# DRUGS, DIMERS, AND MUTATIONS: INVESTIGATING THE EFFECTS OF LIGANDS AND A β2-ADRENERGIC POLYMORPHISM ON HOMO/HETERODIMERIZATION OF β2-ADRENERGIC AND ANGIOTENSIN II TYPE 1 RECEPTORS

by

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DEPARTMENT OF PHARMACOLOGY

The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "DRUGS, DIMERS, AND MUTATIONS: INVESTIGATING THE EFFECTS OF LIGANDS AND A β2-ADRENERGIC POLYMORPHISM ON HOMO/HETERODIMERIZATION OF β2-ADRENERGIC AND ANGIOTENSIN II TYPE 1 RECEPTORS" by Patrick James Holland in partial fulfilment of the requirements for the degree of Master of Science.

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## ABSTRACT

GPCRs are known to form dimeric structures, and this affects their pharmacological properties. The β2AR and AT1aR are GPCRs that are involved the regulation of the adrenergic and renin-angiotensin systems. The β2AR is polymorphic at position 164, affecting its responsiveness to adrenergic ligands. Both of receptors have been shown to form dimers, but little is known on how dimerization affects their trafficking and signalling following ligand treatments. Plasma membrane localization, arrestin-2 recruitment, and G-protein interactions were determined between receptor dimers using molecular biological techniques. This study demonstrates that the formation of heterodimers can change the expected response to ligand treatments, along with associated trafficking events. It was determined that ligands bind to dimers, resulting in conformational changes to the dimeric complexes. Both the β2AR and AT1aR are targeted in cardiovascular disease and this research demonstrates the importance of dimerization when prescribing drug therapies to avoid potential unwanted drug side effects.

## LIST OF ABBREVIATIONS AND SYMBOLS USED

| | |
|---|---|
| A1R | Adenosine A1 Receptor |
| A2AR | Adenosine A2A Receptor |
| AC | Adenylyl Cyclase |
| ACII | Adenylyl Cyclase II |
| ACE | Angiotensin Converting Enzyme |
| ADP | Adenosine Diphosphate |
| AFM | Atomic Force Microscopy |
| AP-1 | Activator Protein-1 |
| AP2 | Adaptor Protein-2 |
| AT1aR | Angiotensin II Type 1a Receptor |
| AT2R | Angiotensin II Type 2 Receptor |
| AT4R | Angiotensin II Type 4 Receptor |
| βAR | Beta Adrenergic Receptor |
| β1AR | Beta 1 Adrenergic Receptor |
| β2AR | Beta 2 Adrenergic Receptor |
| β2ART164I | Polymorphism at position 164, Threonine Replaced by Isoleucine |
| BB2 | Bradykinin B2 Receptor |
| BiFC | Bimolecular Fluorescence Complementation |
| BRET | Bioluminescence Resonance Energy Transfer |
| BSA | Bovine Serum Albumin |
| C-terminus | Carboxy Terminal |
| cAMP | Cyclic Adenosine Monophosphate |

| | |
|---|---|
| CCL19 | C-C Motif Ligand 19 |
| CCL21 | C-C Motif Ligand 21 |
| CCP | Clathrin Coated Pit |
| CCR2 | C-C Motif Chemokine Receptor Type 2 |
| CCR5 | C-C Motif Chemokine Receptor Type 5 |
| CCR7 | C-C Motif Chemokine Receptor Type 7 |
| CCV | Clathrin Coated Vesicle |
| CVD | Cardiovascular Disease |
| CXCR4 | C-X-C Motif Chemokine Receptor Type 4 |
| D1R | Dopamine D1 Receptor |
| D2R | Dopamine D2 Receptor |
| D3 | Dopamine D2 Like Receptor |
| DAG | Diacylglycerol |
| DMEM | Dulbecco's Modified Eagle's Medium High Glucose |
| DTT | Dithiothreitol |
| EE | Glu-Glu Epitope |
| ECL | Extracellular Loop |
| E/DRY | Glutamic acid/Aspartic acid-Arginine-Tyrosine Motif |
| ER | Endoplasmic Reticulum |
| ERK | Extracellular-Signal-Regulated Kinase |
| FBS | Fetal Bovine Serum |
| FRET | Fluorescence Energy Transfer |
| GABA | Gamma-Aminobutyric Acid |

| | |
|---|---|
| GDP | Guanosine Diphosphate |
| GFP | Green Fluorescent Protein |
| GPCR | G-Protein-Coupled Receptor |
| G-protein | Heterotrimeric Guanine Nucleotide-Binding Protein |
| GRK | G-Protein Coupled Receptor Kinase |
| GTP | Guanosine Triphosphate |
| H1 | Histamine H1 Receptor |
| H3 | Histamine H3 Receptor |
| HA | Human Influenza Hemagglutinin Epitope |
| HEK293 | Human Embryonic Kidney Cells 293 |
| ICL | Intracellular Loop |
| IP3 | Inositol-1,4,5-Triphosphate |
| Kir | Inward Rectifying K+ Channels |
| LHR | Luteinizing Hormone Receptor |
| MAPK | Mitogen-Activated Protein Kinase |
| mGluR5 | Metabotropic Glutamate Receptor 5 |
| N-terminus | Amino Terminal |
| NFP | N-formyl peptide receptor |
| NF-κβ | Nuclear Factor Kappa Beta |
| NK1 | Substance P Receptor |
| NHERF1 | NA+/H+ Exchanger Regulatory Factor 1 |
| NS | Non-Stimulated |
| OR | Opioid Receptor |

| | |
|---|---|
| PACAP | Pituitary Adenyl Cyclase-Activating Polypeptide |
| PBS | Phosphate Buffered Saline |
| PCR | Polymerase Chain Reaction |
| PDZ | Post Synaptic Density Protein (PSD95), Drosophila Disc Large Tumor Suppressor (Dlg1), and Zonula Occludens-1 Protein (zo-1) |
| PEI | Polyethylenimine Linear |
| PIP2 | Phosphatidylinositol 4,5-Bisphosphate |
| PKA | Protein Kinase A |
| PLC | Phospholipase C |
| PM | Plasma Membrane |
| RAS | Renin-Angiotensin-Aldosterone System |
| R* | Active GPCR State |
| R | Inactive GPCR State |
| RET | Resonance Energy Transfer |
| RGS | Regulators of G-Protein Signalling |
| RIPA | Radioimmune Precipitation Assay Buffer |
| *R*lucII | *Renilla* Luciferase II |
| Saralasin | [Sar1, Val5, Ala8]-Angiotensin II Acetate Salt Hydrate |
| SDS-PAGE | Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis |
| SNP | Single Nucleotide Polymorphism |
| SNS | Sympathetic Nervous System |
| SSTR5 | Somatostatin Receptor 5 |
| T1R1/T1R3 | Umami Taste Receptor |
| T1R2/T1R3 | Sweet Taste Receptor |

| | |
|---|---|
| TM | Transmembrane |
| Venus 1 | N-Terminal (Amino Acids 1-157) of Venus |
| Venus 2 | C-Terminal (Amino Acids 158-238) of Venus |
| VFT | Venus Fly Trap |
| VV1aR | Vasopressin V1a Receptor |
| VV2R | Vasopressin V2 Receptor |
| WT | Wild Type |
| YFP | Yellow Fluorescent Protein |

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# Chapter 1: Introduction

## *1.1 G-protein-Coupled Receptors*

G-protein-coupled receptors (GPCRs) are a superfamily of membrane spanning proteins that comprise approximately 3-5% of the mammalian genome, encoding for over 800 GPCR genes, making them the largest and most diverse class of proteins[1, 2]. Being the largest family of cell surface receptors, GPCRs are capable of responding to a wide variety of stimuli such as photons, odors, biogenic amines, lipids and peptides, allowing cells to pass extracellular stimuli onto the intracellular milieu[3] (Figure 1.1). GPCRs are widely expressed in the human body and they play a role in the pathophysiology for a broad range of disease states such as cancer, cardiovascular, and those of the central nervous system, which makes them an attractive pharmaceutical target. At this time, of all currently marketed pharmaceuticals, 30% of them directly target GPCRs[4]. Within the past 30 years, GPCRs have drawn attention from academic and pharmaceutical researchers to understand the underlying means by which they signal, with the hope of understanding some of the molecular mechanisms that are associated with certain disease states. New information regarding GPCR trafficking and signalling could provide the necessary information to find potential drug therapeutics, while reducing unwanted drug side effects.

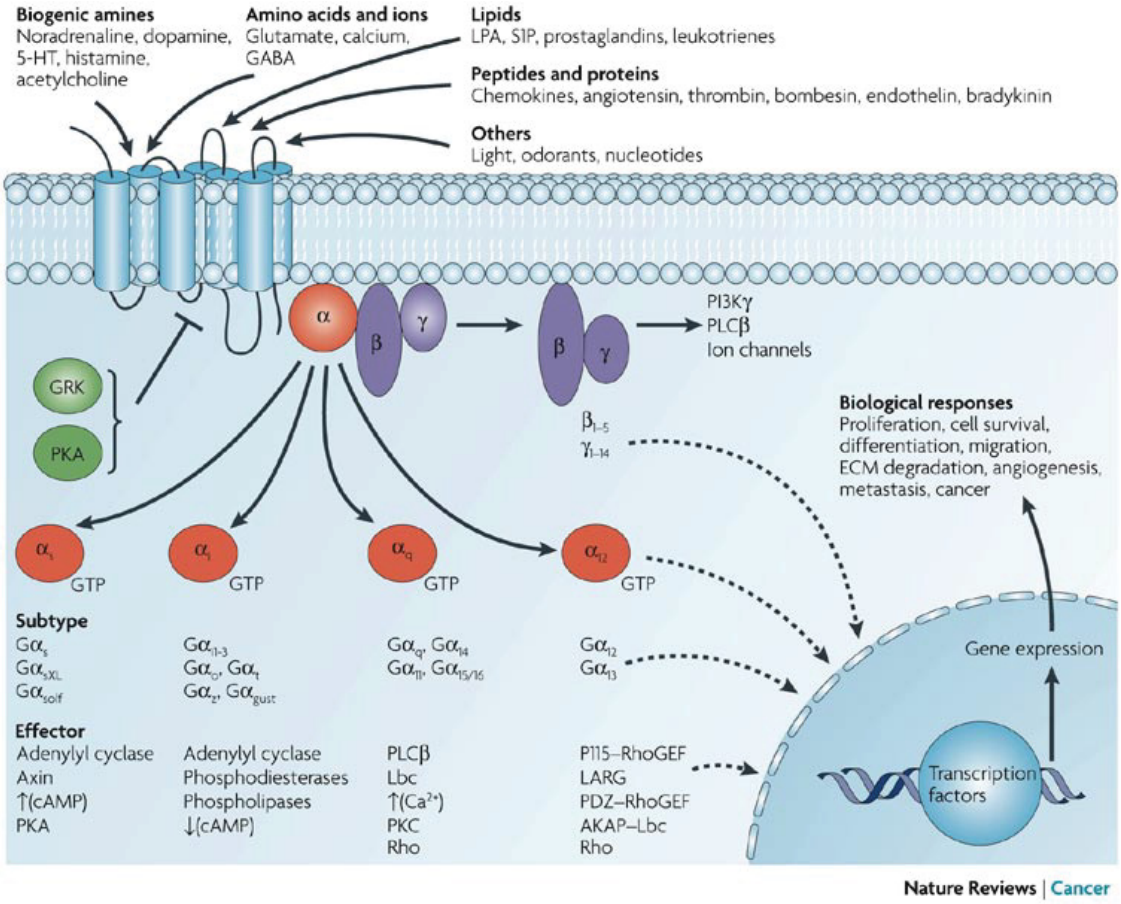



**Figure 1.1 GPCR Signalling Diversity and the Major Intracellular Effectors Involved**

The various extracellular stimuli that can induce GPCRs to promote signal transduction include biogenic amines, amino acids and ions, lipids, peptides and proteins, and synthetic therapeutics. The G-protein dependent mechanisms of signal transduction involve the heterotrimeric Gαβγ subunits. The four classes of the Gα subunit; $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{12}$ and the Gβγ subunit mediate the activity of key intracellular signal transducing molecules such as adenylyl cyclase (AC), phospholipases, and protein kinases. The effects of GPCRs can be fast acting, such as the activation of intracellular calcium stores, or can have longer term effects such as the alteration of gene transcription in order to elicit a biological effect [Dorsam and Gutkind, *Nature Reviews Cancer* 2007; 7(2):79-94, with permission].

### *1.2 GPCR Classification*

GPCRs were originally classified into six phylogenetic groups, A-F, based on sequence homology, with each group sharing around 20% similarity. With the completion of major genomic advances such as the Human Genome Project in 2004, over 800 GPCR encoding genes were discovered and a new classification system was needed to encompass both genetic and structural aspects of GPCRs. GPCRs are now classified into one of three families, with each family having conserved structural motifs. Class A (Rhodopsin-like) is the largest GPCR family, consisting of approximately 700 different members[5]. Amino acid sequence similarity is generally low between different GPCRs in class A, however there are certain key residues that are conserved in the transmembrane (TM) domains, which suggests that they have a key role in maintaining the structural/functional integrity of the receptor itself. One of the conserved amino acids in class A GPCRs is the arginine residue in the second intracellular loop (ICL) in the glutamic acid/aspartic acid-arginine-tyrosine (E/DRY) motif[6]. Most class A GPCRs also contain a conserved disulfide bridge that links the first and second extracellular loops (ECL)[7]. Some of the important ligands that bind to class A GPCRs include biogenic amines, sex hormones, olfactory odorants, and neuropeptides. Furthermore, since the discovery of the structure of bovine rhodopsin, many pharmaceutical therapeutics target class A GPCRs since they are the most abundant GPCRs present in the human body.

Class B (secretin-like) GPCRs contain approximately 15 different members, and are characterized by a large amino (N) terminal extracellular domain (~160 amino acids) that plays a role in ligand binding. Class B GPCRs have little conservation present in their TM domains when compared to class A GPCRs[5, 8]. There are however, a number of

cysteine residues that contribute to disulfide bridge formation in the ECLs. Ligands that bind to class B GPCRs are naturally occurring endogenous peptide hormones such as parathyroid hormone, glucagon, and vasoactive intestinal peptide. Interest in class B GPCRs has grown in the past few years from researchers and pharmaceutical companies due to their role in maintaining homeostasis in key physiological systems through the use of hormones, such as calcitonin regulation of bone; however, drug design for these receptors has been hampered by proteases, which rapidly degrade any synthetic peptides *in vivo*[5, 9].

Class C (glutamate-like) GPCRs contain a large extracellular Venus Fly Trap (VFT) domain along with a large carboxy (C) terminal tail. The VFT domain is thought to play a significant role along with the N-terminal in ligand binding and activation of the receptor. Some of the ligands responsible for binding class C GPCRs include $Ca^{2+}$ ions, sugars, glutamate, and taste flavours such as umami. The gamma-aminobutyric acid (GABA) GPCR requires the formation of heterodimers between $GABA_{B1}$ & $GABA_{B2}$ receptors in order to form a functional heterodimer that is localized at the cell surface[1, 6]. As well, there are two other classes of GPCR families, which are the adhesion and frizzled/taste2. The adhesion class of GPCRs have a long extended N-terminal that is thought to play a role in cell contact. In addition, the adhesion class does not have any drugs that target them, and a vast majority remain as 'orphan' receptors[10]. There is more than 140 'orphan' GPCRs that have not been classified into any of the GPCR superfamilies[11]. Orphan GPCRs are named so because the endogenous ligand that binds and activates them has not been determined. Often, these receptors show similar homology and structure to other GPCR classes. Orphan GPCRs are of particular interest,

however, because mouse knockout models have revealed that orphan GPCRs do play a role in physiological and pathological homeostasis. GPCR 48, an orphan GPCR, was knocked out in mice to reveal its role in bone formation and remodeling[12]. Being able to target orphan GPCRs may lead to new therapeutics to treat disease states.

### *1.3 GPCR Structure*

Despite the great diversity between the sequence homology of GPCRs and the ligands that bind to them, GPCRs have similar protein architecture that is conserved. GPCRs have a benchmark structure that is made up of a bundle of seven TM α-helices, which consists primarily of hydrophobic amino acid residues. Within the TM α-helical region, the ligand binding pocket is sheltered[13]. The seven TM α-helical core is connected by three ECLs bound by an extracellular N-terminus and three ICL which are bound by an intracellular C-terminus[14]. Although a select assortment of motifs that denote GPCR function are present, such as the NPXXY motif in the seventh TM domain of many GPCRs that is involved in receptor internalization[15], the TM region shows the greatest sequence homology. However, the N- and C-termini along with the ECLs show the greatest variability in their amino acid length and sequence amongst the GPCR families[13, 16]. For example, the second ICL of the human adenosine-A2A receptor (A2AR) is helical in its three dimensional structure, compared to the human beta-2 adrenergic receptor (β2AR) which has a coil like structure for its second ICL, illustrating the architectural diversity between two GPCRs of the class A family[17]. Proper GPCR structural conformations are vital for the appropriate functioning of the receptor and its ability to elicit specific intracellular molecular mechanisms, and instances of aberrant structure

often result in states of disease[14]. For example, the vasopressin V2 receptor (VV2R) is a GPCR that functions in the kidneys to concentrate urine and this receptor is polymorphic at a variety of amino acids. Polymorphisms such as tyrosine mutated to histidine at amino acid position 205 can lead to the disease nephrogenic diabetes insipidus[18]. However, structural diversification within GPCRs has given pharmacological advantages when designing drugs to target specific locations on certain receptors.

Visualization of GPCR structure has been quite a difficult task up to date. There are a variety of factors that contribute to the complexity of structure visualization. Firstly, the large size of GPCRs makes it difficult for current technologies such as x-ray crystallography and nuclear magnetic resonance to produce a clear image of GPCRs. Secondly, GPCRs are integral membrane bound proteins and when they are outside of the cellular membrane, instability becomes an issue. Furthermore, GPCRs are capable of attaining many different conformations that give them flexibility, which further complicates visualizing their structure[13, 14]. The first GPCR crystal structure produced was that of bovine rhodopsin, which was determined in the year 2000[19]. Other GPCR structures have been deciphered since bovine rhodopsin including the *Meleagris gallopavo* (Turkey) beta-1 adrenergic receptor (β1AR), the human β2AR, the human A2AR, the ligand free form of rhodopsin (opsin), the dopamine D2 –like receptor (D3), and the C-X-C Motif Chemokine Receptor Type 4 (CXCR4)[20-24]. Although few GPCR structures are known, these studies have shown that GPCRs do in fact maintain general structure in their TM α-helices, but differ in other regions such as the N- and C-termini. Pharmacological techniques that utilize partial and inverse agonists along with antagonists have helped to enhance the thermal stability of GPCRs so that the crystal

structure can be determined. Molecular biological techniques, such as site directed mutagenesis, have also proved to be useful tools, allowing researchers to alter the binding pockets of GPCRs so that they can bind ligands with higher affinities than they normally would. This has been done while maintaining the integrity and function of the receptor so that it can be crystallized, revealing the three dimensional structure. As with any technological advance, one can only expect that more GPCR crystal structures will be determined. Using the well characterized rhodopsin GCPR as a homology model, researchers are actively screening new ligands with the hope of determining new GPCR structures[25]. Understanding the structure of a GPCR is a major achievement because this allows chemists to design therapeutics that will specifically be able to target GPCRs in a very precise manner. Developing therapeutics that specifically targets a particular GPCR is valuable in producing new therapeutics with reduced side effects.

### *1.4 GPCR Activation & Signal Transduction*

Cellular communication is a vital component to any multicellular life form to ensure its synchronized functioning. GPCRs represent the largest family of proteins that transduce signals from the extracellular environment to intracellular effectors. The biological effect of a ligand binding to a GPCR can be either short or long term, ranging from activation of a second messenger such as calcium, to the alteration of gene transcription. The classical view of GPCR activation has consisted of a ligand binding to a GPCR, which causes a single altered ligand-receptor conformational state and the activation of the heterotrimeric guanine nucleotide-binding protein (G-protein). The G-protein consists of a Gβ, Gγ, and Gα subunit and is directly coupled to the GPCR in a

dependent manner. There are 15 Gα, 5 Gβ, and 12 Gγ subunits that all play roles in modulating specific signalling pathways[26]. To illustrate the signal specificity of the different Gβ and Gγ subunits, their effector activity has been summarized in Table 1.1. The $G\beta_{1\text{-}4}$ subunits share 78-88% sequence similarity, except for the $G\beta_5$ subunit, which is distinct in its structure, compared to the Gγ subunits, which are more diverse in their structure[27]. An inactive GPCR has the Gα subunit bound to guanosine diphosphate (GDP) and is associated with the obligate Gβγ dimer. The Gα –GDP complex then exchanges GDP for guanosine triphosphate (GTP), and can then dissociate from the Gβγ subunits to activate downstream effectors[28, 29]. However, the idea that the G-protein subunits dissociate to form a Gβγ dimer and Gα-GTP monomer is highly scrutinized, as some propose that rearrangement may occur amongst the Gβγα subunits rather than complete dissociation[30]. G-proteins are classified into four basic categories based upon sequence homology of the Gα subunit: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_q$, and $G\alpha_{12/13}$. Normally, $G\alpha_s$ stimulates AC increasing levels of cyclic adenosine monophosphate (cAMP) where $G\alpha_{i/o}$ inhibits AC. $G\alpha_q$ binds to isoforms of phospholipase C (PLC), cleaving phosphatidylinositol-4,5-bisphosphate (PIP2) and generating secondary messengers inositol triphosphate (IP3) and diacylglycerol (DAG). The exact role of the $G\alpha_{12/13}$ subunits is rather diverse. They have been implicated in the control of regulator of G-protein signalling (RGS) proteins, which bind to Gα subunits and accelerate GTP hydrolysis[29]. As well, the $G\alpha_{12/13}$ subunits have also been shown to mediate cytoskeletal rearrangements through Rho proteins, which affects cell shape and migratory abilities[31]. Studies in drosophila and zebrafish have also implicated the $G\alpha_{12/13}$ subunits with the developmental process gastrulation[32, 33]. It is

important to note that GPCRs maintain signal specificity by coupling to specific G-proteins, affecting certain downstream pathways.

**Table 1.1 Role of Gβ and Gγ Subunits Effector Activity**

| G-Protein Subunit | Effector Activity | Reference |
|---|---|---|
| $G\beta_1$ | Muscarinic M4 and voltage-dependent calcium channel coupling | 34 |
| $G\beta_2$ | Involvement in chemotactic response to complement 5a mediated release of histamine | 35 |
| $G\beta_3$ | Involvement in the inhibition of calcium channel current in galanin receptors | 36 |
| $G\gamma_2$ | Involvement in the inhibition of calcium channel current in galanin receptors | 36 |
| $G\gamma_3$ | Somatostatin receptor and voltage-dependent gated calcium channel coupling | 37 |
| $G\gamma_7$ | Involvement in AC activity following isoproterenol stimulation | 38 |

Recently, there has been intense investigation into GPCR signalling, proposing new ideas that challenge the classical model of GPCR activation. New methods of GPCR activation propose that signal activation can occur independently of the G-proteins, hence the naming G-protein independent signalling (Figure 1.2). One of the extensively studied methods of G-protein independent signalling involves the non-visual arrestins, arrestin-2 (β-arrestin) and arrestin-3 (β-arrestin1). Arrestin-2 and arrestin-3 are expressed throughout the human body, and are not confined to the photoreceptors in the eye, unlike arrestin-1 and arrestin-4. In this model, GPCRs are able to signal through arrestin-2 and arrestin-3, proteins that were originally characterized in receptor desensitization and internalization following agonist stimulation[28]. Both arrestin-2 and arrestin-3 are responsible for uncoupling GPCRs from their associated heterotrimeric G-proteins following agonist stimulation in order to cease GPCR activation and signalling, following

phosphorylation at serine and threonine residues at the C-tail via the G-protein coupled receptor kinases (GRK)[39, 40]. When either arrestin-2 or arrestin-3 is recruited to the C-tail of a GPCR, they are able to modulate receptor desensitization through steric hindrance between the receptor and its cognate G-protein, thus terminating any further signal transduction. In the classical model of GPCR activation, when a ligand binds to a GPCR, the two will interact to either activate or inhibit signal transduction through the heterotrimeric G-proteins, whether the ligand is an agonist or antagonist. However, in the G-protein independent model of signal transduction, ligands are still able to bind to GPCRs, but will instead induce signal transduction through the use of scaffold proteins such as arrestin-2 or arrestin-3, or any other protein such as the GRKs, which act independently of the G-proteins. The occurrence of GPCR independent signalling has been observed in the β2AR when it is bound to the selective β2AR antagonist ICI 118, 551. ICI 118, 551 is able to phosphorylate extracellular-signal-regulated kinase (ERK) independently of the G-protein through arrestin-2 and arrestin-3[41, 42].

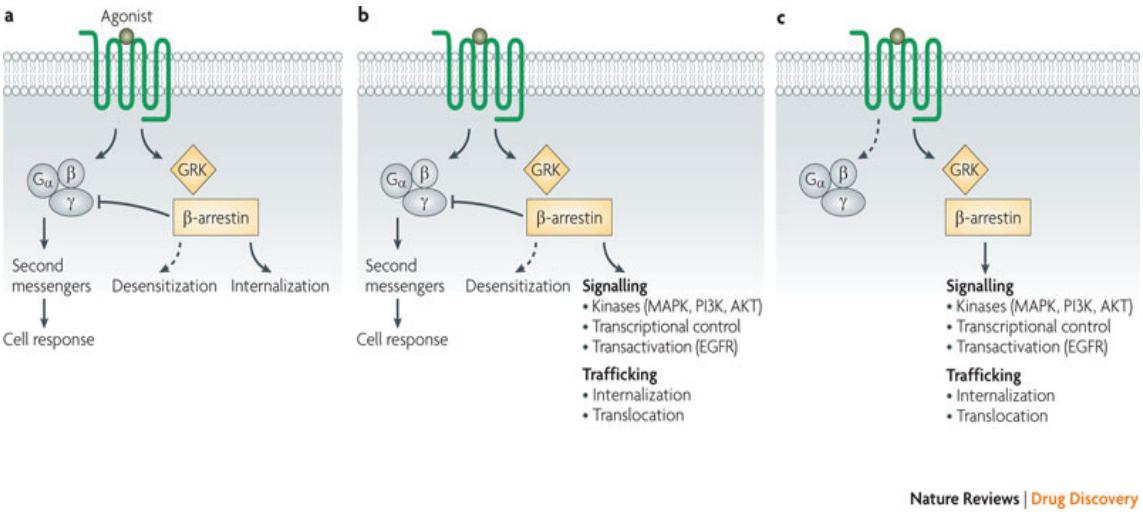



**Figure 1.2 G-protein Dependent and G-protein Independent mechanisms of Intracellular Signal Activation**
**a)** The classical model of G-protein dependent signal activation using the heterotrimeric G-proteins, where desensitization and internalization occurs through the GRKs and arrestins-2,3. **b)** Current models suggest that when a ligand binds to a GPCR, it will activate signalling cascades using both G-protein dependent and G-protein independent mechanisms. **c)** Ligands that are classified as 'biased' will bind to a GPCR and preferentially signal through arrestins-2,3, independently of the G-proteins [Rajagopal, Rajagopal, and Lefkowitz, *Nature Reviews Drug Discovery* 2010; 9(5):373-386, with permission].

The phenomenon of biased agonism has led to the investigation of receptor-ligand interactions, specifically looking at how therapeutics can be designed to elicit desired cellular responses. Many ligands that have been synthetically designed are derivatives of an endogenous peptide and they often possess biased ability to signal using specific pathways such as ERK, through the use of arrestins-2,3. For example, the synthetic analog of angiotensin II, SII angiotensin, is capable of binding the AT1aR and activating arrestin-2 mediated signalling[43]. GRKs have also been shown to be involved in ligand bias. The T and B lymphocyte receptor, C-C chemokine receptor type 7 (CCR7) can bind its endogenous ligands C-C motif ligand 19 (CCL19) and C-C motif ligand 21 (CCL21)

leading to G-protein dependent and G-protein independent activation. The binding of CCL19 results in the recruitment of arrestin-3, which is mediated by both GRK3 and GRK6. However, when CCL21 binds to CCR7, it only activates GRK6 and promotes a weaker interaction with arrestin-3[44]. GPCRs themselves can also show preference for unbiased ligands. An unbiased ligand may bind to a receptor that shows preference to signal through arrestins-2,3 or can activate signal cascades using G-protein dependent mechanisms. Furthermore, ligands have been shown to elicit different effects depending upon the signalling pathway that is being observed, and this is known as protean agonism. An example of this can be seen in the histamine $H_3$ (H3) receptor, which is a GPCR. Proxyfan is a synthetic ligand that targets the H3 receptor and it was shown to act as a partial agonist, a ligand that binds to a receptor and results in a submaximal response. Proxyfan can also antagonize a full agonist, when observing its effect on ERK signal activation and cAMP production. Proxyfan was also capable of acting as a partial inverse agonist, a ligand that binds to a receptor and decreases the signalling activity of the receptor compared to the unbound state, for arachidonic acid release[45]. The ability of proxyfan to bind to the H3 receptor and generate the aforementioned cellular responses shows the importance of testing multiple signalling pathways when attempting to classify (i.e. agonist, partial agonist, inverse agonist, and antagonist) newly designed therapeutics, because a ligand may not activate a single signalling cascade; it could affect multiple pathways and produce unwanted signalling, which is often the cause of many drug side effects.

### *1.5 GPCR Desensitization & Internalization*

The current model used to describe GPCR internalization is based on studies of the β2AR. When an agonist binds to a GPCR it causes a conformational change, which activates intracellular signalling cascades and cellular effectors. After receptor activation has occurred, signal transduction must be terminated (Figure 1.3). Continuous signal activation is often associated with disease states, such as the case with sustained catecholamine induced β adrenergic (βAR) stimulation, which results in the loss of adrenergic receptor responsiveness to catecholamines causing a decrease in cardiac contractility[39]. Agonist bound GPCRs are phosphorylated by GRKs, which selectively phosphorylate residues located on the third ICL and C-tail of the receptor, a process known as homogenous desensitization. The phosphorylation of specific serine and threonine residues on either the third ICL or C-tail will establish what downstream signalling cascades are activated. There are seven different genes that encode GRKs and they are expressed in specific tissues of the body. GRKs differ in their N-terminal region, which is responsible for receptor recognition and in their C-terminal domain, which determines subcellular localization[39]. For example, GRK2 is expressed throughout the body but is highly concentrated in cardiac tissue and interacts with the βARs and angiotensin II type 1a receptor (AT1aR)[46]. GRK3 is also expressed throughout the body and it targets the α-adrenergic receptors and plays a role in airway smooth muscle contractions and also chronotropic effects in cardiac tissue[47]. Finally, GRK6 is also expressed throughout the human body and it plays a role in a variety of systems, such as locomotor effects in the central nervous system. Originally, GRKs were simply thought to be part of a well coordinated process that was involved in receptor desensitization.

However, GRKs have been shown to play a prominent role in different disease states. Increased expression of GRK2 in cardiomyocytes is involved in the molecular dysregulation that occurs in heart failure, which is associated with increased catecholamine levels. When the βAR antagonist carvedilol is prescribed, GRK2 levels decrease in a dose dependent manner, helping to alleviate symptoms of heart failure[48]. Receptor phosphorylation can also occur via second messengers such as protein kinase A (PKA) in the absence or presence of an agonist, which is known as heterologous desensitization. The addition of phosphate groups to the receptor acts to recruit cytosolic accessory proteins, such as the non-visual arrestins (arrestins-2,3), up to the GPCR to promote internalization. Arrestins-2,3 act as a scaffold, forming a complex with adaptor-protein2 (AP2), c-Src kinase, and also clathrin, which targets receptors for clathrin-coated pit (CCP) internalization. During the formation of CCPs, clathrin is recruited from the cytoplasm to the receptor at the plasma membrane (PM) where it can bind to AP2 and arrestins-2,3 to form early CCPs[49]. AP2 plays a vital role in CCP formation by assembling clathrin at the PM and also in selecting what receptors will be internalized to form the clathrin-coated vesicles (CCV). Dynamin is a GTPase, which is another crucial regulator of CCP internalization, as it is involved in pinching the CCVs from the plasma membrane so they can be brought inside the cell. Once internalization has occurred, the receptor can be dephosphorylated and recycled back to the PM. This results in resensitization of receptor mediated signaling. The receptor may also be degraded by the lysosomes where proteolytic cleavage occurs and results in down regulation of receptors, which is regulated by post synaptic density protein (PSD95), Drosophila disc large tumor

suppressor (Dlg1), and zonula occludens-1 protein (zo-1) (PDZ) proteins such as Na+/H+ exchanger regulatory factor 1 (NHERF1)[2, 40, 49].

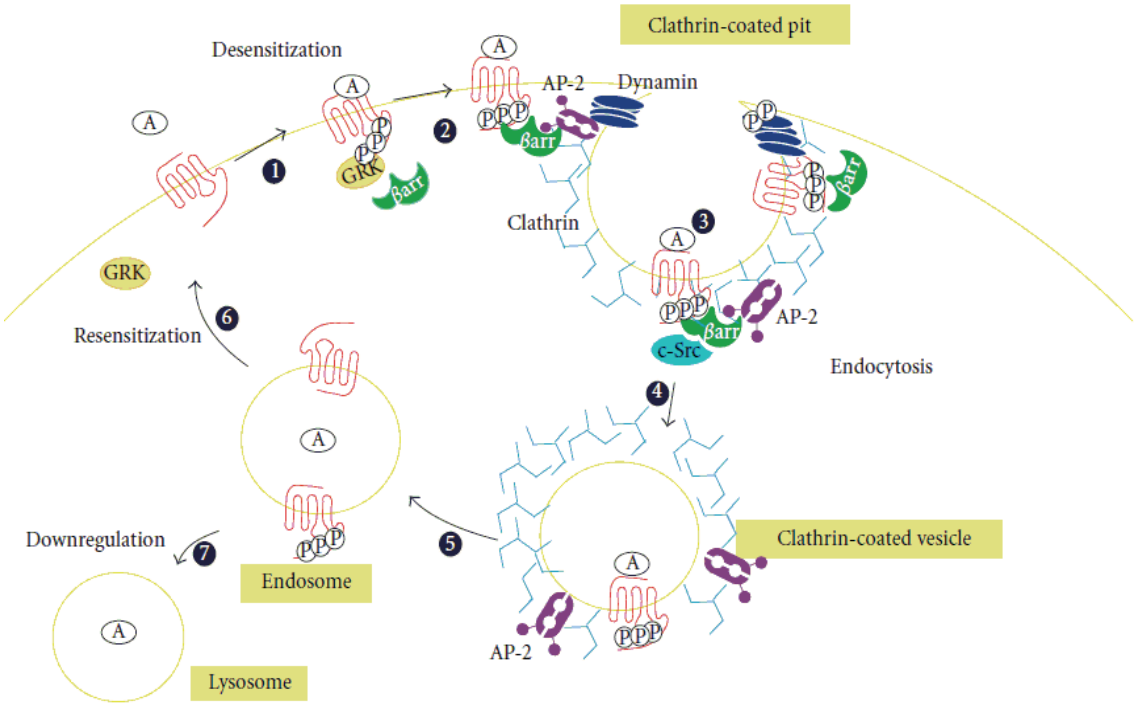



**Figure 1.3 The Steps Involved in GPCR Desensitization, Internalization, and Recycling**
**Step 1)** Following agonist binding, GPCRs are phosphorylated by GRKs and arrestins-2,3 are recruited. **Step 2)** Arrestins-2,3 interact with clathrin and AP-2 to target the GPCR to CCPs. **Steps 3,4)** Arrestins-2,3 can then interact with c-Src kinase and dynamin to pinch the CCP from the PM. **Step 5)** The internalized receptor is located in the endosomes and can be recycled back up to the PM following dephosphorylation **(Step 6)** or can be degraded in the lysosomes **(Step 7)** [Delom and Fessart, *International Journal of Cell Biology* 2011; 2011:246954, open access].

### *1.6 GPCR Dimerization*

The traditional view of GPCR function and activity is that GPCRs were acting as monomeric entities, where a ligand can bind to a receptor and activate a single G-protein. However, there has been intense investigation into whether GPCRs are capable of

forming higher order dimeric and oligomeric complexes. The first example of GPCR dimerization came in 1997 when researchers were investigating the $GABA_B$ receptor, which is a member of GPCR family C. The $GABA_{BR1}$ gene was found to produce a GPCR that was not expressed at the PM due to impaired trafficking. On the other hand, the $GABA_{BR2}$ gene was known to produce a receptor that was expressed at the PM, but was not capable of binding $GABA_B$ ligands. However, when both the $GABA_{BR1}$ and $GABA_{BR2}$ were co-expressed together, a functional receptor was formed at the PM that was capable of $GABA_B$ ligand binding[50]. Further research on the $GABA_B$ heterodimeric receptor complex showed that $GABA_{BR1}$ on its own contained a C-terminal RSRR endoplasmic reticulum (ER) retention motif that is masked when both $GABA_{BR1}$and $GABA_{BR2}$ dimerize, forming a functional $GABA_B$ heterodimer that is expressed at the PM[51]. Research in other family C GPCRs has revealed that there are other constitutive dimers that need to be formed in order to have a functional receptor. For example, the sweet (T1R2/T1R3) and umami (T1R1/T1R3) taste receptors will not form a functional receptor when expressed on their own, but when expressed in heterodimeric pairings they are capable of signal transduction[52]. Another family C GPCR, the metabotropic glutamate receptor 5 (mGluR5), was also shown to form obligate homodimers[53]. Exploration of dimerization in both family A and B GPCRs is an area of considerable research that is attempting to understand the consequences of dimerization between different subtypes of receptors and how this influences the activation of signalling cascades. Given the fact that dimerization between different subsets of receptors has been demonstrated in mice, such as the β2AR/AT1aR heterodimer, it is important in the development of therapeutics in targeting disease states.

One of the main criticisms that have arisen relating to GPCR dimerization is that fact that many experimental approaches rely on the use of *in vitro* cell culture systems, which may not present an accurate representation of dimerization *in vivo*. However, a study which looked at disc membranes from retinal outer rod segments using atomic force microscopy (AFM) showed that rhodopsin can be arranged in organized pairs, which suggests that higher order oligomerization is occurring in native tissue *in vivo*[54]. Further evidence for *in vivo* GPCR dimerization arose when a group studying the luteinizing hormone receptor (LHR), a family A GPCR that influences gonadal development, examined the role of a binding deficient and signalling deficient LHR receptor. When either the binding or signalling deficient receptor was expressed on its own *in vivo*, gonadal phenotypic effects mimicked that of the LHR knockout mice. However, when both the binding and signalling deficient LHRs were co-expressed *in vivo*, gonadal phenotypic development was restored to that of the wild type (WT) mice[55]. This study suggests that dimerization is in fact occurring within native tissues, and that GPCR dimerization has an effect on how receptors will respond to a ligand. In the case of the LHR study, the initial step to investigate the binding and signalling deficient receptors involved the utilization of an *in vitro* cell culture model with human embryonic kidney (HEK) 293 cells. This was a fundamental step in establishing that both the binding and signalling deficient receptors were able to be properly expressed at the PM, and that co-expression was able to restore their function. *In vitro* cell culture work is the first line of action of many experimental designs. The *in vitro* experimental approach allows for the understanding of the basic molecular mechanisms that are associated with a receptor, such as trafficking and signal activation. As well, basic pharmacology can be utilized in

cell culture models, such as ones that investigate GPCR dimerization. For example, HEK293 cells were used to study molecular cross-inhibition in the β2AR/AT1aR heterodimer. One protomer of the heterodimer was inhibited with an antagonist, while the other protomer was stimulated with an agonist to determine if signal transduction was still attainable[56]. Once these interactions have been investigated, the next step can be taken to move into an *in vivo* model. The progression from an *in vitro* model to an *in vivo* model can often be compared to building a house. The foundation of a house is similar to that of an *in vitro* model. You cannot progress in any matter of construction unless you have a foundation, and the same can be said about an *in vitro* model. Understanding the basic mechanisms that occur in cell culture models is pertinent before moving up to the systemic level of an *in vivo* model, such as a mouse.

### *1.7 Molecular Techniques to Study GPCR Dimerization*

Initial experiments that were conducted to examine GPCR dimerization utilized co-immunoprecipitation assays, which were essential in laying the groundwork for further research in this rapidly growing branch of receptor biology. In early co-immunoprecipitation experiments, receptors that were thought to dimerize were tagged with different immunological epitopes, such as the human influenza hemagglutinin (HA), so the receptors were able to be targeted through the use of primary antibodies and studied using *in vitro* cell culture systems. Co-immunoprecipitation experiments can also be performed on samples of native tissue to determine if dimerization is occurring between different GPCR subtypes. After the initial *in vitro* studies showing dimerization of the $GABA_{BR1}$ and $GABA_{BR2}$ receptors, co-immunoprecipitation studies on brain tissue

further confirmed dimerization[57]. One of the pitfalls of co-immunoprecipitation experiments is that the cells have to be lysed to release the GPCR dimers from the PM into solution. With the advent of technology, new techniques have arisen which allow further examination of GPCR dimerization. Bimolecular fluorescence complementation (BiFC) involves tagging separate GPCRs with N-terminal and C-terminal halves of a fluorescent protein and determining the level of fluorescence that occurs when the receptors are co-expressed, which is an indication of dimerization. Bioluminescence resonance energy transfer (BRET) and fluorescence energy transfer (FRET) are both methods that make use of energy transfers that occur between two proteins that are in close proximity and this has been applied to GPCR dimerization research. The limitations of some of the resonance energy transfer (RET) techniques are that they require fluorescent fusion proteins, which provide roadblocks when attempting to use them in an *in vivo* model. BiFC, BRET, FRET, and AMF are advantageous to study dimerization because they can be used in living cells and have helped to further our knowledge and demonstrate that dimerization occurs[54, 58-61]. Although it is well established in many studies that GPCR dimerization does occur, difficulty arises when attempting to determine the stoichiometry of higher level receptor oligomers.

### *1.8 Pharmacological Consequences of Dimerization*

With the discovery of GPCR dimers and higher order oligomeric receptor complexes, the traditional view of ligand-receptor pharmacodynamics must be able to incorporate the changes that are associated with the formation of higher order GPCR structures. GPCR oligomerization is of important clinical relevance, because it

complicates the actions of therapeutics, and their design may subsequently be altered as a result of dimerization. Despite the complications that GPCR dimerization adds to an already intricate system, it opens up many avenues to design therapeutics that will be able to act on dimeric complexes, where a ligand may selectively act on a certain receptor complex over another. This type of novel therapeutic specificity would help to alleviate side effects, which are often associated with non-specific signal transduction[62]. The classical theory on receptor-ligand binding is one where the receptor is in an equilibrium between an active (R*) and an inactive state (R). The binding of ligands will shift the equilibrium between the R* or R state[63]. Inverse agonists are able to shift the equilibrium towards the R state and agonists have the ability to favor the R* state. On the other hand, antagonists are neutral and do not shift the equilibrium between the R and R* state[64]. With the introduction of G-protein independent models of signalling, the two state R and R* does not hold true. Thus, a multiple active state model has been proposed where each separate active state is responsible for coupling GPCRs to specific pathways (Figure 1.4). This has been shown in the type-1 pituitary adenyl cyclase-activating polypeptide (PACAP), where the binding of the agonist PACAP-27 promotes cAMP production through $G\alpha_s$ coupling but is not capable of coupling to $G\alpha_q$ to produce IP3. However, the agonist PACAP-38 promotes $G\alpha_q$ coupling and IP3 production but not $G\alpha_s$ coupling and cAMP production[65]. This further illustrates the concept of biased agonism, showing how two agonists that target the same receptor are capable of eliciting separate cellular signalling events. Multiple active states also apply to GPCR dimerization, because the formation of dimers may introduce the ability to form multiple active states that were not

previously attainable, changing the signalling events that are associated with different ligands.

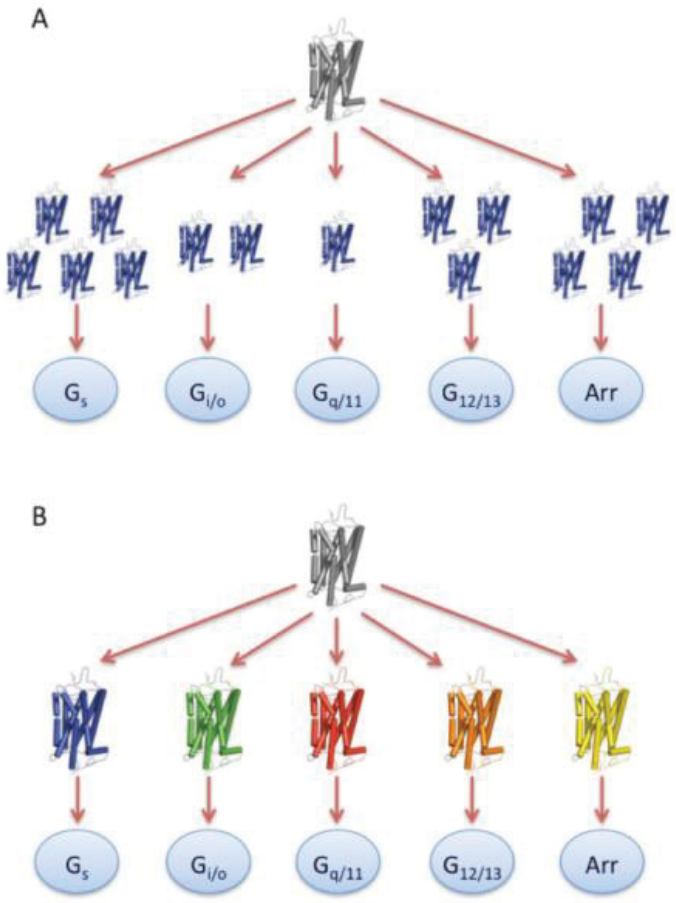



**Figure 1.4 The Diversity of Conformational States in GPCRs that Promote Signal Transduction**
**a)** The two state R and R* model of GPCR signal activation. A GPCR can attain the R* active state and depending upon how many other GPCRs are in a similar R* active state, that will determine whether G-protein dependent ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{12/13}$) or G-protein independent (Arrestins, GRKs) signal pathways are activated. **b)** In the multiple active state model, a GPCR can adopt multiple R* active conformational states (illustrated by different receptor colors) and that will determine what G-protein dependent ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{12/13}$) or G-protein independent (Arrestins, GRKs) signal pathways are activated [Park, *Current Medicinal Chemistry* 2012; 19(8):1146-54, with permission].

The binding of a ligand to a receptor occurs either at an orthosteric site, one where the endogenous ligand can bind, or at an allosteric site, one where a ligand can bind and modulate the activity of the receptor. GPCR dimerization can result in the formation of an orthosteric (or allosteric) ligand binding site that is different from the monomeric form of the receptor(s) and will change the ligand-receptor dynamics, challenging the current classification of therapeutics[66]. GPCR dimerization can affect the potency and affinity of ligands that bind specific receptors depending upon whether the targeted receptors are capable of forming homo- or heterodimers with other receptors, which would change the clinical profile of a therapeutic[62]. Early studies on the opioid receptor (OR) provide a great example of how the formation of heterodimers affects receptor affinity for a ligand. The μ-OR and δ-OR were examined individually to determine the affinity of highly selective agonists, DAMGO and DPDPE respectively, on each of the monomeric units. Each of these synthetic agonists has a very high affinity for their respective receptor. However, when μ-OR/δ-OR heterodimers were formed, there was a noted tenfold decrease in the affinity to both the DAMGO and DPDPE agonists, which suggested that heterodimerization may have resulted in the formation of an allosteric binding pocket that altered the activity of the ligand-receptor complex[67].

There are other examples of heterodimerization involving the μ-OR, such as with the A2AR, which increases the response of the μ-OR/A2AR heterodimer to μ-OR agonists. However, when agonists of both the μ-OR and A2AR receptor are given simultaneously, it decreases the μ-OR/A2AR heterodimers ability to activate signal cascades[68]. Thus, the μ-OR/A2AR heterodimer preferentially binds μ-OR agonists, which further illustrates how GPCR dimerization can lead to different active states and ligand

bias. A unique clinical aspect of GPCR dimerization occurs in the A2AR/dopamine D2 (D2R) heterodimer; adenosine receptor antagonists can control the effects of dopaminergic neurotransmission. Adenosine has been shown to inhibit D2R signalling and increases the activity of the striatopallidal GABA neurons, which leads to Parkinson-like motor incoordination. A2AR/D2R heterodimerization has been translated to a clinical setting to treat Parkinson's disease, where an A2AR antagonist is administered and a D2R agonist, L-DOPA, is co-administered which helps to alleviate Parkinson's-like symptoms[69]. Another fascinating aspect of receptor dimerization is that it opens up the possibility of the receptor complex to 'preferentially' determine what ligand will be able to bind. For example, the δ-OR/κ-OR heterodimer will show selectivity towards the 6'-GNTI opioid agonist over δ-OR and κ-OR homodimers[70]. This type selective agonist binding can also be seen in the dopamine D1 receptor (D1R)/D2R heterodimer, where the agonist SKF-83959 will bind to the heterodimer over D1R and D2R homodimer pairings[71]. It is clear that GPCR dimerization shines new light onto the way that we view pharmacology, specifically in how we study basic pharmacological properties of therapeutics and how they can be used to specifically target various receptor subtypes in a ligand dependent manner.

### *1.9 The Molecular Dynamics of GPCR Dimerization*

GPCR dimerization not only affects the pharmacology of the receptor dimers, but it also changes the molecular dynamics that are associated with the receptor complex. There is growing evidence that the formation of GPCR dimers is an event that occurs early during biosynthesis of the receptors. For example, many family A GPCR members

require N-terminal glycosylation before they are released from the golgi apparatus during the formation of dimers, which acts as a control step to ensure proper receptor expression[72]. Studies that have looked at immature GPCRs (ie: no N-terminal glycosylation) have shown that not only are the immature receptors not able to form at the PM, when they are expressed as a dimer they will also prevent the proper expression of a WT receptor. This has been shown to occur in the D2R, C-C Motif Chemokine Receptor Type 5 (CCR5), and also in the VV2R[73-75]. The β2AR was also shown to form oligomers in HEK293 cells and in cardiomyocytes[76]. It should be noted that GPCR monomers are relatively stable, but some have questioned whether GPCR dimers are able to form stable entities. Until recently, there was no way to determine how long GPCRs were forming stable complexes at the PM. However, a study investigating the N-formyl peptide receptor (NFP), which is a GPCR, was able to utilize specialized combinations of RET technology to look at the ratio of monomeric to dimeric NFP receptors. This group was able to demonstrate that there is a dynamic equilibrium that exists at anytime between monomeric and dimeric forms of NFP in live cells at the PM. As well, they reported that monomeric NFP receptors are continually converted to dimers every ~150ms and that the NFP dimers are then broken back down to monomers every ~91ms[77]. GPCRs thus form a short transient complex that associates long enough to permit signal transduction. Further evidence supports the idea of GPCRs forming dimers during their maturation and trafficking in the ER, as the oxytocin and vasopressin GPCRs were found to be present as both homo- and heterodimers in the ER. Furthermore, treatment of either receptor with an agonist or antagonist did not affect dimer formation[78]. There are important molecular chaperones that help contribute to dimer formation during

their synthesis, which will also preferentially interact with specific homo- and heterodimer complexes. The molecular chaperone ERp57 was shown to be important in the production of AT1aR homodimers and in β2AR/AT1aR heterodimer formation, but not for β2AR homodimer synthesis[79]. Proper GPCR dimer synthesis and trafficking to the PM is an important process, and instances where mistakes occur and PM localization of a GPCR is altered can result in disease states.

GPCR dimerization also presents an interesting occurrence known as molecular crosstalk. During crosstalk, ligand binding can activate one or both of the receptors in the GPCR dimer, which results in structural conformational changes. The conformational changes that occur following ligand binding can be thought of as an information processing event to decide what signalling effectors will be activated or inhibited (Figure 1.5). GPCR molecular crosstalk is evident in neural transmission with the A2AR/adenosine A1 receptor (A1R) heterodimer, where different concentrations of the ligand adenosine produce different affinities for the heterodimer. At lower concentrations of adenosine the A1R protomer of the A2AR/A1R heterodimer is activated, and at higher adenosine concentrations the A2AR protomer is activated in the A2AR/A1R heterodimer. The activation of either the A2AR or A1R is thought to cause steric membrane interactions that antagonize the opposite protomer in the A2AR/A1R heterodimer[80]. Molecular crosstalk is also seen in β2AR/AT1aR heterodimers, where the inhibition of either protomer through the use of an antagonist will result in the uncoupling of its reciprocal receptor from its heterotrimeric G-proteins through a process of trans-inhibition[56]. This method of trans-inhibition was also observed in mice cardiomyocytes. Inhibition of the βARs resulted in a decrease in angiotensin II induced contractility. The

reverse also had similar effects; blocking the AT1aR reduced the contractile response of the βARs in the heart to catecholamines. The model of trans-inhibition of GPCR dimers is very important because it offers the potential to provide a therapeutic benefit, but in certain instances it could account for detrimental results such as side effects, through either activation or inhibition of different signalling cascades. The clinical relevance of this is clear, because patients that suffer from cardiovascular disease (CVD), such as heart failure often receive combinational therapies that target the adrenergic and renin-angiotensin-aldosterone systems (RAS). The use of βAR blockers to treat heart failure on their own has been shown to decrease mortality rates in patients[81]. However, AT1aR blockers prescribed on their own will not decrease mortality in patients that experience heart failure[82]. The Valsartan Heart Failure Trial was a large study that investigated the effect of combinational therapies in patients that experienced heart failure. In this study, 92% of the patients were already taking an angiotensin converting enzyme (ACE) inhibitor, 85% were taking a diuretic, and some were also taking digoxin and/or βAR blockers. Patients were then given an AT1aR antagonist, valsartan, and a two year follow up showed that 19% of the patients who were taking combinational therapies had died[83]. It should also be noted that, in each class of drugs (AT1aR blockers, βAR blockers etc.) used in combinational therapies, there are a number of different therapeutics to choose from and that some combinations work better over others. The mechanism of GPCR dimer trans-inhibition could be used to explain increased morbidities associated with heart failure combinational therapy. Administration of βAR and AT1aR antagonists could completely render β2AR/AT1aR heterodimers unresponsive to any endogenous agonists, which could affect signalling cascades and contribute to a higher mortality rate.

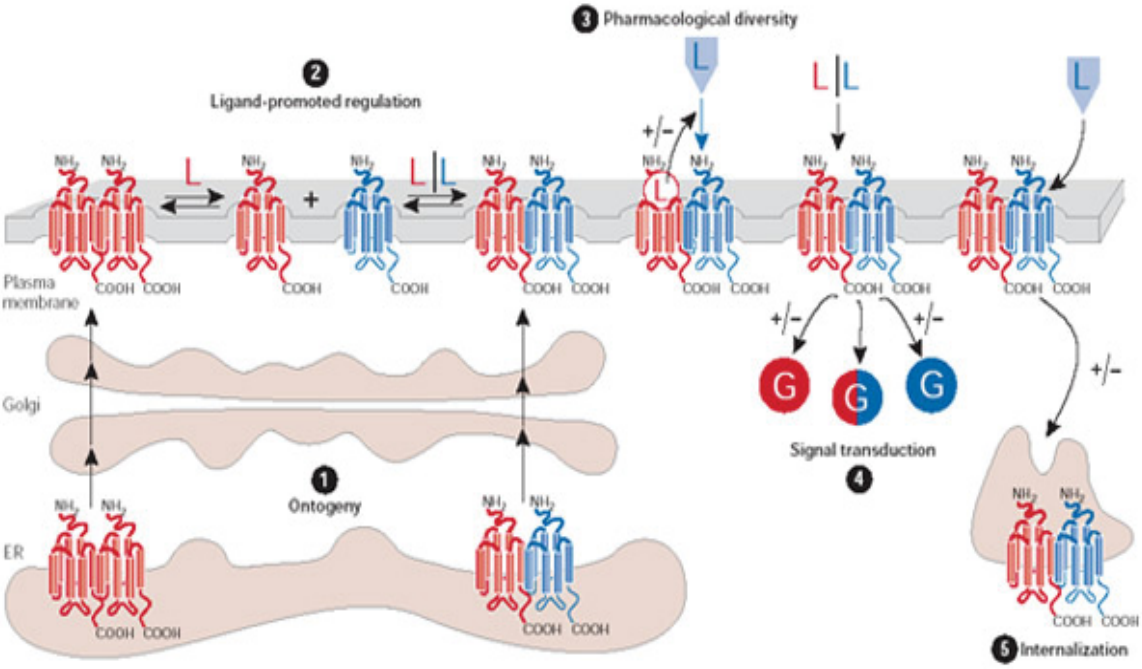



**Figure 1.5 The Role of Molecular Crosstalk in GPCR Dimer Lifecycles**
**1)** GPCR dimerization plays a prominent role in receptor PM localization, such as the case in the $GABA_B$ receptor. **2)** When expressed at the PM, GPCR dimers become targets for ligand binding and regulation. **3)** GPCR dimers can interact with each other, known as molecular crosstalk, which can lead to positive (+) and negative (-) binding cooperativities. **4)** Signal transduction can be relayed (+) or inhibited (-). **5)** Stimulation of a single protomer in a GPCR dimer is sufficient to internalize the whole receptor complex, or if one protomer in the GPCR is resistant to internalization, it can inhibit the entire receptor complex from internalization [Terrillon and Bouvier, *EMBO Reports* 2004; 5(1):30-4, with permission].

There is also mounting evidence that GPCR dimers are capable of binding to a variety of G-proteins during ligand activation. For example, the δ-OR/μ-OR heterodimer was found to couple to G-proteins that were not associated with the individual receptors themselves. When pertussis toxin, a bacterial toxin that prevents the coupling of $G\alpha_i$ to GPCRs through adenosine diphosphate (ADP) ribosylation, was administered to δ-OR/μ-OR heterodimers they were still capable of signalling to inhibit cAMP production, which

suggests a switch from the $G\alpha_i$ subunit to the $G\alpha_z$ subunit[69]. The switching in coupling of Gα subunits is also seen in D1R/D2R heterodimers, where the D2R receptor is usually coupled to $G\alpha_{i/o}$ but when D1R is activated it can switch and couple to $G\alpha_{q/11}$[71]. The formation of AT1aR/bradykinin receptor B2 (BB2) heterodimers was shown to increase the coupling of the AT1aR to $G\alpha_{q/i}$ in response to binding of its endogenous ligand angiotensin II[84]. GPCR dimerization has also been proposed to affect how receptor complexes are internalized following ligand activation. The βARs and AT1aR bind to arrestins in a similar manner during internalization, however, the formation of a βAR/AT1aR heterodimer is thought to affect how the receptor complex is internalized and recycled. The formation of β2AR/δ-OR heterodimers have been shown to internalize in response to isoproterenol, an agonist of the βARs, but internalization did not occur when β2AR/κ-OR heterodimers were exposed to isoproterenol[85]. It is clear that the association of GPCRs to form different receptor dimer pairings results in new avenues of signal activation and receptor trafficking. GPCR oligomerization can generate new pharmacological properties which makes them extremely attractive therapeutic targets.

### *1.10 Cardiovascular Disease*

CVD encompasses a wide variety of dysregulation that occurs in the heart and circulatory system. Examples of CVD include, but are not limited to ischemic disease, heart failure, hypertension, and atherosclerosis. CVD is a very serious condition, because the heart acts as the 'motor' of the body, responsible for ensuring proper distribution of blood throughout the body to distribute nutrients and perfuse various organ systems. With increasing lack of exercise, poor nutrition and diet, and sedentary lifestyles, CVD was the

second leading cause of death in Canada in 2008, responsible for 21% of all deaths[86]. There are two main systems in the body that are dysregulated in CVD, and these are the sympathetic nervous system (SNS) and the RAS. In various instances of CVD, both the SNS and RAS are activated in a compensatory manner to help restore cardiovascular function, which is detrimental to the body[87]. In the SNS, the βARs are activated and in the RAS it is the AT1aRs that are activated. Therapeutics that are used to treat CVD will often antagonize these receptors, and the most common classes of drugs used are the AT1aR blockers, βAR blockers, and ACE inhibitors (Figure 1.6). The use of a single class of antagonists has shown improvement in treating the symptoms of CVD, but in many cases patients will be prescribed a combinational therapy, which utilizes all three classes of blockers (AT1aR, βAR, ACE). As mentioned earlier, patients that are given certain combinational therapies have been shown to be at risk for increased mortality, and the underlying mechanisms of this are unknown[83].

The RAS functions to manage blood pressure and fluid homeostasis in the body and the effector of the RAS is angiotensin II. Angiotensin II controls fluid homeostasis by acting on the AT1aR to increase water and sodium reabsorption, or indirectly by promoting the release of aldosterone from adrenal glands[88]. The production of angiotensin II begins with the enzymatic cleavage of angiotensinogen by renin to form angiotensin I. Angiotensin I is then cleaved by ACE, to produce functional angiotensin II which can bind to its effectors, AT1R and angiotensin II type 2 receptor (AT2R). Angiotensin II can further be cleaved by aminopeptidase A to form angiotensin III, which can also bind to the AT1R and AT2R. Angiotensin III can be cleaved by aminopeptidase N to form angiotensin IV, which binds to its receptor in the brain, angiotensin II type 4

receptor (AT4R) (Figure 1.6a)[89]. The binding of angiotensin IV to AT4R is thought to affect memory formation. On the other hand, the SNS is the other system in CVD that is involved in cardiac homeostasis through enzymatic modulation of phenylalanine and tyrosine. After enzymatic modulation, phenylalanine and tyrosine will produce the catecholamines dopamine, norepinephrine, and epinephrine that can bind and activate the adrenergic receptors (Figure 1.6b).

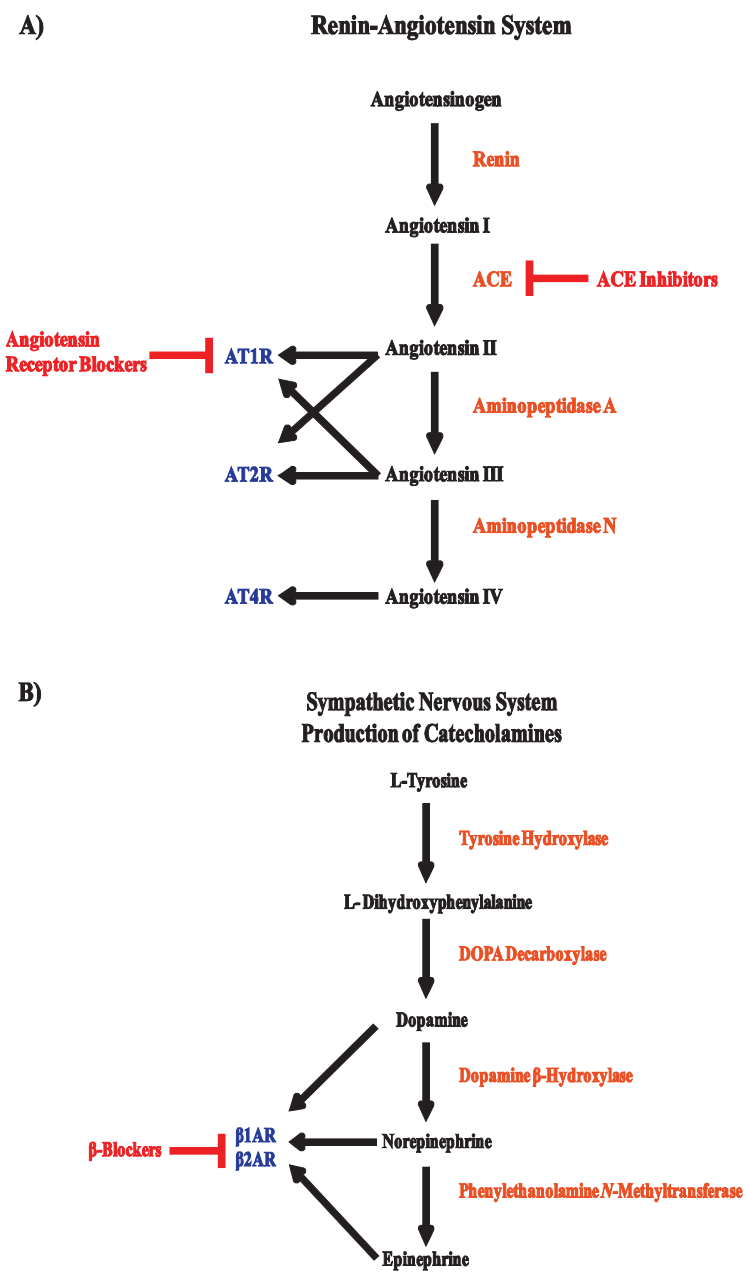



**Figure 1.6 The Role of The Renin-Angiotensin and Sympathetic Nervous Systems in Cardiovascular Disease**

**a)** The renin-angiotensin systems produces the effector molecular angiotensin II, which can bind to the AT1R and AT2R. In states of cardiovascular disease, ACE inhibitors and/or angiotensin receptor blockers are prescribed. **b)** The sympathetic nervous system produces catecholamines dopamine, norepinephrine, and epinephrine, which bind the adrenergic receptors. In states of cardiovascular disease, β-blockers are prescribed.

### *1.11 The RAS and the AT1aR*

The peptide hormone angiotensin II elicits its biological effect by binding to the AT1R and AT2R receptors which are part of the RAS. There are two subtypes of the AT1R receptor (AT1aR, AT1bR) and they are thought to be very similar in the signalling cascades that they activate and the ligands that they are capable of binding. However, the AT1aR receptor is the subtype that is predominantly expressed in various body tissues over the AT1bR, which seems to be expressed mainly in the adrenal and pituitary glands[90]. As well, the AT1aR is involved in maintaining vascular tone, sodium reabsorption, and binds to angiotensin II. Unlike the AT1R, the role of the AT2R is not well defined. Studies that examined the role of the AT2R demonstrated that when the receptor binds to angiotensin II, it decreases renal sodium reabsorption[91]. It is also interesting to note that the AT2R fails to recruit arrestins after it is stimulated with angiotensin II[92]. It has been observed that the AT1R and AT2R function in an opposing manner, but the underlying mechanism by which they do so is still not fully understood[93]. The AT1aR is widely distributed throughout the human body, in the brain, heart, peripheral vasculature, adipose tissue, pancreas, and kidney[88]. The binding of angiotensin II to the AT1aR elicits a variety of cellular effects such as vasoconstriction, cell proliferation, cell differentiation, vascular smooth muscle cell hypertrophy, and cardiovascular remodelling at higher levels of circulating angiotensin II[94]. The AT1aR is able to modulate intracellular cascades by coupling to the $G\alpha_{q/11}$, which can activate PLC. The activation of PLC produces second messengers DAG and IP3, which can then bind to its receptor (IP3R) on the sarcoplasmic reticulum. The binding of IP3 to the IP3R allows calcium to efflux into the cytoplasm, which results in smooth muscle contraction[95]

(Figure 1.7). The AT1aR has also been shown to signal by coupling to $G\alpha_i$, which inhibits AC and reduces cAMP production. Since cAMP is involved in activating downstream signalling pathways that are involved in smooth muscle contractions, decreases in cAMP levels will decrease vasoconstriction.

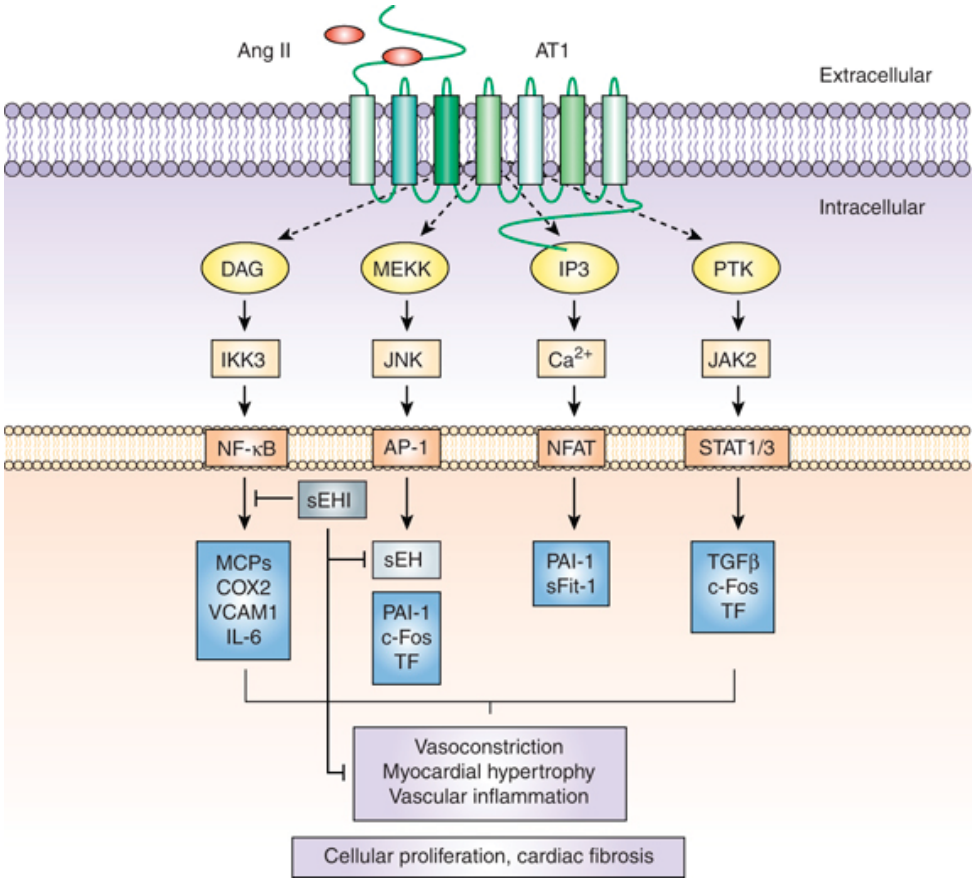



**Figure 1.7 Signalling Pathways Associated with Angiotensin II Binding to AT1aR**
The binding of the endogenous agonist angiotensin II to the AT1aR activates several signalling pathways such as DAG and IP3. This results in upregulation of transcriptional factors such as nuclear factor (NF)-κβ, activating protein 1 (AP-1), which results in the physiological effects of angiotensin II binding, such as vasoconstriction and vascular inflammation [Ai, Shyy, and Zhu, *Kidney International* 2010; 77(2):88-92, with permission].

### *1.12 The Adrenergic System and the β2AR*

The adrenergic system is composed of the α and β receptors, with two different subtypes of the α adrenergic receptors, α1AR (A, B, D) and α2AR (A, B, C), and the βARs (β1, β2, β3)[96, 97]. All of the adrenergic receptors will respond to the same type of circulating catecholamines; however, they will differ in the cellular response that they will elucidate. All three subtypes of the βAR are expressed in the heart at varying levels and are the main type of adrenergic receptors present, but only the α1AR subtype is expressed in the heart[98, 99]. The expression of the βARs throughout the body is rather ubiquitous, with the β1AR dominating in the heart and both the β1AR and β2AR being expressed in the kidney, responsible for stimulating renin release. The β2AR is also expressed in the peripheral vasculature, controlling vasodilation[100]. The signalling cascade of the β1AR and β2AR is well defined. After ligand activation, they can couple to $G\alpha_s$, and they can then activate AC, which then activates PKA. PKA activation is associated with phosphorylation of L-type calcium channels, which modulate cardiac and smooth muscle contractility through calcium influx[97]. However, the role of the β3AR is not well defined, and it is proposed to couple to $G\alpha_i$ and inhibit AC activity, which will then decrease contractility[101]. This theory is supported by a decrease in β1AR expression and an up-regulation of β3AR expression in states of CVD. The β2AR is capable of switching the G-protein it is coupled to. For example, the β2AR can couple to $G\alpha_s$ or it can switch to couple to $G\alpha_i$, which inhibits AC but activates the mitogen-activated protein kinase (MAPK) through the Gβγ subunits in a PKA dependent manner to increase contractility[102]. While the β1AR is the predominantly expressed receptor in the failing and non-failing heart, when it is over-expressed in the myocardium of mice, it was shown

to cause hypertrophy and fibrosis and led to cardiovascular dysfunction[103]. However, studies have shown that β2AR overexpression can enhance cardiac function without causing insult to the cardiovascular system[97]. It is clear that there is a fine balance of βAR receptor expression, specifically between the β1AR and β2AR in maintaining proper cardiovascular function.

### *1.13 The Consequences of the β2ART164I Polymorphism*

As with any protein that is expressed in the body, there are instances of error that can occur during protein transcription and synthesis. These errors can have a range of effects such as the formation of a non functional protein, or more extreme instances can result in the death of an organism. In the adrenergic system, both the β1AR and β2AR are capable of forming single nucleotide polymorphisms (SNP), which affect cardiovascular function. In the β2AR, there have been over 80 polymorphisms that have been identified and 45 of these were found to exist as SNPs[104]. In this research project, we are interested in a SNP that occurs in the fourth TM domain, at codon 164 where the polar residue, threonine, is replaced by a non polar residue isoleucine (β2ART164I) (Figure 1.8). The β2ART164I allelic frequency has been shown to occur between 0.5% - 5%, which is relatively low[105, 106]. The β2ART164I only occurs in a heterozygous form, as the homozygous form has never been observed and is assumed to be lethal. In a clinical study performed on patients that had ischemic and/or idiopathic cardiomyopathies or were healthy individuals, patients that were healthy were found to have a heterozygous β2ART164I allelic frequency of 2.9% compared to patients experiencing heart failure whose β2ART164I heterozygous allelic frequency was 3.9%[106]. There is also no

difference in the occurrence of the β2ART164I allelic frequency between male and females, and prior medical history such as smoking, diabetes, and hypertension was determined not to play a role in affecting the allelic frequency[107]. In terms of patients that harbour the β2ART164I SNP, they are not faced with an increased risk of developing cardiovascular disease. However, it has been shown that patients who possess the β2ART164I SNP and develop CVD will have a more aggressive prognosis. Following CVD diagnosis, patients harboring the β2ART164I SNP were shown to have a 42% one year survival rate, whereas their WT β2AR counterparts experienced a 76% one year survival rate following CVD diagnosis[106, 107].

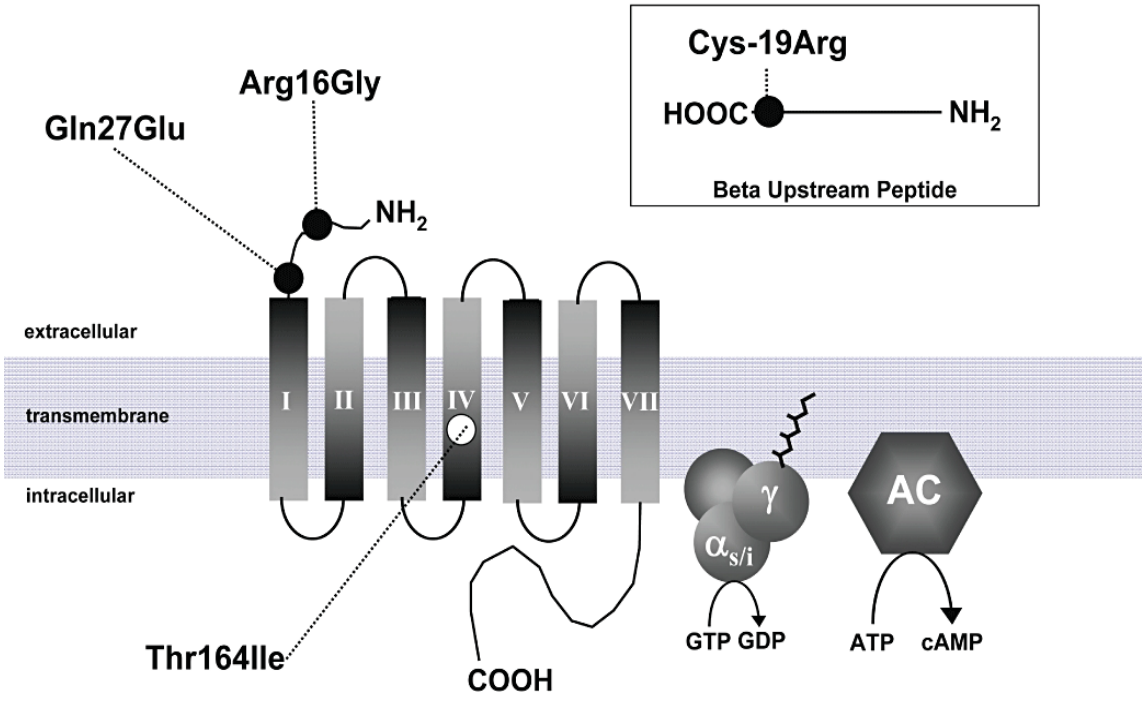



**Figure 1.8 The β2ART164I Single Nucleotide Polymorphism**
The β2AR is polymorphic at a variety of locations, which can be seen above. The β2ART164I polymorphism occurs in the fourth TM domain and affects the coupling to the $G\alpha_s$ subunit of the heterotrimeric G-protein. The β2ART164I also decreases ligand binding of both agonists and antagonists [Leineweber and Heusch, *British Journal of Pharmacology* 2009; 158(1):61-9, with permission].

The molecular affects of the β2ART164I SNP are quite numerous. The β2ART164I shows a decreased affinity to endogenous ligands such as epinephrine and norepenephrine as well as the βAR agonist, isoproterenol[108]. Also, the β2ART164I exhibits decreased basal and agonist stimulated levels of AC compared to the WT β2AR. When expressed in the heart, it also exhibits a reduction in internalization following agonist stimulation that is due to a decrease in coupling to the stimulatory $G\alpha_s$ subunit, which affects the contractility of the heart [109]. In terms of an *in vivo* affect, the β2ART164I SNP affects heart rate and contractility by blunting cardiomyocyte responsiveness to catecholamines and also promotes vasodilation, impairing vascular tone to that of vasoconstriction[107, 110]. It is also important to recognize that the β2ART164I downregulates its expression levels compared to that of the WT β2AR[108]. The clinical significance of the β2ART164I SNP is very serious in patients that develop CVD. To further complicate the matter, the β2ART164I presents decreased affinities for some βAR antagonists such as propanol and ICI 118, 551, which are used to treat CVD[108]. However, given that the allelic frequency of the β2ART164I is so low, there has not been a lot of investigation into the signalling pathways that are associated with this SNP and how dimerization with another WT β2AR or AT1aR may affect its ability to respond to ligands. It is also important to recognize, as mentioned earlier, that the β1AR is down regulated in CVD, and the heart is thought to become more dependent on the β2ARs to mediate catecholamine induced function. Thus, a dysfunctional β2AR such as the β2ART164I may not provide sufficient levels of function to maintain cardiac contractility, contributing to higher levels of mortality in patients that develop CVD. Given these details, the β2ART164I presents a perfect opportunity to investigate

pharmacogenomics, which determines how to direct therapeutic treatments towards patients with specific genetic variations. For example, if a patient harbored the heterozygous β2ART164I SNP and they were given a βAR blocker and were unresponsive, studies like the one performed in this thesis will provide the groundwork to determine another therapeutic to use which will be more responsive.

### *1.14 New Ideas About GPCR Dimerization*

The classical view of GPCR ligand interactions was that a single monomeric receptor was present at the PM, and a ligand could bind to it and activate a variety of signaling cascades. However, significant research has been conducted concerning the formation of GPCR homo- and heterodimers in many different receptors subtypes involved various systems of the human body, such as the A2AR, δ,κ,u-ORs, D1R, D2R, somatostatin receptor 5 (SSTR5), C-C motif chemokine receptor 2 (CCR2), CCR5, β2AR and AT1R[56, 69, 71, 111, 112]. However, there is still much to be learned about dimeric GPCRs and how they bind to ligands and activate signalling cascades. Since GPCRs are capable of signal transduction using G-protein dependent and independent pathways, it is important to examine the role of dimerization in homo- and heterodimers of the β2AR and AT1aR and how they will affect the activation of signalling cascades, since both receptors are targeted in CVD. That is why a variety of βAR and AT1aR agonists and antagonists are being investigated, so that we can see how different ligands will elicit specific effects depending upon the GPCR dimers that they will bind to. In order to determine the molecular signalling events that occur in GPCRs, it is important to look at

the PM localization of the dimers to determine trafficking that occurs following ligand binding. This includes the internalization of the homo- and heterodimer pairings, along with the recruitment of the non-visual arrestins. These proteins play an important role in regulating the signalling events that occur, and understanding what role each plays in response to different ligand binding will further our knowledge about GPCR dimerization and how we can apply this to a clinical setting to allow the treatment of disease states where dimerization plays a role. To further complicate the matter, we are also interested in observing what affect the β2ART164I SNP will play in the formation of heterodimers with the β2AR and AT1aR. We want to determine if the formation of heterodimers with WT β2AR and AT1aR receptors is sufficient to help rescue the β2ART164I SNP, which would have clinical applications in how therapeutics would be prescribed to treat CVD in patients that harbored the β2ART164I SNP. A better understanding of the basic molecular interactions that occur between GPCR homo- and heterodimers and ligands could lead to the development of novel therapeutics, which target dimer specific receptors. By investigating currently used agonists and antagonists of the β2AR and AT1R, this will give insight into the mechanisms behind signal transduction, which would be beneficial to help eliminate unwanted drug side effects, which are often the result of aberrant signalling.

### *1.15 Objectives*

This research project is broken into two main Objectives:

1. Examine the effect of different βAR and AT1aR ligands (agonists & antagonists) on homo/heterodimeric receptors in both the β2AR and AT1aR.

2. Determine the effect of a naturally occurring β2AR polymorphism (T164I) on homo/heterodimeric receptor βAR and AT1aR ligand binding in both the β2AR and AT1aR.

# Chapter 2: Materials and Methods

## *2.1 Reagents*

Reagents used were obtained from the following sources: Heat Inactivated Fetal Bovine Serum (FBS) and Penicillin-Streptomycin (PS) were from Invitrogen (Etobicoke, ON, Canada). Polyethylenimine Linear (PEI) was purchased from Polysciences, Inc. (Warrington, PA, United States of America). Dulbecco's Modified Eagle's Medium High Glucose (DMEM), Protein A-Sepharose, (−)-Isoproterenol hydrochloride, Salbutamol, ICI 118, 551, Angiotensin II human, [$Sar^1$, $Val^5$, $Ala^8$]-Angiotensin II acetate salt hydrate (Saralasin), monoclonal anti-Green Fluorescent Protein (GFP), and all other chemicals, unless otherwise noted, were from Sigma-Aldrich (Oakville, ON, Canada). EZ-Link Sulfo-NHS-LC-Biotin and Streptavidin Agarose Resin were from Thermo Scientific (Rockford, IL, United States of America). Complete EDTA-free cocktail protease inhibitor tablets were purchased from Roche Applied Science (Laval, QC, Canada). Polyclonal anti-GFP, monoclonal anti-p-ERK, and all horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, United States of America). Bovine serum albumin was from Bio Basic Inc. (Markham, ON, Canada). Coelenterazine-*h*, polyclonal anti-ERK, monoclonal anti-HA, and polyclonal anti-glu-glu epitope (EE) primary antibodies were from Cedarlane Labs (Hornby, ON, Canada).

### *2.2 Molecular Constructs*

The previously characterized constructs were used for this project: pcDNA3.1 AT1aR-venus 1, pcDNA3.1 AT1aR-venus 2, pcDNA3.1 β2AR-venus 1, pcDNA3.1 β2AR-venus 2, pcDNA3.1 $G\gamma_2$ and pcDNA3.1 HA-arr2[79, 113, 114]. pcDNA3.1 $G\gamma_2$ and pcDNA3.1 HA-$G\alpha_s$ were obtained as described earlier[115]. pcDNA3.1 Flag-$G\beta_1$, pcDNA3.1 $G\alpha_i$-EE, pcDNA3.1 $G\alpha_q$-EE were obtained from the UMR cDNA resource center. β2ART164I-venus1 was cloned using a two step polymerase chain reaction (PCR). The β2ART164I FWD oligonucleotide (5'GGATTGTGTCAGGCCTTATCTCCTTCTTGCCCATT-3') and BGH RVS primer (5'-TAGAAGGCACAGTCGAGG-3') were used to amplify part of the receptor, while the T7 FWD primer (5'-TAATACGACTCACTATAGGG-3') and β2ART164I RVS oligonucleotide (5'-AATGGGCAAGAAGGAGATAAGGCCTGACACAATCC-3') were used to amplify the remaining receptor portion. The two PCR products were purified, annealed, filled, and a second round of PCR was performed using T7 and BGH primers. The β2ART164I PCR product was then cloned into a pcDNA3.1 vector that contained venus 1 using NheI-ClaI restriction endonucleases. Bi-directional sequencing was used to confirm that proper construct orientation was achieved. The following constructs were generously provided by Dr. Michel Bouvier (Université de Montréal): pcDNA3.1 arrestin-2-*R*lucII, pcDNA3.1 $G\alpha_s$67-*R*lucII, pcDNA3.1 $G\alpha_{i1}$91-*R*lucII, and pcDNA3.1 $G\alpha_q$118-*R*lucII. The receptors studied (AT1aR, β2AR, β2ART164I) were expressed in a pcDNA 3.1 vector that contained either an N-terminal fragment (venus 1, amino acids 1-157) or C-terminal fragment (venus 2, amino acids 158-238) of a yellow fluorescent protein (YFP), venus, as previously described[79]. When either fragment of the

venus protein is expressed on its own, they remain non-functional and do not fluoresce when exposed to light or bind to an anti-GFP antibody. However, when two receptor pairings dimerize independently of the venus tags, it allows the venus fragments to come into close proximity, reconstituting the full length functional venus fluorescent protein that is detectable by an anti-GFP antibody (Figure 2.1). Receptor construct expression levels were confirmed using Western Blot analysis of HEK293A total cell lysates.

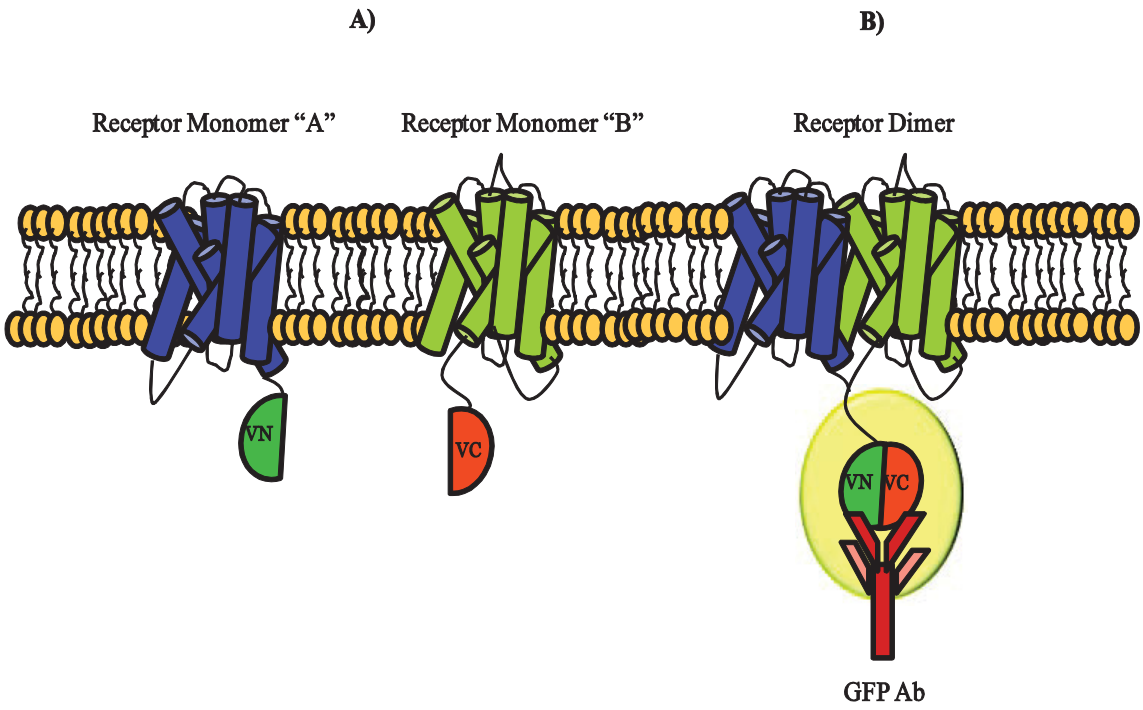



**Figure 2.1 Bimolecular Fluorescence Complementation (BiFC)**
**a)** Each receptor is tagged with either an N-terminal (VN, amino acids 1-157) or C-terminal (VC, amino acids 158-238) fragment of the YFP variant, venus. The venus1/venus2 fragments when expressed alone are non-functional and do not fluoresce or bind the anti-GFP antibody. **b)** If the receptor pairings dimerize independently of the venus tags, it allows the venus fragments to come into close proximity, reconstituting the full length functional venus fluorescent protein which is detectable by an anti-GFP antibody.

### *2.3 Cell Culture and Transfection*

HEK293A cells were cultured using DMEM supplemented with 10% heat inactivated FBS and 2% penicillin-streptomycin in 100 $mm^2$ cell culture plates at 37°C and 5% $CO_2$. Cells were passaged when they were 70-80% confluent. For experiments, HEK293A cells were plated in 6-well plates and were transfected with each cDNA using PEI, with a ratio of 3:1 PEI to DNA. 24 hours post transfection, cell culture media was changed to DMEM only, and all experiments were performed 48 hours post transfection at 37°C and 5% $CO_2$.

### *2.4 Biotin-Streptavidin Cell Surface Assay*

The biotin-streptavidin cell surface assay was used to isolate cell surface proteins from a heterogeneous cell lysate, due to the high affinity binding of streptavidin to biotin. Whole HEK293A cells were labeled with EZ-Link Sulfo-NHS-Biotin, a water soluble long-chain ester that forms stable bonds with primary amines, which is not membrane permeable. Proteins have several primary amines that can be located on side chains of lysine residues and also at the N-terminal of the polypeptide itself. Due to the relatively small size of biotin and a long flexible spacer, steric hindrance is minimized, allowing for conjugation to primary amines while maintaining biological activity. Cells are then lysed and streptavidin-agarose beads are added to the whole cell lysate. Streptavidin and biotin form a high affinity non-covalent interaction, which allows for biotinylated cell surface proteins in the cell lysate to bind to the streptavidin-agarose beads. The biotin-streptavidin conjugate can be washed several times in order to eliminate any proteins that

were not bound to biotin. Cell surface proteins can be purified using Western Blot analysis by disrupting the bond between streptavidin and biotin bound proteins by adding dithiothreitol (DTT), which will release the biotin bound polypeptide (Figure 2.2).

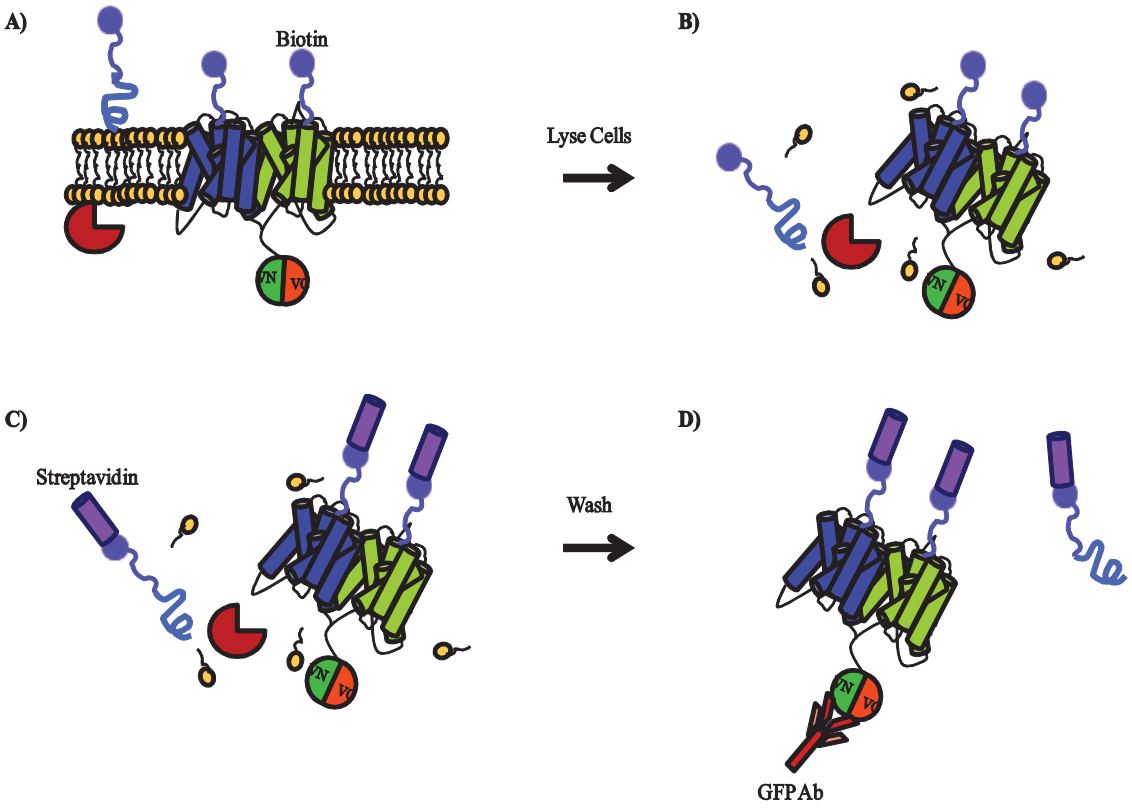



**Figure 2.2 Biotin-Streptavidin Cell Surface Assay**
**a)** Following ligand stimulation, whole HEK293A cells were treated with EZ-Link Sulfo-NHS-Biotin (purple), a water soluble long-chain ester that forms stable bonds with primary amines on the cell surface (blue and green), which is not membrane permeable. **b)** Biotinylated proteins are lysed with RIPA buffer. **c)** Streptavidin-agarose beads (pink) are added to the whole cell lysate, and will bind to biotinylated proteins. **d)** The biotin-streptavidin conjugated proteins are washed to eliminate proteins that are not biotinylated, and the cell surface biotinylated proteins can be isolated using an anti-GFP antibody (brown) via western blot analysis.

HEK293A cells that expressed a receptor dimer pairing (2 μg of each receptor AT1aR, β2AR, β2ART164I) were treated with βAR or AT1aR ligands and washed with 1X cold phosphate buffered saline (PBS) 48 hours post transfection. Cells were then resuspended in 0.9 mM EZ-Link Sulfo-NHS-Biotin and incubated at 4°C for 30 minutes. Cells were then washed twice with cold 1X PBS + 100mM glycine, quenching any unbound biotin. To lyse the cells, radioimmune precipitation assay (RIPA) buffer (50mM Tris, pH 7.5, 10mM $MgCl_2$, 150mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, complete protease inhibitors) was used along with 25 μL of Protein A-Sepharose beads containing bovine serum albumin (BSA), 2 μL of DNase I, and was then incubated at 4°C for 30 minutes. Whole cell lysates were obtained by centrifugation at 13,200 RPM and were incubated with 75 μL of streptavidin-agarose resin overnight at 4°C. Samples were then washed three times with cold RIPA buffer and then incubated in 2.8M DTT-Laemmli sample buffer for one hour at room temperature, eluting bound proteins. Western Blot analysis was then performed and immunoblots were probed with an anti-GFP antibody (1:2000) and a horseradish peroxidase-conjugated secondary antibody (anti-rabbit, 1:5000) was used to detect receptor cell surface expression levels.

### *2.5 Immunoprecipitation Assays*

HEK293A cells that expressed a receptor dimer pairing (AT1aR, β2AR, β2ART164I) along with arrestin-2 were treated with a ligand and washed with 1X cold PBS 48 hours post transfection. To lyse the cells, RIPA buffer was used along with 25 μL of Protein A-Sepharose beads containing BSA, 2 μL of EDTA and DNase I, and was

incubated at 4°C for 30 minutes. Whole cell lysates were then obtained by centrifugation at 13,200 RPM and were incubated with a polyclonal anti-GFP primary antibody or monoclonal anti-GFP primary antibody (1:2000) for thirty minutes at 4°C depending on the experiment. 50 µL of Protein A-Sepharose beads were then added to the samples and incubated overnight at 4°C. Samples were then washed three times with cold RIPA buffer and incubated in 710 mM β-mercaptoethanol-Laemmli sample buffer for 20 minutes at room temperature, eluting bound proteins. For experiments quantifying p-ERK, where immunoprecipitation was not required, whole cell lysates were mixed with 710 mM β-mercaptoethanol-Laemmli sample buffer and resolved using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to determine protein levels via Western Blot analysis with an anti-p-ERK (1:500) primary antibody and then an anti-ERK (1:2000) primary antibody. For experiments that were observing the Gα subunits of the heterotrimeric G-protein, anti-HA (HA-$G\alpha_s$), anti-Glu-Glu ($G\alpha_i$-EE, and $G\alpha_q$-EE) primary antibodies were used (1:2000) to immunoprecipitate the receptor signalling effector complex.

### *2.6 Bioluminescence Resonance Energy Transfer (BRET) Assays*

HEK293A cells that expressed a receptor dimer pairing (1.5 µg of each receptor AT1aR, β2AR, β2ART164I) along with 0.5 µg of $G\beta_1$, Gγ2 and the appropriate Gα subunit ($G\alpha_{i1}$91-*R*lucII, $G\alpha_q$118-*R*lucII, $G\alpha_s$67-*R*lucII) were treated with a ligand and washed with 1X cold PBS 48 hours post transfection for experiments that observed heterotrimeric G-protein interaction with the receptor dimers. For experiments looking at

arrestin-2 recruitment following ligand treatment, HEK293A cells expressed a receptor dimer pairing (1.5 μg of each receptor AT1aR, β2AR, β2ART164I) along with 0.5 μg of arrestin-2-*R*lucII. *Renilla* luciferase II (*R*lucII), a variant of *Renilla* luciferase, catalyzes the oxidation of its substrate, coelenterazine, using oxygen which creates light emission or a source of energy. The receptor pairings themselves form a functional variant of a YFP, venus, which acts as an energy acceptor from *R*lucII, causing excitation and light emission from venus if the donor and acceptor are in close proximity (Figure 2.3). Cells were then resuspended in 90 μL of 1X PBS and placed into 96-well microplates (White Optiplate; PerkinElmer Life Sciences). All of the experiments that were carried out using BRET assays were done so using BRET[1], which uses coelenterazine *h* as the substrate at a concentration of 5μM and the YFP variant, venus, as the energy acceptor. Protein interactions were monitored using a Luminoskan Ascent Microplate Luminometer (Thermo Scientific) to measure donor peak excitations (405 nm, Bandwidth 30 nm) and acceptor peak excitations (510 nm, Bandwidth 30 nm) with the appropriate filters. To determine whether protein-protein interactions were occurring, the BRET ratio was calculated from the emission of light from *R*lucII, passing through the 405 nm filter to the emission of light from venus, which passed through the 510 nm filter. In order to ensure that expression levels of *R*lucII and venus were maintained at constant levels, transfection efficiencies were kept consistent in order to avoid any variation or fluctuation in the expression of *R*lucII and venus. Negative controls used consisted of the receptor dimer pairings fused to venus, co-expressed with *R*lucII, since the receptor dimer pairings and *R*lucII do not interact physiologically.

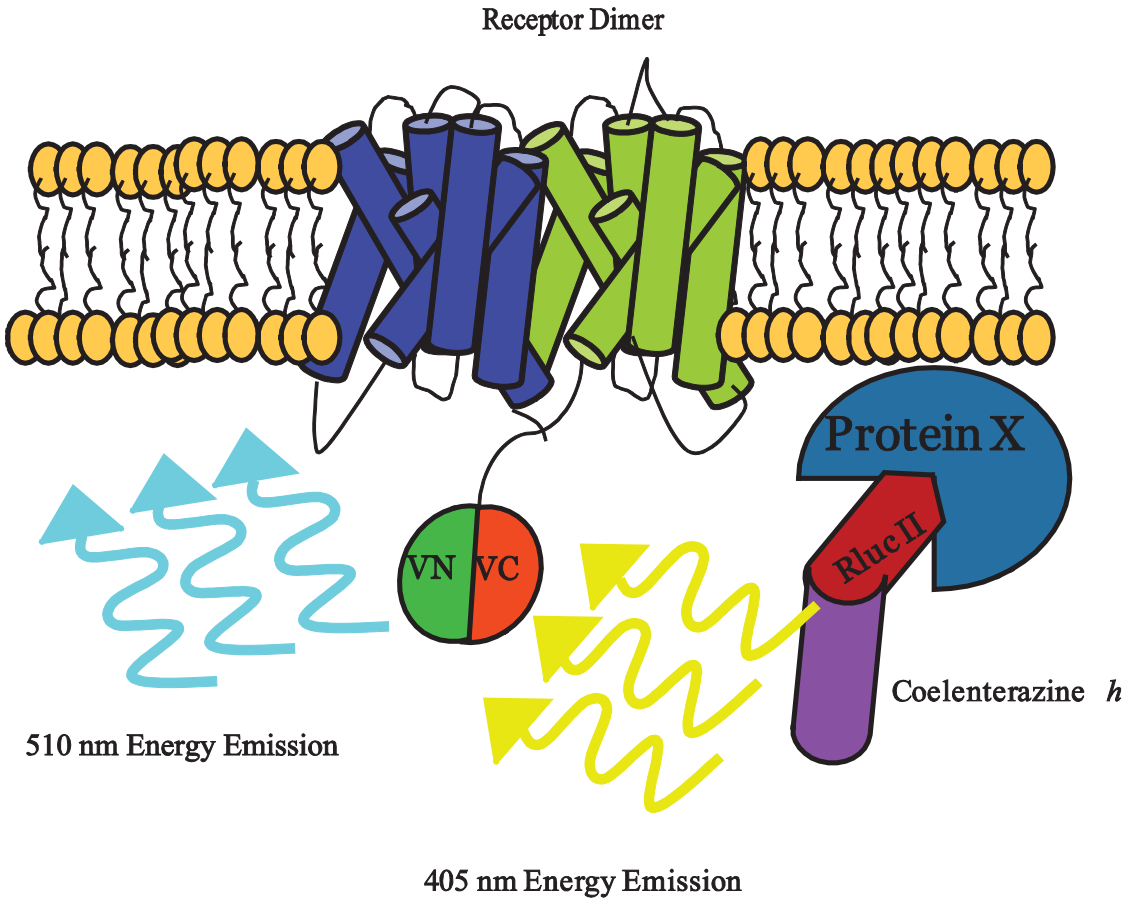



**Figure 2.3 Bioluminescence Resonance Energy Transfer Assay**
The receptor dimer pairings form a functional variant of the YFP, venus (green and orange), which acts as an energy acceptor from *R*lucII (red). *R*lucII catalyzes the oxidation of the substrate, coelenterazine *h* (purple), using oxygen which creates a source of energy (405 nm emission). If venus and *R*lucII are in close proximity, the energy can be transferred to venus and then emitted (510nm emission) and read using a Luminometer.

### *2.7 Analysis and Quantification of Western Blots*

Western blots were quantified using densitometry with the ImageJ[116] software package. Western blot bands were individually selected to determine their respective image densities. Non-stimulated (NS) cells were then normalized to 100% and ligand treated cells were then compared to NS cells for any observable differences. All western blots performed were also normalized to a loading control, which was obtained by harvesting 15uL of the whole cell lysate and blotting against the appropriate protein.

### *2.8 Statistical Analysis*

Statistical testing was performed using a two-tailed unpaired Student's t-test. All figures are representative of the mean ± SEM (error bars) of at least three independent experiments. *$p<0.05$; **$p<0.01$; ***$p<0.001$ for ligand treated cells compared to NS cells.

# Chapter 3: Results

### *3.1 Expression of cDNA Constructs in HEK293A Cell Line*

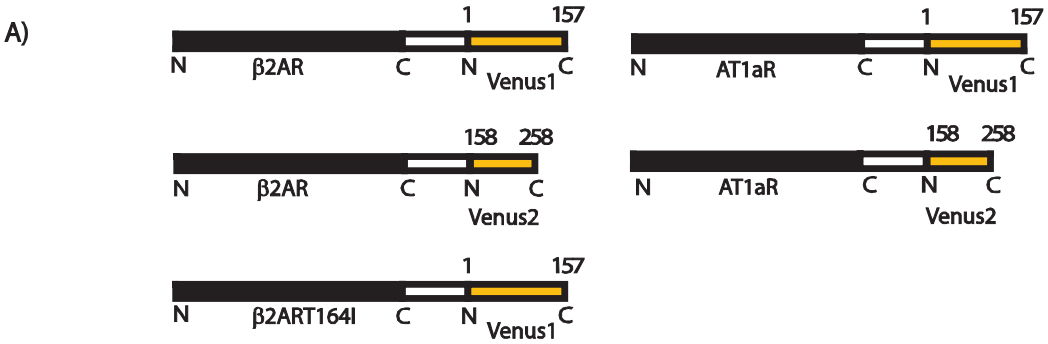
The cell line that we chose to perform our *in vitro* studies was HEK293A; one that is commonly used in molecular biology because it is easily transfectable, allowing the expression of recombinant vectors containing proteins of interest. HEK293A cells are also relatively inexpensive and are able to be used in high throughput studies, permitting rapid data collection. Initially, we wanted to validate that our constructs containing our receptors of interest (Figure 3.1a) would be expressed in HEK293A cells to form functional homo- and heterodimers (Table 3.1). When the receptor constructs were paired as in Table 3.1, we were able to observe the dimerization of our specific receptor pairings using a GFP antibody in Western Blot immunoprecipitations, as the GFP antibody would only bind to the reconstituted venus-receptor complex (Figure 3.1b). Previous experiments performed in our lab optimized the receptor venus pairings that generated sufficient venus reconstitution. Some receptor pairings do not adopt the proper conformation to allow functional fluorescence complementation (i.e. AT1aR-venus1/β2AR-venus2) and will not be bound by the GFP antibody. Therefore, these receptor combinations were not used. The receptor dimer pairings were also visualized using fluorescent microscopy, which demonstrated their PM localization on whole HEK293A cells, using the GFP antibody to target the reconstituted venus (Figure 3.1c).

**Table 3.1 Homo- and Heterodimer Pairings of the β2AR, AT1aR, and β2ART164I**

| Receptor Construct Pairings | Dimer Formed |
|---|---|
| β2AR-venus1 + β2AR-venus2 | β2AR-venus1/β2AR-venus2 |
| AT1aR-venus1 + AT1aR-venus2 | AT1aR-venus1/AT1aR-venus2 |
| β2ART164I-venus1 + β2AR-venus2 | β2ART164I-venus1/β2AR-venus2 |
| β2AR-venus1 + AT1aR-venus2 | β2AR-venus1/AT1aR-venus2 |
| β2ART164I-venus1 + AT1aR-venus2 | β2AR T164I-venus1 /AT1aR-venus2 |

**Figure 3.1: Expression of cDNA constructs in HEK293A Cell Line**

A) HEK293A cells were transfected with β2AR-venus1, β2AR-venus2, β2ART164I-venus1, AT1aR-venus1, or AT1aR-venus2 cDNA constructs. B) 48 hours post-transfection, cells were harvested and washed with 1X PBS and lysed with RIPA buffer. Cell lysates were run on a 10% SDS-PAGE gel and then transferred to nitrocellulose membranes and probed with an anti-GFP antibody (1:2000). Only complementary dimer pairings were detectable. C) Fluorescent microscopy images of HEK293A cell PM localization of i) AT1aR-venus1/AT1aR-venus2, ii) β2AR-venus1/β2AR -venus2, iii) β2AR-venus1/AT1aR-venus2, iv) β2ART164I-venus1/β2AR-venus2, v) β2ART164I-venus1/AT1aR-venus2. Results are representative of 3 independent experiments. Figures 3.1 B) i) and C) i), ii), and ii) from [Hammad and Dupré, *Journal of Molecular Signaling* 2010; 2010: 102(25):9050-5, open access].


A)
1
157
N
β2AR
C
N
Venus1
C
1
157
N
AT1aR
C
N
Venus1
C
158
258
N
β2AR
C
N
C
Venus2
158
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Venus1
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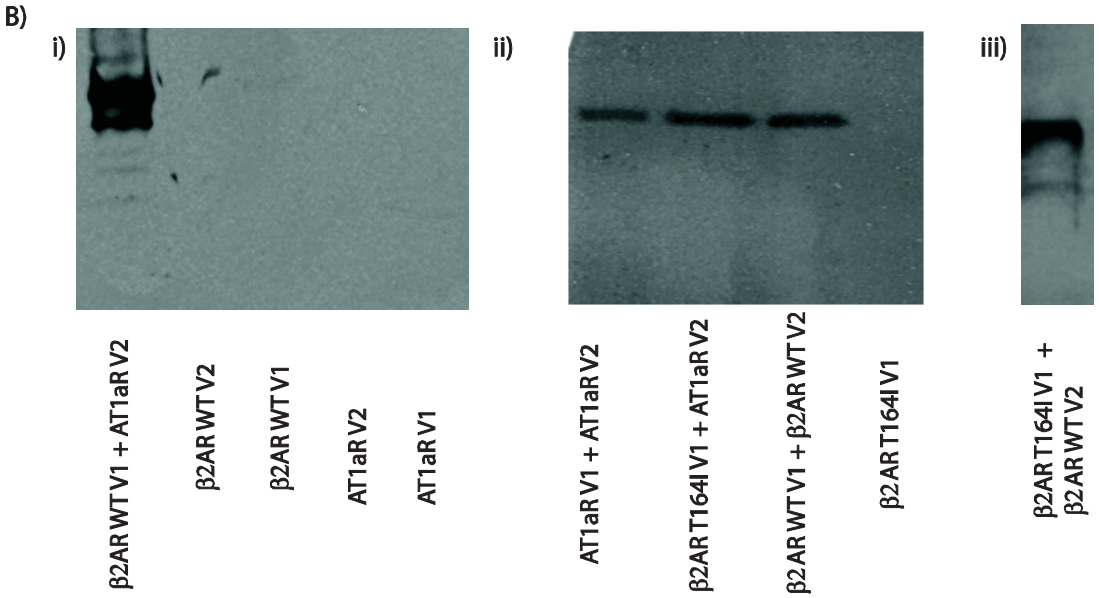
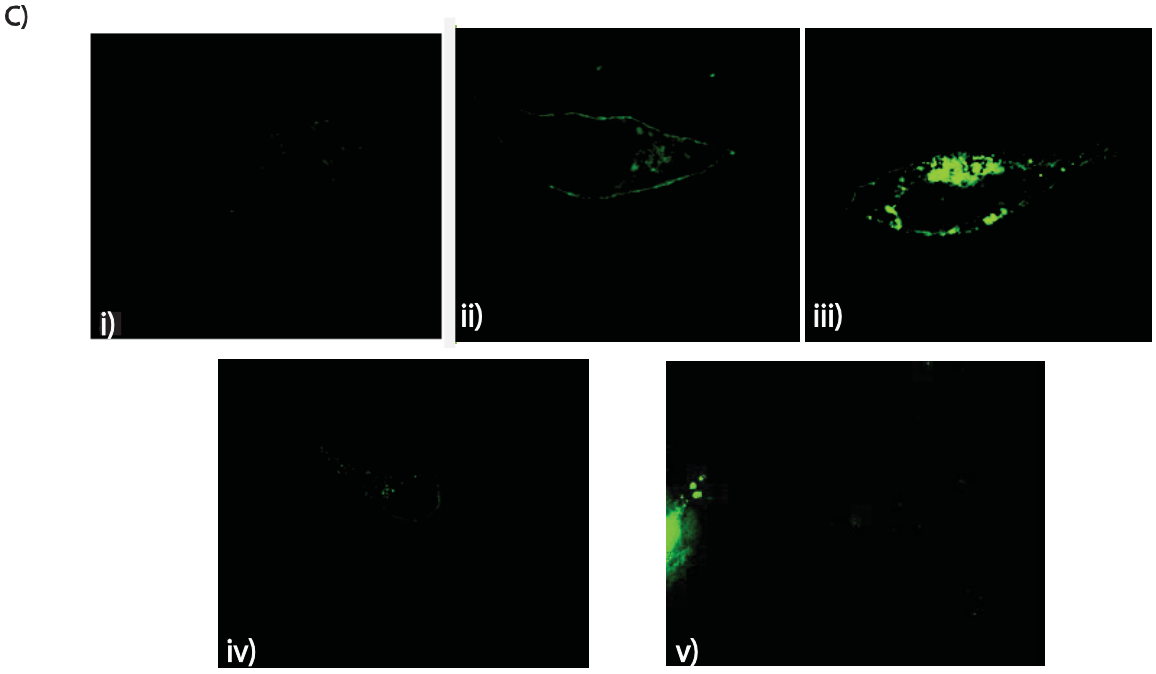
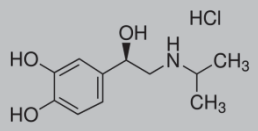
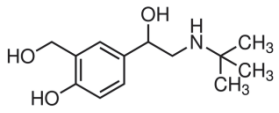
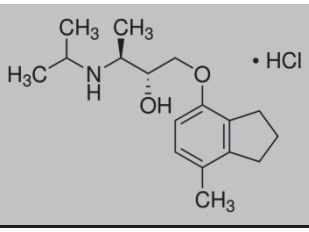

B)
i)
ii)
iii)
β2AR WT V1 + AT1aR V2
β2AR WT V2
β2AR WT V1
AT1aR V2
AT1aR V1
AT1aR V1 + AT1aR V2
β2AR T164I V1 + AT1aR V2
β2AR WT V1 + β2AR WT V2
β2AR T164I V1
β2AR T164I V1 +
β2AR WT V2


C)
i)
ii)
iii)
iv)
v)

### *3.2 The Effect of Isoproterenol, Salbutamol, and ICI 118, 551 Treatment on Plasma Membrane Localization of β2AR, β2ART164I, and AT1aR Homo- and Heterodimers*

It has been previously shown that the formation of β2AR and AT1aR heterodimers affects how the receptor complex can activate signalling pathways in response to treatment with agonists and antagonists of either receptor that is in the heterodimeric complex[56]. Thus, we were interested in determining trafficking events that occur following the treatment with βAR ligands in the β2AR, β2ART164I, and AT1aR homo- and heterodimer receptor pairings. The dimerization of a WT receptor with either a mutated or different receptor has been shown to affect the trafficking of the receptor complex following ligand binding. Furthermore, there is also evidence that occupancy of either protomer in a receptor dimer is sufficient to induce internalization of the entire dimeric complex[117]. Studies have also investigated the role of dimerization between a WT receptor and one that is not functional due to a mutation. The formation of a dimer consisting of a WT β2AR and a constitutively desensitized mutant β2AR (C341Gβ2AR) was shown to rescue the non-functional C341Gβ2AR, allowing it to regain its functionality when exposed to an agonist[118]. Thus, we chose to investigate the β2ART164I SNP because of its impaired ligand binding and ability to promote signal activation. We were interested in determining if the formation of dimers with WT β2AR and AT1aR would be able to restore regular function of the β2ART164I SNP. The βAR ligands used on the receptor dimer pairings were isoproterenol, salbutamol, and ICI 118,551 and their structure, classification, and function is illustrated in Table 3.2.

**Table 3.2 βAR Ligands used to Treat Receptor Dimer Pairings**

| Ligand | Structure | Classification | Function |
|---|---|---|---|
| Isoproterenol |  | βAR non-selective agonist | Can be used to treat bradycardia and asthma by acting on the β1AR and β2AR in a similar manner to catecholamines |
| Salbutamol |  | β2AR selective agonist | Used to treat bronchospasms in patients suffering from asthma. Acts on the β2AR causing bronchodilation |
| ICI 118,551 |  | β2AR selective antagonist | Although not used in clinical settings in humans, it is commonly used in research involving the β2AR |

HEK293A cells were transfected to express all of the receptor pairings described in Table 3.1, except the AT1aR homodimer, to determine the effect of the βAR ligands described in Table 3.2 on receptor PM localization after a one hour treatment. A previous study of the β2AR has shown that after a one hour exposure to an agonist, internalized β2ARs will start to recycle back to the PM, which is why we chose that treatment time[119]. In order to determine a working concentration of the β2AR agonists, dose response curves were produced using isoproterenol and salbutamol to measure the $EC_{50}$ for PM localization of the WT β2AR homodimer (Figure 3.2a). The $EC_{50}$ is the concentration where 50% of the maximal effect of an agonist can be observed. The $EC_{50}$ for isoproterenol was determined to be $1.7x10^{-7}$M and $3.2x10^{-8}$M for salbutamol. In order to ensure that a maximal response was obtained with each agonist, isoproterenol was used at

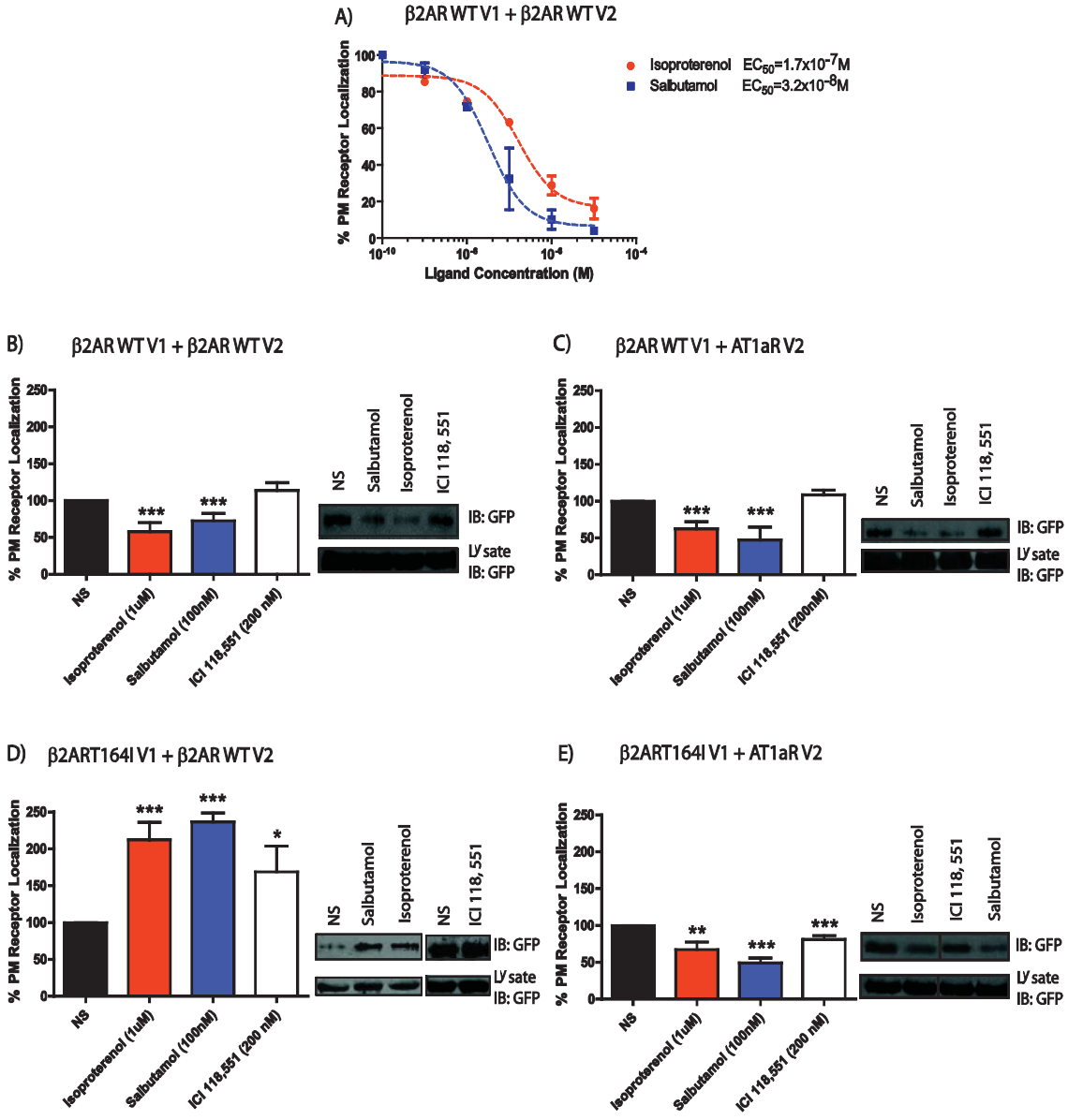
a concentration of $1.x10^{-6}$M and salbutamol at a concentration of $1.0x10^{-7}$M, which is a ~10 fold increase over both the determined $EC_{50}$ values. Dose response curves were not generated for ICI 118, 551 because differences in ligand concentration did not cause significant changes in PM receptor localization, and a concentration of $2.0x10^{-7}$M was used that was determined using reported literature $IC_{50}$ values for ICI 118,551 in HEK293 cells[120]. The $IC_{50}$ value represents a ligand concentration where 50% of the maximal inhibition is observed. In the WT β2AR homodimer, treatment with isoproterenol (42.3±12.5%) and salbutamol (34.9±9.4%) produced a significant decrease in PM localization, while treatment with ICI 118, 551 resulted in a non-significant increase in PM localization (5.4±10.5%) when compared to NS cells (Figure 3.2b). The effect of the βAR ligands on the β2AR WT/AT1aR heterodimer followed similar trends, with isoproterenol (37.5±9.7%) and salbutamol (52.8±17.6%) treatments resulting in significant decreases in PM localization (Figure 3.2c). ICI 118, 551 treatment did not result in any difference (8.7%±6.4%) in PM localization following ligand treatment (Figure 3.2c).

As mentioned earlier, we are interested in studying the β2ART164I SNP because it exhibits a decreased affinity to βAR agonists and antagonists, and we are using two βAR agonists and an antagonist in this study to characterize these interactions. The β2ART164I also exhibits a decrease in agonist induced receptor internalization, altering its trafficking following ligand exposure. Since the β2ART164I has impaired function when expressed on its own, we wanted to determine how expressing the β2ART164I in dimers with the WT β2AR and AT1aR would influence the signalling and trafficking of heterodimeric complexes. To our knowledge, this is something that has never been done

before. PM localization of the β2ART164I/β2AR WT heterodimer following βAR ligand treatment was examined. Isoproterenol (112.5±23.8%), salbutamol (136.8±11.8%), and ICI 118, 551 (68.9±34.9%) treatments resulted in a significant increase in PM localization (Figure 3.2d). When examining β2ART164I/AT1aR heterodimer PM localization following βAR ligand treatment, incubation with isoproterenol (32.5±10.0%), salbutamol (50.7±6.6%), and ICI 118, 551 (18.7±5.1%) treatments all resulted in a significant decrease in PM localization (Figure 3.2e). This shows that PM localization does not change following βAR ligand treatment between the WT β2AR homodimer and the β2AR WT/AT1aR heterodimer. However, the formation of β2ART164I/β2AR WT heterodimers increases the PM localization following βAR ligand treatment, which suggests that receptor internalization may not be occurring properly and that receptor dimers being synthesized and transported to the PM accumulate faster than the rate at which they are being internalized. When β2ART164I/AT1aR heterodimers are formed, the PM localization following βAR ligand treatment seems to mimic that of the β2AR WT/AT1aR heterodimer. This suggests that the AT1aR may result in a structural interaction with the β2ART164I receptor when dimers are formed, allowing receptor internalization to occur. Another possibility is that βAR ligands are able to elicit their effect by acting on the AT1aR. It should be noted that NS cells were normalized to 100% in each graph, and ligand treated cells represent a percentage of PM localization in comparison to their NS equivalent.

**Figure 3.2: The Effect of βAR Ligand Treatments on Plasma Membrane Localization of β2AR, βART164I, and AT1aR Homo- and Heterodimers**

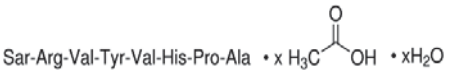
β2AR, β2ART164I, and AT1aR homo- and heterodimer PM localization following one hour 1μM isoproterenol, 100nM salbutamol, and 200nM ICI 118,551 treatments. Experiments were performed 48 hours post-transfection using a biotin-streptavidin cell surface assay to isolate proteins expressed on the PM. Following one hour ligand treatment, HEK293A cells were harvested and washed with 1X PBS and incubated with biotin for 30 minutes. Cells were then lysed with RIPA buffer and streptavidin was added. Samples were then run on a 10% SDS-PAGE gel, transferred to nitrocellulose membranes and probed using an anti-GFP antibody (1:2000). **A)** Dose response curves for isoproterenol and salbutamol in the β2AR WT homodimer. **B)** Effect of βAR ligand treatment on PM localization of the β2AR WT homodimer. **C)** Effect of βAR ligand treatment on PM localization of the β2AR WT/AT1aR heterodimer. **D)** Effect of βAR ligand treatment on PM localization of the β2ART164I/β2AR heterodimer. **E)** Effect of βAR ligand treatment on PM localization of the β2ART164I/AT1aR heterodimer. Results are representative of 3 independent experiments. *$p<0.05$; **$p<0.01$; ***$p<0.001$ using a two-tailed unpaired Student's t-test.


A)
β2AR WT V1 + β2AR WT V2
Isoproterenol EC50=1.7x10-7M
Salbutamol EC50=3.2x10-8M
% PM Receptor Localization
Ligand Concentration (M)
B)
β2AR WT V1 + β2AR WT V2
C)
β2AR WT V1 + AT1aR V2
D)
β2ART164I V1 + β2AR WT V2
E)
β2ART164I V1 + AT1aR V2
NS
Salbutamol
Isoproterenol
ICI 118, 551
IB: GFP
LY sate
IB: GFP
Isoproterenol (1uM)
Salbutamol (100nM)
ICI 118,551 (200 nM)

### *3.3 The Effect of Angiotensin II and Saralasin Treatment on Plasma Membrane Localization of β2AR, β2ART164I, and AT1aR Homo- and Heterodimers*

To investigate the function of the AT1aR in receptor dimers with the β2AR and β2ART164I, we used the AT1aR ligands angiotensin II and saralasin to evaluate their role in receptor dimer pharmacology. The structure, classification, and function of these ligands is illustrated in Table 3.3.

**Table 3.3 AT1aR Ligands used to Treat Receptor Dimer Pairings**

| Ligand | Structure | Classification | Function |
|---|---|---|---|
| Angiotensin II |  | AT1aR agonist | A potent vasoconstrictor of systemic circulation. Involved in aldosterone release. |
| Saralasin |  | AT1aR partial agonist | Used in clinical settings to manage hypertension |

HEK293A cells expressing AT1aR homodimers, β2AR WT/AT1aR or β2ART164I/AT1aR heterodimers were used to evaluate the effect of angiotensin II and saralasin on receptor PM localization. In order to determine a working concentration of the AT1aR agonist, angiotensin II, a dose response curve was generated using the AT1aR homodimer. The $EC_{50}$ for angiotensin II was found to be $1.0x10^{-8}$M, and a concentration of $1.0x10^{-6}$M was used to ensure that maximal response to the ligand would be achieved following one hour stimulation (Figure 3.3a). Saralasin was used at a concentration of $1.0x10^{-6}$M, which was found in prior literature that studied the AT1aR using an *in vitro*

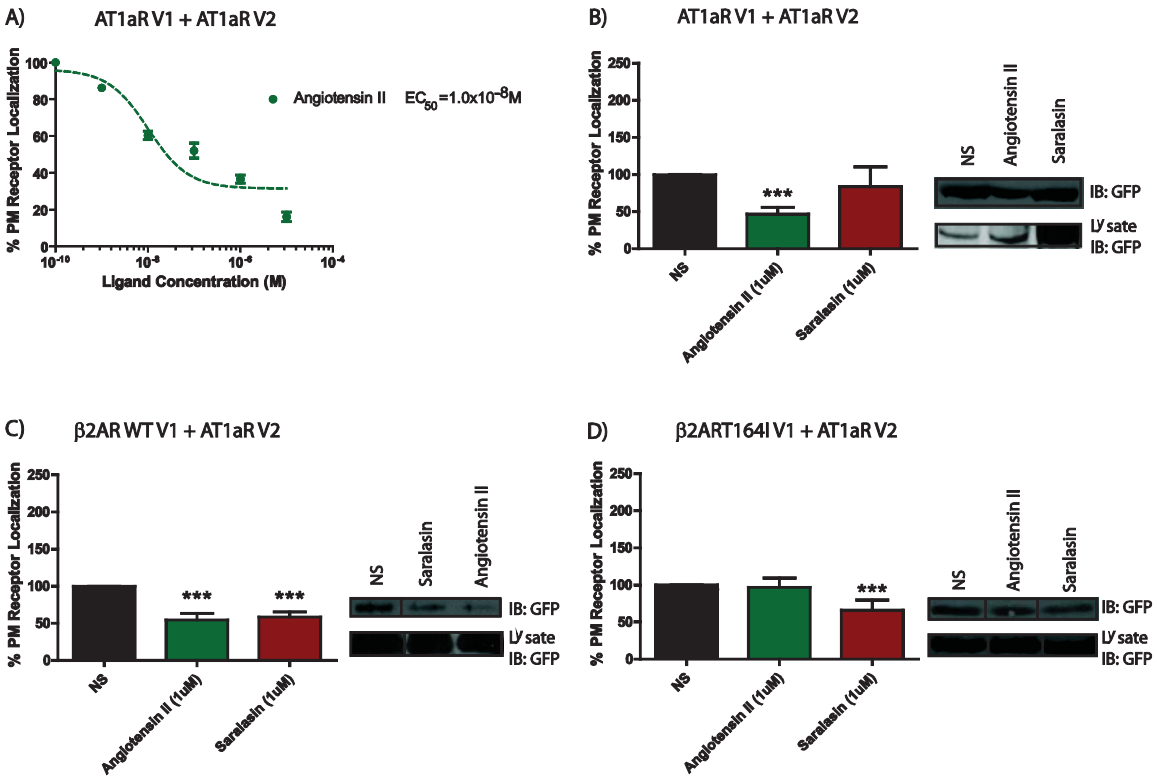
cell culture model[121]. In the AT1aR homodimer, treatment with angiotensin II produced a significant decrease (53.3±9.3%) in PM localization, whereas saralasin (16.4±26.7%) treatment did not significantly affect PM localization (Figure 3.3b). When looking at PM localization of the β2AR WT/AT1aR heterodimer following AT1aR ligand incubation, both angiotensin II (45.4±8.8%) and saralasin (41.6±7.1%) treatments resulted in significant decreases in PM localization (Figure 3.3c). However, the PM localization of the β2ART164I/AT1aR heterodimer did not change when treated with angiotensin II (3.2±12.5%) whereas saralasin (34.1±13.8%) treatment resulted in a significant decrease in PM localization (Figure 3.3d).

When comparing the AT1aR homodimer PM localization to that of the β2AR WT/AT1aR heterodimer following angiotensin II treatment, there is no difference that is observed. However, when the β2AR WT/AT1aR heterodimer is treated with saralasin it results in a decrease in PM localization, which is not observed in the AT1aR homodimer. The β2ART164I/AT1aR heterodimer PM localization is not affected when treated with angiotensin II, but there is a decrease in PM localization when treated with saralasin. What this suggests is that the β2AR and AT1aR protomers are interacting in the β2AR WT/AT1aR heterodimer that permits a response to treatments with angiotensin II and saralasin. When the mutant β2ART164I forms a heterodimer with the AT1aR, we see that the heterodimer is no longer able to respond to angiotensin II binding, but instead is able to bind to saralasin and decrease the PM localization of the receptor dimer. This illustrates how the formation of heterodimers can alter the pharmacological properties of a receptor, so that certain ligands will be able to bind and activate the receptor complex.

Although saralasin is classified as an antagonist, in heterodimer pairings it seems to possess partial agonist activity.

**Figure 3.3: The Effect of AT1aR Ligand Treatments on Plasma Membrane Localization of β2AR, β2ART164I, and AT1aR Homo- and Heterodimers**

β2AR, β2ART164I, and AT1aR homo- and heterodimer PM localization following one hour 1μM angiotensin II and 1μM saralasin treatments. Experiments were performed 48 hours post-transfection using a biotin-streptavidin cell surface assay to isolate proteins expressed on the PM. Following one hour ligand treatment, HEK293A cells were harvested and washed with 1X PBS and incubated with biotin for 30 minutes. Cells were then lysed with RIPA buffer and streptavidin was added. Samples were run on a 10% SDS-PAGE gel, transferred to nitrocellulose membranes and probed using an anti-GFP antibody (1:2000). **A)** Dose response curve for angiotensin II in the AT1aR homodimer. **B)** Effect of AT1aR ligand treatment on PM localization of the AT1aR homodimer. **C)** Effect of AT1aR ligand treatment on PM localization of the β2AR WT/AT1aR heterodimer. **D)** Effect of AT1aR ligand treatment on PM localization of the β2ART164I/AT1aR heterodimer. Results are representative of 3 independent experiments. ***$p<0.001$ using a two-tailed unpaired Student's t-test.




### *3.4 The Effect of Isoproterenol, Salbutamol, and ICI 118, 551 Treatment on Arrestin-2 Recruitment in β2AR, β2ART164I, and AT1aR Homo- and Heterodimers*

In most cases, internalization of GPCRs is accompanied by the recruitment of arrestin-2 to the GRK phosphorylated receptor. In section 3.2, we examined the PM localization of receptor dimer parings (β2AR WT homodimer, β2ART164I/β2AR WT, β2AR WT/AT1aR, β2ART164I/AT1aR) that were treated with βAR ligands. Since we saw changes in receptor dimer localization levels at the PM following ligand treatment, we wanted to investigate arrestin-2 recruitment, which is a major role player in receptor

internalization. We used the same receptor dimers in Table 3.1 except for the AT1aR homodimer. Arrestin-2 was also overexpressed to determine interaction levels with the receptor dimers following βAR ligand treatment. Dose response curves were generated using the β2AR WT homodimer co-expressing arrestin-2, treated with isoproterenol and salbutamol for 15 minutes. The recruitment of arrestins following ligand binding is rapid, which has been shown in prior literature to be maximal at 15 minutes[122]. The $EC_{50}$ determined for isoproterenol was $1.7x10^{-7}$M and $6.2x10^{-9}$M for salbutamol. The working concentrations of isoproterenol ($1.0x10^{-6}$M) and salbutamol ($1.0x10^{-7}$M) were both above their $EC_{50}$ values to ensure maximal responses were obtained (Figure 3.4a). The same concentration of ICI 118, 551 ($2.0x10^{-7}$M) was used as in experiments that examined PM localization following ligand binding. HA-tagged arrestin-2 recruitment was determined in the β2AR WT homodimer following 15 minute treatment with βAR ligands using co-immunoprecipitation experiments, where an HA-tag was used to immunoprecipitate arrestin-2, and a GFP antibody that binds to the venus tagged dimers was used to determine levels of the receptor complex formed. Treatment with isoproterenol (52.9±16.7%), salbutamol (23.0±11.6%) and ICI 118, 551 (79.0±22.8%) resulted in a significant increase in arrestin-2 recruitment (Figure 3.4b). Arrestin-2 recruitment was also determined using a BRET assay, where arrestin-2 was tagged with *R*lucII. If arrestin-2-*R*lucII comes in close proximity to the receptor dimer (i.e. is being recruited) following ligand treatment, we would see an increase in the BRET ratio. This is because the *R*lucII will transfer energy to the YFP variant, venus, which is formed with the receptor dimer pairings. Similar trends were observed in the β2AR WT homodimer as in co-immunoprecipitation experiments, with isoproterenol (0.07±0.01) and ICI 118, 551

(0.07±0.01) treatments resulting in a significant increase in the BRET ratio while salbutamol (0.06±0.02) resulted in a non-significant increase in the BRET ratio (Figure 3.4f).

When observing HA-arrestin-2 recruitment in the β2AR WT/AT1aR heterodimer, both isoproterenol (22.6±14.4%) and salbutamol (31.3±22.5%) treatments resulted in significant increases in HA-arrestin-2 recruitment (Figure 3.4c). However, treatment with ICI 118, 551 did not result in any changes (5.3±37.3%) in HA-arrestin-2 recruitment (Figure 3.4c). Similar trends were observed when using the arrestin-2 *R*lucII BRET assay. Isoproterenol (0.03±0.01) treatment resulted in a non-significant increase in BRET ratio while salbutamol (0.06±0.03) treatment resulted in a significant increase in BRET ratio and ICI 118, 551 (0.01±0.01) treatment did not result in any change (Figure 3.4g).

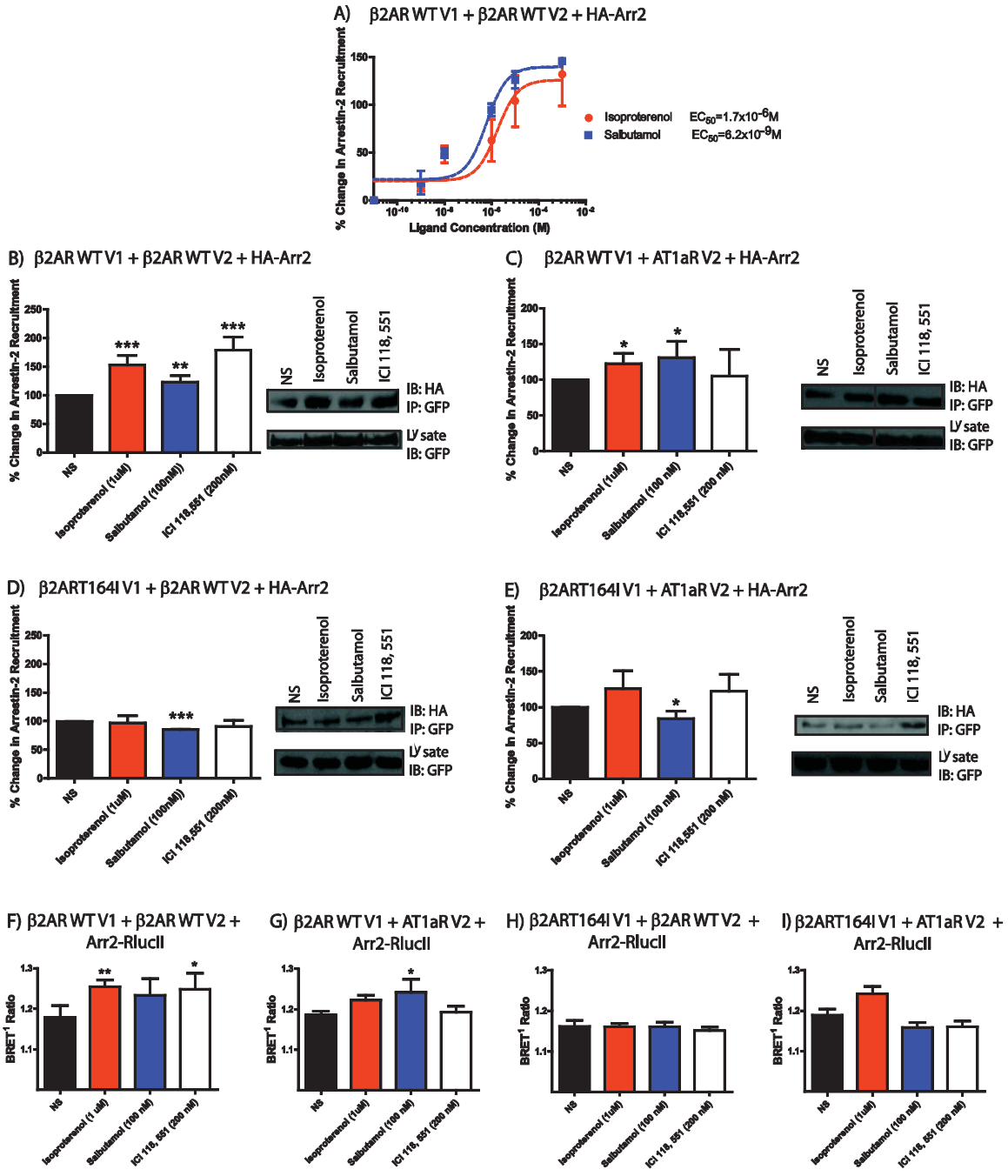
HA-arrestin-2 recruitment was then examined in the β2ART164I/β2AR WT heterodimer, and treatment with isoproterenol (3.1±12.7%) and ICI 118, 551 (8.9±10.3%) did not result in any significant changes in HA-arrestin-2 recruitment (Figure 3.4d). However, there was a significant decrease in HA-arrestin-2 recruitment with salbutamol treatment (14.7±1.1%); although consistent through experimental repetitions, the reduction levels were very low (Figure 3.4d). Again, similar results were obtained when using the arrestin-2 *R*lucII BRET assay; where isoproterenol (0.02±0.01), salbutamol (0.02±0.01), and ICI 118, 551 (0.01±0.01) treatments did not have any effect on the BRET ratio (Figure 3.4h).

HA-arrestin-2 recruitment was also examined in the β2ART164I/AT1aR heterodimer, and treatment with isoproterenol (25.6±25.3 %) and ICI 118, 551

(22.4±23.4%) resulted in no significant change in HA-arrestin-2 recruitment, whereas salbutamol (15.7±10.3%) treatment resulted in a significant decrease in HA-arrest-2 recruitment (Figure 3.4e). Similar trends were observed using the arrestin-2 *R*lucII BRET assay; where isoproterenol (0.05±0.02) had no significant effect on the BRET ratio while both salbutamol (0.03±0.01) and ICI 118, 551 (0.03±0.01) treatments resulted in a decrease in the BRET ratio (Figure3.4i). The differences that are seen between salbutamol and ICI 118, 551 treatments between the BRET and co-immunoprecipitation experiment are more than likely due to conformational changes that occurred following arrestin-2 *R*lucII binding. The results that were obtained in this section correlate with those in section 3.2 that evaluated the same receptor dimer PM localization following βAR ligand treatment. It is interesting to see that the arrestin-2 recruitment profile following βAR ligand treatment differs between the β2AR WT homodimer and β2AR WT/AT1aR heterodimer, which helps to confirm that the formation of receptor dimers affects receptor pharmacology. Furthermore, when the β2ART164I forms heterodimers with the β2AR WT and AT1aR, it also changes the arrestin-2 recruitment profile, suggesting that certain ligands may be able to interact better than others with the β2ART164I when it forms dimers with the β2AR WT and AT1aR.

**Figure 3.4: The Effect of βAR Ligand Treatments on Arrestin-2 Recruitment in β2AR, β2ART164I, and AT1aR Homo- and Heterodimers**

β2AR, β2ART164I, and AT1aR homo- and heterodimer arrestin-2 recruitment following 15 minute 1μM isoproterenol, 100nM salbutamol, and 200nM ICI 118, 551 treatments performed 48 hours post-transfection. HEK293A cells used for BRET assays were harvested following ligand treatment and washed with 1X PBS and resuspended in 90μl 1X PBS and coelenterazine *h* was added (5μM). For co-immunoprecipitations, HEK293A cells were harvested and washed with 1X PBS and then lysed with RIPA buffer and anti-GFP antibody was added (1:2000). Samples were run on a 10% SDS-PAGE gel, transferred to nitrocellulose membranes and probed using an anti-HA antibody (1:2000). **A)** Dose response curves for isoproterenol and salbutamol in the β2AR WT homodimer. **B)** Effect of βAR ligand treatment on HA-arrestin-2 recruitment in the β2AR WT homodimer. **C)** Effect of βAR ligand treatment on HA-arrestin-2 recruitment in the β2AR WT/AT1aR heterodimer. **D)** Effect of βAR ligand treatment on HA-arrestin-2 recruitment in the β2ART164I/β2AR heterodimer. **E)** Effect of βAR ligand treatment on HA-arrestin-2 recruitment in the β2ART164I/AT1aR heterodimer. **F)** Effect of βAR ligand treatment on arrestin-2-*R*lucII recruitment in the β2AR WT homodimer. **G)** Effect of βAR ligand treatment on arrestin-2-*R*lucII recruitment in the β2AR WT/AT1aR heterodimer. **H)** Effect of βAR ligand treatment on arrestin-2-*R*lucII recruitment in the β2ART164I/β2AR WT heterodimer. **I)** Effect of βAR ligand treatment on arrestin-2-*R*lucII recruitment in the β2ART164I/AT1aR heterodimer. Results are representative of 3 independent experiments. *$p<0.05$; **$p<0.01$; ***$p<0.001$ using a two-tailed unpaired Student's t-test.


A) β2AR WT V1 + β2AR WT V2 + HA-Arr2
% Change In Arrestin-2 Recruitment
Ligand Concentration (M)
Isoproterenol EC50=1.7x10-6M
Salbutamol EC50=6.2x10-9M
B) β2AR WT V1 + β2AR WT V2 + HA-Arr2
% Change In Arrestin-2 Recruitment
NS
Isoproterenol (1uM)
Salbutamol (100mM))
ICI 118,551 (200nM)
Isoproterenol
Salbutamol
ICI 118, 551
IB: HA
IP: GFP
LY sate
IB: GFP
C) β2AR WT V1 + AT1aR V2 + HA-Arr2
% Change In Arrestin-2 Recruitment
NS
Isoproterenol (1uM)
Salbutamol (100 nM)
ICI 118,551 (200 nM)
Isoproterenol
Salbutamol
ICI 118, 551
IB: HA
IP: GFP
LY sate
IB: GFP
D) β2ART164I V1 + β2AR WT V2 + HA-Arr2
% Change In Arrestin-2 Recruitment
NS
Isoproterenol (1uM)
Salbutamol (100mM))
ICI 118,551 (200nM)
Isoproterenol
Salbutamol
ICI 118, 551
IB: HA
IP: GFP
LY sate
IB: GFP
E) β2ART164I V1 + AT1aR V2 + HA-Arr2
% Change In Arrestin-2 Recruitment
NS
Isoproterenol (1uM)
Salbutamol (100 nM)
ICI 118,551 (200 nM)
Isoproterenol
Salbutamol
ICI 118, 551
IB: HA
IP: GFP
LY sate
IB: GFP
F) β2AR WT V1 + β2AR WT V2 + Arr2-RlucII
BRET1 Ratio
NS
Isoproterenol (1 uM)
Salbutamol (100 nM)
ICI 118, 551 (200 nM)
G) β2AR WT V1 + AT1aR V2 + Arr2-RlucII
BRET1 Ratio
NS
Isoproterenol (1uM)
Salbutamol (100 nM)
ICI 118, 551 (200 nM)
H) β2ART164I V1 + β2AR WT V2 + Arr2-RlucII
BRET1 Ratio
NS
Isoproterenol (1uM)
Salbutamol (100 nM)
ICI 118, 551 (200 nM)
I) β2ART164I V1 + AT1aR V2 + Arr2-RlucII
BRET1 Ratio
NS
Isoproterenol (1uM)
Salbutamol (100 nM)
ICI 118, 551 (200 nM)

### *3.5 The Effect of Angiotensin II and Saralasin Treatment on Arrestin-2 Recruitment in β2AR, β2ART164I, and AT1aR Homo- and Heterodimers*

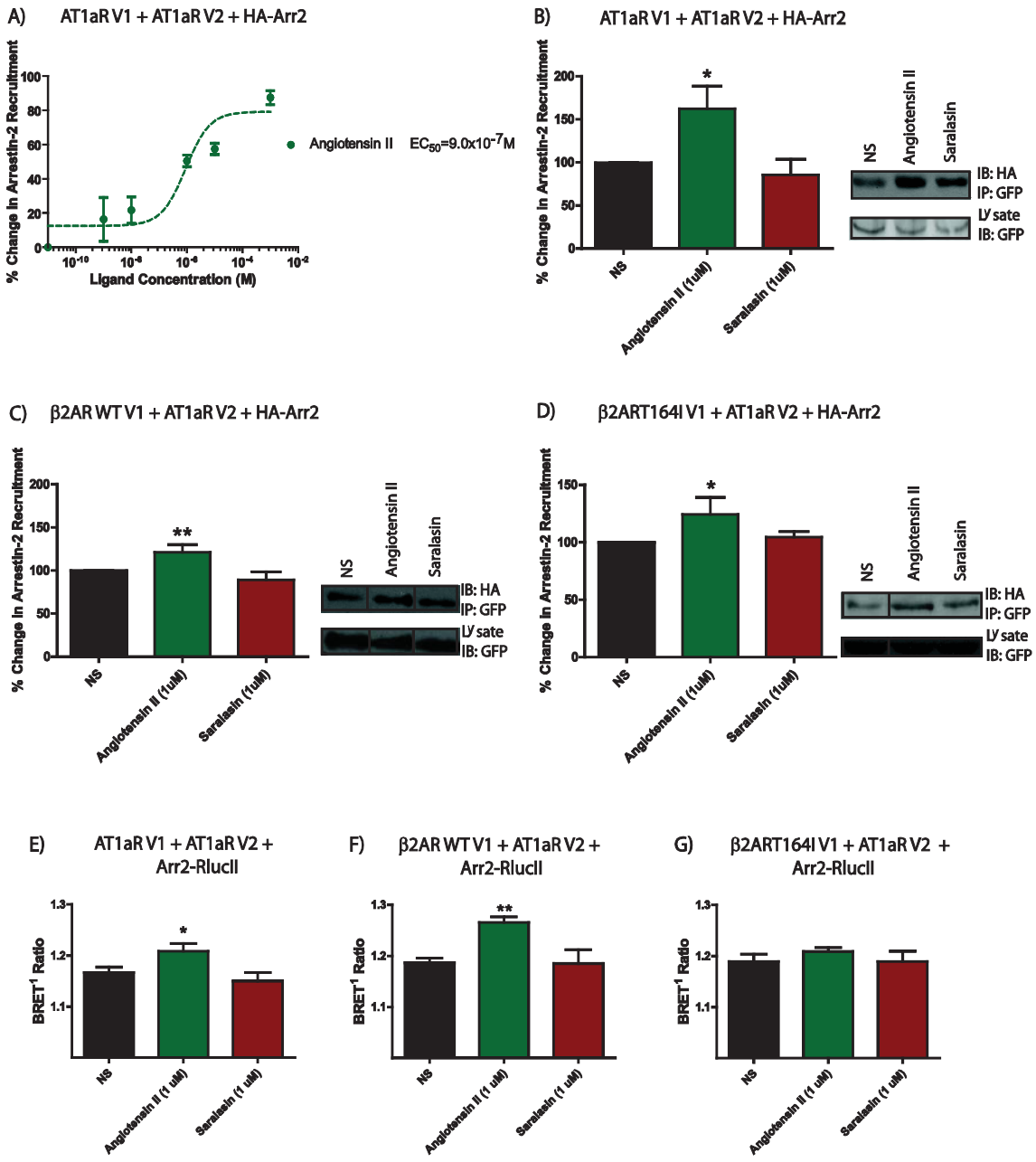
The receptor dimer pairings described in Table 3.1, along with arrestin-2 were co-expressed in HEK293A cells in order to determine what effect angiotensin II and saralasin treatments had on the interaction of arrestin-2 with the receptor dimer pairings. Dose response curves were generated using 15 minute angiotensin II incubations with the AT1aR homodimer co-expressing HA-arrestin-2. The $EC_{50}$ for angiotensin II was determined to be $9.0x10^{-7}$M, and the working concentration used was $1.0x10^{-6}$M, which is above the $EC_{50}$ value to ensure that a maximal response was obtained (Figure 3.5a). The same concentration of saralasin ($1.0x10^{-6}$M) was used as in experiments that examined PM localization following ligand treatment. Examining HA-arrestin-2 recruitment in the AT1aR homodimer, we found that treatment with angiotensin II produced a significant increase in HA-arrestin-2 recruitment (62.3±26.1%) while saralasin did not result in a significant change (14.3±17.9%) (Figure 3.5b). These results were confirmed using the arrestin-2-*R*lucII BRET assay; with angiotensin II treatment resulting in a significant increase (0.04±0.01) in the BRET ratio and saralasin treatment causing no significant changes (0.02±0.02) in the BRET ratio (Figure 3.5e).

When examining HA-arrestin-2 recruitment in the β2AR WT/AT1aR heterodimer, angiotensin II treatment produced a significant increase (21.3±8.6%) in arrestin-2 recruitment, while saralasin produced a non-significant decrease (10.8±9.3%) (Figure 3.5c). Similarly, using the BRET assay to observe arrestin-2-*R*lucII activity, angiotensin II treatment resulted in a significant increase in the BRET ratio (0.08±0.01) while saralasin did not have any effect (0.01±0.03) (Figure 3.5f).

To characterize the effect of the β2ART164I SNP in β2ART164I/AT1aR heterodimers, angiotensin II and saralasin treatment was administered. Angiotensin II was able to significantly increase (24.3±14.7%) HA-arrestin-2 recruitment while saralasin (4.5±4.9%) did not have any effect (Figure 3.5d). This result was again confirmed using the BRET assay to observe arrestin-2-*R*lucII recruitment following ligand treatment. Angiotensin II treatment non-significantly increased (0.02±0.01) the BRET ratio while saralasin treatment did not have any effect (0.0±0.01) (Figure 3.5g). The results that were obtained here correlate with PM localization levels of the dimers observed in section 3.3 when they were treated with angiotensin II and saralasin. In this case, the arrestin-2 recruitment profile was not significantly altered when angiotensin II and saralasin were administered to HEK293A cells that were expressing homo- or heterodimer receptor pairings.

**Figure 3.5: The Effect of AT1aR Ligand Treatments on Arrestin-2 Recruitment in β2AR, β2ART164I, and AT1aR Homo- and Heterodimers**

β2AR, β2ART164I, and AT1aR homo- and heterodimer arrestin-2 recruitment following 15 minute 1μM angiotensin II and 1μM saralasin treatments, performed 48 hours post-transfection. HEK293A cells used for BRET assays were harvested following ligand treatment and washed with 1X PBS and resuspended in 90μl 1X PBS and coelenterazine *h* was added (5μM). For co-immunoprecipitations, HEK293A cells were harvested and washed with 1X PBS and then lysed with RIPA buffer and anti-GFP antibody was added (1:2000). Samples were run on a 10% SDS-PAGE gel, transferred to nitrocellulose membranes and probed using an anti-HA antibody (1:2000). **A)** Dose response curves for angiotensin II in the AT1aR homodimer. **B)** Effect of AT1aR ligand treatment on HA-arrestin-2 recruitment in the AT1aR homodimer. **C)** Effect of AT1aR ligand treatment on HA-arrestin-2 recruitment in the β2AR WT/AT1aR heterodimer. **D)** Effect of AT1aR ligand treatment on HA-arrestin-2 recruitment in the β2ART164I/AT1aR heterodimer. **E)** Effect of AT1aR ligand treatment on arrestin-2-*R*lucII recruitment in the AT1aR homodimer. **F)** Effect of AT1aR ligand treatment on arrestin-2-*R*lucII recruitment in the β2AR WT/AT1aR heterodimer. **G)** Effect of AT1aR ligand treatment on arrestin-2-*R*lucII recruitment in the β2ART164I/AT1aR heterodimer. Results are representative of 3 independent experiments. *$p<0.05$; **$p<0.01$ using a two-tailed unpaired Student's t-test.


A)
AT1aR V1 + AT1aR V2 + HA-Arr2
% Change In Arrestin-2 Recruitment
Ligand Concentration (M)
Angiotensin II EC50=9.0x10-7M
B)
AT1aR V1 + AT1aR V2 + HA-Arr2
% Change In Arrestin-2 Recruitment
NS
Angiotensin II (1uM)
Saralasin (1uM)
NS
Angiotensin II
Saralasin
IB: HA
IP: GFP
Lysate
IB: GFP
C)
β2AR WT V1 + AT1aR V2 + HA-Arr2
% Change In Arrestin-2 Recruitment
NS
Angiotensin II (1uM)
Saralasin (1uM)
NS
Angiotensin II
Saralasin
IB: HA
IP: GFP
Lysate
IB: GFP
D)
β2ART164I V1 + AT1aR V2 + HA-Arr2
% Change In Arrestin-2 Recruitment
NS
Angiotensin II (1uM)
Saralasin (1uM)
NS
Angiotensin II
Saralasin
IB: HA
IP: GFP
Lysate
IB: GFP
E)
AT1aR V1 + AT1aR V2 + Arr2-RlucII
BRET-1 Ratio
NS
Angiotensin II (1 uM)
Saralasin (1 uM)
F)
β2AR WT V1 + AT1aR V2 + Arr2-RlucII
BRET-1 Ratio
NS
Angiotensin II (1 uM)
Saralasin (1 uM)
G)
β2ART164I V1 + AT1aR V2 + Arr2-RlucII
BRET-1 Ratio
NS
Angiotensin II (1 uM)
Saralasin (1 uM)

### *3.6 The Inhibition of a Single Protomer in β2AR, β2ART164I, and AT1aR Heterodimers Renders Near Complete Inhibition of Receptor Complex*

It has previously been shown that the β2AR WT and AT1aR are capable of forming heterodimers, and that the use of βAR and AT1aR ligands can have effects on the activation of signalling pathways associated with the heterodimeric complex. One of the profound effects is that the binding of either a β2AR or AT1aR antagonist can render the receptor dimer unable to activate signalling cascades when it is further incubated with a β2AR or AT1aR agonist[56]. We were interested in performing a similar experiment; however, we wanted to determine if the βAR and AT1aR ligands that we used (Table 3.2 and Table 3.3) were capable of eliciting the same effect on the heterodimer, since we were using different ligands. The same concentrations of the βAR and AT1aR ligands (isoproterenol $1.0x10^{-6}$M, salbutamol $1.0x10^{-7}$M, ICI 118,551 $2.0x10^{-7}$M, angiotensin II $1.0x10^{-6}$M, and saralasin $1.0x10^{-6}$M) were used as in prior experiments that observed PM localization and arrestin-2 recruitment following ligand treatment. HEK293A cells were transfected with the β2AR WT/AT1aR heterodimer, and as experimental controls isoproterenol, salbutamol, and angiotensin II were administered for 5 minutes to determine any changes in phospho-ERK levels. ERK phosphorylation occurs quite rapidly following ligand binding, and 5 minutes is a time that has been used in prior literature conducting a similar experiment[56]. When treating HEK293A cells with ICI 118, 551 (15.0±12.5%), there was no significant change in phospho-ERK levels, but saralasin resulted in a consistent minor decrease (11.3±9.2%) that was non-significant (Figure 3.6a, Figure 3.6c, Table 3.4). However, isoproterenol (99.3±16.4%), salbutamol (33.4±9.3%), and angiotensin II (42.8±16.1%) treatments resulted in a significant increase in phospho-ERK levels (Figure 3.6a, Figure 3.6c, Table 3.4). HEK293A cells

were then pre-treated with ICI 118, 551 or saralasin for 30 minutes. To the cells that were pre-treated with ICI 118, 551, angiotensin II was administered for 5 minutes to determine any changes in phospho-ERK levels. To the cells that were pre-treated with saralasin, isoproterenol or salbutamol was administered for 5 minutes to determine any changes in phospho-ERK levels. Treatment with ICI 118, 551 will inhibit the WT β2AR protomer in the β2AR WT/AT1aR heterodimer, and treatment with saralasin will inhibit the AT1aR protomer in the β2AR WT/AT1aR heterodimer. After ICI 118, 551 pre-treatment, the addition of angiotensin II resulted in no significant difference (2.1±15.1%) in phospho-ERK levels when compared to NS cells (Figure 3.6a, Figure 3.6c, Table 3.4). The same result was obtained when pre-treating with saralasin; the addition of isoproterenol (5.3±18.9%) and salbutamol (3.1±18.9%) did not significantly change phospho-ERK levels compared to NS cells (Figure 3.6a, Figure 3.6c, and Table 3.4).

**Table 3.4 The Effect of Ligand Treatment on β2AR WT/AT1aR Heterodimer phospho-ERK Activity**

| Receptor Dimer Pairing | Ligand Treatment | Bar Graph Color in Figure 3.6a | p-ERK Change from Basal Levels |
|---|---|---|---|
| β2AR WT/AT1aR | NS | Black Bar | N/A |
| | ICI 118, 551 | White Bar | None |
| | Angiotensin II | Green Bar | Increase |
| | ICI 118, 551 pre-treatment followed by angiotensin II treatment | Gray Bar | None |
| | Saralasin | Red Bar | Decrease |
| | Salbutamol | Blue Bar | Increase |
| | Saralasin pre-treatment followed by salbutamol treatment | Pink Bar | None |
| | Isoproterenol | Orange Bar | Increase |
| | Saralasin pre-treatment followed by isoproterenol treatment | Yellow Bar | None |

We were then interested in examining the effect of the β2ART164I SNP on ERK phosphorylation in heterodimer pairings with the AT1aR. HEK293A cells were transfected with the β2ART164I/AT1aR heterodimer, and as experimental controls isoproterenol, salbutamol, and angiotensin II were administered for 5 minutes to determine any changes in phospho-ERK levels. When treating HEK293A cells with saralasin (23.8±31.7%), there was no significant change in phospho-ERK levels, but ICI 118, 551 resulted in a consistent minor decrease (22.3±3.7%) that was significant (Figure 3.6b, Figure 3.6d, Table 3.5). However, isoproterenol (75.2±44.8%), salbutamol (206.8±93.2%), and angiotensin II (304.3±53.1%) treatments resulted in an increase in phospho-ERK levels, with angiotensin II being significant (Figure 3.6b, Figure 3.6d, Table 3.5). HEK293A cells were then pre-treated with ICI 118, 551 or saralasin for 30 minutes. To the cells that were pre-treated with ICI 118, 551, angiotensin II was administered for 5 minutes to determine phospho-ERK level changes. To the cells that were pre-treated with saralasin, isoproterenol or salbutamol was administered for 5 minutes to determine phospho-ERK level changes. Since the β2ART164I SNP has previously shown impairment to βAR antagonist and agonist binding, we want to see if treatment with ICI 118, 551 will inhibit the β2ART164I protomer in the β2ART164I/AT1aR heterodimer. As well, we also wanted to determine if treatment with saralasin would be able to inhibit the AT1aR protomer in the β2ART164I/AT1aR heterodimer. After ICI 118, 551 pre-treatment, the addition of angiotensin II resulted in a non-significant increase in (83.4±57.2%) in phospho-ERK levels when compared to NS cells (Figure 3.6b, Figure 3.6d, Table 3.5). The opposite result was obtained when pre-treating with saralasin; the addition of isoproterenol resulted in a non-significant decrease

(30.8±14.0%) in phospho-ERK levels. Pre-treatment with saralasin followed by the addition of salbutamol resulted in a significant increase (152.6±51.4%) in phospho-ERK levels compared to the NS cells (Figure 3.6b, Figure 3.6d, and Table 3.5). The western blots (Figure 3.6c and Figure 3.6d) shown are representative of three independent experiments that examined phospho-ERK level changes in response to the different ligand treatments that were previously described.

**Table 3.5 The Effect of Ligand Treatment on β2ART164I/AT1aR Heterodimer phospho-ERK Activity**

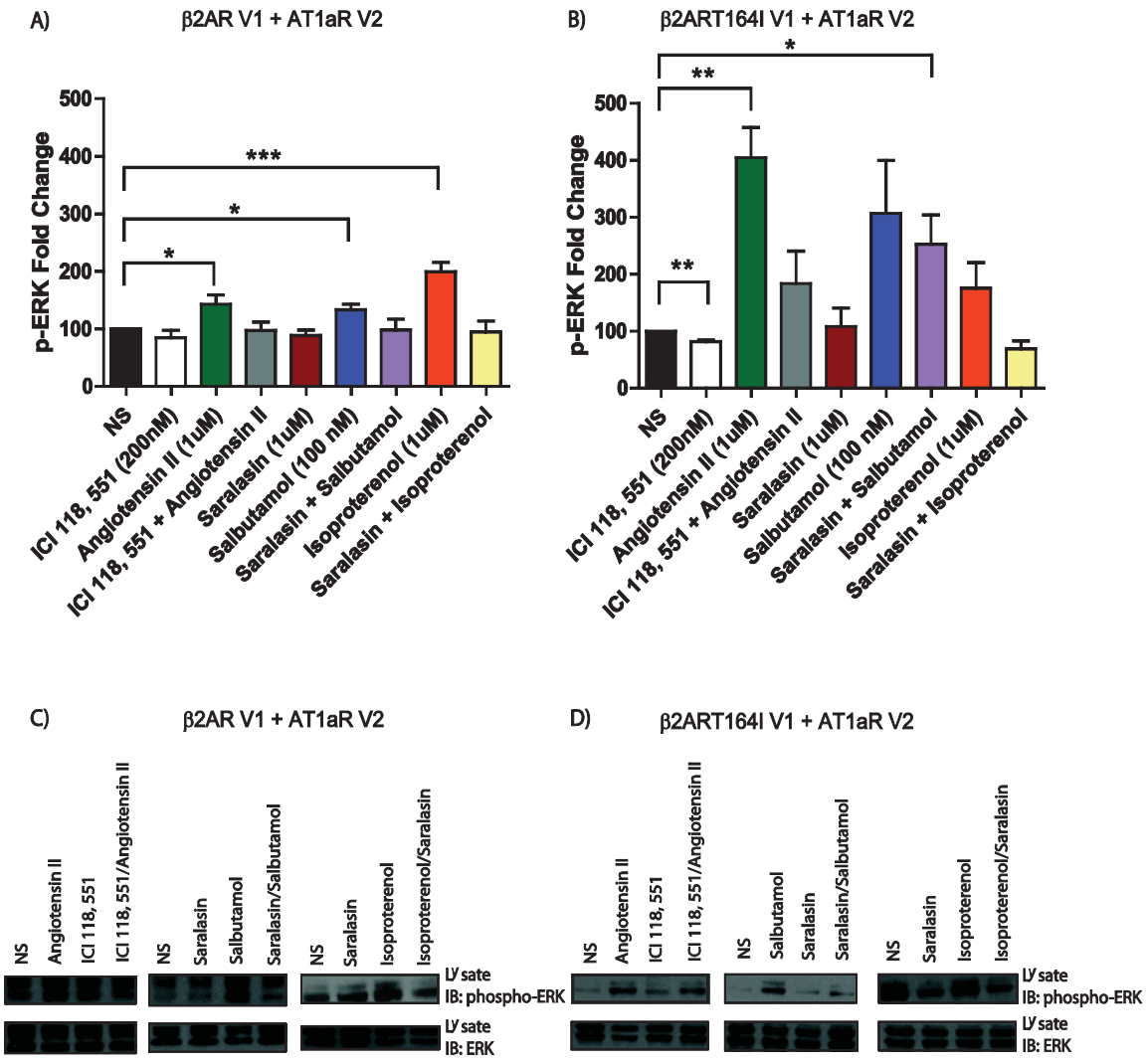
| Receptor Dimer Pairing | Ligand Treatment | Bar Graph Color in Figure 3.6b | p-ERK Change from basal levels |
|---|---|---|---|
| β2AR T164I/AT1aR | NS | Black Bar | N/A |
| | ICI 118, 551 | White Bar | None |
| | Angiotensin II | Green Bar | Increase |
| | ICI 118, 551 pre-treatment followed by angiotensin II treatment | Gray Bar | None |
| | Saralasin | Red Bar | Decrease |
| | Salbutamol | Blue Bar | Increase |
| | Saralasin pre-treatment followed by salbutamol treatment | Pink Bar | None |
| | Isoproterenol | Orange Bar | Increase |
| | Saralasin pre-treatment followed by isoproterenol treatment | Yellow Bar | None |

The results show that in the β2AR WT/AT1aR heterodimer, phospho-ERK activation can be obtained by treating the β2AR WT protomer with a βAR agonist or the AT1aR protomer with an AT1aR agonist. However, when either an antagonist of the β2AR WT or AT1aR protomer is administered, it renders both receptors in the β2AR WT/AT1aR heterodimer unable to further bind βAR and AT1aR agonists, preventing phospho-ERK activation. When examining the β2ART164I SNP in β2ART164I/AT1aR heterodimers, we see that treatment with the AT1aR agonist angiotensin II and βAR

agonists, isoproterenol and salbutamol, result in phospho-ERK activation, possibly by acting on the AT1aR protomer. However, when pre-treated with the β2AR antagonist ICI 118, 551, the addition of angiotensin II is capable of phospho-ERK activation, which was not seen in the β2AR WT/AT1aR heterodimer, indicating impaired ligand binding of the β2ART164I. Similarly, pre-treatment with saralasin followed by the addition of salbutamol also increased phospho-ERK activation. On the other hand, pre-treatment with saralasin followed by the addition of isoproterenol did not increase phospho-ERK levels. The results demonstrate that in heterodimeric receptor pairings such as the β2AR and AT1aR, there is molecular cross talk that occurs, which affects the activity of specific ligands on signal activation. We were also able to show that the formation of β2ART164I/AT1aR heterodimers alters molecular cross talk, permitting signal transduction in response to ligand treatment.

**Figure 3.6: Trans Inhibition of β2AR/AT1aR Heterodimers is Affected by the β2ART164I SNP for ERK Signal Activation**

β2AR, β2ART164I, and AT1aR heterodimer phospho-ERK activation experiments were performed 48 hours post-transfection. HEK293A cells were treated with 1μM isoproterenol, 100nM salbutamol, and 1μM angiotensin II for 5 minutes. Cells pre-treated with ICI 118, 551 for 30 minutes were then incubated with angiotensin II for 5 minutes. Cells pre-treated with saralasin for 30 minutes were then incubated with either isoproterenol or salbutamol for 5 minutes. Following ligand treatments, cells were harvested and washed with 1X PBS. Cells were then lysed with RIPA buffer and samples were run on a 10% SDS-PAGE gel, transferred to nitrocellulose membranes and probed using an anti-phospho-ERK antibody (1:500) and then an anti-ERK antibody (1:2000). **A)** Effect of ligand treatments on β2AR WT/AT1aR heterodimer phospho-ERK signalling. **B)** Effect of ligand treatments on β2ART164I/AT1aR heterodimer phospho-ERK signalling. **C)** Representative western blots showing phospho-ERK and ERK levels following ligand treatments in the β2AR WT/AT1aR heterodimer. **D)** Representative western blots showing phospho-ERK and ERK levels following ligand treatments on the β2ART164I/AT1aR heterodimer. Results are representative of 3 independent experiments. *$p<0.05$; **$p<0.01$; ***$p<0.001$ using a two-tailed unpaired Student's t-test.


A)
β2AR V1 + AT1aR V2
p-ERK Fold Change
500
400
300
200
100
0
***
*
*
NS
ICI 118, 551 (200nM)
Angiotensin II (1uM)
ICI 118, 551 + Angiotensin II
Saralasin (1uM)
Salbutamol (100 nM)
Saralasin + Salbutamol
Isoproterenol (1uM)
Saralasin + Isoproterenol
B)
β2ART164I V1 + AT1aR V2
p-ERK Fold Change
500
400
300
200
100
0
*
**
**
NS
ICI 118, 551 (200nM)
Angiotensin II (1uM)
ICI 118, 551 + Angiotensin II
Saralasin (1uM)
Salbutamol (100 nM)
Saralasin + Salbutamol
Isoproterenol (1uM)
Saralasin + Isoproterenol
C)
β2AR V1 + AT1aR V2
NS
Angiotensin II
ICI 118, 551
ICI 118, 551/Angiotensin II
NS
Saralasin
Salbutamol
Saralasin/Salbutamol
NS
Saralasin
Isoproterenol
Isoproterenol/Saralasin
Ly sate
IB: phospho-ERK
Ly sate
IB: ERK
D)
β2ART164I V1 + AT1aR V2
NS
Angiotensin II
ICI 118, 551
ICI 118, 551/Angiotensin II
NS
Salbutamol
Saralasin
Saralasin/Salbutamol
NS
Saralasin
Isoproterenol
Isoproterenol/Saralasin
Ly sate
IB: phospho-ERK
Ly sate
IB: ERK

### *3.7 The Effect of Isoproterenol, Salbutamol, and ICI 118, 551 Treatment on the Heterotrimeric G-proteins of β2AR and AT1aR Homo- and Heterodimers*

In the classical view of GPCR activation, a ligand will bind to the GPCR and activate the heterotrimeric G-protein so that signal transduction can be carried out by the Gα and Gβγ subunits. Using a BRET based assay, we wanted to examine the potential interactions that occur between the receptor dimers and the Gα subunits following βAR ligand treatment. When our receptor pairings dimerize, they form a functional YFP variant, venus, which is able to act as an energy acceptor. The Gα subunits were tagged with *R*lucII, which acts as an energy donor upon the addition of its substrate, coelenterazine *h*. If the Gα subunits come in close proximity to the receptor, then we will observe this as an increase in the BRET ratio, and if they move farther apart we see this as a decrease in the BRET ratio. The same concentrations of the βAR ligands (isoproterenol $1.0x10^{-6}$M, salbutamol $1.0x10^{-7}$M, and ICI 118,551 $2.0x10^{-7}$M) were used as in prior experiments in sections 3.2-3.6. HEK293A cells were transfected with the β2AR WT homodimer co-expressing Gαs-*R*lucII and $G\beta_1\gamma_2$ subunits. When comparing NS and βAR ligand treated cells to the negative control (β2ARv1/β2ARv2 + *R*lucII), there was a significant increase in the BRET ratio, which indicates Gαs-*R*lucII interaction with the β2AR WT homodimer (Figure 3.7a). Treatment with isoproterenol (0.07±0.02) produced a non-significant increase in the BRET ratio, while salbutamol treatment did not differ from the BRET ratio compared to NS cells (0.01±0.05) (Figure 3.7a). On the other hand, treatment with ICI 118, 551 caused a decrease (0.04±0.02) in the BRET ratio compared to NS cells (Figure 3.7a). To determine whether there were conformational changes that occurred following ligand treatment, co-immunoprecipitation experiments were performed. The β2AR WT homodimer was expressed with the $G\beta_1\gamma_2$ subunits along

with an HA-tagged Gαs subunit. A GFP antibody was used to determine the levels of receptor dimer complex formed, and an HA antibody was used to immunoprecipitate any bound HA-tagged Gαs subunit. Treatment with isoproterenol, salbutamol, and ICI 118, 551 did not result in any changes in the amount of precipitated HA-Gαs when compared to the NS cells (Figure 3.7b). Given the significant increase in the BRET ratio for NS and βAR ligand treated cells compared to the negative control, and the constant amount of precipitated HA-Gαs bound to the receptor complex, this suggests that there were conformational changes occurring following isoproterenol, ICI 118, 551, and salbutamol binding. The βAR ligands resulted in the β2AR WT homodimer venus tag changing its proximity to the *R*lucII tag on the Gαs subunit.

HEK 293A cells were transfected to co-express the β2AR WT homodimer along with Gαi-*R*lucII and $G\beta_1\gamma_2$ subunits. When comparing NS and βAR ligand treated cells to the negative control (β2ARv1/β2ARv2 + *R*lucII), there was a significant increase in the BRET ratio, which indicates Gαi-*R*lucII interaction with the β2AR WT homodimer (Figure 3.7c). Treatment with isoproterenol (0.02±0.01) and ICI 118, 551 (0.02±0.02) produced non-significant increases in the BRET ratio, while salbutamol treatment did not differ from NS cells (0.02±0.01) (Figure 3.7c). The β2AR WT homodimer was expressed with the $G\beta_1\gamma_2$ subunits along with an EE-tagged Gαi subunit, using co-immunoprecipitation techniques to characterize their interaction following ligand incubation. Treatment with isoproterenol, salbutamol, and ICI 118, 551 did not change the amount of precipitated EE-Gαi bound to the receptor complex when compared to the NS cells (Figure 3.7d). There were no significant changes in the BRET ratio when treating with isoproterenol, salbutamol, and ICI 118, 551 but there was a significant

increase in the BRET ratio for NS and βAR ligand treated cells compared to the negative control. Precipitated EE-Gαi also remained constant following ligand treatment, which suggests there was a prior interaction between the β2AR homodimer and Gαi-*R*lucII. The βAR ligands resulted in the β2AR WT homodimer venus tag changing its proximity to the *R*lucII tag on the Gαi subunit.

We were then interested in determining whether the formation of β2AR WT/AT1aR heterodimers affect the interaction with the heterotrimeric G-proteins following βAR ligand treatment. HEK293A cells were transfected to co-express the β2AR WT/AT1aR heterodimer along with Gαs-*R*lucII and $G\beta_1\gamma_2$ subunits. When comparing NS and βAR ligand treated cells to the negative control (β2ARv1/AT1aRv2 + *R*lucII), there was a significant increase in the BRET ratio, which indicates Gαs-*R*lucII interaction with the β2AR/AT1aR heterodimer (Figure 3.7e). Treatment with isoproterenol resulted in a non-significant increase (0.05±0.03) in the BRET ratio, while salbutamol (0.02±0.03) and ICI 118, 551 (0.001±0.004) treatments did not change the BRET ratio in comparison to NS cells (Figure 3.7e). The β2AR WT/AT1aR heterodimer was expressed with the $G\beta_1\gamma_2$ subunits along with a HA-tagged Gαs subunit, using co-immunoprecipitation techniques to characterize their interaction. Treatment with isoproterenol, salbutamol, and ICI 118, 551 did not change the amount of precipitated HA-Gαs when compared to NS cells (Figure 3.7f). There were no significant changes in the BRET ratio when treating with isoproterenol, salbutamol, and ICI 118, 551 but there was a significant increase in the BRET ratio for NS and βAR ligand treated cells compared to the negative control. Precipitated HA-Gαs also remained constant following ligand treatment, which suggests there was a prior interaction between the β2AR/AT1aR

heterodimer and Gαs-*R*lucII. The βAR ligands resulted in the β2AR WT/AT1aR heterodimer venus tag changing its proximity to the *R*lucII tag on the Gαs subunit.

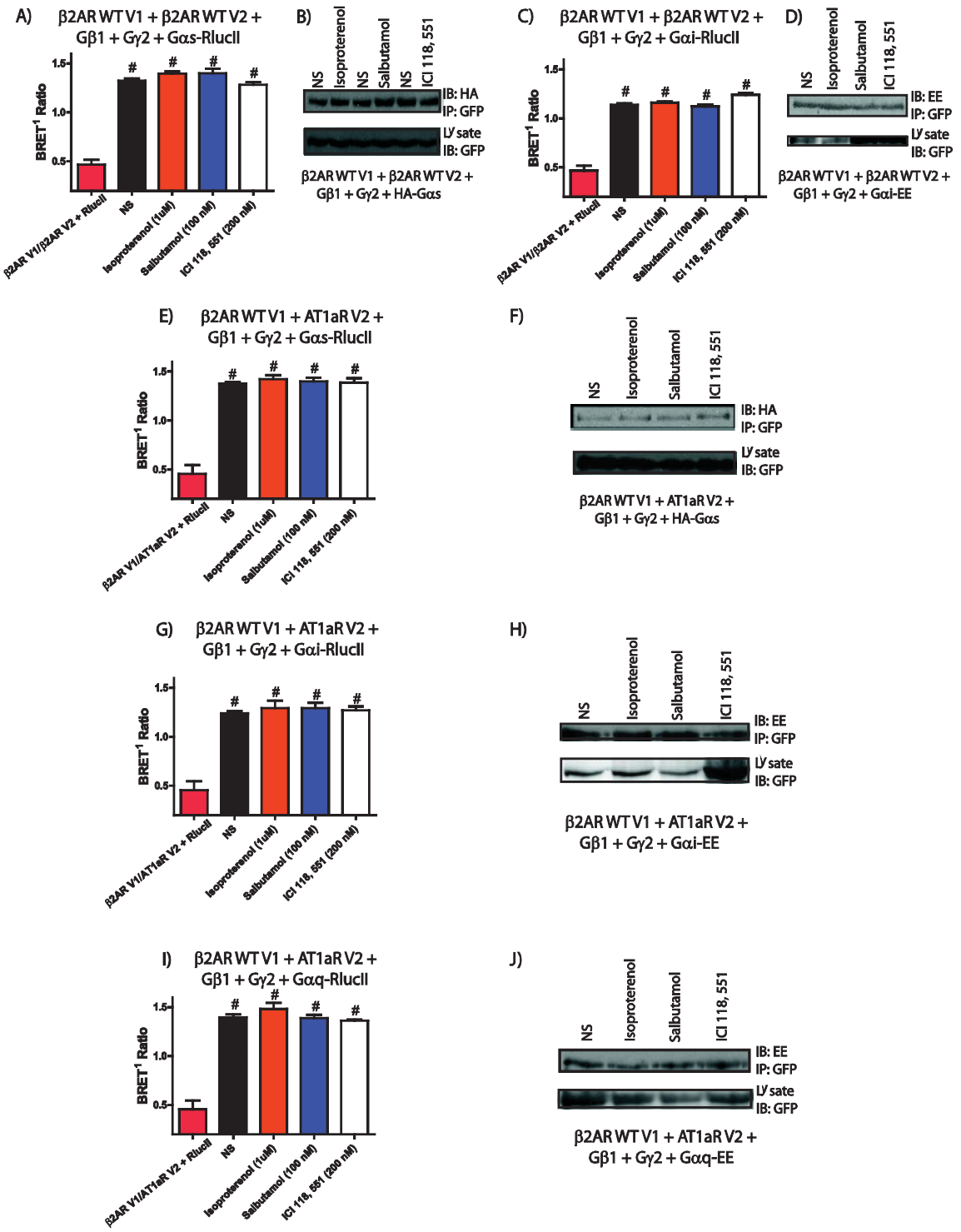
The β2AR WT/AT1aR heterodimer was co-expressed in HEK293A cells along with Gαi-*R*lucII and $G\beta_1\gamma_2$ subunits. When comparing NS and βAR ligand treated cells to the negative control (β2ARv1/AT1aRv2 + *R*lucII), there was a significant increase in the BRET ratio, which indicates Gαi-*R*lucII interaction with the β2AR/AT1aR heterodimer (Figure 3.7g). Treatment with isoproterenol (0.05±0.07), salbutamol (0.05±0.05) and ICI 118, 551 (0.03±0.04) resulted in non-significant changes in the BRET ratio compared to NS cells, although there were tendencies towards an increase in BRET ratio with isoproterenol and salbutamol treatments (Figure 3.7g). The β2AR WT/AT1aR heterodimer was then co-expressed with the $G\beta_1\gamma_2$ subunits along with an EE-tagged Gαi subunit, using co-immunoprecipitation techniques to characterize their interaction following ligand treatment. Treatment with isoproterenol, salbutamol, and ICI 118, 551 did not change the amount of precipitated EE-Gαi when compared to the NS cells (Figure 3.7h). There were no significant changes in the BRET ratio when treating with isoproterenol, salbutamol, and ICI 118, 551 but there was a significant increase in the BRET ratio for NS and βAR ligand treated cells compared to the negative control. Precipitated EE-Gαi also remained constant following ligand treatment, which suggests there was a prior interaction between the β2AR/AT1aR heterodimer and Gαi-*R*lucII. The βAR ligands resulted in the β2AR WT/AT1aR heterodimer venus tag changing its proximity to the *R*lucII tag on the Gαi subunit.

Although the β2AR WT does not couple to Gαq, we wanted to examine the β2AR WT/AT1aR heterodimer to determine if it would be able to interact with the Gαq subunit

following isoproterenol, salbutamol, and ICI 118, 551 treatments. The β2AR WT/AT1aR heterodimer was co-expressed in HEK293A cells along with Gαq-*R*lucII and $G\beta_1\gamma_2$ subunits. When comparing NS and βAR ligand treated cells to the negative control (β2ARv1/AT1aRv2 + *R*lucII), there was a significant increase in the BRET ratio, which indicates Gαq-*R*lucII interaction with the β2AR/AT1aR heterodimer (Figure 3.7i). Treatment with isoproterenol (0.08±0.06) resulted in a non-significant increase in the BRET ratio, while salbutamol (0.01±0.03) did not have any effect on the BRET ratio (Figure 3.7i). ICI 118, 551 treatment resulted in a non-significant (0.04±0.01) decrease in the BRET ratio compared when compared to NS cells (Figure 3.7i). The β2AR WT/AT1aR heterodimer was co-expressed with the $G\beta_1\gamma_2$ subunits along with an EE-tagged Gαq subunit, using co-immunoprecipitation techniques to characterize their interaction following ligand treatment. Incubation with isoproterenol, salbutamol, and ICI 118, 551 did not change the amount of precipitated EE-Gαq when compared to NS cells (Figure 3.7j). There were no significant changes in the BRET ratio when treating with isoproterenol, salbutamol, and ICI 118, 551 but there was a significant increase in the BRET ratio for NS and βAR ligand treated cells compared to the negative control. Precipitated EE-Gαq also remained constant following ligand treatment, which suggests there was a prior interaction between the β2AR/AT1aR heterodimer and Gαq-*R*lucII. The βAR ligands resulted in the β2AR WT/AT1aR heterodimer venus tag changing its proximity to the *R*lucII tag on the Gαq subunit, which suggests that conformational changes are occurring following βAR ligand treatment between the receptor dimer pairings and the heterotrimeric G-proteins.

**Figure 3.7: The Effect of βAR Ligand Treatments on Heterotrimeric G-Protein Interactions with β2AR and AT1aR Homo- and Heterodimers**

The characterization of β2AR and AT1aR homo- and heterodimer pairing interactions with the heterotrimeric G-Protein subunits Gβ1, Gγ2, Gαs, Gαi, and Gαq following 15 minute 1μM isoproterenol, 100nM salbutamol, and 200nM ICI 118, 551 treatments. HEK293A cells used for BRET assays were harvested following ligand treatments and washed with 1X PBS and resuspended in 90μl of 1X PBS and coelenterazine *h* was added (5μM). For co-immunoprecipitations, HEK293A cells were harvested and washed with 1X PBS and lysed with RIPA buffer, followed by incubation with an anti-GFP antibody (1:2000). Samples were run on a 10% SDS-PAGE gel, transferred to nitrocellulose membranes and probed using an anti-HA antibody (1:2000) or an anti-EE antibody (1:2000). **A)** Effect of βAR ligand treatment on Gαs-*R*lucII interaction with the β2AR WT homodimer. **B)** Effect of βAR ligand treatment on HA-Gαs interaction with the β2AR WT homodimer. **C)** Effect of βAR ligand treatment on Gαi-*R*lucII interaction with the β2AR WT homodimer. **D)** Effect of βAR ligand treatment on EE-Gαi interaction with the β2AR WT homodimer. **E)** Effect of βAR ligand treatment on Gαs-*R*lucII interaction with the β2AR WT/AT1aR heterodimer. **F)** Effect of βAR ligand treatment on HA- Gαs interaction with the β2AR WT/AT1aR heterodimer. **G)** Effect of βAR ligand treatment on Gαi-*R*lucII interaction with the β2AR WT/AT1aR heterodimer. **H)** Effect of βAR ligand treatment on EE-Gαi interaction with the β2AR WT homodimer. **I)** Effect of βAR ligand treatment on Gαq-*R*lucII interaction with the β2AR WT/AT1aR heterodimer. **J)** Effect of βAR ligand treatment on EE-Gαq interaction with the β2AR WT homodimer. Results are representative of 3 independent experiments. #$p<0.001$ using a two-tailed unpaired Student's t-test compared to negative controls.


A)
β2AR WT V1 + β2AR WT V2 +
Gβ1 + Gγ2 + Gαs-RlucII
BRET-1 Ratio
β2AR V1/β2AR V2 + RlucII
NS
Isoproterenol (1uM)
Salbutamol (100 nM)
ICI 118, 551 (200 nM)
B)
NS
Isoproterenol
NS
Salbutamol
NS
ICI 118, 551
IB: HA
IP: GFP
Lγ sate
IB: GFP
β2AR WT V1 + β2AR WT V2 +
Gβ1 + Gγ2 + HA-Gαs
C)
β2AR WT V1 + β2AR WT V2 +
Gβ1 + Gγ2 + Gαi-RlucII
BRET-1 Ratio
β2AR V1/β2AR V2 + RlucII
NS
Isoproterenol (1uM)
Salbutamol (100 nM)
ICI 118, 551 (200 nM)
D)
NS
Isoproterenol
Salbutamol
ICI 118, 551
IB: EE
IP: GFP
Lγ sate
IB: GFP
β2AR WT V1 + β2AR WT V2 +
Gβ1 + Gγ2 + Gαi-EE
E)
β2AR WT V1 + AT1aR V2 +
Gβ1 + Gγ2 + Gαs-RlucII
BRET-1 Ratio
β2AR V1/AT1aR V2 + RlucII
NS
Isoproterenol (1uM)
Salbutamol (100 nM)
ICI 118, 551 (200 nM)
F)
NS
Isoproterenol
Salbutamol
ICI 118, 551
IB: HA
IP: GFP
Lγ sate
IB: GFP
β2AR WT V1 + AT1aR V2 +
Gβ1 + Gγ2 + HA-Gαs
G)
β2AR WT V1 + AT1aR V2 +
Gβ1 + Gγ2 + Gαi-RlucII
BRET-1 Ratio
β2AR V1/AT1aR V2 + RlucII
NS
Isoproterenol (1uM)
Salbutamol (100 nM)
ICI 118, 551 (200 nM)
H)
NS
Isoproterenol
Salbutamol
ICI 118, 551
IB: EE
IP: GFP
Lγ sate
IB: GFP
β2AR WT V1 + AT1aR V2 +
Gβ1 + Gγ2 + Gαi-EE
I)
β2AR WT V1 + AT1aR V2 +
Gβ1 + Gγ2 + Gαq-RlucII
BRET-1 Ratio
β2AR V1/AT1aR V2 + RlucII
NS
Isoproterenol (1uM)
Salbutamol (100 nM)
ICI 118, 551 (200 nM)
J)
NS
Isoproterenol
Salbutamol
ICI 118, 551
IB: EE
IP: GFP
Lγ sate
IB: GFP
β2AR WT V1 + AT1aR V2 +
Gβ1 + Gγ2 + Gαq-EE

### *3.8 The Effect of Angiotensin II and Saralasin Treatment on the Heterotrimeric G-proteins of AT1aR and β2AR Homo- and Heterodimers*

In a similar manner, the same BRET based assay was used to examine the potential interactions that occur between the AT1aR and β2AR WT receptor dimers and the Gα subunits following AT1aR ligand treatment. Co-immunoprecipitation experiments were used to further characterize these interactions. The same concentrations of the AT1aR ligands (angiotensin II $1.0x10^{-6}$M and saralasin $1.0x10^{-6}$M) were used as in prior experiments in sections 3.2-3.6. HEK293A cells were transfected with the AT1aR homodimer co-expressing Gαq-*R*lucII and $G\beta_1\gamma_2$ subunits. When comparing NS and AT1aR ligand treated cells to the negative control (AT1aRv1/AT1aRv2 + *R*lucII), there was a significant increase in the BRET ratio, which indicates Gαq-*R*lucII interaction with the AT1aR homodimer (Figure 3.8a). Treatment with angiotensin II produced a non-significant increase (0.04±0.02), while saralasin treatment did not have any effect (0.01±0.01) on the BRET ratio compared to NS cells (Figure 3.8a). The AT1aR homodimer was co-expressed with the $G\beta_1\gamma_2$ subunits along with an EE-tagged Gαq subunit. A GFP antibody was used to determine the levels of receptor complex formed, and an EE antibody was used to immunoprecipitate any bound EE-tagged Gαq subunit. Treatment with angiotensin II and saralasin did not change the amount of precipitated EE-Gαq when compared to NS cells (Figure 3.8b). There were no significant changes in the BRET ratio when treating with angiotensin II and saralasin but there was a significant increase in the BRET ratio for NS and AT1aR ligand treated cells compared to the negative control. Precipitated EE-Gαq also remained constant following ligand treatment, which suggests there was a prior interaction between the AT1aR homodimer and Gαq-

*R*lucII. The AT1aR ligands resulted in the AT1aR homodimer venus tag changing its proximity to the *R*lucII tag on the Gαq subunit.

We were then interested in determining whether β2AR WT/AT1aR heterodimers affect coupling to the heterotrimeric G-proteins following angiotensin II and saralasin treatments. HEK293A cells were transfected with the β2AR WT/AT1aR heterodimer co-expressing Gαs-*R*lucII and $G\beta_1\gamma_2$ subunits. Although the AT1aR does not usually couple to the Gαs subunit, we were interested to see if the formation of a heterodimer will produce Gαs coupling following AT1aR ligand treatment. When comparing NS and AT1aR ligand treated cells to the negative control (β2ARv1/AT1aRv2 + *R*lucII), there was a significant increase in the BRET ratio, which indicates Gαs-*R*lucII interaction with the β2AR/AT1aR heterodimer (Figure 3.8c). Angiotensin II treatment did not have any effect (0.02±0.01) on the BRET ratio, however saralasin treatment produced a significant decrease (0.08±0.01) in the BRET ratio compared to NS cells (Figure 3.8c). To further characterize this interaction, a co-immunoprecipitation was performed. The β2AR WT/AT1aR heterodimer was co-expressed with the $G\beta_1\gamma_2$ subunits along with an HA-tagged Gαs subunit. Treatment with angiotensin II and saralasin did not affect the amount of precipitated HA-Gαs when compared to the NS cells (Figure 3.8d). There were no significant changes in the BRET ratio when treating with angiotensin II, but there was a significant decrease when treating with saralasin compared to NS cells. There was also a significant increase in the BRET ratio for NS and AT1aR ligand treated cells compared to the negative control. Precipitated HA-Gαs also remained constant following ligand treatment, which suggests there was a prior interaction between the β2AR WT/AT1aR

heterodimer and Gαs-*R*lucII. The AT1aR ligands resulted in the β2AR WT/AT1aR heterodimer venus tag changing its proximity to the *R*lucII tag on the Gαs subunit.

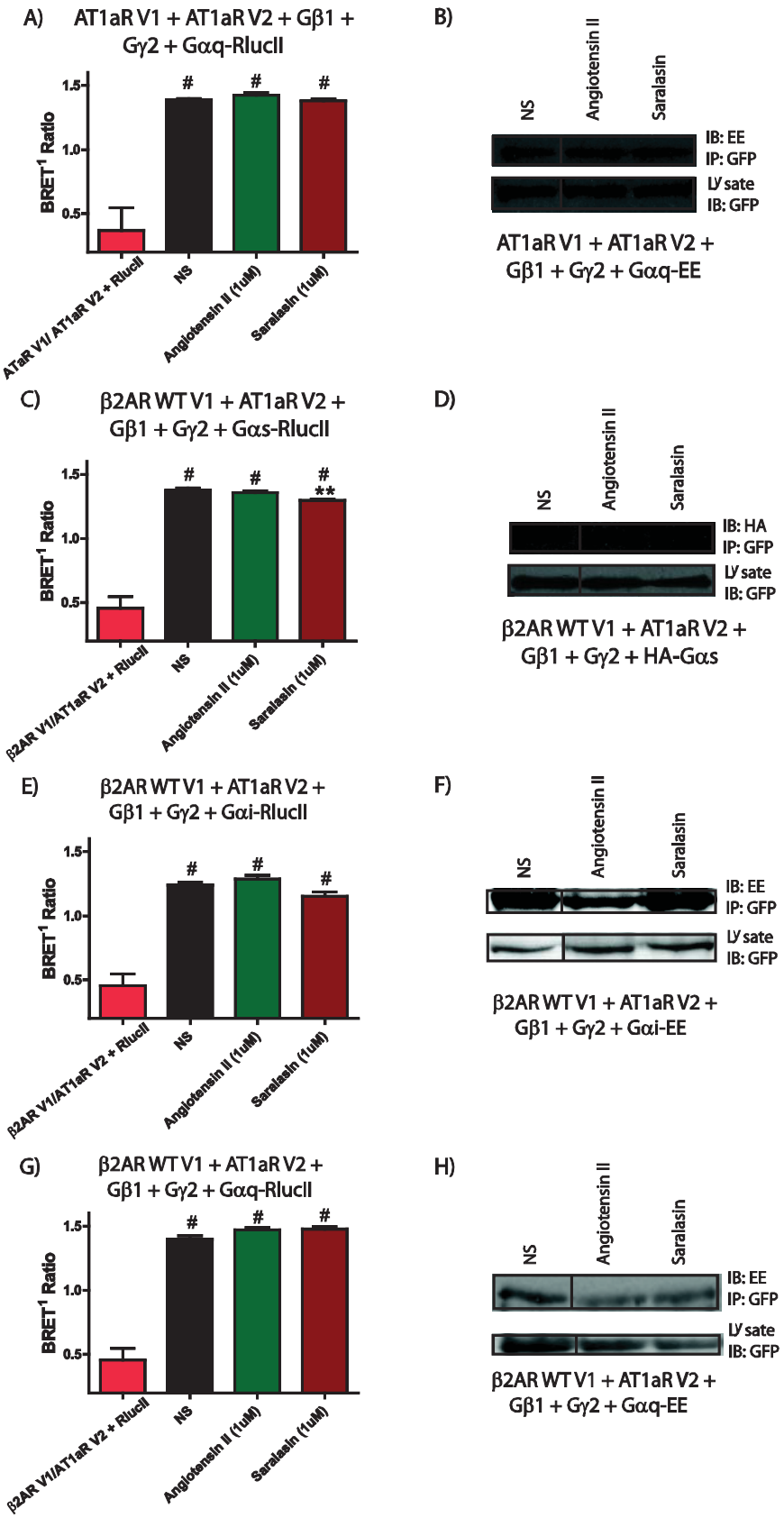
HEK293A cells were transfected with the β2AR WT/AT1aR heterodimer co-expressing Gαi-*R*lucII and $G\beta_1\gamma_2$ subunits. When comparing NS and AT1aR ligand treated cells to the negative control (β2ARv1/AT1aRv2 + *R*lucII), there was a significant increase in the BRET ratio, which indicates Gαi-*R*lucII interaction with the β2AR/AT1aR heterodimer (Figure 3.8e). Angiotensin II treatment produced a non-significant increase (0.05±0.02) in the BRET ratio while saralasin treatment produced a non-significant decrease (0.09±0.03) in the BRET ratio (Figure 3.8e). To further characterize this interaction, a co-immunoprecipitation was performed. Treatment with angiotensin II and saralasin did not affect the amount of precipitated EE-Gαi when compared to the NS cells (Figure 3.8f). Both angiotensin II and saralasin treatment caused significant changes in the BRET ratio when compared to NS cells, and there was also a significant increase in the BRET ratio for NS and AT1aR ligand treated cells compared to the negative control. Precipitated EE-Gαi also remained constant following ligand treatment, which suggests there was a prior interaction between the β2AR WT/AT1aR heterodimer and Gαi-*R*lucII. The AT1aR ligands resulted in the β2AR WT/AT1aR heterodimer venus tag changing its proximity to the *R*lucII tag on the Gαi subunit.

HEK293A cells were then transfected with the β2AR WT/AT1aR heterodimer co-expressing Gαq-*R*lucII and $G\beta_1\gamma_2$ subunits. When comparing NS and AT1aR ligand treated cells to the negative control (β2ARv1/AT1aRv2 + *R*lucII), there was a significant increase in the BRET ratio, which indicates Gαq-*R*lucII interaction with the β2AR/AT1aR heterodimer (Figure 3.8g). Both angiotensin II (0.08±0.02) and saralasin

(0.08±0.01) treatments resulted in a non-significant increase in the BRET ratio (Figure 3.8g). To further characterize this interaction, a co-immunoprecipitation was performed. The β2AR WT/AT1aR heterodimer was co-expressed with the $G\beta_1\gamma_2$ subunits along with an EE-tagged Gαq subunit. Treatment with angiotensin II and saralasin did not affect the amount of precipitated EE-Gαq when compared to NS cells (Figure 3.8h). Only saralasin treatment caused significant changes in the BRET ratio when compared to NS cells, and there was also a significant increase in the BRET ratio for NS and AT1aR ligand treated cells compared to the negative control. Precipitated EE-Gαq also remained constant following ligand treatment, which suggests there was a prior interaction between the β2AR WT/AT1aR heterodimer and Gαq-*R*lucII. The AT1aR ligands resulted in the β2AR WT/AT1aR heterodimer venus tag changing its proximity to the *R*lucII tag on the Gαq subunit, which suggests that conformational changes are occurring following AT1aR ligand treatment between the receptor dimer pairings and the heterotrimeric G-proteins.

**Figure 3.8: The Effect of AT1aR Ligand Treatments on Heterotrimeric G-Protein interactions with β2AR and AT1aR Homo- and Heterodimers**

The characterization of β2AR and AT1aR homo- and heterodimer pairing interactions with the heterotrimeric G-Protein subunits Gβ1, Gγ2, Gαs, Gαi, and Gαq following 15 minute 1μM angiotensin II and 1μM saralasin treatments. HEK293A cells used for BRET assays were harvested following ligand treatment and washed with 1X PBS and resuspended in 90μl of 1X PBS and coelenterazine *h* was added (5μM). For co-immunoprecipitations, HEK293A cells were harvested and washed with 1X PBS and lysed with RIPA buffer, followed by incubation with an anti-GFP antibody (1:2000). Samples were run on a 10% SDS-PAGE gel, transferred to nitrocellulose membranes and probed using an anti-HA antibody (1:2000) or an anti-EE antibody (1:2000). **A)** Effect of AT1aR ligand treatment on Gαq-*R*lucII interaction with the AT1aR homodimer. **B)** Effect of AT1aR ligand treatment on EE-Gαq interaction with the AT1aR homodimer. **C)** Effect of AT1aR ligand treatment on Gαs-*R*lucII interaction with the β2AR WT/AT1aR heterodimer. **D)** Effect of AT1aR ligand treatment on HA-Gαs interaction with the β2AR WT/AT1aR heterodimer. **E)** Effect of AT1aR ligand treatment on Gαi-*R*lucII interaction with the β2AR WT/AT1aR heterodimer. **F)** Effect of AT1aR ligand treatment on EE-Gαi interaction with the β2AR WT/AT1aR heterodimer. **G)** Effect of AT1aR ligand treatment on Gαq-*R*lucII interaction with the β2AR WT/AT1aR heterodimer. **H)** Effect of AT1aR ligand treatment on EE-Gαq interaction with the β2AR WT/AT1aR heterodimer. Results are representative of 3 independent experiments. **$p<0.01$ using a two-tailed unpaired Student's t-test. #$p<0.001$ using a two-tailed unpaired Student's t-test compared to negative controls.


A)
AT1aR V1 + AT1aR V2 + Gβ1 + Gγ2 + Gαq-RlucII
BRET1 Ratio
1.5
1.0
0.5
#
#
#
ATaR V1/ AT1aR V2 + RlucII
NS
Angiotensin II (1uM)
Saralasin (1uM)
B)
NS
Angiotensin II
Saralasin
IB: EE
IP: GFP
Lysate
IB: GFP
AT1aR V1 + AT1aR V2 + Gβ1 + Gγ2 + Gαq-EE
C)
β2AR WT V1 + AT1aR V2 + Gβ1 + Gγ2 + Gαs-RlucII
BRET1 Ratio
1.5
1.0
0.5
#
#
#
**
β2AR V1/AT1aR V2 + RlucII
NS
Angiotensin II (1uM)
Saralasin (1uM)
D)
NS
Angiotensin II
Saralasin
IB: HA
IP: GFP
Lysate
IB: GFP
β2AR WT V1 + AT1aR V2 + Gβ1 + Gγ2 + HA-Gαs
E)
β2AR WT V1 + AT1aR V2 + Gβ1 + Gγ2 + Gαi-RlucII
BRET1 Ratio
1.5
1.0
0.5
#
#
#
β2AR V1/AT1aR V2 + RlucII
NS
Angiotensin II (1uM)
Saralasin (1uM)
F)
NS
Angiotensin II
Saralasin
IB: EE
IP: GFP
Lysate
IB: GFP
β2AR WT V1 + AT1aR V2 + Gβ1 + Gγ2 + Gαi-EE
G)
β2AR WT V1 + AT1aR V2 + Gβ1 + Gγ2 + Gαq-RlucII
BRET1 Ratio
1.5
1.0
0.5
#
#
#
β2AR V1/AT1aR V2 + RlucII
NS
Angiotensin II (1uM)
Saralasin (1uM)
H)
NS
Angiotensin II
Saralasin
IB: EE
IP: GFP
Lysate
IB: GFP
β2AR WT V1 + AT1aR V2 + Gβ1 + Gγ2 + Gαq-EE

### *3.9 The Effect of Isoproterenol, Salbutamol, ICI 118, 551, Angiotensin II, and Saralasin Treatment on the Heterotrimeric G-proteins of β2ART164I, β2AR and AT1aR Heterodimers*

The β2ART164I polymorphism as mentioned earlier, was unable to promote coupling to the Gαs subunit of the heterotrimeric G-protein and showed impairment in both agonist and antagonist binding. We wanted to characterize what effect βAR and AT1aR ligand treatment will have on the coupling of the heterotrimeric G-proteins to β2ART164I/β2AR WT and β2ART164I/AT1aR heterodimers. HEK293A cells were transfected with the β2ART164I/β2AR WT heterodimer co-expressing Gαs-*R*lucII and $G\beta_1\gamma_2$ subunits. When comparing NS and βAR ligand treated cells to the negative control (β2ART164Iv1/β2ARv2 + *R*lucII), there was a significant increase in the BRET ratio, which indicates Gαs-*R*lucII interaction with the β2ART164I/β2AR heterodimer (Figure 3.9a). Both salbutamol (0.23±0.02) and ICI 118, 551 (0.08±0.01) treatments decreased the BRET ratio, with ICI 118, 551 being significant (Figure 3.9a). Isoproterenol treatment did not have any effect (0.02±0.03) on the BRET ratio compared to NS cells (Figure 3.9a). HEK293A cells were then transfected with the β2ART164I/β2AR heterodimer co-expressing Gαi-*R*lucII and $G\beta_1\gamma_2$ subunits. When comparing NS and βAR ligand treated cells to the negative control (β2ART164Iv1/β2ARv2 + *R*lucII), there was a significant increase in the BRET ratio, which indicates Gαi-*R*lucII interaction with the β2ART164I/β2AR heterodimer (Figure 3.9b). Isoproterenol treatment resulted in a non-significant increase (0.04±0.01) in the BRET ratio, whereas salbutamol (0.02±0.02) and ICI 118, 551 (0.01±0.02) treatments did not have any effect on the BRET ratio compared to NS cells (Figure 3.9b). The formation of the β2ART164I/β2AR WT heterodimer is not able to increase the BRET ratio when co-expressing the $G\beta_1\gamma_2$ subunits along with Gαs or Gαi, when treated with any of the βAR ligands. This could be due to the impaired ability

of the β2ART164I SNP to couple with heterotrimeric G-proteins following ligand treatment.

We then wanted to determine if the formation of β2ART164I/AT1aR heterodimers that were treated with both βAR and AT1aR ligands would show any differences in their ability to couple to the heterotrimeric G-proteins. We again used our BRET based assay to characterize these interactions. HEK293A cells were transfected with the β2ART164I/AT1aR heterodimer co-expressing Gαs-*R*lucII and $G\beta_1\gamma_2$ subunits. When comparing NS and ligand treated cells to the negative control (β2ART164Iv1/AT1aRv2 + *R*lucII), there was a significant increase in the BRET ratio, which indicates Gαs-*R*lucII interaction with the β2ART164I/AT1aR heterodimer (Figures 3.9c, d). Treatment with isoproterenol (0.01±0.02) did not produce a BRET ratio that was different from NS cells, but salbutamol (0.03±0.01), angiotensin II (0.06±0.01), and saralasin (0.05±0.01) treatments produced significant increases the BRET ratio compared to NS cell (Figures 3.9c,d). ICI 118, 551 treatment resulted in a non-significant decrease (0.02±0.003) of the BRET ratio compared to NS cells (Figure 3.9c). The results suggest that salbutamol, angiotensin II, and saralasin treatment are promoting a conformational change that brings the *R*lucII tag on the Gαs subunit closer to the β2ART164I/AT1aR heterodimer venus tag. Similarly, ICI 118, 551 treatment seems to promote a conformational change that increases the distance between the *R*lucII tag on the Gαs subunit and β2ART164I/AT1aR heterodimer venus tag.

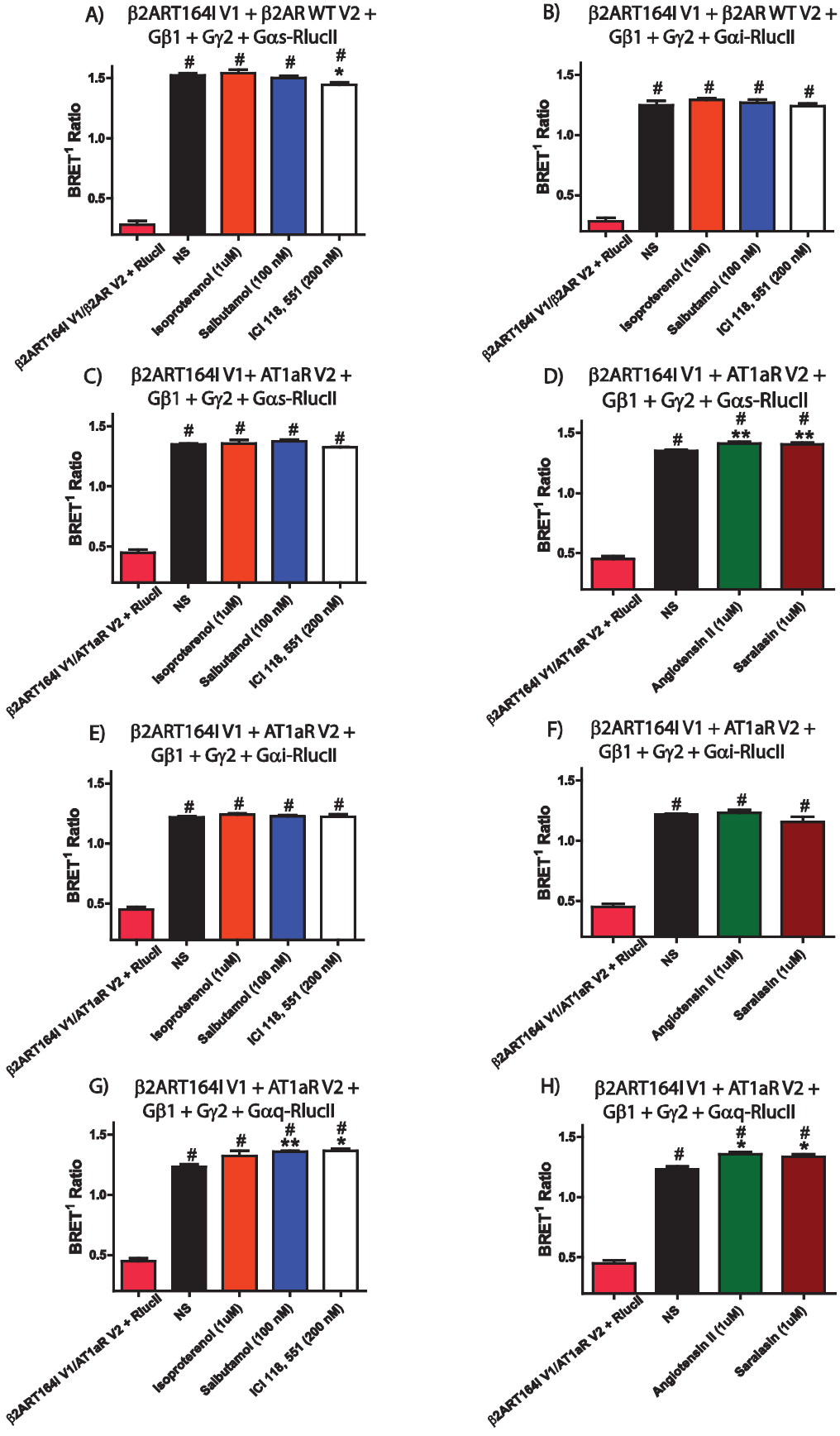
HEK293A cells were transfected with the β2ART164I/AT1aR heterodimer co-expressing Gαi-*R*lucII and $G\beta_1\gamma_2$ subunits. When comparing NS, βAR, and AT1aR ligand treated cells to the negative control (β2ART164Iv1/AT1aRv2 + *R*lucII), there was a

significant increase in the BRET ratio, which indicates Gαi-*R*lucII interaction with the β2ART164I/AT1aR heterodimer (Figures 3.9e, f). Treatment with isoproterenol (0.01±0.01), salbutamol (0.003±0.01), angiotensin II (0.003±0.02), saralasin (0.07±0.04) and ICI 118, 551 (0.01±0.02) did not cause any change in the BRET ratio compared to NS cells (Figures 3.9e, f). βAR and AT1aR ligand treatment did not have any effect on the β2ART164I/AT1aR heterodimer venus tag in relation to the *R*lucII tagged Gαi subunit.

HEK293A cells were transfected with the β2ART164I/AT1aR heterodimer co-expressing Gαq-*R*lucII and $G\beta_1\gamma_2$ subunits. When comparing NS and ligand treated cells to the negative control (β2ART164Iv1/AT1aRv2 + *R*lucII), there was a significant increase in the BRET ratio, which indicates Gαq-*R*lucII interaction with the β2ART164I/AT1aR heterodimer (Figures 3.9g,h).Treatment with isoproterenol (0.09±0.04), salbutamol (0.13±0.01), ICI 118, 551 (0.13±0.01), angiotensin II (0.12±0.01) and saralasin (0.10±0.02) all increased the BRET ratio significantly when compared to NS cells, except for isoproterenol (Figures 3.9g,h). This shows that βAR and AT1aR ligand interactions with the β2ART164I/AT1aR heterodimer results in conformational changes which brought the β2ART164I/AT1aR heterodimer venus tag in closer proximity to the *R*lucII tag on the Gαq subunit.

**Figure 3.9: The Effect of βAR and AT1aR Ligand Treatments on Heterotrimeric G-Protein interactions with β2AR, β2ART164I, and AT1aR Heterodimers**

The characterization of β2AR, AT1aR, and β2ART164I heterodimer pairing interactions with the heterotrimeric G-Protein subunits Gβ1, Gγ2, Gαs, Gαi, and Gαq following 15 minute 1μM isoproterenol, 100nM salbutamol, 200nM ICI 118, 551, 1μM angiotensin II, and 1μM saralasin treatments. HEK293A cells used for BRET assays were harvested following ligand treatment and washed with 1X PBS and resuspended in 90μl of 1X PBS and coelenterazine *h* was added (5μM). **A)** Effect of βAR ligand treatment on Gαs-*R*lucII interaction with the β2ART164I/β2AR WT heterodimer. **B)** Effect of βAR ligand treatment on Gαi-*R*lucII interaction with the β2ART164I/β2AR WT heterodimer. **C)** Effect of βAR ligand treatment on Gαs-*R*lucII interaction with the β2ART164I/AT1aR heterodimer. **D)** Effect of AT1aR ligand treatment on Gαs-*R*lucII interaction with the β2ART164I/AT1aR heterodimer. **E)** Effect of β2AR ligand treatment on Gαi-*R*lucII interaction with the β2ART164I/AT1aR heterodimer. **F)** Effect of AT1aR ligand treatment on Gαi-*R*lucII interaction with the β2ART164I/AT1aR heterodimer. **G)** Effect of βAR ligand treatment on Gαq-*R*lucII interaction with the β2ART164I/AT1aR heterodimer. **H)** Effect of AT1aR ligand treatment on Gαq-*R*lucII interaction with the β2ART164I/AT1aR heterodimer. Results are representative of 3 independent experiments. *$p<0.05$; **$p<0.01$ using a two-tailed unpaired Student's t-test. #$p<0.001$ using a two-tailed unpaired Student's t-test compared to negative controls.


A) β2ART164I V1 + β2AR WT V2 + Gβ1 + Gγ2 + Gαs-RlucII
B) β2ART164I V1 + β2AR WT V2 + Gβ1 + Gγ2 + Gαi-RlucII
C) β2ART164I V1+ AT1aR V2 + Gβ1 + Gγ2 + Gαs-RlucII
D) β2ART164I V1 + AT1aR V2 + Gβ1 + Gγ2 + Gαs-RlucII
E) β2ART164I V1 + AT1aR V2 + Gβ1 + Gγ2 + Gαi-RlucII
F) β2ART164I V1 + AT1aR V2 + Gβ1 + Gγ2 + Gαi-RlucII
G) β2ART164I V1 + AT1aR V2 + Gβ1 + Gγ2 + Gαq-RlucII
H) β2ART164I V1 + AT1aR V2 + Gβ1 + Gγ2 + Gαq-RlucII
BRET-1 Ratio
β2ART164I V1/β2AR V2 + RlucII
β2ART164I V1/AT1aR V2 + RlucII
NS
Isoproterenol (1uM)
Salbutamol (100 nM)
ICI 118, 551 (200 nM)
Angiotensin II (1uM)
Saralasin (1uM)

# Chapter 4: Discussion

### *4.1 General Overview*

GPCRs are the largest family of proteins that are present on the cell surface, and they are widely expressed throughout the body and play an integral role in maintaining physiological homeostasis. Given their high abundance in a multitude of organ systems, it is no surprise that GPCRs are highly targeted to treat disease states through the use of therapeutics. Progress in the last 10 years has demonstrated for various GPCRs that they are capable of forming dimers and higher order structures. There has been a lot of research that has focused on how specific receptors are capable of dimerizing with each other, but there are still aspects that remain unknown about dimerization. For example, the trafficking events following ligand binding to GPCR dimers such as PM localization and arrestin recruitment, along with the effectors that are involved is not fully understood in many GPCR dimers. In addition, characterizing the interactions that occur between heterotrimeric G-proteins and GPCR dimers to activate signalling cascades still remains largely unknown. The research that has been conducted in this body of work attempts to differentiate these issues. Characterizing dimerization between the β2AR WT, AT1aR, and the β2ART164I polymorphism, we were able to demonstrate that dimerization affects receptor trafficking following ligand binding by examining PM localization and recruitment of arrestin-2 in various receptor pairings. Interactions between receptor dimers and the heterotrimeric G-proteins were also investigated; addressing differences in how GPCR dimers can couple to different Gα subunits in homo- and heterodimers following βAR and AT1aR ligand treatments. This could offer new and improved avenues for pharmacological therapies to treat CVD.

The traditional model of GPCR pharmacology was that a ligand is able to bind to a monomeric receptor, which will either elicit or inhibit a biological response by coupling to the heterotrimeric G-proteins. Early studies were able to show that homodimerization was occurring in receptors such as the D2R, mGluR5, and the δ-OR[53, 123, 124]. Further research into the formation of GPCR dimers was able to demonstrate that heterodimers were also able to develop between such receptors as the $GABA_{B1}$ and $GABA_{B2}$ receptors, as well as in the taste receptors T1R1/T1R3 and T1R2/T1R3[51, 52]. The list of GPCRs that are capable of forming heterodimers has extensively grown, which can be seen in Table 4.1. This has been an emerging field of research that has been able to show that the formation of GPCR heterodimers alters the function and pharmacological properties of these receptors, which is why this area of research is of particular interest, for finding new therapeutics to treat various disease states.

Table 2
Heterodimerization of GPCRs

| Receptor pairs |
|---|
| *Heterdimerization between subtypes* |
| GABABR1 and GABABR2 |
| T1R2 and T1R3 |
| T1R1 and T1R3 |
| D2 and D3 dopamine |
| M2 and M3 muscarinic |
| delta- and kappa-Opioid |
| delta- and mu-opioid |
| SSTR1 and SSTR5 |
| SSTR2A and SSTR3 |
| SSTR2 and SSTR3a |
| 5-HT1B and 5-HT1D |
| beta1-Adrenergic and beta2-adrenergic |
| MT1R and MT2R |
| TRHR1 and TRHR2 |
| CCR2 and CCR5 |
| CCR2 (V64I) and CCR5 |
| CCR2 (V64I) and CXCR4 |
| S1P1 and S1P2 |
| S1P1 and S1P3 |
| S1P2 and S1P3 |
| *Heterdimerization between different receptors* |
| SST2A and mu-opioid |
| AT1 angiotensin and B2 bradykinin |
| SSTR5 and D2 dopamine |
| delta-Opioid and beta2-adrenergic |
| kappa-Opioid and beta2-adrenergic |
| A2A adenosine and mGluR5 |
| A2A adenosine and D2 dopamine |
| A1 adenosine and D1 dopamine |
| A1 adenosine and P2Y1 purinergic |
| mGluR and CaR |

**Figure 4.1 Heterodimerization of Various GPCR Receptor Subtypes**
GPCRs involved in similar and different physiological systems are capable of forming heterodimers [Bai, *Cellular Signalling* 2004; 16(2):175-186, with permission].

The physiological functions of the β2AR and AT1aR are modulated by their localization and activation at the PM. In states of CVD, each of these receptors is targeted either individually or in tandem by therapeutics, in order to treat symptoms that are associated with cardiovascular dysfunction. Compensatory mechanisms by the SNS increase circulating catecholamines, resulting in hyperactivity of the βARs, which increases heart rate and results in an increase in oxygen demand, ischemia, and oxidative stress[125]. Peripheral vasoconstriction is also common in CVD, and this causes an increase in blood pressure, which increases the workload of the failing heart. Salt and water retention is increased through aldosterone secretion, a hormone part of the RAS, which places further stress on the heart[126]. The β2AR is targeted using 'β blockers' and the AT1aR with 'angiotensin receptor blockers'. Both of these classes of therapeutics are antagonists for their respective GPCR[82, 83, 125, 127]. The ultimate goal with pharmacologic intervention in CVD is to reduce signal transduction from these receptors to their downstream effectors, which will help alleviate the symptoms of CVD.

Both the AT1aR and β2AR were originally shown to form homodimers. Studies investigating homodimerization in the AT1aR were able to demonstrate that it was occurring constitutively, independent of agonist and antagonist treatment, which were unable to affect AT1aR homodimer formation[128]. The β2AR was also initially shown to form homodimers through investigation of the TM domains. Specifically, TM domain VI was shown to be important in the formation of β2AR homodimers. This was confirmed using a peptide that was derived from TM VI. Pre-treatment of the β2AR homodimer with the TM VI peptide inhibited β2AR AC activity when exposed to an agonist[59]. This effect was not seen in the D1R when pre-treated with the TM VI peptide and stimulated

with an agonist, suggesting that TM VI is important for β2AR homodimer formation. Further work was able to demonstrate that the AT1aR and β2AR were capable of forming heterodimers, and that heterodimerization altered the pharmacology of the β2AR/AT1aR receptor complex[56]. With growing evidence supporting GPCR heteromerization, it is clear that heterodimers are in fact biologically relevant. However, the main issue is there are few studies that have tried to characterize how heterodimers themselves function. The formation of heterodimers can generate new pharmacological properties, where specific receptor dimers can be targeted that will provide important information about ways to modulate signal cascades to direct physiological function. For example, the synthetic agonist SKF83959 is capable of binding only the D1/D2 heterodimer, but not the D1 or D2 receptors individually[71]. Another example occurs in the opioid receptors, where the opioid agonist 6'-GNTI is only capable of activating OR heterodimers, and not OR homodimers[70]. The use of therapeutics that can specifically target tissue specific dimers will help to reduce collateral effects, which are often the result of non specific signal transduction[57].

As with other genes encoded by the human genome, the β2AR gene, *ADRB2*, is polymorphic. There are three important variants of the ADRB2. One variant occurs at amino acid position 16, where glycine is substituted for arginine (Arg16Gly). This polymorphism has been associated with the occurrence of asthma, specifically affecting the responsiveness of the β2AR to agonists, which affects the bronchial tone[129]. The second variant occurs at amino acid position 27, which has glutamine in the place of glutamic acid (Gln27Glu). This polymorphism is associated with a positive effect in the cardiovascular system. Patients that experienced idiopathic heart failure were less likely

experience death or undergo heart transplantation with the Gln27Glu polymorphism[130]. The third important variant occurs at amino acid position 164, where threonine is replaced by isoleucine (Thr164Ile). It is the polymorphism at this position that has been chosen to be investigated by this study because it displays a decrease in binding affinity for catecholamines along with β2AR agonists and antagonists[108]. As well, this polymorphism has been implicated in a decreased survival rate in patients that develop congestive heart failure. Patients that possess the β2ART164I polymorphism and are diagnosed with congestive heart failure experience a 42% one year survival rate, whereas those with WT β2AR that are diagnosed with congestive heart failure experience a 76% one year survival rate[106]. We sought to determine how physical interactions between the β2ART164I, WT β2AR, and AT1aR can affect the signalling of these receptors to see what functional consequences would arise. Previous studies have investigated the interactions of a WT and mutated GPCR. For example, truncated versions of the VV2R were co-expressed with the WT VV2R, forming heterodimers, and the truncated versions acted in a dominant negative manner, causing intracellular retention of the heterodimer[75]. This phenomenon was also observed in CCR5 heterodimers, where the expression of a truncated receptor (CCR5Δ32) along with WT CCR5, resulted in the intracellular retention of the entire dimer[73]. As well, a non-palmitoylated mutated version of the β2AR (C341Gβ2AR) when expressed on its own was shown to have a decreased response to agonists and decreased desensitization compared to the WT β2AR. However, when co-expressing the C341Gβ2AR along with the WT β2AR as a heterodimer, agonist response and desensitization was comparable to that of the WT β2AR, suggesting that dimerization had led to the rescue of the C341Gβ2AR[118]. Understanding the role of polymorphisms in

GPCR dimers has clinical significance. For example, patients that posses the CCR5Δ32 mutation in a heterozygous form have been shown to have a slower onset of human immunodeficiency virus, which uses CCR5 as a means of viral entry. As well, it is important to understand how a polymorphism will affect a patient's ability to respond to therapeutic intervention. This brings to light the concept of pharmacogenetics, which links a patient's genetic background to tailored therapies to ensure efficacious results.

### *4.2 The Formation of β2AR, β2ART164I, and AT1aR Homo- and Heteromers Affects Trafficking Following Ligand Treatments*

GPCR dimerization can influence the trafficking of receptors, and one of the well known examples of this occurs in the $GABA_B$ receptor. The $GABA_B$ receptor forms obligate heterodimers in order to allow PM localization, by masking an ER retention motif on $GABAB_{B1}$ that occurs during dimerization with $GABA_{B2}$. This was an early example that demonstrated how the formation of GPCR heterodimers was important for the proper expression of the $GABA_B$ heterodimer during its anterograde trafficking. Previous work investigating anterograde trafficking of β2AR and AT1aR homo- and heterodimers was able to demonstrate that the molecular chaperone ERp57 was only able to associate with AT1aR homodimers and β2AR/AT1aR heterodimers, but not β2AR homodimers[79]. As discussed earlier, the formation of heterodimers was shown to alter the pharmacology of the individual receptors in the signalling complex. One aspect involved in heterodimer formation is the differences in patterns of internalization that occur, which varies, depending upon the receptors involved. The β2AR was shown to dimerize with the δ-OR, and the heterodimer was able to internalize in response to the binding of either opioid or adrenergic ligands while conserving ligand selectivity[85]. However, when the

β2AR formed heterodimer pairings with the κ-OR, a GPCR that does not internalize, adrenergic agonist binding was unable to result in the endocytosis of the receptor complex. This shows how dimerization can not only alter the pharmacology of the receptors, but it also alters trafficking following ligand binding. The μ-OR and substance p receptor (NK1) are also able to form heterodimers. This heterodimer pairing also exhibits altered trafficking in response to agonist stimulation. When either receptor in the heterodimer is individually targeted with an endogenous agonist, it was demonstrated to be sufficient to promote internalization of the entire heterodimeric complex[57]. Formation of GPCR heterodimers can also generate disadvantageous properties as well. Heterodimerization between the β1AR and β2AR causes the β2AR to become resistant to internalization following isoproterenol stimulation, much like the β1AR is when expressed on its own. Furthermore, β1AR/β2AR heterodimers were unable to activate ERK1/2 signalling after isoproterenol stimulation, again in a similar manner to the β1AR[131].

As mentioned earlier, the β2AR was shown to dimerize with the AT1aR, both *in vitro* and in mouse cardiomyocytes. However, there have not been studies to date that have looked at the trafficking of these two receptors as a heterodimer following ligand binding. βAR and AT1aR ligands were used to determine if there were differences in trafficking of the β2AR/AT1aR heterodimer. PM localization of the heterodimer was observed, and this was used as an indication of the rate of receptor internalization that was occurring following ligand treatments. Isoproterenol (non selective βAR agonist), salbutamol (β2AR selective agonist), ICI 118, 551 (β2AR antagonist), angiotensin II (AT1aR agonist), and saralasin (AT1aR partial agonist) were the ligands used to

determine if differences in heterodimer PM localization occurred following ligand treatment. In the AT1aR homodimer, treatment with angiotensin II was sufficient to promote a decrease in PM localization, whereas saralasin treatments did not have any effect. However, the β2AR/AT1aR heterodimer when treated with both angiotensin II and saralasin exhibited a decrease in the PM localization of the heterodimer complex. This reiterates the fact that the heterodimers can generate new pharmacological properties, and in this case we see this as an altered trafficking itinerary.

There have been a few studies that have investigated the effects of β2AR SNPs in heterodimer pairings. The study by Hebert *et al*., 1996, investigated the effect of a β2AR (Cys341Gly) mutation, which exhibits decreased sensitivity and internalization following agonist binding. They were able to demonstrate that when heterodimers were formed between the WT β2AR and Cys341Gly β2AR, it was able to rescue the mutated receptor so that it was able to respond to agonist stimulation and internalize as one would expect[118]. Another study investigating the insertion of an alanine residue into the c-tail of the β2AR, β2AR-ala, found that it promoted lysosome degradation, rather than recycling of the receptor back to the PM following agonist binding. β2AR-ala was then expressed with the WT β2AR to form a heterodimer, but in this case co-expression with the wild type heterodimer was not sufficient to rescue the function of the β2AR-ala, the entire heterodimeric complex was shown to undergo degradation rather than recycling[132]. The β2ART164I polymorphism was previously shown to have decreased affinity for agonists, and also exhibit a decrease in receptor internalization following the binding of agonists. However, given the prior information about dimerization influencing the trafficking of mutated β2ARs, we wanted to determine if heterodimers formed with the β2AR and

AT1aR would alter trafficking of the β2ART164I following ligand binding. When β2ART164I/ WT β2AR heterodimers were treated with βAR ligands, there was a noticeable increase in PM localization of the heterodimer, which could be due to newly synthesised heterodimer complexes reaching the PM faster than the agonist bound complexes are being internalized. However, the formation of β2ART164I/AT1aR heterodimers treated with βAR ligands do show a decrease in PM localization. The formation of β2ART164I/AT1aR heterodimers may be sufficient to decrease PM localization by permitting the ligands to act on the AT1aR in the heterodimer complex, promoting receptor complex internalization. This again demonstrates how the formation of heterodimers consisting of different receptor subtypes can influence the pharmacology of the receptor complex by exhibiting different biological effects following the binding of different classes of ligands. This demonstrates the importance of dimerization, and how it can alter the effects that specific ligands are capable of eliciting.

The recruitment of arrestins following the activation of GPCRs is well documented[92, 133]. The recruitment of arrestin-2 is a crucial step in the desensitization and recycling process, as it acts as a scaffold protein to facilitate receptor internalization. However, arrestin recruitment in GPCR dimers is an area of GPCR research that is not fully understood. One of the well documented examples involves the vasopressin V1a receptor (VV1aR) and VV2R heterodimers. The formation of the V1aR/VV2R heterodimer alters the arrestin recruitment profile compared to the individual receptors when expressed on their own. The VV1aR when expressed alone, has a transient interaction with arrestin-2 following agonist binding, and then rapidly dissociates from it. On the other hand, the VV2R forms a stable complex with arrestin-2 following agonist

binding. When the VV1aR/VV2R heterodimers are formed and treated with an agonist, they follow the arrestin-2 recruitment profile of the VV2R receptor[134].

Since the β2AR and AT1aR had been shown to form heterodimer pairings previously, we were then interested to determine how this may alter the arrestin recruitment profile following ligand treatments. When observing βAR ligand treatments on the β2AR homodimer and β2AR/AT1aR heterodimer, ICI 118, 551 was able to increase the amount of arrestin-2 recruitment in the β2AR homodimer, but not the β2AR/AT1aR heterodimer. ICI 118, 551 has previously been characterized to act as a protean agonist. It has demonstrated inverse agonist activity for coupling to Gαs and agonist activity for Gαi coupling in the β2AR[135]. Computational biochemical studies have shown that ICI 118,551 is able to stabilize the β2AR receptor confirmation to that of the inactive state, which illustrates that ICI 118,551 is acting independently of the G-protein dependent mechanisms to recruit arrestins up to the dimeric receptor complexes[42, 136]. Differences in the arrestin-2 recruitment profile were also observed when comparing the β2ART164I/β2AR WT and β2ART164I/AT1aR heterodimers. βAR ligand treatment on the β2ART164I/β2AR WT heterodimer was unable to promote arrestin-2 recruitment; however isoproterenol and salbutamol treatments were able to increase arrestin-2 interactions with the β2ART164I/AT1aR heterodimer. When treating the AT1aR homodimer, β2AR WT/AT1aR and β2ART164I/AT1aR heterodimers with angiotensin II or saralasin, there were no differences in arrestin-2 recruitment; except that AT1aR homodimer interactions with arrestin-2 was more pronounced following angiotensin II treatment. Thus, the formation of heterodimer pairings has an effect on trafficking of the receptor complexes following ligand binding.

### *4.3 The Inhibition of β2AR and AT1aR Heterodimers by a Single Antagonist is Disrupted when β2ART164I and AT1aR Heterodimers are Formed*

Originally, the β2AR and AT1aR were thought to act as separate entities in the RAS and adrenergic systems, which are both involved in regulation of cardiovascular function. However, the β2AR and AT1aR have been shown to form heterodimers, interconnecting the RAS and adrenergic systems. Barki-Harrington *et al*. 2003, were able to demonstrate that pre-treatment with an antagonist of either the β2AR or AT1aR rendered the heterodimeric complex unable to further activate ERK phosphorylation following agonist treatment. These types of studies attempt to understand how physical interactions resulting from dimerization will affect the ability of the receptor complex to bind ligands and elicit biological effects. The term 'crosstalk' has been coined to describe the interactions that can occur as a result of two receptors affecting the signalling of another. However, this term has also been used loosely to describe GPCRs that dimerize and affect how each will function in the larger receptor complex. The extent of GPCR interactions that can occur is rather diverse, ranging from conformational changes to swapping of domains and modulation of signal transduction. The histamine H1 (H1) receptor is able to form homodimers, and it was demonstrated that during homodimer formation TM domains VI and VII are able to be swapped between reciprocal H1 receptors in the homodimer[137]. Furthermore, the occurrence of cross-inhibition between GPCR dimers can affect the pharmacology of the receptor complex. When a dimer is formed, the orthosteric ligand binding site of one protomer can act as an allosteric binding site for the reciprocal protomer. For example, receptor 'A' and receptor 'B' can form heterodimers. The orthosteric ligand binding site on receptor 'A' can act as an allosteric binding site for receptor 'B', generating new pharmacological properties. This

type of activity has been demonstrated in D2R dimers, where binding of the D2R antagonist raclopride results in negative cooperativity for its own binding and also to that of the D2R antagonist spiperone[138]. In the study that investigated heterodimer formation between the β2AR WT and β2AR C341G, they were able to show that agonist occupancy of either protomer in the heterodimer was sufficient to promote internalization of the dimer, indicating that cross-inhibition may be ocurring[117]. It was also shown that the inactivation of either protomer in the AT1aR homodimer resulted in cross inhibition of the reciprocal AT1aR protomer, rendering it unable to bind its G-protein[139]. The aforementioned studies show how diverse GPCR dimer cross-inhibition can be, ranging from a variety of receptors involved in different physiological systems.

Activation of ERK1/2 can be accomplished by different GPCR signal inducers. A variety of GPCRs can contribute to G-protein dependent and G-protein independent mechanisms of signal transduction, which all seem to converge at ERK1/2. Thus, ERK1/2 can be used as a measure of GPCR receptor activation resulting from ligand binding. The method that was used to investigate ERK1/2 activation was based on an overexpression system using β2AR/AT1aR heterodimers. Although endogenous GPCRs present in HEK293A cells may contribute to ERK1/2 phosphorylation, we can expect that the majority of ERK1/2 activation will result from the overexpression of the specific receptor pairings that were transfected. When the β2AR WT/AT1aR heterodimer was pre-treated with a β2AR (ICI 118,551) or AT1aR (saralasin) antagonist, subsequent agonist (isoproterenol, salbutamol, or angiotensin II) treatment was unable to activate ERK1/2 in that heterodimer pairing. However, βAR or AT1aR agonist treatment on its own was sufficient to result in ERK1/2 activation. Our results suggest that a mechanism

of transinhibition is occurring through the inhibition of either protomer in the β2AR WT/AT1aR heterodimer. This could be a result of receptor cross-inhibition in the heterodimer, whereby the inhibition of a single receptor is sufficient to inactivate the entire signalling complex. The phenomenon of receptor transinhibition was also shown to occur in the $B_1$ bradykinin and $B_2$ bradykinin heterodimers. The inhibition of either the $B_1$ or $B_2$ protomer in the $B_1/B_2$ heterodimer was sufficient to prevent the receptor complex from further activating cell mediated signalling events[140].

The β2ART164I SNP was then paired with the AT1aR to determine how a β2AR with compromised ability to promote signal transduction would affect the ability of the heterodimer to activate ERK1/2 signalling. Previous studies that observed a mutated GPCR were able to show that in most cases, a heterodimer pairing was sufficient to restore regular activity. However, the formation of β2ART164I/AT1aR heterodimers was unable to do the same. Treatment with the βAR agonists isoproterenol and salbutamol, along with the AT1aR agonist angiotensin II, were able to activate ERK1/2. However, pre-treatment of the β2ART164I/AT1aR heterodimer with the β2AR antagonist ICI 118,551, followed by angiotensin II treatment did not prevent further ERK1/2 phosphorylation as was seen in the β2AR WT/AT1aR heterodimer. This effect was also observed when pre-treating the β2ART164I/AT1aR heterodimer with the AT1aR antagonist saralasin, followed by salbutamol treatment did not prevent further ERK1/2 activation. The formation of the β2ART164I/AT1aR heterodimer renders the signalling complex unable to become inactivated by either βAR or AT1aR antagonist treatment. The presence of the β2ART164I SNP may result in a conformational arrangement with

the AT1aR that will not promote the inactive state of the receptor complex, allowing further agonist treatment to continue to activate ERK1/2 signalling.

### *4.4 βAR and AT1aR Ligand Promoted Interactions Between β2AR, AT1aR, and β2ART164I Homo- and Heterodimers Reveal Pre-Assembly of Receptor Signalling Complexes*

G-protein activation is initiated by a ligand binding to a receptor, which causes the exchange of GDP for GTP on the Gα subunit of the heterotrimeric G-protein. Depending upon the Gα class that is coupled to the GPCR, ligand binding may result in either inhibition (i.e. Gαi) or activation (i.e. Gαs) of signalling pathways such as the case with Gαs,i in the production of cAMP. GPCRs usually couple to a specific Gα subunit and we were interested in determining if the formation of β2AR and AT1aR homo- and heterodimers would alter the coupling of Gα subunits, as well as allow coupling to Gα subunits that they do not usually interact with. The formation of dimers in other GPCRs has been shown to alter their ability to bind to different Gα subunits. For example, the formation of CCR2/CCR5 heterodimers promotes the coupling to Gαq/11, which is unique to the heterodimer because CCR2 and CCR5 do not normally couple to the Gαq/11 subunit[111]. Similar findings were observed in D1/D2 heterodimers. D1 GPCRs couple to Gαs or Gαi, but when the D1/D2 heterodimer is formed it is able to couple to Gαq/11 and activate signalling cascades independently of the Gαs and Gαi subunits[141].

Trafficking of GPCR dimers along with the signalling complexes that they interact with were initially thought to occur independently of each other. Recent work investigating the trafficking of GPCR dimers has shown otherwise. Inward rectifying $K^+$ channels (Kir) are involved in maintaining the resting membrane potential of excitable

and non-excitable cells. Members of the Kir3 family are associated with GPCRs, and Kir3 can be activated in response to ligand stimulation of the GPCR itself[142]. There are four subtypes to the Kir3 family, Kir3.1-Kir3.4, and these are expressed as homo- and heterooligomeric structures. It was determined that Kir3.1 was only able to reach the PM if was able to form heterodimeric complexes with Kir3.2 or Kir3.4. However, Kir3.1 was found to interact with GPCRs in the absence of Kir3.2 or Kir3.4 before it was localized at the PM, suggesting that an interaction is occurring during the trafficking of the signalling complex[143]. A study of the β2AR and adenylyl cyclase II (ACII) along with the heterotrimeric G-proteins was also able to demonstrate that all of these signalling partners were being trafficked together before their PM localization[144]. The concept of pre-assembled signalling complexes was solidified by using the dominant negative version of Rab1, and Sar1, both of which are GTPases that can block transport from the ER to golgi apparatus. When Rab1 or Sar1 were expressed with β2AR, ACII, and the heterotrimeric G-proteins, they did not affect β2AR, ACII, or Gβγ interactions. The Gαs subunit was however, blocked, but this suggests that it is added to the signalling complex at a later trafficking stage. Taken together, the results from these studies suggest that interaction between GPCRs and their signalling complexes can occur early during their synthesis in the ER, and that they are pre-packaged together during their transport to the PM. These interactions were also shown to occur free of agonist or antagonist activity, showing pre-assembly of the receptor and effector signalling complexes is occurring constitutively.

Since the β2AR and AT1aR are known to dimerize, we wanted to investigate how the formation of β2AR and AT1aR homo- and heterodimers treated with βAR and AT1aR ligands would affect coupling to the Gα subunit of the heterotrimeric G-protein.

Experiments were carried out that utilized two main techniques. The first technique employed a BRET-based assay, where the Gαs subunit was tagged with *R*lucII and the receptor dimer pairings form a functional YFP variant, to observe how the physical proximity of the receptor tagged YFP and Gαs tagged *R*lucII changes when treated with βAR and AT1aR ligands. In all receptor dimer pairings co-expressing the heterotrimeric G-protein subunits, there was a significant increase in the BRET ratio of basal level interactions and also after ligand treatment when compared to the negative controls (receptor dimers and *R*lucII only). This result suggested that the receptor dimer pairings, along with the Gβγα subunits are being pre-packaged and trafficked to the PM, where ligand binding can induce conformational changes to affect signal transduction. For example, in the β2AR WT homodimer co-expressed with $G\beta_1\gamma_2$ and Gαs-*R*lucII, ICI 181,551 treatment induces a decrease in the BRET ratio. When the β2AR WT/AT1aR heterodimer is co-expressed with $G\beta_1\gamma_2$ and Gαi-*R*lucII and treated with angiotensin II and saralasin, we observe a significant increase and decrease respectively, in the BRET ratio. This further suggested that ligand treatment was resulting in conformational changes between the receptor dimers and the heterotrimeric G-protein subunits as a way to modulate intracellular signal cascades. To confirm this, co-immunoprecipitation experiments were able to reveal that interaction levels between the receptor dimer pairings and the Gα subunits remained constant following ligand treatments. For example, the β2AR WT/AT1aR heterodimer co-expressed with $G\beta_1\gamma_2$ and EE-Gαi following angiotensin II and saralasin treatments did not increase or decrease the interaction levels with Gαi following ligand treatments. Furthermore, similar to D1/D2 heterodimers, we were able to demonstrate that the formation of β2AR WT/AT1aR

heterodimers is able to promote the coupling to Gα subunits that the β2AR WT and AT1aR do not couple to on their own. The β2AR WT/AT1aR heterodimer co-expressed with $G\beta_1\gamma_2$ and Gαq-*R*lucII treated with isoproterenol results in an increase in the BRET ratio, which suggests that isoproterenol is able to induce conformational changes between Gαq tagged *R*lucII and the YFP tagged β2AR WT/AT1aR heterodimer.

Similarly, β2ART164I/β2AR WT and β2ART164I/AT1aR heterodimers were treated with both βAR and AT1aR ligands to look into interactions between the heterotrimeric G-proteins and the receptor dimer pairings. For this study, only the BRET-based assay was performed. Again, ligand treatment was able to result in conformational changes between the YFP tagged receptor dimers and the *R*lucII tagged Gα subunits. For example, salbutamol and ICI 118, 551 treatments on the β2ART164I/ β2AR WT heterodimer co-expressing $G\beta_1\gamma_2$ and Gαs-*R*lucII were able to significantly decrease the BRET ratio. In the β2ART164I/ AT1aR heterodimer co-expressing $G\beta_1\gamma_2$ and Gαq-*R*lucII, angiotensin II and saralasin treatments both promoted an increase in the BRET ratio. The differences observed in the BRET ratios are also thought to be a result of conformational changes that occur as a result of ligand binding, however to ultimately confirm this, co-immunoprecipitation experiments are necessary to confer protein interaction levels between the receptor dimer pairings and the Gα subunits.

### *4.5 Clinical Significance of Dimerization Between the β2AR, β2ART164I, and AT1aR*

This study was able to demonstrate that differences exist between β2AR, β2ART164I, and AT1aR homo- and heterodimers in terms of their ability to interact with βAR and AT1aR ligands. Given the fact that investigation into GPCR dimerization has

increased over the past 10 years, the information that we are able to contribute to the field is imperative for the increase of this knowledge base. Having a solid grasp on the fundamentals associated with β2AR and AT1aR homo- and heterodimerization and how a polymorphism such as the β2ART164I can change these interactions is important. Our findings show that GPCR dimerization has to be taken into account in terms of clinical medicine. For example, in states of CVD the first line of treatment is often a combination of β-adrenergic and AT1aR antagonists. Given that β2AR/AT1aR heterodimers have been demonstrated in mouse cardiomyocytes[56], the use of an antagonist for either receptor may cause the inhibition of the entire signalling complex, as we were able to confirm. This poses a double-edged sword, where a therapeutic benefit could exist by only having to prescribe a single antagonist to inhibit an entire signalling complex; a single β2AR or AT1aR antagonist could be used to prevent signal transduction associated with β2AR/AT1aR heterodimers. However, this could also result in unwanted inhibition of signalling pathways, which could have serious outcomes. The β2AR is expressed throughout the body in the liver, lung, brain, heart, and muscle and the AT1aR is expressed in the brain, heart, peripheral vasculature, adipose tissue, pancreas, and kidney[88, 145]. Given the widespread expression profile for both receptors and their ability to form dimers, if an AT1aR antagonist were given, this could cause the β2AR to become unresponsive to endogenous agonists in the peripheral vasculature, and potentially result in vasoconstriction. A study of valsartan, an AT1aR antagonist, was performed in heart failure patients that were receiving a combination therapy of β-adrenergic antagonists, AT1aR antagonists, and ACE inhibitors. Their study found a high rate of adverse events associated with combination therapies[83]. Combination therapies may inhibit signalling in

the adrenergic and RAS to a level that is unsustainable. Not accounting for GPCR dimerization between the β2AR and AT1aR could further inhibit signalling, which could be a potential causation for the high rate of adverse events associated with combination therapies to treat CVD.

The formation of heterodimers creates the opportunity to activate a broader range of signalling cascades because GPCR heterodimers are able to couple to different Gα subunits. This presents the ability to create pharmacological agents that are directed at specific GPCR dimers to either activate or inhibit certain signalling pathways to alleviate disease symptoms. Understanding the physiological effects that are associated with dimerization is important. In this case, the formation of β2AR WT/AT1aR heterodimers can promote coupling to Gα subunits that the individual receptors may not couple to, increasing the diversity of signal transduction opportunities. Like any structure, we must start from the bottom and build our way up when attempting to understand GPCRs. Dimerization adds a complex dimension to an already multifaceted subject matter. Understanding how the formation of dimers can change the biological responses obtained is extremely valuable. In some cases, interacting partners in GPCR dimers may result in an opposite response to what is observed with the monomeric receptor subtypes expressed on their own. However, a fundamental understanding of the molecular interactions of GPCR dimerization will allow therapeutics to directly target specific dimeric receptor complexes. Heterodimerization affects the response of the receptor signalling complexes to drugs, which has particular clinical relevance because heterodimers show altered receptor pharmacology, signalling, and trafficking. Eventually,

specific therapies may be tailored to certain receptor subtypes so that drug side effects can be minimized.

The next step for this body of work would be working towards an animal model. In an animal model, a variety of techniques could be implemented to study dimerization between the β2AR and AT1aR. Knockout mice of the β2AR and AT1aR could be used and then the β2AR and AT1aR could be co-expressed as dimers in the animal to gain an understanding of how dimerization affects not only the response to ligands, but also on a systemic level of physiological functioning. Moving toward a whole organism will most certainly lead to a more complex system, but it will allow a thorough understanding of GPCR dimerization. The animal model also lends itself to developing therapeutics that may specifically act against GPCR dimers to treat different disease states while maintaining the physiological role of the non-pathological GPCRs, effectively reducing unwanted therapeutic effects that are often the case of drug side effects.

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