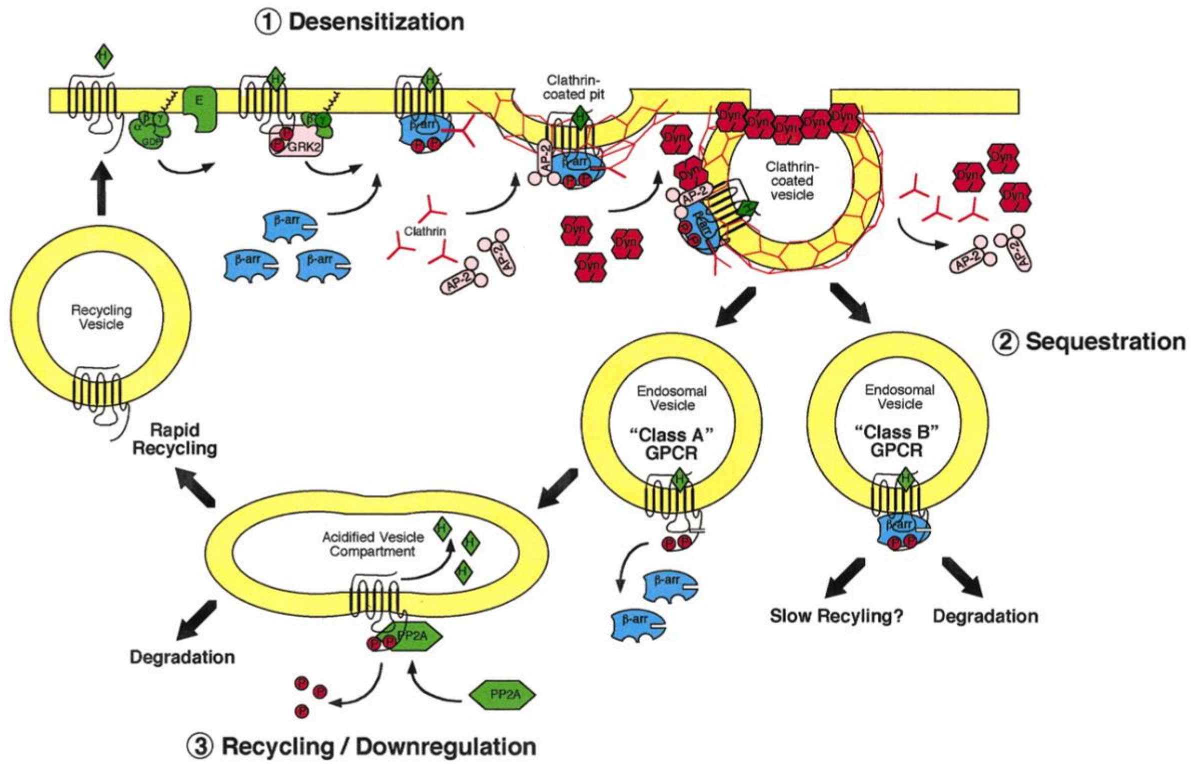

① Desensitization
Clathrin-coated pit
GRK2
β-arr
Clathrin
AP-2
Dyn
Clathrin-coated vesicle
② Sequestration
Recycling Vesicle
Rapid Recycling
Endosomal Vesicle
"Class A" GPCR
"Class B" GPCR
Acidified Vesicle Compartment
PP2A
Degradation
Slow Recyling?
Degradation
③ Recycling / Downregulation

Figure 1.4. Role of β-arrestin in GPCR trafficking. The primary roles of β-arrestin involve the desensitization and internalization of stimulated G-protein-coupled receptors. β-arrestin can be recruited to active GPCRs in order to block the coupling of G-proteins hence attenuating the signal transmission. It can also be recruited to phosphorylated GPCRs which leads to recruitment clathrin-coated pits that lead to the internalization of the receptor. Depending on the interaction between the internalized receptor and the β-arrestin, the internalized receptor can either be degraded, if the interaction is strong, or otherwise recycled back to the plasma membrane. The strength of the interaction between the β-arrestin and the internalized receptor can be determined by the identity of the ligand or the phosphorylation of the receptor. Figure is modified from (55).

1.3. Opioid Crisis

For the last 10 years, North America has witnessed a public health crisis in the form of opioid drugs misuse, which led to high levels of hospitalizations and overdose deaths (56,57). According to a report published in 2016 (58), there has been a recent increase of opioid related mortalities and morbidities in North America. From January 2016 to September 2018, there has been more than 10,300 opioid-related deaths in Canada. This staggering number of deaths is more than the number of deaths related to other premature deaths such as motor vehicle accidents and homicides (59). There was a similar opioid death crisis in British Columbia in the early 1990s; however, the current one affects the whole Canadian population from coast to coast, leading to greater number of opioid-related mortality (60).

The drugs that are responsible for the current opioid crisis are powerful opiates such as fentanyl and carfentanil, which mediate their effects through the opioid receptors. In combination with their analgesic effects, these drugs are powerful sedatives that depress the central nervous system (CNS) activity. Furthermore, the CNS is responsible for the ability of an individual to breath in and out. Consequently, drugs that depress the CNS will impair the ability of the individual to breathe normally, which leads to a respiratory depression and eventually, if not treated, death (61).

Major efforts brought about by the Canadian government to contain this opioid death crisis proved unsuccessful. In fact, some of these efforts exacerbated the effects of the crisis. For example, the Canadian government tried to limit the supply of prescription opiates in order to lower their related overdose deaths. However, the country was flooded with illicit, more powerful opiates (e.g. fentanyl), leading to even higher number of deaths due to overdose (60).

One of the ways this opioid crisis issue can be addressed involves the molecular targets of opioids: the opioid receptors. There is needs to be a way to design and manufacture new drugs targeting the opioid receptors that maintain the analgesic effects while, at the same time, eliminating or significantly reducing side effects. This is an area that is actively being researched at the moment and it involves the ability of opioid receptors to be functionally selective in their signaling pathways. This study focuses on this part of the intervention.

1.4. The Opioid System

The opioid system constitutes a set of molecular receptors and their endogenous peptides that are responsible for the reception and management of pain in mammals. The drugs that target these receptors, opiates, are used to treat severe pain. Most commonly used opiate in treatment of pain is morphine; it is used to treat acute pain such as post-surgery care as well as severe chronic pain (e.g. cancer). Although morphine related drugs are only opioids that are clinically used to treat pain, they are associated with severe side effects such as respiratory depression, constipation, tolerance and addiction (62). In the early 1970s, three opioid receptors were identified that serve as molecular targets for opiates in mammals: μOR (MOR), δOR (DOR), and κOR (KOR) (63-65). The sequence homology between MOR, DOR and KOR within the TM domains is 76%, 73% and 74% respectively whereas the overall homology is about 60% (6). Opioid receptors are part of the rhodopsin family (class A) of G-protein-coupled receptors, characterized by a seven transmembrane α-helices that span the plasma membrane and are important for the recognition and binding of extracellular ligands (66). The structures of all three receptors in this subfamily of class A have been solved; the MOR has been crystallized in both the active and inactive forms (26,67). Similarly, the KOR has also being crystallized in its active and inactive forms (25,68). To

date, the DOR has only been crystallized in its inactive form: inactive mouse DOR structure was solved in 2012 (69) and high resolution inactive human DOR in 2014 (70).

The three opioid receptors are anatomically localized in various areas of the brain including the cortex, limbic system and brain stem (71). Although the expression of these opioid receptors overlaps in some structures, their individual expression levels in these areas varies to some extent. For example, the DOR is mostly expressed in the olfactory tract, striatum and the amygdala, while the KOR is mainly localized in the basal anterior forebrain (striatum, preoptic area, olfactory tubercle, hypothalamus and pituitary). The MOR; however, is most abundantly expressed in the mesencephalon, some basal stem nuclei as well as the amygdala (71).

The μ opioid receptor (MOR) is the prototypical opioid receptor, which is named after its exogenous ligand, morphine. It is stimulated by endogenous opioid peptides named β-endorphins such as endomorphin-1 and endomorphin-2. Deletion of the MOR gene, OPRM1, in mice leads to the elimination of both the rewarding and the deleterious effects of morphine (72). This finding suggests that the MOR is responsible for the pain relieving and the side effects associated with morphine. This receptor is usually targeted for the treatment of severe post-operative, and cancer pain, in addition to, acute and chronic neuropathic pain (73).

The kappa opioid receptor (KOR) is endogenously stimulated by the dynorphins. Ketocyclazocine was the first molecule that was identified as a KOR agonist hence the name of this receptor. The activation of the KOR leads to undesirable effects such dysphoria and hallucinations (74). The third receptor in the opioid receptor family is the delta-opioid receptor (DOR), which was first identified in the mouse vas deferens hence the δ designation. Deletion of the gene of this receptor, OPRD1, in mice revealed their involvement in anxiolytic and antidepressant activities (75). DOR has also been identified to be involved in mood disorders and

can be targeted for the treatment of illnesses such as depression. In addition, they can also serve as a target for the treatment of chronic pain even though it is not as effective as the MOR.

The opioid receptor family is coupled to the inhibitory family of Gα proteins ($G\alpha_{i/o}$), which allows them to translate their extracellular signals by activating downstream effectors. The $G\alpha_{i/o}$ family of proteins inhibit adenylyl cyclase, and therefore, indirectly decrease the cAMP levels in the cell.

1.5. Ligands: Orthosteric and Allosteric

Ligands targeting GPCRs can be divided in two main categories mostly based on their action site. The orthosteric refers to the primary binding site, where the natural ligand binds and activates the receptor. Synthetic ligands that target the orthosteric site are categorized according to the pharmacological profile: full agonists, partial agonists, super agonists, antagonists and inverse agonists (76). The active and inactive conformations of the receptor are in an equilibrium in the absence of a ligand. A sub population of the receptors can become active without binding to any ligand. This phenomenon is known as the constitutive activity of the receptor. The full agonist refers to any ligand that fully activates the receptor in a manner that is similar to the natural peptide. Therefore, the full agonist is said to have 100% efficacy. It stabilizes the active conformation of the receptor. The partial agonist, however, doesn't fully activate the receptor so it has less than 100% efficacy compared to a super agonist, which has more than 100% efficacy. The neutral antagonist blocks the binding of other ligands and doesn't activate the receptor. It stabilizes the inactive conformation of the receptor. Lastly, the inverse agonist can bind to both the active and inactive conformations of the receptor but will favorize the inactive conformation. This leads to a

decrease in the basal or constitutive activity of the receptors. Therefore, the inverse agonists produce the opposite effects of agonists to some degree (76,77).

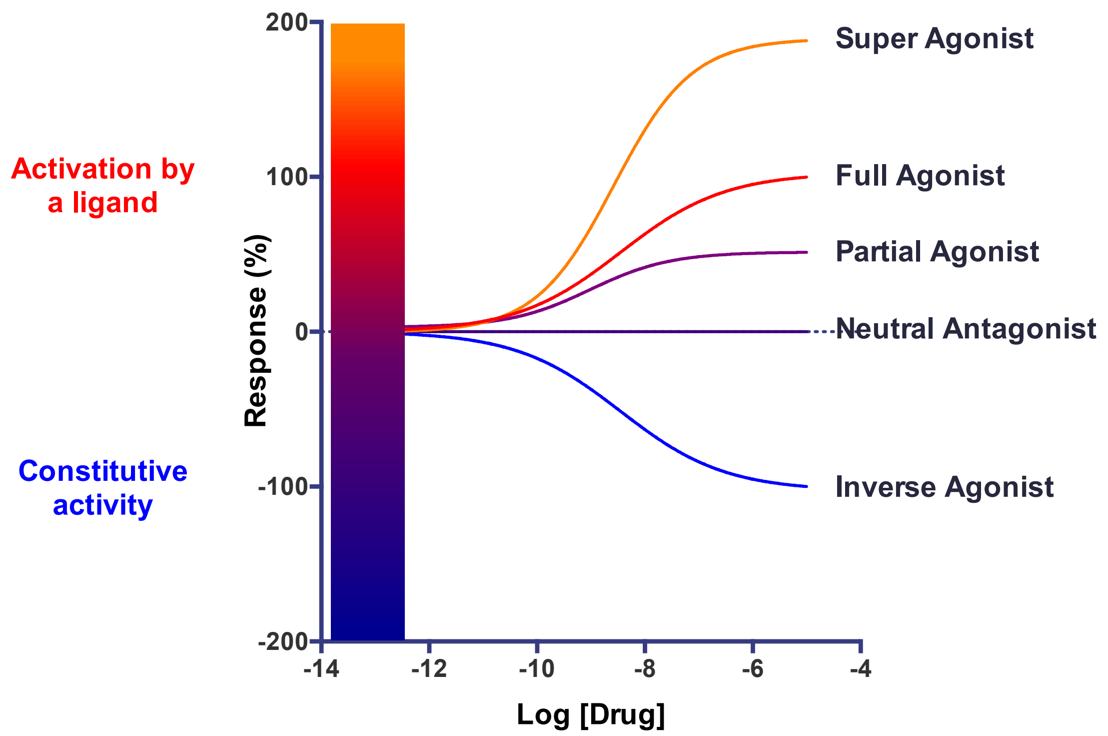

Activation by a ligand
Constitutive activity
Response (%)
200
100
0
-100
-200
-14
-12
-10
-8
-6
-4
Log [Drug]
Super Agonist
Full Agonist
Partial Agonist
Neutral Antagonist
Inverse Agonist

Figure 1.5. Ligands that target the orthosteric site of GPCRs. This diagram highlights the different kinds of ligands that bind to GPCRs. The red, orange purple curves show an example of three different types of agonists that bind and active GPCRs. The black curves illustrates the neutral antagonist that doesn’t activate the receptors. The blue curve highlights the inverse agonist, which bind to both active and inactive conformations of the receptors but only stabilize the inactive conformation.

The allosteric site, however, refers to a topographically distinct site that modulates the activity of the receptor. Ligands that target allosteric sites are referred to as Allosteric Modulators (AM). Pure allosteric modulator regulates the efficacy and/or the affinity of the receptor for an orthosteric ligand but do not processes any intrinsic efficacy. There can be Positive Allosteric Modulators (PAM), Negative Allosteric Modulators (NAM), or Neutral Allosteric Modulators (SAM). PAM increases the efficacy and/or potency, NAM decrease the efficacy and/or potency while SAM doesn't have any activity at all but can compete with PAM or NAM targeting the same site (78).

The allosteric site might potentially serve as a secondary potential drug target site that can be used to modulate the behavior of a receptor. There is a number of advantages of using allosteric modulators compared to orthosteric ligands. Allosteric modulator can serve as tools that can be used to crystallize GPCRs. It is usually difficult to crystallize GPCRs, specially the active formations, and nanobodies are mostly used for their crystallizations. The presence of allosteric modulators might help stabilize GPCRs in the presence of agonists and thus facilitate their crystallizations in the active conformations. In addition to this, the allosteric modulators might provide alternate ways to design drugs for GPCRs that are not favourable drug target to their large orthosteric ligands such as the chemokine receptors. Most importantly for our study, the use of allosteric modulators might prevent the chronic activation of opioid receptors by synthetic opioid, which is believed to be responsible for their adverse side effects such as tolerance, dependence and overdose. Despite these advantages of allosteric modulators, their mechanism of action in GPCRs is poorly understood, mostly because of the lack of compound tools to study them.

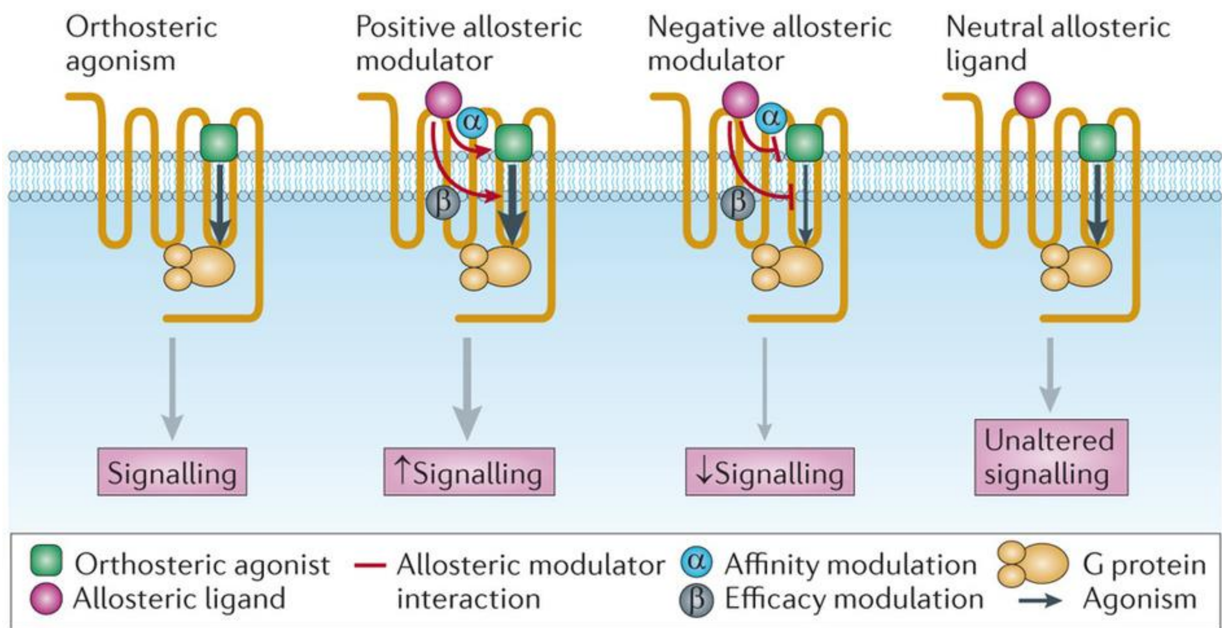

Orthosteric agonism
Positive allosteric modulator
Negative allosteric modulator
Neutral allosteric ligand
α
β
Signalling
↑Signalling
↓Signalling
Unaltered signalling
Orthosteric agonist
Allosteric ligand
Allosteric modulator interaction
Affinity modulation
Efficacy modulation
G protein
Agonism

Figure 1.6. Orthosteric and allosteric ligands that target GPCRs. This diagram highlights the two types of ligands that interact with GPCRs: orthosteric and allosteric ligands. The orthosteric ligand binds to the same site as the endogenous ligand while the allosteric ligands bind to a site within the receptor that is topographically distinct from the orthosteric site. Allosteric modulators can modulate the activity of the orthosteric ligands in three ways: positive, negative and neutral. The positive allosteric modulators increase the affinity and/or efficacy of the orthosteric ligand. negative allosteric modulators decrease the affinity and/or efficacy of the orthosteric ligand. The neutral allosteric modulators don't have an effect on the affinity and/or efficacy of orthosteric ligands. It only competes with the positive and negative allosteric modulators.

1.6. Sodium ion binding site

Fenalti and Giguere et al, have identified a conserved, sodium-dependent allosteric site in the δ-OR that is involved in the modulation of its various signaling pathways, especially the β-arrestin pathway where the amino acid residues that interact and coordinate with the sodium ion act as efficacy switch for this pathway (70). This work also highlighted that the binding of the sodium ion stabilizes the inactive conformation of the receptor. The reasons for this phenomenon are currently unknown and they are being heavily investigated by taking advantage of the availability of the high-resolution crystallography structure for δ-OR. The major advantage of having the crystal structure is that it allows for doing structure-activity-relationship (SAR) studies on the amino residues that constitute the allosteric site through site-directed mutagenesis, which leads to the identification of the amino acid residues that are responsible for specific behavior of ligands that bind to the sodium pocket. β-Arrestin recruitment at opioid receptors was found to be, at least in part, responsible for some of the negative side effects that are associated with drugs that target the opioid receptors such as respiratory depression and constipation. The role of this pathway for the dependence is still not clear (70). The sodium ion-binding pocket is crafted by 16 amino acid residues which are part of the 34 very well conserved residues in class A GPCRs. Only one residue, the asparagine at position 3.35 is not well conserved within class A GPCRs but is present in all three opioid receptors (79).

(A)

(B)

(C)

Figure 1.7. Conserved sequences of sodium ion binding site in G-protein coupled receptors. This figure highlights the sequences that make up the sodium ion binding allosteric site at the A_{2A} adenosine receptor. A) The crystal structure of the A_{2A} adenosine receptor showing the amino acid residues with more 50% sequence identity. B) A focus on the allosteric site in the presence of the sodium ion, showing some of the amino acid residues that make up the pocket. C) All 16 amino acid residues that make up the sodium pocket and their relative conservation among some class A GPCRs (48).

The coordination of the sodium ion within the DOR is quite different in comparison to that of the A_{2A} adenosine receptor (48). There is a Leucine at position 3.35 at the A_{2A} adenosine receptor, whereas the DOR has an asparagine at that position. The $Asn131_{3.35}$ at this position directly coordinates the sodium ion in addition to the conserved $Asp95_{2.50}$ and $Ser135_{3.39}$. The $Asn131_{3.35}$ also serves as connection between the allosteric site and the orthosteric site through its interaction of $Asp128_{3.32}$, which is part of the orthosteric site and interacts with all DOR ligand. The $Asp_{3.32}$ is present and essential to all aminergic receptors through interaction with a charge amine present in the ligand. This explain why opioid receptor are sometimes called aminergic peptide receptor. The highly conserved $Asp95_{2.50}$ forms a salt bridge with the sodium ion and it is very important for the stabilization of the inactive conformation of the receptor by sodium. There are five oxygen atoms that stabilize the sodium ion in the allosteric pocket: three of the oxygens come from $Asp95_{2.50}$, $Ser135_{3.39}$ and $Asn131_{3.35}$ while the other two come from nearby water molecules. Furthermore, the sodium ion also interacts with three nearby residues, two of which are part of highly conserved structural motifs that are important for GPCR activation: the $Trp274_{6.48}$ of the CWxP motif in helix VI and Asn314 of the NPxxY motif in helix VII (70).

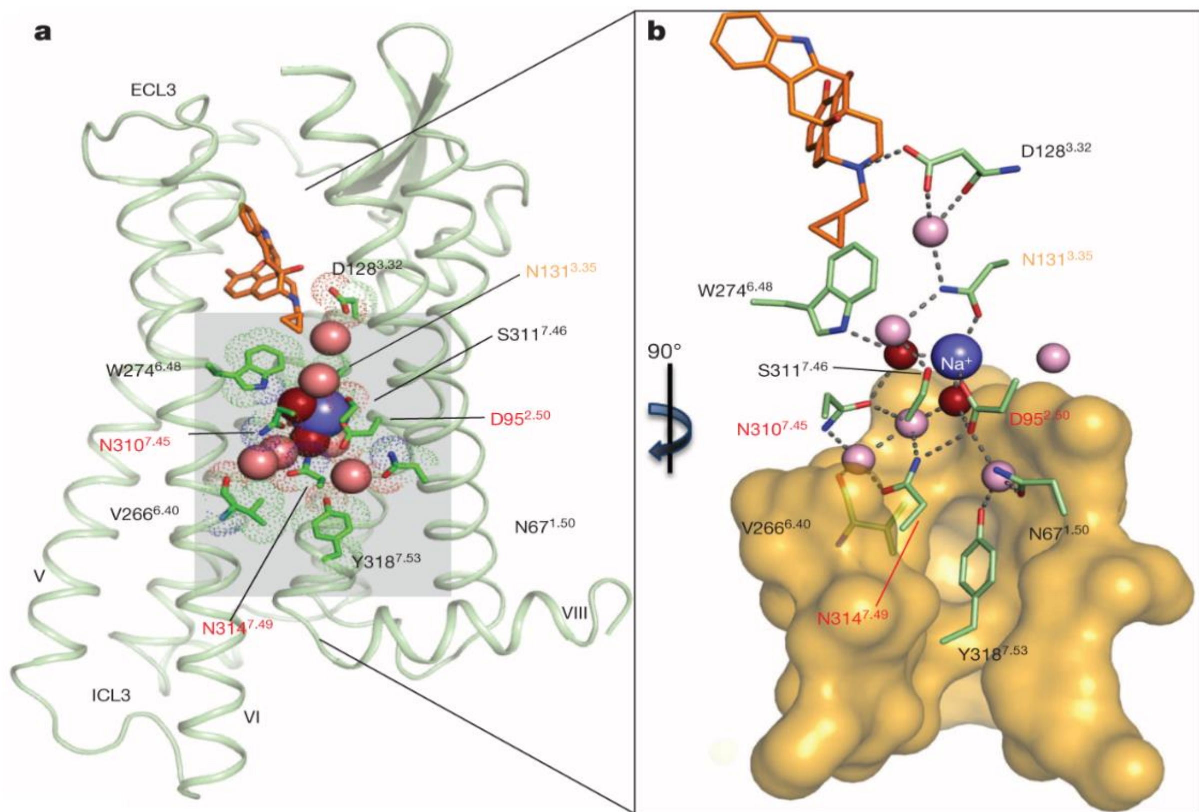

a
ECL3
D128^3.32
N131^3.35
S311^7.46
W274^6.48
D95^2.50
N310^7.45
V266^6.40
N67^1.50
Y318^7.53
V
VIII
N314^7.49
ICL3
VI
b
D128^3.32
N131^3.35
W274^6.48
90°
Na+
S311^7.46
N310^7.45
D95^2.50
V266^6.40
N67^1.50
N314^7.49
Y318^7.53

Figure 1.8. High resolution crystal structure of the DOR highlighting a sodium ion in the presence of naltrindole. The figure illustrates the 1.8 Å crystal structure of the DOR in complex with an antagonist, naltrindole (orange sticks). It also shows the coordination of a sodium ion (blue sphere) by near amino acid residues and water molecules.

1.7. Exploitation of the GPCR's allosteric potential with amiloride derivatives

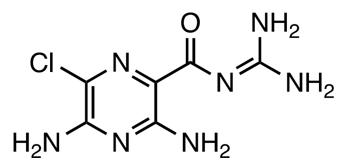
The design and the development of new class of drugs that would target the Na^{+} binding site to allosterically modulate the activity of opioid receptors might reduce the side effects that are associated with orthosteric binding opiates that are currently on the market. This Na^{+} binding allosteric site can accommodate small molecules that are as big as 200-300 Daltons (70). Although we have access to the atomic structure of the allosteric site, its functional role in GPCRs largely remains a mystery. Some preliminary studies of the Na^{+} binding site suggested that amiloride and its derivatives act as non-selective allosteric modulators in GPCRs (80). Amiloride is an FDA approved drug under that is mostly sold under the trade name, Midamor (Merck Frosst). It is a diuretic drug that reduces the excretion of potassium while, at the same time, preventing the reabsorption of sodium in distal tubule of the kidney and thus leading to the loss of sodium and water from the body. This drug directly blocks epithelial sodium channels as well as the Acid-sensing ion channel 3 (ASIC3) (81). It can also block Na^{+}/H^{+} exchangers-1 which lowers the re-perfusion injury in ischemic attacks. This is quite useful because it can used to treat edema that is associated with heart failure or liver cirrhosis (81).

It was proposed that amiloride and its derivatives can act as allosteric modulators in GPCRs with affinity that is less than 10uM (82). Follow up studies found that amiloride and its derivative had allosteric activity at several GPCRs including the Adenosine ($A_{2A}AR$)(83-85), Adrenergic ($\beta_{2}AR$)(86,87), Dopamine (D2R)(88,89) and Serotonin ($5HT_{2c}$)(89). The amiloride and its derivatives have a guanidium group in common, which was proposed to bind the Aspartic acid ($Asp_{2.50}$) in the sodium ion binding allosteric site that is also believed to bind the sodium ion (82). Despite this, the allosteric effects of amiloride and its derivatives on GPCR signaling has not been

thoroughly investigated using functional assays, but only with biophysical assay such as binding assays. The allosteric site is in close proximity to the orthosteric site, which raises the possibility of creating bitopic ligands that can bind simultaneously to both the orthosteric and allosteric sites of GPCRs (90). These studies suggest that the sodium allosteric site can serve as a druggable target that can be used to modulate the activity of some GPCRs.

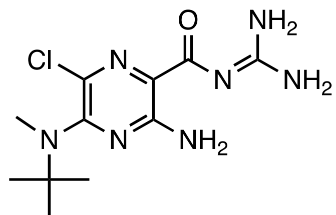
Little is known about this recently discovered sodium cavity and there needs to a thorough investigation to understand its functional importance in class A GPCRs and unlock its potential. Initially, studies heavily focused on the effects of the sodium ion on the binding of orthosteric ligands to GPCRs and, therefore, the role of the sodium cavity on receptor signaling still remains elusive. Our preliminary studies looked at the effects of amiloride derivatives on most of class A GPCRs and found that the amiloride derivatives have a functional allosteric activity at new GPCRs including the opioid receptors. Their allosteric activity was quite profound at the DOR, which is selected for further investigation. Two amiloride derivatives and a sodium channel inhibitor that is structurally related to amiloride were chosen to conduct pharmacological characterization at the DOR. As shown in **Figure 1.9**, the chemical structures of the amiloride derivatives (MIA and HMA) closely resemble the structure of the non-amiloride compound, zoniporide. Although amiloride itself doesn't have an effect on the opioid receptors at non-toxic concentrations, two of its derivatives, MIA and HMA, show the strongest allosteric effect. MIA and HMA were first designed to be high-affinity sodium channel inhibitors in comparison to amiloride. Although zoniporide is a non-amiloride, it is also a sodium channel inhibitor similar to MIA and HMA and it might also target the same sodium allosteric site.

Amiloride



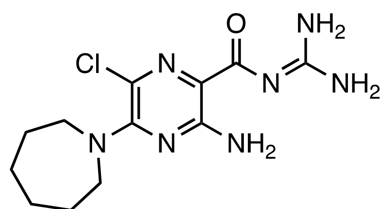
MIA

5-(N-Methyl-N-isobutyl) amiloride



HMA

5-(N,N-Hexamethylene) amiloride



Zoniporide

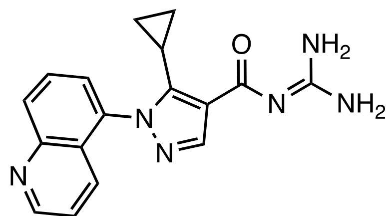


Figure 1.9. Chemical structure of the DOR allosteric modulators: MIA, HMA and zoniporide. This figure highlights the structure of the parent molecule, amiloride, as well as two of its derivatives: MIA and HMA. It also shows the structure of the non-amiloride, zoniporide. All three compounds are sodium channel inhibitors and have a guanidium group.

1.8. Study Objectives

Little is known about sodium mediated molecular control of G-protein-coupled receptors at the moment. The sodium ion binding allosteric site might play an important role in controlling the signaling pathways of GPCRs. We can exploit this allosteric site by developing new allosteric modulators that will modulate the activity of these receptors. Following the discovery of the sodium cavity, initial set of allosteric modulators were identified as amiloride-derivatives (MIA and HMA) and non-amiloride molecule (zoniporide).

A mutation of a conserved aspartic acid residue at position 2.50 was found to abolish the activity of the allosteric modulators in binding experiments. These binding experiments were done at very high concentration (>100μM), which is not physiologically relevant and toxic. In fact, the allosteric modulators at this concentration are too toxic for cell-based assays. For this reason, our lab has performed a GPCRome wide screening with the allosteric modulators at non-toxic concentration in order to find GPCRs that have amiloride-mediated functional allosterism. From this screening, it was found there were six GPCRs that showed amiloride-mediated allosterism including two of the classical opioid receptors. The only classical opioid receptor that didn't show any activity in response to the allosteric modulators was the KOR.

This led to the hypothesis that MIA, HMA and zoniporide can serve as allosteric modulators that can be used to characterize the sodium cavity and identify any critical amino acid residues that might be responsible for their binding and functional activity at the opioid receptors. Two studies are presented in this thesis. The first one deals with the characterization of the sodium pocket in order to find the determinants of the allosteric modulation activity. The second one is based on the development of a sensitive and simple biomedical tool that can used to monitor the recruitment of G-proteins and β-arrestins at the opioid receptors.

For this study, the following objectives will be addressed:

Objective #1: Characterize the sodium pocket of the δ-OR with amiloride-derivatives and small molecules by performing Structure Activity Relationship (SAR) studies on the sodium pocket. For this objective, we will introduce single point mutations in the sodium activity and characterize its effects on MIA, HMA and zoniporide using Tango, GloSensor and binding assays.

Objective #2: Development of real-time pharmacological tool for G-proteins β –Arrestins recruitment to the opioid receptors and use it to establish the recruitment profiles of opioids, which might potentially shed light on the molecular mechanisms of action for these opioids.

2.0. Methodology

2.1. Materials

General Reagents and kits

- Coelenterazine H, Gold Biotechnology, Canada
- Furimazine, Promega, WI
- D-Luciferin, Gold Biotechnology, Canada
- Poly-L-Lysine(PLL), Sigma, CA
- Hank's Balanced Salt Solution (HBSS), 1X pH 7.4 (Life Technologies, Paisley, CA)

Tissue culture reagents

- Dulbecco′s Modified Eagle′s Medium (DMEM), Corning, CA
- Bovine calf serum (BCS), Corning, CA
- Fetal Bovine serum (FBS), Wisent, CA
- Hygromycin, Sigma, CA
- Trypan Blue stain (0.4%), Thermo Fisher Scientific, CA
- Puromycin, KSE Scientific, CA
- Blasticidin S HCl, Gold Biotechnology, CA
- Antibiotic Antimycotic solution 100X, Wisent Inc, CA
- Penicillin and Streptomycin 100X, Wisent Inc, CA
- Ampicillin, Bio Basic Canada Inc, CA

Ligands, drugs, and allosteric modulators

- 5-(N-Methyl-N-isobutyl) amiloride (MIA), Millipore Sigma, CA
- 5- (N, N-Hexamethylene) amiloride (HMA), Millipore Sigma, CA

- Zoniporide Hydrochloride hydrate (zoniporide), Sigma, CA
- D-Ala2, D-Leu5 – Enkephalin- (DADLE), Bachem, CA
- BW373U86, Tocris Bioscience, CA
- Met-Enkephalin, American Peptide, CA
- Leu-Enkephalin, American Peptide, CA
- Naltrindole Hydrochloride, Tocris Bioscience, CA
- 3-Iodobenzoylnaltrexamide (IBNTXA), Purdue Pharma, CA
- 1-(3′,4′-Dihydroxyphenyl)-2-isopropylaminoethanol hydrochloride (Isoproterenol), Sigma, CA

Radioligands

- ^{3}H-DADLE, ARC (American Radiolabeled chemicals Inc), CA
- ^{3}H- Naltrindole, ARC (American Radiolabeled chemicals Inc), CA

2.2. Methods: Molecular Biology

2.2.1. Plasmids

Codon optimized human *δ-OR* cDNA was amplified from the DOR-Tango construct, which is in pcDNA3.1+, that is used in our TANGO assay. The reverse primer used to amplify the cDNA contained the 11-amino acid SmBit that would be attached to the C-terminus of the *δ-OR*. Since the N-terminus of GPCRs are located on the extracellular side, it wouldn't be reasonable to attach the SmBit on the N-terminus hence the cloning of the SmBit solely at the C-terminus. Restriction sites (NOTI at the N-terminus and XbaI at the C-terminus) were also incorporated into the primers to enable the cloning of *δ-OR-SmBit* fragment into a pcDNA3.1+ vector. The *δ-OR-SmBit* fragment and the pcDNA3.1+ were digested with NOTI and Xbal restriction enzymes,

followed by a dephosphorylation of the pcDNA3.1+ vector. Next, the two fragments were then ligated, which was followed by a transformation, colony picking and screening for positive clones via sequencing. The same protocol was followed to build the plasmids for *μ-OR-SmBit* and *Vasopressin receptor 2 (V2)-SmBit* in pcDNA3.1+. For the *κ-OR-SmBit*, however, we were able to clone it by digesting the KOR-TANGO and *V2-SmBit* with NotI and AgeI. This process would remove the V2 from *V2-SmBit* and KOR from the KOR-TANGO. Therefore, we cloned the KOR into the empty (no receptor) SmBit-pcDNA3.1+. *μ-OR-SmBit was also cloned in in pLenti-blast vector in order to produce lentivirus used to generate stable HEK293T.*

Next, we cloned the LgBit portion of the nanoluc at both the N- and C-termini of the Arr2. Unlike the 11-amino acid SmBit, the LgBit is too large to be incorporated into the primer. Therefore, it was amplified as a separate fragment from pBit1.1C (Arr2-LgBit) vector (Promega) with primers containing the BamHI and Xbal restriction sites at the 5' and 3', respectively. The Arrestin2 was amplified from a codon optimized pcDNA3.1-myc-arrestin2-YFP with NOTI and BamHI restriction sites at the 5' and 3', respectively. The LgBit and Arr2 fragments were then cloned in a pcDNA3.1+ vector, where positive cloned were determined by sequencing. This process didn't work well for the LgBit-Arr2 so we used a slightly different approach. The LgBit and the Arr2 were amplified in two separate PCR reaction; each of them with complementary ends that would overlap. In a third PCR reaction, the LgBit and Arr2 were combined without primers for 5 cycles to allow the two fragments to anneal and become a single fragment. A forward primer for the 5' of the LgBit and a reverse primer for the 3' of the Arr2 were added to the third PCR reaction. This reaction amplified a single fragment of LgBit-Arr2 with NOTI and Xbal restriction

sites. The LgBit-Arr2 fragment was cloned into pcDNA3.1+ vector and positive clones were selected by sequencing.

For the G-protein recruitment assay, we have cloned the LgBit on mini-G proteins. These LgBit-miniG-proteins (LgBit-mGx) were initially developed and published for another study (91). Mini-G proteins are basically modified G-proteins that are missing the α-helical domain as well as the part that anchors G-proteins to the membrane. Therefore, mini-G proteins are cytosolic. In terms of function, these small proteins act like the nucleotide-free form of G-protein heterotrimers that are bound to the receptor hence their ability to be recruited to the receptors. Our lab received LgBit-miniGproteins from Dr. Lambert's lab who developed them as chimeras (mini-Gαsx: most of the protein is from mini-Gαs while the last 14 amino acids are modified to either Gαo, Gαz, and Gαi). LgBit-mGαo, LgBit-mGαi, and LgBit-mGαz were synthesized at Biobasic and further clones into pcDNA3.1+.

2.3. Methods: Functional Assays

2.3.1. Cell Culture

Human Embryonic Kidney cells (HEK293T) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 5% bovine calf serum (BCS) and 100µg/ml of penicillin and streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. HTLA cells (provided by R.Axel), which are basically HEK293T stably expressing human β-arrestin fused to Tobacco Etch Virus (TEV) protease and luciferase reporter gene were maintained in DMEM supplemented with 5% FBS, 5% BCS, 100µg/ml of penicillin and streptomycin, 2.5µg/ml of puromycin and 50µg/ml of hygromycin. HEK293T cells stably expressing MOR-smBiT, the Mu opioid receptor (MOR) tagged with the small fragment of the nanoluc (smBiT), were cultured in the same media as the HEK293T cells, supplemented with 5µg/ml of blasticidin.

2.3.2. Transfection

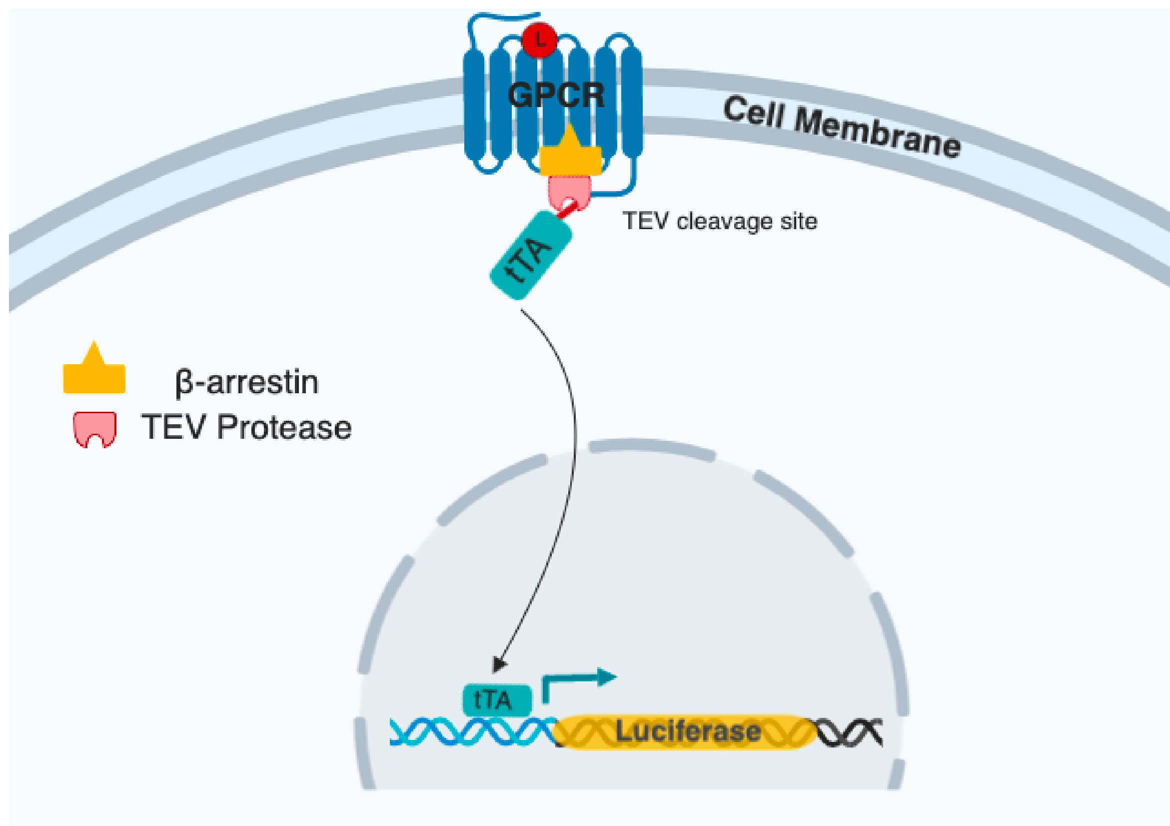
Cells were transiently transfected with different constructs of pCDNA3.1 vector with the coding DNA (cDNA) of the receptor of interest (DOR wild-type or mutants) and cDNA which encodes the yellow fluorescent protein (YFP) to follow the transfection efficiency. Calcium phosphate precipitation method was used as described by Jordan and al. in 1996 (92). In brief, HTLA and HEK293 cells are subcultured in 15cm dishes at a density of $12x10^6$ cells and $15x10^6$ cells, respectively in 25ml of media to reach a 50% confluency the day of the transfection. On the following day, for each 15cm dish a total of 1ml mixture (900µl TE 0.1X, 100µl 2.5 M $CaCl_2$, and 18µg of plasmid of interest (DOR-T, DOR or mutants) with 2 µg of YFP are mixed by vortexing.

An equal volume of HBS 2X (Hepes buffer solution 2X, pH 7.05) is added to the mixture and gently mixed. After one minute incubation at room temperature, the whole solution is added dropwise to the cells. Following a 16-18 hr incubation at 37 °C, cells are detached using trypsin 0.05%, counted and seeded to either 96 or 384-well plate depending on the experiment.

2.3.3. β-arrestin recruitment: Tango Assay

The β-arrestin recruitment assay that we use in our lab is based on the TANGO reporter assay performed as described previously in the original Tango assay (93) **(Figure 2.1).** In this assay, HTLA cells were plated on either 150 mm (12 million cells) or 100 mm (5 million cells) petri dishes and allowed to grow overnight in DMEM containing 10% FBS at 37 °C with an atmosphere of 95% air and 5% CO_2. On the next day, the cells were co-transfected with coding DNA (cDNA) that encodes for the receptor of interest (δ-OR wild type and mutants, in this case) as well as another cDNA that encodes for YFP (20μg in total) using the calcium phosphate precipitation method (92). The calcium phosphate solution precipitated the DNA complex onto the cell membranes of the HTLA cells, allowing the plasmid containing the cDNA to enter the cells passively. On the next day, the cells were transferred on PLL coated, clear bottom 384-well plate (22,000 cells/well) and starved overnight in DMEM containing 1% dialyzed FBS serum. The starvation serves as a way to increase the signaling of the cells once stimulation with a drug. In the following day, the cells were stimulated with ligands that were prepared in filtered assay buffer (20 mM HEPES, 1× Hanks' balanced salt solution (HBSS), pH 7.40) at a concentration of 3.5x (10μM; final concentration) and incubated overnight (16-18 hours). In the final day, the drug solution and the media mix were removed and Bright-Glo reagent (Promega) was added to each well at a dilution of 1:20 in assay buffer. The plates were then incubated at room temperature for

5 minutes prior to counting the luminescence on a luminescence reader (MicroBeta). The data generated by the MicroBeta was subjected to a non-linear regression analysis using the sigmoidal dose curve function found in Graphpad Prism 7.0.



L
GPCR
Cell Membrane
TEV cleavage site
tTA
β-arrestin
TEV Protease
tTA
Luciferase

Figure 2.1. Figure 2.1: Design and principle of the Tango Assay.

Our lab uses HEK293T that stably express two important proteins: the fusion protein β-Arrestin2-TEV protease and the reporter gene luciferase controlled by the tetracycline-operon ($TetO_7$), and these cells are known as HTLA. The receptor is expressed as a fusion protein containing the TEV cleavage site followed by the tetracycline-transactivator protein (tTA), which is created by a fusion of the tetracycline repressor (TetR), with the activation domain of VP16. When the receptor is activated, the β-Arrestin2-TEV is recruited to the activated GPCR and then the TEV protease cleaves the TEV cleavage site leading to the release and translocation of the transcription factor (tTA) into the nucleus, which allowed the transcription of the luciferase. Following 16 hours of incubation, cells are lysed with a lysis buffer containing the Luciferin (Luciferase substrate) and stabilizer following by luminescence read on a microplate reader (94).

2.3.4. Gαi/o activation: cAMP (GloSensor) assay

This is an experimental assay that was used to study the Gαi/o signaling pathway of G-protein-coupled receptors. **(Figure 2.2)** The protocol for this assay is similar to the Tango assay; however, there were few significant differences between the two, which is going highlighted in this subsection. For this assay, HEK293T cells were co-transfected with plasmids containing the wild type or various mutants of δ-OR along with another plasmid containing a gene that encodes for split luciferase-based cAMP biosensor (GloSensor; Promega) and were incubated overnight. The cells were then split and seeded onto PLL-coated clear bottom 384-well plates (18,000 cells/well) with DMEM containing 1% dialyzed FBS and were incubated overnight. In the following day, the media was removed from the 384-well plates before the cells were stimulated with a filtered assay buffer (20 mM HEPES, 1x HBSS, pH 7.40) that contained allosteric modulators and luciferin at 1x, which were incubated at RT for 45 minutes. After the 45-minute period, the cells were immediately stimulated with the drug (δ-OR agonists/antagonists) at 3x and were then incubated at RT for 15 minutes. This was followed by the addition of isoproterenol (a β_2-adrenergic receptor agonist) at a final concentration of 400nM or Forskoline at 5µM. Since the stimulation of Gαi inhibits the formation of cAMP within cells and thus decreases the [cAMP], the isoproterenol initially increases the cAMP levels within cells, by activating Gαs through the β_2-adrenergic receptor, in order to have a greater window for visualizing the inhibitory activity of Gαi signaling pathway. The luminescence was quantified using a MicroBeta luminescence reader. Data generated by the MicroBeta was subjected to nonlinear regression analysis using the sigmoidal dose curve function and this analysis was performed on GraphPad Prism 7.0 software.

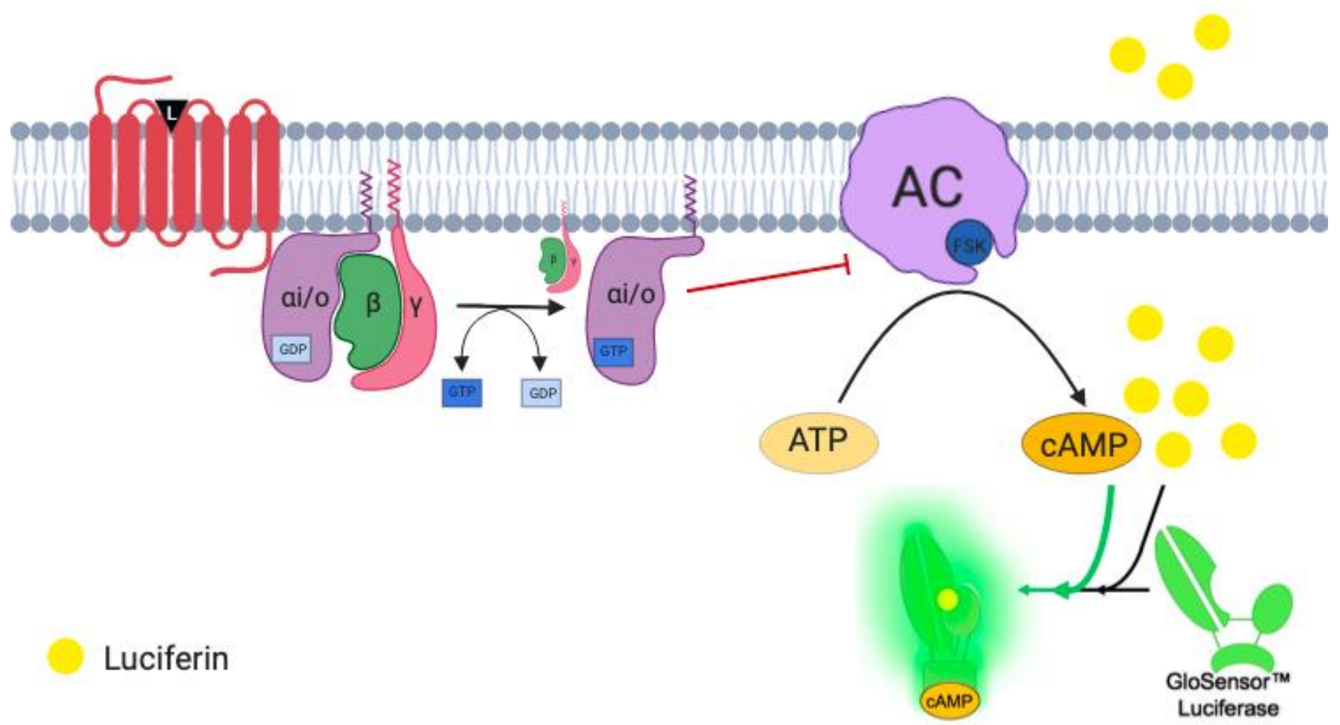

L
αi/o
β
γ
GDP
GTP
GDP
αi/o
GTP
AC
FSK
ATP
cAMP
cAMP
GloSensor™
Luciferase
Luciferin

Figure 2.2. The principle of the GloSensor (cAMP) assay for monitoring Gαi/o activation. This assay measures the cAMP levels inside the cell in response to the activation or inhibition of either **Gα_s** or **Gαi/o** proteins, respectively. Since the opioid receptors are coupled to **Gαi/o**, this assay monitors the reduction of the cAMP level inside the cell. The GloSensor protein is a biosensor that is based on the split firefly luciferase. It has two binding sites; one for cAMP and another for the substrate, Luciferin. The GloSensor only becomes functional when it is bound to cAMP. Upon binding of cAMP, the functional GloSensor can then interact with Luciferin to produce luminescence, which is then quantified by MicroBeta plate reader.

2.3.5. β-arrestin recruitment and Gαi/o recruitment: NanoBiT assay

This functional assay takes advantage of the recently developed protein complementation reporter assay called Nano Binary Technology (NanoBiT), which can be used to study dynamic protein-protein interactions. This reporter assay is designed from a split nanoluciferase containing an 11-a.a (SmBit) and an 142-a.a segments (LgBit), which catalyzes a bright luminescence reaction once complemented (**Figure 3**). The addition of the 11-residues smBit at the C-terminus of the target GPCR is believed to have less functional disturbance than any actual assays involving large fusion protein such as Tango, BRET or FRET. We have optimized the fusion of LgBit to the β-arrestin-1 and -2 and various mini G-proteins and managed to build a versatile toolbox that can be used to assess the molecular activity of certain opiates including newly developed biased ligands. This assay uses precisely the same receptor fusion to test these drugs at the various signaling pathways (β-arrestin and G-proteins) within the exact same time frame. This, we believe, will provide valuable and reliable information on the mechanisms of drug recognition.

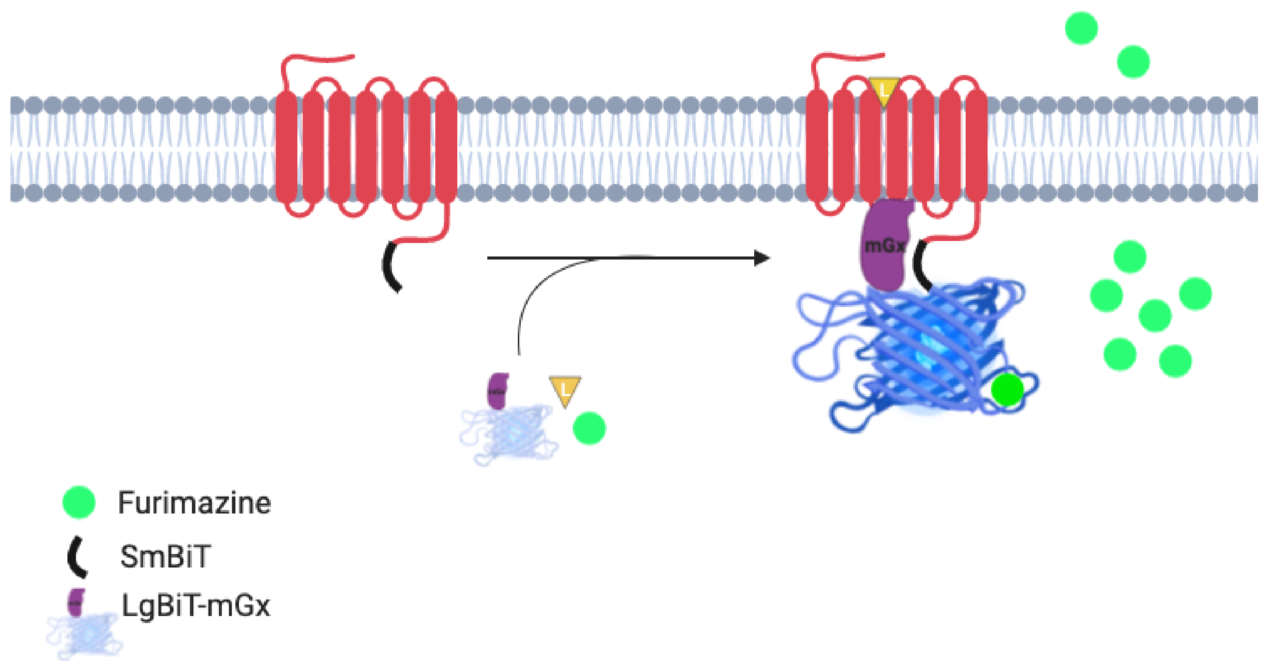

mGx
L
L
Furimazine
SmBiT
LgBiT-mGx

Figure 2.3. Design and principle of the NanoBiT assay for G-protein and β-arrestins recruitment. This figure highlights the principle of the NanoBiT assay for monitoring the recruitment of G-proteins and β-arrestins at the activated opioid receptors. The recruited G-proteins β-arrestins bring the complementary NanoLuc fragments together, which makes it a functional protein that releases bioluminescence.

2.4. Methods: Binding Experiments

2.4.1. Membrane Preparation

HEK293T cells, transiently transfected with the receptor were washed and scraped with PBS and spun down for 3 minutes at speed of 500g. Then, PBS was decanted and cells were resuspended in 8 ml per 15 cm culture dish of hypotonic cold lysis buffer (50 mM Tris-HCl pH 7.4). and incubated for 10 minutes on ice followed by centrifugation at 30,000 x g for 25 minutes. After decanting the supernatant, the pellet was resuspended in 4 ml per 15 cm plate of cold binding buffer (50mM Tris-HCl, 10mM $MgCl_2$, 0.1mM EDTA, pH 7.4). Using a Polytron, membranes were homogenized 4-5 times for 2-3 seconds each. Finally, the protein quantification was done using a BCA kit from Promega. For all binding experiments, 25 to 40 µg of membrane in 75µl of binding buffer per well of 96 well plate was used (95).

2.4.2. Saturation Binding Assay

Saturation binding assay can be used to determine the affinity of a ligand for its recognition site, which is termed as the equilibrium dissociation constant, K_D, for a particular a radioligand. The assay is conducted in 96-well plates with a final volume of 125µl/well. Twenty-five microliters of ^{3}H-DADLE is added to each well of a 96 well plate with a serial dilution from 10nM to 0.08nM, followed by addition of 25µl of antagonist naltrindole at a final concentration of 10µM for nonspecific binding or 25µl of binding buffer (total binding). Finally, 75µl containing 25 to 40µg of membrane protein was added and incubated for two hours in the dark at room temperature. Then, the reaction was stopped by vacuum filtration and washing three times with cold washing buffer (50mM Tris-HCl, pH 7.40) using glass fiber filter, which was previously socked in cold washing buffer containing 0.3% polyethyleneimine (PEI). Filter was dried on hot plate for 2

minutes, and a melt-on scintillation sheet (Melt Lex A, Perkin Elmer) was melted on the top of the filter. Finally, the quenched radioactivity was mesured by using a scintillation microplate reader (MicroBeta). The K_D value was calculated from specific binding (total binding -nonspecific binding) using non-linear fit with one site – specific binding analysis on GraphPad Prism using formula: Y = Bmax * X/(Kd + X) where Y is the specific binding and X is the radioligand concentration.

2.4.3. Displacement Assay

Displacement binding assay is also referred to as a competition assay; it is performed to determine the binding affinity which is the inhibition constant of drug ($Ki=IC50/(1+([L]/K_D)$). The assay procedure is similar to the saturation assay in the previous procedure. It is performed with a final volume of 125µl/well in a suitable binding buffer (50mM Tris-HCl, 10mM $MgCl_2$, 0.1mM EDTA, PH 7.4). The concentration of the hot ligand ^{3}H-DADLE or ^{3}H-naltrindole is at a concentration close to their respective K_D which were calculated previously by using the saturation binding assay. Thus, 25µl of the hot ligand was added in every well (final concentration of 1nM for ^{3}H-DADLE or 2nM for ^{3}H-naltrindole), followed by the addition of 25µl of cold ligand (DADLE or naltrindole as a reference) in serial dilutions starting with 10 µM to 0.1 µM. In the case of the cold ligands MIA, HMA, and zoniporide, they were added in serial dilution, starting with a high concentration of 31.5µM due to a weak binding affinity expectation. The reaction was started by adding 40 ug of the cell membrane in 75 ul of binding buffer in each well. Following 2 hours of incubation period at room temperature in the dark, the rest of procedure is the same as the saturation assay which was previously described (95). The data were analyzed with GraphPad Prism 7.

3.0 Results

3.1 Characterization of the sodium ion-binding cavity in the delta-opioid receptor

The goal of this project is to find ways that can be used to design and develop better and safer analgesics with little to no side effects in comparison to the opiates that are available on the market at the moment. To achieve this goal, we propose to take advantage of the functional selectivity aspect of opioid receptors, which allows compounds to preferentially activate one signaling pathway over another. Essentially, we will be able to design and develop drugs that will have the benefits of analgesics while, at the same time, blocking that pathways that are associated with the side effects. The characterization of sodium ion binding site paves the way for designing allosteric modulators with these unique properties.

3.1.1. Effects of amiloride derivatives and small molecules on the β-arrestin recruitment at the wild type delta-opioid receptor

In this study, we used the tango assay to look at the effects of amiloride-derivatives (MIA and HMA) and small molecule (zoniporide) at the β-arrestin2 recruitment of the δ-OR**.** As shown in **Figure 3.1**, HTLA cells that stably express β-arrestin2 fused with TEV protease were transfected with plasmids containing the δ-OR fused with a TEV cleavage site and tTA (Tango-ized δ-OR. The cells were incubated overnight with either Buffer, or single concentration of MIA, HMA, or zoniporide followed by an increasing concentration of DADLE. The red and green curves in **Figure 3.1** indicates the Ago-PAM activity of MIA and HMA. At low concentration of DADLE (left side of the curve), we observe agonist activity of each modulators. At higher concentration of DADLE, the MIA, HMA and zoniporide seem to increase the efficacy of DADLE, which is a characteristic of positive allosteric modulator (PAM). To validate the intrinsic agonist activity of

MIA through the delta-opioid receptor, we used an opioid antagonist that would stabilize the inactive conformation of the receptor. The antagonist that was used for this experiment was naltrindole: an opioid antagonist that is selective for the δ-OR. As expected, naltrindole completely reversed the intrinsic agonist activity of MIA and HMA (**Figure 3.2**; red and green curves), which suggests that the MIA and HMA stabilize a partially active conformation of the δ-OR. This finding also informs that the effects that we see with amiloride-derivatives and the small molecules is a direct activation of the receptor and not a system effect.

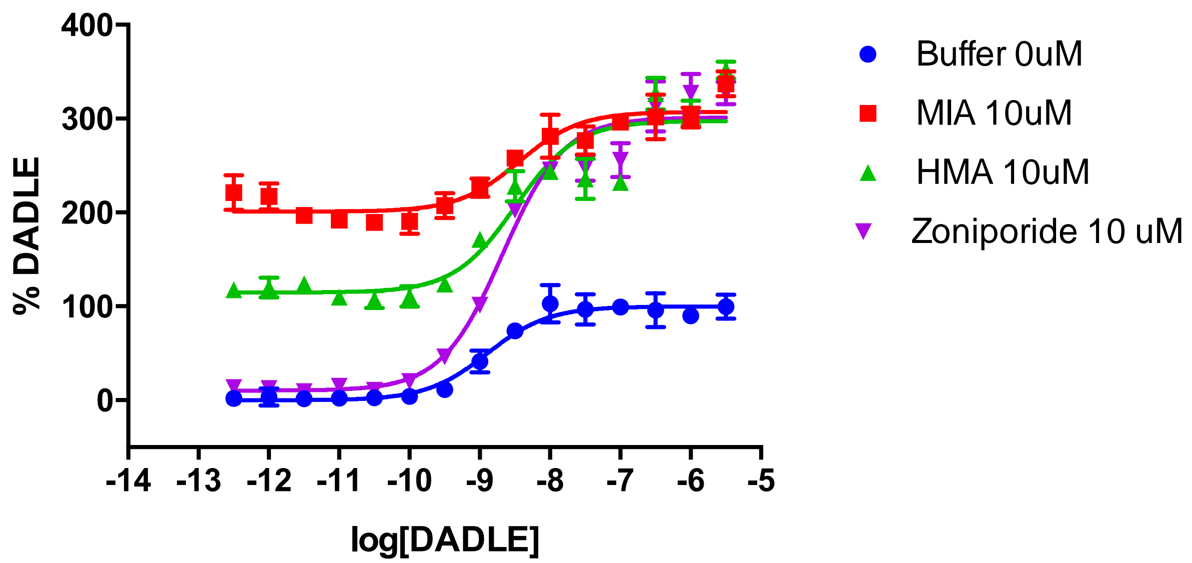

b-arrestin2 recruitment to activated d-OR
400
300
200
100
0
% DADLE
-14 -13 -12 -11 -10 -9 -8 -7 -6 -5
log[DADLE]
Buffer 0uM
MIA 10uM
HMA 10uM
Zoniporide 10 uM

Figure 3.1. Allosteric effects of some amilorides-derivatives & small molecules at the activated δ-OR in TANGO assay. MIA and HMA activate the receptor in the absence of DADLE and, thereby, have intrinsic agonist activity as measured by β-arrestin recruitment. zoniporide have no intrinsic agonist activity. Blue: Buffer with DADLE; RED: MIA (10uM) with DADLE; Green: HMA (10uM) with DADLE; Purple: zoniporide (10uM) with DADLE. The x-axis represents log of drug concentration in Molar. The data is normalized to the orthosteric agonist and presented as %DADLE on the y-axis. Each point is triplicate, and the data is from 3 independent experiments (n=3).

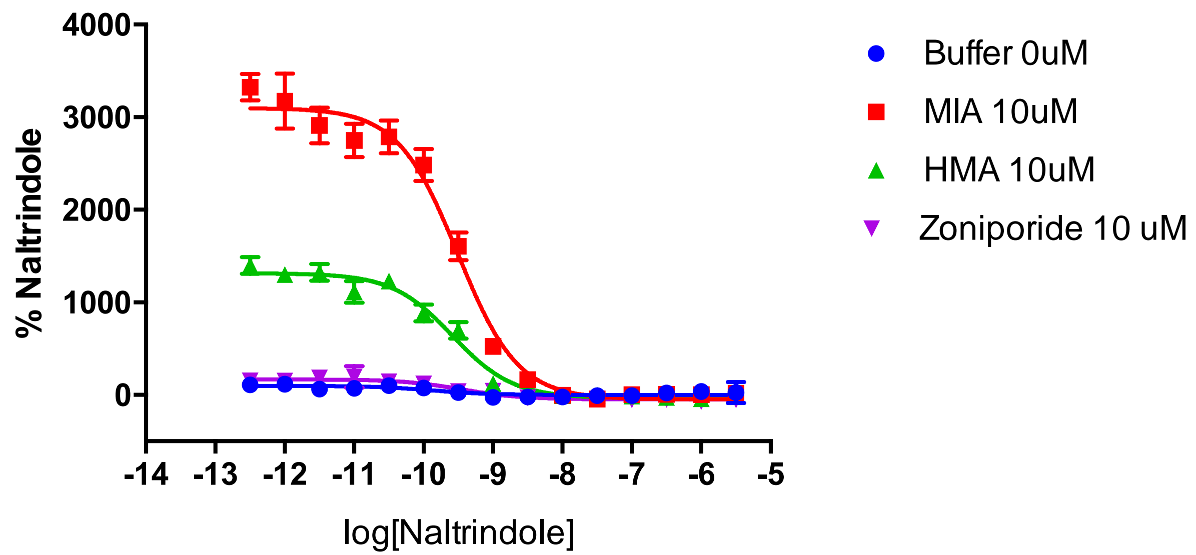

b-arrestin2 recruitment to activated d-OR
4000
3000
2000
1000
0
% Naltrindole
-14
-13
-12
-11
-10
-9
-8
-7
-6
-5
log[Naltrindole]
Buffer 0uM
MIA 10uM
HMA 10uM
Zoniporide 10 uM

Figure 3.2. Naltrindole block and reverse the effects of the two amilorides-derivatives & the small molecule at the activated δ-OR in TANGO assay. This graph shows a dose response curve of naltrindole in the absence or presence of two amiloride derivatives and a small molecule. Naltrindole shows antagonism against the compounds with the intrinsic activity: MIA and HMA. The x-axis represents log of drug concentration in Molar. The data is normalized to the orthosteric antagonist and presented as % naltrindole on the y-axis. Each point is triplicate, and the data is from 3 independent experiments (n=3).

3.1.2 Effects of amiloride derivatives and small molecules on the Gαi activation at the wild type δ-OR

In this study, we used the GloSensor assay to characterize the effects of two amiloride-derivatives and one small molecule on the G-protein pathway at the wild type δ-OR. As shown in **Figure 3.3**, HEK293T cells were co-transfected with two plasmids: one that expressed the wild type δ-OR and another that expressed the split luciferase GloSensor. Following a successful transfection determined by the expression of YFP, the cells were incubated with Luciferin for 45 minutes following by 15 minutes incubation with either buffer, MIA, HMA or zoniporide. The cells were then incubated with an increasing concentration of DADLE for another 15 minutes prior to adding the forskoline (FSK) and recording the luminescence on the plate reader. The FSK was added to increase the cAMP levels in the cells in order to clearly highlight the activation of the inhibitory G-protein (Gi/o) that inhibits adenylyl cyclase and indirectly decreases the cAMP levels inside the cells. All three allosteric modulators activated the wild type δ-OR in the absence of DADLE with MIA being the strongest of all (-12.5 on **Figure 3.3**). However, none of the allosteric modulators show a PAM activity in this G-protein pathway unlike the β-arrestin pathway (-5 on **Figure 3.3**). As previously shown for β-Arrestin pathway, the δ-OR antagonist naltrindole, reverses the ago effects of the amilorides and small molecules that is especially drastic for the MIA that has the strongest agonist activity (**Figure 3.4**). As observed, naltrindole alone (buffer), shows an increase in cAMP level due to its weak inverse agonist activity. It has to be noted that δ-OR has a high-constitutive activity when heterogeneously expressed.

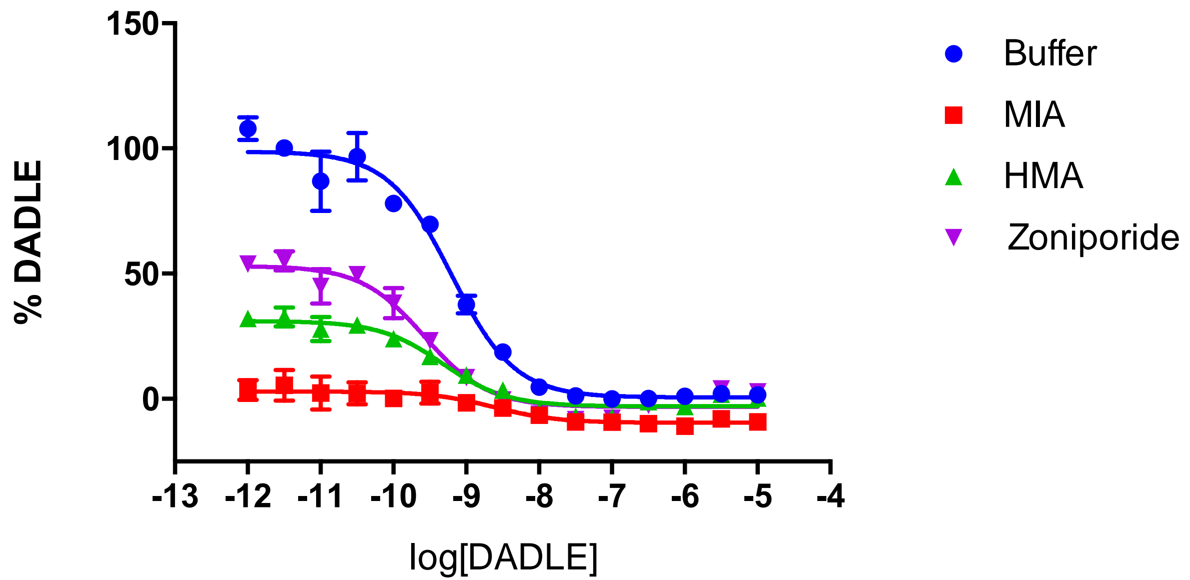

Gαi activation at the δ-OR
150
100
50
0
% DADLE
-13
-12
-11
-10
-9
-8
-7
-6
-5
-4
log[DADLE]
Buffer
MIA
HMA
Zoniporide

Figure 3.3. Allosteric effects of some amilorides-derivatives & small molecules at the activated δ-OR in GloSensor assay. All indicated small molecules have agonist activity at the δ-OR as measured by cAMP modulation. MIA and HMA showed the strongest agonist activity among all. No PAM effect was observed in this assay. The x-axis represents log of drug concentration in Molar. The data is normalized to the orthosteric agonist and presented as %DADLE on the y-axis. Each point is triplicate, and the data is from 3 independent experiments (n=3).

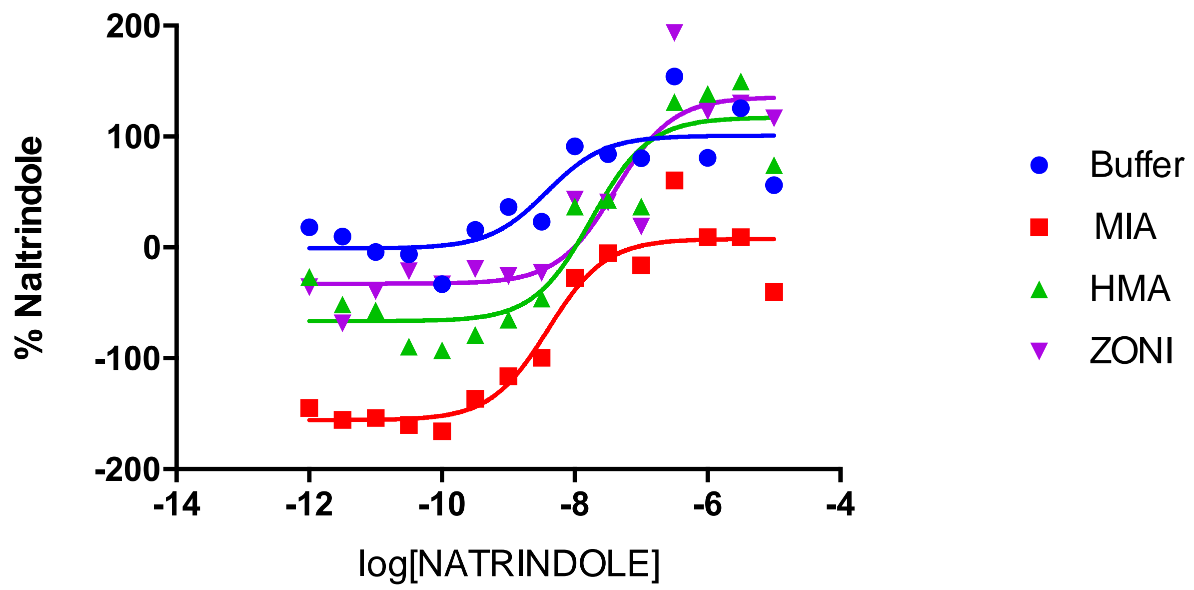

Gαi activation at the δ-OR
% Naltrindole
200
100
0
-100
-200
-14
-12
-10
-8
-6
-4
log[NATRINDOLE]
Buffer
MIA
HMA
ZONI

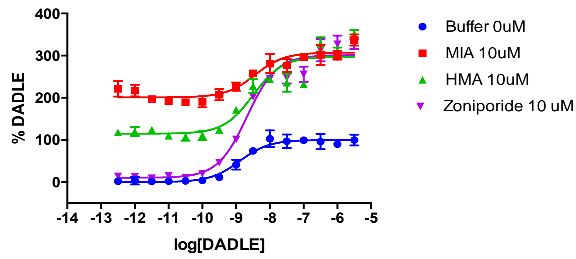
Figure 3.4. Non-competitive antagonism of naltrindole against two amilorides-derivatives & a small molecule at the activated *δ*-OR in GloSensor assay. The figure shows a dose response curve of naltrindole in the absence (buffer) or presence of three allosteric modulators (MIA, HMA, zoniporide). The intrinsic agonist activity of the allosteric modulators can be reversed by the δ-OR antagonist/inverse agonist, naltrindole. The x-axis represents log of drug concentration in Molar. The data is normalized to the orthosteric antagonist and presented as % naltrindole on the y-axis. Each point is triplicate, and the data is from 3 independent experiments (n=3).

3.1.3. Effects of single point mutations within the sodium cavity on the amiloride derivatives and small molecule at arrestin-recruitment using the TANGO assay.

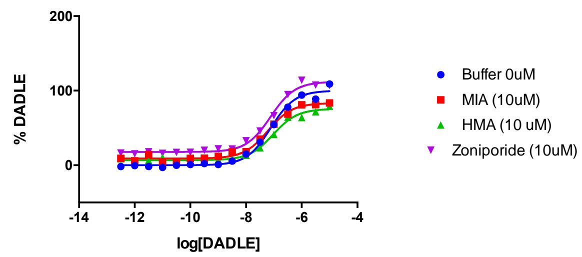
The high-resolution crystal structure of the human delta-opioid receptors was published in 2014, which led to the identification of an allosteric cavity within the receptor that harbours a sodium ion and water molecules (70). There were five residues that were identified to directly coordinate the sodium ion including the highly conserved D95$_{2.50}$ residue. The other four residues are N131$_{3.35}$, N310$_{7.45}$, N314$_{7.49}$ and S135$_{3.39}$. Initially, we focused on these important residues and proceeded to exchange each residue for an Alanine (or Valine in the case of N131) to study its effect on the amiloride derivatives and the small molecule. As highlighted in **Figure 3.5**, each of the tango-ized delta-opioid receptors mutants (D95A, N131V, N310A, N314A and S135A) were transfected in HTLA cells that are stable for β-arrestin2 fused with TEV protease. Following the transfection, the cells were incubated with either buffer, MIA, HMA or zoniporide as well as an increasing concentration of DADLE. Following a 16 hours stimulation, the luminescence from the cells were counted on a MicroBeta plate reader. The five mutants that we focused on for this part of the study showed different profiles for the allosteric (amiloride derivatives and small molecule) and the orthosteric (DADLE) compounds. For the allostery, the D95A mutants seemed to have the largest effect on the allosteric modulators. In that, it significantly reduced the ago-PAM effect of both MIA and HMA while also abolishing the PAM effect of zoniporide. We believe that this aspartic acid residue interacts with the guanidium group of the allosteric modulators; therefore, its drastic effect on the amiloride derivatives and small molecules are to be expected. The other four mutants, however, don't reduce the ago-PAM activity of the amiloride derivatives and the PAM activity of the zoniporide. In fact, the zoniporide seems to be converted into ago-PAM even though

its ago-PAM activity is not as strong as the amiloride derivatives. In addition to the allosteric effects, the mutants also demonstrate effects on the orthosteric compound activity. The S135A and the N310A mutants significantly reduce the potency of the orthosteric ligand, DADLE. Interestingly, the S135A mutant shows a complete loss of efficacy and potency with the agonist DADLE alone. However, the ago-PAM and PAM activity of all three modulators is not affected and even it is enhanced. This strongly support our hypothesis of allosteric modulation by all three modulators but also shows that the receptor is still functional and that the affinity of DADLE is not strongly affected but rather its efficacy. Thus, the allosteric modulators compensate and restore this efficacy loss by DADLE.

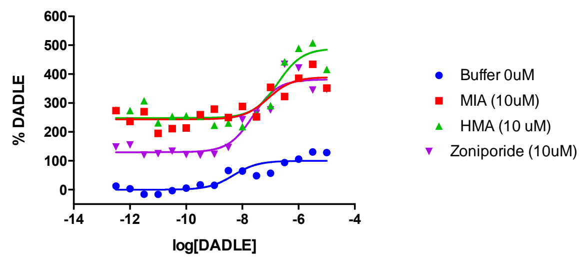
A



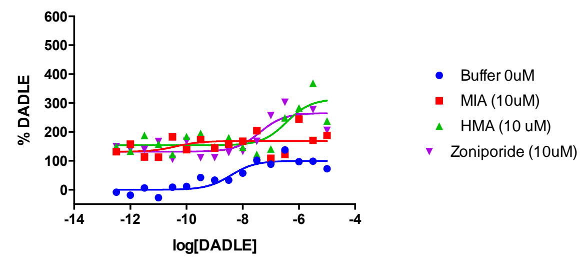
B



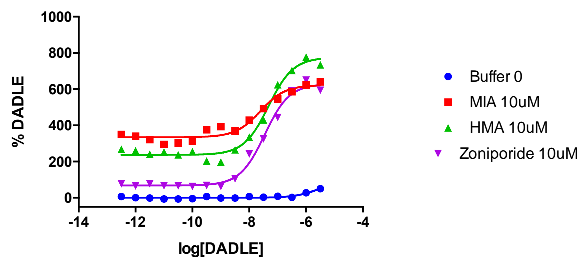
C



D



E



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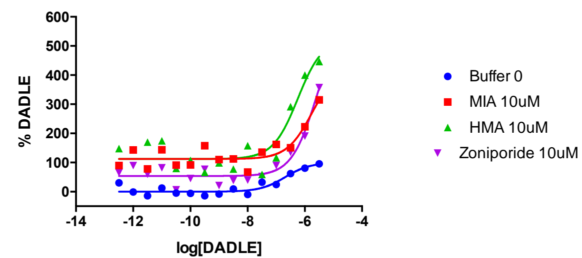
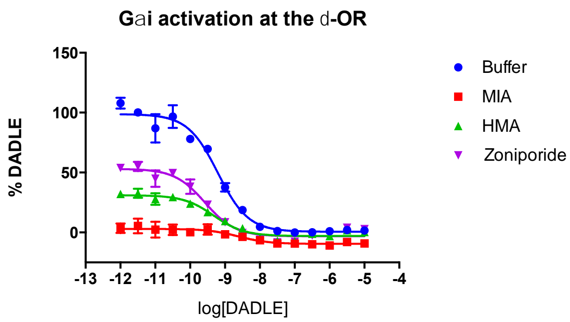
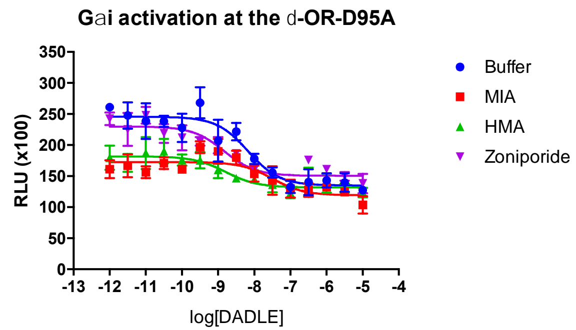


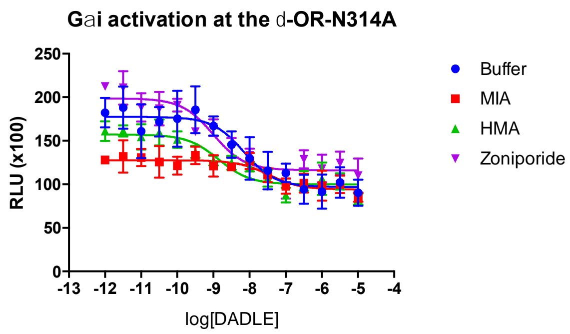
Figure 3.5. Evaluation of critical residues mutation in the sodium binding cavity of the δ-OR. This figure highlights the effects of single point mutation of the critical amino acid residues lining the sodium ion binding cavity on the amiloride derivatives and small molecule. It shows the dose-response curve of the wild type delta-opioid receptor (A) and the mutation of the five amino acids that coordinates the sodium ion in the delta-opioid receptor (B-F) in the absence (buffer) or presence of MIA, HMA and zoniporide. The x-axes represent log of drug concentration in Molar. The data is normalized to the orthosteric agonist and presented as % DADLE on the y-axes. Each point is triplicate and the data for each dose response curve is from 5 independent experiments (n=5).

3.1.4. Effects of single point mutations within the sodium cavity on the amiloride derivatives and small molecule at G-protein activation in the GloSensor assay.

The effects of the mutations within the sodium pocket on the amiloride derivatives and the small molecule in the Gαi-activation pathway was also investigated. HEK293T cells were transfected with plasmids that would express each of the five mutants and another plasmid that expresses the GloSensor protein. The cells were then incubated with the GloSensor substrate (Luciferin) for 30 minutes followed by a 15 minutes incubation with either buffer, MIA, HMA or Zoniporide. Cells were then stimulated with an increasing concentration of DADLE for 15 minutes, followed by the addition of isoproterenol. Following a 15 minutes incubation, the luminescence from the cells were counted on a MicroBeta plate reader. Data suggests that the mutants don't have a major effect on the ability of the allosteric modulators to activate the Gαi activation pathway. Similar to the wildtype DOR, all three allosteric modulators intrinsically activate the receptor with MIA being the strongest of them, followed by HMA and then zoniporide. Furthermore, the PAM effect is also absent for these allosteric modulators at each of the mutants, which is similar to what we have got with the wildtype receptor.


Gαi activation at the d-OR
% DADLE
150
100
50
0
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4
log[DADLE]
Buffer
MIA
HMA
Zoniporide


Gαi activation at the d-OR-D95A
RLU (x100)
350
300
250
200
150
100
50
0
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4
log[DADLE]
Buffer
MIA
HMA
Zoniporide


Gαi activation at the d-OR-N314A
RLU (x100)
250
200
150
100
50
0
-13 -12 -11 -10 -9 -8 -7 -6 -5 -4
log[DADLE]
Buffer
MIA
HMA
Zoniporide

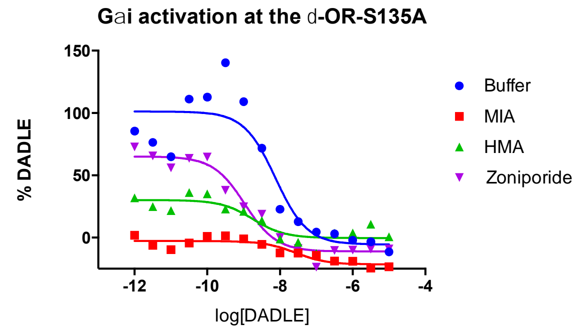
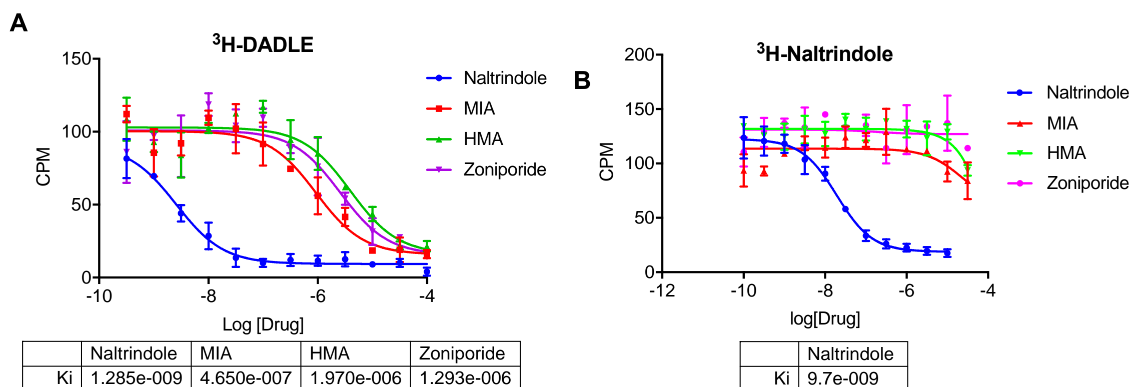

Gαi activation at the d-OR-S135A
% DADLE
150
100
50
0
-12 -10 -8 -6 -4
log[DADLE]
Buffer
MIA
HMA
Zoniporide

Figure 3.6. Allosteric effects of some amilorides-derivatives & small molecules at the activated *δ*-OR mutants in GloSensor assay. The figure highlights the effects of the three allosteric modulators on DOR mutants. All indicated small molecules have agonist activity at each of the δ-OR mutants as measured by cAMP modulation. MIA and HMA showed the strongest agonist activity among all. No PAM effect was observed in this assay. Notice that N314A and N131V mutants are not shown here because they were not functional in this assay. The x-axes represent log of drug concentration in Molar. The data is normalized to the orthosteric agonist and presented as % DADLE on the y-axes. Each point is triplicate, and the data is from 3 independent experiments (n=3).

3.1.5. Binding experiments of allosteric modulators on the wild type δ-OR.

We then aimed to quantify the allosteric equilibrium constant (K_B) at the level of affinity using radioligand binding experiment. However, after preliminary experiments, we found that all tested compounds displace the 3H-DADLE preventing conventional allosteric displacement experiments. Alternatively, we performed radioligand displacement assay using tritiated DADLE and naltrindole with each of the amiloride derivatives and the small molecule. As shown in **Figure 3.7A and B**, naltrindole (blue curve) displaces the radioligands (3H-DADLE and 3H-naltrindole) with at Ki of 1.25nM and 9.7nM respectively. This was expected as the antagonist naltrindole binds to all receptor states and will displace both the agonist DADLE and any antagonists, in occurrence itself. In comparison to the naltrindole, the MIA (red curve), HMA (green) and zoniporide (purple) also displace the agonist radioligand 3H-DADLE with Ki between 465nM and 2000nM (Figure 3.7A). All three compounds failed to displace 3H-naltrindole (Figure 3.7B). This suggests that all three compounds bind to the active conformation of δ-OR. It also suggests that the interaction is non-competitive. Indeed, if it would compete for the orthosteric site, 3H-naltrindole would also be displaced, at least partially. These results also support the allosteric

interaction of these small molecules at the receptor. The fact it can be displaced solely by agonist, suggests that the interaction could be state dependent. One other hypothesis that can be raised is the accessibility to the allosteric side requires the absence of ligand within the orthosteric side. So, only an active conformation with no ligand will positively interact with the modulators. This competition for the ‘’channel’’ access to the allosteric site of the active receptor could explain the displacement of DADLE. The last hypothesis is the modulators stabilize an intermediate state that is not favourable to the interaction of DADLE (low state affinity). Further studies will be needed to clarify those hypotheses.



	Naltrindole	MIA	HMA	Zoniporide
Ki	1.285e-009	4.650e-007	1.970e-006	1.293e-006

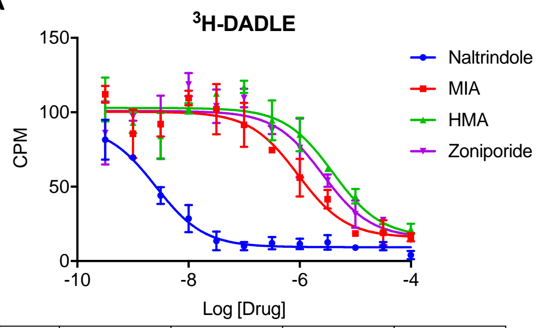
	Naltrindole
Ki	9.7e-009

Figure 3.7. MIA, HMA and zoniporide non-competitively displace 3H-DADLE and 3H-naltrindole. This is a competitive binding experiment where orthosteric-binding radiolabelled (hot) ligands at 1nM are displaced by non-radiolabelled (cold) ligands. The amiloride-derivatives and the small molecule displace both orthosteric ligands (DADLE and naltrindole) with a low apparent affinity at the wildtype δ-OR. The x-axis represents log of drug concentration in Molar. The data is presented as radioactivity counts per minute (CPM) on the y-axis. Each point is triplicate, and the data is from 3 independent experiments (n=3).

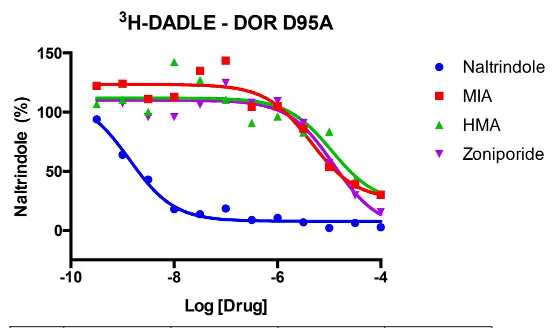
3.1.6. Binding experiments of allosteric modulators on single point mutants of δ-OR.

As mentioned earlier, the sodium ion binding site is the prime candidate site for the binding of amiloride-derivates and the small molecules. Recent work on the A2A receptor suggests that amiloride-derivatives could binding to this allosteric site. As illustrated in **Figure 3.8**, we focused on the single point mutants of the five essential residues within the sodium pocket: D95A, N131V, N310A N314A, and S135A. We performed a radioligand displacement assay with these mutant receptors using MIA, HMA and zoniporide to test if the mutations would prevent their ability to bind to the receptor. The three compounds displace the 3H-DADLE from the mutant receptors at relatively similar affinity to that of the wild type receptor. This data suggests that these amino acid residues are not important for the binding of the amiloride-derivatives and small molecules to the receptor. As shown previously, the D95A mutant completely abrogated the β-arrestin2 recruitment and partially reduces G-protein activation. It is thus not surprising to still have binding to this mutant. However, we do observe a 2-5x reduction in apparent affinity with the D95A mutant that support our functional data. This support the idea that interaction with D95A is important for the function of the modulators but not critical for its interaction (affinity). Moreover, we observe an increase in affinity of about 20 times of the three modulators at the S135A mutants. This is also supported by the function data at this mutant where the allosteric effects were increased. This indicates the S135 residue is not important for the interaction but rather control the receptor activation and consequently the drug's efficacy.

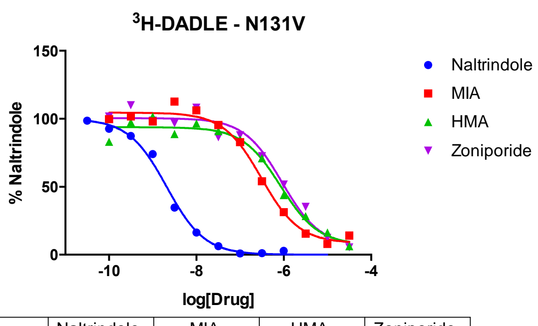
A



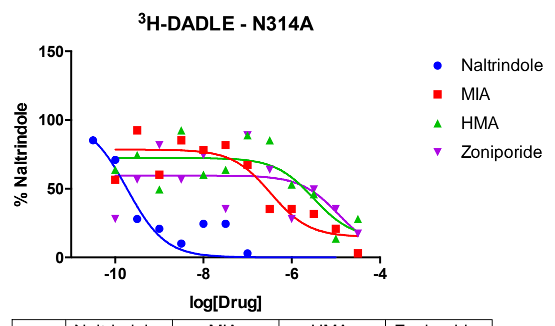
	Naltrindole	MIA	HMA	Zoniporide
Ki	1.285e-009	4.650e-007	1.970e-006	1.293e-006



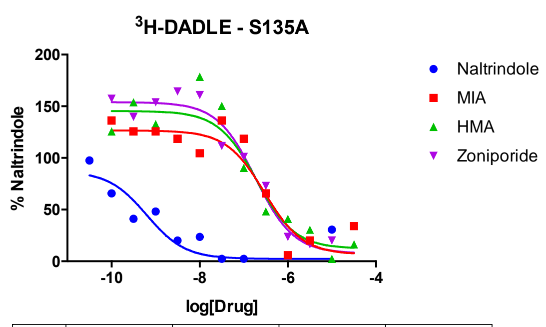
	Naltrindole	MIA	HMA	Zoniporide
Ki	6.855e-010	2.221e-006	5.621e-006	6.051e-006



	Naltrindole	MIA	HMA	Zoniporide
Ki	1.042e-009	1.531e-007	4.238e-007	4.552e-007



	Naltrindole	MIA	HMA	Zoniporide
Ki	9.286e-011	1.679e-007	1.558e-006	5.826e-006



	Naltrindole	MIA	HMA	Zoniporide
Ki	3.143e-010	1.476e-007	9.678e-008	9.149e-008

Figure 3.8. Single point mutations of the amino acid residues (D95, N131, N314, S135) that coordinate the sodium ion have no effect on small molecule binding. Mutations of D95A, N131V, N314A, and S135A of δ-OR do not prevent the displacement of 3H-DADLE by MIA, HMA and zoniporide using radioligand displacement assay. The x-axes represent log of drug concentration in Molar. The data is normalized to the reference non-tritiated ligand and presented as % naltrindole on the y-axes. Each point is triplicate, and the data is from 3 independent experiments (n=3).

3.2. Monitoring GPCR activity

For the scientific community, the ability to monitor GPCR activation and signaling lies at the heart of the drug discovery efforts and unlocking the GPCR mysteries in general. It is very essential to have techniques that are simple, robust, and would require the least amount of reagent additions (miniaturized experiment) (96). There are several methods that are currently available to study GPCR activation and signaling. Some of these methods include, but are not limited to: guanine-nucleotide binding assays, reporter gene assays, cAMP assays, inositol phosphate accumulation assays, and calcium assays (96). The guanine-nucleotide binding assays are based on the measurement of a radiolabelled, non-hydrolysable GTP ([$_{35}$S]GTP$_{\gamma}$S) binding to plasma membranes that express the GPCR of interest after washing off the unbound [$_{35}$S]GTP$_{\gamma}$S (97). This binding assay is generally limited to GPCRs that are coupled to $G_{i/o}$ protein family. The reason behind this is not well understood. The cAMP assay, however, looks at GPCR activation by measuring the levels of intracellular cAMP, which is a second messenger molecule made by adenylyl cyclase as a result of the activation of GPCRs that are coupled to either G_s or $G_{i/o}$ family. G_s has stimulatory effect on adenylyl cyclase (AC) and, therefore, increases intracellular levels of cAMP while $G_{i/o}$ has the opposite effect. For receptors that are coupled to G_s, this assay is fairly easy and straightforward. For the $G_{i/o}$ coupled receptors, however, the cAMP levels must be increased by stimulating the cells with either forskolin or isoproterenol. Since the $G_{i/o}$ family inhibit adenylyl cyclase and reduce the intracellular cAMP, it requires the cAMP to be increased in order to have a reasonable window to visualize its reduction (98). However, as AC can be controlled by numerous others mechanism including calcium, calmodulin, pH, PIP2, and Gβγ dimer, it is sometime difficult to directly correlate the observed cAMP level with G-protein activity.

Cell-based reporter gene assays are also another way of studying GPCR signaling through both the G-protein dependent and independent pathways. G-protein dependent pathways activate or inhibit secondary messengers that will lead to the activation or inhibition of responsive element such as cAMP response element (CRE) that regulates the expression of a selected reporter gene (96). The commonly used reporter gene are luciferase, β-galactosidase, β-lactamase and green fluorescent protein (GFP) (99-102). There are many advantages of using reporter gene assays including its high sensitivity and large signal-to-background ratio. They are also easy to use and cost-effective. However, it also has some disadvantages that include longer incubation periods and high false positive rates. False positives are due to the fact the assay is indirect and the modulation of the second messengers could be trigger by endogenous receptors or any other targets not related to the studied receptor (96). In the case of the G-protein independent pathway, most assay were developed to look at β-arrestin2 recruitment to the receptor. Most of those assays are based on protein-fragment complementation such as pathhunter assay or resonance energy transfer such as bioluminescence resonance energy transfer (BRET) or fluorescence resonance energy transfer (FRET) (103). These techniques have their own share of drawbacks including limited sensitivity in BRET and high background noise in FRET as well as their requirement for bulky donors/acceptors present at the C-terminus of the receptor (104). Another reporter assay developed to track β-arrestin2 recruitment is the PRESTO-Tango described earlier. While very sensitive this assay also has the inconvenient of carrying a bulky fusion at the C-terminus of the receptor and requires long incubation period. We thus aimed to exploit recent development in cell-based assay to develop a sensitive tool that would address most of pitfalls of other assays but more importantly, will incorporate kinetics parameters.

3.3. Development of new pharmacological tool for G-protein dependent and independent pathways

3.3.1. Design of the MOR reporter assay

Nanoluc Binary Technology (NanoBiT) is complementation reporter system that was recently developed by Promega to study protein interaction within living cells. It is based on a very small and bright bioluminescent protein called nanoluciferase found in deep sea shrimp Oplophorus Luciferase. From the original 130kDa luciferase, a 19kDa catalytic fragment, coined 19kOluc, was engineered and used coelenterazine as substrate. Using directed evolution this 19kOluc was evolved to use furimazine as substrate but also to be more stable and soluble. Further, using *in silico* modeling, they generated a protein-fragment complementation assay called NanoBit. The NLuc is split in two inactive fragments: an 11-amino acids fragment called SmBit and a larger fragment of 142-amino acids called LgBit. The fragments have little to no activity on their own. However, the protein becomes functional and releases a bright bioluminescent light once the two fragments complement each other in the presence of the NLuc substrate, furimazine. Therefore, these two fragments can be tagged to two proteins of interest to study their interaction mechanisms. Moreover, the interaction is reversible as the affinity of the two fragments is not too high allowing the study of dynamic interaction (association/dissociation)(105).

For the G-protein recruitment aspect, the assay utilizes mini G-proteins that are quite different from the full G-proteins. The mini G-proteins used in this assay to study the recruitment of G-proteins to GPCRs are modified in four different ways. First, they have a truncated N-terminus, which effectively removes the βγ-binding surface as well as the part that anchors G-proteins to the cell membrane. Second, the alpha helical domain is completely removed. The third and fourth modifications involve mutations that improve the stability of mini G-proteins *in vitro*, and a C-terminal mutation that stabilizes the binding of G-proteins to GPCRs in the presence of

GTP/GDP. This stabilization is achieved because the C-terminal mutation effectively uncouples the action of nucleotide release from the binding of mini G-proteins to GPCRs (91). Similar to arrestin recruitment assays, the mini G-proteins can be used to report GPCR activation in living cells (106).

3.3.2. Optimization of the real time reporter assay: transfection

Following the successful cloning of the NanoBit constructs, we started testing them in HEK293T cells. Initially, we began with transiently transfecting 1ug of each of the plasmids containing the MOR-smBiT and LgBit-miniGαi protein on the HEK293T cells and ran the assay by quantifying the relative luminescence upon the stimulation of the cells with DAMGO in the presence of the nanoluc substrate, furimazine. According to literature, the complementation of the LgBit and smBit should be very sensitive and produce a bright luminescence light (105). However, the fold increase observed in this initial test was quite low. Therefore, we believed we could improve the signal by changing the transfection ratios between the receptor and the miniGαi protein. Upon testing different transfection ratios, we found that our assumption was correct that the signal could be improved by changing the transfection ratio. As shown in Figure 3.9, the optimal transfection ratio that we found was 0.5 ug of the receptor and 1 ug of the miniGαi protein. As a result, the rest of the NanoBit experiments in this section of the thesis utilize this ratio.

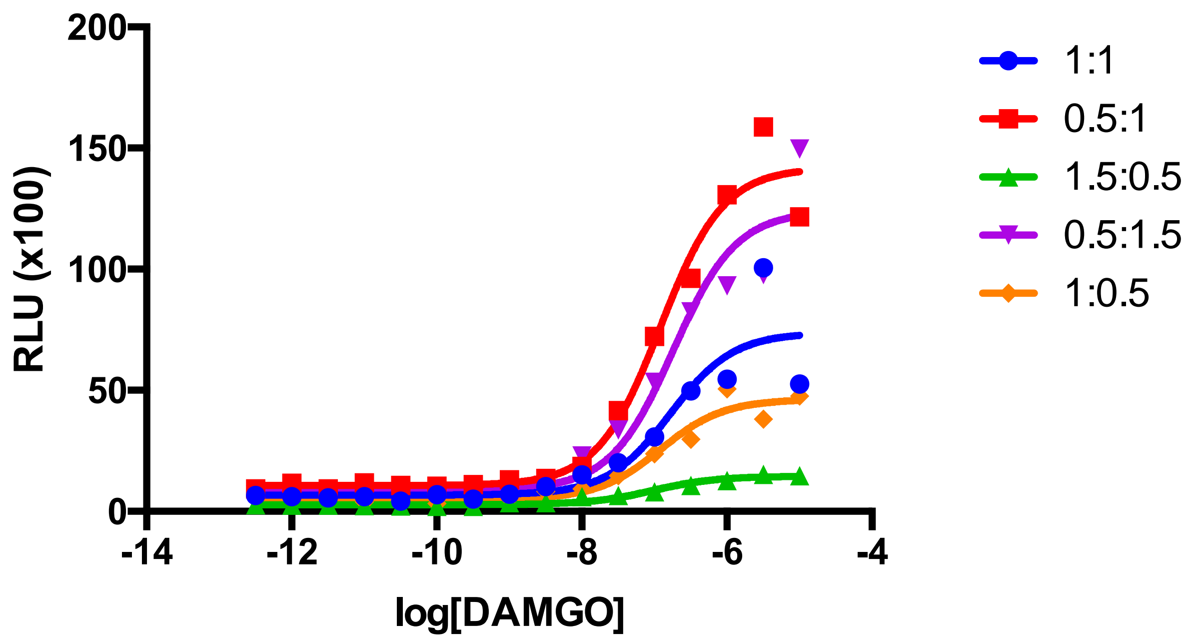
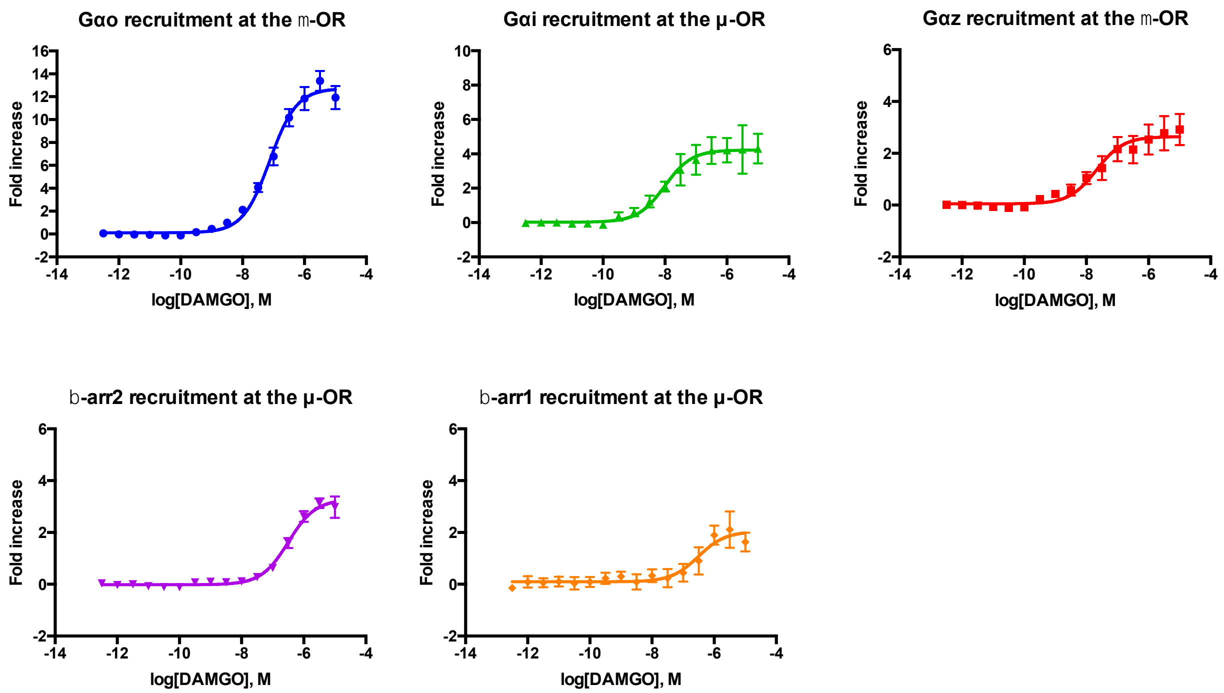

Gαi recruitment at activated μ-OR
RLU (x100)
200
150
100
50
0
-14
-12
-10
-8
-6
-4
log[DAMGO]
1:1
0.5:1
1.5:0.5
0.5:1.5
1:0.5

Figure 3.9. Optimal transfection ratios for mini Gi recruitment. The figure highlights a dose-response curve of DADLE in the recruitment of mini Gi protein at the μ-*OR* with different transfection ratios between the MOR-smBit and LgBit-miniGi. The recruitment of mini G-proteins is dependent on the transfection ratio between the receptor and the mini G-protein. In this case, the optimal transfection ratio seems to be 0.5 ug of MOR-smBit and 1-1.5ug of LgBit-miniGi.

3.3.3. Validation of the real time reporter assay

Following the optimization of the assay, we have used to the optimized transfection parameters for the G-proteins and the β-arrestins to look at their recruitment profiles at the MOR in the presence of the MOR agonist, DAMGO. We co-transfected HEK293T cells with the MOR-SmBit construct and LgBit-$G_{\alpha i}$, $G_{\alpha o}$, $G_{\alpha z}$, β-arr2 or β-arr1 and grew the cells overnight. The HEK293T cells were also transfected with YFP in a separate dish to evaluate the transfection efficiency prior to proceeding with the experiment. If transfection was deemed efficient, the cells were stimulated with HBSS containing 5uM furimazine and incubated for 10 minutes. Following the 10 minutes incubation, the cells were placed in the Fluorescent Imaging Plate Reader (FLIPR) and started reading the plate for two minutes at an interval of 5 seconds to establish a background. After 2 minutes, the cells were stimulated with an increasing concentration of DAMGO and read for an additional 10 minutes post stimulation. The maximum data points in each well were extracted and analyzed in GraphPad Prism to get dose response curves shown in **Figure 3.10**. As illustrated in the figure, the assay is sensitive and robust enough for the recruitment of the G-proteins to the MOR. The β-arr1 and β-arr2, however, require further optimization. Therefore, the recruitment profiles of the G-proteins at the MOR in the presence of a panel of opioids will be selected for further investigation at this moment.

Figure 3.10. Validation of the Real-time β-Arrestins and G-proteins recruitment assay. This figure highlights the recruitment of various G-proteins and β-Arrestins at the activated MOR in the presence of the MOR agonist, DAMGO. The three G-proteins in the $G_{i/o}$ family that are tested here show a very good response to DAMGO with decent fold increase and EC_{50}. The β-Arrestins are right shifted and need more optimization. The x-axes represent log of drug concentration in Molar. The y-axes represent the recruitment of each respective interacting protein to the receptor. Each point is triplicate and the data is from 3 independent experiments (n=3).

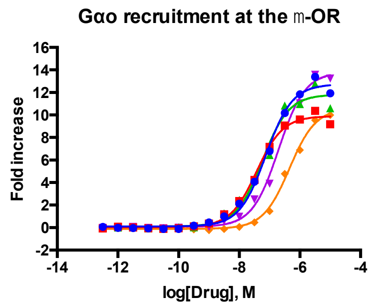


3.3.4. Recruitment profiles of known opiates at the MOR.

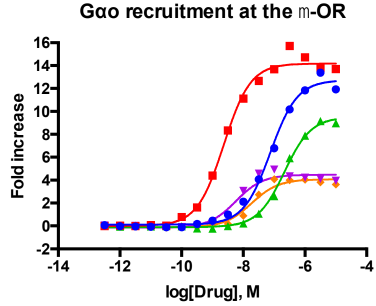
Dose-response curves were obtained for two set of opioids: opioid peptides and non-peptide opioids. Each set of compounds were tested on the MOR and its interacting G-proteins: $G_{\alpha o}$, $G_{\alpha i}$, $G_{\alpha z}$. As shown in **Figure 3.11.**, the opioid peptides seem to have a generally similar recruitment profile for the three G-proteins at the MOR. The Met-enkephalin have a slightly lower potency than DAMGO and the endomorphine-1 and -2. In comparison, the non-peptide opioids lead to drastically different recruitment profiles for the G-proteins at the MOR. Etorphine (Red curve) seems to be the most potent non-peptide opioid in the recruitment of $G_{\alpha o}$ and $G_{\alpha i}$. In the case of $G_{\alpha z}$, the difference in potency between Etorphine (Red curve) and DAMGO (blue curve) is not so accentuated. Although IBNTxA (purple curve) and Buprenorphine (yellow curve) are both as potent as DAMGO, their efficacy is significantly reduced in the recruitment of all three G-proteins. The Buprenorphine seems to have a lower efficacy in the recruitment of $G_{\alpha z}$, $G_{\alpha o}$, and $G_{\alpha I}$ while the potency remains about the same. Morphine, which is a prototypical opioid therapeutic seems to have low potency and efficacy in the recruitment of all G-proteins.

Opioid peptides

Non-peptide opioids

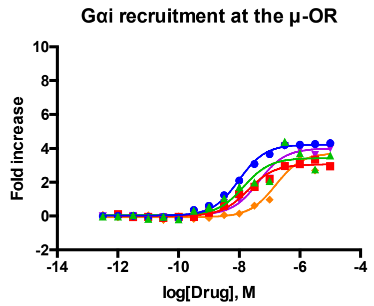


DAMGO
Endomorphine-1
Endomorphine-2
Leu-Enkephalin
Met-Enkephalin

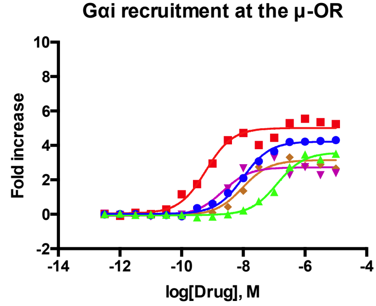


DAMGO
Etorphine
Morphine
IBNTxA
Buprenorphine

Gαo

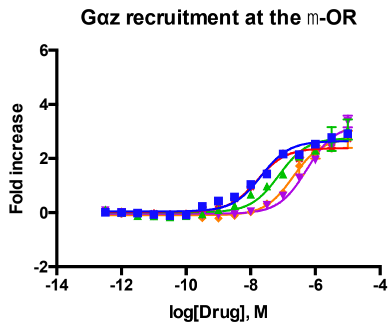


DAMGO
Endomorphine-1
Endomorphine-2
Leu-Enkephalin
Met-Enkephalin

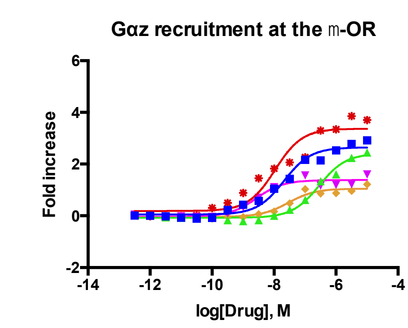


DAMGO
Etorphine
Morphine
IBNTxA
Buprenorphine

Gαi



DAMGO
Endomorphine-1
Endomorphine-2
Leu-Enkephalin
Met-Enkephalin



DAMGO
Etorphine
Morphine
IBNTxA
Buprenorphine

Gαz

Figure 3.11. Recruitment profile of peptide and non-peptide opioids at the MOR. The figure highlights the recruitment of mini G-protein in the $G_{\alpha i/o}$ family to the μ-opioid receptor activated by opioid peptides and non-peptide opioids. This assay was completed using the NanoBit complementation assay by co-transfecting MOR-smBit and LgBit-miniG$_{\alpha}$ proteins. Luminescence reading was performed live on a FLIPR-TETRA. The x-axes represent log of drug concentration in Molar. The y-axes represent the recruitment of each respective interacting protein to the receptor. Each point is triplicate, and the data is from 3 independent experiments (n=3).

4.0.Discussion

The rational of this study is to increase our understanding of the functional selectivity of the delta-opioid receptor through allosteric modulation of the sodium-binding site and explore its drugability. Opioid drugs that are currently available on the market have severe side effects in addition to their therapeutic benefits. Despite their side effects, these drugs are extremely essential for the management of pain and, therefore, cannot be abandoned. Since the use of opioids is very essential, we need to develop new set of opioid analgesics that will retain the therapeutic benefits of opioids and significantly reduce the side effects. For this to happen, we will need to thoroughly study the opioid receptors in the human body and understand their molecular signaling pathways.

G-protein-coupled receptors are the largest and the most druggable protein superfamily in the human genome. They are responsible for the transmission of signals from the extracellular matrix into the intracellular region to elicit cellular responses. Upon activation of G-protein coupled receptors, the signal is transduced via G-protein dependent and G-protein independent pathways. The opioid receptors are part of the rhodopsin (class A) family of GPCRs and they are subdivided into three classical receptors: μ-, δ-, κ-opioid receptor. These receptors are widely targeted for the treatment of chronic pain and addictive disorders using a class of drugs called opioids. Despite their benefits as analgesics, the opioid therapy is associated with severe side effects such as overdose due to respiratory depression, addiction, tolerance and constipation. This current research project focuses on the design and development of a new generation of allosteric modulators that modulate the activity of opioid receptors. The goal of this to eliminate or significantly reduce the side effects associated with the activation of opioid receptors.

The first part of the study focuses on a sodium ion-binding site that was initially identified in a study that published the crystal structure of the human delta-opioid receptor in 2014 (70).

Based on sequence homology, the sodium cavity is believed to be present in more than 95% of class A GPCRs and it harbours a sodium ion and water molecules. The access to this 1.8 Å high-resolution crystal structure of the delta-opioid receptor allows the introduction of mutations in the sodium pocket and study their effects on sodium binding. From these studies, it was found that the sodium ion stabilizes the inactive conformation of the receptor. The sodium pocket seems to modulate the activity of the receptor, which makes it a prime target for the design and development of new generation of drugs. Despite that the majority of the currently available opioid drugs target the orthosteric site of opioid receptors, there are numerous advantages of targeting the allosteric site. It preserves the spatiotemporal control of opioid receptor activation by endogenous ligand, thus allosteric modulators would only modify the behavior of the receptor toward its endogenous ligand. The use of allosteric modulators also provides another level of receptor selectivity between different subtypes of receptor that binds to similar ligand reducing polypharmacology events. Therefore, it holds a great potential for the development of new generation opioids and tool compounds (107,108).

To achieve our goal, we adopted a more strategic approach using known promiscuous modulators such as amiloride compounds, believed to target the sodium cavity as reviewed by Katrich et al.. Amiloride is a compound that is a potent inhibitor of epithelial sodium channels and Na^+/H^+ exchangers in humans (109). In addition to that, amiloride was found to allosterically modulate GPCRs with an affinity >10uM. Mutagenesis studies found that the conserved amino acid residue ($Asp_{2.50}$) abolishes the effects of both sodium and amiloride in binding experiments, when mutated to alanine. This led us to believe that the amiloride targets the same binding site as the sodium ion. Due to low affinity, initial works were done at high concentrations of amiloride

(>100uM). This concentration is good enough for *in vitro* experiments; however, it is toxic to cell cultures and doesn't allow the use of cell-based assays and thus study functional relevance of the interaction.

We thus built a small library of amiloride-derivative and drugs with similar chemotype and activities at sodium channels. Using the presto-tango screening assay, these sodium-channel inhibitors were tested on a large panel of GPCRs leading to the identification of few GPCRs that are modulated at non-toxic concentrations. This primary screening revealed that the opioid receptors MOR and DOR were modulated to different extents. Surprisingly, the KOR, which is very similar to the MOR and DOR in terms of structure and sequence homology, was not affected at all. Upon structural characterization of the sodium pocket in the high-resolution crystal structure of the DOR, the connection was made between sodium ion, amiloride and the conserved aspartic acid residue at position 2.50. Therefore, the amiloride and its derivatives were identified as lead compounds that can be used to design allosteric modulators that will modulate the DOR through its sodium cavity.

The current study aimed to develop a better understanding of the functional and structural features of this pocket by using those modulators and perform structure-activity-relationship studies (SAR). We chose two amiloride derivatives (MIA and HMA) and a small molecule (zoniporide) to characterize the sodium pocket. MIA and HMA are high affinity sodium channel inhibitors in comparison to amiloride. Amiloride itself doesn't have an effect on the DOR at non-toxic concentration. Zoniporide is a Na_+/H_+ Exchanger (NHE-1) that also have an allosteric activity at the DOR. All three allosteric modulator have a guanidium group that, based on modeling, interacts with the asp acid at position 2.50 in the sodium cavity.

The first series of experiment were conducted to address the effect of the three modulators at the functional level. For this, we measured the effect at the β-arrestin2 recruitment and cAMP modulation. As shown in figure 3.1-3.4, we found that all three modulators have a positive allosteric modulator activity (PAM) for the agonist DADLE at the β-arrestin2 recruitment and cAMP level. Both amiloride derivative, MIA and HMA, also have intrinsic partial agonist activity that is not observed for zoniporide. In all case, the effect can be reversed with the selective DOR antagonist naltrindole. This indicates that all effect observed are readily mediated through the activation of the DOR and not by an effect on the assay system. Moreover, most GPCRs are not affected in the same condition reinforcing a selective effect at the DOR.

Following functional characterization, we pursued our goal by performing structure-activity-relationship (SAR) in order to identify critical amino acid residues within the sodium pocket that are responsible for the binding and activity of allosteric modulators at the DOR. Based on a model generated by *in silico* docking, the $D95_{2.50}$ residue was expected to coordinate the guanidium group of all three modulators and thus was a logical mutant to test. We also performed similar assay at all critical amino acid that coordinate the sodium ions within the cavity. All of those residues have their charged side chains pointing within the pocket while most other amino acids crafting the pocket don't. As shown in Figures 3.5 and 3.6, the mutant D95A abolishes most of the allosteric effect at the β-arrestin2 recruitment but only have moderated effect at G-protein activation measured by GloSensor. The other interesting mutants was the S135A.This mutant shows a complete loss of efficacy and potency with the agonist DADLE alone. However, the ago-PAM and PAM activity of all three modulators is enhanced. This strongly support our hypothesis of allosteric modulation by all three modulators but also shows that the receptor is still functional and

that the affinity of DADLE is not strongly affected but rather its efficacy. Thus, the allosteric modulators compensate and restore this efficacy loss by DADLE. The effect of this mutant at the DADLE efficacy is very surprising and was unexpected. We were unable to find an explanation based on the crystal structure as this S135 residue was believed to have a minor role. One hypothesis is that S135 coordinate water molecules within the pocket and could be part of a salt bridge network that control receptor structure and function. It is hypothesized that receptor solvation within the central ‘’channel’’ of the receptor could be, at least in part, important for the communication between the orthosteric site, the allosteric sodium site, and some of the critical micro switches such as the NPxxY and DRY motifs. Unfortunately, we haven’t managed yet to find a critical amino acid residue, in terms of those that coordinate the sodium ion, that would completely abrogate the effect of allosteric modulators while keeping the receptor functional. This is probably the main challenge of performing SAR study by point mutations. All mutant receptors tested herein, are functional, although some effect was observed when tested with DADLE only. The findings of this study shed light on the druggability of the sodium site in order to modulate the effects of the ligand that binds to the orthosteric site. They also show that the amino acid residues within the sodium pocket are important for functionality of the allosteric modulators. Further studies are required to find a molecule that strongly binds to the allosteric site with a higher affinity so that the amiloride-bound receptors can be crystallized at atomic resolution. Once the high definition crystallized receptor is available, we can look at the interactions between the allosteric modulator and the sodium pocket, which will allow us to design better and more efficient allosteric modulators. This is fairly a new effort that is designed to study and understand the various signaling pathways of the opioid receptors in order to design therapeutics that efficient with the lowest possible side effects. The sodium ion binding allosteric site was recently identified

and, therefore, hasn't been a lot of studies that have been done on this pocket at GPCRs so there isn't much that is known about its modulation of these receptors. The main focus of these studies is to investigate this allosteric site at the delta-opioid receptors in order to explore and benefits from its therapeutic potential. Furthermore, we also need to build biomedical tools that can be used to study other opioid receptors as well as the whole GPCR family that have the allosteric sodium cavity.

Kinetics are usually ignored when looking at the functional selectivity of GPCRs (110). Therefore, the second part of this study deals with the development of a robust and easy-to-use biomedical tool that is specifically designed to detect the recruitment of G-proteins and β-arrestins activated receptors over time. This tool is based on a protein complementation technique that was developed by Promega to study protein-protein interactions. The complementary fragments (SmBit and LgBit) for this technique come from the NanoLuc, which is an engineered small luciferase (19 kDa) that was isolated from deep sea luminous shrimp (111). Split fluorescent proteins and β-galactosidase (116kDa), are used to detect protein-protein interaction in living cells. However, such split reporters are either too large or lack the reversibility needed to accurately report protein interaction. Luciferase-based split reporter assay is preferred because they are very sensitive and simple to use. The split NanoLuc that our reporter assay is based on produce very bright luminescence that is easily detected. In addition to that, the protein itself is very small and the association between its fragments is reversible. For these reasons, the impact it has on the natural interaction between the proteins under study is minimized. Despite this, the reporter assay might still have an effect on the dynamics between the GPCR-partner protein (G proteins and β-arrestins) interactions. The mini G-proteins that are used in our assay are truncated at their N terminus ends including the part that would anchor them to the plasma membrane. Therefore, their

recruitment dynamics to GPCRs might not be similar to full G-proteins. However, our assay is not designed to study the dynamics of GPRC-partner protein interactions but to profile different drugs, in our case opioids, in terms of G-proteins and β-arrestins recruitment at the three classical opioid receptors.

The most after-sought areas in GPCR research revolves around the concept of functional selectivity, where a compound can stabilize one of the specific conformations of a receptors and, therefore, lead to the preferential activation of one signaling pathway over another. Theoretically, the therapeutic benefits and the deleterious side effects of a particular GPCR can be discriminated by the discovery of bias compound. Recent studies reported that certain compounds can even achieve functional selectivity between different G-protein subtypes (112,113). However, the current system that is used to calculate bias between G-proteins and arrestins is based on using different cellular based assays (114). For example, the TANGO assay is used to look at the recruitment of arrestins to the activated receptor while GloSensor is used to measure cAMP level (G-protein activation). In one case the receptor is Tango-ized and stimulated 16 hours, and in the other, the receptor is unfused and stimulated 10-30 minutes. Thus, we aimed to build a system where the same receptor, in the same stimulating condition can be used to measure pharmacological behavior of multiple drugs and compare them to a reference. Therefore, we believe that this would be a better estimation of the bias between the various pathways.

So far, the reporter assay that we have built is sensitive enough to characterize the recruitment profiles of G-proteins in the presence of opioids at the MOR. The MOR was chosen to optimize the parameters of the assay before moving on to the other opioid receptors. The reason is that the MOR is a well-defined target for pain with numerous chemotype drugs to be tested including bias agonist, not available for the other opioid receptors. We have generated a

recruitment profile of 4 opioid peptides and 4 non-peptide opioids for the G-protein pathway at the MOR. As shown in Figure 3.12, the recruitment profile for the opioid peptides, including endogenous peptides, is quite similar in each of the G-proteins tested. The Met-enkephalin seems to have a decreased potency compared to the rest of opioid peptides. For the non-peptide opioids, however, the recruitment profiles dramatically vary in each of the G-proteins with etorphine being the most potent. This is was to be expected because etorphine is a powerful opioid that is used to immobilize large mammals such as elephants. As of now, the assay has been optimized and work well for the mini-G-protein at many receptors tested, however, β-arrestin recruitment was found to be more challenging. Further efforts in the lab is ongoing with the ultimate objective to have a normalize system where the same receptor fusion protein can be used to track G protein and β-arrestin signaling.

5.0. Conclusion

Opioid agonists (narcotic analgesics), being the most prescribed class of drugs in Canada, are used for the treatment of chronic pain (115-118), whereas opioid antagonists are generally effective in the treatment of addictive disorders, including substance abuse (opiate, alcohol, amphetamine) (119-122) and non-substance, i.e., behavioral addictions such as gaming (123), gambling (124) and over-eating (125). Given the difficulties associated with opioid agonist treatment, including high risk of death due to overdose (4,460 Canadian victims in 2019), and development of tolerance, addiction, and other life-threatening/life-limiting side effects (respiratory depression, constipation, etc.), there is clearly a need for improved pharmacotherapy for treatment of pain and prevention of relapse in addiction. Opioid receptors belong to the family of G protein-coupled receptors (GPCRs). The plethora of physiological effects controlled by

GPCRs is explained by the fact that they behave as large versatile allosteric machines (45,126). This hallmark allows GPCRs to dynamically sense/bind extracellular cues/ligands, ultimately enabling the formation of a complex propagating a *conformationally restricted* signal inside the cell; this process is termed "functional selectivity". Functional selectivity is generally quantified by comparing the activation of the canonical G protein pathway with β-arrestin recruitment (127). There are three classical opioid receptors: MOR, DOR and KOR. The delta-opioid receptor (DOR), is well described for its role in pain perception and management. In clinical models, it also showed great potential as an anti-depressor and for the treatment of spasmodic movements in Parkinson's disease as well as immunomodulator, as revealed by the immunosuppressive effect of chronic opioid use (128). However, the clinical use of DOR agonists is limited due to the generation of potentially life-threatening side effects (e.g. epileptic-like seizures). The research project proposed seeks to study the pharmacological signature generated by the modulation of a newly identified allosteric modulation site. The ***long-term objective*** is to generate distinct therapeutics that will uniquely modify their pharmacology in a medically meaningful way, increasing their therapeutic efficacy while reducing harmful side effects. The main hypothesis of our research is that sodium plays a key role in regulating the functional selectivity of the DOR and that the cavity housing the sodium ion can be used as a druggable allosteric modulation site. This will allow the generation of better therapeutics for the treatment of mental illness (depression) neurodegenerative disease (Parkinson's) and pain management (narcotic analgesic). A major new insight into the control of GPCR signaling behavior detailed the discovery of a sodium-dependent allosteric regulation site in the DOR (70). This work highlights the presence of a cluster of sodium and water molecules housed in a cavity thought to be present only in the inactive conformation of the receptor. Structure function relationship studies demonstrated the critical involvement of

sodium acting as an allosteric modulator solely at the arrestin recruitment pathway. The sodium pocket is predicted to be present in many GPCRs, but the shape of this allosteric cavity seems to have important structural variation. This low degree of structural homology makes this site optimal for the design of allosteric modulators that will be selective for the DOR toward other opioid receptors (13,48,129). Previous screen in our lab identified sodium ion channel inhibitors as potential allosteric modulators of the DOR. For my research project, I used the three most active modulator to perform functional characterization and structure-activity-relationship to have a better understanding of the sodium-binding allosteric pocket as well as the drugability of this later. Two of the selected compounds are amiloride-derivatives, MIA and HMA, and the other, zoniporide, is not an amiloride-derivative but also contains a guanidium group. This charged group is well known to interact with an aspartic/glutamic acid within sodium channels that normally regulated the cation selectivity. Similarly, we hypothesized this guanidium group also interacts with a conversed aspartic acid that coordinate the sodium found within the sodium-cavity of GPCRs. In the β-arrestin recruitment pathway, the amiloride-based allosteric modulators showed an ago-PAM activity with MIA having the strongest agonist activity compared to HMA. The non-amiloride molecule, zoniporide, only showed PAM activity at this pathway. This is quite interesting because it supports the drugability of the site but also the possibility of making pure allosteric modulators (no intrinsic agonist activity) but also agonist-PAM. In the G-protein pathway, however, all three allosteric modulators have an agonist activity with no observed PAM. However, it has to be mentioned that most allosteric modulators behave the same way when cAMP or calcium level is measured. The exact reason for this is not clear but seems to be related to the fact that we are indirectly looking at G-protein activation via the measurement of second messengers. The other allosteric modulator described for the DOR, BMS 986187, is also showing

similar activity in GloSensor (data not shown) but not in GTPγS binding are described by Burford et al (130). Further characterization of the allosteric site with amiloride-based and non-amiloride allosteric modulators can lead to identification of the determinants that makes a molecule either a pure PAM or an ago-PAM. A follow up study looked at the structure-activity relationship (SAR) on the receptor to identify critical amino acid residues that might be responsible for the ago-PAM or PAM activity at the DOR by introducing single point mutation in the allosteric site. Following a series of experiments, we concluded that single point mutations don't have drastic effect on the binding of allosteric modulators to the receptor. The D95 and S135 position were found to have an important functional role for the activity of those modulators but mutation of neither residues does not abolish interaction.

We also developed a sensitive and simple biomedical tool to detect the recruitment of G-proteins at the opioid receptors. The assay is based on split-luciferase reporter technique called NanoBit that was developed by Promega. The LgBit fragment is tagged to the G-proteins while the SmBit fragment is on the receptors. The complementation assay produces a bright bioluminescence light upon the re-association of SmBit and LgBit fragments, which is signal for the recruitment of G-proteins and β-arrestins. While the actual design works well for G-protein recruitment, it seems that β-arrestins recruitment requires further work. The final goal is to have a system where the same receptor constructs can be used to measure both G-protein and β-arrestins recruitment. This is actually under investigation but not completed at this time. Once completed, this assay will allow us to generate G-proteins or β-arrestins recruitment profile for opioids at the opioid receptors, which will be more accurate to quantify functional bias compare to actual approach.

The current opioid crisis is a huge problem that significantly reduces the quality of life for millions of people in the developed world specially in Canada and the United States. It is also a huge burden on the health care system, costing taxpayers billions of dollars in dealing with it. Development of second-generation drugs for the opioid receptors with little to no side effects might help us deal with this crisis, which would improve the quality of life of those affected while reducing its burden on our healthcare system.

6.0 References

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