L-PHENYLALANINE DIFFERENTIALLY ACTIVATES G PROTEIN-COUPLED RECEPTOR SIGNALING PATHWAYS

BY

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Table of Contents

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Abstract

The most abundant cell surface receptor in eukaryotes, G-protein coupled receptors (GPCRs) couple with heterotrimeric G proteins, utilizing active Gα and Gβγ subunits, to initiate distinct intracellular signaling pathways implicated in cell functions and physiological responses. Several GPCRs are validated therapeutic drug targets, while the majority of GPCRs represent unexplored potential, with many studies aiming to define their signal transduction response, identify ligands, and determine their roles in diseases and disorders. Amino acids have become an interest of studies aiming to elucidate their role as ligands, with previous findings demonstrating that *L*-phenylalanine activates 12 Class A GPCRs. The aim of this study was to characterize the signal transduction response in multiple established signaling pathways for the *L*-phenylalanine -responsive GPCRs. This was investigated utilizing mammalian cells expressing a target GPCR and luciferase reporter for one of the $G\alpha_{12/13}$, $G\alpha_{q/11}$, Gβγ, $G\alpha_{i/o}$, and $G\alpha_{s}$ signaling pathways. Cells were treated with either *L*-phenylalanine or a control vehicle prior to luciferase assay examinations of their response. *L*-phenylalanine differentially activated GPCR signaling pathways, in a manner that was receptor-dependent, to preferentially increase or decrease cellular responses. Moreover, this response was not always limited to one preferential pathway, and not all receptors previously identified to be activated by *L*-phenylalanine were observed to significantly signal through the examined pathways. Elucidation of *L*-phenylalanine's role in the regulation of cellular communication and function must be explored through independent receptor characterization due to its promiscuous receptor-dependent activation of GPCRs.

List of Figures

(visualized as a dotted line) as fold change. Data was collected in biological triplicates and shown as mean +/- SEM. Statistical significance (*$p < 0.05$) in activation relative to the vehicle was evaluated using two-way ANOVAs and corrected using Holm-Šídák statistical hypothesis testing.

Figure 3.3. Receptor-dependent activation of class A orphan GPCRs induced by *L*-Phe. HEK293 cells expressing a luciferase reporter for G protein signaling pathways were transfected with the receptor candidates GPR3, GPR6, GPR12, GPR34, GPR35, GPR88, or GPR142. Receptors were treated with a Opti-MEM vehicle, 0.8- or 5 mM *L*-Phenylalanine (PHE). For investigation of the $G\alpha_{i/o}$ pathway, cells were additionally treated with forskolin (concentration) to activate adenylyl cyclase and induce pathway target pathway activation. Luciferase/β-galactosidase activity (RLU) was determined and expressed relative to the vehicle (visualized as a dotted line) as fold change. Data was collected in biological triplicates and shown as mean +/- SEM. Statistical significance (*$p < 0.05$) in activation relative to the vehicle was evaluated using two-way ANOVAs and corrected using Holm-Šídák statistical hypothesis testing.

Figure 3.4. Receptor-dependent activation of class A lipid metabolism GPCRs induced by *L*-Phe. HEK293 cells expressing a luciferase reporter for G protein signaling pathways were transfected with the receptor candidates CLTR2, GPBA, MC4R, S1P1, or S1P4. Receptors were treated with a Opti-MEM vehicle, 0.8- or 5 mM *L*-Phenylalanine (PHE). For investigation of the $G\alpha_{i/o}$ pathway, cells were additionally treated with forskolin (concentration) to activate adenylyl cyclase and induce pathway target pathway activation. Luciferase/β-galactosidase activity (RLU) was determined and expressed relative to the vehicle (visualized as a dotted line) as fold change. Data was collected in biological triplicates and shown as mean +/- SEM. Statistical significance (*$p < 0.05$) in activation relative to the vehicle was evaluated using two-way ANOVAs and corrected using Holm-Šídák statistical hypothesis testing.

List of Figures and Tables in Appendix

Appendix A. Signaling Treatment Time Optimization. HEK293 cells either transiently or stably expressing a luciferase reporter for G protein signaling pathways were transfected with pBSK. Cells were treated with Opti-MEM (VEH) and either 20% Fetal Bovine Serum or 10 ng/mL PMA & 1 μM Ionomycin (POS) as a positive control for pathway activation. Treatment times of 24-, 18-, 12-, 6-, 4-, 2 hours were observed. Luciferase/β-galactosidase activity (RLU) was determined and expressed relative to VEH as fold change. Data was collected in biological triplicates and shown as mean +/- SEM. Statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) was evaluated using two-way ANOVAs and corrected using Holm-Šídák statistical hypothesis testing.

Appendix B. Normalized posterior probability score. PRED-COUPLE 2.0 predicted coupling specificity of 12 human GPCRs to the G proteins $G\alpha_{q/11}$, $G\alpha_{i/o}$, $G\alpha_{s}$, and $G\alpha_{12/13}$. Positive predictions in bold type.

Appendix C. Location, length, and orientation of predicted GPCR transmembrane helices. TMHMM-2.0 predicted transmembrane domains based on a hidden Markov model, from receptors' FASTA formatted amino acid sequences. Orientation is indicated as outside (o) to inside (i) or inside to outside of predicted transmembrane domains.

List of Abbreviations and Symbols Used

7TM – Seven Trans-Membrane

AC – Adenylate Cyclase

AMP – Ampicillin

ANOVA – Analysis of Variance

cAMP – Cyclic Adenosine Monophosphate

CaSR – Calcium-sensing Receptor

CRE – cAMP Response Element

DAG – Diacylglycerol

DMEM – Dulbecco's Modification Eagle's Medium 1X Mod.

DMSO – Dimethyl Sulfoxide

FBS – Fetal Bovine Serum

GDP – Guanosine diphosphate

GFP – Green Florescent Protein

GPCR – G-Protein Coupled Receptor

GTP – Guanosine Triphosphate

HEK293 cells – Human Embryonic Kidney 293 cells

HTS – High-Throughput Screen

IP_3 – Inositol Trisphosphate

IUPHAR – International Union of Basic and Clinical Pharmacology

FBS – Fetal Bovine Serum

L-Phe – L-Phenylalanine

MAPK – Mitogen Activated Protein Kinase

NFAT – Nuclear Factor of Activated T-cells

NFAT-RE – Nuclear Factor of Activated T-cells Response Element

PBS – Phosphate Buffered Saline

pBSK – empty vector pBluescriptSK

PEI – Polyethyleneimine

PMA – Phorbol 12-Myristate 13-Acetate

PLC – Phospholipase C

PRESTO-Tango – Parallel Receptor-ome Expression & Screening *via* Transcriptional Output-Tango

RhoA – Ras Homolog Family Member A

RLU – Relative Light Units

SEM – Standard Error of the Mean

SRE – Serum Response Element

SRF – Serum Response Factor

SRF-RE – Serum Response Factor Response Element

Veh – Vehicle

β-arrestin – Beta-arrestin

β-gal – Beta-galactosidase

1. Introduction

1.1. Signal Transduction

Signal transduction is an essential form of cellular communication, by which cells adapt to their external environment for survival. Diverse molecules, including lipids, peptides, ions, photons, and amino acids act as extracellular signals to facilitate communication between cells and their surroundings.[3] Cells must interpret these signals through intracellular signaling cascades that disseminate this molecular communication within the cell to elicit cellular responses.[4] These signaling pathways can reach the nucleus of a cell to target transcription factors and effectively regulate gene expression.[5] Signal transduction pathways coordinate and regulate nearly all physiological responses, including cell death, development, differentiation, metabolism, and proliferation.[3]

Extracellular signaling molecules that bind to protein receptors at the cell surface are called ligands.[6] Upon ligand binding, receptors undergo a conformational change to activate target proteins and stimulate the release of second messenger molecules; second messengers amplify and cascade signals by altering the activities of downstream target effector proteins to produce cellular responses.[6] The same signal molecule can activate multiple pathways, which produce distinct responses.[6] Further, signaling pathways can be linear and have single targets; however, many are branched complex networks with the capability of participating in crosstalk, producing a multitude of biochemical responses.[3] As such, many proteins and second messengers participate in more than one signaling pathway to interact with several upstream and downstream molecules.[3] Ligands and receptors exhibit a selective binding affinity for one another, though some demonstrate promiscuous binding, indiscriminately activating intracellular signaling pathways.[6]

The nature of receptor activity is affected by the type and location of ligand binding.[7] Most ligands directly activate a receptor, binding directly at the orthosteric site.[7] Alternatively, ligands can bind to allosteric sites and alter receptor conformation, indirectly affecting its signaling activity.[7] Allosteric ligands change the conformation of all binding sites, modulating their binding affinity.[7] Further ligands are categorized based on their effect on the receptor, as agonists, antagonists, or inverse agonists.[7] Agonists stabilize the receptor to induce activation and therefore initiate signal transduction.[7] Inverse agonists have a negative efficacy on activity, producing opposing effects, compared to the agonist.[7] Antagonists, while they do not affect a receptor's constitutive activity (ligand-independent activity), bind and impede on agonist-induced activation.[7]

The rapidly evolving knowledge surrounding signal transduction has allowed for significant advances in pharmacology and drug design. Cell surface receptors are common targets for drugs which can manipulate and imitate signaling responses for therapeutic benefits.[8] Approaches in drug design rely heavily on the characterization of receptor-specific signal transduction, the determination of the biological impact of signaling, and the identification of ligands which elicit the desired response.[7] Large scale elucidation of the signal transduction of receptors using high-throughput screening (HTS) methods has been advantageous for academics and pharmaceutical groups seeking to identify new drug targets; however, many receptors remain uncharacterized.[8]

1.2. G-Protein Coupled Receptors

G-protein coupled receptors (GPCRs) are the most abundant cell surface receptor in eukaryotes.[9] GPCRs transmit signals intracellularly *via* coupling with heterotrimeric guanine nucleotide-binding proteins (G proteins).[10] There are over 800 GPCRs identified in humans, based

on their distinguished seven-transmembrane (7TM) α-helices structure and topology. They mediate a range of signal transduction events, transmitting signals using their extracellular N-terminus and intracellular C-terminus.[9] Due to their implications in cell functions and physiological responses, GPCRs have been heavily researched and validated as therapeutic drug targets.[11] GPCRs are targets for ~30% of all drugs currently approved across the global therapeutic market, and are the focus of approximately 2/3rds of recent clinical trials[12]. These targets constitute only a fraction (~15%) of the superfamilies' total members.[13] The majority of GPCRs represent unexplored potential, with many studies aiming to define their signal transduction response, identify ligands, and determine their roles in diseases and disorders.[13]

Diverse and relatively unconserved, GPCRs are functionally classified using an A-F system[9]: The rhodopsin-like family (A), the secretin receptor family (B), the metabotropic glutamate receptor family (C), the fungal pheromone receptors (D), cAMP receptors (E) and frizzled/smoothed receptors (F).[14] Classes A, B, C, and F, are found in humans.[9] Approximately 80% of all GPCRs fall within class A which includes hormone, light, and neurotransmitter receptors[14]; additionally, roughly half of the class is comprised of olfactory sense receptors.[14] Class A receptors are known to bind with a diverse range of ligands and are related to a vast number of physiological functions and diseases.[9,15]

The International Union of Basic and Clinical Pharmacology (IUPHAR) officially names GPCRs based on their endogenous ligand.[8] However, several GPCRs have unidentified or disputed endogenous ligands, which are termed "orphans", particularly found within classes A and C.[16] GPCRs become de-orphanized when researchers identify a ligand for the receptor with a physiological link to its activity, and the result is reproducible.[16] In pharmacology, de-orphanization often leads to advances in identifying novel physiological processes and

therapeutics.[16] There are currently several HTS methods available for identifying and validating endogenous ligands.[17]

1.3. G-Protein Signalling

GPCRs coupled with heterotrimeric G proteins act as signal transducers.[10] These heterotrimeric G proteins are comprised of guanosine diphosphate (GDP)-bound Gα subunits and a Gβγ heterodimer.[1] Based on the sequence of their Gα subunit, G proteins divide into four subfamilies: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$.[1] Upon activation, a change in the conformation of the GPCR activates the Gα subunit to exchange GDP for guanosine triphosphatase (GTP).[1] This causes a conformational change in the Gα subunit and the dissociation of the Gβγ heterodimer.[1] Utilizing either the active Gα subunit or free Gβγ, GPCRs initiate distinct intracellular signaling pathways (Figure 1.1).[1] Pathways will propagate signals to regulate the activities of downstream target effectors and transcriptional machinery.[18] Signaling terminates following the reassociation of Gβγ and the hydrolysis of GTP to GDP as a result of GTPase activity.[1]

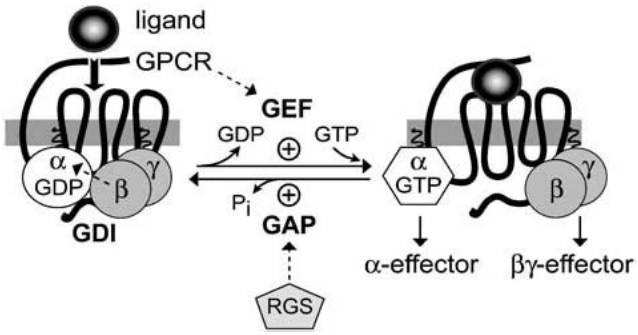


Figure 1.1. Standard ligand induced activation of GPCR signaling pathways. Ligand binding initiates intracellular receptor coupling with heterotrimeric G proteins to transmit signals downstream through target effectors.[1]

The canonical signaling targets of the 4 Gα subunits are well characterized; however, the subsequent interactions and crosstalk within their signaling pathways are often complex, and remain relatively unclear.[1] The $G\alpha_s$ pathway regulates intracellular levels of cyclic adenosine

monophosphate (cAMP) through activation of the enzyme adenylate cyclase (AC).[2] This pathway induces gene transcription via terminal effectors binding to the cAMP response element (CRE).[2] The $G\alpha_{i/o}$ pathway signals through its α subunit to oppose the $G\alpha_s$ pathway by inhibiting the activity of AC.[1] The $G\alpha_q$ pathway activates phospholipase C (PLC) to generate the secondary messengers inositol trisphosphate (IP_3) and diacylglycerol (DAG), to induce Ca^{2+} production.[10] This pathway induces gene transcription via the nuclear factor of activated T-cells response element (NFAT-RE).[2] The $G\alpha_{12/13}$ pathway is currently the least characterized α-subunit pathway; however, it is known to affect the GTPase RhoA. This pathway induces gene transcription via the serum response factor response element (SRF-RE).[2] Further, GPCRs can use their Gβγ heterodimer to initiate signaling, activating mitogen-activated protein kinases (MAPKs). The Gβγ subunit induces gene transcription via activation of gene promotors containing serum response element (SRE).[2]

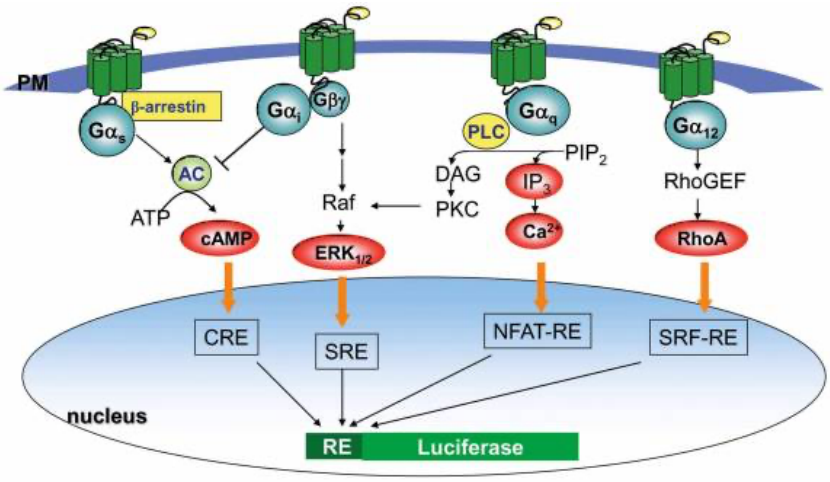


Figure 1.2. The major GPCR signaling pathways $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, $G\alpha_{12/13}$, $G\beta\gamma$. GPCR activation induces cascades of intracellular signaling that terminate in transcriptional activation at pathway-specific response elements. Luciferase genes can be stably or transiently added to cell lines to couple each pathway to expression of a luciferase enzyme.[2]

GPCRs exhibit biased signaling, as distinctive ligand-dependent receptor conformations selectively activate downstream signaling pathways.[19] When activated, GPCRs are often capable

of adopting multiple, ligand-dependent, conformations, each producing a distinct response and downstream effect[19]; each GPCR induced conformational state may initiate a downstream signal through a specific G protein pathway.[7] The reasoning for a receptor's coupling specificity is not fully understood, and may be dependent on extrinsic factors such as subcellular location.[20].

The termination of signal transduction is coordinated and regulated by β-arrestins via a desensitization and sequestration processes.[21] Following activation, G protein coupled receptor kinases phosphorylate the intracellular GPCR loops.[21] Immediately following the phosphorylation of active GPCRs, β-arrestins are recruited to the receptor, binding with a high affinity and causing selective dampening of transduction due to steric hindrance.[21,22] Subsequently, the C terminus of β-arrestin interacts with clathrin proteins to facilitate internalization, degradation, and recycling of the receptor complex *via* endocytosis.[23]

HTS luciferase reporters are commercially available to characterize receptor/G protein pathway activation downstream of each canonical pathway.[2] Promotors for G protein pathways followed by a firefly luciferase gene can be stably or transiently added to cell lines to couple each pathway to expression of a luciferase enzyme. Luciferases are oxidative enzymes that produce bioluminescence through catalysis of a luciferin substrate.[2] If pathway activation occurs, transcription factors corresponding to each pathway transcribe pathway-specific response elements in the nucleus (CRE, NFAT-RE, SRE, SRF-RE). The resultant increase in gene expression and luciferase protein activity is measured as an increase in light that corresponds with the magnitude of pathway activation.[2]

1.4. Amino Acid Signaling

The potential of metabolites, and to a greater extent amino acids, as extracellular signaling molecules has just begun to be elucidated.[24] Amino acid sensing in the body is vital for

coordinating protein abundance with metabolism, and is associated with various signaling pathways.[25,26] Amino acid sensors preferentially recognize the natural *L*-isomers; The *L*-configuration is the predominant form for amino acids, and the only configuration capable of protein biosynthesis in the human body.[26,27] Amino acid sensing receptors have been found abundant in the gastrointestinal tract and were first identified within the class C family of GPCRs, for-example taste receptors, GABA receptors, and the calcium sensing receptor (CaSR).[28] The taste receptors TAS1R1 and TAS1R3, which form the dimer responsible for mediating the umami flavour, are activated by most amino acids.[28] TAS1R1-TAS1R3 receptors are further expressed in tissues throughout the body, where its suggested their activation by amino acids may be linked to regulating autophagy.[28] Studies suggest that amino acid sensing receptors have a limited selectivity or enhanced sensitivity for aromatic amino acids.[26] While there is some progress on the functions of amino acid signaling in regulating metabolism, the signal transduction pathways are largely unknown.[24]

1.5. Rationale for the Current Study

An essential amino acid, as it is not synthesized metabolically and must be taken up in the diet, *L*-phenylalanine (*L*-Phe), serves important physiological roles in metabolic pathways and the biosynthesis of proteins.[27] *L*-phe is the *L*-enantiomer of the large hydrophobic aromatic amino acid phenylalanine. Aromatic amino acids such as *L*-phe, and its derivative *L*-tyrosine, have been recently proposed as an endogenous ligand for GPCRs found within the gastrointestinal tract.[24,29] This is the case for CaSR, and the class A orphans GPR142 and GPR139.[29,26,27]

We have shown in previous studies that the essential amino acid *L*-Phe can activate multiple class A GPCRs. These studies from our lab aimed to investigate the receptor activation of 72 orphan receptors and 72 receptors with known roles in lipid and energy homeostasis,

following treatments with essential and non-essential amino acids mixtures, using a HTS β-arrestin recruitment assay method (**Figure 1.3)**.[32] Following investigations with essential and non-essential amino acid mixtures, we found that the amino acid *L*-Phe had the most significant impact on the activity class A receptor candidates **(Figure 1.4**). Class A orphan receptor candidates GPR3, GPR6, GPR12, GPR34, GPR35, GRP88, GPR142, and class A lipid receptor candidates CLTR2, GPBA, MC4R, S1P1, S1P4 were found to be significantly activated by 0.8 mM L-Phenylalanine. The results suggested that, through activation of GPCRs, the role of *L*-Phe in cell function may extend beyond its role as a building block in proteins to include regulation of physiological significant cellular communication.

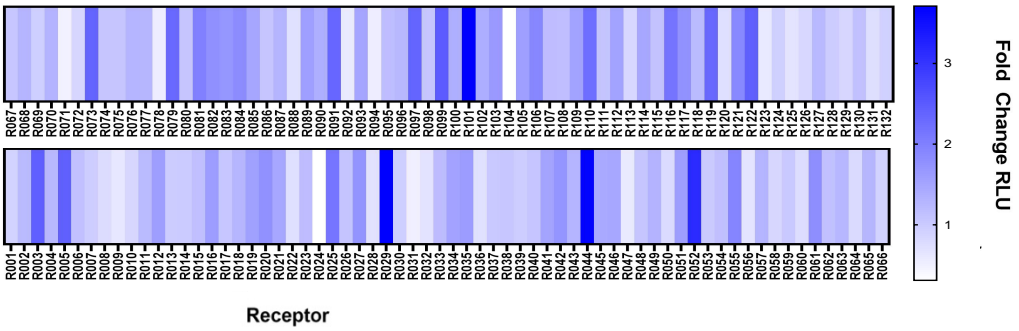


Figure 1.3. Heat map of amino acid-treated orphan and lipid receptors screen. The fold change RLU of the change in luminescent signaling of 144 lipid and orphan receptors treated with a mixture of 20 essential and non-essential amino acids relative to vehicle treatment in a ß-arrestin recruitment assay. (Data courtesy of Madeline Power and Madeline Russell, 2019).

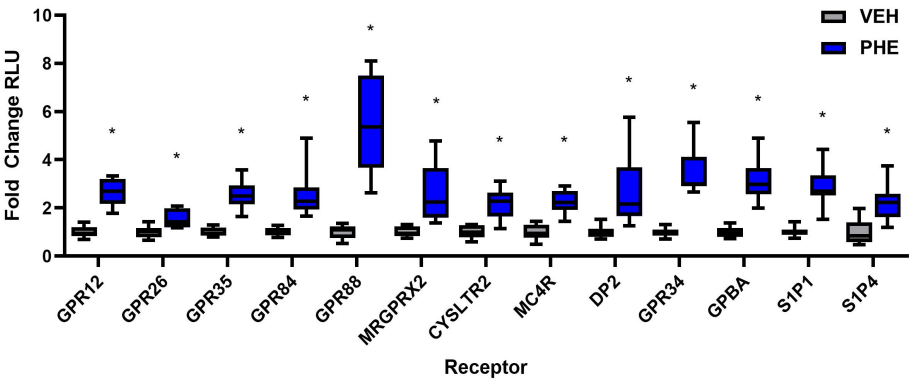


Figure 1.4. *L*-Phe activation of lipid and orphan GPCRs. Fold change RLU of receptor candidates significantly activated in screen treated with vehicle (VEH, gray) and 0.8 mM *L*-Phe (PHE, blue). Data shown as min to max with line as median, * $p < 0.05$. (Data courtesy of Madeline Power and Madeline Russell, 2019).

1.6. Summary

The aim of this study was to characterize the signal transduction response in multiple established signaling pathways for the *L*-Phe-responsive GPCRs. This will be accomplished by individually transfecting 12 class A orphan and lipid GPCRs into mammalian cells stably or transiently expressing vectors for luciferase reporter genes specific to $G\alpha_{12/13}$, $G\alpha_{q/11}$, $G\alpha_iG\beta\gamma$, $G\alpha_{i/o}$, or $G\alpha_s$ signaling pathways. Cells will then be treated with either *L*-phenylalanine or a control vehicle prior to conducting luciferase assays to examine activation responses. Through this project we expect to create 12 receptor profiles, characterizing the transduction response in each major G-protein pathway. We hypothesize that activation of the identified receptors will result in GPCR signal transduction in multiple downstream pathways and that these may differ depending on the receptor. The findings from this project will identify whether these receptors produce biologically relevant responses to *L*-Phe. This is a necessary first step in the longer-term goal to determine the impact and potential these signal transduction pathways and receptors have on health, and metabolism, and diseases.

2. Materials and Methods

2.1. Cell Culture

For Gβγ coupled pathway experiments, human embryonic kidney 293 (HEK293) cell lines stably expressing SRE responsive MAPK reporters, containing a firefly luciferase gene, were utilized.[33] For $G\alpha_{q/11}$ and $G_{12/13}$ pathway experiments, HEK293-T17 cell lines were utilized, and reporters were added by transient transfection. All cells were grown at 37°C and 5% CO_2. Cell growth medium contained Dulbecco's Modification Eagle's Medium (DMEM; Corning, 10-013-CV), with 10% fetal bovine serum (FBS; Wisent Biproducts, 080-150). Cells were passaged when cultures reached 70-80% confluency (approximately every 48 hours), at a sub-cultivation ratio of 1:10. During passaging, cells were washed using 1X phosphate buffered saline (PBS) wash buffer, detached from the plate with 0.25% trypsin/2.21 mM ethylenediaminetetraacetic acid (EDTA; Corning, 25-053-CL), and centrifuged at 300 rcf for 5 minutes, prior to resuspension in a 10 cm cell culture dish. HEK293 cell lines expressing MAPK reporters were additionally maintained in 0.04 μg/mL puromycin (Bioshop, PUR333.25).

2.2. Plasmid Preparation

Plasmids containing the coding sequences of GPCRs were obtained from the PRESTO-Tango library (Roth Lab, Addgene Kit #1000000068), and propagated using standard procedures in *E.coli*. Transfection-grade plasmid DNA was isolated with NucleoBond Xtra Midi Plus kits (Macherey-Nagel, 740412.50) for high-yield purification, and NucleoSpin Plasmid Transfection-grade kits (Macherey-Nagel, 740490.25) for low-yield purification. Additionally, pCMV-beta-galactosidase (β-gal), empty vector pBluescriptSK (pBSK), and pEGFP-N1 expressing green fluorescence protein (GFP) plasmids were a gift from C. Sinal (Dalhousie University). β-gal and GFP were used as internal controls to quantify and observe the efficiency of transfections

respectively. DNA plasmid concentration and purity was quantified using Nanodrop spectrum analysis software.

2.3. Luciferase Assay

A summary of the luciferase assay experimental timeline is shown in **Figure 2.1.**

On Day 1 of $G\alpha_{q/11}$ and $G_{12/13}$ pathway experiments, HEK 293 cells were plated at 12,000 cells/well in DMEM containing 10% FBS in 96 well plates. For Gβγ coupled pathway experiments, MAPK cell lines were plated in the same medium conditions at 35,000 cells/well in 24 well plates.

On Day 2 of experiments HEK 293 cells were transiently transfected with the following components prepared in Opti-MEM: 50 ng/well signaling reporter, or pBSK if reporter was already expressed; 25 ng/well β-gal; 100 ng/well GPCR candidate DNA; using 2.8 μg/well polyethyleneimine (PEI) transfection reagent. Volumes were increased fourfold for Gβγ coupled pathway experiments. Additional control used either pBSK or GFP in place of receptor DNA. pBSK was used to provide a negative transfection control to verify the efficacy of β-gal vector and to elucidate endogenous receptor signaling effects. Transfected cells were incubated at 37°C and 5% CO_2 for 15 hours post-transfections, following which medium was removed and cells were then serum-starved for 24 hours in Opti-MEM (Gibco, 31985-07).

On Day 4 of experiments technical triplicates were treated with 3X treatments of 0.8 mM, 5 mM *L*-Phe (TCI America, P0134), or a control vehicle (Opti-MEM). Treatment times for experiments were 4 hours unless otherwise indicated (**Appendix C**). Positive controls were additionally given 20% FBS (for cell expressing MAPK or SRF) or 10 ng/mL PMA and 1 μM Ionomycin (for cells expressing NFAT). Cells were lysed using 20 μL/well (100 μg/well for Gβγ

pathway experiments) 1X Firefly lysis buffer (Biotium, 30028) and stored at -80°C following treatments.

β-gal assays were performed by combining 30 µL of 2X β-gal assay buffer (0.1 M sodium phosphate buffer (pH 7.3), 1 mM $MgCl_2$, 0.067% (w/v) ortho-nitrophenyl- β-galactosidase (ONPG), 50 mM β-mercaptoethanol) and 30 µL of cell lysate. Absorbance was quantified at 420 nm using a BioTek Synergy HT luminescence reader and Gen5 software, 10 minutes following the addition of β-gal assay buffer. The luciferase assay was performed using 10 µL luciferase assay buffer (Biotium, 30028L) containing *D*-luciferin (Biotium, 9907) and 20 µL of cell lysate. 5 min following the addition of luciferase buffer with oscillation, luminescence (RLU) was quantified using a BioTek Synergy HT luminescence reader and Gen5 software.

All experiments contained technical triplicates of receptors with individual treatments and were performed a minimum of three times.

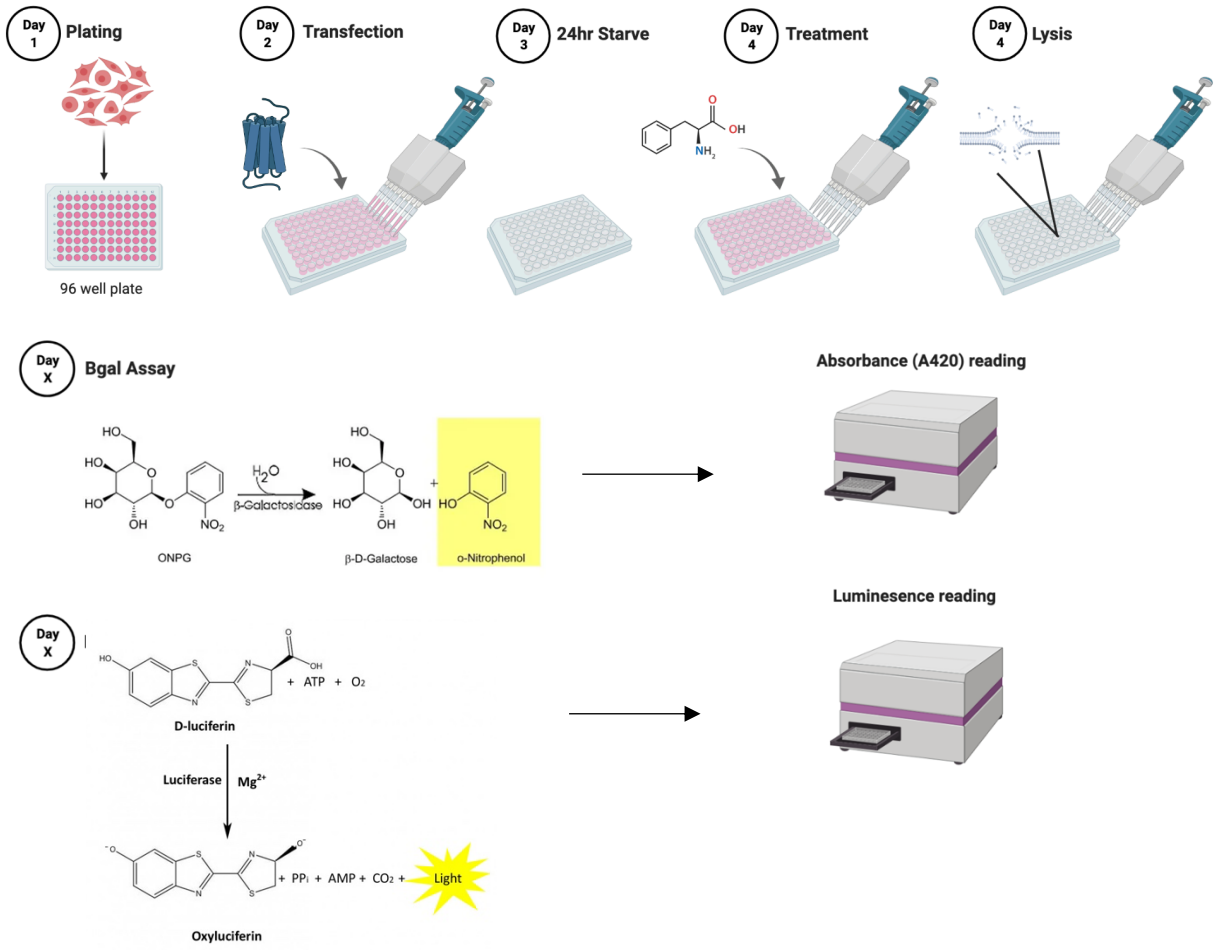


Figure 2.1. Schematic outlining the experimental timeline of Signaling Reporter Assays (Day 1) HEK 293 cells were plated in 96- or 24 well plates. (Day 2) Cells were transfected, β-gal, *L*-Phe activated GPCRs, pBSK, and signaling reporter (for $G\alpha_s$ and $G_{i/o}$ experiments) using PEI. (Day 3) Cells were serum-starved for 24 hours in Opti-MEM. (Day 4) Cells were treated with 0.8 mM *L*-Phe, 5 mM *L*-Phe, vehicle (Opti-MEM), or positive control for designated treatment length; Cells were lysed and stored at -80°C following treatments. (Day X) Receptor activity was quantified *via* luciferase and β-gal assays using a BioTek Synergy HT luminescence reader and Gen5 software. Method illustrations created with www.Biorender.ca.

2.4. Statistical Analyses

Raw data compiled from experiments was analyzed using Microsoft Excel and standardized as RLU/A420. Data was corrected for background noise using assayed 0.1X Firefly lysis buffer as blank measurements and transformed as average fold change with respect to vehicle treatments. Additionally, analogous data from experiments performed on $G\alpha_s$ and $G_{i/o}$ pathways

were consolidated for figure presentations; this data was contributed by master's student Madeline Power. Statistical analysis and data visualization was achieved using Prism 9 (GraphPad Software). A two-way analysis of variance (ANOVA) was performed, with multiple comparisons between the mean of individual treatments to the mean of the vehicle treatments. The analysis was corrected using Holm-Šídák statistical hypothesis testing, which adjusted p values for multiplicity. The significance level for all statistical analyses was set at $p < 0.05$ and indicated in figures with an asterisk. All data was presented in figures as the mean ± standard error of the mean (SEM).

2.5. Bioinformatics

PRED-COUPLE 2.0 was used to predict the normalized posterior probability score, indicating coupling specificity, of investigated GPCRs to the G proteins α subunits: $G\alpha_{q/11}$, $G\alpha_{i/o}$, $G\alpha_{s}$, and $G\alpha_{12/13}$ (**Appendix B**). Further, TMHMM-2.0 was used to predict GPCR transmembrane domain location and orientation, based on a hidden Markov model, from FASTA formatted amino acid sequences (**Appendix C**).

3. Results

3.1. Luciferase assays can be used to inspect GPCR pathway activation

Methods first needed to be validated to ensure significant responses could be measured within target pathways for subsequent examinations of *L*-Phe induced activation of signaling pathways among class A GPCRs. Preliminary screenings of luciferase assays were performed using pBSK as a control for pathway activation and assay function in the absence of GPCR-dependent interference. To determine the extent and nature of pathway signaling we examined the activation of luciferase reporter expression downstream of $G\alpha_{12/13}$, $G\alpha_{q/11}$, $G\beta\gamma$ $G\alpha_{i/o}$, and $G\alpha_{s}$. To do so, HEK293 cells stably or transiently expressing vectors for luciferase reporter genes under the control of the CRE, NFAT, SRE, or SRF-RE response elements were transfected with pBSK and treated with OptiMEM vehicle or known positive controls for pathway activation (**Figure 3.1**). For all pathways, treatment with their respective pathway activation control resulted in significantly increased fold-change luminescence relative to the untreated vehicle. Positive control treatments induced increases of at least 2-fold in all investigated pathways, with the $G\beta\gamma$ pathway demonstrating the most sensitive response with the magnitude of activation over 30-fold. Variation among pathway responses could be attributed to the differences in the number of cells plated for signaling reporter assays, which were presumed from prior transfection optimization procedures. These findings demonstrate that the procedures and equipment are sufficient to investigate signaling in these pathways and ready to use for the investigation of *L*-Phe signaling of GPCRs through these pathways.

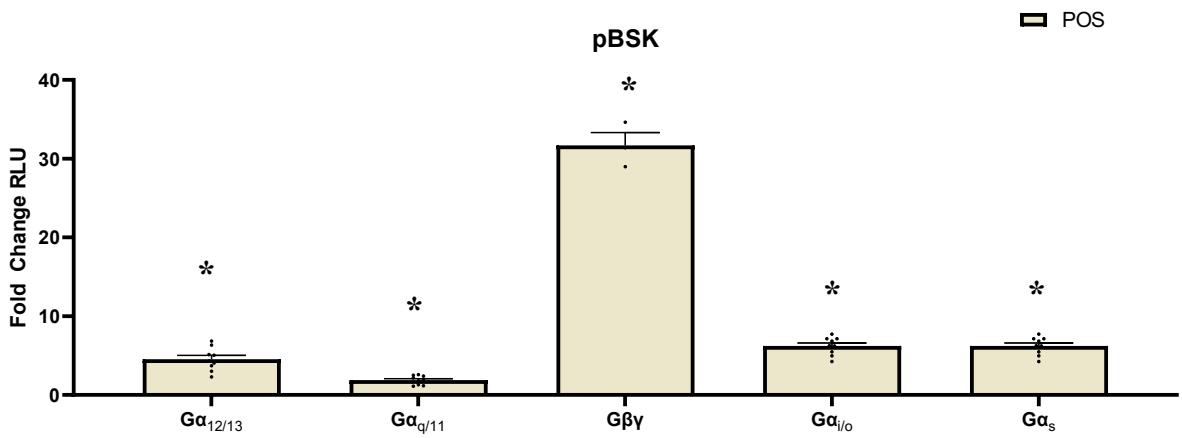


Figure 3.1. Validation of Signaling Reporter Assays. HEK293 expressing a luciferase reporter for G protein signaling pathways were transfected with the empty vector plasmid pBSK. Cells were treated with a Opti-MEM vehicle or a positive control (POS) for activation (controls were 20% FBS for $G\alpha_{12/13}$, $G\beta\gamma$ pathways; 10 ng/mL PMA & 1 μM Ionomycin for $G\alpha_{q/11}$; and forskolin for $G\alpha_{i/o}$ and $G\alpha_s$ pathways). Luciferase/β-galactosidase activity (RLU) was determined and expressed relative to the vehicle as fold change. Data was collected in biological triplicates and shown as mean +/- SEM. Statistical significance (*$p < 0.05$) in activation relative to the vehicle was evaluated using one-way ANOVAs and corrected using Holm-Šídák statistical hypothesis testing.

3.2. Investigation of Endogenous L-Phe signaling

Following assay validation, luciferase assays were interrogated to distinguish between activation of endogenous expressed receptors in HEK293 cells and exogenously expressed GPCRs of interest. Given that HEK293 cells have the potential to endogenously express/interact with signaling proteins, and downstream luciferase assays indiscriminately respond to pathway activation, it is necessary to determine the impact of *L*-Phe treatment in the absence of GPCR expression.[34] To assess whether *L*-Phe could activate endogenous signaling pathways, luciferases assays were performed in response to 0.8- and 5 mM *L*-Phe treatments, using reporter cells transfected with pBSK (**Figure 3.2**). *L*-Phe significantly induced signaling relative to the vehicle across both treatment doses within the $G\alpha_{q/11}$ pathway, and the $G\alpha_{12/13}$ pathway in response to the higher treatment exposure.

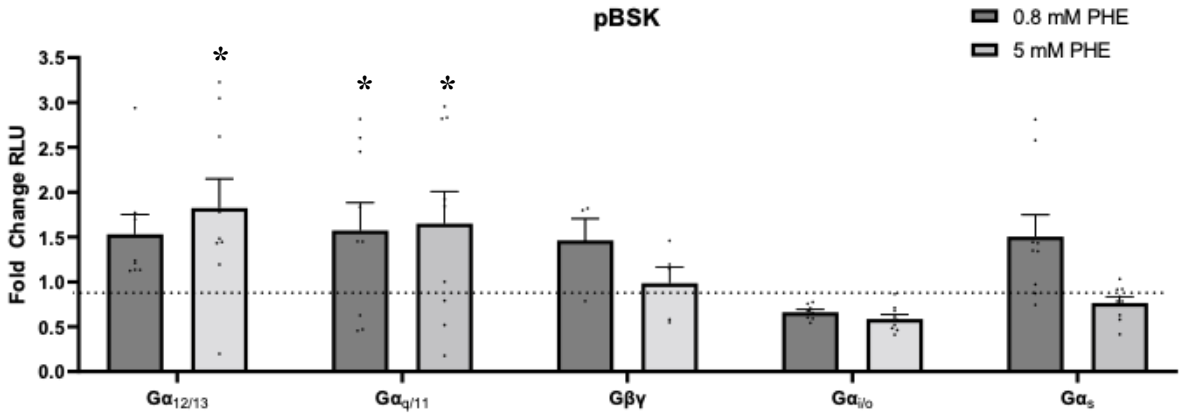


Figure 3.2. GPCR-independent activation of signaling pathways induced by *L*-Phe. HEK293 cells expressing a luciferase reporter for G protein signaling pathways were transfected with the empty vector plasmid pBSK. Cells were treated with a Opti-MEM vehicle, and 0.8- or 5 mM *L*-Phenylalanine (PHE), for optimal treatment times (See Appendix C). $G\alpha_{i/o}$ investigated cells were additionally treated with a forskolin mixture to induce signaling within the target pathway. Luciferase/β-galactosidase activity (RLU) was determined and expressed relative to the vehicle (visualized as a dotted line) as fold change. Data was collected in biological triplicates and shown as mean +/- SEM. Statistical significance (*$p < 0.05$) in activation relative to the vehicle was evaluated using two-way ANOVAs and corrected using Holm-Šídák statistical hypothesis testing.

3.3. L-Phe differentially impacts signaling through class A orphan GPCRs

To determine which signaling pathways *L*-Phe activates in class A orphan GPCRs, luciferase assays were individually conducted for luciferase reporters expressed in $G\alpha_{12/13}$, $G\alpha_{q/11}$, $G\alpha_iG\beta\gamma$, $G\alpha_{i/o}$, and $G\alpha_s$ pathways. Receptor candidates GPR3, GPR6, GPR12, GPR34, GPR35, GPR88, GPR142 were treated with 0.8 mM and 5 mM *L*-Phe (**Figure 3.3**). Fold change compared to vehicle treatments of *L*-Phe yielded a significant change in pathway activity, exclusively among GPR12, GPR34, and GPR35. *L*-Phe differentially activated the signaling of these receptors to significantly increase or decrease response element activity compared to the vehicle. Moreover, except in the case of GPR12, *L*-Phe inhibited signaling responses through a single significant pathway and was dose dependent. In the case of GPR12, *L*-Phe treatments had differing pathway effects, inhibiting activity within $G\alpha_{12/13}$ and $G\alpha_{q/11}$ pathways, this response was dose dependent

for the latter. Further, *L*-Phe induced increases in signaling through $G\alpha_{i/o}$ over both treatment concentrations.

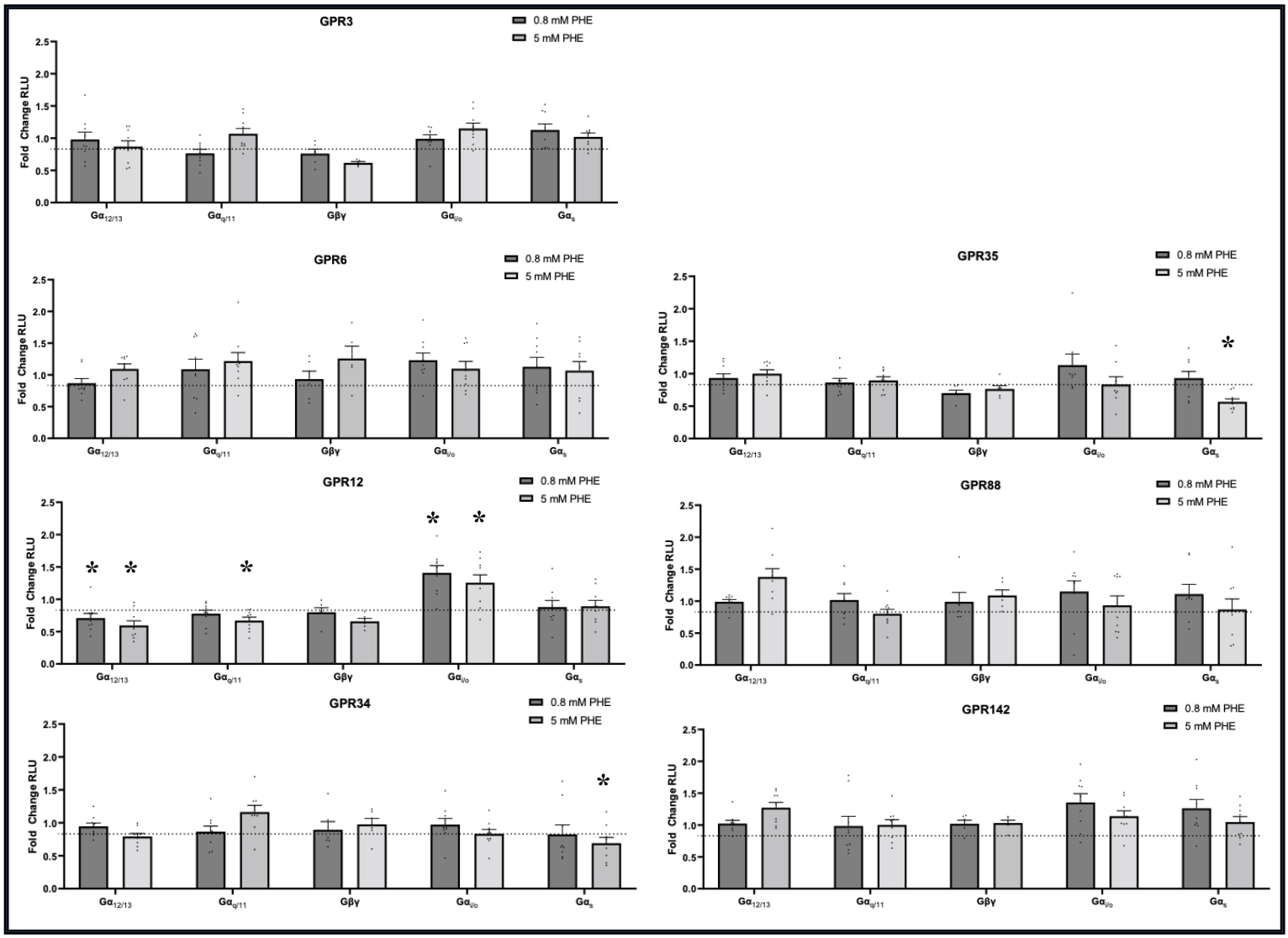


Figure 3.3. Receptor-dependent activation of class A orphan GPCRs induced by *L*-Phe. HEK293 cells expressing a luciferase reporter for G protein signaling pathways were transfected with the receptor candidates GPR3, GPR6, GPR12, GPR34, GPR35, GPR88, or GPR142. Receptors were treated with a Opti-MEM vehicle, 0.8- or 5 mM *L*-Phenylalanine (PHE). For investigation of the $G\alpha_{i/o}$ pathway, cells were additionally treated with forskolin (concentration) to activate adenylyl cyclase and induce pathway target pathway activation. Luciferase/β-galactosidase activity (RLU) was determined and expressed relative to the vehicle (visualized as a dotted line) as fold change. Data was collected in biological triplicates and shown as mean +/- SEM. Statistical significance (*$p < 0.05$) in activation relative to the vehicle was evaluated using two-way ANOVAs and corrected using Holm-Šídák statistical hypothesis testing.

3.4. L-Phe differentially impacts signaling through class A lipid metabolism GPCRs

To determine which which signaling pathways were activated by *L*-Phe in class A lipid metabolism GPCRs, the above experiment was repeated with the receptor candidates CLTR2, GPBA, MC4R, S1P1, S1P4 (**Figure 3.4**). Similar to the unexpected findings among orphan GPCRs, *L*-Phe differentially activated the signaling of GPCRs involved in lipid metabolism, significantly increasing or decreasing response elements compared to the vehicle. *L*-Phe induced significant dose-dependent responses among pathways for GPBA, MC4R, and S1P4. Both GPBA and MC4R had decreased activity when treated with 0.8 mM *L*-Phe, within the $G\alpha_{12/13}$ and $G\alpha_{q/11}$ pathways respectively. Alternatively, treatments of 5 mM *L*-Phe increased receptor activity of S1P4 with the $G\alpha_{q/11}$ pathway.

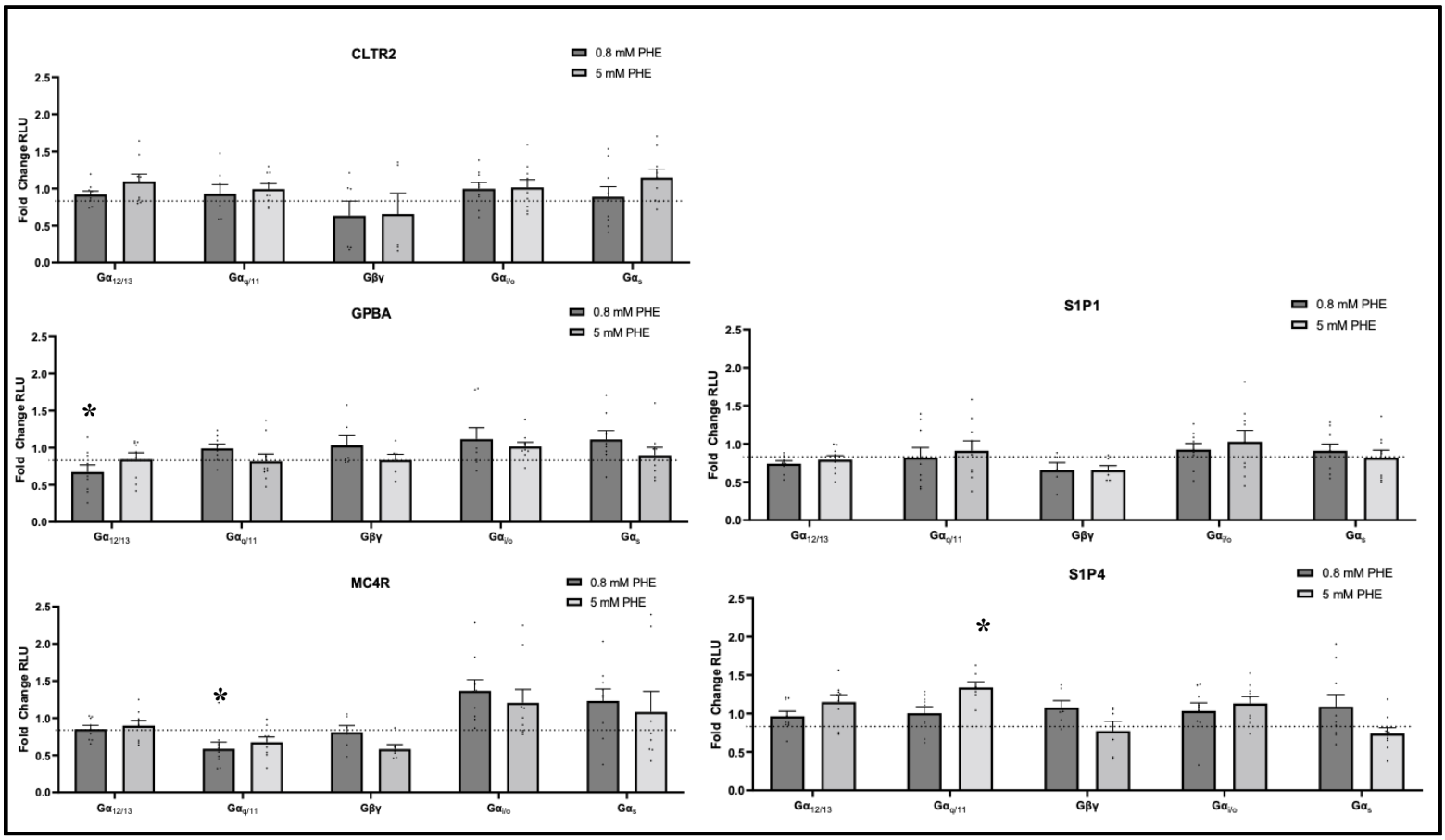


Figure 3.4. Receptor-dependent activation of class A lipid metabolism GPCRs induced by *L*-Phe. HEK293 cells expressing a luciferase reporter for G protein signaling pathways were transfected with the receptor candidates CLTR2, GPBA, MC4R, S1P1, or S1P4. Receptors were treated with a Opti-MEM vehicle, 0.8- or 5 mM *L*-Phenylalanine (PHE). For investigation of the $G\alpha_{i/o}$ pathway, cells were additionally treated with forskolin (concentration) to activate adenylyl cyclase and induce pathway target pathway activation. Luciferase/β-galactosidase activity (RLU) was determined and expressed relative to the vehicle (visualized as a dotted line) as fold change. Data was collected in biological triplicates and shown as mean +/- SEM. Statistical significance ($*p < 0.05$) in activation relative to the vehicle was evaluated using two-way ANOVAs and corrected using Holm-Šídák statistical hypothesis testing.

4. Discussion

Given the results of previous investigations which concluded that *L*-Phe can significantly activate GPCRs by assessing via β-arrestin recruitment, this study aimed to characterize the signal transduction within established signaling pathways of *L*-Phe- responsive GPCRs. This study provides further evidence and insight into *L*-Phe's role as a signaling molecule through the study of 12 orphan and lipid class A GPCRs. This study assessed *L*-Phe induced using HTS luciferase assay methods.[2] Following the validation of signaling reporter assays we showed that *L*-Phe treatments can significantly activate endogenous GPCR signaling pathways relative to vehicle controls, and differentially activate signaling pathways in 6 of the assessed receptors. These data support the conclusion that *L*-Phe has a role in the regulation of cellular communication and function. Additionally, *L*-Phe evaluations exhibit a promiscuous ligand binding profile indicating that complete characterization of signaling must be further explored through independent receptor investigations.

4.1. L-Phe Activates Endogenous GPCR Signaling Pathways

Initial investigations using pBSK demonstrated that *L*-Phe can induce endogenous signaling through $G\alpha_{12/13}$ and $G\alpha_{q/11}$ pathways (**Figure 3.2**). This demonstrates that HEK293 cells endogenously contain the signaling repertoire necessary to activate luciferase reporters, therefore these results will allow us to distinguish between endogenous and exogenous signaling. As such, due to the similarity in observed significance, activation of the $G\alpha_{q/11}$ pathway within receptor S1P4, identified in **Figure 3.4**, may be attributed to endogenous signaling rather than the exogenous receptor. However, as luciferase reporter vectors illustrate isolated activation at the terminal end of the signaling pathway it is unclear what *L*-Phe is acting on endogenously to produce the observed responses.[2] *L*-Phe may be acting on GPCRs endogenously expressed in these

cells, effectors within these signaling pathways, intracellular targets participating in pathway crosstalk, or a combination of all suggested mechanisms simultaneously.[34] In order to characterize the extent of *L*-Phe signaling and attribute its effects to a endogenous signaling mechanism it will be necessary to explore upstream methods of quantifying activation such as utilizing inhibitors throughout target pathways to dissect activation. To address this, further studies from this lab aim to explore upstream activation of *L*-Phe *via* nano-luciferase techniques detecting G protein-ligand specific interactions.[35]

4.2. L-Phe Differentially Activates GPCR Signaling Pathways

Receptor activation in **Figure 3.3 & 3.4** highlights that *L*-Phe does not induce a universal signaling response among all orphan and lipid receptors. Further, dose-dependent effects of *L*-Phe were ambiguous with preference dependent on the signaling pathway and receptor. Results differed from the hypothesis, based on typical biased signaling of GPCRs, that *L*-Phe treatments would elicit activation within a receptor-dependent preferential pathway. Significant activation through signaling pathways was only determined in half of the receptors investigated. Further, receptor activation did not correspond to predicted receptor coupling to G proteins (**Appendix B**), and there was no identified structural basis for activation among receptor transmembrane domains (**Appendix C**). Interestingly signaling activation was not always limited to one signaling pathway within receptors, as seen in the case of GPR12. Activation within the $G\alpha_s$ pathway consistently inhibited signaling responses, however all other pathways varied in their effect on signal transduction. This implies that *L*-Phe has diverse receptor-dependent properties as an agonist, altering effects on the efficacy of receptor activity. Moreover, *L*-Phe did not induce significant activation through the $G\beta\gamma$ pathway among any receptor. The $G\beta\gamma$ pathway is associated with cellular responses such as cell proliferation, differentiation, and development, suggesting *L*-Phe is

not linked to regulation of the cell cycle.[36] These results established that *L*-Phe differentially activates GPCR signaling pathways, in a manner which is receptor- dependent, to preferentially increase or decrease cellular responses.

4.3. L-Phe is a Promiscuous Ligand

Molecular promiscuity is the ability of a molecular to affect multiple targets, hence a promiscuous ligand displays a lack of selectivity over its interactions with receptors.[37] These interactions can be functional with desired biological effects or non-functional producing non-specific effects of no physiological significance.[37] Non-functional ligand promiscuity can cause issues for HTS assay methods by yielding false-positive signals that mimic typical responses.[37] In contrast, promiscuity is the basis of "polypharmacology" which aims to take advantage of ligand promiscuity to design efficient drugs which have multitarget-dependent affects.[38] One example of a promiscuous drug is Aspirin which acts on multiple receptor targets and is used to treat or prevent several disorders including inflammation, fever, heart attacks, etc.[38] The nature of a ligand's promiscuity can be due to its specific characteristics or the similarity of binding pockets among proteins.[39]

L-Phe activates GPCR signaling pathways in a manner that is receptor-dependent, suggesting the signaling molecule has the potential to affect distinct biological functions. Considering literature which proposes *L*-Phe is an endogenous ligand for GPR142 and GPR139, combined with our previous studies suggesting it signaling potential extends far beyond those two receptors, our results present compiling evidence that *L*-Phe may be a promiscuous ligand due to its lack of selectivity. *L*-Phe exhibits a lack of selectivity for a receptor, a preferential signaling pathway, and a particular effect on agonism. This case for promiscuity is further supported by literature which identifies a strong structural basis between aromaticity and a molecule's

promiscuity.[39] It is unclear whether the receptors *L*-Phe had no significant signaling effect on provides evidence that the molecule has non-functional promiscuous properties or whether there is an alternative technical or biological explanation for this observation.

One biological hypothesis is that *L*-Phe is interacting with different binding sites in a receptor-dependent manner; in these receptors *L*-Phe may act on allosteric sites to affect the binding affinity and signaling activity of an orthosteric ligand which was not present under the experimental conditions. Allosteric modulators bind and alter receptor confirmations to affect positively or negatively the signaling response attributed to the orthosteric ligand. This phenomenon has been exhibited in the amino acid sensing receptor CaR, where *L*-amino acids serve as positive allosteric regulators.[40] Experiential investigations exploring the effect of *L*-Phe on receptor signaling in the presence of a known agonist may address this hypothesis.[40]

A further explanation is that *L*-Phe may be interacting in signaling crosstalk among pathways, similar to theories proposed for the endogenous activation, which result in the inhibition of downstream activation. As previously proposed, elucidating the upstream mechanisms of *L*-Phe activation is a necessary step to addressing its functionality as a promiscuous ligand. As well, *L*-Phe may act on GPCR oligomers, meaning has a higher affinity for functional binding sites formed by receptor pairings.[41] If *L*-Phe requires a multimeric receptor complex to signal canonically, this may result in active β-arrestin recruitment but no observed downstream signaling if part of the receptor oligomer is absent. Oligomerization of GPCRs can be detected via coimmunoprecipitation.[41]

Lack of observed significance within receptors could also be attributable to sensitivity limitations of the luciferase assay and technical equipment measuring luminescence. Further

biological replicates and/or conducting *L*-Phe dose response curves for receptor may help validate this theory.

Finally, given that our study demonstrated that *L*-Phe activation is receptor-dependent, each receptor will need its own independent characterization of activation to elucidate signaling. Within relevant parameters of GPCR signaling, future studies would benefit from performing assays using primary cells from tissues that are biologically significant to the individual receptor. Tissue distribution of studied GPCRs can be found within the IUPHAR GPCR database (https://www.guidetopharmacology.org/).

4.4. Conclusion & Future Directions

Through this study, we determined that *L*-Phe differentially activates GPCR signaling pathways, in a manner that is receptor- dependent. Further, not all receptors previously identified to be activated by *L*-Phe were observed to significantly signal through any of the examined G protein signaling pathways. These results suggest that the role of *L*-Phe in cell function may extend beyond its role as a building block in proteins to include regulation of cellular communication through receptor-dependent activation of GPCRs. Additionally, *L*-Phe may exhibit a promiscuous binding profile as a ligand, with various hypothetical implications as a non-functional or functional ligand.

As this study examined receptor activation using reporter vectors downstream of established signaling pathways, future studies should explore the effect of *L*-Phe upstream of target pathways to fully elucidate activation. Additionally, individual receptor characterizations unveiling relationships among ligands, receptors, and effectors are necessary to investigate allosterism, agonism, biased signaling, crosstalk, and multimeric receptor pharmacology.[7]

Overall, the knowledge acquired from this study helps to build pharmacological profiles for both *L*-Phe and investigated receptors. The relationship explored between *L*-Phe and the physiological signalling responses in cells provides support for the importance of *L*-amino acids as extracellular signaling molecules through the activation of GPCRs. Finally our findings also support the recent shift in understanding that the biochemical characterization of GPCR signaling is not a simple paradigm, but rather a diverse cascade involving complex extracellular receptor systems.

5. Reference List

1. McCudden CR, Hains MD, Kimple RJ, Siderovski DP, Willard FS. G-protein signaling: back to the future. *Cell Mol Life Sci*. 2005;62(5):551-577. doi:10.1007/s00018-004-4462-3

2. Cheng Z, Garvin D, Paguio A, Stecha P, Wood K, Fan F. Luciferase Reporter Assay System for Deciphering GPCR Pathways. *Curr Chem Genomics*. 2010;4:84-91. doi:10.2174/1875397301004010084

3. Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P. General Principles of Cell Communication. *Mol Biol Cell 4th Ed*. Published online 2002. Accessed April 1, 2022. https://www.ncbi.nlm.nih.gov/books/NBK26813/

4. Catozzi S, Di-Bella JP, Ventura AC, Sepulchre JA. Signaling cascades transmit information downstream and upstream but unlikely simultaneously. *BMC Syst Biol*. 2016;10(1):84. doi:10.1186/s12918-016-0303-2

5. Brivanlou AH, Darnell JE. Signal Transduction and the Control of Gene Expression. *Science*. 2002;295(5556):813-818. doi:10.1126/science.1066355

6. Heldin CH, Lu B, Evans R, Gutkind JS. Signals and Receptors. *Cold Spring Harb Perspect Biol*. 2016;8(4):a005900. doi:10.1101/cshperspect.a005900

7. Basith S, Cui M, Macalino SJY, et al. Exploring G Protein-Coupled Receptors (GPCRs) Ligand Space via Cheminformatics Approaches: Impact on Rational Drug Design. *Front Pharmacol*. 2018;9:128. doi:10.3389/fphar.2018.00128

8. Przydzial MJ, Bhhatarai B, Koleti A, Vempati U, Schürer SC. GPCR ontology: development and application of a G protein-coupled receptor pharmacology knowledge framework. *Bioinformatics*. 2013;29(24):3211-3219. doi:10.1093/bioinformatics/btt565

9. Lundstrom K. Structural genomics of GPCRs. *Trends Biotechnol*. 2005;23(2):103-108. doi:10.1016/j.tibtech.2004.12.006

10. Neves SR, Ram PT, Iyengar R. G Protein Pathways. *Science*. 2002;296(5573):1636-1639. doi:10.1126/science.1071550

11. Zhao J, Deng Y, Jiang Z, Qing H. G Protein-Coupled Receptors (GPCRs) in Alzheimer's Disease: A Focus on BACE1 Related GPCRs. *Front Aging Neurosci*. 2016;8:58. doi:10.3389/fnagi.2016.00058

12. Hauser AS, Attwood MM, Rask-Andersen M, Schiöth HB, Gloriam DE. Trends in GPCR drug discovery: new agents, targets and indications. *Nat Rev Drug Discov*. 2017;16(12):829-842. doi:10.1038/nrd.2017.178

13. Insel PA, Sriram K, Gorr MW, et al. GPCRomics: An Approach to Discover GPCR Drug Targets. *Trends Pharmacol Sci*. 2019;40(6):378-387. doi:10.1016/j.tips.2019.04.001

14. Hu GM, Mai TL, Chen CM. Visualizing the GPCR Network: Classification and Evolution. *Sci Rep*. 2017;7(1):15495. doi:10.1038/s41598-017-15707-9

15. Tang X long, Wang Y, Li D li, Luo J, Liu M yao. Orphan G protein-coupled receptors (GPCRs): biological functions and potential drug targets. *Acta Pharmacol Sin*. 2012;33(3):363-371. doi:10.1038/aps.2011.210

16. Laschet C, Dupuis N, Hanson J. The G protein-coupled receptors deorphanization landscape. *Biochem Pharmacol*. 2018;153:62-74. doi:10.1016/j.bcp.2018.02.016

17. Martin AL. *Constitutive Activity in Orphan G Protein Coupled Receptors*. Ph.D. Missouri University of Science and Technology. Accessed November 22, 2021. https://www.proquest.com/docview/1698252514/abstract/C402B9E9E58B4253PQ/1

18. Furge LL. Biochemistry of signal transduction and regulation, 4th Ed. by G. Krauss. *Biochem Mol Biol Educ*. 2008;36(5):385-385. doi:10.1002/bmb.20226

19. Seyedabadi M, Ghahremani MH, Albert PR. Biased signaling of G protein coupled receptors (GPCRs): Molecular determinants of GPCR/transducer selectivity and therapeutic potential. *Pharmacol Ther*. 2019;200:148-178. doi:10.1016/j.pharmthera.2019.05.006

20. Gutkind JS. The Pathways Connecting G Protein-coupled Receptors to the Nucleus through Divergent Mitogen-activated Protein Kinase Cascades *. *J Biol Chem*. 1998;273(4):1839-1842. doi:10.1074/jbc.273.4.1839

21. Luttrell LM, Lefkowitz RJ. The role of β-arrestins in the termination and transduction of G-protein-coupled receptor signals. *J Cell Sci*. 2002;115(3):455-465. doi:10.1242/jcs.115.3.455

22. Grundmann M, Merten N, Malfacini D, et al. Lack of beta-arrestin signaling in the absence of active G proteins. *Nat Commun*. 2018;9(1):341. doi:10.1038/s41467-017-02661-3

23. Peterson YK, Luttrell LM. The Diverse Roles of Arrestin Scaffolds in G Protein–Coupled Receptor Signaling. *Pharmacol Rev*. 2017;69(3):256-297. doi:10.1124/pr.116.013367

24. Husted AS, Trauelsen M, Rudenko O, Hjorth SA, Schwartz TW. GPCR-Mediated Signaling of Metabolites. *Cell Metab*. 2017;25(4):777-796. doi:10.1016/j.cmet.2017.03.008

25. He F, Wu C, Li P, et al. Functions and Signaling Pathways of Amino Acids in Intestinal Inflammation. *BioMed Res Int*. 2018;2018:e9171905. doi:10.1155/2018/9171905

26. Conigrave AD, Hampson DR. Broad-spectrum l-amino acid sensing by class 3 G-protein-coupled receptors. *Trends Endocrinol Metab*. 2006;17(10):398-407. doi:10.1016/j.tem.2006.10.012

27. Lopez MJ, Mohiuddin SS. Biochemistry, Essential Amino Acids. In: *StatPearls*. StatPearls Publishing; 2022. Accessed January 18, 2022. http://www.ncbi.nlm.nih.gov/books/NBK557845/

28. Wauson EM, Zaganjor E, Cobb MH. Amino acid regulation of autophagy through the GPCR TAS1R1-TAS1R3. *Autophagy*. 2013;9(3):418-419. doi:10.4161/auto.22911

29. Zhang C, Huang Y, Jiang Y, et al. Identification of an l-Phenylalanine Binding Site Enhancing the Cooperative Responses of the Calcium-sensing Receptor to Calcium. *J Biol Chem*. 2014;289(8):5296-5309. doi:10.1074/jbc.M113.537357

30. Lin HV, Efanov AM, Fang X, et al. GPR142 Controls Tryptophan-Induced Insulin and Incretin Hormone Secretion to Improve Glucose Metabolism. *PLOS ONE*. 2016;11(6):e0157298. doi:10.1371/journal.pone.0157298

31. Liu C, Bonaventure P, Lee G, et al. GPR139, an Orphan Receptor Highly Enriched in the Habenula and Septum, Is Activated by the Essential Amino Acids l-Tryptophan and l-Phenylalanine. *Mol Pharmacol*. 2015;88(5):911-925. doi:10.1124/mol.115.100412

32. Kroeze WK, Sassano MF, Huang XP, et al. PRESTO-Tango as an open-source resource for interrogation of the druggable human GPCRome. *Nat Struct Mol Biol*. 2015;22(5):362-369. doi:10.1038/nsmb.3014

33. Rourke JL, Dranse HJ, Sinal CJ. CMKLR1 and GPR1 mediate chemerin signaling through the RhoA/ROCK pathway. *Mol Cell Endocrinol*. 2015;417:36-51. doi:10.1016/j.mce.2015.09.002

34. Atwood BK, Lopez J, Wager-Miller J, Mackie K, Straiker A. Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. *BMC Genomics*. 2011;12:14. doi:10.1186/1471-2164-12-14

35. Laschet C, Dupuis N, Hanson J. A dynamic and screening-compatible nanoluciferase-based complementation assay enables profiling of individual GPCR–G protein interactions. *J Biol Chem*. 2019;294(11):4079-4090. doi:10.1074/jbc.RA118.006231

36. Zhang W, Liu HT. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res*. 2002;12(1):9-18. doi:10.1038/sj.cr.7290105

37. Feldmann C, Miljković F, Yonchev D, Bajorath J. Identifying Promiscuous Compounds with Activity against Different Target Classes. *Molecules*. 2019;24(22):4185. doi:10.3390/molecules24224185

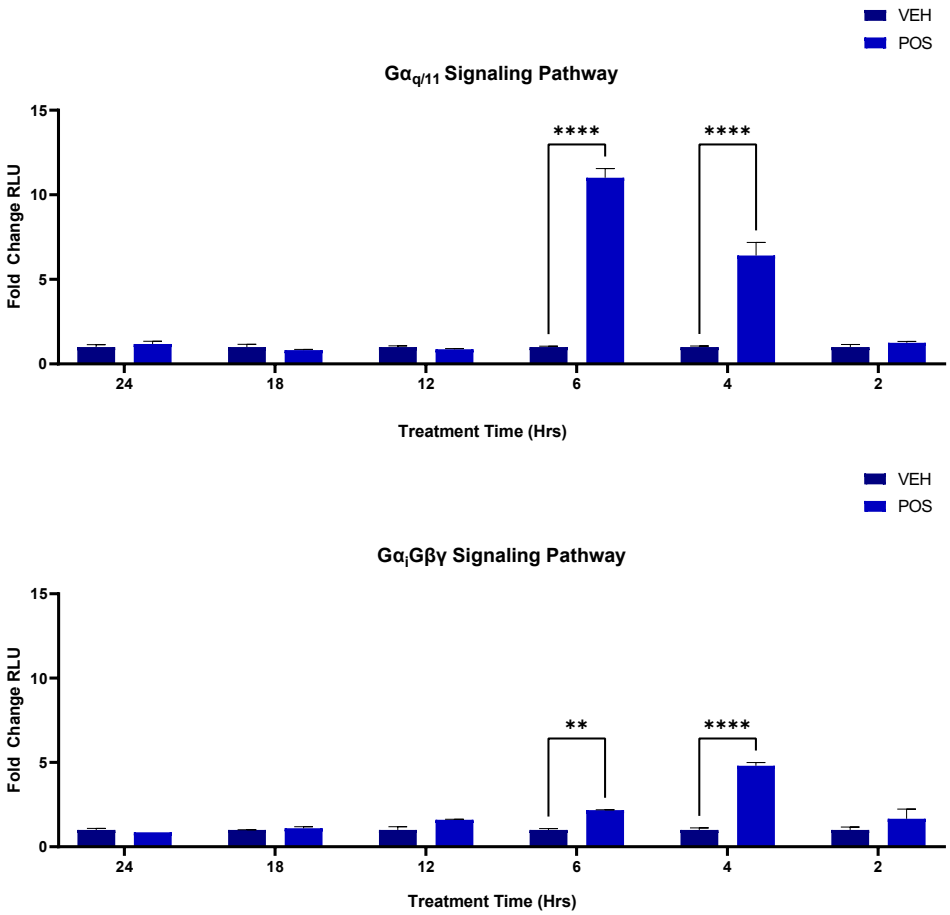
38. Reddy AS, Zhang S. Polypharmacology: drug discovery for the future. *Expert Rev Clin Pharmacol*. 2013;6(1):10.1586/ecp.12.74. doi:10.1586/ecp.12.74

39. Sturm N, Desaphy J, Quinn RJ, Rognan D, Kellenberger E. Structural Insights into the Molecular Basis of the Ligand Promiscuity. *J Chem Inf Model*. 2012;52(9):2410-2421. doi:10.1021/ci300196g

40. Kobilka B. Allosteric activation of the CaR by l-amino acids. *Proc Natl Acad Sci*. 2000;97(9):4419-4420. doi:10.1073/pnas.97.9.4419

41. Agnati LF, Guidolin D, Vilardaga JP, Ciruela F, Fuxe K. On the expanding terminology in the GPCR field: the meaning of receptor mosaics and receptor heteromers. *J Recept Signal Transduct Res*. 2010;30(5):287-303. doi:10.3109/10799891003786226

6. Appendix



Appendix A. Signaling Treatment Time Optimization. HEK293 cells either transiently or stably expressing a luciferase reporter for G protein signaling pathways were transfected with pBSK. Cells were treated with Opti-MEM (VEH) and either 20% Fetal Bovine Serum or 10 ng/mL PMA & 1 μM Ionomycin (POS) as a positive control for pathway activation. Treatment times of 24-, 18-, 12-, 6-, 4-, 2 hours were observed. Luciferase/β-galactosidase activity (RLU) was determined and expressed relative to VEH as fold change. Data was collected in biological triplicates and shown as mean +/- SEM. Statistical significance (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) was evaluated using two-way ANOVAs and corrected using Holm-Šídák statistical hypothesis testing.

Appendix B. Normalized posterior probability score. PRED-COUPLE 2.0 predicted coupling specificity of 12 human GPCRs to the G proteins $G\alpha_{q/11}$, $G\alpha_{i/o}$, $G\alpha_{s}$, and $G\alpha_{12/13}$. Positive predictions in bold type.

Score: < 0.3 negative

> 0.3 positive

	$G\alpha_{q/11}$	$G\alpha_{i/o}$	$G\alpha_{s}$	$G\alpha_{12/13}$
MC4R	0.05	0.05	0.99	0.00
GPR6	0.02	0.30	0.99	0.00
GPR3	0.15	0.79	0.90	0.05
GPBA	0.66	0.91	0.97	0.46
S1P1	0.24	0.98	0.03	0.00
S1P4	0.73	0.99	0.32	0.95
GPR12	0.00	0.52	0.97	0.00
GPR142	0.95	0.83	0.22	0.07
GPR88	0.00	0.93	0.96	0.00
CysLTR2	0.99	0.11	0.00	0.00
GPR34	0.45	0.94	0.14	0.00
GPR35	0.98	0.85	0.14	0.89

Appendix C. Location, length, and orientation of predicted GPCR transmembrane helices. TMHMM-2.0 predicted transmembrane domains based on a hidden Markov model, from receptors' FASTA formatted amino acid sequences. Alternating orientation of domains is indicated as outside (o) to inside (i) or inside to outside of predicted transmembrane domains.

	Orientation	**Location**						
		TM I	**TM II**	**TM III**	**TM IV**	**TM V**	**TM VI**	**TM VII**
CLTR2	o-i	58-80	92-114	129-151	172-194	219-241	331-350	370-392
GPR3	o-i	43-65	78-100	110-132	153-175	195-217	245-267	277-299
GPR6	o-i	75-97	110-132	142-164	185-207	227-246	277-299	309-331
GPR12	o-i	47-69	82-104	114-136	157-179	199-221	249-271	281-303
GPR34	o-i	57-79	91-113	128-150	171-193	217-239	268-290	310-329
GPR35	o-i	23-45	58-80	95-112	133-155	170-192	219-241	-
GPR88	i-o	34-56	119-141	162-179	194-216	284-306	316-338	-
GPR142	o-i	160-182	194-216	236-258	278-300	315-337	357-379	394-416
GPBA	o-i	18-40	53-75	85-107	128-150	165-187	229-251	261-283
MC4R	o-i	50-69	81-103	123-145	166-188	192-214	245-267	282-304
S1P1	o-i	47-69	81-103	118-140	160-182	202-224	252-274	289-311
S1P4	o-i	46-68	81-103	118-140	162-184	204-226	254-276	291-309