SECTION 5: THESIS DETAILS

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DEDICATION
To my family,

ACKNOWLEDGMENTS*

I would firstly like to present my deepest gratitude to my supervisor Assoc. Prof. Dr. Çağdaş D. SON, without whom this thesis study would be neither started nor completed. I owe him a lot for his endless moral support, guidance, encouragement, tolerance, patience and understanding. I will always be grateful to him in my entire academic life.

I would also like to thank the members of my thesis examining committee; Assist. Prof. Dr. Özge Şensoy, Assoc. Prof. Dr. Tülin Yanık, Assoc. Prof. Dr. Sreeparna Banerjee, Assoc. Prof. Dr. Nihal Terzi Çizmecioğlu for their invaluable suggestions and useful critiques to complete this thesis better.

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I also want to thank my roommate Osman Kayahi, my team mates Ekin Berkyürek, Mertcan Pamuk, my lab mate Furkan Oflaz, and my friends Onur Demir, Gökçe Abay, Ezgi Güleç. They always supported me in my life and make myself a better person in life.

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ANALYSIS OF INTERACTIONS BETWEEN BETA-2 ADRENERGIC RECEPTOR & BETA ARRESTIN-2 USING FÖRSTER RESONANCE ENERGY TRANSFER (FRET) METHOD IN LIVE CELLS

A THESIS SUBMITTED TO THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES OF MIDDLE EAST TECHNICAL UNIVERSITY

BY [HÜSEYİN EVCI]

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN BIOCHEMISTRY

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ANALYSIS OF INTERACTIONS BETWEEN BETA-2 ADRENERGIC RECEPTOR & BETA ARRESTIN-2 USING FÖRSTER RESONANCE ENERGY TRANSFER (FRET) METHOD IN LIVE CELLS

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Cells communicate with their environment and with other cells using receptors. Receptor takes signal from outside and transmits it into the cell. After transmitting the signal, receptors could be desensitized and/or endocytosed to stop signal transduction. Following receptor activation many receptors got phosphorylated which will trigger additional protein-protein interactions that could lead to receptor internalization. In the case of Beta-2 Adrenergic receptor ($\beta_2$AR), key protein recognising the phosphorylation is Beta Arrestin-2. It is known that in the C terminus of the $\beta_2$AR, 4 amino acids get phosphorylated and play role in the interaction between $\beta_2$AR and beta arrestin-2.

In this thesis, we planned to further investigate the importance of each one of these 4 amino acids on the interaction by site directed mutagenesis. FRET technique will be used for the quantitative analysis of the interaction in live cells. For this purpose, mutations were done on $\beta_2$AR. After mutations, both receptor and the $\beta$-arrestin 2 protein tagged with both mEGFP and mCherry. Receptor was tagged from 5 different places and $\beta$-arrestin 2 was tagged from both N and C terminus. Receptor
and β-arrestin 2 were cotransfected together to *Mus musculus* Neuroblastoma-2a (N2a) cells as various combinations. Images were taken via spinning disc confocal microscopy to analyze the possible interactions, and FRET efficiency was calculated by using pixFRET Plugin for ImageJ software. Single residue mutations were had a slightly increased FRET signal than wild-type receptor except S355A. One of the two residue mutations (S355A-S356G) was had no interaction with β-arrestin 2 while other one was had the same FRET signal with wild type receptor. Further mutations and experiments can be done to understand which residue is more important for the interaction between β2AR and β-arrestin 2.

Keywords: Beta-2 Adrenergic Receptor, Beta-Arrestin 2, Receptor Internalization, FRET, Site-Directed Mutagenesis

Bu tezde bu 4 amino asidin önem, amino asitleri tek tek mutasyona uğratarak daha iyice araştırılacaktır. Etkileşimin nicel analizini yapmak için FRET tekniği uygulanacaktır. Bu sebepten ötürü \( \beta_2 \)AR üzerinde mutasyonlar yapılmıştır. Mutasyonlardan sonra reseptör ve \( \beta \)-arrestin 2 proteini, mEGFP ve mCherry floresan proteinleri ile işaretlenmiştir. Reseptör 5 farklı bölgeden işaretlenirken, \( \beta \)-arrestin 2

Anahtar Kelimeler: Beta-2 Adrenerjik Receptör, Beta-Arrestin 2, Receptör İnternalizasyonu, FRET, Bölgeye Yönelik Mutasyon
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>Adenylyl Cyclase</td>
</tr>
<tr>
<td>AD</td>
<td>Activator Domain</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphate</td>
</tr>
<tr>
<td>AGE</td>
<td>Agarose gel electrophoresis</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>AP2</td>
<td>Adaptor protein 2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>β2AR</td>
<td>Beta-2 Adrenergic Receptor</td>
</tr>
<tr>
<td>Barr2</td>
<td>Beta-Arrestin 2</td>
</tr>
<tr>
<td>BiFC</td>
<td>Bimolecular Fluorescence Complementation</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BRET</td>
<td>Bioluminescence Resonance Energy Transfer</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>CCV</td>
<td>Clathrin-Coated Vesicles</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA Binding Domain</td>
</tr>
<tr>
<td>D-MEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>D-PBS</td>
<td>Dulbecco’s Phosphate Buffered Saline</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EE</td>
<td>Early Endosomes</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor Receptor</td>
</tr>
</tbody>
</table>
FBS  Fetal Bovine Serum
FRET  Förster Resonance Energy Transfer
GEF  GTP Exchange Factors
GFP  Green Fluorescent Protein
GPCR  G Protein Coupled Receptors
GRK  GPCR Related Kinase
GRK2  GPCR Related Kinase 2
kb  kilo base pair
LB  Luria Bertani
MAPK  Mitogen-Activated Protein Kinase
mCherry  Monomeric Cherry
mEGFP  Monomeric Enhanced Green Fluorescent Protein
MVB  Multivesicular Bodies
N2a  Neuro2a
NMR  Nuclear Magnetic Resonance
PBS  Phosphate Buffered Saline
PCR  Polymerase Chain Reaction
PKA  Phosphokinase A
PLC  Phospholipase C
PPI  Protein-Protein Interaction
RTK  Receptor Tyrosine Kinases
SDM  Site-Directed Mutagenesis
SE  Sorting Endosomes

xx
CHAPTER 1

INTRODUCTION

1.1. G Protein-Coupled Receptors

There are cells that are capable of living by themselves like bacteria, yeast, etc. and there are cells that come together to make more complex organisms. Whether in these more complex organisms or as unicellular organisms, cells have to communicate with other cells to organize functions such as movement, feeding, protection and many more. They use small protein structures called receptors for communication. Through receptors, cells can communicate with the environment, with nearby cells, even with cells that are very far apart. Using these receptors they can react to stimulus around them. G protein-coupled receptors (GPCRs) are the largest receptor family in eukaryotes, having nearly 800 different types in humans alone (Insel et al., 2019). All of the receptors in this family have 7 transmembrane α helical domains while some of them may have additional structures. Another common feature shared by GPCRs is that all of these receptors transmit the signal across the membrane via a guanine nucleotide-binding protein which is commonly referred as G protein (Seyedabadi, Ghahremani, & Albert, 2019). On the other hand, some of the GPCRs can also transmit the signal independent from the G proteins (Sun, McGarrigle, & Huang, 2007). GPCRs are a large family so they are divided into several classes. Originally they are divided into 6 categories such as class A, class B, class C, class D, class E, and class F. Recent studies divide GPCRs in vertebrates to five main branches called GRAFS. These five branches cover Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2 and Secretin receptors.
(Bjarnadóttir et al., 2006; Sahin, Can, & Son, 2014). Many different extracellular ligands like odors, hormones, light, small molecules, and ions can act through these receptors (Angers, Salahpour, & Bouvier, 2001), meaning that these receptors take part in cellular differentiation, immune response, inflammation, cell growth. After a ligand binds to GPCRs, G proteins are activated with GTP Exchange Factors (GEF), wherein this case GPCR acts as GEF. Activated G proteins interact with effector molecules such as ion channels, phospholipase C (PLC), adenylyl cyclase (AC) (Kankanamge et al., 2019). After that secondary messengers like cAMP and calcium ions deliver the signal to interior parts of the cell.

![Figure 1.1 Classic G Protein signaling pathway (taken from (Frooninckx et al., 2012))](image)

GPCRs have roles in so many different cellular functions that they are targeted in most of the drug research. In the market, 34 % of the drugs that are approved by the FDA, target the GPCRs and approximately 321 agents are currently in clinical trials, of which ~20 % target 66 potentially novel GPCR targets that do not currently have
an approved drug (Hauser, Attwood, Rask-Andersen, Schiöth, & Gloriam, 2017). Because of that, GPCRs remain the main target for drug design and research.

1.1.1. Beta-2-Adrenergic Receptor (β2AR)

Beta-adrenoceptors are GPCRs that belong to rhodopsin-like subfamily and the β2AR is one of the 3 β-adrenoceptors (Chan, Filipek, & Yuan, 2016). Like all GPCRs it has an N-terminus and a C terminus as well as 3 extracellular loops and 3 intracellular loops. The Cysteine amino acid at the 341st position is palmitoylated. This acts as an anchor to bind this amino acid to the cell membrane. Amino acids between the 341st amino acid and 7th transmembrane (spinning domain) create an α-helix sometimes called 4th intracellular loop or the 8th helix (Johnson, 2006).
Figure 1.2 A snake diagram of β2AR. Black circles with white letters indicate disordered residues not included in the model created by Rasmussen and his friends. Grey letters and circles indicate residues not included in the β2AR365 construct used for crystallography. Red letters indicate amino acids for which side-chain electron density was not modeled. Yellow residues indicate amino acids implicated in ligand binding from mutagenesis studies. Orange residues indicate the conserved DRY sequence. Green residues from the Fab5 epitope and pink residues are packed against the Fab5 constant domain in the lattice. Small blue circles indicate glycosylation sites. Red lines indicate ten-amino-acid increments (Rasmussen et al., 2007).
β2AR can activate many events in the cell-like cell growth (Hen, Axel, & Obici, 1989), providing fuel as glucose and respiration (Chan et al., 2016), the mobilization of stored fatty acids and stimulation of the specialized process of adaptive nonshivering thermogenesis (Collins, Cao, & Robidoux, 2004). β2AR generally couples with Gαs protein at the membrane. Upon activation of the receptor with the binding of a ligand, Gαs protein is activated by replacement of GDP with GTP resulting in dissociation of Gα subunit from Gβγ subunit. Then Gα subunit couples with adenyl cyclase to increase cAMP concentration and finally PKA is stimulated (Johnson, 2016). On the other hand, there are studies that show β2AR can also couple with Gαi protein (Rosenbaum, Rasmussen, & Kobilka, 2009).

Following continuous agonist stimulation of the receptor, PKA phosphorylates the receptor from its C tail. This phosphorylation prevents the coupling of Gαs protein with β2AR and receptor couples with Gαi protein. In this case Gβγ subunit of heterotrimeric G protein activates the mitogen-activated protein (MAP) kinase. MAPK stimulation by the β2-adrenergic receptor is mediated by the Gβγ subunits of pertussis-toxin-sensitive G proteins through a pathway involving the non-receptor tyrosine kinase c-Src and the G protein Ras (Daaka, Luttrell, & Lefkowitz, 1997). PKA phosphorylation is the key in these events because it terminates the signal with phosphorylating the receptor and starts another signaling cascade with the same modification.

1.2. Receptor Desensitization

Receptors are one of the main proteins on the membrane that are responsible for communication of the cell with their environment. Receptors can take the signal from the environment, from other cells or even from their own cell and then transmit it to the cytosole. After transmitting the signal, the job of the receptor is finished but
the ligand still might remain around the receptor and could keep the receptor in an active state. The signal must be terminated, one of the ways cells can regulate the receptor is by endocytosis to terminate the signal. With internalization of the receptor, the signal is attenuated and then receptor can be recycled back to the membrane or can be degraded in the lysosomes (Lee, Ferguson, George, & O’Dowd, 2010). Until recent years, it is believed that by internalization the signal that comes from the receptor is attenuated because it is separated from other proteins like G proteins but studies showed that receptors can signal even when they are inside of the endosomes after internalization (Calebire et al., 2009). Internalization starts with phosphorylation of the receptor after ligand binding. Serine, Threonine and/or Tyrosine amino acids are phosphorylated as they have an OH- group that can react with phosphate easily.

GPCRs are phosphorylated from their Serine and Threonine residues by the second messenger-dependent protein kinases or GPCR related kinases (GRK) (Walker, Premont, Barak, Caron, & Shetzline, 1999). Phosphorylation by GRKs starts internalization of receptors while receptor can retain the ability to signal (Foster & Bräuner-Osborne, 2017). After the phosphorylation of the receptors, a scaffold of proteins interacts with each other to start the internalization of the receptor. βArrestins interact first with the phosphorylated GPCRs and then they form clusters with the help of clathrin. After forming Clathrin-Coated Vesicles (CCVs), the receptor can be recycled back to the plasma membrane or can be sent to the lysosome for degradation (Rajagopal & Shenoy, 2018). E3 ubiquitin ligases ubiquitinate the specific lysine residues on the receptor to target it to the lysosome for lysosomal degradation. For GPCRs NEDD4 (E3 ubiquitin ligase) ubiquitinates the receptors for protein degradation in the lysosome.
1.2.1. Arrestin 3 (β-Arrestin 2)

Arrestins belong to a small protein family that regulates the GPCRs by binding and initiating the receptor internalization process (Shenoy & Lefkowitz, 2011). Arrestin 1 and 4 have roles in the visual context in the rod and cone cells. They prevent the phototransduction by inhibiting the GPCRs. Arrestin 2 and 3 (known as β-Arrestin 1 and 2, respectively) are non-visual arrestins and they are ubiquitously expressed and regulate the GPCR trafficking (Kang, Tian, & Benovic, 2013). Arrestins have two major domain composed of antiparallel β-sheets that are connected with short flexible loops. These 2 major domains are called N-domain and C-domain. Between these two major domain, there is a hinge region that provides the connection. C tail of the protein is connected by a flexible linker to the C-domain and contains a short β strand that interacts with a lateral β strand of the N-domain. Protein has a polar core with buried salt bridges that are important for stabilization and by a three-element interaction involving the first β strand, an α-helix in the N-domain and the C-terminal tail (Han, Gurevich, Vishnivetskiy, Sigler, & Schubert, 2001). The polar core is comprised of charged residues from the amino terminus (Asp-27 in β-arrestin-2), N-domain (Arg-166), C-domain (Asp-291 and Asp-298), and C-terminal tail (Arg-393) thus bringing different parts of the molecule together to maintain a basal conformation (Vishnivetskiy et al., 2000). All of these charged residues of the polar core is conserved in all arrestins suggesting that these residues are very important in the function of the arrestin proteins. It has been hypothesized that interaction between these charged residues of arrestin with phosphate groups on the receptor turns arrestin proteins into the active state (Hirsch et al. 1999). Indeed, Arg170 and Asp291 in β-arrestin-2 (Zhan et al. 2011) are particularly important for arrestin selectivity for binding to activated phosphorylated receptors (Vishnivetsky et al. 1999).
Figure 1.3 A Tertiary structure of β-arrestin 1 indicating important regions such as binding sites (taken from (Kang et al., 2013))

β-arrestins main job is regulating the GPCRs after their activation. For the binding of β-arrestins to GPCRs, some signals are needed. The first signal is activation of the receptor with agonist binding and the second signal is phosphorylation of the receptor with GRKs. After the phosphorylation by GRKs, β-arrestins bind to the active receptors and change confirmation which initiates binding of other proteins to the complex for receptor internalization (Lohse & Hoffmann, 2014). The interaction of other proteins like Adaptor protein 2 (AP2) and clathrins aid the receptors internalized into clathrin-coated vesicles. After that receptors can be recycled back to the plasma membrane or targeted to the lysosome for degradation.

Studies show that β-arrestins can also start a signaling pathway independent from G proteins (Shenoy & Lefkowitz, 2011). β-arrestins can gather proteins needed for
ERK mitogen-activated protein kinase cascade like G proteins (Tohgo et al., 2003). When G proteins silenced, ERK 1/2 could be activated following ligand induction (Violin & Lefkowitz, 2007). β-arrestins can act as scaffolds for the kinases after starting the internalization process and induce different signals independent from G proteins. For instance, Arrb2-dependent biased signaling is indispensable for angiotensin II type 1 receptor (AT1R)-induced cardioprotection against ischemic injury (Kim et al., 2012).

1.3. Interaction Between β2AR and β-arrestin 2

β2AR is rhodopsin-like GPCR that can be phosphorylated by either GRKs or the second messenger-dependent protein kinases like PKA. β-arrestin 2 can regulate β2AR after activation. PKA can phosphorylate the receptors from the 261st, 262nd, 345th, and 346th Serine amino acids. For desensitization to occur, all of the Serine amino acids must be phosphorylated by PKA (Serge Moffett, Rousseau, Lagacé, & Bouvier, 2001). At the same time, this receptor can be phosphorylated from 355th, 356th, 360th, and 364th amino acids by GRKs to start the internalization (Krasel et al., 2008). Following phosphorylation of 355th, 356th, 360th, and 364th amino acids by GRK2, β-arrestin 2 binds to the receptor to start the internalization process. After binding of β-arrestin 2 to the receptor, its conformation changes (Krasel et al., 2008). For receptor-arrestin complex to enter the clathrin-coated pits for endocytosis, some adaptor proteins are needed. For β2AR-β-arrestin 2 complexes, adaptor protein 2 (AP2) is needed (Hirst & Robinson, 1998). AP2 directly binds to β-arrestin 2, like clathrin, and starts the internalization process (Laporte, Oakley, Holt, Barak, & Caron, 2000). Phosphorylated receptors gather inside a clathrin-coated pit and then endocytosis starts. Dynamin proteins cleave the membrane to make a clathrin-coated vesicle that has the β2AR-β-arrestin 2 complex inside. The strength of the interaction between receptor and arrestin decide the fate of the receptor, which either recycles to
the membrane or goes to the lysosome for degradation (Oakley, Laporte, Holt, Caron, & Barak, 2000).

**Figure 1.4** Schematic representation of GPCR signaling and internalization by β-arrestin. (taken from (Foster & Bräuner-Osborne, 2017))
1.4. Protein-Protein Interaction Detection Methods

Proteins are important macromolecules in the cell. Proteins can regulate the events in the cell by interacting with other molecules like other proteins, lipids, DNA, (Braun & Gingras, 2012). Thus, protein-protein interactions (PPI) are subject to research as promising targets for rational drug design (Murakami, Tripathi, Prathipati, & Mizuguchi, 2017). The main basis of the PPIs are non-covalent interactions between the chains of the proteins (Ofran & Rost, 2003). These interactions can be obligate or non-obligate, transient or permanent, homo or hetero-oligomeric (Zhang, 2009). There are many methods developed and optimized to detect these various interactions. These detection methods generally separated into 3 categories; in vitro methods, in vivo methods, and in silico methods (Rao, Srinivas, Sujini, & Kumar, 2014). In vitro studies are generally done outside of the cell. Tandem affinity purification, co-immunoprecipitation, are a few examples of in vitro methods to detect PPIs. In vivo studies aim to detect PPIs inside the cell. Yeast-2-hybrid, Bimolecular Fluorescence Complementation (BiFC), Bioluminescence Resonance Energy Transfer (BRET), and Förster Resonance Energy Transfer (FRET) are some of the in vivo methods that are used for detecting PPIs. In silico methods on the other hand, use computers or computer simulations to predict the interaction between the proteins. Programs such as Pathguide, Search tool for the retrieval of interacting genes/proteins (STRING), DIP, MINT are used for predicting the PPIs (Hayes, Malacrida, Kiely, & Kiely, 2016).

In vitro studies are often done in a controlled environment outside a living organism. In Tandem Affinity Purification (TAP) method, proteins are tagged with different epitopes, like STREP and FLAG. Then they are co-transfected to the cells. After transfection, cells are lysed and then proteins are purified using STREP beads to get the proteins that are tagged with STREP epitope or get the proteins that are bound to STREP tagged protein. After the first elution, remaining proteins then purified with
FLAG beads to get the proteins that are tagged with FLAG epitope or get the proteins that are bound to FLAG-tagged proteins. Then using western blot analysis it is possible to show two proteins are still inside the solution meaning that they are interacting with each other (Ma, Fung, & D’Orso, 2017). Using this method, many interactions between proteins are discovered like Gβ subunit interaction with Histone deacetylase 5 (HDAC5), Gβ subunit interaction with T-complex 1 (Campden et al., 2015). One of the disadvantages of the TAP is that it is not possible to observe the localization of the interaction. Also, if the interaction is not stable, some of the samples can be lost in the purification step (Ma et al., 2017).

It is important to detect PPIs in live cells to understand the dynamics of the PPIs in real time. Yeast 2 hybrid method is one of the first methods to detect the PPIs in live cells. In this method, one of the proteins is fused with a DNA binding domain (DBD) and other protein is fused with an activator domain (AD) of a reporter gene. So when both genes co-transfected to yeast, the reporter gene will be expressed if the fusion proteins interact with each other (Mehla, Caufield, & Uetz, 2018). However, sometimes proteins that are fused with DBD can activate the reporter gene which creates a false positive signal. Interactions can be transient or interactions need posttranslational mechanisms that prevent the activation of the reporter gene and detection of the PPIs (Moosavi, Mousavi, Yang, & Yang, 2017). BRET, BiFC, and FRET methods are more suitable to detect PPIs between membrane proteins such as receptors. In the BiFC method, both proteins are tagged with two non-fluorescent fragments of a fluorescent protein e.g., the Venus N-terminal 1–158 amino acid residues, called Venus-N, and its C-terminal 159–239 amino acid residues, named Venus-C, a commonly used pair where co-transfection followed by observation of Venus signal is an indication of PPI. If the proteins are interacting with each other, a fluorescent signal, in this case, Venus signal, is observed under a microscope (Lai & Chiang, 2013) or using a plate reader to read the spectrum. One of the advantages of this technique is that it is very easy to analyze the data because the signal will demonstrate the interaction of the proteins. Also, the signal intensity will be
proportional to the strength of the interaction (Morell, Espargaro, Aviles, & Ventura, 2008). Disadvantages of this method are that maturation time might be longer than the interaction time especially for transient interactions, also it has been reported that the interaction between the non-fluorescent fragments might not be reversible so transient interaction locations cannot be observed correctly (Hu, Chineno, & Kerppola, 2002).

*In silico* methods use computers or computer simulations to make predictions of PPIs. PPIs can be identified with screening a large group of proteins using yeast-2 hybrid and TAP method. NMR, X-ray crystallography or cryo-electron microscopy can identify PPIs between specific proteins in high resolution. However, all these methods have limitations because of post-translational mechanisms (Duan & Walther, 2015), transient interactions (Acuner Ozbabacin, Engin, Gursoy, & Keskin, 2011), proteins with intrinsically disordered regions (Mészáros, Simon, & Dosztányi, 2009). *In silico* methods approaches to detect PPIs and PPI sites for expanding PPI coverage. Also, based on a confidence score of interaction between proteins, they can filter out false positives. A network is generated from experimental data and this experimental data can be used in the simulation to find other PPIs (Murakami et al., 2017). STRING is a network that attempts to integrate many databases into a network. Homology, co-expression, co-occurrence merges with PPI and association databases. These databases are derived from sources such as genomic context, high-throughput experiments (e.g., immunoprecipitation, yeast two-hybrid, co-expression), PPI database imports, and literature co-occurrence. STRING quantitatively integrates interaction data from these sources for a large number of organisms and transfers information between these organisms where applicable (forming a supergenomic network) (Lua et al., 2014). Advantages of the in silico methods are that they are very fast and cost-effective. Information about known PPIs in the literature is already collected to form databases and using this information it is easy to make predictions on PPIs. The disadvantage of in silico
methods is that the known PPIs are incomplete because of limitations in PPI detection methods. Researchers bias some proteins and this lead to incomplete interactome of PPIs (Murakami et al., 2017). It is not easy to detect if the interaction is direct between two proteins or interaction is inside a large protein complex (Snider et al., 2015).

1.4.1. Förster Resonance Energy Transfer (FRET)

Microscopes are indispensable tools in cell biology to study unicellular and multicellular organisms at molecular and organizational levels in their natural environment. With the developments in microscopy, many detailed observations can be done and cellular mechanisms can be studied. However, there is still a limitation to the improvement in the optical resolution. With the classical confocal microscope, resolution in the XY can be as low as 200 nm and even with the super-resolution microscope, the limit is around 50 nm (Sanderson, Smith, Parker, & Bootman, 2014). However, proteins sizes range between 2-10 nm when they are perfectly spherical (Erickson, 2009). Even though they have irregular shapes, it is not possible to observe proteins or PPIs, using confocal microscope because of the resolution limit of light microscopy. Förster Resonance Energy Transfer (FRET) method eliminates this resolution limitation problem and makes it possible to observe PPIs inside the live cells (Piston & Kremers, 2007).

FRET, a quantum mechanical transfer of energy from an optically excited donor to an acceptor over distances usually limited to about 10 nm, is a property of certain pairs of fluorophores (Shaner, Steinbach, & Tsien, 2005a). For this phenomenon to occur, the spectrum of the donor’s emission and acceptor’s excitation must overlap. Another criteria is that donor and acceptor must be in close proximity so that energy can be transferred (below 10 nm) but not a collision between donor and acceptor
would prevail (above 1 nm) (Shrestha, Jenei, Nagy, Vereb, & Szöllősi, 2015). The efficiency of FRET \( (E) \) decreases with the 6th power of the distance between donor and acceptor molecules according to the following formula (Loura, 2012);

\[
i = \left(\frac{1}{t_0}\right)\left(\frac{R_{0i}}{R_i}\right)^6
\]

In the formula \( t_0 \) is the donor’s fluorescence lifetime, \( k_i \) is the rate of dipole-dipole transfer, \( R_i \) is the distance between donor and acceptor, and \( R_{0i} \) is the Förster distance of the FRET pair.

It is very important to select proper fluorophores for FRET experiments. There are some criteria to prioritize. First, the quantum yield of the donor fluorophore must be high in order to transfer energy efficiently (Kempe et al., 2017). Second, the absorbance of the acceptor fluorophore must be high, in order to prevent energy waste. Third and the most important one is that the emission spectrum of the donor and the excitation spectrum of the acceptor must overlap as much as possible. On the other hand, the emission and the excitation spectrum of both donor and acceptor must not overlap if possible. Lastly, the fluorophores must not interact with each other to prevent false signaling (Shaner, Steinbach, & Tsien, 2005b). Originally most of the fluorescent proteins were originated from the GFP protein isolated from jellyfish Aequorea victoria (Shimomura, Johnson, & Saiga, 1962). These fluorescent proteins can have emission maximum at 529 nm and because of that, there were no fluorescent proteins that can emit in yellow, orange, red emissions (Miyawaki, Shcherbakova, & Verkhusha, 2012). Distant homologs of GFP in reef coral and anemone have a red-shifted spectrum (Wall, Socolich, & Ranganathan, 2000). However, fluorescent proteins derived from reef coral and anemone tends to be in tetrameric formation and this can create false positives in FRET experiments. Mutations are introduced to these fluorescent proteins for making them unable to perform dimerization so that they can be used as a FRET pairs (Shaner et al., 2004).
FRET has many advantages over other PPI detection methods. Using FRET method, it is possible to see the PPI by live cell imaging without causing any damage to the cell. The cell will not be stressed and its life will not be threatened with foreign objects. In the yeast-2-hybrid method, the interactions between proteins that interact transiently are hard to detect. FRET eliminates this problem and using FRET method many PPI can be detected in various parts of the cell. Another advantage of FRET is that we can detect the subcellular localization of the PPI (King, Raicu, & Hristova, 2017; Piston & Kremers, 2007; Shrestha et al., 2015).

In this study, mEGFP and mCherry were chosen as a FRET pair. Both are monomeric fluorescent proteins, and this can prevent any dimerization between fluorescent proteins. Also, Donor Spectral Bleed Through (DSBT) and Acceptor Spectral Bleed Through (ASBT) of this pair is very low. There can be a false FRET signal caused by DSBT and ASBT so, it is preferred to have low ASBT and DSBT.

1.5. Aim of the Study

Beta-2 adrenergic receptor is a rhodopsin-like GPCR. After activation of β2AR with ligand binding, β-arrestin 2 binds to the receptor to start receptor internalization. β2AR cannot be phosphorylated or can be over phosphorylated when there are mutations, this will lead to Nephrogenic diabetes insipidus, toxic thyroid adenoma, retinitis pigmentosa. These diseases can be cured by activating β-arrestin 2 in the absence of phosphorylation.

Aim of this thesis is to find residue or residues crucial for the interaction between beta-2 adrenergic receptor and beta-arrestin 2. It has been discovered that there are 4
residues that are responsible for the interaction. Combinations of mutations were done to find residue or residues that can disturb the interaction.

For this purpose, mutations were done on $\beta_2$AR. After mutations, both $\beta_2$AR and $\beta$-arrestin 2 are tagged with mEGFP and mCherry fluorescent proteins. FRET method is used to analyze the interaction between $\beta_2$AR and $\beta$-arrestin 2. This thesis developed the system for the detection of the interaction between arrestin proteins and phosphorylated receptors.
CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial Culture, Media and Conditions

Luria Bertani (LB) medium was prepared according to the instructions given in Appendix B. Ingredients were dissolved in 800 mL distilled water. After dissolving the chemicals, pH was adjusted to 7 using NaOH. Following pH adjustment, volume completed to 1L, LB was sterilized at 121 °C for 20 minutes using Nüve OT 40L autoclave. For selection purposes, ampicillin was added in the LB at a 100 mg/100 mL concentration.

Bacterial cultures were grown either on LB containing 2 % agar at 37 °C in a Nüve® brand incubator or liquid LB at 37 °C with 200 rpm in a Zheiheng shaker incubator.

2.1.2. Neuro2a (N2a) Mouse Neuroblastoma Cell Line and Media

Plasmids that have the fusion protein genes were transfected to Neuro2a (N2a) Mouse Neuroblastoma cell line with transient transfection for expression. The cell line was purchased from ATCC.
For growing the cells the medium was prepared using Dulbecco’s Modified Eagle Medium (D-MEM) with L-glutamine (Invitrogen, Cat#41966029), OptiMEM® Reduced Serum Medium with L-glutamine (Invitrogen, Cat#31985047), Fetal Bovine Serum (Invitrogen, Cat#26140-079), and Penicillin/Streptomycin solution (Invitrogen, Cat#15140-122). Medium contains 44.5 % D-MEM, 44.5 % OptiMEM, 10 % FBS, and 1 % Penicillin/Streptomycin. After adding these ingredients to the solution, the solution was filtered using Millipore Stericup® Filter Units to sterilize the solution.

For removing waste from the cells, Dulbecco’s Phosphate Buffered Saline (D-PBS) powder (Invitrogen, 21300-058) was dissolved in distilled water to make PBS. After dissolving the powder in water, it was autoclaved and then used. For removing adherent cells from the flasks or plates, TrypLE™ Express Stable Trypsin-Like Enzyme with Phenol Red (Invitrogen, Cat#12605-028) was used.

N2a cells reach 70-80 % confluency in about 72 hours; therefore, they were subcultured at every three days by transferring 1/10th of cells into an 8 ml fresh growth medium containing T-25 flask.

Cells were frozen in freezing medium containing 50 % N2a medium, 40 % FBS and 10 % DMSO. To freeze the cells, ~10^7 cells were centrifuged at 1000 rpm for 5 minutes. The cell precipitate was resuspended in 3 ml freezing medium and transferred to a screw-cap cryovial. These cryovials were stored in liquid nitrogen tank at around -150 °C. To thaw frozen cells, cryovials were incubated at room temperature and the content was transferred into a fresh medium containing cell culture flask. All of the chemicals and reagents used for cell culture were cell culture grade.
2.1.3. Neuro2a Cell Culture Conditions

N2a cells were incubated at 37 °C with 5 % CO₂ in Nuve® EC 160 CO2 incubator, at 37 °C with 5 % CO₂. Cell culture studies were carried out in a laminar flow cabinet with a Hepa filter.

2.1.4. Other Chemicals and Materials

The chemicals utilized in this study were purchased from Sigma Chemical Company (NY, USA) and Applichem (Darmstadt, Germany). Molecular biology kits were from Fermentas (Ontario, Canada), QIAGEN (Düsseldorf, Germany) or Invitrogen (CA, USA). DNA polymerases used in polymerase chain reaction (PCR) were from Fermentas (Ontario, Canada). Restriction enzymes and DNA ligases were from New England Biolabs (Hertfordshire, UK). Cell culture media and reagents were all from Gibco®, Invitrogen (CA, USA).

T-75 and T-25 cell culture flasks were purchased from Greiner (Frankfurt, Germany). Sterile serological pipettes were from LP Italiana (Milano, Italy). 35 mm glass bottom dishes used for transfection and live cell imaging experiments were ordered from In Vitro Scientific (CA, USA).

All primer sets used in this study were synthesized by Oligomer Biotechnology Company (Ankara, Turkey). Receptor and protein cDNA clones were obtained from Addgene (Massachusetts, USA), Mark Scott (Paris, France) and Dr. Jonathon M. Willets (Leicester, UK). Enhanced Green Fluorescent Protein (EGFP) and mCherry
cDNA vectors were kindly gifted by Prof. Dr. Henry Lester from California Institute of Technology (CA, USA).

Live cell imaging studies were done using Leica Microsystems CMS GmbH’s DMI4000B confocal microscope with Andor DSD2 spinning differential disc confocal device. PixFRET a plug-in for ImageJ program was used for analysis of images.

2.2. Methods

2.2.1. Polymerase Chain Reaction (PCR)

Primers in Appendix E were designed to add two different cut sites at each end of the gene coding the proteins of interest. After that using PCR, cut sites were introduced to the genes. Conditions are given on Table 2.1.
Table 2.1 Optimized PCR conditions to introduce cut sites of restriction enzymes for a total of 50 µl volume

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclease-Free Water</td>
<td>add up to 50 µl</td>
<td></td>
</tr>
<tr>
<td>5X Phire Reaction Buffer</td>
<td>10 µl</td>
<td>1X</td>
</tr>
<tr>
<td>10 mM dNTPs</td>
<td>1 µl</td>
<td>200 µM</td>
</tr>
<tr>
<td>Primer Forward</td>
<td>1.25 µl</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>Primer Reverse</td>
<td>1.25 µl</td>
<td>0.5 µM</td>
</tr>
<tr>
<td>Template DNA</td>
<td>200 ng</td>
<td></td>
</tr>
<tr>
<td>Phire Hot Start II DNA Polymerase</td>
<td>1 µl</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>98 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Denaturation</td>
<td>98 °C</td>
<td>10 sec</td>
</tr>
<tr>
<td>Annealing</td>
<td>54 °C</td>
<td>30 sec</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
<td>1 min</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72 °C</td>
<td>5 min</td>
</tr>
</tbody>
</table>
2.2.2. Agarose Gel Electrophoresis

After PCR, samples were run on agarose gel for size control. Agarose gel prepared with 1 % agarose (w/v) and Ethidium Bromide was added at 0.5 µg/mL concentration. One lane always loaded with appropriate DNA Ladder and other lanes were filled with samples mixed with 6X DNA loading dye (Fermentas®, Cat#R0611, see Appendix C) with 5:1 DNA to loading dye ratio to get 1X loading dye concentration at the end. The gel was run in 1X TBE at 110 V for 40-45 minutes.

2.2.3. Extraction of DNA from Agarose Gel

To extract PCR products from agarose gel after electrophoresis, QIAGEN® Gel Extraction Kit (Cat# 28704) was used. After the desired band was excised on the UV plate, instruction guide of the kit was followed.

2.2.4. Quantification of DNA Amount

To quantify the amount of DNA after plasmid isolation and gel extraction protocols, BioDrop μLITE spectrophotometer was used. 1 µl of the sample solution was loaded onto the micro-volume pedestal and the measurement was done as directed by the user’s manual.
2.2.5. Restriction Enzyme Digestion

All restriction enzymes used in the course of this thesis work were purchased from New England Biolabs Inc. (NEB). After adding cut sites of restriction enzymes with PCR, samples and plasmids were digested with appropriate restriction enzymes. Double digestion was done to make sticky ends which will place the gene in the correct orientation. 27 µl of the sample which had concentration between 50-200 ng was added in tubes. 15 units of each enzyme was added to the tube. After that 3.3 µl of 10X Cutsmart buffer was added to make final buffer concentration nearly 1X. After mixing the ingredients, samples were placed at 37 °C for 2 hours.

2.2.6. Ligation

After quantification of digested samples, digested products and plasmids were ligated in vitro using 1 µl T4 DNA ligase (NEB, Cat#0202T) in 1X T4 DNA ligase buffer (Appendix D) provided by NEB. For ligation, 1:10 vector to insert ratio was used; and volumes of vector and insert were calculated accordingly, considering their sizes as well. The ligation product waited at room temperature for 3 hours before the transformation.

2.2.7. Preparation of Competent E. coli Cells by RbCl2 Method

XL1-Blue strain of E.coli was grown on LB agar plate with no antibiotics. After 16 hours, the plate was taken from the incubator. A single colony was inoculated in 2.5 ml of LB in a blood tube at 37 °C with 200 rpm for 16 hours. 1 ml of the grown culture was added to 100 ml LB in a 500 ml sterile Erlenmeyer flask and continued to incubate with same conditions. 1 ml of 20 mM MgSO, was added in the
subculture. Flask was placed in a shaker at 37 °C with 200 rpm. OD of the solution was read using spectroscopy and when it reached between 0.4-0.6 OD at 590 nm, the flask was taken from the shaker. It was split into 2, 50 ml sterile Falcon tube. They were centrifuged at 4000 rpm for 5 minutes at 4 °C. Supernatant was thrown and the pellet was resuspended in 0.4th of the original volume in ice cold TFBI (For 100 ml, 40 ml TFBI was used). After resuspension, tubes incubated on ice for 5 minutes. After incubation, they were centrifuged at 4000 rpm for 5 minutes at 4 °C. After supernatant was thrown, the pellet was resuspended in 0.04 original volume ice cold TFBII (For 100 ml use 4 ml TFBII). Cells were incubated on ice for 45 minutes. Then cells were aliquoted in ice cold 1.5 ml eppendorf tubes with 50 μl for each tube. Then cells were stored at -80 °C.

2.2.8. Transformation of Competent E. coli cells with a plasmid

Competent E.coli cells were stored at -80 °C. They were taken from the freezer and put on ice for 10-15 minutes. After that 100 ng DNA or at most 10 % of the ligation solution was added to the cells. Cells were incubated on ice for 30 minutes. Then cells were heat shocked at 42 °C for 45 seconds. They were placed on ice for 5 minutes after heat shock. The volume of the cells was completed to 1 mL by adding LB after 5 minutes. Cells were placed into the shaker after adding LB and they were shaken at 37 °C with 200 rpm for 45 minutes. After incubation on the shaker, cells were centrifuged at 4000 rpm for 5 minutes to precipitate the cells at the bottom of the tubes. 900 μl of the supernatant was taken out and cells were resuspended in 100 μl remaining supernatant and then inoculated to LB agar plates containing ampicillin using glass beads. Agar plates were incubated at 37 °C for 16 hours.

After transformation, a single colony grown on the LB agar plate with ampicillin resistance was picked and inoculated to 4 ml liquid LB with ampicillin broth to grow
overnight at 37 °C. The next day, 4 ml bacterial inoculum was used to isolate plasmid with Fermentas® GeneJETTM Plasmid Miniprep Kit according to the instructor’s manual.

2.2.9. PCR Integration Method

Receptors were tagged from the 3rd intracellular loop and from the C-tail as a backup plan. For this purpose, PCR Integration method was used. First, the fluorescent proteins were amplified with PCR to have nearly 30 bp overhangs that can bind to the receptor gene at targeted sites. Following the first PCR, samples were run on the gel and then extracted from the gel. Then, quantification of second PCR was done. Conditions of the second PCR given in Table 2.2. In the second PCR, the product of the first PCR was used as a primer and plasmid that have wild-type receptor gene used as a template.

Table 2.2 Optimized second PCR conditions to tag the receptor gene from the 3rd intracellular loop and inside the C-tail for a total of 50 µl volume

<table>
<thead>
<tr>
<th>Components</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>2X Phire Green Hot Start II PCR Master Mix</td>
<td>25 µl</td>
</tr>
<tr>
<td>Primer (Product of first PCR)</td>
<td>5X ng</td>
</tr>
<tr>
<td>Template DNA</td>
<td>X ng</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>Up to 50 µl</td>
</tr>
<tr>
<td>Total</td>
<td>50 µl</td>
</tr>
<tr>
<td>Step</td>
<td>Temperature</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Initial Denaturation</td>
<td>95 °C</td>
</tr>
<tr>
<td>Denaturation</td>
<td>95 °C</td>
</tr>
<tr>
<td>Annealing</td>
<td>54 °C</td>
</tr>
<tr>
<td>Extension</td>
<td>72 °C</td>
</tr>
<tr>
<td>Final Extension</td>
<td>72 °C</td>
</tr>
</tbody>
</table>

After the second PCR, the samples were digested using *Dpn I* to remove methylated DNA at 37 °C for 3 hours. Then, the samples were transformed into the competent *E. coli* cells using SOC instead of LB. Schematic representation of the cloned gene after Integration PCR was demonstrated on Figure 2.1.

![Figure 2.1](image.png)  

**Figure 2.1** Schematic representation of Integration PCR
2.2.10. Site-Directed Mutagenesis (SDM)

For mutating the amino acids that are responsible for phosphorylation, site-directed mutagenesis was used. First, primers with the desired mutations were prepared. Then second PCR of integration PCR method was done using Phire II hot start enzyme. After PCR, the samples were digested with Dpn I to remove methylated DNA at 37 °C for 3 hours. At the next step, samples were transformed into the competent E.coli cells using SOC instead of LB. Process was shown on Figure 2.2.

![Figure 2.2 Schematic representation of Site-Directed Mutagenesis (SDM) ("Site-directed Mutagenesis Libraries - Creative Biostructure," 2009)](image)

2.2.11. Passage of the N2a cells

Cells transferred to another flask after reaching 70-80 % confluence to make sure that there is enough space for growth. Generally, passage of the cells was done twice a week. First, the medium was removed from the flask. Then 3 ml of PBS was added
to the flask for rinsing the flask and removing waste and the dead cells. After removing the PBS, 0.5 ml of TrypLE™ Express Stable Trypsin-Like Enzyme with Phenol Red was added to the cells. Cells were incubated in the incubator at 37 °C with 5 % CO₂ for 5 minutes. In the meantime, 9 ml of N2a medium was added in a new flask. After 5 minutes, 9.5 ml of N2a medium was added in the old flask and pipetting was done. 1 ml of medium was taken from the old flask and added into the new flask. Finally, the new flask was incubated in the incubator at 37 °C with 5 % CO₂.

2.2.12. Transfection of N2a cells with pcDNA 3.1 (-) eukaryotic expression vector

Constructs were transfected to N2a cells using Lipofectamine™ LTX with Plus™ reagents from Invitrogen®. At first, cells were seeded to glass bottom dishes following passage. 75.000 cells were seeded in each dish with 2 ml of N2a medium. Transfection was done, next day after initial seeding. 500 ng of the DNA was diluted in 100 µl of OptiMEM. Then, 4 µl of Plus™ reagent was added to the mixture and incubated for 15 minutes. While waiting for 15 minutes, in another tube 4 µl of Lipofectamine LTX™ was diluted with OptiMEM. When the time is up, two tubes were mixed and an additional 15 minutes of incubation was completed. During waiting time, medium of the cells was removed and they were rinsed with PBS. Next step was adding the mixture, on top of the cells and then 800 µl OptiMEM was added. Cells were grown for 3 hours in the incubator at 37 °C with 5 % CO₂. 2 ml of N2a medium was added on to the cells. Next day, the medium was removed from the cells and 2 ml of N2a medium was added to the cells. Imaging of the transfected N2a cells were done on the following day.
2.2.13. Imaging Transfected N2a Cells with Laser Scanning Confocal Microscope

For each FRET analysis, three transfection reactions were done. In the first dish, $\beta_2$AR tagged with EGFP plus wild type $\beta$-arrestin 2 plus wild type GRK2 were transfected to N2a cells. In the second dish, wild type $\beta_2$AR plus $\beta$-arrestin 2 tagged with mCherry plus wild type GRK2 were transfected to N2a cells. In the third dish, $\beta_2$AR tagged with EGFP plus $\beta$-arrestin 2 tagged with mCherry plus wild type GRK2 were transfected to N2a cells.

EGFP channel excites the cells using an excitation band pass filter between 464-500 nm and emission was collected between 500-550 nm. mCherry channel excites the cells using an excitation filter between 561-605 nm and emission was collected between 600-650 nm. The FRET channel excites the cells using an excitation filter between 464-500 nm and emission was collected between 600-650 nm. For calculating donor spectral bleed-through, images of the first dish was taken using EGFP and FRET channel. For calculating acceptor spectral bleed-through, images of the second dish was taken using mCherry and FRET channel. For calculating the net FRET signal, images of the third dish was taken using EGFP, mCherry, and FRET channel. All FRET analyses were done by using PixFRET plug-in of ImageJ software (Feige, Sage, Wahl, Desvergne, & Gelman, 2005). Settings of the confocal microscope was shown in Figure 2.3.
Figure 2.3 Settings of the confocal microscope for FRET

2.2.14. Functional Analysis

Constructs that were used in FRET analysis were checked for functionality using Promega’s cAMP-Glo™ (Catalog number V1501, WI, USA) assay. Assay measures cAMP levels in the cells via monitoring remaining ATP levels at the end of a specific signaling cascade. After activation or deactivation of adenylate cyclase (AC) by a GPCR, AC modulates cAMP levels in the cell accordingly. cAMP molecule
activates Protein Kinase A (PKA) which in return phosphorylates its substrate by the use of ATP. Remaining ATP levels are then determined by presence of luciferase which consumes ATP to produce luminescence. Thus, cAMP concentration and luminescence value are inversely proportional. Figure 2.4 shows how the cAMP is generated inside the cell and how cAMP-Glo™ assay measures the change in the cAMP concentration.

Figure 2.4 Schematic representation of cAMP production inside the cell and measurement of the change in cAMP concentration (taken from www.promega.com)
First, 150000 cells were seeded in 35 mm glass bottom dishes. Next day, constructs that were used in FRET experiments were transfected to cells. Following day, cells were imaged first to make sure that they were transfected. After that, cells were suspended with a scratcher. Suspended cells were seeded in a white 96 well plate with 10000 cells per well concentration. The cells were incubated at 37 °C with 5 % CO₂ for one day. Then protocol for the assay was applied to the cells.

2.2.15. Image Analysis with Pix-FRET

During FRET analysis crosstalk area of the spectrum can create false FRET signals. Donor spectral bleed-through (DSBT) and Acceptor spectral bleed-through (ASBT) signals could mix with FRET signal so these spectral bleed-through signals must be separated from the actual FRET signal. Pix-FRET contains an algorithm to overcome this problem.

To be able to use Pix-FRET, three experimental sets should be prepared. The first set is for donor bleed-through and it involves only donor fluorophore. Two images from this set were taken with the indicated order with the following channels;
- FRET Channel
- EGFP Channel
For acceptor bleed-through, only acceptor fluorophore should be present and two images should be taken with the following order;
- FRET Channel
- mCherry Channel
For FRET, two fluorophores must be present at the same time and three images should be taken, this time with the following configuration;
- FRET Channel
- EGFP Channel
- mCherry Channel

Using bleed-through images, Pix-FRET outputs three equations; constant, linear and exponential; that fit and comprise most of the pixel intensity values. The best line from these was chosen to normalize the bleed-through. Afterwards, the FRET image is analyzed according to this normalization.

2.2.16. Statistical Analysis

Graphs were drawn using Graphpad Prism Version 7.00 showing mean±SEM. The nonparametric Kolgomorov-Smirnov test was applied to test the significances.
CHAPTER 3

RESULTS AND DISCUSSIONS

3.1. Cloning of Wild Type Beta-2 Adrenergic Receptor and Beta-Arrestin 2 Coding Sequences to pcDNA 3.1(-)

The coding sequence of β₂AR was purchased from Addgene (Gene ID number is #14697) and the coding sequence of β-arrestin 2 was kindly gifted by Mark Scott (CNRS Research Associate, Paris, France). They were placed in pcDNA 3.1(-) mammalian expression vector using restriction enzymes. PCR was done in order to add specific restriction sites to the genes. Following PCR, the inserts were digested with the proper restriction enzymes and ligated into the pcDNA 3.1(-) plasmid. Results of the size confirmations were shown in Figure 3.1 and Figure 3.2.

β₂AR gene has 1230 bp while β-arrestin 2 gene has 1242 bp. According to Figure 3.1 and Figure 3.2, bands are between 1000 bp and 1500 bp. Size control strongly suggest that both wild type β₂AR and β-arrestin 2 coding sequences are cloned to pcDNA 3.1(-). For β-arrestin 2, 3 colonies were selected in order to increase the chance. Also, correct sized bands are shown with white arrow. Other bands are the rest of the plasmid when it is digested with two restriction enzymes.
**Figure 3.1** Agarose gel image of the digested pcDNA 3.1(-) which has the β2AR gene. First well have the plasmids that have the wild type receptor gene. Second well have the empty plasmid for negative control. GeneRuler 1 kb plus DNA Ladder was used as a DNA marker.
Figure 3.2 Both BamHI I and Hind III digested pcDNA 3.1(−) that has β-arrestin 2 gene in lane 1, 2, and 3. GeneRuler 1 kb DNA Ladder was used as a DNA marker.
3.2. Making Phosphorylation Deficient β2AR by Mutating Specific Residues

After cloning wild type receptor coding sequence into the pcDNA 3.1(-), mutations were done in residues to make the phosphorylation-deficient receptor. It is known that 355th, 356th, 360th, and 364th amino acids are phosphorylated by GRK2 to start receptor internalization by β-arrestin 2 (Krasel et al., 2008). Primers were designed (see Appendix E) to use EcoRV cut site inside of the receptor gene. In the reverse primer, mutations were introduced, and EcoRV cut site was added. PCR was done and after that PCR product was digested with both of Nhe I and EcoRV. Also, pcDNA 3.1(-) which has β2AR gene was digested with both of Nhe I and EcoRV. Following digestions, ligation was done, and ligated products were transformed into the competent XL1 Blue strain of E.coli. Single colonies were selected and inoculated in 4 ml of LB for plasmid isolation. After isolation, plasmids were sent to sequencing and mutations are confirmed. Figure 3.3 shows the sequence chromatograms of the wild-type and phosphorylation-deficient receptor.
Figure 3.3 Results of the sequence chromatograms. A) The chromatogram of the wild type β2AR gene sequence. B) The chromatogram of the phosphorylation-deficient β2AR. Underline regions are coding sequences of the residues that are phosphorylated by GRK2. First bases of each residue were changed into the Guanine that resulted in changing residues to Alanine or Glycine.
3.3. Obtaining Combination of Mutations on β2AR

After making phosphorylation-deficient β2AR gene, various combinations of these resudues were tested to see the individual importance of these sites. One of the constructs which have 355th and 356th residues mutated was done using EcoR V cut site. Other constructs were done using Site-Directed Mutagenesis (SDM). After plasmid isolation, all constructs were sent for sequencing and the mutations were confirmed using sequence chromatogram. All residues were mutated separately. 355th and 360th residues were changed to alanine while 356th and 364th residues were converted to glycine. β2AR-S355A, β2AR-S356G, β2AR-T360A, and β2AR-S364G, β2AR-S356G-S364G was obtained using SDM. A total of 6 mutated receptors (Except negative control β2AR-PD that has all the mutations) were generated for the further experiments.

3.4. Tagging of β2AR from C-Tail with mEGFP and mCherry Protein Gene

When wild type gene of the receptor cloned into the pcDNA 3.1(-), next step was tagging the receptors with fluorescent proteins. Firstly, we cloned the mEGFP and mCherry protein gene sequences into pcDNA 3.1(-) between EcoR I and BamH I restriction cut sites. Reverse primer with EcoR I cut site and removal of the stop codon was designed for the target gene. Nhe I cut site was added to forward primer. PCR was done using primers. Figure 3.4 indicates that the receptor was tagged with both mEGFP and mCherry gene, from its C-tail.

Receptor gene has 1230 bp and fluorescent protein gene has about 700 bp. When the receptor is tagged, fusion protein gene becomes 1930 bp. Bands that are shown with
white arrows are near the 2000 bp so the size confirmation was successful. Other bands are the rest of the plasmid when it is digested with two restriction enzymes. They were transfected to N2a mammalian cells for observation of their expression and localization.

**Figure 3.4** Gel image of the ligation products that are digested with both of Nhe I and BamH I restriction enzymes. First well is for the plasmids that have the receptor
tagged from its C-tail with mEGFP. Second well is for the plasmids that have the
receptor tagged from its C-tail with mCherry. Last well is for the empty plasmid for
control. The ladder is the GeneRuler 1 kb plus DNA Ladder for the confirmation of
the sizes.

3.5. Tagging of Beta-Arrestin 2 with mEGFP and mCherry

β-arrestin 2 was planned to tag from its C-terminus and N-terminus. Samples were
digested to check if the fusion protein cloned to the pcDNA 3.1(-) vector. Result of
the tagging was shown in Figure 3.5. Beta-arrestin 2 protein gene has 1242 bp and
when it is tagged with EGFP or mCherry, it has nearly 2000 bp. Like in previous
result, bands are near 2000 bp so tagging from both termini was successfully done.
White arrows on Figure 3.5 show the bands that have the correct size. Other bands
are the rest of the plasmid when it is digested with two restriction enzymes.
Figure 3.5 Gel image of the plasmids that have the fusion protein of β-arrestin 2 with mEGFP or mCherry fluorescent proteins. First well is for the empty plasmid for the negative control. Second well have the plasmids that have β-arrestin 2 tagged with mEGFP from its C-terminus. Third well have the plasmids that have β-arrestin 2 tagged with mCherry from its C-terminus. Fourth well have the plasmids that have β-arrestin 2 tagged with mEGFP from its N-terminus. Fifth well have the plasmids that have β-arrestin 2 tagged with mCherry from its N-terminus. Generuler 1 kb plus DNA ladder is used as a DNA marker.
3.6. Tagging of β2AR From Intracellular Loops with Integration PCR

The receptor was tagged from its C-tail. Receptor is also tagged from its 3rd loop and inside the C-tail if the FRET signal was not high enough using the receptor constructs that are tagged from its C-tail. The plasmids were digested with Nhe I and EcoR I to check whether the tagging process is successful. Figure 3.6 shows the results. mEGFP gene has 720 bp while mCherry gene has 711 bp. On the other hand, β2AR gene has 1242 bp and when it is tagged with mEGFP or mCherry, the fusion protein gene has 1962 and 1953 bp, respectively. For receptors that are tagged after 229th amino acid, 3 colonies were selected. For other constructs, 4 colonies were selected for control. Figure 3.6 suggest that the tagging process was a successful considering the sizes of the expected and observed bands. White arrows on the figure 3.6 show the constructs that have the correct size. Other bands are the rest of the plasmid when it was digested with two restriction enzymes.
Figure 3.6 Both of Nhe I and EcoR I digested products of Integration PCR methods were run on the agarose gel. 1st, 2nd, and 3rd wells are for tagging of the receptor after 229th amino acid with mEGFP. 4th, 5th, and 6th wells are for tagging of the receptor after 229th amino acid with mCherry. 7th, 8th, 9th, and 10th wells are for tagging of the receptor after 252nd amino acid with mEGFP. 11th, 12th, 13th, and 14th wells are for tagging of the receptor after 252nd amino acid with mCherry. 15th, 16th, 17th, and 18th wells are for tagging of the receptor after 332nd amino acid with mEGFP. 19th, 20th, 21st, and 22nd wells are for tagging of the receptor after 332nd amino acid with mCherry. 23rd, 24th, 25th, and 26th wells are for tagging of the receptor after 374th amino acid with mEGFP. 27th, 28th, 29th, and 30th wells are for tagging of the receptor after 374th amino acid with mCherry.
3.7. Tagging of Mutated β₂AR from C-Tail with mEGFP and mCherry

Following mutagenesis, mutated receptors were tagged with mEGFP and mCherry from their C-tail. The process was the same with tagging wild type receptor from its C-tail as it was mentioned in chapter 3.5. Figure 3.7 in the below shows the results. mEGFP gene has 720 bp while mCherry gene has 711 bp. On the other hand, β2AR gene has 1242 bp and when it is tagged with mEGFP or mCherry, the fusion protein gene has nearly 2000 bp. For all constructs, 2 colonies were selected to increase the chance of having the correct construct. According to Figure 3.7, it was shown that all the receptors which have mutations were tagged with fluorescence proteins from their C-tail. White arrows on the figure 3.6 show the constructs that have the correct size. Other bands are the rest of the plasmid when it is digested with two restriction enzymes.
**Figure 3.7** Gel image of digested pcDNA3.1(+) plasmids that have gene sequence of fusion proteins of mutated β2AR and mEGFP, mCherry fluorescent proteins. Lane 1 and 2 are for β2AR-S355A-mEGFP, 3 and 4 are for β2AR-S355A-mCherry, 5 and 6 are for β2AR-S356G-mEGFP, 7 and 8 are for β2AR-S356G-mCherry, 9 and 10 are for β2AR-T360A-mEGFP, 11 and 12 are for β2AR-T360A-mCherry, 13 and 14 are for β2AR-S364G-mEGFP, 15 and 16 are for β2AR-S364G-mCherry, 17 and 18 are for β2AR-S355A-S356G-mEGFP, 19 and 20 are for β2AR-S355A-S356G-mCherry, 21 and 22 are for β2AR-S356G-S364G-mEGFP, 23 and 24 are for β2AR-S356G-S364G-mCherry, 25 and 26 are for β2AR-PD-mEGFP, 27 and 28 are for β2AR-PD-mCherry.
3.8. Functional Analysis of the Constructs

After tagging of the β2AR and β-arrestin was complete, constructs were checked for functionality. When using cAMP-Glo™ assay, ratio of the luminescence values was measured. For each transfection, highest luminescence value was set as 1 and other luminescence values were divided to highest value to get the ratios. Bar graph on Figure 3.8 was drawn using these ratios.

It is known that β2AR interact with the Gαi and increases cAMP concentration. According to graph on Figure 3.8, all tagged construct showed similar response with the wild types. Ligand activated the receptor and increased cAMP concentration and resulted with the decrease in luminescence values. The change in cAMP concentration can be clearly seen from the graph. When using tagged wild type receptor, β-arrestin 2 that is tagged from its N-terminus showed a higher change in cAMP concentration than others. On the other hand, β-arrestin 2 that is tagged from its C-terminus showed a similar change in cAMP concentrations with the non-tagged constructs. Thus, β-arrestin that is tagged from its C-terminus was used in the FRET experiments.
**Figure 3.8** Bar graph of the Functional Analysis. Each value represents the mean ±S.E.M. of one experiments performed in triplicate. R stands for β2AR, A stands β-arrestin 2, K stands for GRK2, mR stands for β2AR–PD, tR stands for tagged β2AR, mtR stands for tagged β2AR–PD, tA stands for N-terminus tagged β-arrestin 2, At stands for C-terminus tagged β-arrestin 2 and LIG stands for 10 μm Isopreterenol.

### 3.9. Transfection of the Constructs to the N2a Cell Culture

After preparing all the constructs, they were transfected to the N2a cells. For transfection, 75000 cells were seeded in 35 mm glass bottom dish 24 hours before transfection. After transfection, cells were incubated for 48 hours to express the fusion proteins. After 48 hours, images of the cells were taken using the confocal
microscope. Localization of the receptor was observed on the cell membrane after it was tagged with fluorescent proteins from its C-tail in Figure 3.9.

**Figure 3.9** Images of the $\beta_2$AR-mEGFP and $\beta_2$AR-mCherry. A) Image of the N2a mammalian cells transfected with $\beta_2$AR-mEGFP taken with EGFP Channel. B) Image of the N2a mammalian cells transfected with $\beta_2$AR-mCherry taken with mCherry Channel. (Magnification 63X, Scale line is 10 $\mu$m)
The sequences of the tagged receptors were checked after the tagging process. According to sequence chromatograms, there was no mutation and the sequences of the constructs were correct. When the functional analysis of the receptor was checked using the cAMP-Glo assay, the tagged receptors were found to be functional. After imaging the constructs that are tagged C-tail, constructs that are tagged from inside were transfected to N2a cells. Figure 3.10 presents the images of the N2a cells transfected with receptors that are tagged from inside. Some of the constructs were localized on the cell membrane. Receptors tagged with mCherry fluorescent proteins were also localized inside of the cells. Their localization on the cell membrane was clear so it is believed that the signals that are inside of the cells are due to early maturation of mCherry (Merzlyak et al., 2007).
Figure 3.10 Images of the cells transfected with the constructs that were done with Integration PCR methods. A) Image of the N2a mammalian cells transfected with β2AR-229-mEGFP taken with EGFP Channel. B) Image of the N2a mammalian
cells transfected with β₂AR-229-mCherry taken with mCherry Channel. C) Image of the N2a mammalian cells transfected with β₂AR-252-mEGFP taken with EGFP Channel. D) Image of the N2a mammalian cells transfected with β₂AR-252-mCherry taken with mCherry Channel. E) Image of the N2a mammalian cells transfected with β₂AR-332-mEGFP taken with EGFP Channel. F) Image of the N2a mammalian cells transfected with β₂AR-332-mCherry taken with mCherry Channel. G) Image of the N2a mammalian cells transfected with β₂AR-374-mEGFP taken with EGFP Channel. H) Image of the N2a mammalian cells transfected with β₂AR-374-mCherry taken with mCherry Channel. (Magnification 63X, Scale line is 10 μm)

3 of the 8 constructs were localized inside the cell, instead of the cell membrane. It was realized that, the receptor that is tagged after 332nd residue was not localized on the membrane. Data suggest that the confirmation of protein after tagging process was not correct so it was not localized on the membrane. Fluorescent protein may disturb a secondary structure that is not predicted or identified by an experimental data. Similarly, β₂AR-374-mEGFP did not localize on the cell membrane. When this construct was sequenced it was discovered that amino acids between 332-374 were deleted. This deletion might be the reason why β₂AR-374-mEGFP did not go to the cell membrane. The sequences of all constructs were checked and other than β₂AR-374-mEGFP, all constructs had the correct sequence. The functionality of the best constructs according to their microscopy images were carried out using the cAMP-Glo assay kit. Localization of the single residue mutated receptors was observed on the cell membrane after it was tagged with fluorescent proteins from its C-tail in Figure 3.11.
Figure 3.11 Images of the cells transfected with the constructs that were done with Integration PCR methods. A) Image of the N2a mammalian cells transfected with β2AR-S355A-mEGFP taken with EGFP Channel. B) Image of the N2a mammalian cells transfected with β2AR-S355A-mCherry taken with mCherry Channel. C) Image of the N2a mammalian cells transfected with β2AR-S356G-mEGFP taken with EGFP Channel. D) Image of the N2a mammalian cells transfected with β2AR-
S356G-mCherry taken with mCherry Channel. E) Image of the N2a mammalian cells transfected with β2AR-T360A-mEGFP taken with EGFP Channel. F) Image of the N2a mammalian cells transfected with β2AR-T360A-mCherry taken with mCherry Channel. G) Image of the N2a mammalian cells transfected with β2AR-S364G-mEGFP taken with EGFP Channel. H) Image of the N2a mammalian cells transfected with β2AR-S364G-mCherry taken with mCherry Channel. (Magnification 63X, Scale line is 10 μm)

The functionality of the receptor that has all the mutations was checked and reported in the literature (Krasel et al., 2008). In this study same four mutations and individual mutations of these four residues were done. As the receptor with four mutations was functional in our study it is assumed that the receptor with single residue mutants should be functional. β2AR-S355A-S356G-mEGFP, β2AR-S355AS356G-mCherry, β2AR-S356G-S364G-mEGFP, β2AR-S356G-S364G-mCherry were transfected to N2a cells.

The tagged receptors were localized on the cell membrane as seen in Figure 3.12. After checking the localization of the receptors, tagged β-arrestin 2 proteins transfected to N2a cells. Figure 3.13 shows the images of the N2a cells transfected with the tagged β-arrestin 2 proteins.
Figure 3.12 Images of the cells transfected with the tagged β-arrestin 2. A) Image of the N2a mammalian cells transfected with β₂AR-S355A-S356G-mEGFP taken with EGFP Channel. B) Image of the N2a mammalian cells transfected with β₂AR-S355A-S356G-mCherry taken with mCherry Channel. C) Image of the N2a mammalian cells transfected with β₂AR-S356G-S364G-mEGFP taken with EGFP Channel. D) Image of the N2a mammalian cells transfected with β₂AR-S356G-S364G-mCherry taken with mCherry Channel. (Magnification 63X, Scale line is 10 μm)
**Figure 3.13** Images of the cells transfected with the tagged β-arrestin 2. A) Image of the N2a mammalian cells transfected with Barr2-mEGFP (500 ng) taken with EGFP Channel. B) Image of the N2a mammalian cells transfected with Barr2-mCherry (500 ng) taken with mCherry Channel. C) Image of the N2a mammalian cells transfected with mEGFP-Barr2 (500 ng) taken with EGFP Channel. D) Image of the N2a mammalian cells transfected with mCherry-Barr2 (500 ng) taken with mCherry Channel. (Magnification 63X, Scale line is 10 μm)

In normal conditions, β-arrestin 2 is a cytosolic protein so its localization is cytoplasm and it does not have nuclear localization signal or does not interact with any nuclear protein, so it cannot go to the nucleus of the cell. All tagged arrestins were in the cytoplasm as seen in Figure 3.13. These constructs also were sent to sequencing and it was confirmed that sequences of all constructs were correct. Their functionality was checked with cAMP-Glo assay and it was observed that they were functional as well. β-arrestin 2 can localize to the membrane upon activation of β₂AR. Tagged β-arrestin 2 constructs transfected to cells with wild type receptor and
wild type GRK2 which was a kind gift from Dr. Jonathon M. Willets. Cells were imaged using EGFP and mCherry channel of the confocal microscope, results presented in Figure 3.14.

When the cells were transfected with the plasmids (the tagged Beta-arrestin 2, the wild-type receptor, and the wild-type GRK2 proteins), it was observed in Figure 3.14 that β-arrestin 2 went to the cell membrane. The problem was that there are 3 plasmids. All plasmids must be transfected to the same cell, but the transfection efficiency was not 100% so, some cells did not get receptor or GRK2 protein. This was the reason why β-arrestin 2 did not go to the cell membrane in some cells. After addition of Isoproterenol at 10 μM concentration, the receptor was activated and β-arrestin 2 translocated to the cell membrane to start the internalization process. Images clearly show that there were β-arrestin 2 proteins on the cell membrane after addition of ligand.
Figure 3.14 Images of the cells that were transfected with wild type receptor, tagged β-arrestin 2 and wild type GRK2. For activating the receptor, Isopreterenol was used as a ligand at 10 μM concentration. A) Image of the N2a mammalian cells transfected with Barr2-mEGFP taken with EGFP Channel. B) Image of the N2a mammalian cells transfected with Barr2-mCherry taken with mCherry Channel. C) Image of the N2a mammalian cells transfected with mEGFP-Barr2 taken with EGFP Channel. D) Image of the N2a mammalian cells transfected with mCherry-Barr2 taken with mCherry Channel. (Magnification 63X, Scale line is 10 μm)
3.10. Analysis of the Interaction Between Wild Type β₂AR-β-arrestin 2 & Phosphorylation Deficient β₂AR-β-arrestin 2

After confirming the localization of receptor and β-arrestin 2, transfection of tagged receptor and β-arrestin 2 was done to the N2a cells. For detection, the interaction between receptor and arrestin protein, wild type GRK2 was also transfected to the cells. The receptors that were tagged from its 3rd intracellular loop were used in Figure 3.15 and 3.16 to observe the interaction between the receptor and Beta-arrestin 2.

Figure 3.15 N2a cells transfected with Barr2-mEGFP, β₂AR-229-mCherry, and wild-type GRK2. A) Image taken using EGFP Channel. B) Image taken using mCherry Channel. C) Image taken using FRET Channel. D) FRET signal efficiency using PixFRET. (Magnification 63X, Scale line is 10 μm)
Figure 3.16 N2a cells transfected with Barr2-mEGFP, β2AR-252-mCherry, and wild-type GRK2. A) Image taken using EGFP Channel. B) Image taken using mCherry Channel. C) Image taken using FRET Channel. D) FRET signal efficiency using PixFRET. (Magnification 63X, Scale line is 10 μm)

When the images were taken, it was observed that β-arrestin 2 did not go to the membrane. There may be some interaction between the 3rd intracellular loop of β2AR and β-arrestin 2 that when the receptor is tagged from its 3rd intracellular loop, the interaction is broken so β-arrestin 2 cannot bind to the receptor which results with no signal of β-arrestin 2 in the cell membrane. For the observation of the interaction, β2AR-374-mCherry was used and the results were presented in Figure 3.17.
Figure 3.17 N2a cells transfected with Barr2-mEGFP, β₂AR-374-mCherry, and wild-type GRK2. A) Image taken using EGFP Channel. B) Image taken using mCherry Channel. C) Image taken using FRET Channel. D) FRET signal efficiency using PixFRET. (Magnification 63X, Scale line is 10 μm)

When the receptor was tagged from inside of the C-tail, β-arrestin 2 can go to the cell membrane. Although β-arrestin 2 localized on the membrane, the FRET signal was so low to analyze, thus receptors that are tagged from their C-tail was decided to be used in the future experiments. Similarly, β₂AR-mEGFP was used to observe the interaction and images were presented in Figure 3.18.
Figure 3.18 N2a cells transfected with Barr2-mCherry, β2AR-mEGFP, and wild-type GRK2. A) Image taken using EGFP Channel. B) Image taken using mCherry Channel. C) Image taken using FRET Channel. D) FRET signal efficiency using PixFRET. (Magnification 63X, Scale line is 10 µm)

According to figure 3.18, Beta-aretin 2 was localized on the membrane after addition of the ligand. When the receptor was tagged from its C-tail, there was a significant FRET signal on the plasma membrane. When the FRET efficiency was calculated, it was also significant compared to the other constructs. This set-up was used as a positive control in the future experiments. After deciding the positive control, β2AR-PD-mCherry was used in Figure 3.19 to test the effects on the interaction.

When the ligand was added, localization of the Beta-aretin 2 remain the same as seen in Figure 3.19. After calculating the FRET efficiency, it was clear that there is no interaction between phosphorylation-deficient receptor and β-aretin 2. This represents that the mutated residues are important for the interaction between the
receptor and β-arrestin 2. This construct was used as a negative control later in the thesis.

Figure 3.19 N2a cells transfected with Barr2-mEGFP, β2AR-PD-mCherry, and wild-type GRK2. A) Image taken using EGFP Channel. B) Image taken using mCherry Channel. C) Image taken using FRET Channel. D) FRET signal efficiency using PixFRET. (Magnification 63X, Scale line is 10 μm)

Analysis of the FRET signal was calculated the FRET efficiencies of positive and negative control were represented as line graphs in Figure 3.20.
**Figure 3.20** Graphical representation of the interaction between β2AR and β-arrestin 2. Each value represents the mean ± S.E.M. of two experiments performed in triplicate. The red line is the analysis of the FRET signal between Wild-type β2AR and β-arrestin 2. The green line is the analysis of the FRET signal between Phosphorylation-Deficient β2AR and β-arrestin 2.

From the graph in Figure 3.20, it was observed that most of the FRET signal between Phosphorylation-Deficient β2AR and β-arrestin 2 belongs to the 1-2 % FRET Efficiency. This can be considered as random interaction between mEGFP and mCherry. The FRET signal between Wild-type β2AR and β-arrestin 2 has less pixels than Phosphorylation-Deficient β2AR that belongs to the 1-2 % FRET Efficiency. This can be interpreted as when Phosphorylation-Deficient β2AR and β-arrestin 2 co-transfected, there is no interaction and FRET signal is mostly occurring from random interaction. However, the wild-type receptor is interacting with β-arrestin 2. The FRET Efficiency of the wild-type receptor is low. This can be explained with low transfection efficiency. 3 plasmids (β2AR, β-arrestin 2, and
GRK2) must be transfected into the same cell to observe the interaction. However, low transfection efficiency eliminates the possibility of interaction in most of the cells. Even though the FRET signal is low, there is an interaction between the wild-type receptor and β-arrestin 2 according to the graph. When the Kolmogorov-Smirnov test was applied, it was observed that the FRET efficiencies are significantly different from each other while p<0.0001.

3.11. Analysis of the Interaction Between Mutated β₂AR-β-arrestin 2

After observing the interaction between the wild-type receptor and β-arrestin 2, interaction between mutated β₂AR-β-arrestin 2 were analyzed. Single residue mutated receptors were transfected to N2a cells and imaged. Representative images of the results were presented in Figure 3.21 to 3.24. The interaction between receptor and β-arrestin 2 was evaluated in order to comment on the importance of the individual residues.
Figure 3.21 N2a cells transfected with Barr2-mEGFP, β2AR-S355A-mCherry, and wild-type GRK2. A) Image taken using EGFP Channel. B) Image taken using mCherry Channel. C) Image taken using FRET Channel. D) FRET signal efficiency using PixFRET. (Magnification 63X, Scale line is 10 μm)
Figure 3.22 N2a cells transfected with Barr2-mCherry, β₂AR-S356G-mEGFP, and wild-type GRK2. A) Image taken using EGFP Channel. B) Image taken using mCherry Channel. C) Image taken using FRET Channel. D) FRET signal efficiency using PixFRET. (Magnification 63X, Scale line is 10 μm)
Figure 3.23 N2a cells transfected with Barr2-mCherry, β2AR-T360A-mEGFP, and wild-type GRK2. A) Image taken using EGFP Channel. B) Image taken using mCherry Channel. C) Image taken using FRET Channel. D) FRET signal efficiency using PixFRET. (Magnification 63X, Scale line is 10 μm)
Figure 3.24 N2a cells transfected with Barr2-mEGFP, β2AR-S364G-mEGFP, and wild-type GRK2. A) Image taken using EGFP Channel. B) Image taken using mCherry Channel. C) Image taken using FRET Channel. D) FRET signal efficiency using PixFRET. (Magnification 63X, Scale line is 10 μm)

When images were analyzed, it was found that all constructs have the same FRET efficiency similar to the positive control except β2AR-S355A. β2AR-S355A had lower FRET efficiency than the positive control. It suggests that Serine residue positioned at 355, is more crucial for the interaction between receptor and β-arrestin.
2 than other residues. Double mutated receptors were used in Figure 3.25 and Figure 3.26.

According to Figure 3.25, the FRET efficiency of $\beta_2$AR-S355A-S356G was very low when compared to the positive control. On the other hand, the FRET efficiency of the $\beta_2$AR-S356G-S364G was same with the positive control. After taking images with the spinning disc confocal microscopes, the FRET signal was calculated to observe if there will be any significance between mutated receptor and wild-type receptor.

Figure 3.25 N2a cells transfected with Barr2-mCherry, $\beta_2$AR-S355A-S356G-mEGFP, and wild-type GRK2. A) Image taken using EGFP Channel. B) Image taken using mCherry Channel. C) Image taken using FRET Channel. D) FRET signal efficiency using PixFRET. (Magnification 63X, Scale line is 10 $\mu$m)
**Figure 3.26** N2a cells transfected with Barr2-mCherry, β₂AR-S356G-S364G-mEGFP, and wild-type GRK2. A) Image taken using EGFP Channel. B) Image taken using mCherry Channel. C) Image taken using FRET Channel. D) FRET signal efficiency using PixFRET. (Magnification 63X, Scale line is 10 μm)
The FRET efficiency of all the constructs were represented as a line graph in Figure 3.27. From the graph in Figure 3.27, it was observed that the $\beta_2$AR-S355A construct had a FRET signal between wild-type and phosphorylation-deficient receptor. While $\beta_2$AR-S356G, $\beta_2$AR-T360A, and $\beta_2$AR-S364G had the same FRET signal compared to the wild-type. $\beta_2$AR-S355A-S356G had a FRET signal similar with the phosphorylation-deficient. $\beta_2$AR-S356G-S364G had a FRET signal the same with wild-type. After taking images and calculating the FRET signals, the Kolmogorov-Smirnov test was applied to observe if these FRET signals have a significant difference from the FRET signal of the wild-type receptor. Table 3.1 shows the comparison of constructs with the wild-type.
Figure 3.27 Graphical representation of the interaction between all $\beta_2$AR constructs and $\beta$-arrestin 2. Each value represents the mean ±S.E.M. of two experiments performed in triplicate.

According to Table 3.1, $\beta_2$AR-S355A and $\beta_2$AR-S355A-S356G constructs showed a significantly different FRET signal than wild-type receptor. After that $\beta_2$AR-S355A and $\beta_2$AR-S355A-S356G compared with the phosphorylation-deficient receptor. It is observed that $\beta_2$AR-S355A construct also had significantly different FRET signal than negative control. On the other hand, $\beta_2$AR-S355A-S356G had the same FRET signal with the negative control. These results suggest that it is enough to break the interaction between receptor and $\beta$-arrestin 2 by mutating Serine residues at 355th and 356th position on the receptor. Also, when Serine residue at 355th position is mutated, the interaction is disturbed but not completely broken.
Table 3.1 Comparison of the all β₂AR constructs with wild-type β₂AR using the Kolgomorov-Smirnov test.

<table>
<thead>
<tr>
<th>Comparisons</th>
<th>Significant</th>
<th>Summary</th>
<th>Adjusted P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-Type vs. Phosphorylation Deficient</td>
<td>Yes</td>
<td>****</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Wild-Type vs. S355A</td>
<td>Yes</td>
<td>*</td>
<td>0.0132</td>
</tr>
<tr>
<td>Wild-Type vs. S356G</td>
<td>No</td>
<td>ns</td>
<td>&gt;0.9999</td>
</tr>
<tr>
<td>Wild-Type vs. T360A</td>
<td>No</td>
<td>ns</td>
<td>0.7227</td>
</tr>
<tr>
<td>Wild-Type vs. S364G</td>
<td>No</td>
<td>ns</td>
<td>0.7227</td>
</tr>
<tr>
<td>Wild-Type vs. S355A-S356G</td>
<td>Yes</td>
<td>****</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Wild-Type vs. S356G-S364G</td>
<td>No</td>
<td>ns</td>
<td>0.7227</td>
</tr>
<tr>
<td>Phosphorylation Deficient vs. S355A</td>
<td>Yes</td>
<td>*</td>
<td>0.036</td>
</tr>
<tr>
<td>Phosphorylation Deficient vs. S355A-S356G</td>
<td>No</td>
<td>ns</td>
<td>0.4647</td>
</tr>
</tbody>
</table>
CHAPTER 4

CONCLUSION

The aim of this thesis was to identify residues of Beta-2 Adrenergic receptor which are important for its interaction with β-arrestin 2. Förster Resonance Energy Transfer (FRET) technique was used for quantitative analysis of the interaction between β2AR and βarrestin 2 in live cells.

β2AR was mutated and then the wild-type receptor was tagged from 5 different positions using both mEGFP and mCherry. β-arrestin 2 was tagged from both N and C termini with mEGFP and mCherry. Reciprocal setups, like β2AR-mEGFP and Barr2-mCherry or β2AR-mCherry and Barr2-mEGFP, were used to observe FRET. Other than β2AR-332-mEGFP, β2AR-332-mCherry, and β2AR-374-mEGFP, all other receptor constructs were correctly localized on the cell membrane. Localization of the β-arrestin 2 constructs was in the cytoplasm after transfection to N2a cells. On the other hand, mutated receptors were only tagged from their C terminus, since it was observed that this position gives the highest FRET signal when experimented. Their localization was also on the cell membrane, as expected.

The sequence of the constructs was checked, and it was confirmed that they were correct. Only the best constructs were further checked for functionality and it was confirmed that they were functional. It was also verified that, after all residues of β2AR were mutated in order to make the receptor phosphorylation-deficient, the receptor was still functional. The residues that are targeted for mutations are known to be responsible only for phosphorylation. It was therefore assumed that when these residues are mutated, either separately or in combinations, the receptor would still remain functional.
The interaction between the wild-type receptor and β-arrestin was observed when β2AR-mEGFP, Barr2-mCherry, and GRK2 were co-transfected to N2a cells. Other constructs were also co-transfected, but they did not give any significant FRET signal. Therefore, β2AR-mEGFP & Barr2-mCherry pair was used as a positive control setup. The FRET signal was low, probably due to the transfection efficiency. 3 plasmids were needed to be transfected into the same cell; this may be the reason for the FRET signal. When there is no GRK2 protein, the receptor is not phosphorylated and β-arrestin 2 cannot bind. However, receptor and β-arrestin 2 can interact through random chance. This can decrease the high FRET signal to low FRET signal. This problem can be solved using double promoter vectors. If GRK2 and one of the fusion proteins cloned into a double promoter vector, it will be certain that GRK2 is transfected inside of the cell. β2AR-PD-mCherry, Barr2-mEGFP, and GRK2 were also cotransfected in order to observe whether the interaction was disrupted. When the FRET signal was calculated, it was observed that most of the FRET signal was at 1-2% of FRET Efficiency. This can be considered as a random interaction between mEGFP and mCherry. It can, therefore, be concluded that there is no interaction between β2AR-PD-mCherry and Barr2-mEGFP. For the comparison of the two curves in the graph, the Kolmogorov-Smirnov test was chosen as a non-parametric test. The difference between the wild-type receptor and phosphorylation-deficient receptor was significant when the Kolmogorov-Smirnov test was applied.

Mutated receptors were transfected into N2a cells together with β-arrestin 2 to observe the interaction between the receptor and β-arrestin 2. Afterward, the FRET signals were calculated. It was observed that β2AR-S355A had a FRET signal lower than wild-type. β2AR-S356G, β2ART360A, and β2AR-S364G had the same FRET signal with the wild-type. β2AR-S355A-S356G had a FRET signal comparable to the phosphorylation-deficient receptor while β2AR-S356G-S365G had similar FRET signal when compared to the wild-type receptor. According to these results, it was sufficient to mutate Serine residues positioned at 355th and 356th on β2AR to disrupt
the interaction between β2AR and β-arrestin 2. On the other hand, mutating Serine residues at 356th and 364th on β2AR does not affect the interaction. These results suggest that, among the four residues that had been discovered, Serine 355 is relatively more important for the interaction between β2AR and β-arrestin 2.
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APPENDICES

A. COMPOSITIONS OF CELL CULTURE SOLUTIONS

Table A. 1 Composition of D-MEM with high glucose

<table>
<thead>
<tr>
<th>COMPONENT</th>
<th>CONCENTRATION (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amino Acids</strong></td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>30</td>
</tr>
<tr>
<td>L-Arginine hydrochloride</td>
<td>84</td>
</tr>
<tr>
<td>L-Cysteine 2HCl</td>
<td>63</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>580</td>
</tr>
<tr>
<td>L-Histidine hydrochloride-H2O</td>
<td>42</td>
</tr>
<tr>
<td>L-Isoleucine</td>
<td>105</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>105</td>
</tr>
<tr>
<td>L-Lysine hydrochloride</td>
<td>146</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>30</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>66</td>
</tr>
<tr>
<td>L-Serine</td>
<td>42</td>
</tr>
<tr>
<td>L-Threonine</td>
<td>95</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>16</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>72</td>
</tr>
<tr>
<td>L-Valine</td>
<td>94</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
</tr>
<tr>
<td>Choline chloride</td>
<td>4</td>
</tr>
<tr>
<td>D-Calcium pantothenate</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-------</td>
</tr>
<tr>
<td>Folic acid</td>
<td>4</td>
</tr>
<tr>
<td>Niacinamide</td>
<td>4</td>
</tr>
<tr>
<td>Pyridoxine hydrochloride</td>
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</tr>
<tr>
<td>Riboflavin</td>
<td>0.4</td>
</tr>
<tr>
<td>Thiamine hydrochloride</td>
<td>4</td>
</tr>
<tr>
<td>i-Inositol</td>
<td>7.2</td>
</tr>
</tbody>
</table>

**Inorganic Salts**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Calcium chloride</td>
<td>264</td>
</tr>
<tr>
<td>Ferric nitrate</td>
<td>0.1</td>
</tr>
<tr>
<td>Magnesium sulfate</td>
<td>200</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>400</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>3700</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>6400</td>
</tr>
<tr>
<td>Sodium phosphate monobasic</td>
<td>141</td>
</tr>
</tbody>
</table>

**Other components**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose (Dextrose)</td>
<td>4500</td>
</tr>
<tr>
<td>Phenol Red</td>
<td>15</td>
</tr>
<tr>
<td>Sodium pyruvate</td>
<td>110</td>
</tr>
</tbody>
</table>

**Table A. 2 Composition of 1X Phosphate Buffered Saline (PBS) solution**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8 g/L</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g/L</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>1.44 g/L</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.24 g/L</td>
</tr>
</tbody>
</table>

The components are dissolved within dH₂O. After adjustment of pH to 7.4, solution is autoclaved.
B. COMPOSITION AND PREPARATION OF BACTERIAL CULTURE MEDIUM

Luria Bertani (LB) Medium

10 g/L Tryptone
5 g/L Yeast Extract
5 g/L NaCl

All of the components are dissolved in distilled H$_2$O. 20 g/L agar is added for solid medium preparation. The pH of the medium is adjusted to 7.0.
C. COMPOSITIONS OF BUFFERS AND SOLUTIONS

1X NEB-CutSmartTM Buffer:

50 mM Potassium Acetate
20 mM Tris-acetate
10 mM Magnesium Acetate
100 µg/ml BSA
pH 7.9 at 25°C

1X T4 DNA Ligase Reaction Buffer:

50 mM Tris-HCl
10 mM MgCl2
1 mM ATP
10 mM Dithiothreitol
pH: 7.5 at 25°C
**6X Loading Dye:**

- 10 mM Tris-HCl (pH: 7.6)
- 0.03 % Bromophenol Blue
- 0.03 % Xylene Cyanol FF
- 60 % Glycerol
- 60 mM EDTA

1X TAE (Tris Base, Acetic acid, EDTA) Buffer

- 40mM Tris
- 20mM Acetic Acid
- 1mM EDTA

Dissolved in dH2O.
**Table C.1** Composition of TFB I and TFB II.

<table>
<thead>
<tr>
<th></th>
<th>Solution Concentration</th>
<th>Prepared Stock</th>
<th>For 100 mL Solution take from prepared stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOAc</td>
<td>30 mM</td>
<td>300 mM</td>
<td>10 mL</td>
</tr>
<tr>
<td>RbCl</td>
<td>100 mM</td>
<td>1000 mM</td>
<td>10 mL</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>10 mM</td>
<td>1000 mM</td>
<td>1 mL</td>
</tr>
<tr>
<td>MnCl$_2$</td>
<td>50 mM</td>
<td>1000 mM</td>
<td>5 mL</td>
</tr>
<tr>
<td>Glycerol</td>
<td>15 %</td>
<td>87 %</td>
<td>17.2 mL</td>
</tr>
</tbody>
</table>

Complete the solution to 100 mL with distilled water and adjust pH to 5.8. After adjusting the pH, autoclave the solution or filter the solution using 0.45 mm filter.

<table>
<thead>
<tr>
<th></th>
<th>Solution Concentration</th>
<th>Prepared Stock</th>
<th>For 10 mL Solution take from prepared stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPS/PIPEC</td>
<td>10 mM</td>
<td>1000 mM</td>
<td>0.1 mL</td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>75 mM</td>
<td>1000 mM</td>
<td>0.75 mL</td>
</tr>
<tr>
<td>RbCl</td>
<td>10 mM</td>
<td>1000 mM</td>
<td>0.1 mL</td>
</tr>
<tr>
<td>Glycerol</td>
<td>15 %</td>
<td>87 %</td>
<td>1.7 mL</td>
</tr>
</tbody>
</table>
Complete the solution to 100 mL with distilled water and adjust pH to 5.8. After adjusting the pH, autoclave the solution or filter the solution using 0.45 mm filter.
D. PLASMID MAPS

**Figure D. 1** Map of pcDNA 3.1 (-) (taken from Invitrogen® Life Technologies)
### E. PRIMERS

**Table E.1** Primers designed for cloning of wild-type gene sequences of $\beta_2$AR and $\beta$-arrestin 2 into the pcDNA 3.1(-) and tagging of $\beta_2$AR and $\beta$-arrestin 2 with mEGFP and mCherry fluorescent proteins.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Enzyme</th>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
</table>
| $\beta_2$AR  | Nhe I  | $\beta_2$AR-Nhe I F | 5’-GTT GTT GTT GCT AGC ATG  
|              |        |             | GGG CAA CCC GGG AAC GGC-3’ |
|              | EcoR I | $\beta_2$AR-EcoR I R | 5’-GTT GTT GTT GAA TTC CAG  
|              |        |             | CAG TGA GTC ATT TGT ACT AC-3’ |
|              | EcoR I | $\beta_2$AR-EcoR I with stop codon R | 5’-GTT GTT GTT GAA TTC CTA CAG  
|              |        |             | CAG TGA GTC ATT TGT ACT AC-3’ |
| Arrestin 3   | Nhe I  | Barr2-Nhe I F | 5’-GTT GTT GTT GCT AGC ATG  
|              |        |             | GGG GAG AAA CCC GGG ACC AGG-3’ |
|              | EcoR I | Barr2-EcoR I R | 5’-GTT GTT GTT GAA TTC GCA GAG  
|              |        |             | TTG ATC ATC ATA GTC GTC-3’ |
|              | BamH I | Barr2-BamH I F | 5’-GTT GTT GTT GGA TCC ATG  
|              |        |             | GGG GAG AAA CCC GGG ACC AGG-3’ |
|              | Hind III | Barr2-Hind III R | 5’-GTT GTT GTT AAG CTT CTA GCA  
|              |        |             | GAG TTG ATC ATC ATA GTC GT-3’ |
Red letters are bases used for creating an extention so that restriction enzymes can have a pace to land. Blue letters are bases for the restriction cut sites. Black letters are bases that can bind to the gene.

**Table E.2** Primers designed for making mutated β<sub>2</sub>AR.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β&lt;sub&gt;2&lt;/sub&gt;AR-Nhe I F</td>
<td>5’-GTT GTT GTT GCT AGC ATG GGG CAA CCC GGG AAC GGC-3’</td>
</tr>
<tr>
<td>β&lt;sub&gt;2&lt;/sub&gt;AR-PD-N R</td>
<td>5’-GTT GTT GTT GAT ATC CAC C[CT GCT CCC CTG C[GT TGC CGT TGC C[GG C[GT AGC CAT TCC-3’</td>
</tr>
<tr>
<td>β&lt;sub&gt;2&lt;/sub&gt;AR-S355A-S356G R</td>
<td>5’-GTT GTT GTT GAT ATC CAC TCT GCT CCC CTG TGT TGC CGT TGC C[GG C[GT AGC CAT TCC-3’</td>
</tr>
</tbody>
</table>

Red letters are bases used for creating an extention so that restriction enzymes can have a pace to land. Blue letters are bases for the restriction cut sites. Black letters are bases that can bind to the gene. Green highlighted regions are the bases that are mutated.

**Table E.3** Primers designed for SDM.

<table>
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<th>Primer Name</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>β&lt;sub&gt;2&lt;/sub&gt;AR-S355A F</td>
<td>5’-GCT ACG CCA GCA ACG GCA ACA CAG-3’</td>
</tr>
<tr>
<td>β&lt;sub&gt;2&lt;/sub&gt;AR-S355A R</td>
<td>5’-GCT GGC GTA GCC ATT CCC ATA GGC-3’</td>
</tr>
<tr>
<td>β&lt;sub&gt;2&lt;/sub&gt;AR-S356G F</td>
<td>5’-CTC C[CG CAA CGG CAA CAC AGG GGA G-3’</td>
</tr>
<tr>
<td>Protein</td>
<td>Position</td>
</tr>
<tr>
<td>-----------</td>
<td>----------</td>
</tr>
<tr>
<td>β2AR-S356G R</td>
<td>229</td>
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<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>β2AR-T360A F</td>
<td>252</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
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<td>β2AR-T360A R</td>
<td>332</td>
</tr>
<tr>
<td>β2AR-S364G F</td>
<td></td>
</tr>
</tbody>
</table>

Green highlighted regions are the bases that are mutated.

**Table E.4** Primers designed for Integration PCR.
| 374 | EGFP Forward | 5’- GGA TAT CAC GTG GAA CAG GAG AAA GAA AAT ATG GTG AGC AAG GGC GAG GAG-3’ |
|     | EGFP Reverse | 5’- CGT GCC TGG GAG GTC TTC ACA CAG CAG TTT CTT GTA CAG CTC GTC CAT GCC G-3’ |
F. CODING SEQUENCES OF FUSION PROTEINS

Coding Sequence of wild-type β₂AR:

ATGGGGCAACCCGGGATCGGACCCCTCTGACCCATAGAGAAGCCATTGCGCCCGGACACCA
CGTACACGGCAGGAAAGGAGAGGATCGGTGGTTGGGAGGATCCGTCATGGCTCTACTGCTG
CCATCCTGTTTGGCAATGTGCTGGTCATCAAGACCACTTGGCGAGGCTATGGCAGCAGCTAC
AACTTCTTCTGCTGGGCTGTCATTCGCTTGGGCTGTCAGTGGCGGCGGTGGCAGTAGA
TGCTGCTGGCAGTGGCCCGCATAGATCGGCCCTTGGGATCGCCAGTTGAGCGGACTTCCTT
TCGAGCTCATTTGATCCAAGCTGCACTGATGCTGCTGATGCTGCTGCTGCTGCTGCTGCT
CTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT

Coding Sequence of wild-type β-arrestin 2:

ATGGGGGAGAACCAGGACACCCGGGCCACAGGCTTCCAAAGGATCGGAGCCCTCAACTGCTG
CGCAAGCAGGACTCTCAGATCACCCTGACAAAGTGGGACCTGATGGCGGTGTGGTGGGAC
CTGACTCCGAGGACCCGAGATTCCGCTGAGGCTATCCGCTGCTGCTGCTGCTGCTGCTG
CTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT

105
CCACAAAGGAAACTCTTGTGGCGGTGTGATCCGAAAGGTCAGCTCCGCCCAGAAGAACCCGCGC
AGCCCTCACCAGGAAACCACACGCGACACTCTCCTCTAGTGTGACCGGT7CCCTGCAACCTCGAGGCTTCCCTG
GACAAGGAGCTGTACTACCATGTTGGGAGCCCTCATCAATG?AAATGTCCACGTCACCCAAACACTCCACCAAA
GACCGTTCAAGAAT?CAAAATGCCTCAGTAGACAG7ACGCCGACATGCTGCTTTCAAGCGCGGAGT
ACAAGTGTGCTGTGGCTACATCGCAAAAGAGTACCGAGG7ATCTCCAGCTCCACATTCTGTG?AAGGGT
TACACCCTAACCCACCAGCTCAAGGAGACACCGGAGGAAGCGGGGTTCGCCCCCTGAGGTGGAAA
GCACGAGGACACACCTGGCTTCCACCCACATCTTGAAGAGGGGTGCAACAAAGGGAGGTGCTGGGAA
TCTGTGTGTCTCTACCGG24CAAGTTGCTGTTGCTGCTGCTGAGGGGTGCTGCTCTGAGCTGTG
CCTTTGTTCTTATGCAACCCCAAGGCCAACACGACCATTCCTCTTTGCCACACTCTTTGAGGTCTT
GGAGACGAGAATGCTCCTCCTACCATGTCCACAAACTTTGAGTTGATACATTGCTGATCGAGTAC
TTGTGTTTGGAGACCTTTGGGCCGCTTCGGTGAAGGGGA7GAAGGATGACGACTATGATGATCAACTC
TGCTAG

**Coding Sequence of β₂AR-mEGFP:** Black sequence corresponds to β₂A receptor; blue sequence shows restriction cut site while green sequence represents mEGFP sequence.

ATGGGGCAACCGGGGAAAGGAGCGGCCGCTTTCTTGCTGGCACCACCAAT?AGAAGCCATGCGCCGGACGACA
CGTCACGCGACAAAGGGAGGAGGTGGTGGTGGGCTATGGCA?CTGCTATGTCTCTACATCGTCTCTGG
CCTCAGTGTCTGGACAGTACCCCATGCTACACGACCATTTGCCACAGTGCACGCAGGGTTCTGACGAGTAC
AACTACTCCTCACTCACTTGGGCTTCTGCTGATCGTCTCGTCCATCTGGCGACGTGGCGCTTTTTGGG
CCGCCCATTATCTTATTGGAATGTGGGAGCTTTTGCGACACCTGCTGAGGATCTGGGACTTTTGAGTT
TGCTGTGCTGGCTACGACCCACAGATTGAGACCTTTGCTGTCTGGTGCTAGGATGACTGGACGGCTTTgTGCAT
TCACCCCTTCAGATCGTACCCCATGCAAAGAATAPSGGGCCGGGTGATATTCTGATGTTGGCTTTCC
ATCGTGTCTCTCTAGTTCCCTCTTGCTGTATCGTACCGAGGTCTTCTTCAAGGACGCAA
AAGGCAGCTCCAGACGAGTGAACATCTGAGGGGCTTTCCCGATCTCGGATGTTGATGCTGATCGAGTGAGC
AGGATGGGGAGCAGGAGCTTCGAGGAGCAGCTTTCCACAGTCTGTTGAAAGAGCCACAAAGCCTC
AAGAGCTTGGAGCCTACCATGCGGGCCTTCTCTCGTCTGCTGCTCTTCTTACTAGTACTGAG
GCATGTGTACCGGATAAAGTCCATCGTGAAAGGTATCTGCTCTCCTGTTATGAGGCTGATATTGA
ATTGCTGGTCTTACATCTAATGCGACCCGAGGAGACATTCGCAATGGCCCTCCGAGGAGCTTCTG
TGCTGGCGAGGTTCTTCTGAGGCGCTATGGGAAATGCTACTCCAGCAACGGCAACAGGAGGAGCA
Coding Sequence of β2AR-mCherry: Black sequence corresponds to β2A receptor; blue sequence shows restriction cut site while red sequence represents mCherry sequence.

```
GAGTGGATATCACGTGGAAACAGGGAGAAAGAAAAATTAAACTGCTGTGTAAGACCTCCAGGCGACAGGAAG
ACTTTCTGGGCCCATCAAGTCTGCTTGCCTAGAGATACATGAGTACACCAAAGGAGATTTGAGTACA
AATGACACTGACTGGAAATTTCAAGCTGAGGGAGCCAGGAGATTTGAGGAGCTTCCAGCCGGGTCCTGCTCCAC
GTGGTGGTGGGAGGCCCAGAGTGGTGGGAGGCCCAGAGTGGTGGGAGGCCCAGAGTGGTGGGAGGCCCAGAGTGGT
CTTCAAGTCCCGCATGCGGGACATGCTGACCGAGGCGACCACACCTTTCTTCTCAGGAGACCGGCACTCTTCTGA
AGAAGCCCCCGCCGAGGATTTCCGAGGGAGGCGACCACCTTTCTTCTCAGGAGACCGGCACTCTTCTGA
TACACCTCGCGATGCGGTGACACGTCCAGGAGGCGACCACCTTTCTTCTCAGGAGACCGGCACTCTTCTGA
GACAGGCTCGCTGGGACACCAGAAACAGGCAACTACGGGAGACCGGCACTCTTCTCAGGAGACCGGCACTCTTCTGA
TACACCTCGCGATGCGGTGACACGTCCAGGAGGCGACCACCTTTCTTCTCAGGAGACCGGCACTCTTCTGA
ACCAGAGCTCTGGGACACCAGAAACAGGCAACTACGGGAGACCGGCACTCTTCTCAGGAGACCGGCACTCTTCTGA
GACAGGCTCGCTGGGACACCAGAAACAGGCAACTACGGGAGACCGGCACTCTTCTCAGGAGACCGGCACTCTTCTGA
TACACCTCGCGATGCGGTGACACGTCCAGGAGGCGACCACCTTTCTTCTCAGGAGACCGGCACTCTTCTGA
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**Coding Sequence of β2AR-229-mEGFP:** Black sequence corresponds to β2A receptor while green sequence represents mEGFP sequence.

```
GAGTGGATATCACGTGGGAACAGGAGAAAGAAAATAAACTGCTGTGTAAGACCTCCAGCCACCGGAAG
ACTTTTGTGGGCACTACAGGGTCTGCTGGGCTAGGATACCAAACGAGGAGAATTGGTTAGTCA
AATGACTCAGCTGCGTAATTCATGGTGAAGGAGGCGAGGAGGAGAACATGCCACATCTCAAGGGAGTT
CATCCGCTTCAAGGTCGACAGGCCGACAGGCCTGGAATTGGTGAGCGCCACTGACGGGACGACT
AGGGGCGGCCTAAGGAGGCGGCCAGCCAGCGCGAAGCTGAAAGATGACCGAAGGAGGCTGCCCTTC
GCCCTGGACTACTTGGAAAGCTGTTCTTCCCGAGGCTTCAATGGGAGCGCGTATGAAACTTCGAGGACG
GCGCGTGTTGGCAGATTTGACCCGATCTCCTGCTCTGGGAGAGGAGGCAGCTTAGCTACAGGAGTAC
GCGCGCACTACAAGCTGAGTGCAAGACCCACTACAAGAGCAAGGCGACATGCCGCTGCCCCCGGCC
TACACAGCTCAACACTCAGATGAGCACTACCTCCCAACACCAAGGAGGACTTACACATCTGATTGAAACAGTACGA
ACGCGCGAGGGCGCCACTCCACCCGCGGACTGGACGACTGCTACAAAGTAA
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108
AGCAGAAGAACGGCATCAAGGTGAACCTCAGATCGACACCCACCCACACATCGGGACGGACGCCTGACTC
CGGACACACTACAACGAGAACACCCCCACATCGGACGGCCCGCTGCTCCGCCAAGAACACACTACCT
GACACCCCACTCCGCCCTAGCAAAAGACCCCTACCCACGAAGCCACCACTCGATCGTTCAACTTCAG
GAGCCGCTCTCCACGTGCAAAGAACCTATTACGCCAGTTGAGAGCATGCTGGCAGGACGGCTGACTTCC
ATCTCTCAAGTCCTGCTGTAAGGCAACAACACCTCTACAGCTATGCACTGCTGCTCGCTGGGACTTCA
CCCTCTGCTGGCTCCCTCTCCTCCTGTAGTTGAGTGGGACTGTTGCTGTCGTCTCTGCTGGCTAG
GAAGTTTACATGCTCTCTAAAATGGATGCTGATGCTGATTCTCTACATGGACTTTCAAGATCCTACT
GAGCCGAGTATTAGACTTACATAGAGAGGCGAGCTTCTGTGCGCTGCGCGAGTTCTCTTGAAGAGATATG
CGAGTAACTCTACCCAGCAAGACCGCAACGGGGCAGAGCTGATGAGTACGATACCCGGGAGGGGAGGAA
AATAAAGACGTGTTGCTGAGAACCTCTCCAGGACGGAGGAGAGACTTTGCTGGCCATATCAAGGTACTGCTGCTAG
CGATAAACATTTAGCACAAGGAGGAGATTGATGACAAATAGACTACTGCTGTA

**Coding Sequence of β2AR-229-mCherry:** Black sequence corresponds to β2A receptor while red sequence represents mCherry sequence.

ATGGGGCAACCCGGGAAACGGCGACGCAGCCTTCTCTGCTGGACACCCCAATAGAACGACATCGGCGCCGACAGA
CGTCAAGGGCAACAGGACGGAGGTTGAGGTGGGTGGGAGCTGGGCCAGTGGCATGATCTGCTTCTGG
CCATCTGTTGTTGCAATCTGCTTGCTCATCACAGGATTGGCGCTGCAAGCTGAGTCTGGCGACGATCCACCTG
AAGTACTCTCTTCTACCTCTACCTGCGCTGTCTGGATCTTGTGGCTATGGGGCTCTACAGTGAGGCTGTTGG
CCCGCATATTCTTTAGGATTTTGCGACTTTTCTGCTGCGGAGTTTGGGATTTTGGACTTTTCTCATGATG
TGGCTGCTCGTACCGCCACAGGTGAGACCTCTGCGCGGAGCTGATCTGCTTCTTGCGAATATGGACGTAC
TCACCCTTTCAAGTACACAGGACCTGCTGACCCAAGAATAGGGCCCCGGGTGATCAATCTGGTGGTGGAT
TGTTGAGGGGCTCTTACCTCTTTTCTGCGTCTCGACTCTACATGGCACTGGCCACACCCAGGAGGCA
TCAACCTCTGATCGATGCCACATTGAGGCTGACGCTTCTCTGAGCAAACTGACCGCTGCGACACGTAA
AAAGGCAAGAGGTGAGACGAGGGCAGGGGAGGATACGATCGAGGCGGAGGCGGAGGCGGCGGCCCTTAC
GAGGGCCACACCAAGGCAGTTGAGCAAGCAAGGGCCGTCGCCCTGCCCTTGCGCTGGAGAACATCCT
CTCAGCTCTTTATTGTACGCTGGCTCCACCGCTAGTGAGACGACCCCGCGACACTCTCTGAG
AGCTCTCTTCCAGAGGGGCTCTAAATGAGGGCCGGGCGGATGAACTTTGCAGGAGGCGGCGGCTTGACC
TGGAACCAGGACCTCTCTCTGGAGGAGGTCTCATACTACAGTTGAGGGCGCACAACACTT
CCCTCCAGGGCCCGGTGATGACAAAGAAAGGACATGGGCTGGAGGGCGCTGGGACGAGTACC
CCAGGGCAAGGGCCCTCTGAGGCGCGAGATCAAGCAGAGGCTGAAGCTGAGGACGGCGCCACTACGAC

109
Coding Sequence of β2AR-252-mEGFP: Black sequence corresponds to β2A receptor while green sequence represents mEGFP sequence.
CGCCGACCACCTACGAGCAGAAACACCCCCCATCGGGGACGGGCCCCGTGTAGTGCCCGGAAACCACCTACCC
TGACCAACCCAGTCGGCCCTGACGCAACAGCCCAACGAGAGGCCGATCACTGCTGTGGGAGCTTCC
GTGACCCCGCGCGGAATCACCCTCTCCGGCATGGAAGAGCTGTCACAAGCGGACGGGATGGAGACTCCGCAG
ATCTTCGGAATTGGCTGTAAGGGAGCAAGCGCCTCCAAGACCCGCAAGGCCGATCACTGCTGTGGGAGCTTCC
CCCTCTGTGGCTGGCCCTTCTCTCATGTAACATGTGGCAAATGCATGTAACACTGCTCCCCTTATCTACTGGCC
GAGCCCGAGATTCCAGGATCCCTCCAGGACGCTTCTGTGGGCTGGCCAGAGAGCTTCTTTGAAGGCCCTATG
GGAATGCGCTACTCCGACCGGACACACAGGACAGATTGGAATGTAACGTGGGAAAGAGGAGAAAGA
AAATAACTCGCTGGTGAGACACCTCCCGACGGAAGAAAGACTTCTGTGGGCTACAGATGACTGTGCCTAG
CGATAAACATTGATTCACACAGGAGGAATTGTCATACACAGACTGCTCCCTTGAA

**Coding Sequence of β2-AR-252-mCherry:** Black sequence corresponds to β2A receptor while red sequence represents mCherry sequence.

ATGGGGCAACCGGGAACGGCAGAACGGCGCTTCTTCTGTGGCCACCCGAAATAGAAGCGCAATGGGCCGGGACCACGA
CGTCAGCAAGAACCAAGGGACAGCTGTTGGTGGCGGACATGGCTCATCTCTGCTCTGGG
CACAATAGTCTTCTTCAGAAAATGAAGGACTTTTGTCCGAGATTTGGGACATCTCC
AACATATTCTATACAGCCCTTCTCTTCTTGCCCATTCTAGATGCACGTGGTACCAGGCACCCACCAGAGGAAGCCA
TCACTGCTAGTAAGACCAATGAGGACCACTTCCTACCTCTGCCAGAAACGCTTTGATAGCATGGTGGAT
TGATGCAGCATCTACCTCCTGCTGCAGTACATGCATGACGCTCTTTGGGCCAAGCTTGAGAAGCGAAC
AGGCAGCTCTCAGAAGATTAACACTTGAGGGCCGCTTCTAGCTGTCCAGAACCCTACAGGGGAGGAG
AGATGGGAATGCGAGCACAGGGAGGAAGAAACCATGGCCAATCTCAAGAGGTCTACGTAGCTCGCTCAAG
GTCGACCATGGGAGGGTCCGGATTGAACCGGAACTGGGTGAGATAGCCAGGCGGGGAGGCGGCGGCGGGCCCCTA
CGAGGGCAACCGGACACGCAAGCTGGAAGTGCCAAAGGTGGCGCCCTCTGGCCCTCGGCTTGACATCC
TGTCCTCCCTCATAGTCTACGGCTCAAGGCGATCCGTTGAGAATATTGGAGAGCGGCGGCTGGTGAC
CGTGACCCAGGACCTCTCCCTCCGAGCGAACGAGGTGTCACTTAAGAAGCAGATGGTACGATGGCAGGCACAATC
TCCCTCCGAGGCCCGGTAATGCAAGAGAACACATGGGGTGGGAGCGCCCTCTCTCGGAGCGGATGC
CCCGAGGACCGGCGCCCTGGAAGGGCGAGATCCAGACAGAGGCTGAACTGGAAGAGCCGGCGCACTACGA
CGCTGAGTGTCAGACACACCTCATCAAGGCAAGCGGCGCTTGTGACGATGGCCCGGCGGCTACTACAGA

111
TCAAGTTGGACATCACCTCCCAACACGAGGACTACACCCATCGTGGAACAGTACGACGCGGGCCGCAGG
CCGCACTCCACCGGGGCAATGGAAGCGACTGCTACCACGAGATCTTCCCAAT
GTTCTGTCTGAAGAGAACCAAAAGCCCTCAAGAAGCTTTAGGCAATCATCATGCGGCCTTATCCACCTCTG
GGTGCCCTTATCCTATGTTAATCTGTGCTGATTCAAGCTCCATCTCGTAGGACTCTTGAAGGCTTAT
ATCTTCAATATCTAGTATGACTATCAGTCTTGTGCTACCTTCTATGCTGGG
CCATCGTGTCTGGCAATGTCTGTCATACACAGCCATTTGAAGCTTTGCGACGTCTGCGAGACGTCACC
ACTGACTTCTACTGCTCATCGCCTGTGCCTGGAATCTGCTCCAGTGTGAGGCTCCCTTTGGGC
CGCCATATTTCTTAAGAAATGTTGGACTTTTGGCAACTCTCTGTGCGATTTTTGGGACTCTCATATG
TGCTGTTGCGTGCCAGGATTCATGTGGACACTTTCTGCTGCTATGAGGATTTGTTCTTGACATCT
TCACCTTTCAAGTACAGAGACCTGCTGAGCAACAAATAGCGGAGCTACATTGATGGTATG
TGCTGAGAGGCTTATCCTCTCCCTCTTTGCCCATTGAGAGCTCAGGACTTTGCTGAGGCAACA
CTAGCTGCTATGCAGCTAGCTGATTCCACAGCTTCTGCTGCACCTTCTCTGTACATG
ATCGTGTCTGTCTTTCTAGTTCTCTCTTTGCCCATTGAGAGCTCAGGACTTTGCTGAGGCAACA
AAGGCAGTCTCAGAAGATTTGAACAAATCTGAGGAGCCTTCCCATGTTCCAGAACTTACCCAGTGGAC
AGAATGGGGCAGGGCACTAGGACAGACTCTCTGCTTGGACGACCGGACATTTTATGAG
ATCTGTTTCTCACTCCCTATCTACTGCGGAGCCAGATTTCTATGGTGCAAGCCGGAGGAGGCTG
TTCCAGTTGAGGTGTCGACAGACCGACGCGGAACAGTGCAAAAGCCGCAACTTACGGCT
CGGGAGGCCCAGGGGCACTGACCAACAGCTTCTGACTCTGCCACCTTACCCCGTGGAGGCTAC
GACCACTAAGAAGCAGACAGACTATTTCTTCAAATGCGCCCTGCGAGAGTCTACGTCAGGAGGCACAT
CTTCTCTCAGGAGGCACACTCAAGACCCGCGGCGAGGGTGAGATTTTGAGGAGGCGACACCTTGTTGQA
ACCCGATCGAGCTGAAGGGCACTCGACTTCAAGAGGAGACCGGAACACACTTCTGGGCGACAAAGTCGAGTAC

Coding Sequence of β2AR-332-mEGFP: Black sequence corresponds to β2A receptor while green sequence represents mEGFP sequence.

ATGGGGCAACCCCGGAACGCCAGCCCTCTTTGCTGGACCCCAATTGAAAGCCATTGCCCGGACCAGGA
CGTCAGCGAGAAAAGGCCAGGGTGTTGGGTGGTGGCAGAGTCCGTTGCTCATGCTCTCATGCTGGC
CCATCGTGTCTGGCAATGTCTGTCATACACAGCCATTTGAAGCTTTGCGACGTCTGCGAGACGTCACC
ACTGACTTCTACTGCTCATCGCCTGTGCCTGGAATCTGCTCCAGTGTGAGGCTCCCTTTGGGC
CGCCATATTTCTTAAGAAATGTTGGACTTTTGGCAACTCTCTGTGCGATTTTTGGGACTCTCATATG
TGCTGTTGCGTGCCAGGATTCATGTGGACACTTTCTGCTGCTATGAGGATTTGTTCTTGACATCT
TCACCTTTCAAGTACAGAGACCTGCTGAGCAACAAATAGCGGAGCTACATTGATGGTATG
TGCTGAGAGGCTTATCCTCTCCCTCTTTGCCCATTGAGAGCTCAGGACTTTGCTGAGGCAACA
CTAGCTGCTATGCAGCTAGCTGATTCCACAGCTTCTGCTGCACCTTCTCTGTACATG
ATCGTGTCTGTCTTTCTAGTTCTCTCTTTGCCCATTGAGAGCTCAGGACTTTGCTGAGGCAACA
AAGGCAGTCTCAGAAGATTTGAACAAATCTGAGGAGCCTTCCCATGTTCCAGAACTTACCCAGTGGAC
AGAATGGGGCAGGGCACTAGGACAGACTCTCTGCTTGGACGACCGGACATTTTATGAG
ATCTGTTTCTCACTCCCTATCTACTGCGGAGCCAGATTTCTATGGTGCAAGCCGGAGGAGGCTG
TTCCAGTTGAGGTGTCGACAGACCGACGCGGAACAGTGCAAAAGCCGCAACTTACGGCT
CGGGAGGCCCAGGGGCACTGACCAACAGCTTCTGACTCTGCCACCTTACCCCGTGGAGGCTAC
GACCACTAAGAAGCAGACAGACTATTTCTTCAAATGCGCCCTGCGAGAGTCTACGTCAGGAGGCACAT
CTTCTCTCAGGAGGCACACTCAAGACCCGCGGCGAGGGTGAGATTTTGAGGAGGCGACACCTTGTTGQA
ACCCGATCGAGCTGAAGGGCACTCGACTTCAAGAGGAGACCGGAACACACTTCTGGGCGACAAAGTCGAGTAC

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Coding Sequence of β₂AR-332-mCherry: Black sequence corresponds to β₂A receptor while red sequence represents mCherry sequence.
Coding Sequence of β₂AR-374-mEGFP: Black sequence corresponds to β₂A receptor while green sequence represents mEGFP sequence.
Coding Sequence of β2AR-374-mCherry: Black sequence corresponds to β2A receptor while red sequence represents mCherry sequence.

ATGGGCCAACCAGGGAAAGCGGAGCTGCGGTGGTGGGTGGCACTGGGTCATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
CTCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTC
AATGGCGGCAACCAGGGAAAGCGGAGCTGCGGTGGTGGGTGGCACTGGGTCATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
CTCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTC
AATGGCGGCAACCAGGGAAAGCGGAGCTGCGGTGGTGGGTGGCACTGGGTCATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
CTCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTC
AATGGCGGCAACCAGGGAAAGCGGAGCTGCGGTGGTGGGTGGCACTGGGTCATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
CTCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTC
AATGGCGGCAACCAGGGAAAGCGGAGCTGCGGTGGTGGGTGGCACTGGGTCATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
CTCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTC
AATGGCGGCAACCAGGGAAAGCGGAGCTGCGGTGGTGGGTGGCACTGGGTCATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
CTCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTC
AATGGCGGCAACCAGGGAAAGCGGAGCTGCGGTGGTGGGTGGCACTGGGTCATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
CTCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTC
AATGGCGGCAACCAGGGAAAGCGGAGCTGCGGTGGTGGGTGGCACTGGGTCATGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
CTCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTCACTACCTC
AAGCTGAAAGCAGCGCCACCTACGACGTAGCTAGACACCACACTCAACAGCAAGGCCCAGCCGCTGCA
GCTGCCGGCGCCCTACAACTGCTAAGCATCCTAGTGGACATCACTTCTCCACACAGCAGGACTACACCATCG
TGGAAACAGTACGACAGCAGCAGGAGGCGCCCGGACCTCCACCCGCCGCTACGAGCTGCTAGCTACAGATACAGT
CTGTGTAAGACTCCACGACCGCGAGAAGTCAGTGCTGCTGAAGCTAGATAAACAT
TGATTCACAGGAGGAAATGTAGTACCAAAATGACTCTGACTGCTGTA

**Coding Sequence of Barr2-mEGFP**: Black sequence corresponds to β-arrestin 2; blue sequence shows restriction cut site while green sequence represents mEGFP sequence.

ATGGGGGAGAAGACCCCGAGACGAGGCTGACTGAGGCCCTAACTGCGAGCTCACCCGTGACTT
GGCGAAGCGCCGCCTGCTAGATCACCTGAGAAGATGGACCCCTGTAGATGGCGTGCTGTGGACCC
CTGACTACCTGAGACCCGCAAAGTGTGTGAGACCCTACCTGCGCCCTCCGCTATGCGCGGTAAGAC
CTGGATGTGGCTGGCTGTCCTTGCGAAGACCTGAGTCTGGCCACCCCTTCCCGGTGACCCCGT
GCCAACCCACCCCGCCCTGACGAGCGGCGCTGGAGAGCTGGGACGACGATTCCACGCAGGACGAT
ACAGAAAGGGCATCGGGGTAGACTTTGGAGATTCGAGCCTTCTGTGCTGCTAAATCATAGAAGAGAAAG
CACCAAAAGGGACTCTTGGGCGGTGTGCTGGACGAGGAGAAAAAGTGTCGCGCCGAGAAGAGAACG
AGCCTTCAGCCGAAACCCACGCACTTCAATGTGCTGACCGGCTCCGCTACCTGCCGGTACGT
GACAAGGACTGTCATACCAATGGGAGCCCTCAATGATAAATGTCCAGGTCAACAAACTCTGCCAAGT
GACCGTGAAGAGTAAGTTCTCTTGAGAGACTAGCCGACATCGTCCCTCTTGCAGCACCAGGCCCAGT
ACAAAGTTGCTCTGGGCTCAACTCGAAGAACAGAGACCAGGTACTCCAGTCACCTAATCTGAGCTG
TACACCATAACCCCGCTCGCCCGACACCGGAGGATCGGGGTGTGCGCCCTGGATGGGAAAGTCAG
GCCAGGGCTAACAACCTCGGGCTGGAGCTACGGCAAGGAGAATGGGCTGAGGAGAAGAGAAGC
TCCCTGGTGTCCTACAGGGTCAAGGCTGAGTCTGGCTGTGGCTGCTGAGGGGATGCTCTGCTGAGCTG
CCTTTTGTGTCTATTGACCCCCAAGCCCGCAGAACTACCCCTCCACCGCCAGGGAGCAGCCGCTCC
GGACAGACATGGCTTCCACGACACCAACTCTTTGATTGATACCAAACTATGGCAAGATGATGACA
TTGTTTTAGAGACTTTCGCCGCTTGGGCTGAAGGGAGTGAAGGATGAAGACTATGATGACCAACTCT
TGCGAATTACATCTAGTGACAGCAGCGCCAGGAGCTGTCCAGCGGGGTCGCTGTCGAGTGGGA
GGCGCAGCTAACAACGCGCACAAGTTTCAGCGTGGCTCCGGCGAGGCGAGGCGATGCGACCTACGCGGACG
TGACCCTGCGAATCCGCGAGGTAGCCTCCGCCTGCGCCACCCCGCTGAGGACGACCTGCGT
ACCTCGCGCGTGGCAGCTGCTCAGGGGCTTACCCCGACGACATGGAAGCAGACGACTTCTCTAAGCTCCG
CATGGCCGAGGCTAGCTGGCCAGGGCCCACTGCATCTGTTCAAGGAGCAGCGGAACTACAGAACAGCGC
CCAGGGTGAGAGTGCTGGAGGCCGACACCTCTGTTAGAAAGCCAGATCGAGCTGAAGGGCATCGACTTCAAGGAG
Coding Sequence of mEGFP-Barr2: Black sequence corresponds to β-arrestin 2; blue sequence shows restriction cut site while green sequence represents mEGFP sequence.
AGCACCCATCGTGAAGGAGGTGCCAACAAAGGAGGTGCTGGGAATCTCTGTGTGCTCACAGGTCAGCT
GAAGCTGGTGTTGCTTCTGAGGCGCTGGGATGTCTCTGTGGACTTGCTTCCTTTGTTCTTATGCAACCCGAGCC
CCCAGCACCACATCCCACTCTCACCACTGACACAGTATGTCCCTGTGGACACC
AACCTCTATGAAATGTGATACCTAATGCTGACAGTATGACATATGTGTTGTGGAGACTTTGCCCAGGCT
TCGGCTGAAGGGGTGAAGATGACAGCATTATGATGATCAACTCTGCTAG

**Coding Sequence of Barr2-mCherry:** Black sequence corresponds to β-arrestin 2; blue sequence shows restriction cut site while red sequence represents mCherry sequence.

ATGGGGAGAAGACCCCGAGCCAGGCTTTCTTCAAGAAGTGCAGGCTCCCTAACACTGCAAGCTCAACCGTGTACCT
GGCCAAAGCGGAGCTTCTGTAGATCACCTGGACAATAAGTGGAACCCCTGTAGATGTGGCTGGGGTCTGGACC
CTGACTACCTGAAAGCGCAAGAGTGTTTGACCCCTACCTGCGCCTTCCGCTATGGCGCTGAGAC
CTGGAGTGCTGGGCTGGCCTCTTCCGCCAAAGACCTTTCTGCTACCGACAAGCTCTTCTCACGGCCTTCCCGGT
GCCAACCACCCCGCCGCCACCCCGCTGAGGACGAGCTCCTGAGAGGCGCCAGATGCCC
ACCCCTTCTTTTCTTCACCA?ACCCCAAGAATCTTCTTCTATGCTCTGCTACACATGACGCCACGGCGAAGATGC
ACAGGAAGGGCTCGGGAAGTACTTTTGGATTTGAGATTGCAGCCTCTCTGCTGCTAAATACCTAGAAGAAGAAGAC
CCACAAAGGAATCTCTGTGGCTGGATCTGGAAAGGAGTTGCAAGTGGGCGCCAGAAACCCCGGCCCT
AGCCTTCACCAGAACCCAGCGCACTCTCTCATGTCTGACCGGCTCCCTGACCTCGAGGCTTCCCTG
GACAAGGAGCTGATCTACTCACATGCGGCAGGCCCTTCAATGTAATTGTCCACGTCACCAACAACACTCCACAA
GAGCCTCAAGAGATCAAGAGCTCTGTGTGAGACAGA?AGGGAGCTTA?CTGCCCTTTGAGAGCCAGGACGT
ACAGAGCTCTGTGCTCAGTCAAGAACAGATGACAGAGGATCTCTCAGCTCAAATTCTGTAGGAGGTG
TACACCATAACCCGACTGAGCGGACAAACCAGGGAAGGCGGCTTCTGCGGCTCTGAGTGAGGAAACTCA
GCCAGAGCGCAACCCACCTCTGCGTACCATCGACACATGCTGAAGGAGGCTGCGGACCCACAAAGGAGGTGCG
TCCTGGTGCTCTACAGCGTGAAATGCTGTGCTGCTGTCGGAGGGGGTGCTGGAGTCTGCTGGCCTG
CCTTCTGCTTATCTGACCCCAAGCCACCCGAGCCACGAACTCCCTCCCGGACTCCCGGACGCTC
GGAGACAGATGTTGCTGGAGCAACACACCCTATGATGATGATGACAGATGACATGTAACAATC
TGCCGAAATCTGAGTGAGCAAGGCGGAGGAGGTTAACAGTGCCCATCTCAAGGAGTTCTGTACCA
GGTGCACAGTGAGGGCTCGTGAAACGCGCAGAATCCGAGATGAGGGCGAGGCGAGGGCGAGGGCGGCCT
ACAGAGGCGACCCACCCGGCAAGGCAGAGTGAGCAAGGGTGGGCGCTCCCTGGGCTTCCCTGGGAGCATC
CTGTCCCTTCTTGATGGATGACCGGCTCAGTTCAATCGCAGAAGGCTCTGTCCGAGGCAACCGGAGTTCCTTCGAAGGCACCCGAGCC
118
TTCCCTCCGACGCGCCCGGTAATGCAAGAAGAAGCCATCGGGCTGGGAGCCCTCGTCGAGGTGATGTA
CCCGAGACCGCCGCACTGGAGGATCCAGATCAAGAGGCTAAGGGCGGCGAGCTGGAAGGGCGGCGGCACCCTACGG
ACGCTAGGTTCAAGGCCACCTACGCAAGCCAGTGCGCTGCGGTGCTCGCGGCGCCCTAGAAGCCTCAAC
ATCAAGTGGACATCACCCACACGCAGGAGCTACCAAGCAGCGACGGCTGGAAGCAGCGGCGCAGGCGGACCGCCAGG
CCGCCACTCACCAGGCGGACCATGGGAGACTGCTAACAGATGAAG

**Coding Sequence of mCherry-Barr2:** Black sequence corresponds to β-arrestin 2; blue sequence shows restriction cut site while red sequence represents mCherry sequence.

ATGTTAGCAAGGCGGGAGGATATGAATTGGCCATCTCAAGGTTGAGTTGACAT
GGAGGCTCCGTTGAGAGCCGCACTGGCAAGGCGGCGGAGCTGGAAGGGCGGCGGCACCCTACGG
CCCGAGACCGCCGCACTGGAGGATCCAGATCAAGAGGCTAAGGGCGGCGAGCTGGAAGGGCGGCGGCACCCTACGG
ACGCTAGGTTCAAGGCCACCTACGCAAGCCAGTGCGCTGCGGTGCTCGCGGCGCCCTAGAAGCCTCAAC
ATCAAGTGGACATCACCCACACGCAGGAGCTACCAAGCAGCGACGGCTGGAAGCAGCGGCGCAGGCGGACCGCCAGG
CCGCCACTCACCAGGCGGACCATGGGAGACTGCTAACAGATGAAG

ATGCGACCTTACTGCAAGCGCCATGAGGGACCCAGGCGGCCCTCCGCTGCGCTGCGGTGCTCGCGGCGCCCTAGAAGCCTCAAC
ATCAAGTGGACATCACCCACACGCAGGAGCTACCAAGCAGCGACGGCTGGAAGCAGCGGCGCAGGCGGACCGCCAGG
CCGCCACTCACCAGGCGGACCATGGGAGACTGCTAACAGATGAAG

119
GTGAAGGAGGGTGCCCAACAGGAGGTGCTGGGAAACCTCTGGTGCTCACAGGGATGGTGAAGGCTGGTGGTGTCTCGAGGGGATGCTCTGTGGAGCTGCTCTTTTGTCTTTATGCACCCCCAAGCCCCACGACCACATCCCCCTCCCCCAGACCCCGTCTCCGAGACAGATGTCCTCTGTGGACACCCACTCTCTTGAATTTGATACCAACTATGCCACAGATGACATTGTGTGTTGAGGACTTTGCCCCGCTTCGGCTGAAAGGGATGAAGGATGACGACTATGATGATCAACTCTGCTAG