

AN ASSESSMENT OF ORPHAN G PROTEIN-COUPLED
RECEPTOR ACTIVATION WITH L-PHENYLALANINE
BY
ERIN SARAH YAUNG

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Abstract

As the largest family of cell membrane receptors in the human genome, G protein-coupled receptors (GPCRs) exist in a wide variety of body tissues with a wide range of cellular signaling functions. A large subset of these GPCRs are orphans with no determined or validated endogenous ligand, which limits studies of their function and their potential pharmacological applications. Among many possible activating ligands, amino acids are able to activate some GPCRs. We hypothesized that L-Phenylalanine (L-Phe) specifically would activate numerous GPCRs, including some orphan GPCRs. To study this, a high throughput activation screen of 277 GPCRs that included 64 orphan GPCRs was conducted with a 3 mM L-Phe treatment and compared to a vehicle treatment. This L-Phe treatment significantly activated 44 of 64 orphan GPCRs, where the GPCRs GPR88, GPR45, GPR31, CXCR7, and GPR32 exhibited the highest magnitude in activation from baseline activity levels at above seven-fold. A database search showed that the tissue distributions of these orphan GPCRs were also variable, with highest GPCR expression reported in the brain and the gastrointestinal system. These findings support the idea that L-Phe is a GPCR ligand and that it potentially deorphanizes some of these orphan GPCRs. By looking at GPCR activation with L-Phe, we can gain a greater understanding of the biological implications of L-Phe beyond protein assembly.

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Table of Contents

Abstract.....	i
Acknowledgements	ii
List of Abbreviations	iv
1. Introduction.....	1
1.1 G Protein-Coupled Receptors.....	1
1.2 PRESTO-Tango Assays.....	3
1.3 The Biological Role of Amino Acids.....	5
1.4 Amino Acid-Activated GPCRs	6
1.5 L-Phenylalanine	7
1.6 Experiment Rational.....	8
2. Methods.....	8
2.1 HTLA Cell Culturing	8
2.2 DNA Plasmid Preparation.....	9
2.3 DNA Transfection and L-Phe Treatment	10
2.4 PRESTO-Tango Assay	11
2.5 Understanding GPCR Tissue Distribution	13
2.6 Creation of an Opentrans Protocol for PRESTO-Tango Assays.....	14
3. Results	14
3.1 L-Phe Activates Numerous Orphan GPCRs	14
3.2 GPCRs Are Highly Expressed in the Brain and Gut.....	19
3.3 Preliminary Opentrans Protocol Design.....	20
4. Discussion.....	22
4.1 L-Phe Causes High Activation of Most Class A Orphan GPCRs.....	23
4.2 GPR88	24
4.3 GPR45	25
4.4 GPR31	25
4.5 CXCR7 (ACKR3).....	26
4.6 GPR32	27
4.7 L-Phe as a GPCR Signaling Molecule in PKU and Gut Function.....	27
4.8 Challenges in GPCR Deorphanization with L-Phe.....	29
4.9 Conclusion and Future Directions.....	30
5. References.....	31

6. Appendix.....	42
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List of Abbreviations

AVPR2.....	human arginine vasopressin 2 receptor
cAMP	cyclic Adenosine Monophosphate (AMP)
β -gal.....	pCMV- β -galactosidase
CCK.....	Cholecystokinin
DMEM	Dulbecco's Modified Eagle Media
FBS	Fetal Bovine Serum
GFP.....	Green Fluorescent Protein
GIP.....	Gastric Inhibitory Polypeptide
GLP-1.....	Glucagon-Like Peptide-1
GPCR.....	G Protein-Coupled Receptor
IUPHAR	International Union of Basic and Clinical Pharmacology
L-Phe	L-Phenylalanine
L-Trp.....	L-Tryptophan
L-Tyr.....	L-Tyrosine
MAPK/ERK	Mitogen-Activated Protein Kinase/Extracellular Signal- Regulated Kinase
mTOR	Mechanistic Targeting of Rapamycin
ONPG	<i>Ortho</i> -nitrophenol- β - <i>D</i> -galactopyranoside
PBS	Phosphate Buffered Saline
PKU	Phenylketonuria
PRESTO-Tango	Parallel Receptor-ome Expression and Screening via Transcriptional Output-Tango
RLU	Relative Light Units
tTA	Tetracycline-controlled Transactivator
TEV.....	Tobacco Etch Virus

1. Introduction

1.1 G Protein-Coupled Receptors

G protein-coupled receptors (GPCRs) are the largest family of cell surface receptors in mammals, with approximately 800 GPCR genes encoded in the human genome and over 350 of those genes coding for non-olfactory GPCRs^{1,2}. GPCRs play a role in various signaling pathways, modulating cellular responses from input stimuli like odorous compounds, light photons, hormones, and other metabolites and molecules³. This receptor superfamily is classified into five families of human GPCRs based on phylogenetic structural similarities, known as GRAFS classification: metabotropic **G**lutamate receptors (Class C), **R**hodopsin-like receptors (Class A), **A**dhesion receptors (Class B2), **F**rizzled/Taste2 receptors (Class F), and **S**ecretin receptors (Class B)⁴. Though this is one of the more recent and common methods of classification, GPCRs are also categorized based on common ligand-binding sites within classes, allowing for the creation of additional groupings⁴. Class A rhodopsin-like GPCRs are the most abundant and diverse family, with approximately 80% of human GPCRs falling into this category⁵; consequently, they are also the most studied and best characterized GPCR family.

The structure of a GPCR consists of seven transmembrane α -helices, with three loops and the protein's N-terminal located extracellularly, and three intracellular loops and the protein's C-terminus located intracellularly⁶. Each GPCR is associated with a heterotrimeric G protein made of α , β , and γ subunits that is usually biased towards a specific signaling pathway, dissociating into α and $\beta\gamma$ subunits to trigger signaling cascades⁷. G proteins can be classified into one of four classes based on the α subunit: $G\alpha_i$, $G\alpha_s$, $G\alpha_{12/13}$, and $G\alpha_q$ ⁸. Each G protein generally regulates a specific second messenger cascade, dependent on what conformational changes occur in a given ligand-GPCR complex. These intracellular signaling pathways that are generally associated with a certain G protein may be cyclic adenosine monophosphate (cAMP), calcium ions, or Mitogen-Activated Protein Kinase/Extracellular Signal-Regulated Kinase (MAPK/ERK) as a secondary messenger (Figure 1), which then triggers various downstream signaling cascades to promote or inhibit cellular processes like DNA transcription^{9,10}.

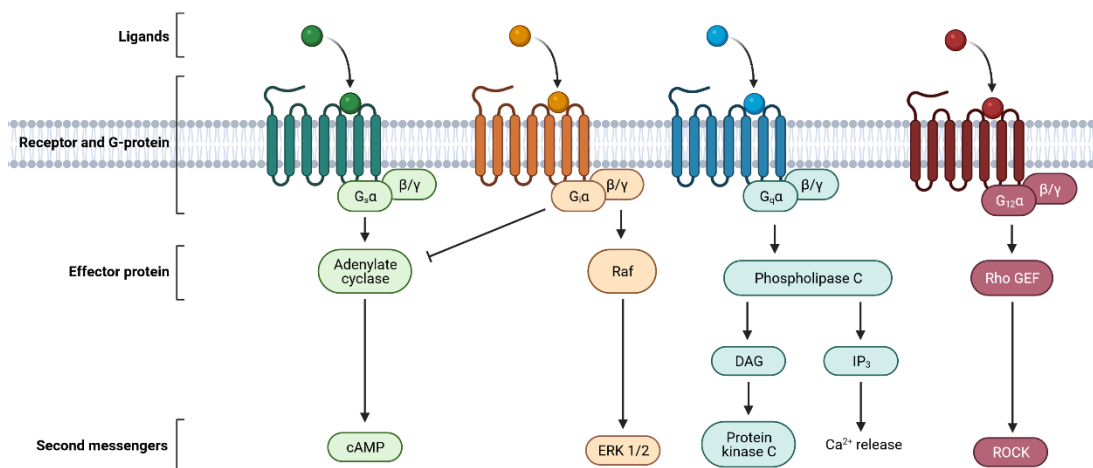


Figure 1: A simplified diagram of the general signaling pathways of each G protein. Modified from the GPCR Effector Pathways template on BioRender.

Alternatively, the GPCR can recruit a G protein-independent arrestin protein and instigate a different signaling pathway⁷. The β -arrestin family consists of four proteins: the visual β -arrestins (arrestin1 and arrestin4) that are present mainly in the rods and cones of the eye, while the non-visual β -arrestins (β -arrestin2 and β -arrestin3) are highly conserved in mammals and found in most tissues and cell types¹¹. β -arrestins, like β -arrestin2, are able to regulate GPCR activation by uncoupling the GPCR from its associated G protein. This occurs when a GPCR kinase (GRK) phosphorylates the GPCR with the binding of a ligand, causing the GPCR to become active¹². The β -arrestin is recruited to interact with the phosphorylated GPCR at the membrane, which can block the G protein from interacting with the phosphorylated GPCR¹². This means that a GPCR signaling cascade can be propagated by a G protein, β -arrestin, or a combination of a G protein and β -arrestin to result in downstream signaling¹². β -arrestins also activate second messenger cascades like the MAPK/ERK pathway, and can do so in a manner that is independent of G proteins^{12,13(p5)}.

Given their involvement in a number of cellular and metabolic pathways with a variety of possible endogenous and exogenous ligands, GPCRs are highly valued in the development of many pharmaceutical treatments for health issues surrounding obesity, metabolic syndrome, and others, with approximately 40-50% of approved drugs targeting these receptor types⁵. However, GPCRs may only act as pharmacological targets if they have been well studied, with their roles in metabolism and overall physiology determined and a ligand discovered. For many GPCRs,

endogenous ligands are not well characterised or are not validated, and their intracellular signaling pathways and GPCR roles within metabolism may not yet be well defined¹⁴. Such proteins are termed orphan GPCRs, and they are the focus of much GPCR research, especially in the field of pharmacology, because of their great potential in regulating a wide variety of functions such as in hormone signaling, the pathophysiology of various neurodegenerative, cardiovascular, and immune diseases, and cancers^{15,16}. According to the International Union of Basic and Clinical Pharmacology (IUPHAR), there are approximately 121 orphan GPCRs that remain to be deorphanized, and approximately 61 of these orphan GPCRs with proposed but currently unvalidated endogenous ligands¹⁷.

1.2 PRESTO-Tango Assays

GPCR activation can be difficult to quantify for a large number of GPCRs at once based on the fact that there are so many variances in G protein-coupling and downstream pathways. As such, there has been a recent push to figure out how to accurately measure GPCR activation for multiple receptors simultaneously in an efficient manner. One such protocol that is aimed at screening numerous GPCRs at once is the Parallel Receptor-ome Expression and Screening via Transcriptional Output-Tango protocol, known in short as PRESTO-Tango, developed by Kroeze et al. in 2015 (Figure 2)¹. The PRESTO-Tango methodology was modified from the original Barnea et al. (2008) Tango protocol and uses β -arrestin recruitment to generate a luciferase reporter transcriptional output. As previously mentioned, β -arrestin recruitment can act independently of G protein coupling. This means that the PRESTO-Tango assay can work for a wide variety of GPCRs, including orphan GPCRs whose coupled G protein is still relatively unknown in comparison to GPCRs whose known ligand(s) allow for characterization of their G protein signaling pathways.

Barnea et al. (2008) developed a stable cell line called HTLA cells from human embryonic kidney (HEK)-293t cells that contained specific components necessary for the Tango (and PRESTO-Tango) assay to function^{1,18}. HTLA cells produce a luminescent signal that is directly proportional to GPCR activation. This is accomplished through targeted genetic modification of both the cells and the GPCRs of interest. To produce this cell line that stably expresses the luminescence reporter gene, Barnea et al. transfected RSV-puro virus and a tetracycline-controlled transactivator (tTA)-luciferase gene into HEK293t cells, which generated the HTL cell line¹⁸. This tTA-dependent luciferase reporter gene was created by incorporating a

tTA promoter sequence upstream to the firefly luciferase gene before transfection into the original HEK293T cells¹⁸. A β -arrestin2-TEV protease fusion gene was then transfected into the HTL cells to create the final, stable HTLA cells to be used for GPCR plasmid transfection¹⁸.

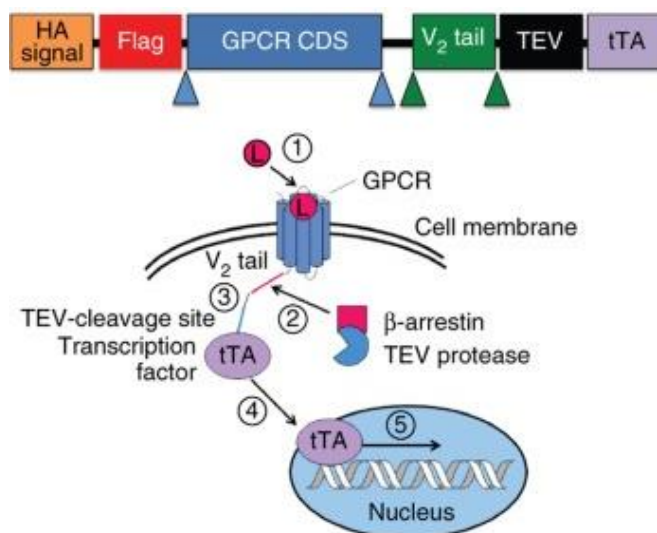


Figure 2: PRESTO-Tango β -arrestin recruitment to an activated PRESTO-Tango-ized GPCR. The general plasmid scheme is shown at the top of the diagram, outlining the hemagglutinin sequence for membrane localization \rightarrow FLAG epitope for visualization \rightarrow codon-optimized GPCR coding sequence \rightarrow human arginine vasopressin 2 tail for β -arrestin recruitment \rightarrow cleavable Tobacco Etch Virus (TEV) cleavage sequence \rightarrow tTA transcription factor. The blue and green arrows represent restriction enzyme cutting sites for easy removal of added components.

The general cascade of events following GPCR transfection into a cell are as follows: 1) A ligand binds to an activating site on the optimized GPCR; 2) The cell-expressed β -arrestin fused to a TEV protease is recruited to the GPCR's vasopressin tail; 3) the TEV protease cleaves the TEV cleavage sequence to free tTA; 4) The released tTA travels to the nucleus; 5) tTA allows for transcription of the luciferase gene. Diagram modified from Kroeze et al.¹.

The original Barnea et al. plasmid designs had an added sequence from the C-Terminus of the human arginine vasopressin 2 receptor (AVPR2), to promote recruitment of the HTLA cell's expressed β -arrestin protein to the membrane-localized GPCR¹⁸. Kroeze et al. modified these original plasmids to be able to include or exclude modules as needed, and this was done for all druggable (non-olfactory) GPCRs¹. The specific modifications to Barnea et al.'s GPCR plasmids by Kroeze et al. include the addition of a cleavable human hemagglutinin (HA) signal sequence to the 5' end of the GPCR DNA sequence¹. This was done to promote GPCR membrane localization post-transfection into HTLA cells as based on a human β_2 -adrenergic

receptor translocation study by Guan et al.^{1,19}. A FLAG epitope tag was also added to the DNA sequence downstream to the HA sequence so that membrane localization could be visualized and confirmed with immunofluorescence¹. At the 3' end of the GPCR DNA sequence, Kroeze et al. kept the design of Barnea et al.'s original Tango plasmids¹. There, a Tobacco Etch Virus (TEV) gene was fused by polymerase chain reaction (PCR) to the DNA sequence for the cleavable tTA protein^{1,18}.

To conduct the PRESTO-Tango assays, the optimized GPCR plasmid is transfected into HTLA cells and allowed to localize to the membranes for GPCR membrane expression after the transfection has occurred. These optimized plasmids therefore allow for the study of individual GPCRs of interest, independent of the endogenous GPCRs in the human cells. When the desired GPCR agonist is added to the extracellular environment, it binds to a ligand-binding site on the GPCR, activating a change in GPCR conformation. During this, the HTLA-expressed β -arrestin2-TEV protease fusion protein is recruited to the AVPR2 sequence on the GPCR, cleaving the TEV sequence and allowing the tTA transcription factor to migrate to the nucleus^{1,18}. Once at the nucleus, the tTA activates transcription of the luciferase gene, and with the addition of a D-luciferin substrate, the luciferase transcriptional output can be quantified in the form of bioluminescence.

1.3 The Biological Role of Amino Acids

Amino acids are most well known for their essential role in protein synthesis as the building blocks of a protein product. There are twenty amino acids used by humans in protein synthesis, nine of which cannot be synthesized by the body and are categorized as nutritionally 'essential,' which must be taken in through the diet; these are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine²⁰⁻²². The other eleven amino acids are deemed nutritionally 'nonessential' since the human body can synthesize them in adequate amounts for growth and survival²¹. L-amino acids are the configuration of amino acids used in mammals and most other forms of life, while the D-amino acid enantiomers are more commonly found within the Bacterial Domain as key components of peptidoglycan formation, and are not used within mammalian protein synthesis²³.

In addition to being protein building blocks, amino acids also regulate gene expression through the amino-acid-sensing mechanistic targeting of rapamycin (mTOR) component of mTOR Complex I-dependent signaling pathways^{24,25}. Various amino acids, such as leucine, also

have roles in stimulating glucose homeostasis and hormone release like insulin²⁶. The branched chain amino acids (leucine, isoleucine, and valine) and their associated catabolic enzymes are also highly researched as biomarkers for various diseases that reportedly use mTOR signaling²⁷. This includes diseases like diabetes mellitus type 2 where insulin resistance is affected through mTOR signaling, cardiovascular diseases, and even cancers associated with the mTOR pathway²⁷. With a potentially wider role in metabolism and cellular response becoming more evident, research into the roles of specific amino acids as more than just protein building blocks is required to address the gap in knowledge about how these processes regulate cell physiology. Such research would also show the huge therapeutic potential in causing targeted disruptions of steps within cellular signaling pathways.

1.4 Amino Acid-Activated GPCRs

One of the current fields advancing GPCR research is focused on finding out how cells sense amino acids in their environments. One such way that cells may sense amino acids is through GPCR signaling, with amino acids as the activating ligand. Metabotropic glutamate GPCRs are so named because of their glutamate-sensitive modulation of neurotransmitter release, which have implications in neurodegenerative diseases like Parkinson's disease and Alzheimer's disease²⁸. Amino acids also activate some dimeric Class C GPCRs, like taste receptor GPCRs^{29,30}. Recently, multiple researchers have reported that the aromatic amino acids L-Tryptophan (L-Trp) and L-Phenylalanine (L-Phe) activate the GPCR GPR139³¹. Expressed in the striatum, habenula, and hypothalamus of the brain, this specific Class A orphan GPCR is now believed to have a role in regulating food intake and energy expenditure^{32(p1)}. GPR142, another Class A GPCR, also has a role in sensing amino acids³³. It is generally expressed in the enteroendocrine β - and α -cells of the pancreas, with activation by the amino acid L-Trp in particular³³. This means that GPR142 is implicated in insulin and glucagon regulation, and also stimulates the release of Gastric Inhibitory Polypeptide (GIP), Glucagon-Like Peptide-1 (GLP-1), and cholecystokinin (CCK)³³.

This research on amino acids as signaling molecules is what led to our initial research hypothesis, that amino acids activate GPCRs. The expression of numerous GPCRs throughout the body, orphan or otherwise, makes the study of their activation with amino acids like L-Phe very interesting and leads to a better understanding of amino acids' roles within metabolism as signaling molecules. With this research also comes the potential to use this knowledge as a basis

for developing treatments for metabolic and genetic issues like diabetes or phenylketonuria (PKU).

In previous Rourke Laboratory research, studies were conducted to better understand the potential role of amino acids in GPCR signaling. For one past honours research project, an initial GPCR assessment screen found that an essential + nonessential amino acid solution activated numerous GPCRs beyond baseline activity levels³⁴. Upon further investigation in this honours project, L-Phe was identified as the amino acid causing the highest levels of GPCR activation from baseline measurements of activity, prompting more questions about its role in cellular processes³⁴. With our new research hypothesis being that L-Phe can activate multiple GPCRs, we wanted to know how many GPCRs L-Phe can activate.

1.5 L-Phenylalanine

L-Phe is an essential amino acid and is a metabolite generated from the breakdown of proteins, implicated in metabolism and protein synthesis. It is also modified by phenylalanine hydroxylase to make the amino acid L-Tyrosine (L-Tyr), which can be further modified to make dopamine and the catecholamines epinephrine and norepinephrine, playing a role in neurotransmitter regulation^{35,36}. Aside from being a product of protein breakdown, L-Phe is also a product of the breakdown of the artificial sweetener aspartame, and is therefore an amino acid that is very prominent in our diets³⁷.

It has been proposed that L-Phe has a modulating effect on gut hormone secretion, with GPCRs triggering these responses with the detection of L-Phe and free fatty acids in the gastrointestinal tract^{38,39}. In particular, L-Phe activates the Class C calcium-sensing receptor CaSR to stimulate GLP-1 and peptide YY secretion and cause a decrease in plasma ghrelin in rats³⁸. Similar results were found for CaSR in a human sample cohort, with an additional finding that D-Phenylalanine did not cause a pre-prandial increase in insulin like L-Phe did³⁹.

This suggestion of L-Phe as a hormone modulator within metabolism also means that it should be of interest in the studies of metabolic disorders like obesity, the metabolic syndrome, and diabetes. L-Phe is also an amino acid of interest because of its large role in genetic diseases such as PKU, where a deficiency in the enzyme phenylalanine hydroxylase causes a damaging build-up of L-Phe and L-Phe by-products in the brain, since studies have shown that L-Phe is able to cross the blood-brain barrier through a large neutral amino acid transporter⁴⁰. Classic PKU is characterized by blood concentrations of L-Phe higher than 1200 $\mu\text{mol/L}$, while milder

cases of PKU experience L-Phe blood concentrations of 600-1200 $\mu\text{mol/L}$ ³⁶. Normal blood concentrations of L-Phe are generally much lower, ranging from 55 – 60 $\mu\text{mol/L}$ in adults⁴¹. While there is no cure for PKU, it can generally be managed by limiting L-Phe intake *via* diet, and it is a disease diagnosed shortly after birth to help prevent severe intellectual impairment, seizures, growth abnormalities, and microcephaly earlier³⁶.

The molecular mechanisms underlying the relationship between high L-Phe levels and PKU-associated intellectual impairments remain unknown. Given the likelihood that L-Phe is a GPCR ligand, there is a chance to discover a function of L-Phe beyond its role as an essential metabolite in protein synthesis.

1.6 Experiment Rational

Research with a focus on amino acids as signaling molecules is still relatively new in the field of GPCR studies. As a result, there remains a gap in knowledge regarding amino acids as GPCR ligands, and a lack of understanding of the role of L-Phe in GPCR signaling. As such, this novel research could greatly improve our understanding of how aromatic amino acids like L-Phe have a role in cellular signaling. Additionally, orphan GPCRs represent an important subset of cellular receptors that have potential in future therapeutic applications, especially when it comes to better understanding their roles in metabolism and genetic diseases like PKU.

We hypothesized that GPCRs, and orphan GPCRs in particular, may be able to sense amino acids, and that L-Phe is one amino acid that will act as a signaling molecule and ligand for orphan GPCRs. This study specifically asks the question: to what extent does L-Phe activate the orphan family of class A GPCRs? The answer to this research question offers the potential to deorphanize some GPCRs, should L-Phe strongly activate the tested orphan GPCRs. In order to elucidate the number of GPCRs that L-Phe can activate, a large GPCR screen with an L-Phe treatment was conducted on 277 different GPCRs. This thesis will focus on 64 orphan GPCRs from this larger screen; the other 213 GPCRs have proposed, validated ligands, and are not considered orphans.

2. Methods

2.1 HTLA Cell Culturing

The HTLA cells created by Barnea et al. and used for our PRESTO-Tango quantifications came from the Roth Lab^{1,18}. These cells were maintained at 37°C and 5% CO₂ in

a complete medium consisting of high glucose (4.5 g/mL) Dulbecco's Modified Eagle Medium (DMEM; Corning, cat. #10-013-CV) supplemented with 10% Fetal Bovine Serum (FBS; Corning, cat. #35-015-CV). Selection antibiotics were added to the complete medium as 2 $\mu\text{g/mL}$ puromycin (BioShop, cat. #PUR333.100) and 100 $\mu\text{g/mL}$ hygromycin B (BioShop, cat. #HYG002.1) for the maintenance of the genetic modifications. Cells were sub-cultured in a 1:20 split ratio once they reached 70-80% confluence, and culture medium was changed every two days between sub-cultures.

To sub-culture, old culturing medium was removed from the cells and a 1X Phosphate Buffered Saline (PBS) wash was added to the cells to remove traces of FBS and other potential impurities. The PBS was removed from the cells and trypsin added (0.25% trypsin, 2.21 mM EDTA; Wisent Inc., cat. #325-043-EL) at 37°C for 2 minutes. The complete medium was added to the lifted cells, and the cells were then centrifuged at 350 rpm for 5 minutes. The supernatant was removed and replaced with fresh high glucose complete medium for replating.

2.2 DNA Plasmid Preparation

PRESTO-Tango optimized plasmids for 277 GPCRs, including the 64 orphan GPCRs of interest were obtained from *Escherichia coli* (*E. coli*) stocks (Addgene, cat. #1000000068), deposited by the Roth lab PRESTO-Tango library. The vector-transformed *E. coli* were cultured at 37°C on LB Agar (BioShop, cat. #LBA408.1) plates containing 100 $\mu\text{g/mL}$ ampicillin for 18 hours. One colony per GPCR vector was then transferred to LB Broth (BioShop, LBL407.1) containing 100 $\mu\text{g/mL}$ ampicillin to propagate for 18 hours at 37°C with oscillation (250 rpm). These GPCR plasmids were then extracted from the liquid cultures using a miniprep kit as per the manufacturer's product protocol (Macherey-Nagel, cat. #740588.50). DNA was extracted from *E. coli* for both pCMV- β -galactosidase (β -gal) and for pBSK with a midiprep kit as per the manufacturer's protocol (Macherey-Nagel, cat. #740410.50) to act as assay controls. To produce high yields of the β -gal plasmid, 20 ng of DNA was transformed into competent DH5 α *E. coli*, followed by 45 seconds of 40°C incubation and immediate incubation on ice prior to propagation for plasmid purification.

The concentration of purified DNA was determined using a NanoDrop ND-1000 spectrophotometer using the DNA-50 double-stranded DNA setting, and a DNA purity ratio between absorbances at 260 nm and 280 nm was also recorded for each receptor. A 260/280 ratio

of near 1.8 is considered pure DNA uncontaminated by RNA, proteins, or other forms of contaminants⁴². All PRESTO-Tango optimized plasmid DNA was diluted with Milli-Q water to 5 ng/ μ L, pBSK and β -gal were diluted to 250 ng/ μ L and 200 ng/ μ L respectively. Diluted samples were stored at -20°C until needed at transfection.

2.3 DNA Transfection and L-Phe Treatment

Once the HTLA cells were 70-80% confluent, they were washed, lifted, and centrifuged as in sub-culturing. Centrifuged cells were resuspended in serum and antibiotic-free 4.5 g/mL glucose DMEM. Cells were counted with a hemocytometer before being plated in 96-well plates at 12,000 cells per well in a volume of 100 μ L, with a one-well-wide barrier around the plate edges filled with sterile PBS to prevent cell media evaporation. The 96-well plates were incubated 24 hours at 5% CO₂ and 37°C.

A transfection mixture containing 50 ng/well pBSK, 25 ng/well β -gal, 100 ng/ μ L of the desired PRESTO-Tango plasmid, 10 μ L OptiMEM (Gibco, cat. #31985070), and branched polyethyleneimine (PEI; Sigma-Aldrich 408727) to a final concentration of 0.4 μ g per well was gently mixed and incubated for 15 minutes at room temperature. After this, 40 μ L/well Opti-MEM was added to the mixtures. Seventy percent of the cell medium was then removed from each well of the 96-well plate and replaced with 50 μ L of the transfection mixtures in triplicate after the 24-hour cell incubation period. Each triplicate contained the same PRESTO-Tango plasmid for either the L-Phe treatment or a vehicle Opti-MEM treatment.

One column (six wells) per plate was transfected with pBSK but not with the PRESTO-Tango plasmids. pBSK is an empty vector that does not express exogenous proteins, and alongside pCMV- β -galactosidase (β -gal) plasmid DNA, this served as the negative control for the luciferase assay. The β -gal plasmid contains the *E. coli* β -galactosidase gene, which allowed us to use pBSK + β -gal as the GPCR expression reference for the other PRESTO-Tango plasmids in the standardizing β -galactosidase assay. Another column on the plate was transfected with the PRESTO-Tango optimized GPCR, LPAR1, which is a non-orphan GPCR that is activated by FBS. LPAR1 with 10% FBS as a ligand functioned as the positive control for the luciferase assay. One well per plate was transfected with pEGFP-C1 DNA (green fluorescent protein; GFP) in lieu of PRESTO-Tango plasmids to check the transfection efficiency 15-18 hours following transfection at 5% CO₂ and 37°C.

If the transfection GFP check after the incubation period confirmed that the plasmids had incorporated into the cells, the transfection mixture was removed completely from the cells, and replaced with either a 3 mM L-Phe solution in Opti-MEM or a vehicle Opti-MEM treatment. This concentration of the L-Phe treatment (3 mM) was chosen to because it follows the serum L-Phe concentration range seen in classic PKU, which is characterized as above 1200 $\mu\text{mol/L}$ ³⁶. The positive LPAR1 control was treated in triplicate with the 10% FBS solution instead of the 3 mM L-Phe, while the negative pBSK control was treated in triplicate with vehicle and with 3 mM L-Phe. The HTLA cells were incubated in their treatment solutions at 5% CO₂ and 37°C for 24 hours before the treatment solutions were completely removed. A 1X lysis buffer (Biotium, cat. #30075-2) was added to the cells (20 μL /well) to destroy cell membrane integrity, and the 96-well plates were then wrapped in parafilm and stored at -80°C for future processing.

Owing to the magnitude of screening 277 receptors, Madeline Power made up the 3 mM L-Phe solution and conducted HTLA cell culturing, transfection, treatment, and lysis steps of this project to ensure consistency in these areas. I cultured all vector *E. coli* and purified all plasmid DNA. I also performed the luciferase and β -galactosidase assays of the PRESTO-Tango assay (below) and conducted preliminary data processing for all 277 GPCR triplicates across three biological replicates, with a detailed analysis of 64 orphan GPCRs.

2.4 PRESTO-Tango Assay

We used a standardizing measurement in the form of a β -galactosidase absorbance assay to quantify the efficiency of the transfection. The β -galactosidase assay is commonly used in transfection experiments, and measures transcriptional output of the bacterial gene *LacZ*, where the produced β -galactosidase enzyme can catalyze a reaction breaking down *ortho*-nitrophenol- β -D-galactopyranoside (ONPG) into a yellow substrate, quantifiable with spectrophotometry⁴³. We transfected the β -gal plasmids necessary for this assay into the HTLA cells as previously stated.

Once ready for assay data collection, the 96-well plates were removed from their -80°C environments and allowed to thaw at room temperature alongside the 2X β -galactosidase buffer (0.2 M sodium phosphate buffer (NaH₂PO₄), 0.002 M magnesium chloride (MgCl₂), 133 mg ONPG, 0.1 M β -mercaptoethanol) stored at -20°C, 10 mg/mL D-luciferin (Biotium, cat. #30075-2) in Milli-Q water stored at -20°C, and luciferase assay buffer (Biotium, cat. #30075-2) stored at

-80°C. When all reagents were equilibrated at room temperature, 180 µL Milli-Q water was added per lysate well in the plates and resuspended on a plate shaker for 2 minutes.

β-Galactosidase assay

The resuspended cell lysate was mixed with pipetting and 30 µL per well was added to a transparent 96-well plate for the β-galactosidase assay. Pipet tips were changed between each tested GPCR. One triplicate of 0.1X lysis buffer from the 1X lysis buffer was added to the assay plate as a blank. With a repeater pipet, 30 µL of 2X β-galactosidase assay buffer was added to each well, and the plate was left to incubate and monitored for approximately 3 minutes until a yellow colour had developed. At this point, the absorbance was measured at 420 nm in a BioTek Synergy HTX microplate reader.

Luciferase assay

After pipetting to mix the cell lysate again, 20 µL of lysate was added to a white 384-well plate, with one row and one column left empty between each sample to avoid inter-well interference in luminescence readings. A triplicate of 0.1X lysis buffer was also added to the 384 well plate as a blank. The repeater pipet with a new syringe tip was used to add 10 µL luciferase assay buffer to each sample well, and the 384-well plate was incubated at room temperature for 5 minutes with shaking. Once incubation finished, the plate luminescence was read from the top in the Biotek Synergy HTX microplate reader and results were recorded.

Data analysis

Data from all assays were compiled into Microsoft Excel, where calculation of Relative Light Units (RLU) and fold-change in RLU took place. RLU for each GPCR triplicate was calculated by first finding the corrected absorbance at 420 nm (A420) from the β-gal assay by subtracting the average of the lysis buffer blanks, and dividing the luciferase value by the resulting A420 for each GPCR. Fold change in RLU for each GPCR was found by taking each individual RLU value and dividing this by an average of the triplicates from the vehicle treatment group. All fold changes for each GPCR's L-Phe treatment and vehicle treatment were then averaged to allow for comparison between the treatments.

Statistical analyses were conducted with R Version 4.0.2 and GraphPad Prism Software Version 9.2.0. To test assumptions, Bartlett's test for homogeneity of variances and Shapiro-Wilk test of normality were used. Multiple unpaired *t*-tests (df = 16) assuming for individual variances across GPCRs were conducted to determine significance in activation for those orphan GPCRs that passed assumptions, while a non-parametric Mann-Whitney test was used for 19 orphan GPCRs that did not pass assumptions even after a logarithmic transformation. The significance level was set at $p < 0.05$ for both the Holm-Šídák method of *t*-test analysis and the Mann-Whitney non-parametric ranked comparisons.

2.5 Understanding GPCR Tissue Distribution

Creating a description of which body tissues each orphan GPCR is expressed in can help us to understand more about each GPCR, and its activation with L-Phe. The IUPHAR GPCR database is one of the most comprehensive GPCR databases accessible to researchers, and provides information about human, mouse, and/or rat GPCR tissue distribution and expression levels, among many other individual GPCR characteristics⁴⁴. The Human Protein Atlas (www.proteinatlas.org) is another protein database that reports the RNA and protein expressions levels detected in various tissues of the human body, including some for GPCRs⁴⁵. The Human Protein Atlas is significantly less comprehensive than the IUPHAR, but provides the possibility of showing the human levels of GPCR expression where the IUPHAR database may not show this.

In order to compile information about GPCR tissue expression, these two databases were searched for each orphan GPCR, and levels that were reported on each database as high (or medium where no high levels were reported) were taken for this project to be representative of each GPCR tissue expression profile. In some cases where human GPCR tissue distributions were absent or were scored as uncertain/unreliable in the Human Protein Atlas, then mouse or rat GPCR tissue distribution was reported (Appendix 1). For tissue distribution reports that did not have designations of high or moderate levels, then quantitative mouse tissue distributions were used, with a logarithmic relative abundance cut-off point of 2 for high expression, and 1 for moderate expression for GPCRs where logarithmic relative abundance above 2 did not exist⁴⁴.

2.6 Creation of an Opentrons Protocol for PRESTO-Tango Assays

Minimizing time and pipetting error is a key goal when conducting a large number of repetitive assays such as those found in the PRESTO-Tango protocol used in this project. It was therefore a prudent idea to create a PRESTO-Tango pipetting protocol for an Opentrons pipetting robot, to allow for the Rourke laboratory to conduct large assays with more pipetting consistency and to increase the feasibility of larger screens in future experiments. A preliminary protocol has been created using the Opentrons protocol design website (<https://designer.opentrons.com/>) for the PRESTO-Tango β -gal and luciferase assays, with the exception of adding luciferase assay buffer to the final 364 well-plate. This was due to an issue with the volume added per well being too small for the robot to aspirate. The luciferase assay buffer must be added manually after the Opentrons protocol is finished running for each 364-well plate.

Custom labware had to be designed for the Opentrons robot protocol for the pipette tips and pipette tip racks used in the Rourke Laboratory for PRESTO-Tango assays, due to a lack of similar pre-set measurements in the protocol designer.

3. Results

3.1 L-Phe Activates Numerous Orphan GPCRs

To determine how many orphan GPCRs are activated in response to L-Phe, HTLA's transfected with one of 64 receptors were assessed with β -galactosidase and luciferase assays to measure GPCR activation. Once the fold-change in GPCR activation was calculated for the L-Phe treatment compared to the untreated GPCR cells, these fold-changes were compiled into a heat map to better visualize the levels of activation with 3 mM L-Phe (Figure 3).

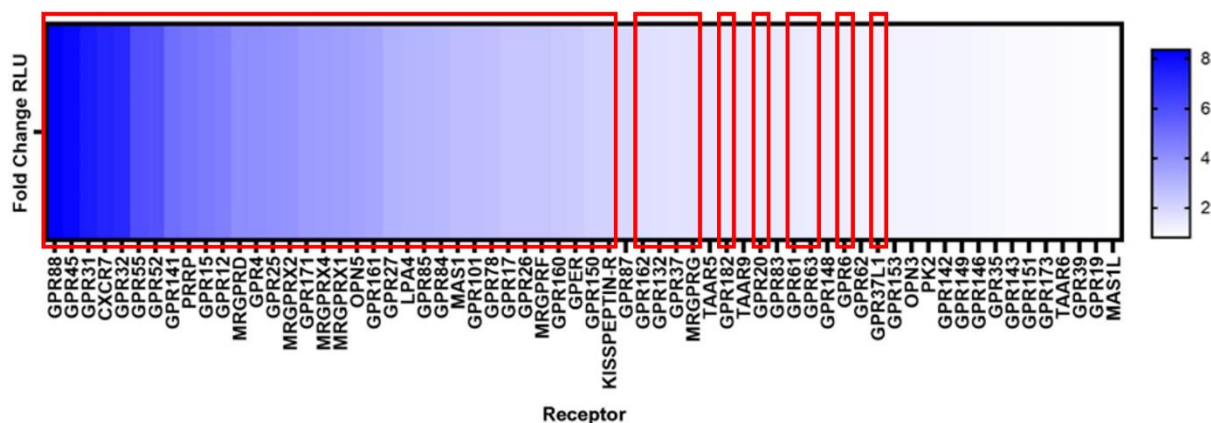


Figure 3: Heat map of fold-change in RLU of 64 orphan GPCRs with 3 mM L-Phe treatment. The L-Phe treatment was added to PRESTO-Tango optimized plasmids transfected into HTLA cells, and GPCR activation (RLU) was measured through luciferase assays standardized with β -galactosidase assays. High activation is represented by dark blue rectangles, little to no activation is represented by white rectangles, and significant changes in GPCR activation are highlighted by the red boxes.

It was found that 34 of the 64 tested GPCRs, or 53%, generated a two-fold change or higher in RLU activation with L-Phe in comparison to baseline activation with the vehicle treatment. Five of the highest responders had a change in RLU seven-fold or higher with the L-Phe treatment compared to the vehicle baseline measurements. These five GPCRs were GPR88, GPR45, GPR31, CXCR7, and GPR32 (Figure 4). Though only 53% of the 64 orphan GPCRs were found to have a fold change in RLU greater than or equal to a two-fold magnitude, a combination of multiple unpaired *t*-tests and unpaired non-parametric Mann-Whitney tests indicated that approximately 69% of receptors, or 44 of the tested 64 GPCRs, had a significant change in activation with L-Phe compared to baseline activation based on *p* values (Table 1).

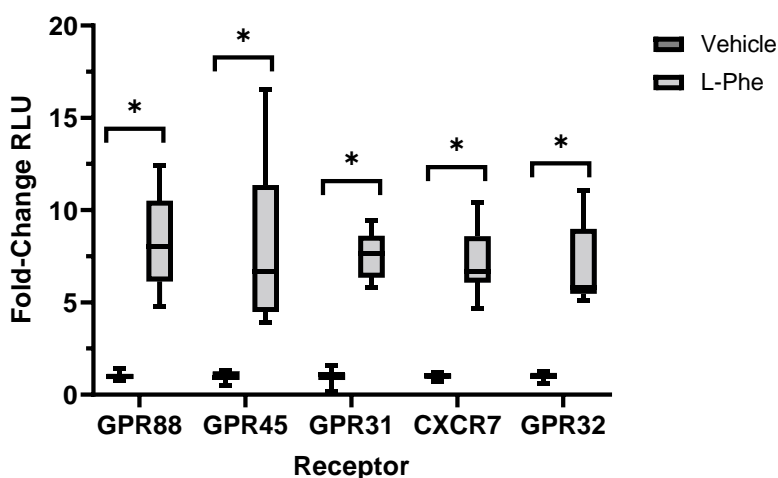


Figure 4: Comparison of fold-change in RLU of the top 5 highest PRESTO-Tango-ized GPCR responders with 3 mM L-Phe compared to vehicle treatment. Bars indicate minimum, maximum, and median fold-change values within collected datasets (* $p < 0.05$).

Table 1: GPCR classes, families, PRESTO-Tango plasmid concentration (ng/ μ L) and 260/280 ratio of extracted plasmids, and significance levels (p value) of activation with 3mM L-Phe for 64 tested orphan GPCRs ($p < 0.05$).

Receptor Name	GPCR Class; Family	[Plasmid] (ng/ μ L)	260/280	p Value
CXCR7	Class A; Chemokine receptors	105.1	1.87	<0.000001
GPR101	Class A Orphans	246.6	1.86	<0.000001
GPR12	Class A Orphans	164.3	1.89	<0.000001
GPR141	Class A Orphans	328.8	1.86	<0.000001
GPR4	Class A Orphans	65.7	1.86	<0.000001
GPR55	Class A	142.2	1.86	<0.000001
GPR88	Class A Orphans	152.6	1.86	<0.000001
GPER	Class A; G Protein-coupled Estrogen Receptor	151.1	1.87	<0.000001
GPR15	Class A Orphans	114.6	1.84	<0.000001
MRGPRD	Class A Orphans	169.6	1.89	<0.000001
GPR26	Class A Orphans	234.0	1.86	0.000011
GPR25	Class A Orphans	108.2	1.88	0.000025
GPR45	Class A Orphans	309.4	1.85	0.000041
GPR160	Class A Orphans	126.7	1.90	0.000047
GPR27	Class A Orphans	223.5	1.88	0.000050
GPR162	Class A Orphans	130.5	1.88	0.000056
GPR150	Class A Orphans	186.0	1.90	0.000086
LPA4	Class A; Lysophospholipid Receptors	89.3	1.88	0.000111
MRGPRF	Class A Orphans	173.8	1.88	0.000112

Receptor Name	GPCR Class; Family	[Plasmid] (ng/μL)	260/280	p Value
GPR78	Class A Orphans	150.5	1.85	0.000130
GPR37	Class A Orphans	584.6	1.79	0.000157
GPR17	Class A Orphans	206.1	1.90	0.000221
PrRP	Class A; Prolactin-releasing Peptide Receptor	163.7	1.87	0.000409
MRGPRX4	Class A Orphans	176.7	1.86	0.000480
GPR85	Class A Orphans	94.1	1.87	0.000604
GPR161	Class A Orphans	49.3	1.87	0.000781
GPR171	Class A Orphans	159.0	1.88	0.000781
GPR31	Class A Orphans	103.4	1.92	0.000781
GPR32	Class A Orphans	265.9	1.89	0.000781
MAS1	Class A Orphans	125.2	1.89	0.000990
GPR20	Class A Orphans	507.4	1.86	0.001119
KISSPEPTIN-R	Class A; Kisspeptin Receptors	570.1	1.82	0.001656
GPR52	Class A Orphans	179.3	1.88	0.002301
GPR132	Class A Orphans	147.1	1.89	0.004188
GPR182	Class A Orphans	139.6	1.86	0.005689
GPR84	Class A Orphans	446.4	1.83	0.006398
GPR61	Class A Orphans	243.8	1.86	0.006546
MRGPRX2	Class A Orphans	488.7	1.84	0.009339
MRGPRX1	Class A Orphans	328.1	1.89	0.014192
GPR37L1	Class A Orphans	563.8	1.80	0.035561

Receptor Name	GPCR Class; Family	[Plasmid] (ng/ μ L)	260/280	p Value
OPN5	Class A; Opsin Receptors	101.9	1.87	0.043026
GPR6	Class A Orphans	285.8	1.87	0.046617
MRGPRG	Class A Orphans	318.6	1.88	0.048310
GPR63	Class A Orphans	109.7	1.86	0.049426
GPR83	Class A Orphans	160.1	1.86	0.077005
GPR87	Class A Orphans	364.0	1.88	0.077005
TAAR5	Class A Orphans	317.7	1.85	0.093105
TAAR9	Class A Orphans	180.2	1.86	0.135911
GPR148	Class A Orphans	128.0	1.88	0.163889
GPR39	Class A Orphans	168.5	1.87	0.234139
GPR19	Class A Orphans	154.6	1.89	0.286169
GPR149	Class A Orphans	75.4	1.91	0.346615
PK2	Class A; Prokineticin Receptors	353.4	1.85	0.386508
OPN3	Class A; Opsin Receptors	192.2	1.89	0.502264
GPR62	Class A Orphans	313.3	1.86	0.530021
GPR146	Class A Orphans	142.3	1.85	0.576111
GPR142	Class A Orphans	161.7	1.85	0.584102
MAS1L	Class A Orphans	162.8	1.88	0.652442
GPR173	Class A Orphans	94.5	1.88	0.725194
GPR151	Class A Orphans	144.5	1.87	0.770926
GPR153	Class A Orphans	128.6	1.88	0.796174

Receptor Name	GPCR Class; Family	[Plasmid] (ng/ μ L)	260/280	p Value
GPR143	Other 7TM Proteins	262.3	1.84	0.860355
TAAR6	Class A Orphans	135.8	1.85	0.866926
GPR35	Class A Orphans	182.9	1.86	0.880880

3.2 GPCRs Are Highly Expressed in the Brain and Gut

To understand the tissue distribution of the 64 studied orphan GPCRs, the IUPHAR GPCR database⁴⁴ and the Human Protein Atlas⁴⁵ (www.proteinatlas.org) were surveyed and the highest-reported tissue distributions compiled (Figure 5). More specific results of this database search can be found in Appendix 1, for all 64 orphan GPCRs.

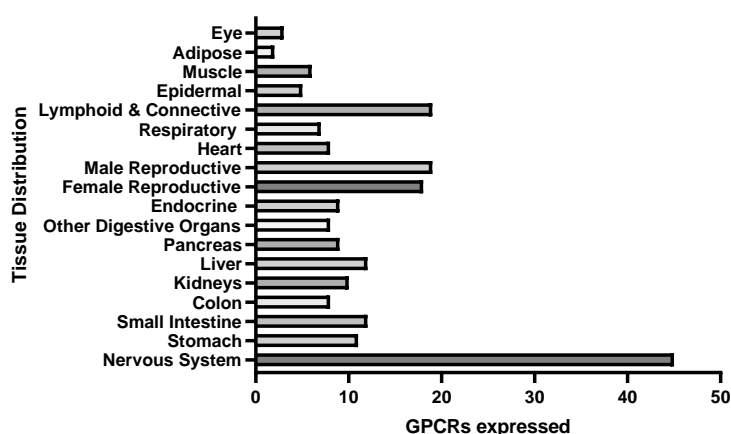


Figure 5: Graphical representation of how many of 64 orphan GPCRs are expressed within the various general tissues of the body, as described in the IUPHAR GPCR database and the Human Protein Atlas^{44,45}.

The nervous system was the most prominent location of orphan GPCR expression and detection as found in the databases, with 45 receptors listing the brain, spinal cord, or dorsal ganglia as primary tissues. This was followed by male reproductive tissues (mainly the testes and seminal vesicles) and lymphoid & connective tissues, both with 19 orphan GPCRs being highly expressed. There were 21 GPCRs with detection in the gastrointestinal system, consisting of a grouping of the esophagus, stomach, small intestine, and colon. One GPCR, MRGPRG, did not have any reportable tissues in which it was expressed, for either mouse, rat, or human samples.

Most of the GPCRs within the accessed databases were also reportedly detected at low levels in additional tissues beyond those described in Appendix 1. However, for brevity, these low-tissue-expression levels were excluded from this analysis of orphan GPCR tissue distribution.

3.3 Preliminary Opentrons Protocol Design

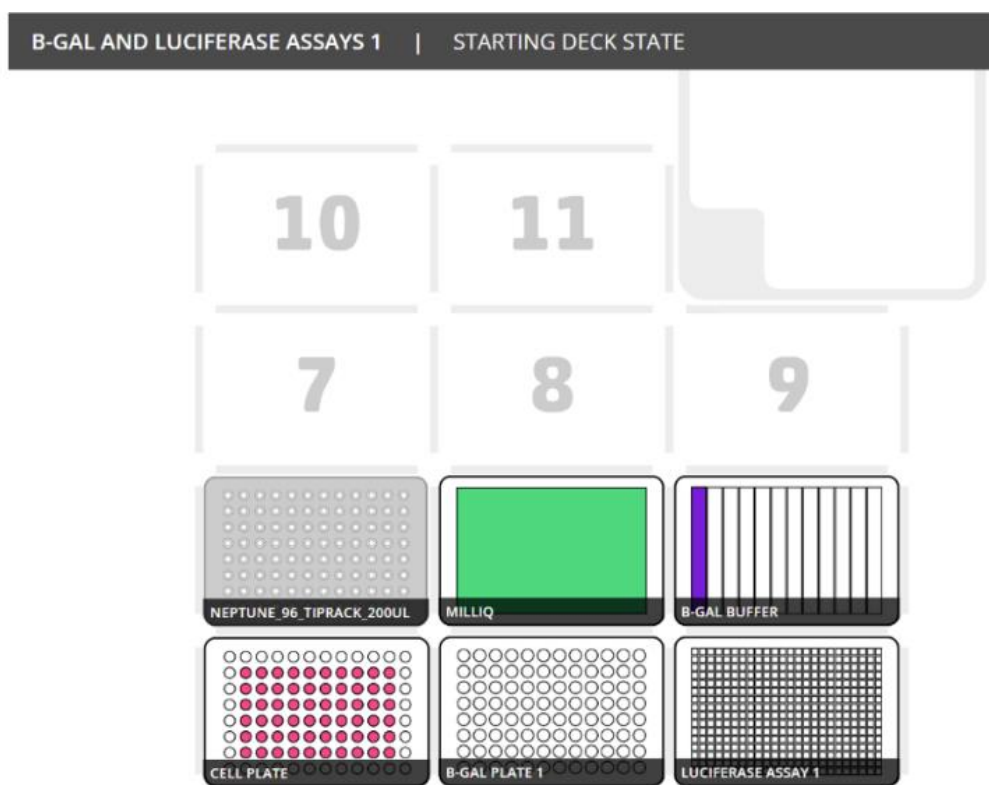
The Opentrons protocol designer website was where I created a series of pipetting steps for both the β -galactosidase and luciferase assays (Figure 6). The current layout of the manually pipetted cell lysate plate (“CELL PLATE” in the protocol deck below) involves a moat around where the cell lysate sits within the 96-well plate. This cell plate design was kept for this initial Opentrons protocol, but may be changed in future Opentrons protocols should a different layout be found to be more effective. As it is right now, there are two rows within the β -galactosidase assay plate that contain both MilliQ water and β -gal buffer without any cell lysate, which could be fixed by adding more rows of cell lysate in the cell plating and transfection steps. Similarly, the luciferase 384-well plate also contains two wells of MilliQ water and no cell lysate. However, because the luciferase buffer must be added manually due to robot pipetting volume issues, there would not be any buffer wastage.

Based closely on the manual β -galactosidase and luciferase pipetting protocol, the Opentrons robot protocol begins by transferring 180 μ L MilliQ water to each cell-lysate well in the cell lysate plate with one set of tips. The first column of cell lysate is then set to mix three times, before transferring 30 μ L of lysate to the β -gal plate and 20 μ L to the luciferase plate. Tips are set to be exchanged between each step of mixing lysate, and kept until the next mixing step. The aspiration and dispensation liquid flow rates were adjusted from the default 94 μ L/s to a much slower 10-15 μ L/s, to avoid splashes and cross contamination for each step. For the MilliQ transfer step and the β -gal buffer transfer step, the height of dispensation was changed from the default 0.5 mm from the bottom of the well to 7 mm from the bottom of the well, to avoid accidentally contaminating shared tips.

This protocol has been designed for only one cell lysate plate at a time. If multiple cell lysate plates are to be used, then the MilliQ water reservoir and the β -gal buffer reservoirs must be monitored and refilled, and the tip rack must be exchanged between each plate as well. The lysis buffer blanks used for the β -galactosidase and luciferase assays must also be pipetted manually after the Opentrons robot protocol is finished.

While this protocol presents promise as a way to minimize pipetting error and improve the feasibility of large GPCR assays, it should be noted that this is a very preliminary design that has not yet been used for β -gal or luciferase assays. There are likely many adjustments to be made to this protocol that will only be discovered when large assays are being conducted. This protocol does, however, provide what is hopefully a good basis for future large-scale GPCR assays, with any changes to be made being small, fast, and easy to fix.

a)



b)

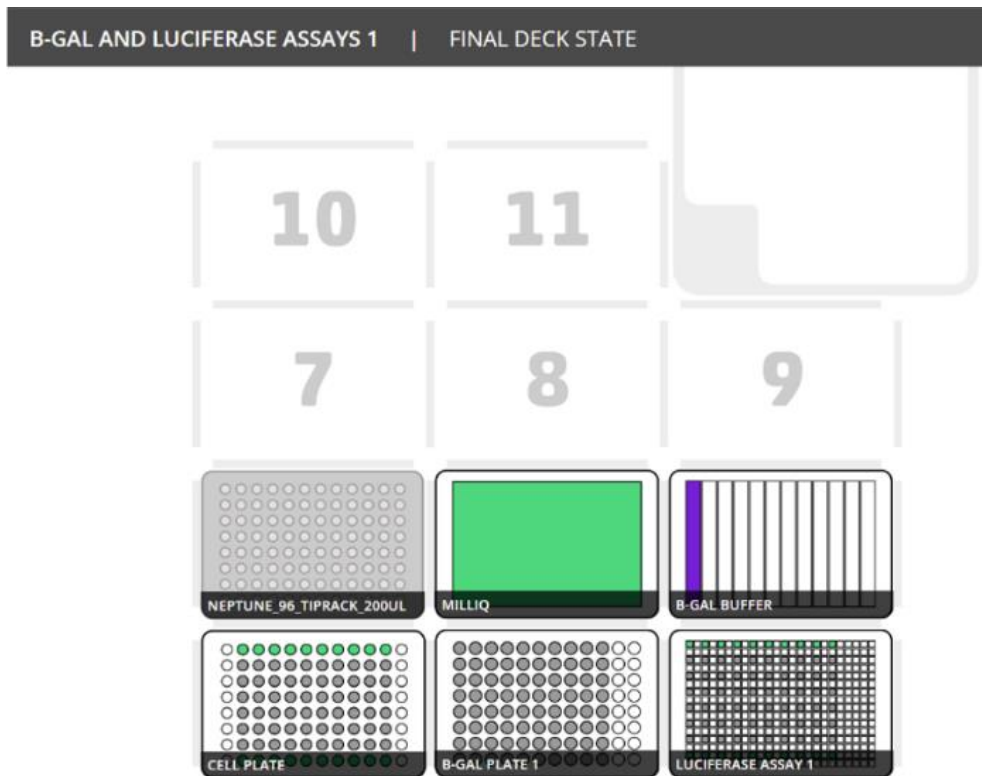


Figure 6: a) Opentrons β -galactosidase and luciferase assay protocol starting deck layout. b) Opentrons β -galactosidase and luciferase assay protocol final deck layout after all pipetting steps are carried out. The green colour represents MilliQ water, purple represents the β -galactosidase assay buffer, and grey wells represent a mixture of solutions.

4. Discussion

To add to a better understanding of how amino acids may act as signaling molecules for membrane receptors like GPCRs, Power and I assessed the L-Phe-induced activation of 277 GPCRs using the PRESTO-Tango interrogation screen developed by Kroeze et al. (2015), based on the Barnea et al. (2008) Tango assay^{1,18}. This thesis focuses on an evaluation and presentation of a 64 orphan GPCR subset of these GPCRs, with no confirmed or validated ligands. We show that L-Phe can cause a significant level of receptor activation relative to each untreated baseline in 44 of the assessed 64 orphan receptors. This represents over half of the orphan receptors in this experiment, with 69% of orphan GPCRs becoming activated. Among these significantly activated orphan GPCRs were five receptors with an increase in activity of at least seven-fold across averaged biological replicates: GPR88, GPR45, GPR31, CXCR7, and GPR32. These data

support the conclusion that L-Phe acts as a ligand for numerous orphan GPCRs. Additionally, the orphan GPCRs evaluated appear to have high expression in both the brain and gastrointestinal tract, which gives a very preliminary starting point for speculation that L-Phe may act as a signaling molecule in these tissues.

4.1 L-Phe Causes High Activation of Most Class A Orphan GPCRs

While 44 of 64 orphan GPCRs were significantly active in the presence of the 3 mM L-Phe solution, there were five highest responders with a change from baseline activation that was on average seven-fold or higher. It should be noted that the fold-change values from this 3 mM L-Phe screen are taken as an average of all triplicate fold-changes in RLU values across the three biological replicates, and that, as seen in Figure 3, there is quite a bit of variation among the obtained fold-change values. However, even with this variation each biological replicate of the L-Phe-treated group was significantly different from the untreated group, showing that with a high enough concentration of L-Phe many GPCRs can be activated.

The high levels of activation observed were somewhat unexpected for this many GPCRs. The last large amino acid screen conducted in the Rourke Laboratory used a lower concentration of L-Phe (0.8 mM) in a mixture of amino acids as well as individually³⁴. Power's initial amino acid screen generally yielded insignificant fold-changes in activation of 2 or less for most orphan receptors³⁴. However, while only 6 GPCRs were found to be significantly activated by the amino acid mixture, these 6 GPCRs (GPR12, GPR26, GPR37L1, GPR84, GPR88, and MRGPRX2) were also found to be activated in the 3 mM L-Phe screen among many more GPCRs³⁴. GPR88 was also the orphan GPCR with the greatest fold-change increase in activation for each of these three screens³⁴. CXCR7 (referred to as CMKOR1 in Power (2020)), GPR31, GPR32, and GPR45 were also not significantly activated in the 0.8 mM L-Phe screen, but were activated in the 3 mM screen³⁴. These differences in activation show that there may be a concentration threshold that must be reached for GPCRs to be significantly activated by L-Phe. Conducting dose response curves with L-Phe and orphan GPCRs may help us to find out what this threshold is for each GPCR.

Our finding of a large number of L-Phe-activated orphan GPCRs is also supported by the findings of a study on the microbiome of the gut. This study conducted a PRESTO-Tango high-throughput screen of non-olfactory, druggable GPCRs in the presence of intestinal microbiota-metabolites, as isolated from samples⁴⁶. What they reported was that L-Phe from various gut

bacteria activated the adhesion orphan GPCRs GPR56 and GPR97, which were not studied in our PRESTO-Tango screen⁴⁶. This study also reported that the concentrations of L-Phe tested for these two GPCRs were above 1 mM, which is comparable to our own L-Phe concentration of 3 mM⁴⁶. Additionally, instances of single molecules activating multiple different receptors in therapeutic treatments is known as drug “polypharmacology,” and is an indicator of a promiscuous ligand⁴⁷. In our experiment, L-Phe activated many orphan GPCRs from different subfamilies (Table 1), and likely has different biological roles in signaling for each activated receptor. This all supports our hypothesis that L-Phe does, in fact, activate GPCRs, and that it is a promiscuous endogenous ligand that likely deorphanizes some orphan GPCRs.

Given this idea of ligand promiscuity, it follows that the orphan GPCRs that are activated by L-Phe have different tissue distributions (as found in the tissue distribution database search) and different signaling functions. This also means that L-Phe likely has diversity in signaling roles that is dependent on each GPCR and its location. However, despite our newly identified unifying characteristic of activation with L-Phe, there is still not very much that is known about our tested orphan GPCRs and research remains limited. What is currently known about our top L-Phe-responders GPR88, GPR45, GPR31, CXCR7, and GPR32, is outlined below.

4.2 GPR88

With high expression in the brain and its reward centres, GPR88 is one orphan GPCR that is implicated in regulating food intake and energy homeostasis in mice^{48(p88)}. In humans, GPR88 has been reported in the gamma-Aminobutyric Acid-ergic (GABAergic) projections of medium neurons in the striatum, where it plays a role in locomotion and locomotion-related neurodegenerative disorders like Parkinson’s disease⁴⁹. GPR88 is therefore an orphan receptor with multiple possible roles within the body in food intake and metabolism, as well as in homeostasis to prevent diseases like Parkinson’s and in psychiatric disorders where dopamine abnormalities play an essential role, such as in schizophrenia⁵⁰. GPR88 is also closely associated with dopamine receptors D1 and D2 due to their expression in the same neurons of the striatum, which strengthens the hypothesis of L-Phe as an activator and potential ligand for GPR88, especially since L-Phe is both a product of food molecule break-down and a precursor to dopamine^{35,49}.

In her honours thesis, Power found that GPR88 showed a significant fold-change of 5.35 with 0.8 mM L-Phe, and had the overall highest magnitude of activation in both the L-Phe and

amino acid mix screens³⁴. This is comparable to the fold-change in activation of approximately 8.36 as calculated for the 3 mM L-Phe screen, where GPR88 was also the highest responder to L-Phe. These findings suggest that L-Phe is a ligand for GPR88, and that it may not require as high a concentration of this amino acid to cause significant activation as do other GPCRs. This hypothesis could be further explored through L-Phe dose response curves.

4.3 GPR45

The orphan GPR45 has a role in regulating obesity in mice through pro-opiomelanocortin (POMC) in POMC neurons of the arcuate nucleus *via* JAK/STAT signaling, where GPR45-mutant mice saw increased fat stores and a decrease in POMC and overall energy expenditure⁵¹. GPR45 is also found in other areas of mouse brains outside of the arcuate nucleus, as well as in the liver and testes⁵¹. This suggests that there is potentially a wider neuronal function of GPR45 in regulating obesity outside of POMC signaling. Additionally, while food intake does not appear to be different in mice with a GPR45 knockout compared to non-mutated mice, this does not preclude the possibility that food intake has an effect on GPR45 signaling⁵¹, potentially from L-Phe from metabolic breakdown.

While GPR45 had the largest variation across our biological triplicates in the PRESTO-Tango assay, each biological replicate for this GPCR was statistically significant, and still yielded on average a seven-fold increase in activity from the baseline. The cause of this variation is somewhat unclear, especially when compared to the much smaller variances seen in the other top four GPCR responders. The cause of this variance may be either a slight difference with the transfection process, or through the manual pipetting of the PRESTO-Tango assay, which is a factor that hopefully will be eliminated in future by the use of the Opentrons robot. Because GPR45 was also the only GPCR with such a wide variation, we also cannot exclude the possibility that L-Phe may be doing something else to the GPCR to cause different levels of activation, such as potentially activating different signaling pathways based on ligand binding site.

4.4 GPR31

GPR31 is likely a lipid receptor with an affinity for the arachidonic acid metabolite 12-(*S*)-Hydroxy-5,8,10,14-eicosatetraenoic acid (12-(*S*)-HETE)⁵². This receptor has a wide description of roles, such as in platelet activation when dimerized with the thrombin-activated

GPCR Proteinase-Activated Receptor 4 (PAR4)⁵³. GPR31 may also be implicated in various cancers, in part due to its presence on platelets, but also due to 12-(*S*)-HETE being involved in cytoskeleton alterations of microfilaments and intermediate filaments to promote cellular chemotaxis, as well as promoting secretion of Vascular Endothelial Growth Factor (VEGF)^{54,55}.

Activation of GPR31 with L-Phe appears somewhat uncharacteristic of this receptor due to the lack of similarity between the aromatic structure of L-Phe and the more linear nature of fatty acids and their resulting metabolites. However, in individuals with PKU, it is not uncommon to see an abnormal blood lipid profile with generally lower levels of lipoproteins and fatty acids in plasma and sera, though there is much variation among the findings of studies conducted to date^{56,57}. This correlation may be due to diet, which is the source of most lipids for people with PKU, though there is also the possibility that L-Phe is acting as a signaling molecule for GPR31 as a signal molecule for abnormalities in lipid and/or L-Phe concentration levels in the extracellular environment. It may also be that L-Phe does not activate GPR31 from the main orthosteric binding site at which lipids have generally been found to activate it. Perhaps L-Phe binds to a yet unidentified orthosteric site to activate this GPCR. A more in-depth look into lipid-sensing GPCRs such as GPR31 and their association with L-Phe would be an intriguing direction to take for future research, where there is much that is unknown or inconclusive.

4.5 CXCR7 (ACKR3)

CXCR7 (also referred to as ACKR3) is an atypical chemokine receptor, with some shared phylogeny with opioid receptors; it is referred to as an intermediate receptor type between the typical chemokine receptors and opioid receptors⁵⁸. As an unusual receptor, CXCR7 has been the focus of much pharmacological research into potential ligands (both endogenous ligands like CXCL12 and exogenous), with the findings that different signaling outcomes resulted from activation with different ligands⁵⁹. This makes studying and understanding such a receptor more difficult, especially when CXCR7 is a regulator in abnormalities including cancers and cardiovascular diseases⁵⁹.

CXCR7's high activation with L-Phe from its baseline levels of activity indicates that L-Phe is a newly demonstrated endogenous ligand among the many already reported. While there is no current published literature that looks specifically at CXCR7 and its response to amino acids or L-Phe in particular, given CXCR7's relatively ambiguous overall role in cellular

signaling, L-Phe represents another opportunity to discover an additional cellular function and lead to a better understanding of this GPCR.

4.6 GPR32

GPR32 is also referred to as the Resolvin D1 (RvD1) Receptor since GPR32 can be activated by RvD1, a derivative of the lipid mediator docosahexaenoic acid (DHA)⁶⁰. RvD1 and DHA have anti-inflammatory roles in cellular signaling, which is an indication that GPR32 also has regulatory roles in inflammation and immunity⁶¹. Unfortunately, due to the lack of a murine homolog of GPR32, the amount of research that can be conducted to learn more about the specific *in vivo* roles and outcomes of GPR32 activation are limited⁶².

With a role in inflammation and immune cells, GPR32's activation with L-Phe is interesting. L-Phe is a product of protein catabolism, so it is possible that during immune responses and inflammation where protein breakdown is occurring, the L-Phe acts as a signaling molecule acting on GPR32 to cause the eventual release of anti-inflammatory molecules, or moderate leukocyte activity at the site of inflammation.

4.7 L-Phe as a GPCR Signaling Molecule in PKU and Gut Function

The high levels of orphan GPCR activation obtained in this PRESTO-Tango screen with 3 mM L-Phe suggest that L-Phe may be a significant signaling molecule in cellular processes at high concentrations. While its overall role in signaling is still not well characterized, it is possible that L-Phe has a role in various disorders of the brain and in gut function.

The disease that first comes to mind when high levels of L-Phe are mentioned is PKU, which is defined by a mutation resulting in a loss of function in the phenylalanine hydroxylase converting enzyme. The high levels of L-Phe that build up in the brain are detrimental to cognitive function and development. After conducting a database search to understand where in the body the various orphan GPCRs are expressed, a large number are found to be expressed in the brain at moderate to high levels, while other GPCRs may be present in the brain at much lower levels. The high level of L-Phe that we mimicked from PKU in our *in vitro* HTLA cell GPCR activation model generated significant receptor activation of 69% of the tested orphan GPCRs. With this knowledge, there are a few possibilities that could be suggested to help in developing an understanding of the potential role of L-Phe in cognitive function in PKU.

The large amounts of L-Phe present in the extracellular environment may result in this amino acid acting on GPCRs because of its abundance. While the signaling pathways and cellular responses of each of these tested orphan GPCRs with L-Phe activation is still not known, this would be a good direction to pursue for an understanding of the role of L-Phe in diseases like PKU and in general as a signaling molecule. Additionally, though we know that toxicity is the end result of high L-Phe levels, how exactly this toxicity occurs remains to be elucidated. Van Spronsen et al. reported on this topic in 2009, suggesting multiple possibilities for the cause of L-Phe-induced toxicity, including a shortage of large neutral amino acid (LNAA) transportation over the blood-brain barrier *via* the LNAA-transporter⁶³. Another study conducted in 2014 found that L-Phe was able to self-assemble into amyloid fibrils like those found in PKU, and suggested that this was a large cause of the toxic symptoms characteristic of PKU⁶⁴. It may well be that a combination of multiple factors like altered neurotransmitter transmission, low levels of LNAAs, formation of L-Phe-induced amyloid fibrils and plaques, and L-Phe triggering GPCR signaling cascades with unknown outcomes could all contribute to the effects of L-Phe toxicity in patients experiencing high levels of L-Phe.

Other studies that look at inflammation also commonly note an abnormal ratio of L-Tyr to L-Phe. One study looking at immune activation and inflammation in cardiovascular diseases found that an increase in neopterin (a marker of immune activation) and high sensitivity C-reactive protein (a marker of inflammation) was correlated with low levels of L-Tyr and higher levels of L-Phe, which in turn corresponded to neuropsychiatric symptoms of disorders like depression⁶⁵. PKU has also been found to have prominent neuropsychiatric comorbidities like depression, anxiety, schizophrenia, and attention deficit hyperactivity disorder among others^{66,67}. Should L-Phe be acting on GPCRs within the brain, it is possible that one of the outcomes of this mechanism is the onset of neuropsychiatric symptoms.

In the gastrointestinal system, it is also known that L-Phe is sensed by GPCRs like CaSR to modulate hormone secretion for glucose homeostasis. If L-Phe is possibly implicated in signaling related to inflammation, L-Phe could have roles in inflammatory disorders of the gastrointestinal system. A study on the irritable bowel disorders ulcerative colitis and Crohn's disease found that lowered levels of the aromatic amino acid L-Trp caused an increase in the severity of bowel inflammation⁶⁸. While not as closely related to L-Trp as to L-Tyr, both L-Trp and L-Phe are aromatic essential amino acids. Decreases in L-Trp in the bowel may be a more

significant cause of inflammation, but it is possible that L-Phe is acting in the background on GPCRs within the gut to mediate symptoms of inflammation. Dopamine and melanin, two downstream products of L-Phe catabolism, have been implicated in various anti-inflammatory processes such as lowered levels of the proinflammatory cytokines tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and interleukin-10, as well as an increase in neutrophil immune response and secretion of leukocytic anti-inflammatory mediators⁶⁹. If L-Phe also exhibits similar anti-inflammatory and pro-immune response properties to dopamine, it is possible that L-Phe acts on GPCRs in the gut to reduce inflammation when not present at toxic levels.

It is also known that aromatic amino acids like L-Trp or L-Phe can act on amino-acid sensing GPCRs like GPR142 or CaSR (respectively) to cause the release of such hormones like CCK and GIP^{33,70}. Given that L-Phe was found in this experiment to activate of 44 orphan GPCRs and given that so many of these orphan GPCRs are found in the digestive tract (Figure 4), it is also possible that L-Phe could cause similar effects on gut hormone secretion in these orphan GPCRs expressed in the gut. However, the orphan GPR142 was included in this 3 mM L-Phe screen and was not found to be significantly activated from the baseline. While GPR142 has been reported to be activated by L-Trp and synthetic phenylalanine-related molecules, it appears that L-Phe itself is likely not a strong activating ligand for this particular receptor³³. An experiment using L-Phe to activate orphan GPCRs in enteroendocrine cells with an analysis of the secreted hormones would be a very interesting direction of study to take, and can help clarify both the role of L-Phe in GPCR signaling and allow us to learn more about each GPCR's role in gut function.

4.8 Challenges in GPCR Deorphanization with L-Phe

While this experiment indicated that L-Phe has an important role in cellular signaling, there are many challenges associated with our particular experimental design for measuring GPCR activation with L-Phe. For this study, we increased the concentration of L-Phe in this PRESTO-Tango assay to 3 mM from Power's original experiment at 0.8 mM to be able to clearly identify activated GPCRs. However, this represents a concentration of L-Phe that is high above the reported concentration boundaries in individuals with PKU, where the highest blood sera concentrations are generally left open ended with a lower boundary of 1200 $\mu\text{mol/L}$ ³⁶. It is assumed that 3 mM (or 3000 $\mu\text{mol/L}$) of L-Phe would likely result in detrimental effects to

human health, so while this concentration of L-Phe was useful in an *in vitro* screen of GPCRs, this data must be interpreted carefully when applied to or cross referenced for *in vivo* models. Additionally, the *in vitro* cellular model lacks the larger interactions seen by cells within a complete organism, so it is probable that there are other factors that influence how GPCRs interact with L-Phe that simply were not present in this experiment. This may include GPCR crosstalk or dimerization, other ligands competing for a binding spot on a GPCR, or more.

That said, our finding that L-Phe is an activator of many orphan GPCRs is a large first step towards deorphanization. There is much additional work that must be done in determining the ligand binding site for L-Phe to understand whether it is a direct agonist, or a secondary, low-affinity ligand for a given orphan GPCR, through methods like x-ray crystallography or *in silico* GPCR-ligand interaction models⁷¹. This can also lead to a better understanding of the overall role of L-Phe in metabolic signaling.

4.9 Conclusion and Future Directions

Through this study, we determined and verified that L-Phe does activate a large number of orphan GPCRs from Class A specifically, and is a very likely endogenous ligand for many of them. With the evaluation of GPCR tissue distribution through database searches and finding high expression in the brain and gastrointestinal tract, we proposed that L-Phe may have a role in mediating inflammation or neuropsychiatric symptoms through GPCR signaling, as well as maintaining a role in signaling for metabolic homeostasis.

With these results, a future direction would be to look more closely at other related aromatic amino acids like L-Tyr to determine why these do not activate GPCRs as strongly despite a similar molecular structure, as initially found by Power³⁴. Additionally, looking at the L-Phe enantiomer, D-Phe, will also further our understanding of how L-Phe works as a signaling molecule at the superficial level and add a new perspective into the D-amino acids as signaling molecules. Dose response curves with concentrations of L-Phe in the normal physiological range for humans should also be conducted in future studies, to better understand how L-Phe acts on GPCRs in the absence of disease. Research on the downstream intracellular signaling cascades of each of these orphan GPCRs in response to L-Phe activation is also a necessary component of understanding each receptor's cellular response, and will further our understanding not only of orphan GPCRs, where little is known about them, but also in L-Phe's larger role in organismal homeostasis.

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6. Appendix

Appendix 1: Description of the tissues that each of 64 orphan GPCRs has been detected in to moderate or high levels (as reported and described in the IUPHAR GPCR Database and the Human Protein Atlas)^{44,45}.

Receptor Name	GPCR Class; Family	Tissue Localization
CXCR7	Class A; Chemokine receptors	Human: thyroid; bronchus; pancreas; kidney; fallopian tubes; placenta; appendix Mouse: bone; brain; heart; kidney; vasculature ⁷²
GPR101	Class A Orphans	Human: brain (hypothalamus, basal ganglia, caudate putamen) ^{73,74}
GPR12	Class A Orphans	Human: endothelium of vasculature (arteries and veins); vascular smooth muscle (arteries and veins, bronchi, uterus, skeletal muscle) ⁷⁵ Mouse: brain (synapses); liver ⁷⁶
GPR141	Class A Orphans	Human and Mouse: bone marrow ⁴⁴
GPR161	Class A Orphans	Human: brain (cerebral cortex, cerebellum, hippocampus); thyroid; stomach; duodenum; small intestine; colon; testes; seminal vesicle; endometrium; spleen; lymph nodes; tonsil ^{77(p161)}
GPR17	Class A Orphans	Human: brain; heart; kidney; endothelial cells of umbilical vein ⁷⁸
GPR171	Class A Orphans	Human: appendix; lymph node; thymus; spleen ^{79(p171)}
GPR26	Class A Orphans	Human (and Mouse): brain (midbrain, cerebral cortex, hippocampus, striatum) ⁴⁴
GPR31	Class A Orphans	Mouse: pituitary gland; testes ⁴⁴
GPR32	Class A Orphans	Human: arterial & venous tissue; parathyroid gland myeloid cells ^{62(p1)}
GPR4	Class A Orphans	Human: kidney; heart; lung ⁸⁰
GPR55	Class A	Human: spleen; thymus; small intestine; brain (caudate nucleus, nucleus accumbens, putamen, striatum) ^{81,82}
GPR78	Class A Orphans	Human: placenta; pituitary gland ⁷⁴

Receptor Name	GPCR Class; Family	Tissue Localization
GPR84	Class A Orphans	Human: bone marrow; peripheral blood leukocytes (neutrophils, eosinophils); lung; brain (cerebellum) ^{83,84(p85)}
GPR85	Class A Orphans	Human: brain (forebrain); testes; small intestine; placenta; spleen ^{85,86(p85)}
GPR88	Class A Orphans	Mouse: brain (striatum, medium division of the central nucleus of the amygdala, piriform cortex) ⁸⁷
OPN5	Class A; Opsin Receptors	Human: retina; brain; testes ⁸⁸
MRGPRX2	Class A Orphans	Human: hypothalamus; thyroid; pancreas; pituitary gland; lung; stomach; ileum; colon; ovary; testis ⁸⁹
GPER	Class A; G Protein-coupled Estrogen Receptor	Human: nasopharynx; lungs; bronchus; stomach; duodenum; colon; liver; epididymis; seminal vesicle; placenta ^{90(p1)}
GPR15	Class A Orphans	Human: small bowel and colonic mucosa; lymph nodes; prostate; testes; liver ⁹¹
LPA4	Class A; Lysophospholipid Receptors	Human: ovary; thymus; pancreas; brain; heart; small intestine; testis; prostate; colon; spleen ^{92(p9)}
MRGPRD	Class A Orphans	Mouse: nonpeptidergic neurons that innervate the epidermis ⁹³
MRGPRG	Class A Orphans	UNKNOWN
GPR132	Class A Orphans	Human: nasopharynx; bronchus; lung; stomach; rectum; gallbladder; kidney; epididymis; fallopian tube; endometrium; cervix; bone marrow ^{94(p132)}
MRGPRF	Class A Orphans	Human: kidney; testis; seminal vesicle; ovary; endometrium; breast; smooth muscle; gastrointestinal tract (ileum, large intestine) ⁹⁵⁻⁹⁷
GPR52	Class A Orphans	Human: brain (caudate region, putamen) ⁹⁸
GPR27	Class A Orphans	Human: brain (caudate nucleus, putamen, hippocampus, subthalamic nucleus); ovary; testis; heart; prostate; pancreas; peripheral leukocytes ⁸⁵

Receptor Name	GPCR Class; Family	Tissue Localization
PrRP	Class A; Prolactin-releasing Peptide Receptor	Human: pituitary gland; adrenal glands (cortex and medulla); brain (cerebellum, hippocampus, pons, medulla oblongata, hypothalamus, thalamus, occipital lobe, temporal lobe, frontal lobe) ^{99,100}
GPR162	Class A Orphans	Human: central nervous system; lungs; reproductive organs ¹⁰¹
GPR160	Class A Orphans	Human: nasopharynx; bronchus; salivary gland; esophagus; stomach; urinary bladder; prostate; vagina; endometrium; cervix; skin; tonsil ^{102(p160)} Mouse: testes ⁴⁴
MRGPRX4	Class A Orphans	Human: dorsal root ganglion ¹⁰³
MAS1L	Class A Orphans	Human: uncertain - cervix ¹⁰⁴
MAS1	Class A Orphans	Human: brain (hippocampus, cerebral cortex); heart ^{105,106}
GPR83	Class A Orphans	Human: brain (cerebral cortex, hippocampus, amygdala, endopiriform nucleus, diagonal band of Broca, thalamus, hypothalamus) ¹⁰⁷
GPR45	Class A Orphans	Human: brain (basal forebrain, frontal cortex, caudate); liver ¹⁰⁸
GPR182	Class A Orphans	Human: heart; skeletal muscle; liver; pancreas; stomach; spleen; lymph node; bone marrow; adrenal gland; thyroid ¹⁰⁹
GPR61	Class A Orphans	Human: brain (cerebral cortex, occipital pole, temporal lobe, frontal lobe, amygdala, hippocampus); testes ¹¹⁰
GPR87	Class A Orphans	Human: esophagus; skin; vagina ^{44,111}
GPR37	Class A Orphans	Human: brain (corpus callosum, substantia nigra, caudate nucleus, amygdala, hippocampus, hypothalamus, thalamus); liver; placenta; pancreas; kidney ¹¹²
GPR150	Class A Orphans	Human: brain; pancreas ^{101,113(p150)}
GPR20	Class A Orphans	Human: brain (thalamus, putamen, caudate); liver; small intestines ^{114,115}
MRGPRX1	Class A Orphans	Human: dorsal root ganglia ^{103,116}

Receptor Name	GPCR Class; Family	Tissue Localization
GPR63	Class A Orphans	Human: brain (thalamus, caudate nucleus); thymus; stomach; small intestine ⁷⁵
GPR25	Class A Orphans	Human: stomach ^{115(p25)} Mouse: brain; thymus ⁴⁴
TAAR9	Class A Orphans	Human: kidney; pituitary gland; skeletal muscle ^{117,118}
KISSPEPTIN-R	Class A; Kisspeptin Receptors	Human: pancreas; placenta ¹¹⁹
GPR6	Class A Orphans	Human: brain (putamen, frontal cortex, hippocampus, hypothalamus) ¹²⁰
GPR37L1	Class A Orphans	Human: brain (cerebellum, cortex, medulla, spinal cord, putamen, occipital lobe, frontal lobe, temporal lobe) ¹²¹
TAAR5	Class A Orphans	Human: skeletal muscle; brain (amygdala, hippocampus, caudate nucleus, thalamus, hypothalamus, substantia nigra) ¹²²
GPR148	Class A Orphans	Human: brain; testes ^{101,123}
GPR149	Class A Orphans	Human: ovaries; brain; digestive tract ¹²⁴
GPR153	Class A Orphans	Mouse: brain (thalamus, cerebral cortex, striatum) ^{125(p153)}
PK2	Class A; Prokineticin Receptors	Human: brain (amygdala, hippocampus, frontal lobe, cerebral cortex); testes; small intestine ^{126,127}
GPR142	Class A Orphans	Mouse: spleen; liver; kidney; testes; brain ¹²⁸
GPR39	Class A Orphans	Rat: liver; stomach; pancreas; colon; adipose ¹²⁹
GPR19	Class A Orphans	Human: brain (caudate nucleus, putamen, thalamus) ¹³⁰
OPN3	Class A; Opsin Receptors	Human: brain; liver; retina ¹³⁰
GPR62	Class A Orphans	Human: brain (frontal cortex, caudate, basal forebrain, thalamus, putamen, hippocampus) ¹³¹
GPR146	Class A Orphans	Mouse: brain; heart; liver; skeletal muscle; white adipose; gallbladder; bone marrow ⁴⁴
TAAR6	Class A Orphans	Human: kidneys; brain (amygdala, hippocampus) ¹¹⁷

Receptor Name	GPCR Class; Family	Tissue Localization
GPR35	Class A Orphans	Human: peripheral leukocytes; spleen; colon; small intestine; stomach ¹³²
GPR173	Class A Orphans	Human: brain; ovaries; small intestine ⁸⁵
GPR151	Class A Orphans	Human: spinal cord; brain; testes; liver; kidney; stomach ¹³³
GPR143	Other 7TM Proteins	Human: brain; skin ^{134(p143)} Mouse: whole eye ⁴⁴
