The Study of 5-HT_{1D} and 5-HT_{1F} Receptor Interactions with G Proteins via BRET Analysis

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Mental illnesses like migraines are prevalent worldwide and cause recurrent suffering in men, women, and children. According to data obtained from self-reported migraine and severe headache responses in the United States, the occurrence of migraines is estimated to be about one in every five Americans, with females affected more than males [1]. Around the world, migraines affect about one in every seven people [2]. These statistics reveal the large impact that migraines have on the human population and stress the importance of understanding how migraines occur to create ways for treating this condition. One way we can seek treatment options for this condition is by identifying specific drugs or substances that target certain receptors implicated in the occurrence of migraines, which include serotonin receptors, specifically 5-HT_{1D} and 5-HT_{1F} [3,4]. Serotonin or 5-hydroxytryptamine (5-HT) is a neurotransmitter that activates serotonin receptors. Serotonin receptors, like the 5-HT_{1D} and 5-HT_{1F} receptors, influence various functions within the human body when activated, including regulation of the sleep-wake cycle, appetite, and moods [5]. Studying the roles of these serotonin receptors in the body will provide information that can be used in potential drug development for an effective cure that provides worldwide relief from migraine pain.

To understand how to treat mental illnesses like migraines, we must first comprehend how the 5-HT_{1D} and 5-HT_{1F} receptors function in the body. Both the 5-HT_{1D} and 5-HT_{1F} receptors are G protein-coupled receptors (GPCRs), a superfamily of proteins used primarily for cell-cell communication. G protein-coupled receptors are membrane proteins that are associated with heterotrimeric proteins called G proteins. These receptors are composed of seven transmembrane domains that span the plasma membrane of a cell, with the N-terminus of the receptor in the extracellular space and the C-terminus of the receptor in the intracellular space of the cell **(Figure 1)**. GPCRs can bind to substances called ligands. Each GPCR has a unique

affinity for a particular ligand which will evoke a certain response from that cell. The site where the ligand binds is on the extracellular portion of the GPCR. In contrast, the site where the associated G protein binds to the GPCR is on the intracellular portion.

The two-state model for GPCRs assumes they exist in equilibrium between an active state and an inactive state. In the active state, GPCRs bind G proteins, activating them, while in the inactive state, GPCRs are uncoupled from G proteins [6]. There are various substances that can bind to and affect the function of the GPCR, including agonists, antagonists, inverse agonists, partial agonists, among others. Agonists are substances that bind to the receptor at the ligand-binding site and cause a conformational change in the receptor to activate it and initiate an effect. Antagonists are substances that compete with the agonist for the ligand-binding site and when bound, prevent the agonist or other substances from binding. Antagonists do not cause an effect on the receptor but instead prevent the action of the agonist on the receptor. Inverse agonists bind to the ligand-binding site and cause an effect opposite to that of the agonist by promoting the inactive state of the receptor to decrease the receptor's activity. Partial agonists bind to the ligand binding site but produce an effect lower than that of an agonist. Substances can be categorized using this terminology which hints at how that substance affects a specific receptor and what the subsequent effect is in comparison to a known effect.

As stated in the name of these receptors, an important component for GPCR function is its association with heterotrimeric G proteins. The main families of heterotrimeric G proteins are: G_s, $G_{i/o}$, G_q, and $G_{12/13}$. A heterotrimeric G protein is a protein complex consisting of three subunits: an alpha (α) subunit, a beta (β) subunit, and a gamma (γ) subunit. The alpha and gamma subunits are bound to and move through the plasma membrane via lipid anchors, making the heterotrimeric G protein membrane-bound [7]. The alpha subunit of the heterotrimeric G

protein binds to either guanosine triphosphate (GTP) or guanosine diphosphate (GDP), which are called guanine nucleotides. Since the alpha subunit binds to either GTP or GDP, the heterotrimeric G protein acts as a molecular switch [7]. In other words, the G protein can be active or inactive depending on which guanine nucleotide is bound to the alpha subunit. When GTP is bound to the alpha subunit of the G protein, the G protein becomes active. When GDP is bound to the alpha subunit of the G protein, the G protein becomes inactive.

Both GPCRs and G proteins are involved in a process called signal transduction. Signal transduction converts the extracellular message from a ligand into an intracellular message to direct a cell's response as illustrated in **Figure 1**. This process involves key components including the ligand, the GPCR, the associated G protein, effector enzymes, and secondary messengers. Signal transduction starts with the binding of a ligand to a GPCR, which causes a conformational change in the GPCR to its activated state. The associated G protein moves through the membrane and binds to the GPCR, and the GPCR triggers the release of GDP from the associated G protein to allow the G protein to bind to GTP [7]. The active G protein can then detach from the GPCR. The alpha subunit dissociates from the beta and gamma subunits of the heterotrimeric G protein complex and binds to effector enzymes such as adenylyl cyclase [7]. The beta and gamma subunits may also bind to other effectors to initiate their own distinct signaling pathways [7]. The binding of the active alpha subunit to the effector enzyme will affect the production of certain second messengers, which are molecules used to create an intracellular message for eliciting a cellular response. However, this message is short-lived because the alpha subunit has an intrinsic ability called GTP hydrolysis, which allows it to cleave GTP to form GDP [7]. When GDP is bound to the alpha subunit of the G protein, the alpha subunit becomes inactive and does not bind to effector enzymes. Instead, the inactive alpha subunit re-associates

with the beta and gamma subunits. Thus, the signal transduction will cease, and the cellular response will stop. General examples of cellular responses created by signal transduction pathways include changes in gene expression or changes in enzymatic activity [8].

Depending on the type of G protein that interacts with the GPCR, a specific signaling pathway will be initiated which will direct the response of the cell by using an intracellular message [7]. Each G protein influences which second messengers are produced and the signaling pathways involved as depicted in **Figure 2**. The G_s family stimulates production of cyclic adenosine monophosphate (cAMP) from adenylyl cyclase whereas the $G_{i/o}$ family inhibits cAMP production from adenylyl cyclase. The G_q family activates phospholipase C to cleave phosphatidylinositol 4,5-bisphosphate (PIP_2) producing diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). The $G_{12/13}$ family leads to activation of another G protein, RhoA. By determining which G protein interacts with a certain GPCR, we can use this information to understand how that signaling pathway affects the physiology of the body. For example, a specific physiological effect can be observed in response to a signaling pathway that is not active or has decreased activity (versus the effect when the signaling pathway is active).

A signaling pathway can be identified by the GPCR and G protein(s) involved in its initiation. Currently, several methods for determining which GPCR interacts with which G protein to initiate a signaling pathway have been employed, but each method has disadvantages. Traditional practices measure differences in concentrations of secondary messengers produced upon receptor activation, which could be misleading. Some ligands may activate multiple types of G proteins, resulting in activation of multiple signaling pathways [9]. In addition, the G proteins that are activated could exert opposing effects on effector enzymes, such as stimulating and inactivating the same effector enzymes [9]. This leads to an ambiguous concentration of the

second messenger, and as such in some cases second messenger assays are unreliable methods for elucidating which signaling pathway is activated by an extracellular signal.

Methods used to directly monitor protein-protein interactions like fluorescence resonance energy transfer (FRET) are amendable to study GPCR: G protein interactions. However, FRET requires excitation of a fluorophore, a molecule that emits light, by an external light source [10]. The fluorophore is therefore susceptible to photobleaching, which reduces the amount of signal or photons measured from the fluorophore [10]. Additionally, the acceptor molecule, which receives a resonance energy transfer from the fluorophore, may also absorb the monochromatic light due to a similar absorption spectrum to the fluorophore [10]. Likewise, this could lead to a misinterpretation of the actual interaction between the receptor and the associated G protein when performing analysis of the data [10].

Instead, to directly analyze the G protein-coupled receptor and G protein interactions, this study reports on the use of a technique called bioluminescence resonance energy transfer (BRET). BRET allows for the measurement of protein-protein interactions in real time and in live cells [11]. **Figure 3** depicts how BRET works. This technique employs a bioluminescent donor molecule and a fluorescent acceptor molecule, and both molecules must be 10 nm in distance or closer to each other for the transfer of resonance energy to occur between the two molecules [11]. A bioluminescent donor is a luciferase which emits light of a certain wavelength in the presence of its substrate. A fluorescent acceptor emits light of a certain wavelength when it receives a transfer of resonance energy from the bioluminescent donor. In our system, the bioluminescent donor molecule is bound to the GPCR (Protein X) whereas the fluorescent acceptor molecule is bound to the G protein (Protein Y). When these two proteins interact, the

transfer of resonance energy occurs since the protein interactions bring the donor and acceptor molecules within 10 nm of each other.

In a BRET assay, the light energy emitted at distinct wavelengths by both the donor and acceptor molecules is measured using a luminometer [11]. A BRET ratio can be calculated using the number of photons emitted by the bioluminescent donor and fluorescent acceptor. The BRET ratio is a value determined by dividing the number of photons emitted by the fluorescent acceptor by the number of photons emitted by the bioluminescent donor [12]. This ratio can be used to determine the relative strength of interaction between the GPCR and the G protein that was studied. A higher BRET ratio correlates with a stronger interaction between the specific G protein and a specific GPCR. Once this quantitative information is known, the associated signaling pathway(s) can be identified.

The purpose of this study was to determine which mini G (mG) proteins couple with the 5-HT_{1D} and 5-HT_{1F} receptors using BRET assays to identify the signaling pathways they initiate when stimulated by serotonin or other ligands. Mini G proteins are smaller versions of the four main families of heterotrimeric G proteins used in signal transduction in cells [13]. These proteins are engineered to only contain the GTPase domain of the alpha subunit and remain in the cytoplasm rather than attached to the plasma membrane [13]. When a receptor is activated at the plasma membrane, the mG protein will translocate from the cytoplasm and bind to the active receptor. This creates a receptor-mG protein complex that can generate more effective BRET measurements since it is more stable compared to a receptor-heterotrimeric G protein complex, which can dissociate quickly based on ambient concentrations of guanine nucleotides [13]. Additionally, since a mG protein is not membrane-bound like a heterotrimeric G protein, less background signals will be measured and interfere with the obtained BRET signals from

receptor-mG complexes [13]. Despite the modification of the G protein, experimental results report that mini G proteins can be used as probes for profiling G protein-coupled receptors as they exhibit the same functionality as their corresponding classes of heterotrimeric G proteins [13].

Ultimately, the information obtained from this project will be documented on the IUPHAR/BPS Guide to Pharmacology database. IUPHAR/BPS database is a merged collection of information recording and describing medicinal ligands and drugs that target receptors [14]. It can be used by students for information on numerous compounds and by researchers when developing clinical drugs [14]. In the near future, the use of the information on the 5-HT_{1D} and 5-HT_{1F} receptors could result in the development of novel drugs to cure migraines for people all around the world.

Procedure Summary [16]

To study such protein-protein interactions using BRET, the genes that encode for the 5-HT_{1D} and 5-HT_{1F} receptors were cloned with the bioluminescent donor in BRET, Nanoluciferase (NLuc). A process called molecular cloning produced plasmids containing the receptor genes tagged with NLuc at the C-terminal end. This process involved the amplification of the genes for the 5-HT_{1D} and 5-HT_{1F} receptors via polymerase chain reaction (PCR) and subsequent purification. A plasmid prep was used to isolate a plasmid containing the NLuc gene, which is designated as pNLuc. A restriction enzyme digest was performed on pNLuc and the amplified receptor genes and following gel electrophoresis, the digested DNA molecules were purified. Then, the receptor genes were ligated into pNLuc, and the ligation reactions were

transformed into *E. coli* JM109 cells. The transformed *E. coli* JM109 cells were screened for the presence of plasmids containing receptor genes using colony PCR. Colonies identified as potential clones were used to isolate plasmid DNA which was subjected to another restriction enzyme digest to check for the presence of both plasmid and receptor DNA. For further confirmation, DNA sequencing was used to check for the correct DNA sequence for both receptor genes.

Afterwards, the plasmids containing the cloned receptor genes were co-transfected into human embryonic kidney (HEK 293) cells along with mG proteins that were tagged with the fluorescent protein Venus, the energy acceptor in BRET. These co-transfected cells were used in BRET assays to measure the GPCR and mG protein interactions. The instrument used for BRET assays, a Mithras LB940 photon-counting plate reader, provided BRET ratios that indicate the degree of interaction between the mG protein and the GPCR. The BRET ratio provided was a measure of the number of photons emitted by Venus (520-545 nm) divided by the number of photons emitted by NLuc (475-495 nm).

Materials

The molecular cloning process required Qiagen kits that contain the manufacturer's procedures for each of the steps in the process as described. In addition, biological compounds from Promega and New England BioLabs were needed. The Qiagen kits and biological compounds were provided through funding from the Center for Undergraduate Research and Scholarship (CURS) at Augusta University.

HEK 293 cells, mG proteins, cell media, substrate for NLuc (furimazine), serotonin (5-HT), methiothepin, and the Mithras LB940 photon-counting plate reader were used for co-transfection and BRET assays. These materials were provided by collaborators in the Department of Pharmacology and Toxicology on the Health Sciences Campus of Augusta University.

Experimental Procedure:

Cloning the 5-HT_{1D} and 5-HT_{1F} Receptor Genes in *E. coli* JM109 Cells

Purification and Restriction Enzyme Digest of Plasmid DNA

Sterile LB media (10 mL) was aliquoted into two flasks, each with added kanamycin (25 µg/mL) and inoculated with *E. coli* JM109 cells that were grown at 37 °C overnight in an incubator. Plasmid DNA containing the gene for Nanoluciferase (pNLuc) from *E. coli* JM109 cells was isolated and purified using the QIAprep spin miniprep kit from Qiagen according to manufacturer's instructions. The plasmid DNA was subjected to restriction enzyme digest at 37 °C overnight using the restriction enzymes according to Table 1 (see Appendix). Agarose gel (1% w/v) electrophoresis was performed using 1X Tris-Acetate-EDTA (TAE) buffer on the restriction digest reactions to observe for proper restriction enzyme cuts.

PCR Amplification of Receptor Genes

Polymerase chain reaction (PCR) was performed on template DNA of the 5-HT_{1D} and 5-HT_{1F} receptors isolated from different colonies of *E. coli* JM109 cells, which harbored

plasmids containing the genes for these receptors, to amplify these receptor DNA molecules. The cells that contained the plasmids for the receptors came from a PRESTO – Tango GPCR kit (Addgene). Each polymerase chain reaction contained 1X Q5 Polymerase Buffer, dNTP mix (400 μM), forward primer Tango-NLN1-F (ACTCAGATCTCGAGCTCAAGCTTCGAATTCGCCACCATGAAGACGATCATCGCCCTGAGC) (0.5 μM), reverse primers specific to the receptor inserts (0.5 μM), 2 U Q5 DNA Polymerase, template DNA of the receptors from plasmids from the PRESTO – Tango GPCR kit (5 μL), and sterile water (see Appendix, Table 2). The reverse primer sequence for 5-HT_{1D} was GACCGGTGGATCCCGGGCCGGCGGTACCCCAGAGGCCTTTCTAAAAGGGACGATCTTCTG. The reverse primer sequence for the 5-HT_{1F} was GACCGGTGGATCCCGGGCCCGCGGTACCCCACACCTGCAGCGAACAAGCTTCTGAAACGC.

The PCR parameters were performed in the following sequence: 1 denaturation cycle at 98 ºC for 30 seconds, 30 cycles of denaturation at 98 ºC for 10 seconds, annealing at 56 ºC for 30 seconds, elongation at 72 ºC for 1 minute, followed by 1 elongation cycle at 72 ºC for 2 minutes. The reactions were stored at 4 ºC. Agarose gel electrophoresis of the PCR products were examined on a 1% (w/v) agarose gel in 1X TAE buffer to observe for correct DNA sizes. A QIAquick PCR purification kit from Qiagen was used according to manufacturer's instructions to purify the PCR products.

Large Scale Restriction Enzyme (RE) Digest for Cloning

Reactions for large scale restriction enzyme (RE) digests were performed for pNLuc and for both receptor inserts. Each reaction contained a NEBuffer specific to the type of restriction

enzymes used for each insert (Buffer 1.1 for 5-HT_{1D} and Buffer 3.1 for 5-HT_{1F}, provided by New England BioLabs), 1X RE buffer, the DNA sample, sterile water, and two restriction enzymes provided by New England BioLabs (see Appendix, Table 3). The restriction enzymes used for 5-HT_{1D} were *XhoI* and *KpnI*. The restriction enzymes used for 5-HT_{1F} were *XhoI* and *BamHI*. RE digests were incubated in a 37 °C water bath overnight. A 1% (w/v) agarose gel electrophoresis was performed using 1X TAE buffer on these reactions to observe for correct DNA sizes. Both the plasmid and insert DNA were excised and purified from the agarose gel using a QIAquick Gel Extraction Kit Protocol from Qiagen according to manufacturer's instructions.

Ligation Reactions of Receptor DNA into Plasmid DNA

The absorbance of 260 nm light by the DNA samples was measured to determine the concentration of the purified DNA inserts and plasmid DNA. The ligation reaction mixtures used double digested 5-HT_{1D} and 5-HT_{1F} inserts and pNLuc respectively. Eight ligation reactions were created with varying concentrations of the receptor inserts (see Appendix, Table 4). Each reaction contained 1X ligase buffer provided by Fisher Bioreagents, double digested plasmid (100 ng), double digested insert at varying concentrations, 5 U T4 DNA ligase, and sterile water. Ligations were incubated overnight at room temperature. The DNA samples in the ligation reactions were transformed into *E. coli* JM109 cells using the standard transformation protocol from Promega. The transformation reactions were plated on agar plates containing the antibiotic kanamycin.

Colony PCR of E. coli JM109 *cells*

To identify which transformed colonies of *E. coli* contained the desired recombinant DNA containing 5-HT_{1D} and 5-HT_{1F} receptors, twelve PCR reactions were created. Each reaction consisted of 1X GoTaq Buffer, dNTPs (200 µM), forward primer Flag-F (ATGAAGACGATCATCGCCCTGAGC, 0.5 µM) from Integrated DNA Technologies (IDT), reverse primer NLucrev (ATCTCCGAGTGTGAAGACCAT, 0.5 µM) from IDT, 2 U GoTaq DNA Polymerase, and sterile water. Six colonies of *E. coli* were chosen from the plate containing 5-HT_{1D} receptor insert ligations, and another six colonies of *E. coli* were chosen from the plate containing the 5-HT_{1F} receptor insert ligations. The PCR parameters were: 1 denaturation cycle at 95 ºC for 5 minutes, 30 cycles of denaturation at 95 ºC for 30 seconds, annealing at 56 ºC for 30 seconds, followed by elongation at 72 ºC for 1 minute, and finally 1 elongation cycle at 72 ºC for 6 minutes. The PCR reactions were stored at 4 ºC. The PCR reactions were analyzed on a 1% (w/v) agarose gel electrophoresis in 1X TAE buffer to observe for the presence of the 5-HT_{1D} or 5-HT_{1F} genes.

Restriction Digests of Purified DNA from Selected Colonies

Four colonies were selected for further analysis based on colony PCR results: two containing a 5-HT_{1D} insert and two containing a 5-HT_{1F} insert. The selected colonies of *E. coli* were used to inoculate kanamycin-containing media, and plasmid DNA was purified using the QIAprep spin miniprep kit from Qiagen according to manufacturer's instructions. Restriction digests were performed on the purified plasmids using corresponding restriction enzymes. Each restriction digest reaction contained 1X NEBuffer provided by New England BioLabs, the

purified plasmid, corresponding restriction enzymes, and sterile water (see Appendix, Table 5). 1% (w/v) agarose gel electrophoresis of the restriction enzyme digests confirmed that the plasmid and insert were both present in the isolated plasmids. The presence of the correct receptor sequences in these plasmids was confirmed by DNA sequencing. Correct plasmids were purified for transfection using a HiSpeed Plasmid Midi Kit from Qiagen according to the manufacturer's instructions.

BRET Analysis

Transfection of Plasmid DNA into HEK 293 Cells

Human embryonic kidney (HEK 293) cells were split into 6-well plates to allow for multiplication of the cells for the BRET assay. Prior to transfection, the growth medium – 1X Minimum Essential Medium (MEM) containing 10% fetal bovine serum (FBS) and the antibiotic 1X Penicillin-Streptomycin-Glutamine – was removed and replaced with fresh growth medium (2 mL). Transfection samples were prepared as follows in separate centrifuge tubes containing diluted polyethylenimine (PEI) in NaCl solution (0.15 M): plasmid DNA harboring either 5-HT_{1D} or 5-HT_{1F} receptor inserts (0.01 µg), Venus-tagged mini G proteins (mG_{si}, mG_{s}, mG_{sq}, mG_{o}, or mG_{12}) or Venus-tagged arrestin-3 (0.75 µg), and pcDNA3.1 (1.25 µg). The 6-well plate was placed in the CO_2 incubator at 37 °C for two to four hours. Afterwards, the growth medium was removed and replaced with fresh growth medium (2 mL), and the 6-well plate was returned to the CO_2 incubator at 37 °C until the BRET assay which was performed approximately 24 hours after transfection.

BRET Assay for Profiling the 5-HT_{1D} and 5-HT_{1F} Receptors

The transfected HEK 293 cells in each well were washed twice using PBS with ETDA (0.5 mL/well). 1X Dulbecco's Phosphate-Buffered Saline (DPBS) (0.5 mL; Thermo Fisher Scientific) and Phosphate Buffered Saline (PBS) with EDTA (0.5 mL) were added to all wells to dislodge the cells from the bottom of each well. A black, opaque 96-well plate was used for the BRET assay. Each well that was used in the 96-well plate contained the transfected HEK 293 cells (100 μL), either a diluted drug solution of serotonin or methiothepin (50 μL of 40 μM drug solution) or 1X DPBS (50 μL), and a 1:1000 diluted furimazine solution in 1X DPBS (50 μL). Upon the addition of diluted furimazine, the 96-well plate was immediately placed into the Mithras LB940 photon-counting plate reader, which was used to measure the quantity of photons emitted by the NLuc donor and the Venus acceptor. From the data, a BRET ratio was calculated by dividing the number of photons emitted by Venus by the number of photons emitted by NLuc. Net BRET ratios were calculated by subtracting 0.135, which represents the baseline value for the light emitted by NLuc, from the computed BRET ratio. The net BRET ratios were analyzed for interactions between the receptors and drugs by comparing the ratios under each condition (DPBS, serotonin, or methiothepin) to each other. This assay was repeated two additional times.

BRET Assay for Dose-Response Curves for the 5-HT_{1D} and 5-HT_{1F} Receptors

Prior to the dose-response BRET assay, the HEK 293 cells were split into 6-well plates for transfection. The cells were transfected as described above but only contained either one of two mG proteins: mG_{si} or mG_o (0.75 μg). These mG proteins were chosen because they displayed relatively higher BRET ratios compared to the other mG proteins in the profiling

BRET assays, indicating greater coupling to the receptors. A serial dilution of serotonin was created using 1X DPBS (1000 mL) in seven centrifuge tubes, starting with a 380 µM serotonin solution through six successive dilutions by 10-fold to a final dilution of 2.2×10^{-4} µM. Each row of the black, opaque 96-well plate contained either 1X DPBS (50 µL) or a specific concentration of serotonin (50 µL). The transfected HEK 293 cells were washed twice with PBS with EDTA (0.5 mL) and dislodged from the surface of each well using 1X DBPS (0.5 mL) and PBS with EDTA (0.5 mL). HEK 293 cells (100 µL) and a 1:1000 furimazine solution (50 µL) was added to all wells. The 96-well plate was placed into the Mithras LB940 photon-counting plate reader to measure the quantity of photons emitted by both the NLuc donor and the Venus acceptor. A dose-response curve was generated in GraphPad Prism software using the net BRET ratios versus the log of the concentration of serotonin added to the cells. Three trials were performed for dose-response data. Net BRET ratios were calculated by subtracting 0.135, which represents the baseline value for the light emitted by NLuc, from the computed BRET ratio.

Results and Discussion:

Two receptor genes from the serotonin receptor family were chosen to be cloned: 5-HT_{1D} and 5-HT_{1F}. Based on **Figure 4**, the bands on the agarose gel indicated successful PCR amplification of the 5-HT_{1D} and 5-HT_{1F} receptor genes. DNA bands of 1.3 kb in size correspond to the expected size of the amplified 5-HT_{1D} and 5-HT_{1F} receptor genes (**Figure 4**). The PCR products of the 5-HT_{1D} and 5-HT_{1F} amplification were purified and used for cloning into the pNLuc plasmid by performing large scale restriction enzyme digests and subsequent ligations. The recombinant DNA was transformed into *E. coli* JM109 cells. Transformed cells were plated on kanamycin-containing agar plates and allowed to grow into colonies that potentially harbored recombinant DNA.

Colony PCR was used to identify possible recombinant DNA from the transformed *E. coli* JM109 cells. Twelve different colonies of *E. coli* JM109 cells were used in colony PCR. Half of the colonies potentially contained the 5-HT_{1D} receptor gene, and the rest possibly contained the 5-HT_{1F} receptor gene. According to **Figure 5**, many of the colonies tested contained the receptor genes of interest, which were approximately 1.3 kb in size. Cells from the colonies positively identified as containing recombinant plasmids were subjected to plasmid DNA purification and subsequent restriction digest.

To confirm the correct location of the gene of interest in the plasmid, the plasmid DNA from the selected colonies was cut using the restriction enzymes used for cloning. The results of the digest are shown in **Figure 6**. The band corresponding to about 4.5 kb was the pNLuc plasmid. The band corresponding to about 1.3 kilobases was the expected size for both the 5-HT_{1D} and 5-HT_{1F} receptor genes. The plasmid DNA samples containing the 5-HT_{1D} and

5-HT_{1F} receptor genes were sent for DNA sequencing to check for accuracy. The results of the DNA sequencing confirmed that both receptor genes were successfully ligated into the pNLuc plasmid.

5-HT_{1D}-NLuc or 5-HT_{1F}-NLuc plasmids as well as mG_x-Venus plasmids were co-introduced into HEK 293 cells via transfection with polyethylenimine (PEI). Transfection reactions consisted of p5HTR-NLuc plasmid, mG_x-Venus plasmid, pcDNA3.1, and diluted PEI in NaCl solution. Each DNA solution was added dropwise into wells containing HEK 293 cells in a 6-well plate to transfect the cells. BRET assays were performed using the transfected cells about 24 hours after transfection. Initial BRET assays required optimization to determine the ideal concentrations of serotonin receptor (5HTR) and mG protein to utilize for profiling assays. To do so, varying concentrations of the 5HTR-NLuc plasmid (1 µg to 0.01 µg) were transfected into HEK 293 cells and tested with a constant concentration of mG_{si}-Venus plasmid (1 µg) since it is known that the 5-HT_1 receptor class couples to the $G_{\alpha i/o}$ proteins [14, 20]. Serotonin was added to stimulate GPCR activation and coupling with mG_{si}, and BRET was measured to determine which concentration of receptor gave the largest BRET ratio relative to the background (no serotonin present). The same method was used for determining the ideal mG protein concentrations to use with each receptor. Results showed that 0.75 µg of mG_x-Venus plasmid and 0.01 µg of 5HTR-Nluc plasmid were optimal experimental conditions for BRET assays. Therefore, these concentrations were used for all subsequent assays.

For the profiling and dose-response experiments, the mG proteins used were: mG_s, mG_{si}, mG_o, mG_{sq}, and mG_{12}. Some of the mG proteins (excluding the mG_s protein) contain an additional "s" in the subscript, indicating that these mG proteins consist of the mG_s protein sequence except with "specificity-determining residues" from the other G protein involved to

give the functionality of the other G protein [13]. In the profiling experiments, three conditions were used: 1X DPBS, 5-HT (10 μM), or methiothepin (10 μM). DPBS was a buffer solution that contained no drug solution. DPBS was used to observe potential coupling in the absence of GPCR activation. 5-HT was the endogenous agonist for the 5-HT_{1D} and 5-HT_{1F} receptors and activated the receptor for mG protein coupling. Methiothepin, an inverse agonist for the 5-HT_{1D} and 5-HT_{1F} receptors, was used in the profiling experiments to provide BRET ratios for when the receptors were inactivated, preventing coupling with the mG proteins [14]. Arrestin-3 was also used in profiling the serotonin receptors. Arrestin-3 can bind to a GPCR that is bound and activated by an agonist, blocking G protein coupling to the active GPCR [18]. Arrestin-3 was used as a positive control to show a difference in ratios when comparing GPCR activity under the three different conditions.

Based on the data obtained from BRET profiling of the receptors, the 5-HT_{1D} receptor and the 5-HT_{1F} receptor coupled well with mG_{si} and mG_o proteins (**Figures 7 and 8**). Upon serotonin activation of the 5-HT_{1D} receptor, the net BRET ratio increased as compared to the baseline (DPBS only) for the mG_{si} and mG_o proteins compared to when the GPCR was coupled to the other mG proteins (**Figure 7**). A higher net BRET ratio in the presence of serotonin can be interpreted as greater interaction or coupling of the mG protein with the GPCR when the GPCR became activated by serotonin. Likewise, in **Figure 8**, serotonin-stimulated 5-HT_{1F} receptor coupled well with the mG_{si} and mG_o proteins, although the net BRET ratio was high in the absence of the agonist (DPBS only). A high BRET ratio in the absence of agonist is a potential sign of constitutive activity where the receptor is in an activated state and binds G proteins without agonist activation. Inverse agonists can be used to determine if a receptor is constitutively active. In the presence of methiothepin with the 5-HT_{1F} receptor, the net BRET

ratios were lower than the net BRET ratios in DPBS for these mG proteins. The results with methiothepin present are consistent with its known mechanism of action – reducing the coupling of mG_{si} and mG_o proteins to the 5HTRs by inactivating the receptor. The results presented in **Figure 7** and **Figure 8** support the current knowledge that the 5-HT_1 receptor class couples primarily to the $G_{\alpha i/o}$ class of G proteins [14, 20].

Dose-response BRET assays were also performed on the 5-HT_{1D} and 5-HT_{1F} receptors to obtain dose-response curves (**Figures 9 and 10**). A dose-response curve exhibits a sigmoidal shape when plotted on a semi-log scale, which represents a graded increase in the response of a receptor to an agonist as the agonist concentration increases. A dose-response curve has a significant value, the half maximal effective concentration (EC_{50}), which is the concentration of an agonist that gives half the maximal response. An EC_{50} value is used as a measure of potency of an agonist, which is defined as the concentration of agonist required to produce an effect of a specific intensity. Potency is used to compare the affinity of various agonists for a specific receptor. An agonist with a lower EC_{50} value has a higher affinity for the receptor and is therefore more potent than an agonist with a higher EC_{50} value. In the dose-response experiments, EC_{50} values were computed using GraphPad Prism software. 5-HT was the only agonist used for activating the serotonin receptors, so potency cannot be discussed because no other agonist was tested in comparison to 5-HT on the serotonin receptors.

In **Figures 9 and 10**, each figure displays two dose-response curves, showing the coupling between the 5HTR with mG_{si} and mG_o proteins as the 5-HT concentration increased. As expected, higher net BRET ratios were acquired as the 5-HT concentration increased which indicated greater 5HTR and mG protein coupling since more receptors were bound and activated by 5-HT. Specifically, the EC_{50} value for the 5-HT_{1D} receptor coupling with mG_{si} protein was

1.81 μM for 5-HT (**Figure 9**). When the 5-HT_{1D} receptor coupled with the mG_o protein, the EC_{50} value was 0.828 μM for 5-HT (**Figure 9**). As for the 5-HT_{1F} receptor, the EC_{50} value for the 5-HT_{1F} receptor coupling with the mG_{si} protein was 1.04 μM for 5-HT and with the mG_o protein, the EC_{50} value was 0.132 μM for 5-HT (**Figure 10**). Interestingly, both receptors coupled to mG_o gave lower EC_{50} values for 5-HT than the receptors coupled to mG_{si}.

One of the GPCRs studied in this study appeared to demonstrate constitutive activity. Constitutive activity is activity of a receptor in the absence of an agonist binding to and activating that receptor. In previous studies of the 5-HT_{1D} and 5-HT_{1F} receptors, 5-HT_{1D} receptors were shown to exhibit constitutive activity through experimentation with inverse agonists [22]. In this study, constitutive activity of 5-HT_{1D} receptors was not observed in profiling experiments. The literature on the 5-HT_{1F} receptor does not suggest constitutive activity. However, the results obtained from BRET profiling and dose-response assays for the 5-HT_{1F} receptor highlighted potential constitutive activity of this receptor (**Figures 8 and 10**). This was evident by the high net BRET ratios observed in the absence of serotonin and the relatively small increase in the net BRET ratio upon receptor stimulation using serotonin. Additionally, the dose-response curves for the 5-HT_{1F} receptor showed very little change in net BRET ratios as the concentration of serotonin was increased. This indicated that the GPCR was possibly already coupled with the mG protein without serotonin and thus the coupling is not as sensitive to changes in the concentration of serotonin. As shown in the profiling experiments, the net BRET ratios from the 5-HT_{1F} receptor coupled to the mG_{si} and mG_o proteins in the presence of methiothepin were lower than the net BRET ratios in the presence of DPBS (**Figure 8**). This indicates that methiothepin prevented the coupling of the 5-HT_{1F} receptor with the mG proteins that occurred in the absence of serotonin, supporting the idea that the 5-HT_{1F} receptor was

possibly pre-coupled to the mG protein due to its constitutive activity. Further research will investigate this potential constitutive activity of the 5-HT_{1F} receptor which has not been previously reported in published journals.

Conclusion:

In this study, the 5-HT_{1D} and 5-HT_{1F} receptors were analyzed for their coupling to mini G proteins upon serotonin stimulation using BRET assays. The results showed that these receptors coupled well with the mG_{si} and mG_{o} proteins, which agrees with what current information on these receptors state regarding coupling with heterotrimeric $G_{i/o}$ proteins [14, 20]. For future direction, the 5-HT_{1F} receptor may be investigated in further detail concerning its possible constitutive activity. Using heterotrimeric G proteins to profile the 5-HT_{1F} receptor and third-party BRET assays may reveal if the possible constitutive activity of 5-HT_{1F} receptor is present.

Additionally, the results of this work indicate that mini G proteins can be used as surrogates for heterotrimeric G proteins to determine which signaling pathways are involved when a ligand binds a GPCR. This assay can be used to identify novel ligands that interact with the 5-HT_{1D} and 5-HT_{1F} receptors and initiate a signaling pathway. This work could aid in the identification of potential drugs for migraines. Currently, the commonly used migraine medications are sumatriptan and other drugs from the triptan class, which are selective agonists of the 5-HT_{1B}, 5-HT_{1D}, and 5-HT_{1F} receptors [19]. However, these appear to have central nervous system (CNS) side effects even though some of the triptans are hydrophilic and cannot cross the brain-blood barrier easily [19]. Few clinical trials have shown migraine-treatment

efficacy of these drugs while other trials using triptans show no relief in this type of pain [19]. The mechanism of action of triptans for specifically relieving migraines is still unknown [19].

Among other drugs, lasmiditan, a selective agonist for the 5-HT_{1F} receptor, could become a drug that relieves acute migraines without constricting blood vessels around the brain or heart [4]. For future studies, lasmiditan may be used as an agonist for the 5-HT_{1F} receptor to study the signaling pathway(s) initiated upon 5-HT_{1F} receptor activation. Knowing this information could be used to develop a novel migraine medication that exerts a therapeutic effect on the user without the negative side effects. However, there is evidence of adverse side effects that affect the CNS as shown in clinical trials, which currently limits the use of lasmitidan in the treatment of migraines [4]. The side effects could be due, in part, to secondary coupling to the other members of the G protein family, which could be identified using the assay described in this report.

Glossary:

Agonist: a substance that binds to the ligand-binding site of a receptor and activates the receptor by inducing a conformational change in the receptor

Antagonist: a substance that binds the ligand-binding site of a receptor but does not activate the receptor

Bioluminescent donor molecule: a molecule that emits light when it catalyzes the oxidation of its substrate

C-terminus: the carboxyl end of the amino acid sequence of a protein

Constitutive activity: activity of a receptor in the absence of an agonist binding the receptor

Deoxyribonucleic acid (DNA): a molecule that stores genetic information in a cell

Domain: an individually stable part of the protein that can perform a specific function

Enzyme: a protein catalyst that is used to speed up the rate of a reaction

Fluorescent acceptor molecule: a molecule that accepts the resonance energy from the bioluminescent donor molecule to emit its own light

Gene: a segment of DNA that encodes for a specific product such as a protein

Gene expression: process of using the information contained in a gene to produce a functional product

GTPase: an enzyme that can catalyze the hydrolysis of GTP

Half-maximal effective concentration (EC_{50}): the concentration of a substance that gives half the maximal response

Insert: the piece of DNA being cloned into the desired plasmid

Ligand: a substance outside the cell that binds to a specific receptor in the plasma membrane to initiate an event in the cell

Ligation: to join two DNA segments together

N-terminus: the beginning of an amino acid sequence of a protein

Neurotransmitter: a chemical substance made and released by nerve cells that is used to relay signals to other nerve cells

Photon: a fundamental particle of light

Plasma membrane: the membrane that surrounds a cell's contents and acts as a barrier of the cell against the external environment

Plasmid: a circular DNA molecule that is present in bacteria and commonly used for gene cloning

Polymerase chain reaction (PCR): a molecular technique used to make many copies of a desired DNA segment that involves repeated cycles of heat treatment to separate the DNA strands, fit complementary primers onto the DNA strands, and synthesize the DNA strands

Restriction enzymes: enzymes that recognize certain DNA sequences and cleave the DNA at those sites

Substrate: a substance that interacts with an enzyme

Superfamily: a large group of proteins that have similar structure and function but different protein sequences

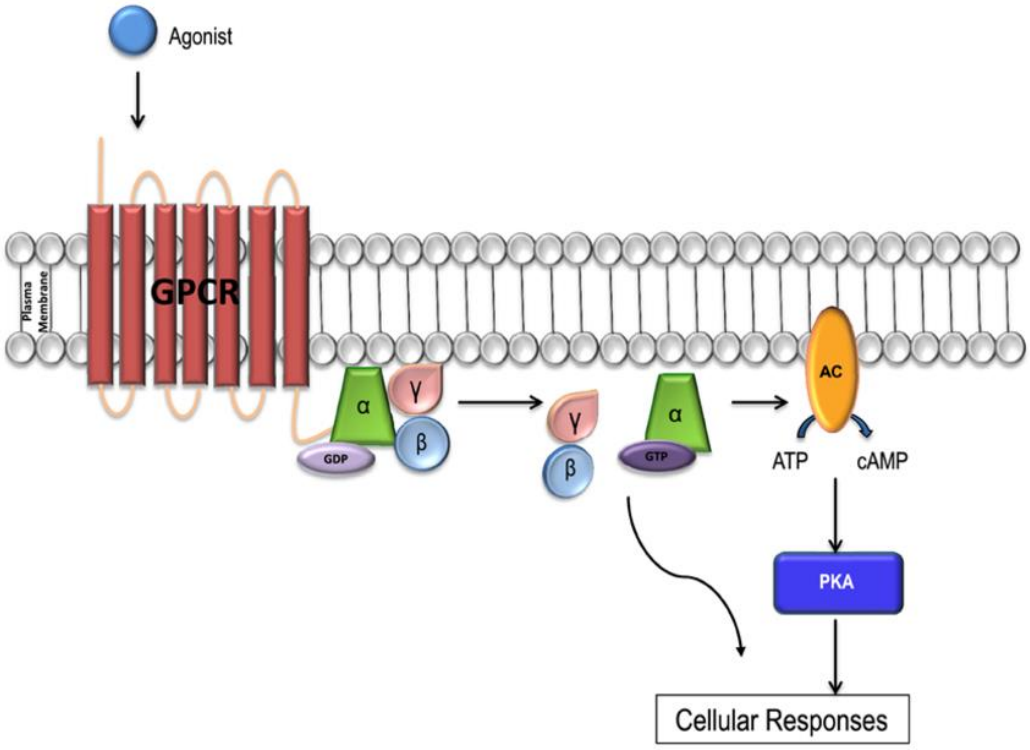

Figure 1. An Example of GPCR Signal Transduction Activation and Its Key Components.

This scheme describes how signal transduction occurs using a GPCR and a specific signaling pathway. Here, the ligand is an agonist, a molecule that binds to and activates the GPCR. The heterotrimeric G protein (green, pink, and pale blue shapes) is attached to the plasma membrane, and it associates with a GPCR when the GPCR is activated. Once the GPCR is bound to a ligand, it changes conformation to its active state which causes the release of GDP and the binding of GTP to the alpha subunit of the G protein (green). The alpha subunit detaches from the GPCR, dissociates from the beta and gamma subunits, and binds to adenylyl cyclase (AC). Adenylyl cyclase, an effector enzyme, produces cyclic adenosine monophosphate (cAMP) from adenosine triphosphate (ATP), increasing the concentration of cAMP in the cell. Increased cAMP concentration leads to activation of protein kinase A (PKA) to continue signal amplification and eventually create an intracellular message for eliciting a cellular response. (Illustration obtained from ref 14)

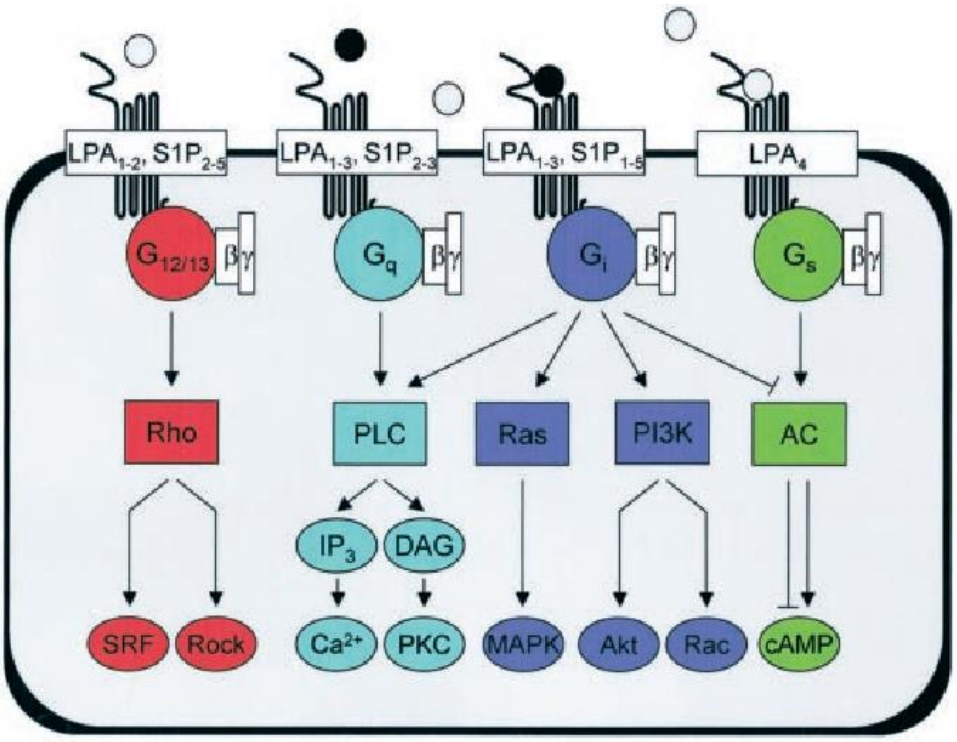

Figure 2. An Illustration of the Various Families of Heterotrimeric G Proteins and Their Effectors.

This illustration depicts various signaling pathways that are associated with the families of G proteins and the lysophospholipid (LPA) or sphingosine 1-phosphate (S1P) receptors. Although the receptors in this figure are not being studied in this project, the G proteins and their signaling pathways function in the same manner. Here, the G_α subunit for each heterotrimeric G protein is labeled based on the subtype: $G_{12/13}$, G_q, G_i, and G_s. Each G_α subunit, when bound to GTP, will activate a downstream effector, such as phospholipase C (PLC) or phosphoinositol 3-kinase (PI3K). Activation of an effector leads to the production of second messengers like calcium or cyclic AMP (cAMP) or can lead to activation of another effector like protein kinase C (PKC), rho kinase (ROCK), or mitogen activated protein kinase (MAPK). This figure also reinforces the idea that signaling pathways are not isolated and that they affect other activated pathways that can simultaneously occur in a cell. (Illustration obtained from ref 20)

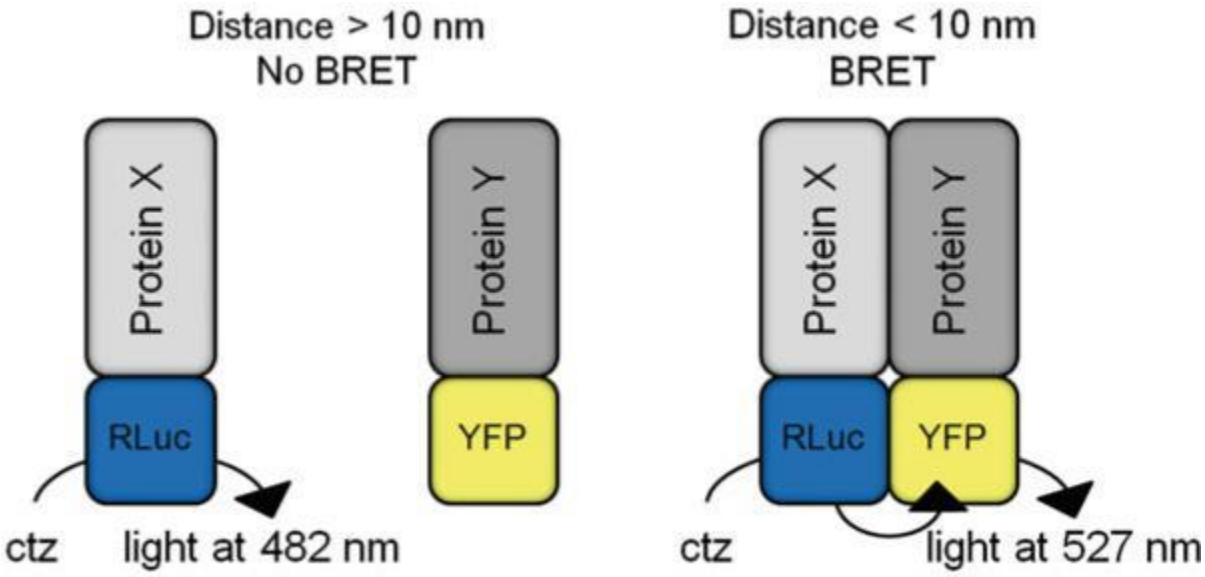

Figure 3. The Process of Bioluminescence Resonance Energy Transfer (BRET).

This diagram shows how BRET is utilized to measure protein-protein interactions. Here, the bioluminescent donor molecule is Renilla luciferase (RLuc) and is tagged onto protein X. When the substrate coelenterazine (ctz) is present, RLuc catalyzes its oxidation, producing blue light at 482 nm [11]. The fluorescent acceptor molecule is a yellow fluorescent protein (YFP) which is tagged onto protein Y. When the two molecules are not interacting, they are more than 10 nm away from each other. Thus, the resonance energy from RLuc is not transferred to YFP. However, when the two proteins interact with each other, the energy donor (RLuc) and the energy acceptor (YFP) are 10 nm or closer together. Therefore, the resonance energy from RLuc is transferred to YFP. YFP then emits its own light of a different wavelength, which can be measured and quantitated. (Illustration obtained from ref 11)

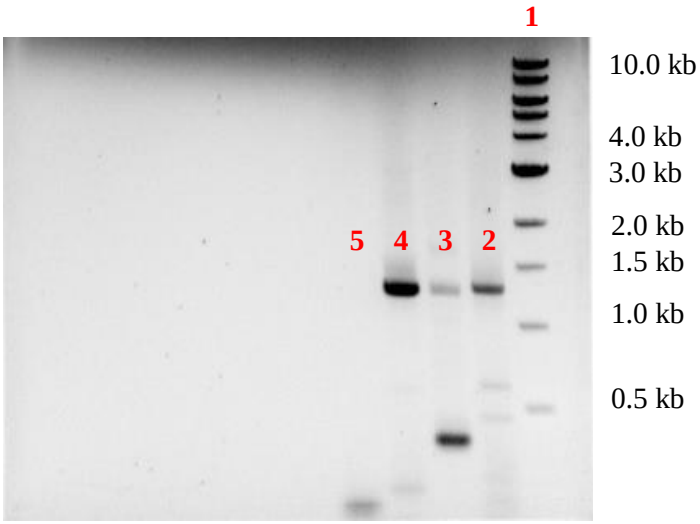

Figure 4. 1% (w/v) Agarose Gel Electrophoresis of PCR Amplification of Receptor Genes.

Lane 1 contained the 1kb DNA ladder. Lane 2 contained the 5-HT_{1D} receptor gene, and lane 4 contained the 5-HT_{1F} receptor gene. Lane 3 contained the 5-HT_{1E} receptor gene, which was not subjected to cloning and will not be discussed in this study. Lane 5 was a negative control containing no template DNA.

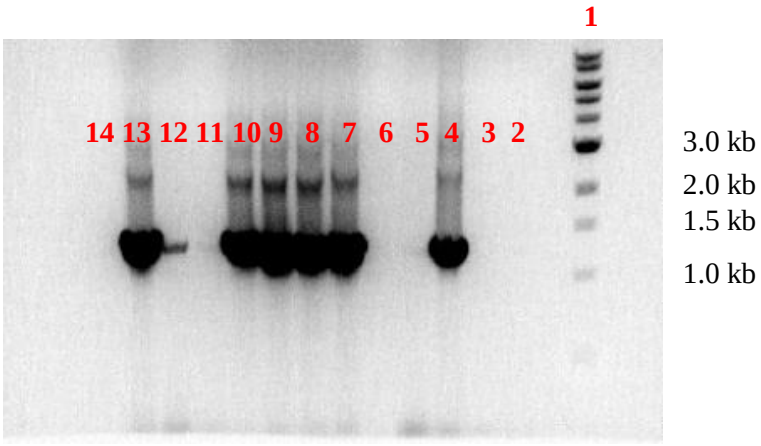

Figure 5. 1% (w/v) Agarose Gel Electrophoresis of Colony PCR Products from Transformed *E. coli* JM109 Cells.

Lane 1 contained the 1kb DNA ladder. Lanes 2 through 7 contained colonies potentially containing the 5-HT_{1D} gene. Lanes 8 through 13 contained colonies potentially containing the 5-HT_{1F} gene. Specifically, lanes 4 and 7 contained the 5-HT_{1D} gene with a band size of about 1.3 kb. Lanes 8 through 10, 12, and 13 contained the 5-HT_{1F} gene with a band size of about 1.3 kb. Lane 14 was a negative control.

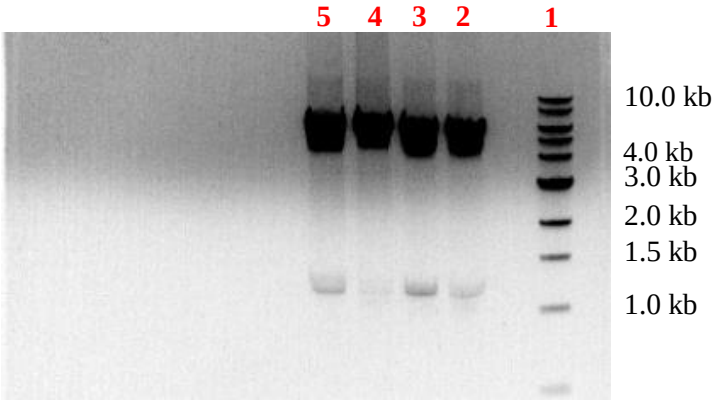

Figure 6. 1% (w/v) Agarose Gel Electrophoresis of Restriction Digests of Potential Recombinant DNA Isolated from Transformed *E. coli* Cells.

Lane 1 contained the 1kb DNA ladder. Lanes 2 through 5 displayed bands for both the plasmid pNLuc and the receptor gene inserts 5-HT_{1D} and 5-HT_{1F}. The size of the plasmid band was about 4.5 kb. Lanes 2 and 3 contained 5-HT_{1D} inserts with a band size of about 1.3 kb. Lanes 4 and 5 contained 5-HT_{1F} inserts with a band size of about 1.3 kb.

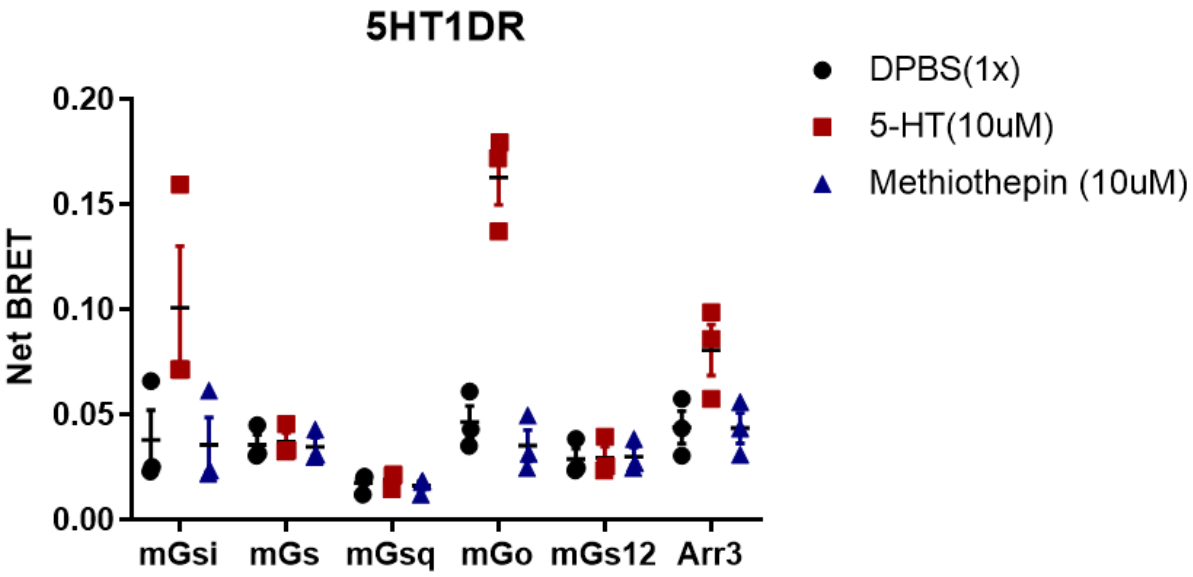

Figure 7. Characterization of 5-HT_{1D} Receptor: mG Interactions via BRET Analysis.

Data from triplicate experiments on profiling the 5-HT_{1D} receptor with various mini G proteins under different conditions (DPBS, 5-HT, or methiothepin). Net BRET ratios were calculated by subtracting 0.135, which represents the baseline value for the light emitted by NLuc, from the computed BRET ratio. The 5-HT_{1D} receptor coupled well to both mG_{si} and mG_o proteins.

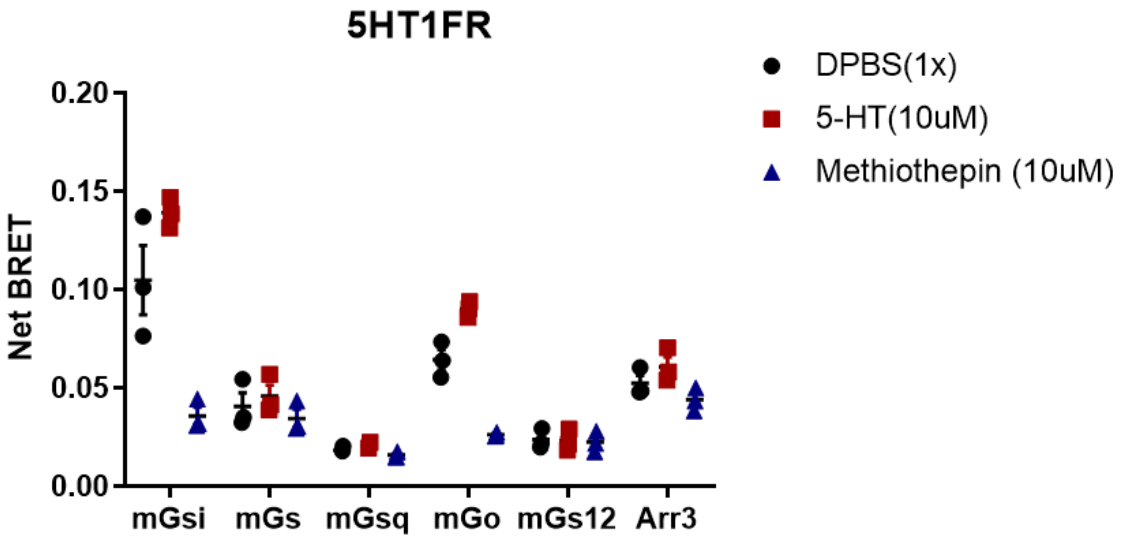

Figure 8. Characterization of 5-HT_{1F} Receptor: mG Interactions via BRET Analysis.

Data from triplicate experiments profiling the 5-HT_{1F} receptor with the mini G proteins under different conditions (DPBS, 5-HT, or methiothepin). Net BRET ratios were calculated by subtracting 0.135, which represents the baseline value for the light emitted by NLuc, from the computed BRET ratio. The 5-HT_{1F} receptor coupled well to both mG_{si} and mG_o proteins.

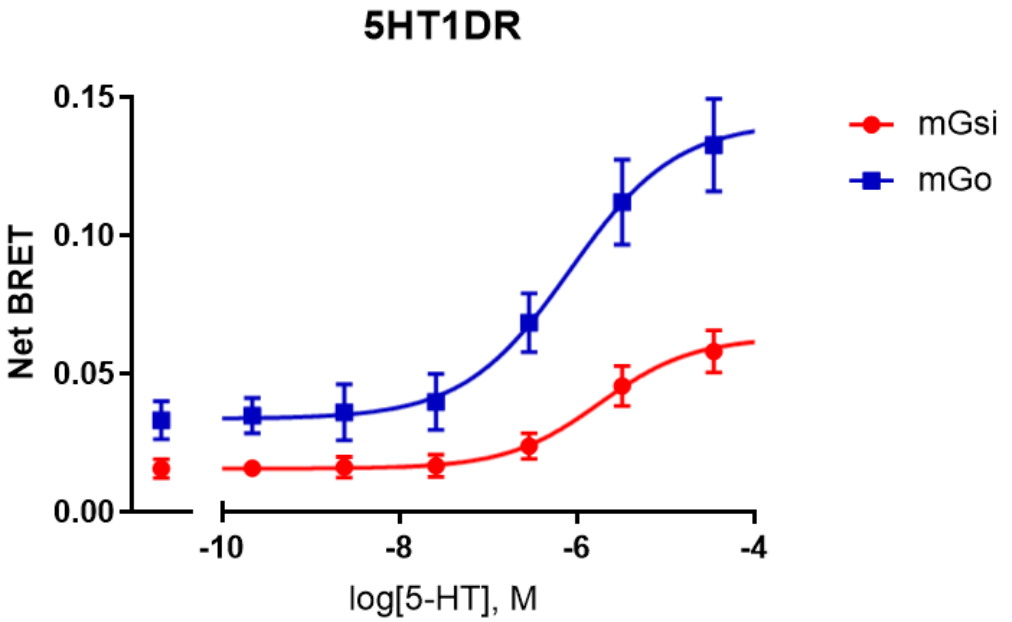

Figure 9. Dose-response Curve for the 5-HT_{1D} Receptor When Coupled with Two Mini G Proteins.

This dose-response curve indicates the EC_{50} values of 5-HT_{1D} receptor coupling with both the mG_{si} and mG_o proteins. Through GraphPad Prism software computations, the EC_{50} values for this receptor with mG_{si} ($EC_{50} = 1.81$ µM) and mG_o ($EC_{50} = 0.828$ µM) were obtained.

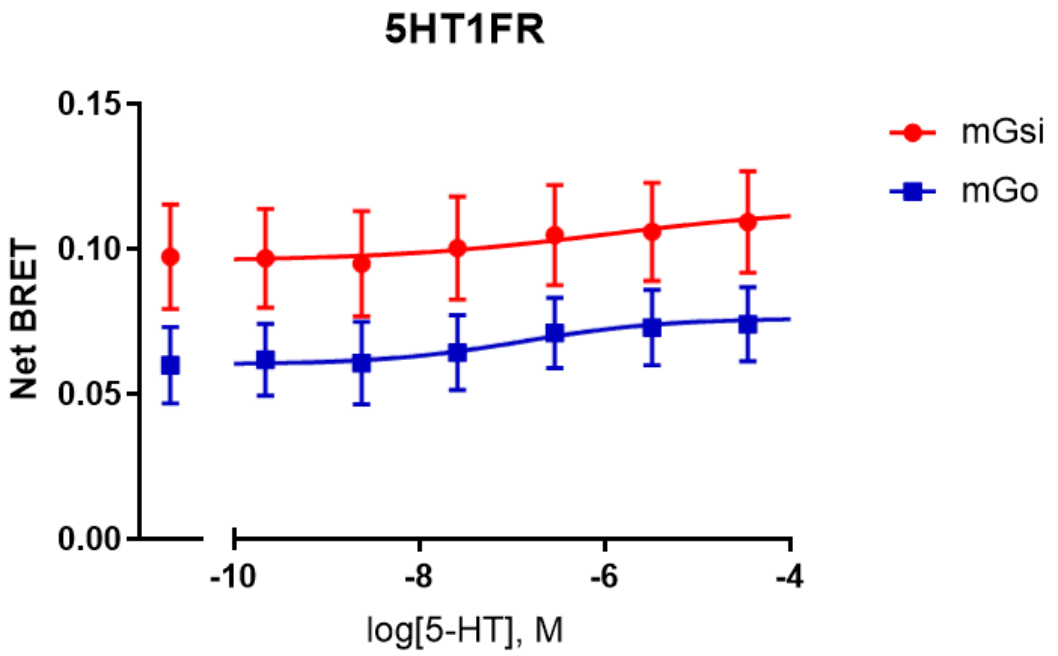

Figure 10. Dose-response Curve for the 5-HT_{1F} Receptor When Coupled with Two Mini G Proteins.

This dose-response curve indicates the EC_{50} values of 5-HT_{1F} receptor coupling with both the mG_{si} and mG_o proteins. Using GraphPad Prism software data, the EC_{50} values for this receptor coupling with mG_{si} ($EC_{50} = 1.04$ µM) and mG_o ($EC_{50} = 0.132$ µM) were calculated.

Appendix:

Table 1. Restriction Enzymes and Digest Sites for the 5HT-1D and 5HT-1F Receptor Genes

Receptor	Forward primer	Reverse primer
5HT-1D	XhoI (5')ACTCAGATCTCGAGCTCAAGCTTCGAATT CGCCACCATGAAGACGATCATCGCCCTGAG C (3')	KpnI (5')GACCGGTGGATCCCGGGCCGGCGGTACCC CAGAGGCCTTTCTAAAAGGGACGATCTTCTG (3')
5HT-1F	XhoI (5')ACTCAGATCTCGAGCTCAAGCTTCGAATT CGCCACCATGAAGACGATCATCGCCCTGAG C (3')	BamHI (5')GACCGGTGGATCCCGGGCCCGCGGTACCC CACACCTGCAGCGAACAAGCTTCTGAAACGC (3')

The underlined sequences are the sequences that are recognized by the above labeled restriction enzyme to cleave the DNA at those sites.

Table 2. PCR Amplification of Receptor Genes

Substance	Reaction 1: 5HT-1D	Reaction 2: HTR1E	Reaction 3: 5HT-1F	Negative Control
5 X Q5 Polymerase Buffer	10 µL	10 µL	10 µL	10 µL
dNTP mix (10 mM)	2 µL	2 µL	2 µL	2 µL
TANGO FP (10 µM)	2.5 µL	2.5 µL	2.5 µL	2.5 µL
Reverse primer: 5HT-1D (10 µM)	2.5 µL	-	-	2.5 µL
Reverse primer: 5HT-1E (10 µM)	-	2.5 µL	-	-
Reverse primer: 5HT-1F (10 µM)	-	-	2.5 µL	-
Q5 DNA Polymerase (2 U/µL)	1 µL	1 µL	1 µL	1 µL
5HT-1D template DNA	5 µL	-	-	-
5HT-1E template DNA	-	5 µL	-	-
5HT-1F template DNA	-	-	5 µL	-
Sterile water	27 µL	27 µL	27 µL	32 µL

Table 3. Large Scale Restriction Enzyme Digest for Cloning

Tube	Type of RE buffer	10X RE buffer (µL)	DNA Sample	Volume of DNA Sample (µL)	Sterile water (µL)	*XhoI* (20 U/µL) (µL)	*KpnI* (10 U/µL) (µL)	*BamHI* (20 U/µL) (µL)
1	NEBuffer 1.1	6	pNluc	30	20	2	2	-
2	NEBuffer 3.1	6	pNluc	30	20	2	-	2
3	NEBuffer 1.1	6	5HT-1D	30	20	2	2	-
4	NEBuffer 3.1	6	5HT-1F	20	30	2	-	2

Table 4. Ligation Reaction Mixtures

Tube	10X Ligase Buffer (µL)	Sterile water (µL)	Type of double digest vector	Double digested vector (µL)	Type of double digested insert	Volume of double digested insert (µL)	T4 DNA ligase (5U/µL) (µL)
L1	1	6	pNluc-XKpI (49.5 ng/µL)	2	5HT-1D (25.5 ng/µL)	0	1
L2	1	5		2		1	1
L3	1	3		2		3	1
L4	1	1		2		5	1
L5	1	6	pNluc-XBaI (62 ng/µL)	2	5HT-1F (30 ng/µL)	0	1
L6	1	5		2		2	1
L7	1	3		2		3	1
L8	1	1		2		5	1

Table 5. Restriction Digests of Purified DNA from Selected Colonies

Tube	Type of 10X buffer	Volume of 10X buffer (µL)	Type of vector in DNA sample	Type of insert in DNA sample	Volume of DNA sample (µL)	Sterile water (µL)	*XhoI* (20 U/ µL) (µL)	*KpnI* (10 U/ µL) (µL)	*BamHI* (20 U/ µL) (µL)
1	NEBuffer (1.1)	1	pNluc	5HT-1D	5	2	1	1	-
2	NEBuffer (1.1)	1	pNluc	5HT-1D	5	2	1	1	-
3	NEBuffer (3.1)	1	pNluc	5HT-1F	5	2	1	-	1
4	NEBuffer (3.1)	1	pNluc	5HT-1F	5	2	1	-	1

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