Pharmacology of novel free fatty acid receptor 4 ligands and their potential use in metabolic studies

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Abstract

Free fatty acids serve as both dietary nutrients and signalling molecules by activating the G protein-coupled receptors of the free fatty acid family. After being deorphanized in 2005, free fatty acid receptor 4 (FFAR4) was shown to be highly expressed in the pancreas, where it was proposed to have a function in the production of insulin. The development of synthetic FFAR4 agonists as a potential therapy for type-2 diabetes mellitus has been founded on this. Recently, there has been research on the function of FFAR4 in the pancreas, specifically in δ-cells, which have high levels of FFAR4 expression. FFAR4 has been shown to be expressed in various types of islet cells, such as α, β, δ, and γ cells, inside the pancreas. FFAR4 plays a crucial role in regulating insulin and glucagon synthesis, as well as suppressing the release of somatostatin in the islets of Langerhans. Thus, FFAR4 serves as a compelling pharmaceutical target for metabolic disorders. Nevertheless, the lack of FFAR4 agonists in clinical trials is primarily attributed to a smaller number of available agonists, challenges with drug selectivity, and suboptimal pharmacokinetic and pharmacodynamic characteristics. This underscores the necessity for increased attention and scientific investigation into the development of these receptor agonists.

The objective of this thesis was to conduct a pharmacological characterization of new FFAR4 ligands and evaluate their potency and efficacy in comparison to the reference agonist, TUG-891. The research aimed to determine the specific location of FFAR4 in pancreatic islets and examine the effects of FFAR4 expression on the function of these cells. Moreover, the objective of the thesis was also to assess the capacity of FFAR4 ligands to induce insulin secretion from pancreatic islets. This research aims to enhance the understanding of FFAR4's involvement in glucose regulation and its potential as a target for treating metabolic diseases. Moreover, this study aims to enhance the knowledge of FFAR4 pharmacology and its physiological roles in the pancreas. By doing so, it will provide vital insights for the creation of new treatment approaches that specifically target this receptor.

In order to analyse the signalling processes of the mouse FFAR4 receptor, functional tests were conducted on cell lines that constitutively express the mouse ortholog of FFAR4. It has been verified that FFAR4 mostly associates with Gαq/11 G proteins, and there is little or no indication of coupling with Gαs or Gαi in cell lines. Out of the ligands tested, FFAR4 Agonist II showed greater efficacy, whereas Merck cpd A and GSK137647A revealed similar or lower efficacy compared to TUG-891, which was used as the standard ligand. Based on the analysis, it was shown that the FFAR4 Agonist II is a superior ligand compared

to TUG-891. FFAR4 Agonist II has the potential to be a useful tool to conduct experiments both *ex vivo* and *in vivo* to validate FFAR4 as a viable target for treating metabolic diseases.

Functional tests have shown that FFAR4 plays a pivotal function in the regulation of hormone production in the pancreas. Although the FFAR4 ligand TUG-891 has a minor impact on the release of insulin from β-cells, FFAR4 is crucial for enhancing insulin secretion caused by the M3 agonist oxotremorine. This effect was not observed in FFAR4-KO islets. Interestingly, the combination of TUG-891 with oxotremorine and FFAR4 antagonist AH7614 resulted in a 2.5-fold reduction in the impact of oxotremorine. In addition, the phosphorylation of the FFAR4 receptor seems to have a role in insulin release. This was shown by comparing the response of islets from a mutant mouse line expressing an FFAR4 variation that is defective in phosphorylation (PD mouse) to wild-type islets when exposed to oxotremorine. In addition, somatostatin release was 2-3 times higher in FFAR4-KO islets than in wild-type islets, demonstrating that FFAR4 regulates somatostatin secretion independently of ligand activation. The results highlight the potential of FFAR4 as a target for treating metabolic diseases.

These findings confirm that FFAR4 is a new and promising target for therapeutic development in the treatment of metabolic diseases, namely T2DM. Existing treatments for T2DM, such as metformin, may lead to adverse effects and may not be successful for specific patient groups. This emphasises the need for new and safer medications in clinical practice. The capacity of FFAR4 to regulate the production of insulin and somatostatin in the pancreas, together with its ability to control glucose homeostasis, emphasises its therapeutic promise. Additional investigation into the precise mechanisms that control FFAR4 activation and signalling pathways has the potential to result in the creation of targeted medications that successfully regulate glucose metabolism and enhance patient outcomes while reducing the adverse effects associated with current medicines.

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Prihandoko, R., Kaur, D., Wiegman, C.H., Alvarez-Curto, E., Donovan, C., Chachi, L., Ulven, T., Tyas, M.R., Euston, E., Dong, Z., **Alharbi, A.G.M.,** Kim, R.Y., Lowe, J.G., Hansbro, P.M., Chung, K.F., Brightling, C.E., Milligan, G. and Tobin, A.B. (2020) 'Pathophysiological regulation of lung function by the free fatty acid receptor FFAR4', Science Translational Medicine, 12(557), pp. 1–14.

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Author's Declaration

"I declare that, except where explicit reference is made to the contribution of others, this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution."

May 2024

Abdulrahman Ghali Alharbi

Definitions/Abbreviations

5-HT2C - Serotonin 2C receptors

AC - Adenylyl cyclase

ACE - Angiotensin-converting enzyme

BRET - Bioluminescence resonance energy transfer

BSA - Bovine serum albumin

cAMP - Cyclic adenosine monophosphate

CHO - Chinese hamster ovary

CP - Long-term pancreatitis

CRD - Cysteine-rich domain

CRHR2 - Corticotropin releasing hormone receptor 2

DAG - Diacylglycerol

DMEM - Dulbecco's Modified Eagle Medium

DMSO - Dimethyl sulfoxide

dNTP - Deoxynucleoside triphosphate

DPP4 - Dipeptidyl peptidase-4

DTT - Dithiothreitol

EC50/80 - Effective concentration at 50/80% maximal response

ECL - Extracellular loop

EDTA - Ethylenediamine tetraacetic acid

EEC - Enteroendocrine cell

ELISA - Enzyme-linked immunosorbent assay

EP2 - Extracellular protein 2

EPA - Eicosapentaenoic acid

EPAC - Exchange protein directly activated by cAMP

EPI - Exocrine pancreatic insufficiency

ERK - Extracellular signal-regulated kinases

eYFP - Enhanced yellow fluorescent protein

FAs - Fatty acids

FFAs - Free fatty acids

FFAR - Free fatty acid receptor

FFAR1 - Free fatty acid receptor 1

FFAR2 - Free fatty acid receptor 2

FFAR3 - Free fatty acid receptor 3

FFAR4 - Free fatty acid receptor 4

FBS - Fetal bovine serum

FRET - Fluorescence resonance energy transfer

FZD - Frizzled

GABA - Gamma-aminobutyric acid

GAIN - GPCR autoproteolysis-inducing domain

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GDP - Guanosine-5'-diphosphate

GIP - Glucose insulinotropic polypeptide

GLP-1 - Glucagon like peptide 1

GLUT2 - Glucose transporter type 2

GLUT4 - Glucose transporter type 4

GPCR - G protein-coupled receptor

GRK - G protein-coupled receptor kinase

GSIS - Glucose-stimulated insulin secretion

GSSS - Gastric somatostatin secretion

GTP - Guanosine-5'-triphosphate

HA - Hemagglutinin

HBSS - Hanks' balanced salt solution

HEK - Human embryonic kidney

HRP - Horseradish peroxidase

HTRF - Homogenous time resolved fluorescence

IBMX - 3-isobutyl-1-methylxanthine

ICC - Immunocytochemistry

ICL - Intracellular loop

IHC - Immunohistochemistry

IKK - Inhibitor complex of nuclear factor-KB kinase

IL - Interleukin

IP1 - Inositol monophosphate

IP3 - Inositol triphosphate

JNK - c-Jun N-terminal Kinase

KO - Knockout

LBD - Ligand binding domain

LCFAs - Long-chain fatty acids

MAPK - Mitogen-activated protein kinase

NAM - Negative allosteric modulator

NEFA - Nonesterified fatty acid

PAM - Positive allosteric modulator

PBS - Phosphate buffered saline

PCR - Polymerase chain reaction

PD - Phospho-deficient

PFA - Paraformaldehyde

PI - Pancreatic insufficiency

PKA - Protein kinase A

PKC - Protein kinase C

PLC - Phospholipase C

PP - Pancreatic polypeptide

PTX - Pertussis toxin

PPAR - Peroxisome proliferator-activated receptor

RIPA buffer - Radioimmunoprecipitation assay buffer

RH - Trafficking homology

RT - Reverse transcriptase

SCFAs - Short-chain fatty acids

SDS-PAGE - Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SMO - Smoothened

SST - Somatostatin

T2DM – Type-2 diabetes mellitus

TAB1 - TGF-beta activated kinase binding protein 1

TAK1 - TGF-beta activated kinase 1

TBST - Tris-buffered saline (TBS) Tween

TM - Transmembrane

V1 - Vasopressin 1

V2 - Vasopressin 2

VFD - Venus fly-trap domain

WB - Western blot

WT - Wild type

β2AR - β2-adrenergic receptor

Chapter I Introduction

1.1 The G protein-coupled receptor superfamily of cell surface receptors

Cell surface receptors known as G protein-coupled receptors (GPCRs) have the remarkable ability to detect and respond to a vast range of stimuli, such as hormones, neurotransmitters, and external signals. Because of this, GPCRs are the most numerous pharmacological drug targets in the world, accounting for more than 30% of all prescription drugs *(Santos et al.,* 2017). They are characterised by their distinct seven transmembrane (7TM) domain structure. This structure is composed of seven α-helical segments that span the cell membrane, connected by alternating intracellular and extracellular loops (Cong *et al.,* 2022; Trzaskowski *et al.,* 2012). The conserved 7TM architecture plays a vital role in converting signals from outside the cell into responses inside the cell, highlighting the significance of GPCRs in cellular communication and regulation (Venkatakrishnan *et al.,* 2013).

On the basis of their structure and function, GPCRs are often divided into six subfamilies, class A-F (Alexander *et al.,* 2021). Class A receptors, also known as “rhodopsin-like” based on the similarity with the visual GPCR rhodsopsin, are the most common receptor class representing ~80% of all GPCRs. The free fatty acid receptor 4 (FFAR4), which is the focus of this thesis, belongs to this group. Receptors of class A GPCRs are the ones most often targeted therapeutically because of the diverse variety of physiological activities that they perform (Yang *et al.,* 2021). Furthermore, this subfamily encompasses receptors that are highly responsive to a wide range of ligands, that extends from small molecules and neurotransmitters to peptides and hormones (Wess, 1993; Milligan *et al.*, 2017a). Class A GPCRs are distinguished by a DRY motif between TM3 and ICL2, as well as an NSxxNPxxY motif in TM7 (Fredriksson *et al.,* 2003; Nomiyama and Yoshie, 2015).

Class B includes receptors for Adhesion and Secretin. Class B adhesion receptors are phylogenetically correlated with class secretin receptors due to their similarity in the TM domain. Nevertheless, they possess a unique, long extracellular N-terminal domain that undergoes autoproteolytic cleavage at a conserved GPCR proteolysis site (GPS) located within a GPCR autoproteolysis inducing (GAIN) domain (Prömel, Langenhan and Araç, 2013; Yang *et al.,* 2021).

Class C includes receptors for gamma-aminobutyric acid (GABA), taste, calcium, and the metabotropic glutamate family. Class C receptors in rodents also include taste type-1 and vasopressin (V2) pheromone receptors, none of which are found in humans *(Alexander et al.,* 2017). Class C GPCRs characterised by a broad and unique extracellular domain (ECD) that is composed of a ligand-binding domain (LBD) and a cysteine-rich domain (CRD, with the exception of the $GABA_B$ receptor) (Chun *et al.,* 2012). The N-termini of glutamate receptors consist of approximately 600 residues. In this case, the ligand attaches to a binding site in the Venus fly trap domain (VFD), utilising two large N-terminal lobes found in the extracellular domain (Kunishima *et al.,* 2000; Wu *et al.,* 2014).

According to Attwood and Findlay (1994), class D receptors include fungal mating pheromone receptors, class E cAMP receptors, and class F Frizzled and Smoothened receptors. Frizzled/Taste2 receptors (class F) are the most recent class of receptor that has been discovered. They consist of frizzled (FZD) and smoothened (SMO) receptors that are stimulated by secreted Wingless/Int-1 (Wnt) glycoproteins and Hedgehog (HH) proteins. Both FZD and SMO receptors have a common N-terminal signal sequence, which is subsequently followed by a highly conserved CRD that plays a crucial role in ligand recognition (Dann *et al.,* 2001; Vinson, Conover and Adler, 1989).

In recent years, a different categorization scheme for mammalian GPCRs termed GRAFS has been proposed as an alternative classification method (Fredriksson *et al.,* 2003). Receptors in this method are categorised into five classes based on phylogenetic sequencing of the human genome: Glutamate (G), Rhodopsin (R), Adhesion (A), Frizzled/Taste2 (F), and Secretin (S) (Figure 1-1). When compared to the A-F classification method, the GRAFS system has classifies the Class B GPCR family into two distinct groups: the Secretin family and the Adhesion family. This divide was established on the basis of early discoveries that described a separate evolutionary history shared by both groups (Hu *et al.,* 2017; Odoemelam *et al.,* 2020). GRAFS is the most widely utilised system and will be used throughout this thesis.

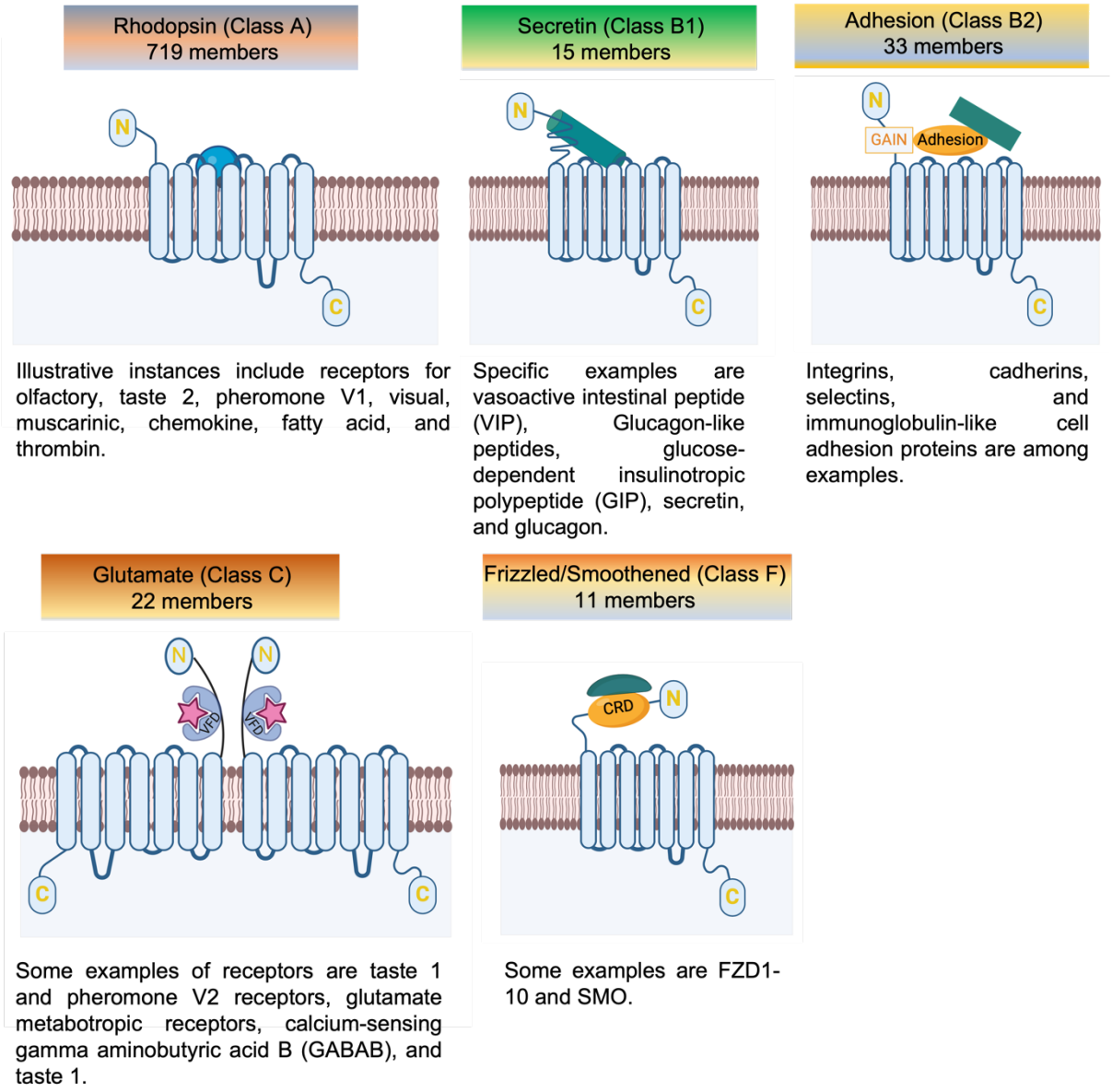


Figure 1-1: Characteristics of GPCR subfamilies and examples. GPCRs consist of a transmembrane domain composed of seven α-helices, an N-terminus located on the outside of the cell, and a C-terminus located on the inside of the cell. The intracellular portions are rather similar, but the extracellular area varies significantly across various subfamilies. Blue shapes indicate the various mechanisms of ligand interaction with certain GPCR families. The majority of glutamate receptors are present in a dimeric structure and use a VFD for binding with ligands. Rhodopsin-like receptors have a short N-terminus, and their orthosteric ligand binding pocket is located deep inside the transmembrane domain. Adhesion GPCRs have a GAIN domain that facilitates the cleavage of the N-terminus, allowing the adhesion domains to be loosely connected to the receptor. Frizzled/smoothened class receptors have a cysteine-rich domain (CRD) at the N-terminus. Each GPCR subfamily in humans is illustrated along with the current number of identified members according to Alexander *et al.* (2021).

Of approximately 800 GPCRs in mammalian systems; there are at least 140 receptors considered to be orphan receptors (Levoye *et al.,* 2006). These receptors represent a category of GPCRs where their natural (endogenous) ligands have not yet been found. When their endogenous ligands are found, these orphan receptors are "deorphanized" and may be appropriately classified within the GPCR family according to their signalling and ligand binding capabilities (Tang *et al.,* 2011). An example would be seen in the Free fatty acid receptors (FFAR1, FFAR2, FFAR3, and FFAR4) as they were first considered to be orphan receptors until their endogenous ligands were discovered. After deorphanizing these receptors, it was determined that short-chain fatty acids (SCFAs) with two to four carbon atoms (C2-C4) activate FFAR2 and FFAR3 (Tazoe *et al.,* 2009), whereas long-chain fatty acids (LCFAs) with 12 to 26 carbon atoms activate FFAR1 and FFAR4 (Hirasawa *et al.,* 2005). This finding properly classified these receptors within the GPCR family and prompted research into their physiological functions and therapeutic potential.

1.2 Structural characteristics of GPCR subfamilies

Structural studies have revealed that the key distinguishing factors among GPCR subfamilies are the unique ligand binding pockets and N-terminal domains. The N-terminus and, in certain instances, the extracellular loops (ECLs) exhibit a significant degree of structural variety because they are often implicated in the function of receptors and the binding of ligands (Kruse *et al.,* 2012; Manglik *et al.,* 2012).

The ECL sequences, particularly ECL2, vary among different families due to the role of ECL2 in recognising and binding ligands (Kruse *et al.,* 2012; Manglik *et al.,* 2012). For example, ECL2 exhibits an α-helical structure in adrenergic receptor structures, while adopting a hairpin structure in all the peptide receptor structures. Various receptors exhibit distinct conformations in their 7TMs to enable diverse ligand binding modes (Basith *et al.,* 2018; Zhang, Zhao and Wu, 2015). On the other hand, the ECL1 and ECL3 typically lack structural features and are relatively short in length (Unal and Karnik, 2012).

1.3 GPCR heterotrimeric G protein signalling

Intracellular signal transduction is initiated when a ligand binds to an extracellular binding site, activating related heterotrimeric guanine nucleotide-binding proteins (G proteins) that are made up of three subunits: Gα, Gβ and Gγ (Figure 1-2) (Hilger *et al.,* 2018). They attribute their name to the chemical that governs their actions, the energy carrier guanosine triphosphate (GTP), and its inactive counterpart guanosine diphosphate (GDP) (Calebiro *et al.,* 2021). There are 16 Gα subunits encoded in the mammalian genome, along with six different Gβ-subunits and 12 Gγ-subunits. Various G subunits have the ability to combine together and create a wide range of heterotrimeric G protein complexes, which serve as a diverse array of signalling mediators. Although Gα subunits can signal on their own, Gβ and Gγ subunits can signal as a heterodimer (Gβγ) (Gilman, 1987). Furthermore, Gα subunits are categorised into four functional families, which are determined by their signalling mechanisms and the degree of sequence conservation. These families include Gαs (Gαs(S), Gαs (XL), and Gαs (olf)), Gαi/o (Gαo, Gαi (1-3), Gαt, Gαz, and Gαgust), Gαq/11 (Gαq, Gα11, Gα14 and Gα15/16), and Gα12/13 (Gα12 and Gα13) (Neer, 1995; Syrovatkina *et al.,* 2016; Wettschureck and Offermanns, 2005).

In response to stimuli or ligand interaction, receptors undergo conformational changes that activate guanine exchange factor activity, exchanging GDP for GTP on the Gα subunit (Figure 1-2). This leads to a rearrangement of the G protein complex and disassociation of the GTP-bound Gα and Gβγ subunits (Janetopoulos, Jin and Devreotes, 2001; Oldham and Hamm, 2008). Disassociated G protein subunits transmit the signal to generate various intracellular second messengers, including cyclic adenosine monophosphate (cAMP), inositol triphosphate (IP3), and diacylglycerol (DAG) (Neves, Ram and Iyengar, 2002). The signalling is stopped when GTP is hydrolysed to GDP, which is facilitated by the Ras-like GTPase domain of the Gα subunit. This process is facilitated by the interaction of regulators of G protein signalling, which promotes the re-association of G protein subunits *(Mann et al.,* 2016).

It was previously believed that GPCRs would only activate a specific G protein pathway when stimulated by an agonist. Nevertheless, the complexity of GPCR biology surpassed initial assumptions, as receptors have the capability to connect with various G protein subtypes (Wootten *et al.,* 2018).

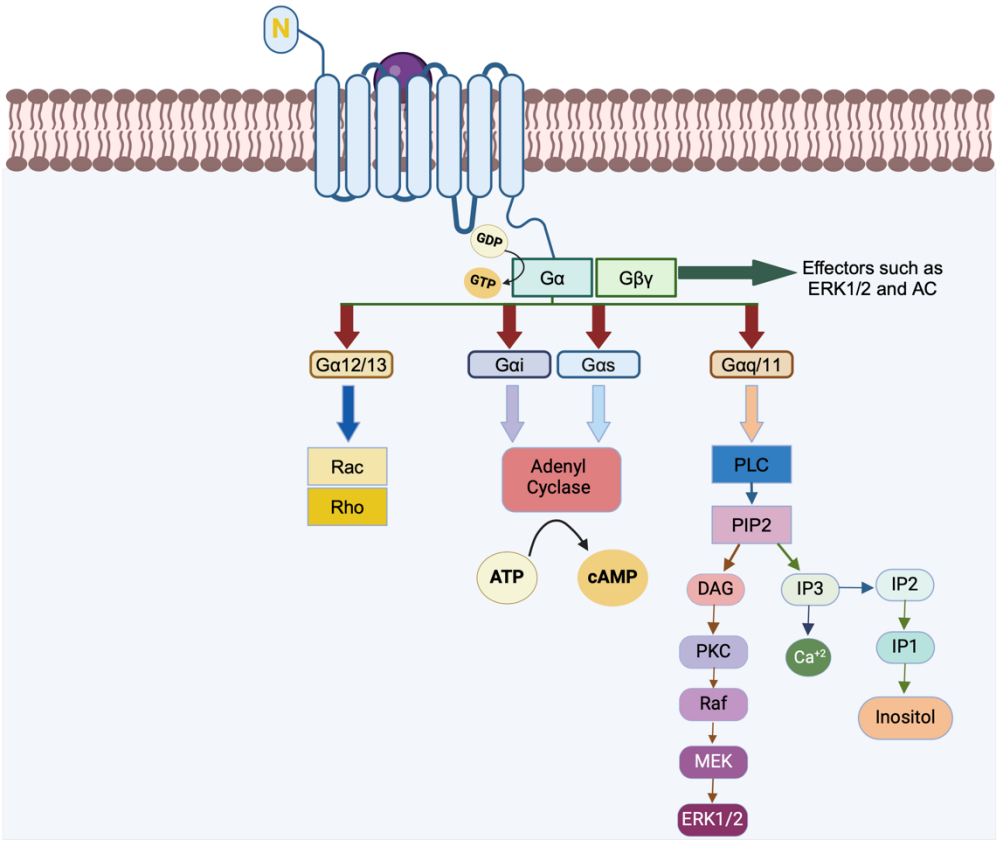


Figure 1-2: Signalling pathways mediated by G proteins coupling. GPCRs undergo structural transformations as a result of ligand binding, which activates the receptors. Structural modifications play a role in guanosine nucleotide exchange in Gα subunits, leading to the separation of Gα and Gβγ subunits. Gα subunits are classified into four families: Gαs, Gαi/o, Gαq, and Gα12/13. The Gα12/13 family stimulates Rho GTPases, whereas the Gαs and Gαi families control adenylate cyclase (AC) activity. The Gαq subunit initiates the activation of phospholipase C (PLC), which breaks down phosphatidylinositol 4,5-bisphosphate (PIP2) into two second messengers: inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). Within the IP3 pathway, the release of Ca^{2+} from intracellular storage is initiated by IP3, which is then dephosphorylated to form IP2, IP1, and inositol in the inositol phosphate pathway. The DAG-PKC-Raf-MEK-ERK1/2 cascade, which plays a vital role in Gαq signalling, progresses in the following manner: DAG, which is generated by PLC, stimulates the activation of protein kinase C (PKC) in the presence of Ca^{2+}. PKC then triggers the activation of Raf (Rapidly Accelerated Fibrosarcoma), which in turn phosphorylates and activates MAPK/ERK kinase MEK. Ultimately, MEK phosphorylates and stimulates the activation of ERK1/2. Furthermore, Gβγ subunits may activate effectors such as ERK1/2, adenylyl cyclase, and Rho GTPases.

1.4 GPCR β-arrestin signalling

1.4.1 Arrestin

Arrestins are groups of intracellular proteins that mediate the function of G protein-coupled receptors (GPCRs); they also mediate trafficking and signalling of these receptors (Peterson and Luttrell, 2017). The majority of mammalian species comprise the following four arrestin subtypes: visual arrestin-1 and -4 (historical names S-antigen, visual or rod arrestin), arrestin-2 (also known as β-arrestin or β-arrestin-1), arrestin-3 (β-arrestin-2), and arrestin-4, all of which are specifically found in photoreceptors of the retina and inhibit light-induced signalling of photopigments in rods and cones (Gurevich *et al.,* 2008; Lohse *et al.,* 1990; Murakami *et al.,* 1993; Pfister *et al.,* 1985).

When arrestins were first discovered, they were described for their function in desensitising GPCRs. Arrestins were found based on their structural similarity to visual arrestin (arrestin-1), which was given its name due to its capacity to "arrest" rhodopsin signalling in the retina of the eye (Wilden *et al.,* 1986). β-arrestin-1 and β-arrestin-2 are two distinct isoforms of arrestins (also referred to as arrestin-2 and arrestin-3, respectively). Both are abundantly distributed in the body and they share 78% sequence identity (Attramadal *et al.,* 1992). In addition, there are two distinct types of arrestin-GPCR complexes known as class A and class B, which are determined by the receptor's affinity for arrestin. Class A receptors have been found to have a brief interaction with arrestin, while class B receptors exhibit a stronger affinity (Kahsai, Pani and Lefkowitz, 2018; Oakley *et al.,* 2000). This classification should not be mistaken for other GPCR family classifications and solely distinguishes receptors based on their interactions with arrestins.

1.4.2 Desensitisation and internalisation of receptors

A two-step mechanism involves the recruitment of β-arrestin to a GPCR, whereby the arrestin not only identifies the transmembrane domains of the active GPCR conformation after agonist activation, but also the locations of receptor phosphorylation. This enables the arrestin to undergo a conformational change, which enables it to bind to the GPCR with a strong affinity (Sommer, Hofmann and Heck, 2014; Tobin, 2008). The relationship between β-arrestin and GPCRs is mainly reliant on interactions with the phosphorylated residues on intracellular residues inside the GPCR. However, there have also been reports of coupling mechanisms that are independent of phosphorylation processes. For instance, β-arrestin

binding in 5-HT2C and β2AR is mediated by the first 10 residues of ICL2 (Marion *et al.,* 2006). Furthermore, it has been proposed that IL3 plays a role in the binding of β-arrestin to various receptors, such as vasopressin (V1) and lutropin receptors, without the need for phosphorylation (Gurevich and Gurevich, 2006; Mukherjee *et al.,* 2002). When β-arrestin binds to the phosphorylated intracellular residues of the receptor, it creates a barrier that prevents G proteins from binding to the receptor. This leads to the inhibition of activation and signalling of G proteins, which eventually results in receptor desensitisation. On the other hand, receptor phosphorylation can happen separately from ligand activation through second messenger kinases and tyrosine kinases. This process, known as heterologous phosphorylation, can directly separate the receptors from their G proteins in heterologous desensitisation events (Burns *et al.,* 2014). β-arrestins play a crucial role in various processes related to GPCRs, including desensitisation, endocytosis, trafficking, and signalling events (Thomsen *et al.,* 2016). When β-arrestin binds to the GPCR, it can trigger the GPCR to be taken inside the cell through clathrin coated pits. This process involves interactions with proteins like clathrin and AP-2, which are part of the endocytotic machinery. The GPCR-β-arrestin complex brings AP-2 and clathrin to the membrane in order to internalise the receptor into clathrin coated pits (Bond *et al.,* 2019; Goodman *et al.,* 1996). Two distinct mechanisms of internalisation can occur depending on the nature of the receptor-β-arrestin interaction. β-arrestins, which have a weak interaction with receptors, may dissociate when the clathrin coated pit pinches off and disconnects from the plasma membrane due to dynamin's action. On the other hand, in situations when the connections are stronger, β-arrestin may stay bonded to the GPCR. Additionally, β-arrestins internalised into endosomes function as scaffolding and adapter proteins via their interactions with several signalling molecules (Laporte *et al.,* 2002; Thomsen *et al.,* 2016). Once β-arrestin dissociates, the GPCR undergoes dephosphorylation and is either degraded or recycled back to the plasma membrane for additional signalling (Thomsen *et al.,* 2016).

1.4.3 The GRK family

Homologous phosphorylation is an agonist-dependent process in which kinases, including G protein-coupled receptor kinases (GRKs), phosphorylate serine/threonine residues located in the C-terminal tail and/or intracellular loops of the target GPCR (Stone *et al.,* 1989; Burns *et al.,* 2014; Prihandoko *et al.,* 2016; Tobin, 2008). On the basis of their sequence similarities, members of the GRK family are categorised into the following three subgroups:

the rhodopsin kinase or visual GRK subgroup comprising GRK1 and GRK7; the 'β-adrenergic' receptor kinase subgroup, GRK2 and GRK3; and the GRK4 subgroup which is comprised of GRK4, GRK5 and GRK6 (Drube *et al.,* 2022). Although similar in terms of targeting GPCRs, the subgroups have their own distinctive regulatory features. The expression of GRK2, 3, 5, and 6 is widespread across mammalian tissues, whereas expression of GRK1, 4, and 7 is limited to certain organs (Gurevich *et al.,* 2012; Sterne-Marr *et al.,* 2013). GRK4 is found in the testes, cerebellum, and kidneys (Penela *et al.,* 2001; Sallese *et al.,* 2000) whilst GRK1 and 7 are found predominantly in the rods and cones of the retina, respectively (Sallese *et al.,* 2000). Modularity is achieved in GRKs by the presence of a short amino-acid terminal α-helical domain (N-helix) and a variable carboxy terminal lipid-binding region (Gurevich and Gurevich, 2019). The catalytic region of GRKs is located inside the regulator of the G protein trafficking homology (RH) region (Claing *et al.,* 2002). The regulated phosphorylation of the vast majority of GPCRs is under the stringent control of the four ubiquitously expressed GRKs (Claing *et al.,* 2002), although the contribution of each may be receptor and cell type specific. Desensitisation, internalisation, and their functional consequences are the results of the engagement of GRKs with particular receptors. Subsequently de-phosphorylation is important to allow receptors to recycle back to the cell surface (Godbole *et al.,* 2017; Matthees *et al.,* 2021). Variation allows diversity, with some GPCRs showing sustained intracellular trafficking, which localises the receptors to particular intracellular compartments, potentially leading to a second wave of endosomal-generated signalling. Such localisation of the receptors is achieved through the GPCRs' ability to bind to their ligands for longer periods of time (Mann *et al.,* 2020). Within a cellular setting, the act of GRK-binding causes active GPCRs to undergo intracellular activation at the places where they are located.

GRKs play not only a vital role as regulators but also determine the actions of β-arrestins by causing ligand-specific GPCR activation or by preferentially coupling to particular active receptor regions (Gurevich *et al.,* 2012). Although the advent structural biology has made significant contributions to our comprehension of the architectural changes that occur in receptors before they engage in contact with G proteins or arrestins, the ubiquitous expression of GRK2, 3, 5, and 6 has made it difficult to understand the functions that each particular GRK plays in the process of receptor activation (Thal *et al.,* 2011). However, the use of selective small molecule GRK inhibitors, such as compound 101 to block GRK 2/3 (Uehling *et al.,* 2021) and compound 18 to block GRK5/6 (Mann *et al.,* 2019), siRNA/shRNA methods (Møller *et al.,* 2020; Thal *et al.,* 2011) or CRISPR/Cas9 approaches

targeting only a specific subset of relevant GRKs (Gurevich *et al.,* 2012; Just *et al.,* 2013), is beginning to unravel the mysteries of these topics. In addition, the use of phospho-site-specific antibodies (Butcher *et al.,* 2011; Divorty *et al.,* 2022; Marsango *et al.,* 2022) and mass spectrometry analysis of the sites of regulated phosphorylation (Briscoe *et al.,* 2006; Butcher *et al.,* 2011; Marsango *et al.,* 2022; Tesmer *et al.,* 2005) are also providing valuable insights. For example, Marsango *et al.* (2022) identified a crucial pair of threonine residues in the medium chain fatty acid receptor GPR84 that are only phosphorylated in response to receptor activation, and this occurs, at least in HEK293 cells, via GRK2/3 (Marsango *et al.,* 2022). This regulation defines efficient interactions with arrestins and allows the separation of G protein-biased and more balanced GPR84 agonists (Marsango *et al.,* 2022). Similarly, Divorty *et al.* (2022) reported phospho-site-specific antisera that act as activation state-specific biomarkers for the orphan metabolite receptor GPR35. Here pre-treatment with the GRK2/3 blocker compound 101 significantly decreased the agonist-induced phosphorylation of human, and particularly mouse, orthologues of GPR35, as detected by these antisera. These studies indicate a critical function for GRK2 and/or GRK3 on key residues to control interactions with arrestins (Divorty *et al.,* 2022).

It is still not known if a particular GPCR is activated by one GRK or by numerous GRKs in a sequential and potentially hierarchical manner. Drube *et al.* (2022) were able to show that various GRKs and second messenger kinases are able to induce diverse outcomes based on the targeted GPCR. For example, the activity of GRKs can be either increased or decreased via the action of protein kinase C (PKC) and the presence of G_q-family G proteins. They recorded a decrease in GRK5- and GRK6-mediated β-arrestin recruitment to the type I angiotensin II receptor (AT1R) when PKC activity was suppressed. Clearly, however, much further analysis will still be required (Drube *et al.,* 2022).

GRK2 and GRK3 are typically found within the cytosol in the absence of GPCR stimulation, but they are nevertheless able to translocate to the cell surface upon stimulation due to their engagement with the βγ-heterodimer of active G proteins (Attramadal *et al.,* 1992). By contrast, GRK5 and GRK6 are routinely membrane-localised (Drube *et al.,* 2022). It is possible, particularly in more complex native cells, that some receptors are found in membrane regions that are inaccessible to GRK5 and GRK6, and this may in part help shape the effects of different GRKs.

1.5 GPCR ligands

A ligand's pharmacological properties define how it will interact with a receptor. The properties of ligands that can be measured include their potencies, effectiveness, and affinity. Affinity refers to the strength of ligand binding to the receptor site, while efficacy is a biological measure that describes the maximum response of the receptor that generates the desired biological effect after ligand binding (Strange, 2008). Measuring ligand binding can help to determine affinity, and there are different methods like using fluorescent or radiolabelled ligands. Sometimes, though, it is not possible to find labelled ligands for the specific receptor of interest. In those cases, potencies are often measured as an alternative (Rosenkilde and Schwartz, 2000; Milligan *et al.*, 2017a). A ligand's potency is defined as the drug concentration necessary to achieve half of the drug's maximum response (EC_{50}) (Salahudeen and Nishtala, 2017). Various functional assays are commonly employed for determining potencies and efficacies or maximal responses. However, it is important to note that values obtained from different assays can vary due to factors such as signal amplification levels and variations in receptor expression in cell lines (Leroy *et al.,* 2007). It is possible that this is due to the fact that efficacy is dependent on the number of drug-receptor complexes that are formed as well as how well receptors are activated to produce cellular responses.

Orthosteric, allosteric, and bitopic are the three types of ligands that may be assigned to the category of receptor ligands, depending on how they impact the function of a receptor or location of their binding site (Figure 1-3) (Mohr *et al.,* 2013). These different binding modes are responsible for the majority of the modulation that may be brought about in the functioning of a receptor protein. In most cases, the orthosteric binding site is occupied not just by endogenous ligands but also by conventional agonists, conventional antagonists, and inverse agonists. Agonists and antagonists are both types of ligands that interact with receptors. Agonists have both affinity and efficacy, meaning they can bind to the receptor and activate it. On the other hand, antagonists also have affinity and can bind to the receptor, but they lack the efficacy to activate it (Salahudeen and Nishtala, 2017). Allosteric ligands are those that have a binding site in the receptor protein positioned somewhere other than the orthosteric binding region (Mohr *et al.,* 2013). Thus, allosteric ligands have a more complicated pharmacological profile. Allosteric modulators may exhibit cooperation with orthosteric ligands in receptors that have numerous binding sites. Following the binding of the allosteric modulator to a second binding site, this cooperativity can appear as a change (increase or decrease) in the affinity of an orthosteric ligand (Bridges and Lindsley, 2008).

Thus, different types of allosteric ligands can be classified based on their properties. These include neutral allosteric antagonists, as well as positive and negative allosteric modulators (PAMs and NAMs). The categorization depends on whether the agonist enhances or reduces the activity of the ligand at the orthosteric binding site (Figure 1-3B, C) (Roche, Gil and Giraldo, 2013).

Orthosteric binding sites for many GPCR families are conserved, which makes it difficult to show selectivity for one family member over another. It is essential to keep in mind that allosteric ligands have the potential to bind to a receptor subtype of interest with a great deal more selectivity than their orthosteric counterparts which called ligand bias (Milligan *et al.*, 2017a; Milligan *et al.,* 2017b). This is due to the fact that the orthosteric binding area is frequently situated in close proximity to or directly at the extracellular loops, which have a low level of sequence conservation between subtypes (Peeters *et al.,* 2011). Lastly, bitopic ligands may be differentiated from other types of ligands because they are capable of binding to two different regions of the receptor. However, they do not necessarily make use of the allosteric and orthosteric binding regions. These molecules bind concurrently to distinct or the same binding regions (including receptors), and they comprise two pharmacophores, either of which may be homobivalent or heterobivalent depending on the conditions that exist (Valant *et al.,* 2012).

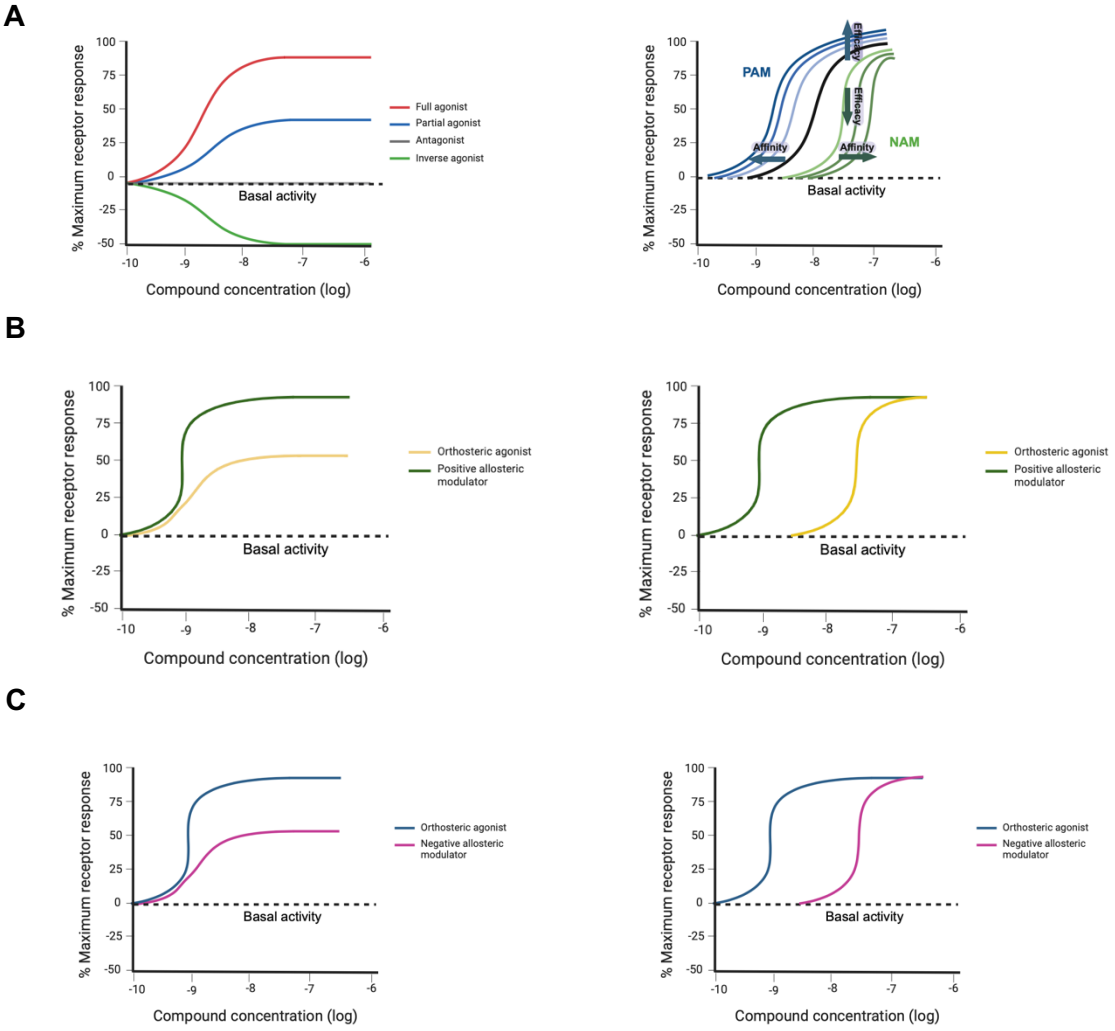


Figure 1-3: Pharmacology of GPCR Ligands. The effectiveness of an orthosteric agonist determines the shape of the concentration response curve it produces. When applied to a receptor, full agonists provide a maximum response, while partial agonists cause a submaximal one. Assuming the receptor exhibits detectable constitutive activity **(A),** inverse agonists cause a reduction in response relative to baseline activity, whereas neutral antagonists do not create any receptor response. **(A)** The black line represents the concentration-response curve of an orthosteric agonist when allosteric ligands are not present. Adding positive allosteric modulators (PAMs) (blue) to an orthosteric agonist enhances its affinity and/or efficacy, whereas adding negative allosteric modulators (NAMs) (green) decreases both. Without orthosteric ligands, allosteric agonists and antagonists act in a manner similar to that of orthosteric agonists and antagonists. Potencies and maximum receptor responses are impacted by PAMs and NAMs, respectively. **(B)** The effects of incubation with PAMs on the potency and maximum response of the orthosteric agonist are enhanced, whereas **(C)** the effects of incubation with NAMs are diminished.

1.6 Biased agonism

Activated GPCRs recruit various transducers, such as heterotrimeric G proteins, GPCR kinases, and β-arrestin, and as a result, may generate a variety of different biological effects. In this sense, biased ligands are those that selectively activate one signalling route over another, and they may be more effective therapeutically (Smith *et al.,* 2018; Tan *et al.,* 2018). Because biased agonists activate just a certain component of a receptor's signalling pathway while suppressing others (Whalen *et al.,* 2011), it is reasonable to anticipate that functional and physiological effects of biased agonists will be distinct from those of traditional balanced agonists. Biased agonism has the potential of producing a whole new class of "smarter" medications that preferentially target therapeutically important signalling route with less adverse effects from non-selective activation or blockage of other signalling pathways. Hence, a few therapies that are currently being used in medical settings have been demonstrated to operate as biased agonists, and this clarify why certain medications in the exact same class have better effectiveness than others (Kim *et al.,* 2008). The dopamine D2 receptor and the μ-opioid receptor (also known as OR) are two examples of biased signalling that have been used in the process of drug development. Initially, it was believed that dopamine D2 receptors affected schizophrenia via Gi/G0, mediated through suppressing the release of cAMP (Girault and Greengard, 2004). On the basis of this knowledge, one would anticipate that blocking the G-protein mediated D2 signalling would be enough to effectively treat schizophrenia. In spite of this, behavioural and biochemical research continues to demonstrate a central role of β-arrestin-2 in signal transduction by D2 dopamine receptors via its control on the AKT-GSK3 pathway (Beaulieu *et al.,* 2007). This is accomplished through the development of a protein complex composed of β-arrestin-2, AKT, and PP2A which stimulates the dephosphorylation of AKT in response to dopamine.

1.7 GPCRs in drug discovery

GPCRs have been of great importance as therapeutic targets of prescribing medications, contributing ~27% of the worldwide share of the marketplace of therapeutic medications, with combined sales for 2011–2015 of around $890 billion in the United States (The IDG Knowledge Management Center, 2016). A number of factors lead to their effectiveness as therapeutic targets. One is that they regulate a huge variety of biological processes in the human body. Additionally, they are the largest group of receptors situated on the cell

membrane (Rask-Andersen, Masuram and Schiöth, 2014). Transmembrane proteins are excellent candidates for drug targeting because drugs can easily reach their targets without having to cross the plasma membrane (Hauser *et al.,* 2017; Sriram and Insel, 2018). Consequently, it should come as no surprise that GPCRs are still a common target for pharmacological research initiatives.

Islet dysfunction, which involves decreased insulin secretion and elevated glucagon secretion, is a key factor contributing to the development of type-2 diabetes mellitus (T2DM) (Cerf, 2013). Free fatty acid receptors (FFARs), such as FFAR1, GPR119, and FFAR4, have become promising targets for treating diabetes and obesity because of their involvement in regulating glucose levels (Milligan *et al.*, 2015; Kimura *et al.*, 2020). Within β-cells, fatty acids affect glucose homeostasis by a mechanism mediated by FFAR1, the most extensively researched FFAR. Certain compounds acting on this receptor can potentially increase insulin secretion, while antagonists may help mitigate the negative effects of excessive stimulation (Li *et al.,* 2018). GPR119 and FFAR4 have also received considerable interest due to their impact on glucose regulation, appetite, and weight management (Sørensen *et al.*, 2021; Alharbi *et al.,* 2022).

Intestinal hormones such as glucose-dependent insulinotropic polypeptide and GLP-1 are essential for maintaining glucose homeostasis (Röder *et al.,* 2016). Studies have demonstrated that GPR119 and FFAR4 can enhance GLP-1 secretion, indicating that drugs targeting these receptors could potentially be useful in managing diabetes and obesity (Patel *et al.,* 2014; Patil *et al.,* 2024; Sundström *et al.,* 2017). With ongoing research into the complex mechanisms of FFARs signalling and its impact on metabolic health, there is hope for the advancement of GPCR-targeted therapies that can effectively address diabetes and obesity.

According to Sriram and Insel (2018), only around 12% of GPCRs have medications that have been approved to target them, despite the fact that GPCRs represent a significant part of pharmacological targets (Sriram and Insel, 2018). Hence, additional investigation is necessary to create suitable medications for these GPCRs in order to enhance the number of pharmacological treatments that are now readily available. The free fatty acid receptor family is one of these receptor families that has not yet been used to its fullest extent for its potential therapeutic value.

1.8 Free fatty acid receptors

1.8.1 Free fatty acids

Fatty acids (FAs) are organic compounds characterised by a carboxyl group and a long aliphatic chain, which can be either saturated or unsaturated (Bloch and Vance, 1977). The majority of naturally occurring fatty acids are composed of a linear carbon chain ranging from 4 to 28 carbons in length, with an even distribution of carbon atoms (Olson, 1966). Fatty acids (FAs) are frequently found in living organisms in the form of three primary ester classes, namely triglycerides, phospholipids, and cholesterol esters. Metabolic intermediates, including fatty acids, are significant not only as nutritional constituents but also as signalling molecules, with their plasma concentration playing a critical role in many different ways. That means, their levels may change based on the pathological and physiological conditions of the body, as well as whether or not the body uses them as a source of energy in addition to providing physiological purposes (Tang and Offermanns, 2015). Saturated fatty acids are free from carbon-carbon double bonds, whereas unsaturated fatty acids possess one or more carbon-carbon double bonds (Alfin-Slater and Aftergood, 1968; Wakil *et al.,* 1983). The existence of unsaturated fatty acids in cis or trans isomers is made possible by the presence of carbon-carbon double bonds, with the cis isomer being the prevailing configuration for the majority of naturally occurring unsaturated fatty acids. Trans fats on the other hand, also known as trans fatty acids (FAs), are not found in their natural configuration and are instead artificially synthesised through a chemical process (Wanders *et al.,* 2017). The variances in geometric configuration among distinct categories of unsaturated fatty acids, as well as between saturated and unsaturated fatty acids, are significant factors in diverse biological processes and the formation of biological structures, such as cell membranes. In the absence of their ester form within the circulating plasma, here FAs are referred to by the terms nonesterified FAs (NEFAs) or free fatty acids (FFAs) (Kimura *et al.,* 2020). FFAs are consistently associated with a transport protein, namely albumin. FFARs present in the bloodstream are a result of the hydrolysis of stored triglycerides (Zechner *et al.,* 2005). Due to their insolubility in aqueous solutions, FAs are conveyed through the bloodstream by associating with plasma albumin. In many vertebrates, including mammals, plasma FFA concentrations are controlled by the number of binding sites on albumin. These organisms employ FFAs as an energy substrate in different tissues (Abdollahi *et al.,* 2022). However, it is important to note that not all organisms use albumin for this purpose. For example, invertebrates and plants do not produce albumin, and they have different mechanisms for transporting and utilizing fatty acids.

The majority of these FFAs have the ability to attach to a particular receptor belonging to the GPCR family, thereby regulating a range of cellular processes. These processes include regulating receptor signalling, gene expression, and maintenance of whole-body fuel energy equilibrium, influenced by different biological conditions (Blad *et al.,* 2012; Peti-Peterdi *et al.,* 2017; Stoddart *et al.,* 2008). One way to identify them is by the presence of a carboxyl group, and as mentioned before they may either be saturated or unsaturated. Research has indicated that certain types of fatty acids play a role in modulating immune system function and metabolic processes. The human body has the ability to transform arachidonic acid into potent signalling molecules, namely leukotrienes and prostaglandins, which play a crucial role in regulating the immune system. The expression of FFA receptors on immune cells has led researchers into their potential therapeutic applications for inflammatory, metabolic and respiratory conditions (Alvarez-Curto *et al.*, 2016; Prihandoko *et al.*, 2020; Croze *et al.*, 2021).

1.8.2 Free fatty acid receptor families

During the early 2000s, it was observed that FFAs have the ability to activate cell signalling independently, serving as natural agonists for four GPCRs, which were initially identified as GPR40, GPR41, GPR43, and GPR120 (Briscoe *et al.,* 2003; Brown *et al.,* 2003; Hirasawa *et al.,* 2005). The finding that free fatty acids may function as signalling molecules eventually resulted in the early identification of cell surface free fatty acid receptors, which were given the names FFAR1-FFAR4 and were each stimulated by a distinct chain length of fatty acid. In general, FFAs are classified into three distinct categories based on the length of their aliphatic tails. These groups include short chain fatty acids, which are composed of 1-6 carbon atoms, medium chain fatty acids, which consist of 7-12 carbon atoms, and long chain fatty acids, which contain over 12 carbon atoms. According to Tazoe *et al.* (2009), short chain fatty acids exhibit specificity towards FFAR2 and FFAR3 activation, while Hirasawa *et al.* (2005) found that medium and long chain fatty acids act as specific agonists for FFAR1 and FFAR4. Each free fatty acid receptor (FFAR) has the ability to function as a FFA sensor that exhibits specificity towards a specific carbon chain length of FFAR, which is obtained from food or metabolites derived from food. The aforementioned receptors for fatty acids are classified as members of the GPCRs of the rhodopsin-like family, as stated by Ichimura *et al.* in 2012.

Sawzdargo *et al.* (1997) initially discovered the genes for FFAR1, FFAR2, and FFAR3 in humans within a cluster of intron-less genes that are tandemly stored and found on chromosome 19q13.1 (Sawzdargo *et al.,* 1997). In 2003, the deorphanisation of FFAR1 was achieved through ligand screening experiments, which coincided with the identification of the activation of FFAR1 upon attaching to long chain free fatty acids, as reported by Briscoe *et al* in 2003. The genomic region 19q13.1 also encompasses the genes that encode for FFAR1 and GPR42. The proteins that have been identified based on these sequences have seven putative transmembrane domains (TMDs), in addition to a wide array of other characteristics that are typical to the class A of GPCRs. Despite exhibiting a 98% homology with FFAR3 as reported by Sawzdargo *et al.* in 1997, GPR42 has been proposed to be a pseudogene in humans according to Brown *et al.*'s findings in 2003. Recent research utilising comprehensive gene sequencing as well as examination of publicly available human genome databases has indicated that the six amino acid variances between FFAR3 and GPR42 may be polymorphisms rather than gene-specific distinctions (Liaw and Connolly, 2009). As a result, a significant number of individuals are expected to exhibit functional GPR42, as reported by Liaw and Connolly in 2009. Regarding FFAR4, the first reason why this G protein-coupled receptor was not included in this class was because it had a low degree of similarity with the other free fatty acid receptors (Stoddart *et al.,* 2008). Subsequently, Fredriksson *et al.* identified it in 2003 through genomic sequencing experiments. Later, in 2005, Hirasawa *et al.* deorphanised the receptor upon discovering that the activation by long chain unsaturated free fatty acids was possible. After the deorphanisation of the free fatty acid receptors, their nomenclature was methodically revised from the GPR receptor classification to the currently employed FFAR1-4 receptors (Davenport *et al.,* 2013; Stoddart *et al.,* 2008).

1.8.3 FFAR1

The expression of FFAR1 is observed in cells and organs that play a crucial role in regulating metabolism, including pancreatic β-cells, monocytes, osteoblasts, osteoclasts, CNS, and enteroendocrine cells (Kimura *et al.,* 2020). Prior research has indicated that FFAR1 signalling is mediated by three distinct G proteins pathways, namely Gq, Gi, and Gs. These G proteins pathways trigger diverse signalling processes, including the activation or inhibition of cAMP, as well as the stimulation of the PLC/IP3/DAG pathway. Consequently, this leads to an elevation of intracellular Ca^{2+} level. The signalling mechanism of this entity involves the activation of Gαq/11 G proteins, which subsequently leads to the stimulation of

insulin secretion, as demonstrated in various studies (Fujiwara *et al.,* 2005; Hauge *et al.,* 2015; Itoh *et al.,* 2003; Kotarsky *et al.,* 2003; Mancini *et al.,* 2015). Of the various disorders under consideration, the role of FFAR1 in stimulating insulin secretion from pancreatic β-cells in response to varying degrees of glucose concentrations has garnered significant interest (Ghislain and Poitout, 2021; Tan *et al.,* 2008). The impact on insulin release is, to some extent, attributed to the augmentation of intracellular calcium signalling through receptor-induced mechanisms (Ghislain and Poitout, 2017; Lin *et al.,* 2012; Lin *et al.,* 2011). This phenomenon is characterised by an increase in intracellular Ca^{2+} levels, triggered by the synthesis of the secondary messenger inositol 1,4,5 trisphosphate (IP3) facilitated by Gq/G11 pathway. Glucose-stimulated insulin secretion (GSIS) in pancreatic β-cells is dependent not only on glucose, but also on other signalling stimuli that are triggered by external calcium, as evidenced by previous studies (Feng *et al.,* 2006; Fujiwara *et al.,* 2005; Itoh *et al.,* 2003). These stimuli that are commonly observed in this context encompass peptide hormones, neurotransmitters, and various other compounds (Carullo *et al.,* 2021). The most common studied FFAR1 ligand is TAK875, a drug developed by Takeda, that underwent clinical trials but was ultimately withdrawn during Phase III due to concerns regarding its potential liver toxicity. This information has been reported in various studies (Defossa and Wagner, 2014; Otieno *et al.,* 2018; Shavadia *et al.,* 2019).

1.8.4 FFAR2/FFAR3

Firstly, the expression of FFAR2 mRNA has been observed in multiple tissues, with the greatest levels of expression occurring in immune cells, including but not limited to neutrophils, eosinophils, monocytes, peripheral blood mononuclear cells, B-lymphocytes, and polymorphonuclear cells. This finding has been documented in various studies, including those conducted by Brown *et al.* (2003), Nakajima *et al.* (2004), Le Poul *et al.* (2003), and Nilsson *et al.* (2003). Le Poul *et al.* (2003) reported the presence of significant levels of FFAR2 mRNA in the bone marrow and spleen. However, it is likely that this observation is attributed to the expression of the receptor by immune cell types. Additionally, Nilsson and colleagues (2003) reported the presence of this receptor in both skeletal muscle and heart. Additionally, FFAR2 mRNA has been documented in adipose tissue (Hong *et al.*, 2005b; Ge *et al.*, 2008), as well as in the distal ileum and colon of rats (Karaki *et al.,* 2006).

Secondly, the expression arrangement of FFAR3 is more extensive compared to that of FFAR2 receptors. Brown *et al.* (2003) reported that preliminary investigations on FFAR3 revealed substantial levels of receptor mRNA in various human tissues, with the most significant expression observed in adipose tissue. A significant expression was also observed in various organs such as the pancreas, spleen, lymph nodes, bone marrow, and blood vessel mononuclear cells (Brown *et al.*, 2003; Le Poul *et al.,* 2003).

Although FFAR2 receptors share tissue expression and activating ligands with FFAR3 receptors, they differ in their signalling pathways. FFAR2 receptors activate $G\alpha_{q/11}$ and $G\alpha_{i/o}$ G proteins and β-arrestin-2, while FFAR3 receptors exclusively signal through the $G\alpha_{i/o}$ pathway. This information has been reported by Le Poul *et al.* (2003), Nilsson *et al.* (2003), and Hudson *et al.* (2012).

1.9 FFAR4

1.9.1 Expression

The present thesis focuses on FFAR4 receptor, which has been detected in various tissue locations (Figure 1-4). Several studies reported that the expression of FFAR4 varies across different body sites and may have distinct physiological functions (Briscoe *et al.,* 2003; Brown *et al.,* 2003; Hirasawa *et al.,* 2005). Hirasawa and colleagues provided evidence that FFAR4 is highly expressed in the gastrointestinal tract and activation of its receptors induces the secretion of glucagon-like peptide-1 (GLP-1) from intestinal cells (Hirasawa et al., 2005). According to Iwakura *et al.* (2010), FFAR4 immunoreactive cells did not exhibit any colocalization in the stomach. However, in the duodenum, a significant level of colocalization was observed with the ghrelin cell population (Iwakura *et al.,* 2010). The FFAR4 receptor is expressed in the ghrelinoma cell line known as MGN3-1, and its role in these cells has yet to be comprehensively elucidated, as reported by Janssen *et al.* (2012). Previous research has demonstrated that FFAR4 is selectively expressed in distinct cellular subtypes, including K cells and brush cells of the intestinal epithelium. Within K cells, FFAR4 has been found to facilitate insulin secretion, as reported by Parker *et al.* in 2009. Janssen *et al.* (2012) showed that FFAR4 was present in the brush cells in a specific area of the mouse stomach, suggesting that FFAR4 could serve as a receptor for detecting long chain fatty acids in the stomach. Furthermore, a different study demonstrated that FFAR4 knockout mice exhibited obesity upon being fed a high-fat diet (Ichimura et al., 2012). Moreover, the knockout mice had glucose intolerance and fatty livers, which further

highlighted the significance of investigating the FFAR4 in those settings. The investigation into the nature of FFAR4 expression in various tissues, including but not limited to pancreas, lung, heart, and skeletal muscle, is still ongoing. Further research is required to fully understand the physiological function of this receptor in these tissues.

Over the last several years, there has been a rise in the number of people diagnosed with T2DM as well as obesity. Consequently, researchers are endeavouring to mitigate these disorders through the development of therapeutic interventions (Hu, 2011). One potential approach involves targeting FFAR4, which serves a crucial function in regulating glucose homeostasis and inducing anti-inflammatory effects. The functions of FFAR4 in the pancreas will be elaborated upon in section 1.10.

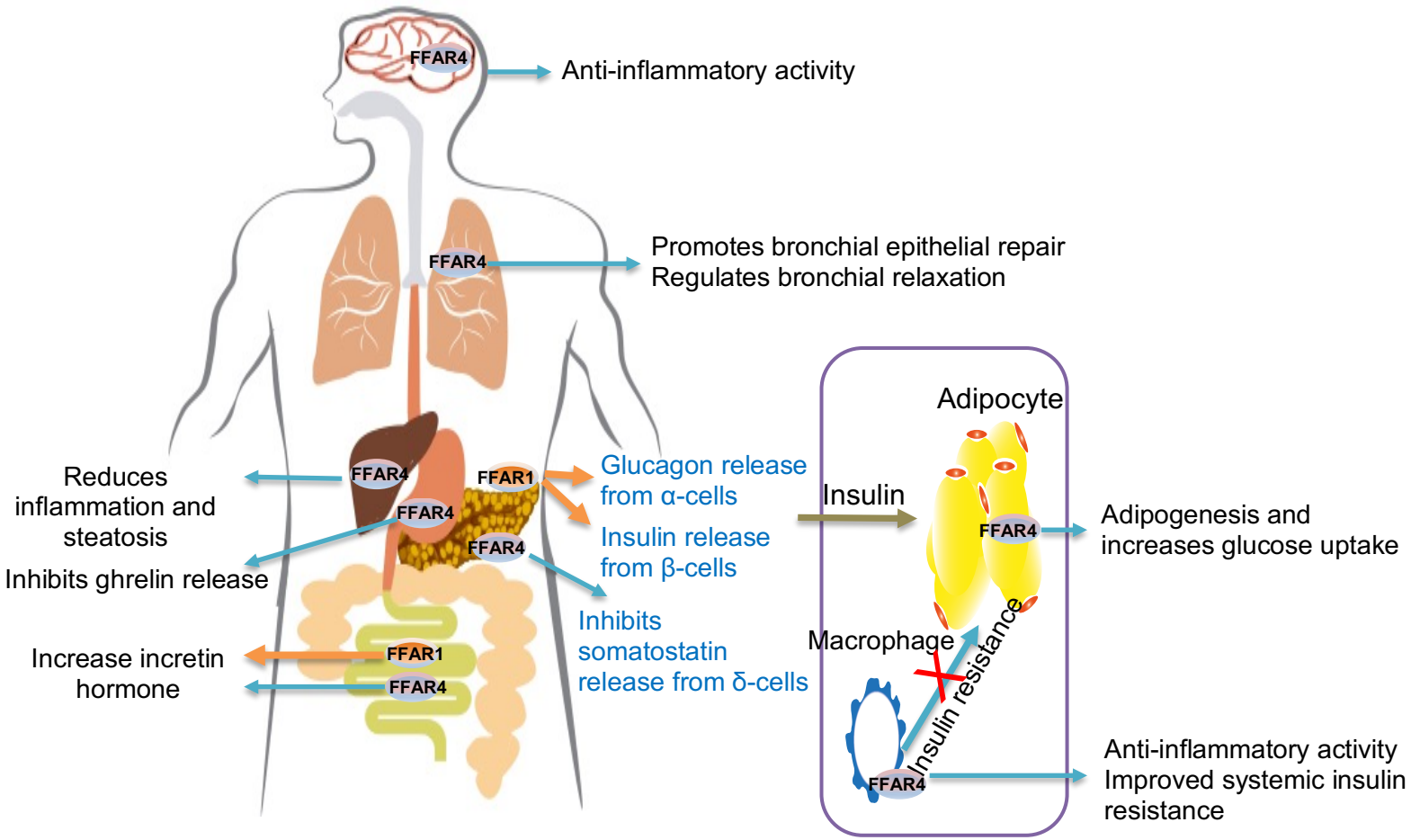


Figure 1-4: Activities that may be regulated by FFAR1 and FFAR4 receptor ligands. Release of glucagon from pancreatic α-cells and insulin from β-cells is induced by FFAR1 that couples to both $G_q/_{11}$ and β-arrestin-dependent pathways. FFAR1 in enteroendocrine cells regulates the secretion of incretin hormones including glucagon-like peptide-1 and cholecystokinin. FFAR4 is also present in enteroendocrine cells, as well as in lungs, brain, white adipose tissue, and the liver. Within adipose tissue, a rise in adipogenesis and glucose uptake is associated with activation of FFAR4. Anti-inflammatory benefits are mostly attributed to the ability of FFAR4 to recruit β-arrestin-2 in an agonist-dependent manner and its subsequent consequences. Orange arrows, illustrates the function of FFAR1 receptors, while blue arrows illustrate the function of FFAR4 receptors in the specified tissue site.

1.9.2 Signalling

Although there is considerable diversity in tissue expression, the activation of three identical intracellular signalling pathways enables the functions of FFAR4 across these distinct tissues mentioned earlier. Receptor interactions with $G_{q/11}$ on FFAR4 are facilitated by ligands, which in turn activate phospholipase C and cause an increase in Ca^{2+} levels. This increase in Ca^{2+} levels leads to the release of L cell-expressed peptide hormones (Figure 1-5) (Moodaley *et al.,* 2017). The importance of the G protein subfamily in the fundamental components of FFAR4 actions is demonstrated by the ability of selective G_q/G_{11} blockers, such as FR900359 and YM-254890, to eliminate such signals (Hudson *et al.,* 2013). Furthermore, this assertion is corroborated by the observation proving that FFAR4 does not elevate inositol phosphates or intracellular Ca^{2+} levels in HEK293 cells, wherein the expression of both Gq and G11 had been ablated via genome editing (Alvarez-Curto *et al.*, 2016). The aforementioned signalling pathway plays a pivotal role in numerous physiological responses mediated by FFAR4 and considered as the primary pathway of this receptor (Alvarez-Curto *et al.,* 2016; Carullo *et al.,* 2021; Milligan *et al.,* 2017b; Son *et al.,* 2021). In contrast, the administration of pertussis toxin, which characterises the role of Gi-family G proteins, eradicates the FFAR4-induced synthesis of the hormone ghrelin (Engelstoft *et al.*, 2013).

Furthermore, it has been observed that FFAR4 is capable of binding to β-arrestin 2, resulting in the internalisation of a complex formed by FFAR4 and β-arrestin. The interaction between agonist-activated FFAR4 and β-arrestins is characterised by rapid and sustained effects, leading to the desensitisation of G protein-mediated functions and receptor (Alharbi *et al.,* 2022; Hudson *et al.,* 2013). The aforementioned observations raise inquiries regarding the possible obstacles in effectively targeting FFAR4 through therapeutic means *(Moodaley et al.,* 2017), which necessitate further comprehension and resolution. Despite the abundance of evidence suggesting the significant anti-inflammatory effects resulting from FFAR4 activation, the underlying mechanisms responsible for these effects remain unclear. Regardless, the fact that there are observable impacts on macrophages, it is evident that these effects are a result of a sequence of events triggered by the β-arrestin-mediated scaffolding of adaptor proteins (Oh *et al.*, 2010; Oh *et al.*, 2014). The significance of arrestin-mediated signalling may vary depending on the type of cell. Alvarez-Curto *et al.* (2016) observed that the impact of arrestins on FFAR4-induced stimulation of ERK1/2 phosphorylation was absent in HEK293 cells that underwent genome editing to eliminate the expression of both of β-arrestin-1 and β-arrestin-2. Despite the fact that HEK293 cells derived from parental

sources are frequently utilised to establish the involvement of ERK1/2 phosphorylation as an arrestin-mediated consequence of heterologously expressed GPCR activation (Alvarez-Curto *et al.*, 2016). In contrast, the elimination of the β-arrestins in arrestin-null cells resulted in the abrogation of desensitisation of G protein-mediated FFAR4 signalling. This suggests that the conventional role of β-arrestins in arresting signalling had been eliminated entirely as reported in a previous study of Alvarez-Curto *et al.* in 2016.

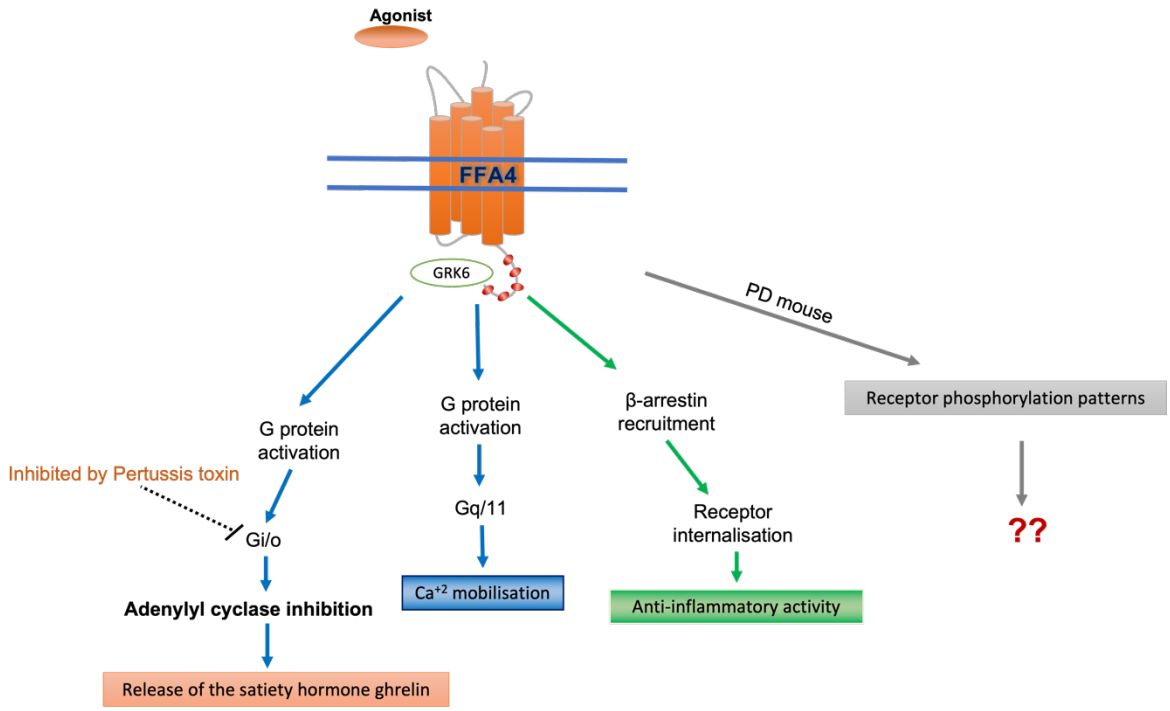


Figure 1-5: FFAR4 engages with a variety of pathways to control signaling and physiological functions. Ligand-induced interactions of FFAR4 with G_q/G_{11} G proteins leads to increased intracellular Ca^{2+} levels. This pathway is fundamental to many of the effects that FFAR4 has in physiological settings. Numerous efforts to develop synthetic ligands of FFAR4 have employed receptor-β-arrestin interaction assays. A key physiological function of regulated FFAR4 engagement with a β-arrestin is the production of anti-inflammatory mediators by macrophages. A number of studies have defined the key sites of agonist-mediated, GRK-dependent (here shown as GRK6) phosphorylation in both human and mouse FFAR4. A transgenic knock-in mouse line expressing a phosphorylation-deficient (PD mouse) form of FFAR4 is available and this will help to assess the specific roles and functions of the phosphorylation of FFAR4, including in mouse models of disease. Studies have also shown the important role for FFAR4 interactions with pertussis-toxin-sensitive G protein to control the release of the satiety hormone ghrelin.

1.9.3 FFAR4 isoforms and orthologs

Two variants of the FFAR4 proteins are produced by alternative splicing of exon 3 of the human FFAR4 gene: a shorter isoform (FFAR4-S) with 361 amino acids and a longer isoform (FFAR4-L) with an extra 16 amino acids in the third intracellular loop (Figure 1-6) (Burns and Moniri, 2010; Cheshmehkani *et al.,* 2017; Moniri, 2016). It is worth mentioning that the extended isoform is exclusive to humans, as it has not been observed in rodents or non-human primates (Moore *et al.,* 2009), with current evidence indicating its detection solely in the colon and in colon cancer cell lines derived from humans (Galindo *et al.,* 2012; Kim *et al.,* 2015). While the long variant's expression seems limited, it functions as a "biased" receptor that cannot activate G protein-mediated signalling systems. However, it can interact with β-arrestins like the short isoform and involve in agonist-dependent internalisation (Watson *et al.,* 2012). The deeper implications of this matter are not yet fully comprehensible as there is no tissue where the extended isoform is exclusively expressed, thereby leading to solely β-arrestin- or non-G protein-mediated signalling.

Studies have demonstrated that the agonism of FFAR4-S exhibits a preference for coupling with Gαq/11 proteins, thereby promoting an elevation in intracellular Ca^{2+} levels. This, in turn, is believed to stimulate diacylglycerol and subsequently trigger the protein kinase C (PKC) signalling cascade (Milligan *et al.,* 2017a; Moniri, 2016; Ulven and Christiansen, 2015). It is noteworthy that the long isoform exhibits an inability to stimulate agonist-mediated Ca^{2+} signals when introduced ectopically in clonal cell lines (Watson *et al.,* 2012). Moreover, there have been accounts of tissue-specific association between FFAR4 and $G\alpha_{i/o}$ and Gαs proteins in pancreatic δ-cells, gastric ghrelin-secreting cells, and intestinal L-cells (Engelstoft *et al.*, 2013; Stone *et al.*, 2014; Tsukahara *et al.*, 2015; Moniri, 2016). Upon stimulation by either endogenous or synthetic agonists, FFAR4-S undergoes quick phosphorylation at its C-terminus by G protein-receptor kinase 6 (GRK6). This event results in a strong association with the essential scaffolding protein β-arrestin-2 (Burns and Moniri, 2010; Burns *et al.,* 2014; Butcher *et al.,* 2014). The physiological importance of FFAR4 is heavily reliant on the aforementioned effect, as β-arrestin-2 functions as a signalling hub that governs several crucial FFAR-signalling outcomes. This has been extensively discussed by other scholars in previous works (Burns and Moniri, 2010; Moniri, 2016; Ulven and Christiansen, 2015).

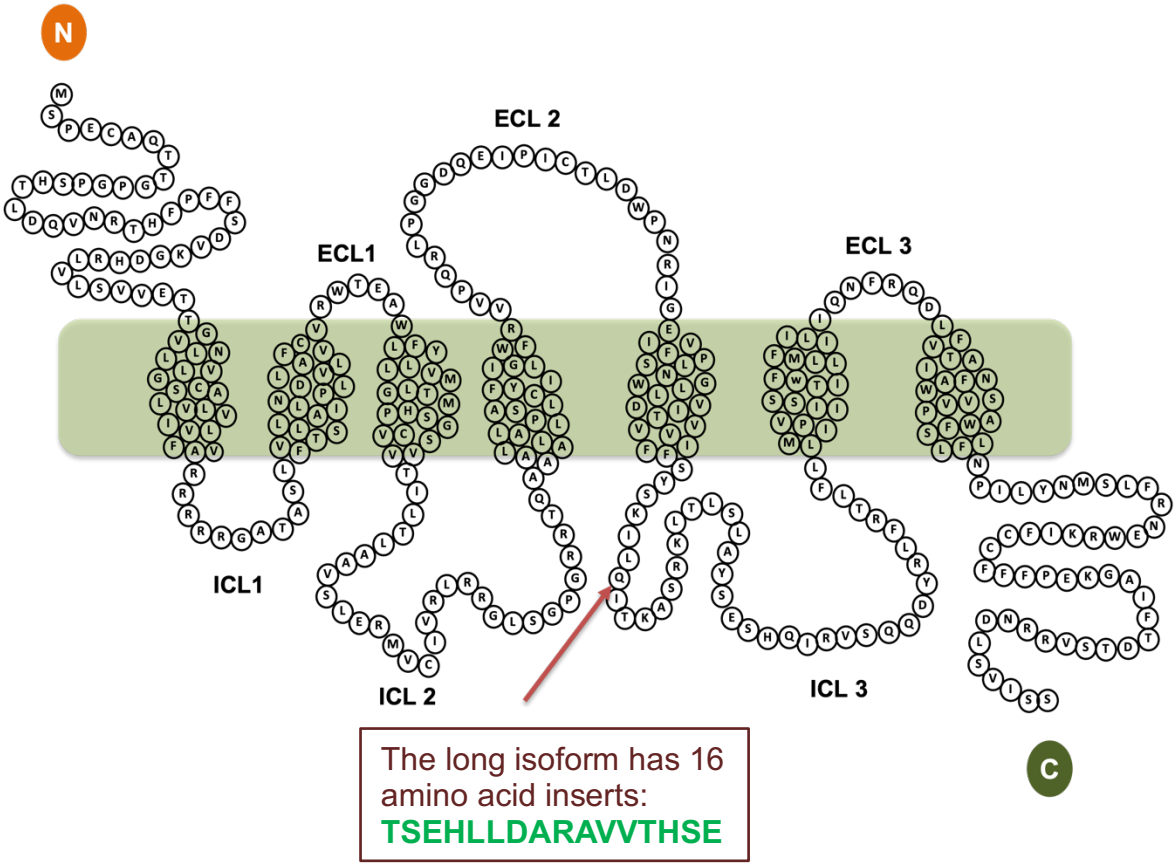


Figure 1-6: Primary amino acid composition of the short isoform of FFAR4. A snake plot is utilised to represent the amino acid residues of the FFAR4 short form isoform. The third extracellular loop has a 16 amino acid insertion that creates the FFAR4 long isoform; its position is shown (after Q position).

1.9.4 FFAR4 agonists and antagonists

Both FFAR1 and FFAR4 are activated by medium- to long-chain free fatty acids, that serve as their natural ligands. Saturated and unsaturated fatty acids, with carbon chains usually between 12 and 22 induce responses from both receptors. due to this overlap in ligand specificity, it is difficult to characterise the existing agonists for these receptors and to distinguish their different functions. It is currently imperative to create and design selective agonists, particularly for FFAR4 receptors, in order to gain a more comprehensive understanding of their unique functions and potential therapeutic applications. Identifying selective agonists helps to unravel the role of different types of receptors and can be employed in studies related to knockout and knockdown strategies. Moreover, it can also help to understand the biological function of these receptors. Despite the fact that FFAR1 and FFAR4 receptors share the same length of fatty chain as the agonist, the receptors are

not closely related. For instance, the initially described FFAR1 receptor agonist, GW9508 has also been shown to have a much higher potency towards the FFAR4 (Briscoe *et al.,* 2006). As such, in the initial absence of selective FFAR4 agonists, GW9508 was used in studies as an agonist for FFAR4 in tissues where FFAR1 is not expressed (Oh *et al.,* 2010). Early studies have described how there has been only limited success in developing a selective FFAR4 receptor agonist, Suzuki *et al.* (2008) synthesised and tested novel ligands as well as ligands that had been previously developed. The compound NCG21, also known as 4-{4-[2-(phenyl-pyridin-2-yl-amino)-ethoxy]-phenyl}-butyric acid, was chosen and modified. It is an active molecule that activates the peroxisome proliferator-activated receptor γ (PPARγ) which showed a limited selectivity for the FFAR4 receptor over the FFAR1. So, researchers put great efforts to develop a selective FFAR4 synthetic agonist that helps to understand the role of this receptor in different tissues.

TUG-891 was the first FFAR4 receptor ligand with greater potency in human cells and tissues, and with more than a 100-fold selectivity for FFAR4 over FFAR1 receptors (Shimpukade *et al.,* 2012). This study was conducted by employing a screen of this compound and several others using β-arrestin-2 bioluminescence resonance energy transfer (BRET) assays that were previously established for the FFAR1 receptor. Compounds that exhibited activity at FFAR4 and demonstrated selectivity towards FFAR1 were subsequently optimised utilising this approach. Of great importance, the compound TUG-891, which is an ortho-biphenyl ligand, has been found to exhibit greater potency and selectivity towards FFAR4 compared to its earlier generations (Shimpukade *et al.,* 2012). This discovery is attributed to the presence of 4-{[4-fluoro-4′-methyl(1,1′-biphenyl)-2-yl]methoxy}-benzenepropanoic acid in the ligand's structure. The aforementioned agonist has undergone extensive research and has been utilised for determining the activity of FFAR4 in various physiological tissues and was recently discovered to have bronchodilatory effect in *ex vivo* and *in vivo* lung tissue samples, suggesting potential relevance for conditions such as asthma (Prihandoko *et al.*, 2020).

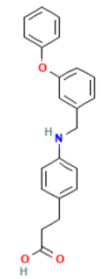
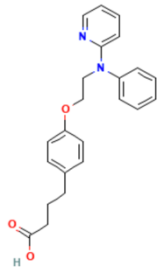
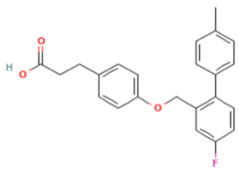
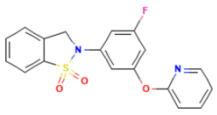
In recent times, several compounds have been documented to exhibit enhanced potency and greater selectivity towards the FFAR4 receptor (Table 1-1). It is not unexpected that the potent activation of FFAR4 can be achieved by chemically modifying these agonists, as reported by Varga *et al.* (2011) and Grygiel-Górniak (2014). Currently, there have been no FFAR4 agonist compounds that have undergone clinical trials. However, it is anticipated that the use of such ligands could result in innovative advantages for individuals with T2DM, as suggested by Scheen (2016) and Suckow and Briscoe (2017).

All of the molecules listed above, much like free fatty acids, have a carboxylate that has been demonstrated directly (or at least simulated) to interact with Arg^{99} residue of FFAR4 (Milligan *et al.,* 2017b). Nevertheless, a couple of recent reports have identified that FFAR4 agonists that contain sulfonamide group, as stated in Sparks *et al.* (2014) and Azevedo *et al.* (2016) as have the ability to selectively activate FFAR4 receptors. An example of these selective FFAR4 agonists is, GSK137647A, which is a compound with the chemical formula (4-methoxy-N-(2,4,6-trimethylphenyl) benzenesulfonamide), which exhibits a selectivity of over 50 times for FFAR4 in comparison to FFAR1, a selectivity maintained across different species. Similar to this, TUG-1197, also known as 2-[3-(pyridin-2-yloxy)phenyl]-2,3-dihydrobenzo[d]isothiazole 1,1-dioxide, is a strong nonacidic sulfonamide FFAR4 agonist that has been reported as having no measurable action at FFAR1 (Azevedo *et al.,* 2016). Although this compound is nonacidic in nature, various mutational and modelling studies suggest that it attaches to the orthosteric binding pocket similar to the carboxylate-containing agonists that bear resemblance to synthetic fatty acids (Azevedo *et al.,* 2016). The development of chemically different types of FFAR4 agonists presents the potential of utilising pairs of compounds originating from distinct series to furnish stronger evidence for particular functions of FFAR4 in different tissues (Hansen and Ulven, 2017).

Despite the absence of clinical trials involving synthetic FFAR4 selective agonists in any disease context, there have been limited clinical investigations utilising naturally occurring FFAR4 active ligands (Alharbi *et al.,* 2022). Two studies were conducted to examine the effects of pine nut oil and olive oil on glucose tolerance and metabolic receptors, including FFAR1, FFAR4, and GPR119, in both healthy and obese individuals (Sørensen, 2018; Sørensen *et al.,* 2021). Pinolenic acid, which is the primary fatty acid constituent of pine nut oil, has been identified as a natural dual agonist of FFAR1 and FFAR4, and has been shown to have similar potency between human and mouse orthologues of both FFAR1 and FFAR4. In rodents, it has been observed to improve glucose tolerance (Christiansen *et al.,* 2015). Nevertheless, it is crucial to take into account that the impact on glucose tolerance may not be solely due to the activation of FFAR4 by pinolenic acid. Both pine nut oil and olive oil have a rich combination of fatty acids and other bioactive compounds, like polyphenols and sterols, that may play a role in the metabolic effects that have been observed.

The only FFAR4 antagonist compound that has been reported to date is AH-7614 (initially known as compound 39), a xanthine derivative of a diarylsulfonamide-based FFAR4 agonist. AH-7614 appears to act as a non-competitive antagonist of FFAR4 and blocks the effects of FFAR4 agonists (Sparks *et al.,* 2014). However, this needs further studies to confirm the exact antagonistic mechanism of this compound. Recently, AH-7614 has been

employed in many studies; for instance, it has been used to indicate the role FFAR4 plays in splenic macrophages which are responsible for the secretion of lysophosphatidic acid that has the capacity to generate systemic resistance to platinum-based anticancer drugs, such as cisplatin and carboplatin (Houthuijzen *et al.,* 2017). Moreover, it has been used to unravel the role of the FFAR4 receptor in activating brown fat (Quesada-López *et al.,* 2016), and to know if the effects of arachidonic acid are mediated by FFAR4 (Villegas-Comonfort *et al.,* 2017). Nevertheless, as reported by Watterson *et al.* (2017), AH-7614 has no antagonist effects on FFAR1, so can be used as a selective antagonist for FFAR4.

Ligand	**Chemical names**	**Chemical structure**	**Actions**	**References**
GW9508	3-(4-((3-Phenoxybenzyl)amino)phenyl)propanoic acid		Prevent fasting-induced plasma ghrelin elevation	(Gong *et al.,* 2014)
NCG21	4-{4-[2-(Phenyl-2-pyridinylamino)ethoxy]phenyl}butyric Acid		Increase plasma GLP-1 levels	(Suzuki *et al.,* 2008)
TUG-891	3-(4-{[5-Fluoro-2-(4-methylphenyl)phenyl]methoxy}phenyl)propanoic acid		Ameliorate inflammation in visceral white adipose tissue and insulin resistance Enhance fat oxidation and reduce fat mass in mice Osteoporosis	(Gao *et al.,* 2015; Gozal *et al.,* 2016; Schilperoort *et al.,* 2018)
TUG-1197	2-(3-fluoro-5-pyridin-2-yloxyphenyl)-3H-1,2-benzothiazole 1,1-dioxide		Type 2 diabetes Inflammatory disorders	(Azevedo *et al.,* 2016)

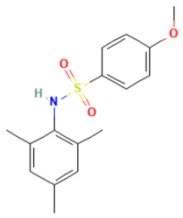
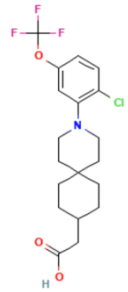
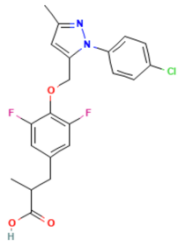
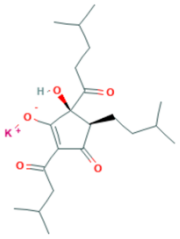
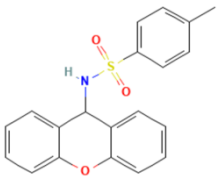
GSK137647A	4-methoxy-N-(2,4,6-trimethylphenyl)benzenesulfonamide		Osteogenic and adipogenic differentiation of bone mesenchymal stem cells Type 2 diabetes	(Wang *et al.*, 2020; Wang *et al.*, 2019)
Merck cpd A	2-(3-(2-Chloro-5-(trifluoromethoxy)phenyl)-3-azaspiro[5.5]undecan-9-yl)acetic acid		Anti-inflammatory effects in macrophages Type 2 diabetes	(Croze *et al.*, 2021; Oh *et al.*, 2014)
Metabolex 36	3-(4-((1-(4-Chlorophenyl)-3-methyl-1h-pyrazol-5-yl)methoxy)-3,5-difluorophenyl)-2-methylpropanoic acid		Study FFAR4 function in pancreatic δ cells	(Stone *et al.*, 2014)
KDT501	potassium;(4R,5S)-5-hydroxy-2-(3-methylbutanoyl)-4-(3-methylbutyl)-5-(4-methylpentanoyl)-3-oxocyclopenten-1-olate		Type 2 diabetes	(Konda *et al.*, 2014)
AH7614	4-Methyl-N-(9H-xanthen-9-yl)benzenesulfonamide		Type 2 diabetes Interstitial cystitis syndrome Non-alcoholic fatty liver disease	(Chen *et al.*, 2018; Kang *et al.*, 2018; Sparks *et al.*, 2014)

Table 1-1: Chemical names of FFAR4 compounds with their actions and chemical structure. Images of chemical structures from PubChem.

1.10 FFAR4 in the pancreas

1.10.1 Background

The pancreas plays an essential part in fuel and energy metabolism, encompassing two primary functions: an exocrine function that facilitates digestion and an endocrine function that governs blood glucose levels (Karpińska and Czauderna, 2022). Moreover, it has a significant impact on the development of diabetes mellitus.

The pancreas is located behind the stomach in the upper left quadrant of the abdominal cavity. Its structure consists of three major parts: the head, body, and tail (Figure 1-7) (Karpińska and Czauderna, 2022). Its biggest part, the head, rests within the duodenal curve; its narrowest part, the body, gradually narrows to the left and becomes the tail next to the spleen. The pancreas is composed of two primary components. The first component is the pancreatic islets, also known as the islets of Langerhans. These islets make up a small percentage of the pancreas and play a crucial role in hormone production. The second component is the exocrine cells, which make up the majority of the pancreas. These cells are responsible for secreting pancreatic juice that contains digestive enzymes (Karpińska and Czauderna, 2022).

The islet cells of the pancreas are spherical clusters of pancreatic endocrine cells that are surrounded by a dense network of acinar exocrine tissue. The endocrine cells of the pancreas comprise approximately 2% of the total pancreatic volume, with the majority of these islets located in the pancreatic tail (Karpińska and Czauderna, 2022; Mense and Rosol, 2018). The pancreatic islets of Langerhans consist of an extensive variety of cellular components, encompassing α, β, δ, polypeptide (PP), epsilon, G, and EC cells (Mense and Rosol, 2018). The β-cells (50%-70%), which secrete insulin, exhibit a polyhedral morphology and are uniformly distributed throughout the pancreatic tissue. α-cells (20%-30%), responsible for glucagon secretion, exhibit a columnar morphology and are primarily located within the body and tail regions of the pancreas (Croze *et al.*, 2021). The δ-cells (10%), which produce somatostatin, possess dendritic structures and exhibit variable distribution. The pancreatic PP cells (2%), secreting pancreatic polypeptide, are located within the cephalic region and uncinate process of the pancreas. Insulin is a hormone responsible for reducing blood glucose levels, whereas glucagon stimulates an increase in blood glucose levels. Additionally, somatostatin hormone functions to inhibit the release of these pancreatic hormones (Mense and Rosol, 2018). A few studies have demonstrated that FFAR4 predominantly promotes the secretion of somatostatin from δ-cells in the pancreas (Stone *et al.*, 2014; Croze *et al.*, 2021).

According to Taneera *et al.* (2012), there is a suggestion that FFAR4 is expressed in human islets and provides protection against apoptosis. However, there is conflicting data regarding the expression pattern of FFAR4 in the endocrine pancreas, as the first findings indicated its absence from both human islets of Langerhans and clonal mouse pancreatic β-cells (Costanzi *et al.,* 2008; Hirasawa *et al.,* 2005). Taneera *et al.* (2012) conducted a systematic genetic approach and found that pancreatic islets of T2DM patients exhibit a significantly low level of FFAR4. Specifically, their research showed that the levels of FFAR4 in the pancreatic islets of T2DM patients were decreased by almost 50% when compared to those without the condition. The significant decrease in FFAR4 expression indicates its probable involvement in the development of T2DM and emphasises the significance of FFAR4 in regulating glucose balance. This knockdown led to a decrease in the eicosapentaenoic acid (EPA), which is an agent that activates the FFAR4 receptor and inhibits the induction of cell apoptosis by palmitic and linoleic acid (Taneera *et al.,* 2012). More recent findings suggested that FFAR4 has been observed to be expressed in islet α, β, δ, and γ cells within the pancreas (Taneera *et al.*, 2012; Stone *et al.*, 2014; Croze *et al.*, 2021). Its activation has been found to alleviate β-cell dysfunction and apoptosis (Taneera *et al.,* 2012), while also modulating islets hormone release (Croze *et al.,* 2021; Zhao, 2022). Stone *et al.* demonstrated that the FFAR4 is not prevalent in the majority of islet endocrine cells, such that their findings align with the initial reports of Hirasawa *et al.* (2005), as they did not observe any indication of the receptor's presence in mouse β-cells. Stone *et al.* previously detected FFAR4 in different clonal rat β-cell lines, indicating the possibility of species variations or that clonal rat β-cell lines may not accurately represent primary cells in terms of FFAR4 expression (Stone *et al.*, 2014).

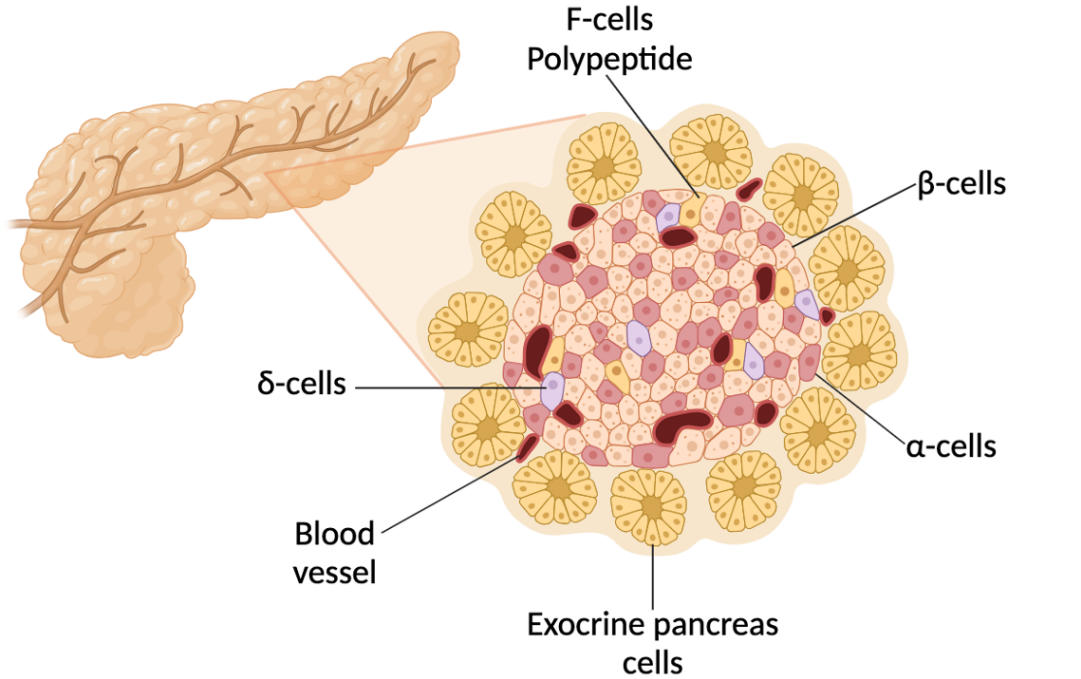


Figure 1-7: The structural composition of the pancreas. The human pancreas is comprised of both exocrine and endocrine components. The endocrine pancreas is structurally composed of clusters known as islets of Langerhans which are composed of several hormone-producing cells, including β-cells, α-cells, δ-cells and F-cells. It represents around 2% of the adult human pancreas. The exocrine pancreas, consisting of acinar and ductal epithelial cells, makes up about 95% of the adult pancreatic mass.

1.10.2 Localisation of FFAR4 in pancreatic cells

The role of long-chain fatty acids (LCFAs) in regulating the release of hormones from pancreatic α-cells has been identified as an important research area of interest. This can be seen as the LCFAs have been found to enhance the release of glucagon from pancreatic islets (Hong *et al.,* 2005a). It has been observed that this effect is more pronounced when saturated variants of LCFAs are used as opposed to unsaturated fatty acids of the same chain length (Hong *et al.,* 2005b). Nevertheless, regardless of whether the LCFAs were saturated or unsaturated, this impact was significantly mitigated in mice who lacked the FFAR4 gene (Hong *et al.,* 2005b). Suckow *et al.* (2014) verified the existence of FFAR4 in pancreatic α-cells and showed that the absence of FFAR4 in mice can lead to compromised glucose haemostasis through alterations in the glucagon axis. According to Stone *et al.* (2014), the detection of FFAR4 in pancreatic δ-cells of mouse islet indicates its role in regulating somatostatin secretion. The promotion of the release of insulin from pancreatic β-cells through intracellular calcium mobilisation has been reported to be facilitated by FFAR4

signalling (Moran *et al.,* 2014; Zhang *et al.,* 2017). Transcriptomic profiling and RT-PCR analyses have revealed that FFAR4 is predominantly expressed in δ-cells, with comparatively lower levels of expression observed in α and β-cells (Taneera *et al.*, 2012; Adriaenssens *et al.*, 2016; DiGruccio *et al.*, 2016; Segerstolpe *et al.*, 2016; Zhao *et al.*, 2020). The confirmation of the favoured expression of FFAR4 in islet δ-cells was achieved through the implementation of a knock-in approach of LacZ reporter into the FFAR4 locus in mice, as reported in literature (Stone *et al.*, 2014). The specific impact of FFAR4 signalling on insulin, glucagon and somatostatin release resulting from its activation in distinct islet endocrine cell types has yet to be determined. Activation of FFAR4 has been reported to enhance glucose-stimulated insulin secretion (GSIS) (Moran *et al.,* 2014; Wu *et al.,* 2021; Zhang *et al.,* 2017), augment glucagon secretion (Suckow *et al.,* 2014; Wu *et al.,* 2021), suppress gastric somatostatin secretion (GSSS) (Stone *et al.*, 2014), and induce pancreatic PP secretion (Zhao *et al.,* 2020).

1.10.3 Pancreatic diseases

The pancreas is an endocrine gland that exerts a significant influence on the systemic physiology of the body. Pancreatic insufficiency (PI) is a condition characterised by the inadequate biosynthesis and/or secretion of digestive enzymes by the pancreas, resulting in an insufficient amount of enzymes to effectively break down and utilise nutrients from food in the intestines (Karpińska and Czauderna, 2022). Pancreatic insufficiency typically arises from pancreatic damage, which may stem from various clinical conditions such as recurrent acute pancreatitis, chronic pancreatitis, diabetes, autoimmune disorders, or post-pancreatectomy surgery. Pancreatic or gastrointestinal cancer can be the underlying cause of such failure.

Inflammatory diseases to the pancreas can be divided to acute and long-term pancreatitis. Long-term pancreatitis (CP) is the prevailing aetiology of exocrine pancreatic insufficiency (EPI) in the adult population, as well as the most prevalent disorder affecting the pancreas (Vege and Suresh, 2022). The malfunctioning of pancreatic cells and premature activation of pancreatic enzymes occur as a consequence of inflammation within the pancreas. The presence of inflammation in the gland hinders the release of insulin, and there is a possibility that digestive enzymes may initiate the degradation of the pancreas and adjacent tissues.

Untreated CP has the potential to result in mortality. From a clinical perspective, various subtypes of CP are identified, each characterised by distinct clinical patterns and morphological imaging characteristics (Barry, 2018; Karpińska and Czauderna, 2022). These subtypes include autoimmune pancreatitis (De Pretis *et al.,* 2018; Finkelberg *et al.,* 2006), paraduodenal pancreatitis (De Pretis *et al.,* 2017; Rebours *et al.,* 2007), and pancreatitis associated with gene mutations (Frulloni *et al.,* 2008).

Acute pancreatitis appears abruptly, devoid of any preceding symptoms. The prevalence of the disease is higher among males and typically manifests within the age range of 30 to 40 years (Karpińska and Czauderna, 2022; Weiss, Laemmerhirt and Lerch, 2019). Patients commonly present with symptoms such as lower abdominal and middle back pain, potentially accompanied by symptoms such as vomiting, dizziness, and increasing sweating. If left untreated, it may become CP which has the potential to induce the development of pseudo cysts within the glandular tissue (Karpińska and Czauderna, 2022). Upon becoming infected, these microorganisms induce the formation of abscesses. In instances of heightened severity, individuals may exhibit symptoms such as blood poisoning, renal dysfunction, respiratory distress, and ultimately progress to a state of shock. In cases of acute pancreatitis, the administration of medications and intravenous fluids may be required (Szatmary *et al.,* 2022). Chronic inflammation may prompt consideration of painkillers, enzyme supplementation, steroid administration, or, in the context of diabetes mellitus, therapeutic intervention. A dietary regimen is essential for the management of both acute and long-term pancreatitis. For the immune system to effectively combat inflammation, it is imperative to maintain a state of equilibrium in the intestinal flora. The immune system is directly influenced by the composition and condition of the gut microbiome, also referred to as gut flora. According to Kuno *et al.* (2003), animal research has demonstrated that a group of medications called angiotensin-converting enzyme (ACE) inhibitors have the potential to alleviate CP and are being considered as a potential treatment option for this condition (Karpińska and Czauderna, 2022). The next section will provide an overview of metabolic diseases with the focus on diabetes and obesity.

1.10.4 Overview of metabolic diseases

In contemporary times, there has been a rise in the incidence of T2DM and obesity cases. As a result, researchers are actively engaged in establishing an effective intervention to mitigate the impact of these medical conditions (Hu, 2011). This can be done through many approaches. One potential approach involves targeting the FFAR4, which is known to play a crucial role in regulating energy homeostasis and promoting anti-inflammatory effects (Oh *et al.*, 2010; Ichimura *et al.*, 2012). The activation of FFAR4 by omega-3 fatty acids has been associated with numerous health benefits, including the modulation of inflammatory effects and potential prevention of metabolic-related conditions (Oh *et al.*, 2010). Abnormal energy homeostasis has been associated with insulin resistance, which typically appears in adipose, liver, and muscle tissues (Matsuzaka and Shimano, 2011). Individuals with insulin resistance, commonly referred to as impaired insulin sensitivity, exhibit a reduced responsiveness to insulin, thereby diminishing the hormone's efficacy (Lee *et al.,* 2022). Consequently, an increased amount of insulin is required to facilitate the uptake of glucose by adipose and skeletal muscle tissues, as well as to maintain its storage in the liver. The aforementioned phenomenon results in an increase in glucose levels in the circulatory system, ultimately leading to the occurrence of hyperglycaemia and glycosuria (Hatting *et al.,* 2018). In response to hyperglycaemia, the pancreatic β-cells increase insulin secretion to maintain glucose homeostasis. Over time, β-cells experience a decline in functionality, leading to an inability to adequately secrete insulin in response to glucose production demands (Prentki and Nolan, 2006). In the end, the body's capacity to sustain glucose homeostasis is compromised, leading to impaired glucose transportation to the liver, muscle, and adipose tissues (Stanford and Goodyear, 2014). The onset of T2DM is linked to the presence of insulin resistance and a malfunction in the secretion of insulin by the pancreas. Predisposing factors for insulin resistance and T2DM encompass obesity, tobacco use, and insufficient physical activity. Furthermore, the prevalence of diabetes is higher among individuals aged 40 years and above, although there has been a rise in the occurrence of the disease among younger populations. The occurrence of those conditions can also be attributed to genetic factors, as evidenced by the higher prevalence rates observed in ethnic groups with a higher risk profile, such as Native American, African American, and Asian groups (Chen *et al.,* 2012).

Moreover, there is a direct correlation between T2DM and obesity. Over the past thirty years, there has been a growing worldwide prevalence of obesity, which has been identified as a key factor contributing to the subsequent epidemic of T2DM. The presence of macrophage-driven chronic inflammation in white adipose tissue is a fundamental aspect of obesity and a crucial determinant in the pathogenesis of insulin resistance and, ultimately, T2DM (Lumeng and Saltiel, 2011; Olefsky and Glass, 2010; Xu *et al.,* 2003). There are multiple indications that suggest the involvement of FFAR4 in the modulation of body weight. The expression of FFAR4 in various organ systems is contingent upon obesity, whereby the expression in the gastrointestinal system exhibits a positive correlation with the individual's body mass index (Little *et al.,* 2014; Widmayer *et al.,* 2012). Obese individuals have shown a notable rise in the levels of FFAR4 expression in both subcutaneous and visceral adipose tissue. Moreover, the strong association between FFAR4 in subcutaneous as well as visceral fat indicates a systemic regulation of this receptor expression. This finding has been reported in a study of Ichimura *et al.* in 2012. In contrast, one investigation discovered that individuals with morbid obesity exhibited reduced levels of the FFAR4 protein and mRNA in their visceral adipose tissue compared to individuals with a lean physique. Furthermore, the expression was observed to be further diminished three hours after the consumption of a high-fat meal in the obese groups, but not in the lean groups (Rodriguez-Pacheco *et al.,* 2014). The aetiology of this discrepancy remains unclear; however, the two studies indicate a correlation between FFAR4 and obesity. The study indicates that mice with a deficiency in FFAR4 receptors and fed with a high-fat diet exhibit a greater propensity towards obesity and metabolic disorders compared to their wild-type counterparts on the same diet (Ichimura *et al.,* 2012). The study findings indicate that there was no notable variance in food consumption among the two groups. However, the basal energy expenditure was considerably reduced in young mice lacking FFAR4, but not in older mice. In rats, it has been observed that a diet high in fat leads to an upregulation of FFAR4 (Cornall *et al.,* 2011). The latest findings indicate that FFAR4 experiences a notable upregulation in the brown adipose tissue of mice following exposure to cold, thereby suggesting the receptor's involvement in energy expenditure (Rosell *et al.,* 2014). The findings indicate that FFAR4's impact on obesity is more probable mediated by metabolic regulation rather than appetite or dietary habits.

1.10.5 Current therapeutic opportunities in metabolic diseases by targeting FFAR4

Moreover, as reported by Khan *et al.* (2020), T2DM accounted for more than one million fatalities globally in 2017. Although there exist contemporary treatments, the demand for more efficient therapies without the unfavourable side effects is clear. The existing T2DM therapies aim to enhance glycaemic control through three distinct mechanisms: (1) augmentation of the release of insulin [via insulin secretagogues], (2) enhancement of insulin action [via insulin sensitizers], and (3) reduction of insulin requirement [via inhibitors of glucose absorption] (Sheehan, 2003). Metformin is the drug of choice for first-line treatment of T2DM because of its ability to lower insulin resistance and has the potential to lower body weight (Goswami *et al.,* 2014). Its mechanism of action involves the reduction of hepatic glucose formation, enhancement of intestinal glucose absorption, and improvement of insulin sensitivity at the hepatic and peripheral tissues, thereby leading to an improvement in glycaemic control (Goswami *et al.,* 2014). However, its usage is frequently restricted since its adverse effects are unbearable and it has numerous contraindications (Goswami *et al.,* 2014). Further agents that aim to target glucagon-like peptide 1 (GLP-1) and glucose insulinotropic polypeptide (GIP), as well as dipeptidyl peptidase-4 (DPP4), have been identified (Goswami *et al.,* 2014). Over the last ten years, GLP-1 has garnered significant interest owing to its impact on regulating glucose through different processes such as augmentation of the release of insulin that is glucose-dependent, slowing down gastric emptying time, management of postprandial glucagon, and decreases food consumption. The phenomenon known as the incretin effect, whereby the insulin secretion response to oral glucose is greater than that to intravenous glucose, serves to demonstrate the involvement of GLP-1 in the maintenance of glucose homeostasis (Nauck *et al.,* 1986). This phenomenon has been observed to exhibit a reduction in individuals diagnosed with T2DM in comparison to those without the condition. This is primarily attributed to a decline in GLP-1 levels (Vilsbøll *et al.,* 2001). GLP-1 is synthesised from the proglucagon gene located in L-cells situated in the small intestine. Its secretion is triggered by the presence of nutrients and its primary function is to stimulate the production of insulin from the pancreatic islets in a glucose-dependent manner (Dungan and Buse, 2005). GLP-1 exhibits an extremely short half-life and undergoes quick degradation to its metabolites through the DPP4 (Demuth *et al.,* 2005). Marín-Peñalver *et al.* (2016) recommend incorporating enhanced dietary and physical activity interventions in conjunction with pharmacological treatments to control T2DM. Accordingly Watterson *et al.* (2014) conducted a comprehensive analysis of T2DM therapies, highlighting the fact that many frequently given

drugs have been linked to negative side effects. Research has shown that certain sulfonylureas and thiazolidinediones might cause weight gain (Krentz, Patel and Bailey, 2008), whilst insulin and sulfonylureas may elevate the likelihood of hypoglycaemia (Zammitt and Frier, 2005).

As stated before in this thesis, the potential of free fatty acid receptors as therapeutic targets for metabolic disorders has garnered significant attention in contemporary times. The variation in response to food intake among different tissues, such as the pancreas and gut, can be attributed to their respective localizations. Due to its high expression in pancreatic β-cells, FFAR1 functions by augmenting the production of insulin caused by high-glucose level in reaction to diverse medium- and long-chain fatty acids (Briscoe *et al.,* 2006; Del Guerra *et al.,* 2010; Itoh *et al.,* 2003). As previously stated in this dissertation, TAK-875 has been found to be unsuccessful in clinical trials due to adverse effects, including liver toxicity, thus resulted in its termination (Kaku *et al.,* 2015). This statement demonstrates the importance of comprehending the exact mechanisms at the cellular level in order to attain the advantageous impact of FFAR1. The evidence suggests that FFAR4 may serve as a promising drug target for T2DM diabetes, as it is associated with both regulation of glucose homeostasis and weight loss (Suckow *et al.,* 2014).

The expression of FFARs receptors in the intestine can be influenced by the existence of obesity, suggesting their significance in the pathophysiology of obesity. Prior research has shown a positive correlation between elevated body mass index (BMI) and heightened expression of human intestinal mRNA FFAR4. Conversely, it was observed that the FFAR1 expression remained unchanged in those same individuals (Little *et al.,* 2014). Additionally, it has been observed that the FFAR1 and FFAR4 receptors exhibit upregulation in the colon of mice with obesity, as reported in a previous study (Peiris *et al.,* 2018). According to the same paper, the mice who had bariatric surgery had the opposite of the expected results, which were associated with the FFAR receptors. The mRNA expression of FFAR1 was observed to be upregulated, whereas that of FFAR4 was downregulated. The authors believed that the observed phenomenon was attributable to the dietary regimen rather than the surgical intervention.

Activation of FFAR1 and FFAR4 have been associated with many health benefits, resulting glucose-dependent release of insulin, protection of pancreatic islets, anti-inflammatory and insulin-sensitizing effects, and release of hormones that regulate appetite and glucose (Christiansen *et al.,* 2015). The confluence of these effects is anticipated to effectively mitigate metabolic disorders. Thus, the simultaneous activation of FFAR1 and FFAR4 seems to be a promising approach for managing metabolic disorders. Dual agonists of FFAR1/FFAR4, even those with moderate potency like pinolenic acid, may have the

ability to produce significant effects as a result of the synergistic activities between the receptors. Pinolenic acid is known to exhibit certain effects that align with the anticipated outcomes of dual FFAR1/FFAR4 agonism (Christiansen *et al.,* 2015).

1.10.6 Mechanism of FFAR4 function in metabolic diseases

Researchers have found and connected two mechanisms that increase the involvement of the FFAR4 receptor in a variety of cell types and tissues (Hudson *et al.,* 2013; Oh *et al.,* 2010; Shimpukade *et al.,* 2012; Sparks *et al.,* 2014). These pathways include the β-arrestin-dependent pathway and the Gq-mediated signalling pathway. The involvement of the β-arrestins pathway in various functions of GPCRs has been reported, along with a particular subset of receptor subtypes, as documented by Miller and Lefkowitz in 2001. Upon stimulation of the receptor, the aforementioned pathway can associate with the cytoplasmic domains of GPCRs and engage in distinct downstream signalling cascades, while also inducing receptor endocytosis, such as the transportation of iron into a mammalian cell (Luttrell and Lefkowitz, 2002). The study conducted by Oh *et al.* (2010) revealed that the recruitment of β-arrestin-2 leads to anti-inflammatory outcomes by means of its association with the transforming growth factor beta (TGF-β)-activated kinase 1 binding protein 1 (TAB1). This association hinders the binding of TAB1 with TGF-β-activated kinase 1 (TAK1), thereby restraining the stimulation of TAK1 and the ensuing signalling to the inhibitory kappa-B kinase beta (IKKβ)/ nuclear factor-kappa-B (NFκB) and c-Jun N-terminal kinase (JNK)/ activator protein 1 (AP1) system. In contrast, it has been suggested that the secretion of hormones and translocation of glucose transporter 4 (GLUT4) are reliant on both Gq/11 and the mobilisation of calcium (Oh *et al.,* 2010).

1.11 Thesis aim

Despite the availability of established therapeutic interventions for metabolic disorders such as obesity and diabetes, the global prevalence of these conditions remains substantial, exerting a significant impact on the well-being of a vast number of individuals. The administration of drug therapies can lead to adverse effects and may exhibit limited efficacy in specific patient groups, thereby underscoring the imperative for the development of innovative and less hazardous therapeutic interventions. As previously stated, FFAR4 presents a promising therapeutic target for T2DM due to its role in the management of

glucose equilibrium in the body. Furthermore, it is possible that the anti-inflammatory benefits produced by FFAR4-mediated β-arrestin-dependent signalling pathways could potentially mitigate mild inflammation in individuals with obesity. The potential anti-inflammatory benefits of these interventions could potentially provide relief from inflammation that contributes to insulin resistance in individuals with T2DM. According to Croze *et al.* (2021), it has been demonstrated that FFAR4 has the ability to enhance the secretion of somatostatin from pancreatic δ-cells, thereby regulating the release of insulin from β-cells and glucagon from α-cells.

The lack of selectivity, effectiveness, and solubility in earlier identified FFAR4 ligands has rendered them unfit for use in clinical settings. Unfortunately, variations in the assay techniques employed have led to inconsistencies in the pharmacological data gathered for FFAR4 ligands. As a consequence, this thesis's overarching goals are to:

- Pharmacologically evaluate FFAR4 ligands in Chinese Hamster Ovary (CHO) or human embryonic kidney (HEK) cells, assessing changes in inositol monophosphate (IP1), cyclic AMP (cAMP), phospho-extracellular signal-regulated kinase 1/2 (pERK1/2), and β-arrestin-2 coupling to describe ligand characteristics and directly evaluate them.
- Identify which pancreatic cell type expresses the FFAR4 receptor *ex vivo* in mice. To do this, mouse lines of FFAR4-WT-HA, FFAR4-KO, and FFAR4-PD will be utilised.
- Conduct *ex vivo* studies to characterise the effects of FFAR4 compounds on the release on insulin and somatostatin from isolated islets from mouse pancreas.

Furthermore, the precise functions of phosphorylation of FFAR4 and the biological consequences of FFAR4-mediated processes that rely on phosphorylation remain unclear. Consequently, further studies were performed using CHO and HEK cell lines, as well as C57BL/6 mice that express a mutant form of the FFAR4 lacking phosphorylation sites. This mutation renders the receptor incapable of activating phosphorylation-dependent signalling pathways. Hence experiments were designed to ascertain the extent to which phosphorylation-dependent signalling pathways contributes to the physiological functions of FFAR4.

Chapter II Materials and methods

2.1 Materials

Water used in the preparation of solutions was miliQ water and the filter was obtained from the ELGA Filtration System (ELGA Labwater, Marlow, U.K.). The following are listings of the essential materials that were employed in this thesis, as well as the respective suppliers; a product identification number (P/N) is also included in the instances when it was feasible to do so.

2.1.1 Ligands Acting on FFAR4

Compound	Supplier
TUG-891	Tocris (P/N: 4601)
TUG-1197	Kind gift from Trond Ulven, Copenhagen University
FFAR4 Agonist II	MedChem Express (P/N: 1234844-11)
Merck compound A	Merck (P/N: 349085-82)
GSK137647A	Tocris (P/N: 5257)
CDT-321	A kind gift from Caldan Therapeutics, 7-11 Melville Street, Edinburgh, EH3 7PE
CDT-347	A kind gift from Caldan Therapeutics, 7-11 Melville Street, Edinburgh, EH3 7PE
CDT-168	A kind gift from Caldan Therapeutics, 7-11 Melville Street, Edinburgh, EH3 7PE
AH7614	Tocris (P/N: 5256)

Table 2-1: List of product number and suppliers of FFAR4 specific compounds. FFAR4 compounds were diluted in DMSO to 10 μM stock concentration and stored at -20°C before use in different experiments.

2.1.2 General Materials and Reagents

2X Laemmli buffer (Invitrogen, P/N: LC2676)

30G x 1/2" Hypodermic Needles (Vet Technology and Solutions, P/N: DE017V)

37% Hydrochloric acid (Thermo Fisher Scientific, P/N: 2042637)

5X Reaction buffer (Invitrogen, P/N: 28025013)

Bradford assay reagent (Pierce™ coomassie plus) (Thermo Fisher Scientific, P/N: 23238)

Deoxynucleoside triphosphate (dNTP) (New England Biolabs, P/N N0447S)

Dithiothreitol (DTT) (Invitrogen, P/N: D1532)

DMEM, low glucose, pyruvate, no glutamine, no phenol red (Thermo Fisher Scientific, P/N: 11054020)

DMSO (Sigma-Aldrich, P/N: D2650)

DNase amplification grade, 10X DNase I reaction buffer and DNase I stop solution (Sigma-Aldrich, P/N: AMPD1-1KT)

Dulbecco's phosphate buffered saline (PBS) (Thermo Fisher Scientific, P/N: 14190094)

Ethanol (Fisher Chemical, P/N: 15242380)

Ethylenediamine tetraacetic acid (EDTA), PH 8.0 (Invitrogen, P/N: 15575-038I)

Fast SYBR green (Sigma-Aldrich, P/N: S9430)

Fatty acid free bovine serum albumin (BSA) (Merck, P/N: 10775835001)

Foetal bovine serum (FBS) (Thermo Fisher Scientific, P/N: 10500064)

Forskolin (Sigma-Aldrich, P/N: F3917)

Glucagon-like peptide I (GLP-1) (Sigma-Aldrich, P/N: G8147-1MG)

Glucose (Sigma-Aldrich, P/N: 137048)

Goat serum (Sigma-Aldrich, P/N: G9023)

Hank's balanced salt solution (HBSS) (Sigma-Aldrich, P/N: H6648-500ML)

HEPES (Gibco, P/N: 15630-080)

Histopaque (Sigma-Aldrich, P/N: 11771)

Hygromycin B solution (Santa Cruz Biotechnologies, P/N: sc-29067)

IBMX (3-isobutyl-1-methylxanthine) (Invitrogen, P/N: PHZ1124)

Igepal CA360 (Thermo fisher Scientific, P/N: J61055.AE)

Immobilon western chemiluminescent horseradish peroxidase (HRP) substrate (Millipore, P/N: WBKLS0100)

L-Glutamine (Gibco, P/N: 25030081)

Liberase™ TL Research Grade 0.1g (Sigma-Aldrich, P/N: 05401020001)

Lipofectamine 2000 (Thermo Fisher Scientific, P/N: 11668019)

Nutrient mixture F-12 ham with L-glutamine and sodium bicarbonate (Sigma-Aldrich, P/N: N6658)

Opti-MEM (Thermo Fisher Scientific, P/N: 31985062)

Penicillin-Streptomycin (Pen-Strep) (10,000U/mL) (Thermo Fisher Scientific, P/N: 15140122)

Phosphatase Inhibitor Cocktail tablets, 20 tablets (PhosSTOP™ EASYpack) (Sigma-Aldrich, P/N: 04906837001)

Precision plus protein All Blue Prestained Protein Standards (Bio-Rad, P/N: 1610373)

Protease Inhibitor Cocktail tablets, 20 tablets (cOmplete™) (Sigma-Aldrich, P/N: 04693116001)

PTX, inhibitor of Gi signalling (Bio-Techne Ltd, P/N: 3097)

Roche Anti-HA High Affinity 50ug (Sigma-Aldrich, P/N: 11867423001)

RPMI 1640 (Gibco, P/N: 11879-020)

Triton X-100 (Sigma-Aldrich, P/N: T9284)

Trypan Blue Solution (0.4%) (Sigma-Aldrich, P/N: T8154)

Tween-20 (Sigma-Aldrich, P/N: P7949)

VECTASHIELD® HardsetTM Antifade Mounting Medium with DAPI (2bscientific, P/N H-1200-10)

YM-254890, potent inhibitor of Gq/11 signalling (Bio-Techne Ltd, P/N: 7352)

2.1.3 Kits

Phospho-ERK (Thr202/Tyr204) cellular kit, 10000 tests (Perkin Elmer, cisbio, P/N: 64ERKPEH)
cAMP - Gs Dynamic kit- 20,000 tests (Perkin Elmer, cisbio, P/N: 62AM4PEC)
IP-One - Gq kit - 20,000 tests (Perkin Elmer, cisbio, P/N: 62IPAPEC)
RNeasy Plus Mini Kit (Qiagen, P/N: 74134)
Rat/Mouse Insulin ELISA (Millipore (U.K.) Limited, EZRMI-13K)
Mouse Somatostatin ELISA Kit (Colorimetric) (Bio-Techne Ltd, NBP2-80270)

2.1.4 Solutions

1X Tris-buffered saline-Tween (TBS-T) (pH 7.4)- 20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween20 (v/v)
Blocking buffer immunohistochemistry- TBS with 0.1% Triton X-100 (v/v), 10% goat serum (v/v) and 1% BSA (w/v)
Citrate buffer (pH 6)- 10 mM Tri-sodium citrate, H_2O until 1000 mL
Insulin buffer- 12.5 mL solution A, 12.5 mL solution B, 2.5 mL 1M HEPES, H_2O until 250 mL
Krebs-Ringer Bicarbonate Buffer (KRBB)- 5.9 mM NaCl, 0.25 mM KCl, 0.15 mM $CaCl_2$ $2H_2O$, 0.05 mM $MgSO_4$ $7H_2O$, 1 mM KH_2PO_4, 25 mM $NaHCO_3$
Radioimmunoprecipitation assay buffer (RIPA) buffer- 25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 (v/v), 1% sodium deoxycholate (w/v), 0.1% SDS (v/v), 1 tablet protease inhibitor (Sigma-Aldrich, P/N: 04693116001), 1 tablet phosphatase inhibitors (Sigma-Aldrich, P/N: 04906837001)
Transfer buffer- 25 mM Tris-base, 192 mM glycine, and 20% methanol (v/v)
Tris-Glycine SDS running buffer- 25 mM Tris, 250 mM glycine, 0.1% SDS (v/v)
Wash buffer immunohistochemistry- Tris-buffered saline (TBS) with 0.1% Triton X-100 (v/v)

2.1.5 List of Primary antibodies

Antibody (Application)	Species	Dilution	Supplier and P/N
GAPDH (WB)	Rabbit	1:5000	Cell Signalling Technology (2118L)
Phospho-44/42 MAPK (ERK1/2) (WB)	Rabbit	1:2000	Cell Signalling Technology (9101S)
Phospho-mFFAR4 (pT347/pS350) (WB)	Rabbit	1:2000	In-House By Prihandoko *et al.* (2016)
Total-p44/42 MAPK (ERK1/2) (WB)	Rabbit	1:2000	Cell Signalling Technology (9102S)
Anti β-galactosidase	Chicken	1:500	abcam (ab9361)
Anti-insulin (IHC)	Rabbit	1:100	Cell Signalling Technology (4590)
Anti-somatostatin (IHC)	Rabbit	1:1000	Fisher Scientific UK Ltd (PA5-82678)
Anti-glucagon (IHC)	Rabbit	1:1000	Cell Signalling Technology (2760)

Table 2-2: Descriptions and catalogues of primary antibodies used in this thesis. Antibodies for Western blotting (WB) were dissolved in a solution of 5% BSA in TBS-T, while antibodies used in immunohistochemistry (IHC) were prepared in blocking buffer IHC.

2.1.6 List of Secondary antibodies

Antibody (Application)	Dilution	Supplier and P/N
IRDye 800CW Donkey anti-Rabbit IgG (H+L) (WB)	1:10000	LI-COR Biotechnology (926-32213)
Goat anti-rabbit (HRP) substrates (WB)	1:5000	Thermo- Fisher Scientific (65-6120)
AlexaFluor™488 Goat anti-Rabbit IgG (H+L) (IHC)	1:400	Invitrogen (A-11008)
Alexa Fluor™ 546 Goat anti-Chicken IgY (H+L) (IHC)	1:400	Invitrogen (A-11040)
Alexa Fluor™ 594 Goat anti-Rabbit IgG (H+L) (IHC)	1:400	Cell Signalling Technology (8889)
Alexa Fluor™ 647 Goat Anti-rat IgG (H+L) (IHC)	1:400	Cell Signalling Technology (4418)

Table 2-3: List and description of secondary antibodies for western blots (WB) and Immunohistochemistry (IHC). The working solutions they were made in is 5% BSA in TBS-T.

2.1.7 Equipment

LI-COR Odyssey Sa Infrared Imaging System (LI-COR Biosciences) Liquid Scintillation Counter (Beckman Coulter, UK LS6500)

FLUOstar OPTIMA Microplate Reader (BMG Labtech)

PheraStar plate reader (BMG Labtech)

Immunoblotting apparatus- power supplies, gel casting apparatus, electrophoresis chamber etc. from the Bio-Rad Mini-PROTEAN range (Bio-Rad Laboratories Ltd.)

QuantStudioTM 5 Real-Time PCR System (Thermo Fisher Scientific)

2.2 Methods

2.2.1 Cell Culture

2.2.1.1 Generation of Cell Lines Stably Expressing the FFAR4

Cell culture techniques were carried out in aseptic environment in class-2 biological safety cabinets and maintained in incubators at 37°C that were supplied with 5% CO_2.

In this thesis I used Chinese hamster ovaries (CHO), stably expressing mFFAR4 with the fluorescent protein eYFP fused to the C-terminal tail (Prihandoko et al., 2016). These cells were generated using the Flp-In™ system, whereby a plasmid (pcDNA5/FRT/TO) containing the FFAR4-eYFP sequence was inserted into parental CHO cells and then Hygromycin B was added into the culture medium to select cells that were stably expressing the receptor.

The plasmid was subjected to a 15-minute incubation period in 500 µL of Opti-MEM, along with the addition of 10 µL of Lipofectamine 2000 at room temperature. Cells that had not undergone transfection were cultured in a 10 cm petri dish and subjected to serum starvation for a minimum of 5 hours. Following this, a transfection mixture containing both the construct and vector was added to the cells. The cells were subjected to overnight incubation at a temperature of 37°C and a carbon dioxide concentration of 5%. Following that, the medium was modified to include a complete growth medium supplemented with hygromycin B. The process of selecting transfected cells was carried out by introducing hygromycin B, as the pcDNA5/FRT/TO plasmid confers resistance to this antibiotic. The medium was replaced every two days until the cells reached confluence. The cells were subsequently rinsed with 2 mL of phosphate-buffered saline (PBS) containing 1 mM ethylenediaminetetraacetic acid (EDTA) and were dislodged from the 10 cm dishes by exposing them to 0.5 mL of PBS containing 1 mM EDTA at a temperature of 37°C and a CO_2 concentration of 5% (v/v) for a duration of 5 minutes. The separated cells were diluted using the appropriate medium and afterward moved to new T-25 flasks that contained new complete medium of FFAR4. (Ham's F12 Nutrient mixture (Fisher Scientific, P/N: 31765) + 10% foetal bovine serum (FBS) + 500 ug/ml hygromycin B + penicillin/streptomycin mix (100 U/mL & 100 µg/mL).

2.2.1.2 Cell line maintenance

CHO cells were cultured at 37°C and 5% CO_2 in a humidified atmosphere and grown to confluence in Nutrient Mixture F12 Ham containing 10% FBS, and penicillin/streptomycin (100 U/mL & 100 µg/mL).
In this study, two CHO cell lines were utilised: a non-transfected cell line and a cell line that exhibited stable expression of the FFAR4 receptor that has an eYFP tag at the C-terminal tail (mFFAR4-eYFP). For stably transfected cell lines (mFFAR4-eYFP) 0.4µg/mL hygromycin (Santa Cruz Biotechnologies) was added to the culture media.

In addition, transiently transfected Human embryonic kidney (HEK) cells were used. The cells were maintained in Dulbecco's modified eagle medium (DMEM) complemented with 10% FBS (v/v), 100 units/mL penicillin, 100 µg/mL streptomycin, and 400 µg/mL hygromycin B (v/v). The cells were subjected to experiments after being treated with 100 ng/ml doxycycline for 24 hours in order to promote the expression of the FFAR4. Cells that were not transfected were maintained in DMEM complemented with 10% FBS (v/v), 100 units/mL penicillin, and 100 µg/mL streptomycin.
The cell lines were cultured until they reached full coverage of the flask, at which point they were transferred to new culture vessels every 3 days. This process involved removing the cell culture medium and rinsing the cells with sterile 1X PBS solution containing 1 mM EDTA. Subsequently, the cells were subjected to incubation with phosphate-buffered saline (PBS) containing 1 mM ethylenediaminetetraacetic acid (EDTA) for a duration of 3 minutes at a temperature of 37°C under a 5% CO_2 environment. This incubation process aimed at separating the cells from the flask. The separated cells were diluted using the appropriate medium and then moved to a new flask that had been sterilised and contained fresh cell culture medium.

2.2.1.3 Agonist stimulation in cells

CHO cells, including non-transfected cells and cells stably expressing mFFAR4, were grown in a 6-well plate and allowed to spread for a period of 2-3 days. Subsequently, the cells were subjected to serum starvation by incubating them overnight with 1 mL of serum-free medium. The cells were subjected to treatment with various concentrations of FFAR4 ligands (Table 2-1) (1 µM or 10 µM) that were dissolved in a 0.01% DMSO vehicle (v/v). Additionally, a control group was treated with 10% FBS (v/v) as a comparison in pERK assays. The cells were subjected to a 5-minute stimulation period at a temperature of 37°C.

Subsequently, the medium was withdrawn, and the cells were lysed in order to prepare for western blot analysis.

2.2.1.4 Cryopreservation of cells in liquid nitrogen/-80°C

The cells were cultivated until they reached approximately 75% confluency, at which point the culture medium was removed through aspiration. The cells were subjected to a washing step using 5 mL of sterile PBS containing 1 mM EDTA prior to being incubated with 2 mL of PBS/1 mM EDTA for a duration of 3 minutes at a temperature of 37°C in an environment with 5% (v/v) CO_2. This incubation was performed to facilitate the detachment of cells from the flask. The detached cells were diluted using the appropriate medium and subsequently transferred to a sterile 15 mL falcon tube in order to undergo centrifugation at a force of 1500 x g for a duration of 3 minutes. The medium was subjected to aspiration, and subsequently, the cells were resuspended in a freezing medium consisting of 1 mL of FBS supplemented with 10% (v/v) DMSO. Subsequently, the cells were carefully transferred into a cryotube and subjected to freezing at a temperature of -80°C, intended for short-term utilisation, or alternatively, they were preserved in liquid nitrogen at -180°C for extended periods.

2.2.1.5 Receptor internalisation

CHO cells that express mFFAR4-eYFP were grown on 20mm glass coverslips placed in a 6-well plate at a density of $3x10^5$ cells/well and allowed to grow overnight. These cells were then serum starved overnight before treatment with 10 µM FFAR4 ligands for 10 minutes. Subsequently, media were aspirated and then fixed with 10% formalin in PBS for 30 minutes at 4°C. The cells were then washed three times with 2mL PBS for 30 minutes. The coverslips were mounted using Vectashield mounting media containing DAPI (1.5 µg/ml) (Vector Laboratories) to see the nucleus. The samples were visualised using an LSM 880 confocal laser scanning microscope (Zeiss) at a 60x objective lens. Image acquisition and analysis were performed using Zen software (Zeiss).

2.2.2 Immunoblotting

2.2.2.1 Sample preparation for Western Blot

After treatment, cultured cells were incubated with 300 µL of RIPA buffer per well, for 30 minutes on ice. The lysates were scraped and subsequently centrifuged at a speed of 12,000 x g at 4°C for 10 minutes in order to eliminate cellular debris. The supernatants were collected, and protein concentration was calculated according to the protocol outlined in the subsequent section. The cell lysates were then combined with 4x Laemmli sample buffer in order to achieve a final concentration of 1x. The samples were subjected to a temperature of 65°C on heat block for a duration of 5 minutes and subsequently underwent a brief centrifugation process to mix the contents prior to being loaded onto a gel.

2.2.2.2 Protein quantification

The quantification of protein in cell lysates was performed using a Bradford assay. Initially, a 10 µL aliquot of lysate/RIPA control was combined with 990 µL of distilled water in a 3 mL cuvette tube. Subsequently, 1000 µL of Bradford reagent was introduced into the mixture. The measurement of absorbance was conducted using a spectrophotometer (Eppendorf BioPhotometer) at a specific wavelength of 595nm. The concentrations were determined by interpolating values from a standard curve, which was generated by measuring the absorbance of standards with known concentrations.

2.2.2.3 SDS-PAGE

The Bio-rad mini-Protean III equipment was utilised to cast polyacrylamide resolving gels. The determination of the ultimate acrylamide percentage was contingent upon the size of the protein under investigation, with a standard of 8% applied to proteins exceeding 60 kDa, and 12% applied to proteins below 60 kDa. The other components used in the experiment included a solution of 375 mM Tris-HCl (pH 8.8), 0.1% SDS (w/v), 0.1% ammonium persulfate (APS) (w/v), and 200 nM N,N,N',N'-tetramethylethylenediamine (TEMED), which were appropriately diluted in distilled water. The gel thickness was 1.5 mm with either 10 or 15 wells. The casting of the stacking gel was performed on the upper surface of the resolving gel subsequent to its solidification. The reagents utilised for the stacking gel preparation had a final concentration of 5% acrylamide, 125 mM Tris-HCl (pH 6.8), 0.1%

SDS (v/v), 0.1% APS (v/v), and 200 nM TEMED. The samples were typically subjected to electrophoresis using a Tris-glycine SDS running buffer. Each lane was loaded with 10 µg of protein, and the gels were run for approximately 1 hour or until the samples migrated to the end of the resolving gel, which typically occurred within 50 minutes at 200V in 1X Tris-Glycine running buffer.

2.2.2.4 Western blot probing and detection

In a semi-dry transfer Transblot machine (BioRad), SDS-PAGE gels were transferred onto nitrocellulose membrane at 25 V for an hour. The membranes were subsequently blocked using a 5% BSA (w/v) solution prepared in TBST pH 7.4 for a duration of 1 hour at room temperature. The membranes were subjected to overnight incubation at a temperature of 4°C with primary antibodies, as specified in Table 2-2. The primary antibodies were prepared in a solution containing 5% BSA (w/v) diluted in TBST. Subsequently, the BSA solution containing the primary antibodies was stored at -20°C for a second use. Additionally, the membrane underwent a series of three washes using TBST, with each wash lasting for a duration of 15 minutes. The LI-COR secondary antibody was prepared by diluting it in TBST and combining it with a 5% BSA. This mixture was then added to the experimental samples, as indicated in Table 2-3. The samples were subsequently incubated in a dark environment at room temperature for a duration of 1 hour. The membranes underwent a triple wash in TBST solution for a duration of 15 minutes each prior to the development process. The experimental procedure was conducted under conditions of low light intensity for the membranes that were subjected to probing with LI-COR antibodies. Membranes were prepared using two distinct methods: the Immobilon western chemiluminescent HRP substrate which added to the membranes at a concentration of 1:5000 for 15 seconds. This method was used in conjunction with an Xomat machine which was used to detect FFAR4 bands on X-ray films. Alternatively, a LI-COR development system with LI-COR secondary antibodies at a concentration of 1:10,000 was used for the detection of all other proteins. The quantification of blot images was performed using ImageJ software. Band intensities were quantified and normalised to the loading control for determining the relative amounts of protein expression.

2.2.3 Pharmacological and functional assays

The assays used in these experiments, including pERK1/2, cAMP, and IP1 assays, utilise the principles of fluorescence resonance energy transfer (FRET) to measure the quantities of particular molecules or the phosphorylation status of proteins. These experiments utilise two distinct antibodies, each labelled with a separate fluorescent marker.

The first antibodies are linked with Eu^{3+}-cryptate, which acts as a luminous donor and emits light at a wavelength of 620 nm. The second antibodies are tagged with a d2 conjugate which functions as the recipient, emitting light at a wavelength of 665 nm. In the pERK1/2 experiment, one antibody selectively detects the phosphorylated motif on the ERK1/2 protein, whereas the second antibody binds to the protein independent of its phosphorylation status.

When phosphorylated ERK1/2 is present, both antibodies attach to the protein, causing the Eu^{3+}-cryptate and d2 labels to become close together. A FRET signal is produced when energy is transferred from the donor to the acceptor due to this close proximity. The magnitude of this FRET signal is closely correlated with the degree of ERK1/2 phosphorylation, offering a quantitative assessment of the protein's activation status.

The cAMP and IP1 experiments, while both relying on FRET, exhibit slight differences in how they work. These assays are based on the principles of competition binding, where the synthesis of naturally occurring cAMP or IP1 molecules competes with the binding of labelled cAMP or IP1 molecules to their corresponding antibodies. As the levels of endogenous cAMP or IP1 rise, they compete out the tagged molecules, causing a reduction in the FRET signal. The reduction in signal strength is directly proportional to the quantity of cAMP or IP1 generated in the sample.

2.2.3.1 cAMP assay (CHO cells)

The assay was performed on CHO cells following the protocol provided by the CisBio cAMP kit. For this experiment, 30,000 cells were placed in each well of a 96-well plate and left to attach overnight. In order to synchronise the cells and reduce the impact of serum-derived factors on the assay, the cells were serum starved overnight with 1mL of serum free media. By implementing a serum starvation step, the cells can be brought to a quiescent

state, effectively minimising any background noise that may interfere with the accuracy of cAMP measurements. After serum starvation, the cells were exposed to different FFAR4 ligands to evaluate their effect on cAMP levels. The stimulation buffer was prepared by adding 0.5 mM IBMX, a non-selective phosphodiesterase inhibitor.

Gαs signalling: Following a 15-minute incubation period in 90 µL of stimulation buffer, 10 µL of serially diluted FFAR4 ligands or forskolin in serum-free medium were added to the cells. This exposure lasted for a duration of 1 hour and 45 minutes, resulting in a final compound concentration range of 0.1 nM to 3.16 µM. After the incubation period with the ligands, the medium was extracted and subsequently 50 µL of lysis buffer was introduced into each well. In order to disrupt the cellular membranes, the plate was subjected to agitation using a plate shaker operating at 600 rpm at room temperature for a duration of 30 minutes, followed by subsequent freezing at a temperature of -20°C.

Gαi signalling: Following a 15-minute incubation period in 90 µL of stimulation buffer, a mixture of serially diluted FFAR4 ligands in serum-free medium and EC80 forskolin concentration was added to the cells. The EC80 forskolin concentration was determined using GraphPad Prism software and values obtained from Gαs studies. The cells were then co-incubated with the mixture for a duration of 1 hour and 45 minutes. After the incubation with the agonist, the medium was removed and 50 µL of lysis buffer was added into each well of the 96-well plate. In order to induce cell lysis, the plate was placed on a plate shaker at a speed of 600 rpm at room temperature for 30 minutes, followed by subsequent freezing at a temperature of -20°C.

2.2.3.2 pERK1/2 assay (CHO cells)

The pERK1/2 assays were conducted on CHO cells following the instructions provided in the CisBio phospho-ERK (Thr202/Tyr204) kit. The cells were seeded into 96-well plates at a density of 30,000 cells per well and incubated overnight. The cell medium was replaced by 90 µL of serum-free medium and the following day the cells were stimulated with the FFAR4 ligands. For the antagonist experiments, 80 µL of the serum free media were utilised, and cells were pre-incubated with 10 µL of FFAR4 antagonist at serial concentrations for 30 minutes. Afterwards, 10 µL of FFAR4 ligands in serum-free medium was added at concentrations ranging from 0.1 nM to 3.16 µM. The cells were incubated for 5 minutes in

concentration-response experiments, or 30 minutes in time-response experiments. The medium was removed, and the cells were incubated in 50 µL of lysis buffer, for 30 minutes at room temperature, on a plate shaker at 600 rpm. Subsequently, the lysed cells were stored at a temperature of -20°C.

2.2.3.3 IP1 Accumulation assay (CHO cells)

The IP1 assays were conducted according to the manufacturer's instructions provided in the CisBio IP1 kit. The determination of Gαq protein signalling involved the measurement of the inositide signalling pathway, specifically by detecting the accumulation of the by-product inositol-1-phosphate (IP1). The cells were distributed at a density of 30,000 cells per well on 96-well plates and incubated overnight before conducting the assay. Following a 30-minute incubation period in 90 µL stimulation buffer per well, cells were exposed to 10 µL of FFAR4 agonists that had been serially diluted in serum-free medium, bringing the total volume to 100 µL per well. This exposure lasted for 1 hour, resulting in a final compound concentration range of 0.1 nM to 3.16 µM. After the incubation with the ligands, the medium was taken out and subsequently, 50 µL of lysis buffer was introduced into each well. In order to lyse the cells, the plate was subjected to agitation on a plate shaker operating at 600 rpm at room temperature for a duration of 30 minutes. Subsequently, the plate was stored at a temperature of -20°C.

2.2.3.4 Pharmacological and functional assays quantification

After performing all the experimental procedures outlined in section 2.2.3, 16 uL/well of the lysed cells were introduced into a 384-well proxiplate (PerkinElmer) following a 10-minute incubation on a shaker operating at 600 rpm. In each well, 4 µL of cell lysate containing a 1:1 mixture of cryptate and d2 conjugate antibodies (HTRF assay kit, CisBio) were introduced. The antibodies were diluted at a ratio of 1:40 in lysis buffer.

The proxiplates were agitated for the duration specified in their respective kits, which was approximately for 2 hours. The plates were analysed using the Pherastar system (BMG biotech) at wavelengths of 665 nm and 620 nm. The calculations for pERK1/2 assays involved determining the percentage of maximal response, which was then normalised to TUG-891 or the appropriate positive control, as indicated in each figure. The data was

graphed in the form of a concentration response curve using GraphPad Prism software. The IP1 and cAMP assays were conducted, and the resulting data was graphed using GraphPad Prism as a concentration response curve. The values were then inverted and normalised to the percentage of the maximal response of TUG-891 or the respective positive control, as indicated in each figure.

2.2.3.5 Bioluminescence resonance energy transfer (BRET) β-Arrestin-2 recruitment assay

The HEK293 cells were initially distributed at a density of 2 million cells per 10 cm petri-dish. The following day, cells were transfected using Lipofectamine 2000 transfection reagent according to the manufacturer's instructions. For each Petri dish, a total of 2 µg of plasmid was used, consisting of FLAG-FFAR4-eYFP and β-arrestin-2 fused with Renilla-luciferase (β-arrestin-2-RLuc) at a 1:4 ratio. The plasmid was diluted in 500 µL of Opti-MEM medium (Gibco). Separately, 3 µL of Lipofectamine 2000 was diluted in 500 µL of Opti-MEM and incubated for 5 minutes at room temperature. The diluted plasmid and Lipofectamine 2000 solutions were then combined, gently mixed, and incubated for 20 minutes at room temperature to allow complex formation. The 1 mL transfection mixture was then added dropwise to each Petri dish containing cells in antibiotic-free growth medium. The transfection medium was replaced with fresh growth medium after 6 hours.

The cells were seeded onto 96-well plates with white bottoms and incubated overnight for growth. The cells underwent two rounds of washing with 200 µL of 1x Hank's Balanced Salt Solution (HBSS) w/o phenol red, calcium chloride, and magnesium sulfate, followed by a 30-minute incubation in 80 µL of HBSS buffer. After incubating the samples in HBSS buffer, a volume of 10 µL of 50 µM Renilla-luciferase substrate coelentrazine-h was added to each well. The samples were then incubated for a period of 10 minutes. A series of logarithmic dilutions was prepared for FFAR4 ligands and forskolin, spanning concentrations from 0.1 nM to 3.16 µM. Subsequently, 10 µL of the ligands were introduced into the wells and subjected to stimulation for a duration of 5 minutes. The plates were analysed using a PheraStar plate reader, with measurements taken at a wavelength range of 475-30/520-30 nm. The readings were determined by calculating the percentage of maximal response, which was then normalised to TUG-891 or the respective positive control, as

indicated in each figure. The data was graphed in the form of a concentration response curve using GraphPad Prism software.

2.2.4 Experimental animals

The study utilised four distinct strains of mice: C57BL/6 mice with a hemagglutinin (HA) tag on the free fatty acid receptor 4 (FFAR4) gene (FFAR4-WT-HA), C57BL/6 mice with phosphorylation deficient (PD) mutations on the FFAR4 gene (FFAR4-PD), and C57BL/6 mice where FFAR4 involves a β-galactosidase knockout (FFAR4-KO). A more comprehensive illustration of the animals can be found in Chapter 4. The animals were housed in a controlled facility at the University of Glasgow, where they were subjected to a 12-hour light and dark cycle, maintained at room temperature, and provided with a standard chow diet. The experimental procedures involved the utilisation of adult mice, both male and female, at the age of 12 weeks. The genotyping of mice was conducted using the Transnetyx platform.

2.2.4.1 Ethics statement

Under the authority of personal licence number 17AEE1FEC, which was held by the author, and project licence number 70/8473 or PP7704105, which was held by Professor Andrew B. Tobin of the University of Glasgow, all animal treatments were carried out in compliance with the Animals (Scientific treatments) Act of 1986.

Given that the experiments conducted were *ex vivo* utilising tissue or primary cells, and no *in vivo* experiments were conducted, it was sufficient to possess a Home Office breeding licence (PP0894775) for the experiment, rather than a Home Office personal licence. The ethical standards were upheld by consistently considering the humane euthanasia of all animals and the principles of Replacement, Reduction, and Refinement (the 3R's). In order to minimise the utilisation of animals, initial studies were conducted on a subset of n=3/4 animals. Additionally, power calculations were conducted on experiments that did not yield statistically significant results, with the aim of determining whether additional experiments should be conducted in the future to achieve statistical significance. Moreover, in order to minimise the number of animals used, multiple tissues were extracted from a single mouse.

2.2.5 Quantitative real time polymerase chain reaction (qRT-PCR)

2.2.5.1 RNA extraction from tissue

RNA was isolated from tissue samples using a Qiagen RNeasy Plus Mini Kit, following the guidelines provided by the manufacturer. In this study, adipose tissue or pancreatic islets obtained from mice were subjected to homogenization using RLT Buffer. The homogenates were subsequently centrifuged at 8,000 x g for a duration of 30 seconds using a gDNA eliminator spin column. The resulting mixture was then maintained at a low temperature on ice. The flow-through was collected and combined with freshly prepared 70% (v/v) ethanol. Subsequently, the mixture was transferred to an RNeasy mini spin column and centrifuged at a speed of 8000 x g for a duration of 15 seconds. The resulting solution was then stored on ice. Subsequently, the flow-through was removed and the column underwent a washing process using wash buffers. Following this, the RNA was eluted in a volume of 20 µL of nuclease-free water. The concentration of RNA was measured using a Nanodrop-1000 spectrophotometer by calculating the absorbance at a wavelength of 260 nm.

A DNAse digestion procedure was conducted remove any potential DNA contaminants in the RNA samples that were obtained during the process of RNA isolation from tissue. The experimental setup included the following components: A total volume of 10 µL was prepared, consisting of 1 µg of RNA diluted in 8 µL of nuclease-free water, along with 2 µL of a 1:1 mixture of DNase amplification grade and 10x DNase I reaction buffer. The entire mixture was subjected to incubation at the outside temperature for a duration of 15 minutes, following which 1 µL of DNase I stop solution was introduced to terminate the reaction.

2.2.5.2 Reverse transcriptase PCR

The RNA obtained after DNase treatment was subjected to thermal cycling at a temperature of 65°C for a duration of 10 minutes to denature the enzyme. A PCR master mix was prepared for each reaction, consisting of 4 µL of a 5x reaction buffer, 2 µL of a 100 mM DTT solution, 1 µL of a 50 ng/µL random hexamers solution, 1 µL of a 10 mM dNTP solution, and 1 µL of nuclease-free water. The DNase-digested RNA was supplemented with a PCR master mix and 200 U of MMVL reverse transcriptase enzyme. The PCR tubes underwent incubation at three different temperatures: 25°C for a duration of 10 minutes,

37°C for a duration of 1 hour, and 70°C for a duration of 15 minutes. The resulting cDNA was mixed with nuclease-free water to achieve a final volume of 100 µL.

2.2.5.3 q-PCR

A q-PCR master mix was made by combining 10 µL of fast SYBR green (Sigma-Aldrich), along with 5 µM of both the forward and reverse primers. The resulting mixture was then adjusted to a final volume of 16.8 µL using nuclease-free H_2O. The qPCR primer specifications can be found in Table 2-4.

Primer	Sequence	Supplier
mFFAR4 Forward Primer	GGCACTGCTGGCTTTCATA	Eurofins
mFFAR4 Reverse Primer	GATTTCTCCTATGCGGTTGG	Eurofins
M3 Forward Primer	ACGAGAGCCATCTACTCCATCG	Quanitect
M3 Reverse Primer	TGTCGGCTTTCCTCTCCAAGTC	Quanitect
HA Forward primer	GTGGTGGCCTTCACGTTTGC	Eurofins
HA Reverse primer	AGCGTAATCTGGAACATCGTAAGGGTA	Eurofins
mGAPDH Forward Primer	CGGATTTGGCCGTATTGGG	Eurofins
mGAPDH Reverse Primer	CTCGCTCCTGGAAGATGG	Eurofins

Table 2-4: List of qPCR primers.

In each well of a 96-well PCR plate (Biorad), 16.8 µL of master mix was introduced, which was followed by the addition of 3.2 µL of cDNA to each well. The plates were securely sealed using a polypropylene plate seal and subjected to PCR amplification using a QuantStudioTM 5 PCR instrument, following the cycling parameters outlined in Table 2-5. The procedure outlined in Step 2 was repeated for a total of 44 cycles.

Stage	Temperature	Time in seconds
Preheating	95^{O}C	20
Denaturing	95^{O}C	1
Annealing and Extension	60^{O}C	20
Melt Curve– Heating	95^{O}C	15
Annealing	60^{O}C	60
Heating	95^{O}C	15

Table 2-5: qPCR cycle parameters. List of qPCR reaction cycles which was repeated 44 cycles.

The QuantStudioTM design and analysis software (Thermo Fisher Scientific) was utilised for calculating comparative cycle threshold (Ct) values. The calculation of ΔCt values involved the subtraction of the Ct value of the target gene from the Ct value of a GAPDH housekeeping gene.

2.2.5.4 *Ex vivo* Pancreatic islets isolation

C57BL/6 mice (FFAR4-WT-HA, FFAR4-PD or FFAR4-KO) aged between 12 and 18 weeks were humanely killed and dissected to isolate pancreatic islets. Briefly, Liberase enzyme was dissolved in HBSS solution to a final concentration of 2.5 mg/mL (w/v). This enzyme solution was injected into mouse common bile ducts, allowing the pancreas to be digested for approximately 5 minutes. The pancreatic tissue was then collected in a 15 mL conical tube containing the Liberase enzyme mixture after removing all the mesenteric tissue. Following that, the conical tube was placed in a water bath at 37°C for 20 minutes until all the tissue was fully digested and homogenous. Homogenised tissue was then washed two times with washing solution (HBSS+ 1% BSA+ 1% Pen/Strep solution and 5 mM HEPES (pH 7)). Samples were centrifuged at 700 x g for 2 minutes to pellet the isolated pancreatic islets, supernatants were discarded and the purified tissues collected. Histopaque was added to the pellet to further digest any mesenteric tissue remaining and the same volume of washing solution was added to the mix to dilute the histopaque. The mix was then centrifuged at 700 x g for 20 minutes and the islets were transferred to a 25 mm Petri dish containing culture media (RPMI 1640 (Gibco, P/N: 11879-020) + 5.5 mM glucose + 10% FBS + penicillin/streptomycin mix (100 U/mL & 100 µg/mL)). Islets were picked under a stereomicroscope and plated out in 12-well plates containing culture media before overnight

incubation at 37°C. A summary of the protocol steps used to isolate mouse islets is shown in Figure 2-1.

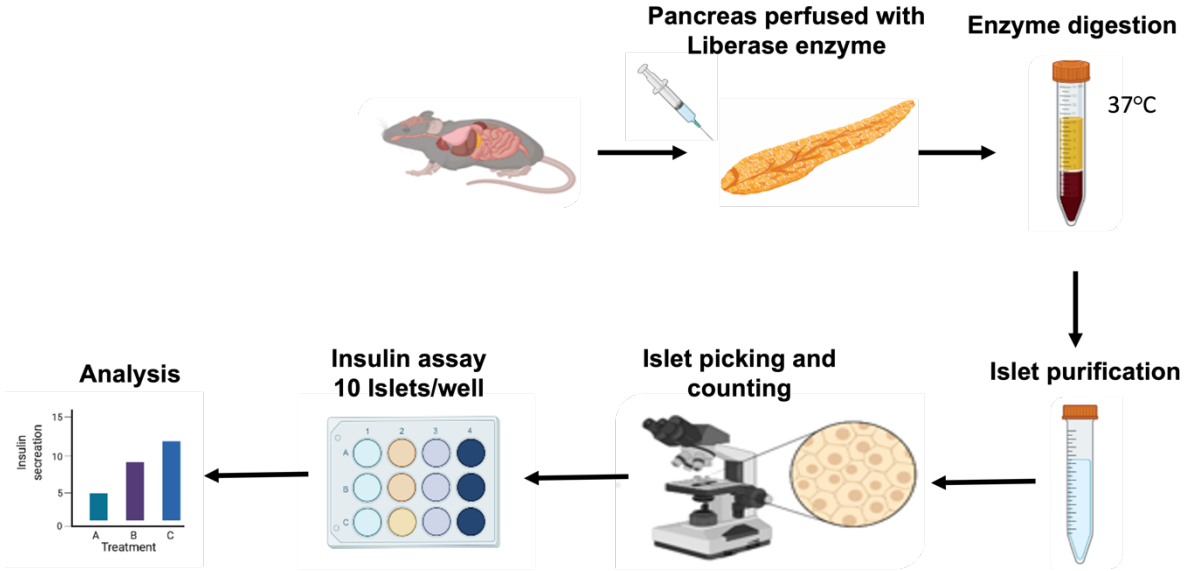


Figure 2-1: A summary of the protocol steps for isolating mouse islets. In order to look for the insulin secretion from mouse islets, Liberase enzyme mixture was perfused through the gall bladder into the pancreas which basically helps the pancreas to be digested better allowing the islets to dissociate from the tissue and once dissociated, they were purified and then hand-picked and finally plated out in 12-well plates to carry out the experiment.

2.2.5.5 *Ex vivo* Pancreatic secretion assay

This method of isolating islets has been previously described in detail by Rossi *et al.* (2015) and Willets *et al.* (2003). After being incubated overnight at a temperature of 37°C, groups of 10 islets were placed in 12-well plates and exposed to insulin buffer with a glucose concentration of 3.3mM for a duration of 1 hour at a temperature of 37°C. The buffer solution was replaced by another insulin buffer but with a glucose concentration of 16.7mM and the islets were incubated for 1 hour at 37°C. During this incubation, two experimental conditions were employed: one with the presence of oxotremorine (M3 agonist) at a concentration of 100 mM, serving as a positive control, and another condition in which the islets were treated with FFAR4 ligands at a concentration of 1 µM and 10 µM. Following the incubation process, the medium was collected for the purpose of measuring insulin levels. Subsequently, the residual insulin present in the islets was extracted through sonication using an acid/ethanol buffer. This buffer was prepared by mixing 1.5% (v/v) of

37% HCl (resulting in a final HCl concentration of approximately 0.56%) with 70% (v/v) ethanol. The 70% ethanol was prepared by diluting 100% ethanol with dH_2O. The insulin secretion during the incubation period was standardised by dividing it by the total insulin content of each well, which included both the islets and the medium. The measurement of islet insulin content was conducted using ELISA kit (Millipore (U.K.) Limited, EZRMI-13K). The samples were assayed following the instructions provided by the manufacturer. Briefly, samples from 3.3mM glucose were assayed undiluted while the samples from 16.7mM glucose were diluted 1 in 25 and the total lysate were diluted 1 in 1000.

Somatostatin concentrations were determined via ELISA Kit (Colorimetric) (Bio-Techne Ltd, NBP2-80270). The assay was performed according to manufacturer instructions. The secretion of somatostatin was measured undiluted from the samples and for the total lysate were 1 in 2000.

The concentrations of insulin and somatostatin were estimated according to the standard curves generated with the standard reagents provided in the kit. In order to calculate the percentage of insulin and somatostatin released from islets (%total), the average concentration values in the supernatant from three relevant replicates were subtracted by the average total concentration of insulin or somatostatin stored, and then multiplied by 100. Data was computed for each individual mouse and subsequently graphed using GraphPad Prism software.

2.2.5.6 Immunohistochemistry

The pancreas tissue obtained from mice was subjected to fixation in a 4% paraformaldehyde (PFA) solution (diluted in PBS) for a duration of 2 hours. The samples underwent processing in paraffin wax and were subsequently sliced into sections with a thickness of 5 μm. Antigen retrieval was conducted by subjecting the samples to sodium citrate buffer (10 mM tri-sodium citrate, pH 6), which was subsequently exposed to full power microwave irradiation for a duration of 10 minutes, with this process being repeated twice. The slides were subsequently placed in a blocking buffer (TBST + 10% BSA + 1% GS) and incubated for a duration of 2 hours at room temperature. The primary antibodies (Table 2-2) were prepared in a blocking buffer and subjected to an overnight incubation at a temperature of 4°C. The samples were subsequently subjected to three washes, each lasting 15 minutes, using a wash

buffer. Following this, the samples were incubated with the appropriate secondary antibodies (as indicated in Table 2-3) for a duration of 1 hour at room temperature. The samples were washed three times for a duration of 15 minutes each in wash buffer. Following this, the samples were mounted on coverslips using Vectashield mounting media that contained DAPI (1.5 µg/ml) to visualise the nucleus. Finally, the samples were imaged using an LSM 880 confocal laser scanning microscope manufactured by Zeiss.

2.2.5.7 Statistical analysis

In order to obtain measures of statistical significance, a minimum of three separate biological replicates were examined in each experiment. The statistical analyses were conducted using the GraphPad Prism software, with the input data consisting of values obtained from biological replicates. In the statistical analysis conducted to examine differences between groups, it was assumed that the data followed a normal distribution. Parametric tests were employed to make comparisons between the groups. In the case of pairs, either independent t-tests or dependent t-tests were conducted. Unpaired t-tests were conducted to analyse data from two distinct and independent groups, while paired t-tests were employed to compare two measurements obtained from the same animal. In experimental settings involving the analysis of three or more groups, such as the comparison of multiple pharmaceutical compounds, a one-way analysis of variance (ANOVA) was conducted. Dunnett's multiple-comparisons post hoc corrections were employed in order to compare the mean of each group with a single reference mean. A repeated measures one-way analysis of variance (ANOVA) was conducted to compare data from three or more groups, where multiple measurements were obtained for the same attribute. In this study, Geisser-Greenhouse post-hoc corrections were applied.

2.2.5.8 Power Calculations

Power calculations were conducted using the preliminary animal data in Chapters 4 to establish the minimal number of experimental animals needed to detect significant effect sizes in subsequent studies. The standard deviation of control conditions obtained from data analysis in GraphPad Prism was entered into sample size calculators for the appropriate statistical analyses using Mini-Tab software. The targeted power was set to 80%, a

commonly acknowledged power number in scientific literature according to Charan and Kantharia (2013). The significance criterion for detecting a significant effect size was set at $p< 0.05$. In Chapter 4, in the mouse pancreatic islets isolation studies, the effect sizes were determined to be significant as a mean difference of 0.75 fold change or a 75% increase between treated and not treated pancreatic islets, as per Croze *et al.'s* (2021) earlier research.

This value was determined based on previous experiments conducted by Psichas *et al.* (2015) and Bolognini *et al.* (2019) in their GLP-1 studies. Analysis indicated that in Chapter 4, 4 experimental animals would be needed to achieve the appropriate power in pancreatic islets studies.

Sample size calculations are essential for planning studies that provide reliable findings while minimising the number of animals used, in accordance with the principles of the 3Rs. These calculations are used to establish the minimal number of animals required to identify a biologically significant impact with a specified degree of statistical power. Inadequate sample sizes may result in studies lacking statistical power, making it difficult to identify significant differences and leading to results that are unclear. Consequently, this might eventually need an increase in the overall number of animals utilised. On the other hand, using an excessively large quantity of animals might give rise to ethical issues and could raise the chances of identifying statistically significant but physiologically insignificant differences. Researchers typically use a maximum of 6-10 animals each experiment, and it is recommended that future research aim for about 10 animals as the maximum number of biological replicates (Bonapersona *et al.,* 2020; Charan and Kantharia, 2013).

Chapter III Pharmacological characterisation of FFAR4 ligands

3.1 Introduction

G protein-coupled receptors (GPCRs) have garnered significant and enduring attention as attractive targets for pharmacological intervention due to their ability to modulate a wide range of physiological processes and their presence as accessible sites for drug binding on the cell surface. Pharmaceuticals that specifically interact with GPCRs constitute approximately 27% of the overall market share for therapeutic drugs worldwide (Hauser *et al.,* 2017). The FFAR GPCRs serve as effective exemplars of receptors that have the potential to offer significant therapeutic advantages to patients through targeted interventions. However, there exist challenges in comprehensively understanding their preclinical pharmacology, designing specific high-affinity synthetic ligands, and successfully translating these discoveries into clinical applications. To date, the predominant emphasis has been placed on investigating FFAR1, which has led to the development of ligands that have advanced to clinical trials for the treatment of T2DM (Patti *et al.,* 2022; Watterson *et al.,* 2014). However, our understanding of the mechanisms and effects of the FFAR4, which is activated by medium and long chain free fatty acids, remains limited. FFAR4 is commonly associated with $G_{\alpha q/11}$ heterotrimeric G proteins, leading to the generation of inositol phosphates and subsequent elevation of intracellular Ca^{2+} levels. However, studies have shown that this receptor can also interact with $G_{\alpha i}$ G proteins (Hirasawa *et al.,* 2005; Briscoe *et al.,* 2006; Engelstoft *et al.,* 2013; Stone *et al.,* 2014; Alvarez- Curto *et al.,* 2016).

It has been difficult to develop potent and selective ligands for FFAR4. Specifically, while FFAR4 and FFAR1 have relatively few similarities in their sequences, it has been challenging to find ligands that exhibit a high level of selectivity for FFAR4 compared to FFAR1. Given that most existing biological evidence indicates that stimulating FFAR4 would be advantageous for treating metabolic and/or inflammatory conditions (Cornall *et al.,* 2014; Hara *et al.,* 2014) the primary research efforts to date have been on finding synthetic FFAR4 agonists. Several chemical classes of FFAR4 synthetic agonists have been described (Figure 3-1), demonstrating varying degrees of selectivity for FFAR4 over FFAR1. In contrast, there has only been one chemical compound to date that acts as an FFAR4 antagonist, although it has not been well characterised. In the past, there has been a scarcity of synthetic ligands that specifically target FFAR4. Consequently, numerous studies

have been limited to utilising different fatty acids, which, despite being the natural ligands, exhibit only moderate affinity for the receptor. However, these investigations have been carried out using a variety of recombinant and endogenous systems, which presents difficulties when attempting to compare the effectiveness and strength of different FFAR4 ligands. For instance, there remains a requirement for novel ligands that exhibit selective activation of the FFAR4, given that TUG-891 has the potential to activate the FFAR1 as well. Therefore, it remains uncertain whether various orthosteric FFAR4 agonists exhibit ligand selectivity.

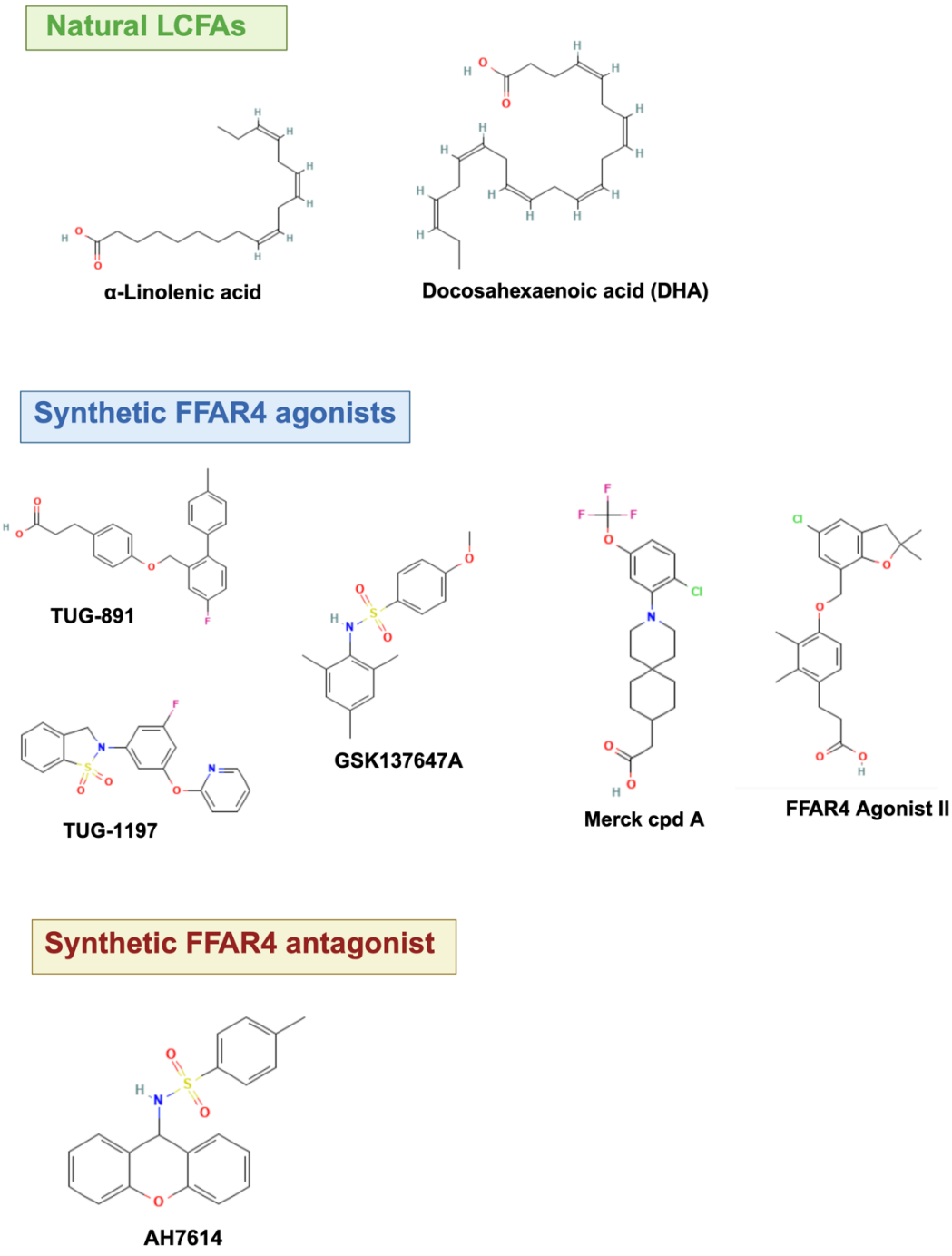


Figure 3-1: Chemical structures of FFAR4-targeting compounds: natural agonists, synthetic agonists, and antagonist. With the carboxylic acid moiety preserved in agonists and aromatic rings and other functional groups added to synthetic molecules, this figure shows the structural variety of FFAR4 ligands, which range from natural long-chain fatty acids to synthetic modulators. Natural FFAR4 agonists, such as α-Linolenic Acid and Docosahexaenoic Acid (DHA), have lengthy carbon chains and numerous double bonds. Synthetic FFAR4 agonists, such as TUG-891, TUG-1197, Merck cpd A, and GSK137647A, demonstrate a variety of structural changes aimed at increasing their effectiveness and selectivity. The compound AH7614 is an antagonist of the FFAR4. The highlighted structural elements include carboxylic acid groups, carbon chains, aromatic rings, and important functional groups. CDT compounds chemical structures from Caldan Therapeutics are currently unavailable. Images of chemical structures from PubChem.

3.2 Objectives

The objectives of this chapter were to:

• Corroborate the expression and location of mouse FFAR4 (mFFAR4) in CHO and HEK293 cells for analysing the function of mFFAR4.

• Evaluate the activation of mFFAR4 receptors following treatment with FFAR4 ligands.

• Evaluate the coupling of mFFAR4 to G protein and β-arrestin-2 signalling pathways after stimulation with FFAR4 ligands.

• Examine the phosphorylation of FFAR4 and determine if it occurs in a ligand concentration-dependent manner.

3.3 Results

3.3.1 Expression of mFFAR4 in CHO cell lines

In order to validate the expression of FFAR4, CHO *Flp-In* cell lines were generated according to the Flp-In™ system protocol from *Life Technologies,* which involved transfecting CHO cells with eYFP fused to the C-terminal tail of the FFAR4 which helps to detect the receptor using the protocol previously reported by Prihandoko *et al.* (2016). Immunocytochemistry (ICC) and Western blot (WB) analyses were conducted on cellular samples, with Figure 3-2 illustrating the results. These experiments aimed to ascertain the presence or absence of the receptor and provide evidence for its expression. First, in ICC the CHO cell line stably expressing mFFAR4 and non-transfected cell line were grown on coverslips, fixed and then treated with DAPI which shows cell nuclei. By using confocal microscopy, receptor expression was verified by the presence of eYFP, which corresponds to mFFAR4-eYFP. Results from the ICC study (Figure 3-2A) identified that the fluorescent protein-tagged receptor was present at the cell surface of CHO mFFAR4-eYFP cells, as expected for a transmembrane GPCR, while no eYFP expression was detected in non-transfected cells (Figure 3-2A). The aforementioned observation was further substantiated through Western blot analyses, where protein samples were resolved on an SDS-PAGE gels and WB were performed. Results revealed that the receptor is absent in proteins derived from the non-transfected cell line, while its presence was detected in proteins obtained from the mFFAR4-eYFP cell line, which again confirms the presence of mFFAR4 (Figure 3-2B).

In this study, phospho-site specific antibodies were employed to identify Thr^{347} and Ser^{350}, following their phosphorylation as these are known to be regulated by agonists (Butcher *et al.*, 2014; Prihandoko *et al.*, 2016). These antibodies were used as a means to detect and measure the activation of FFAR4 in mFFAR4-eYFP cells. In order to ensure accurate measurements, internal loading controls were utilised in these studies. These controls involved the simultaneous detection of GAPDH levels, which migrates in the SDS-PAGE gels at a position similar to that of a 37-kDa marker protein. The mFFAR4-eYFP exhibited a molecular mass of approximately 75 kilodaltons (kDa). It is of importance to mention that the observed band size surpassed the reported molecular mass of mFFAR4, which is documented as 45 kDa in the study conducted by Prihandoko *et al.* (2016). Nevertheless, taking into account the supplementary molecular weight introduced by the eYFP tag, which is approximately 27 kDa, one can confidently infer that the observed molecular mass corresponds with that of mFFAR4-eYFP.

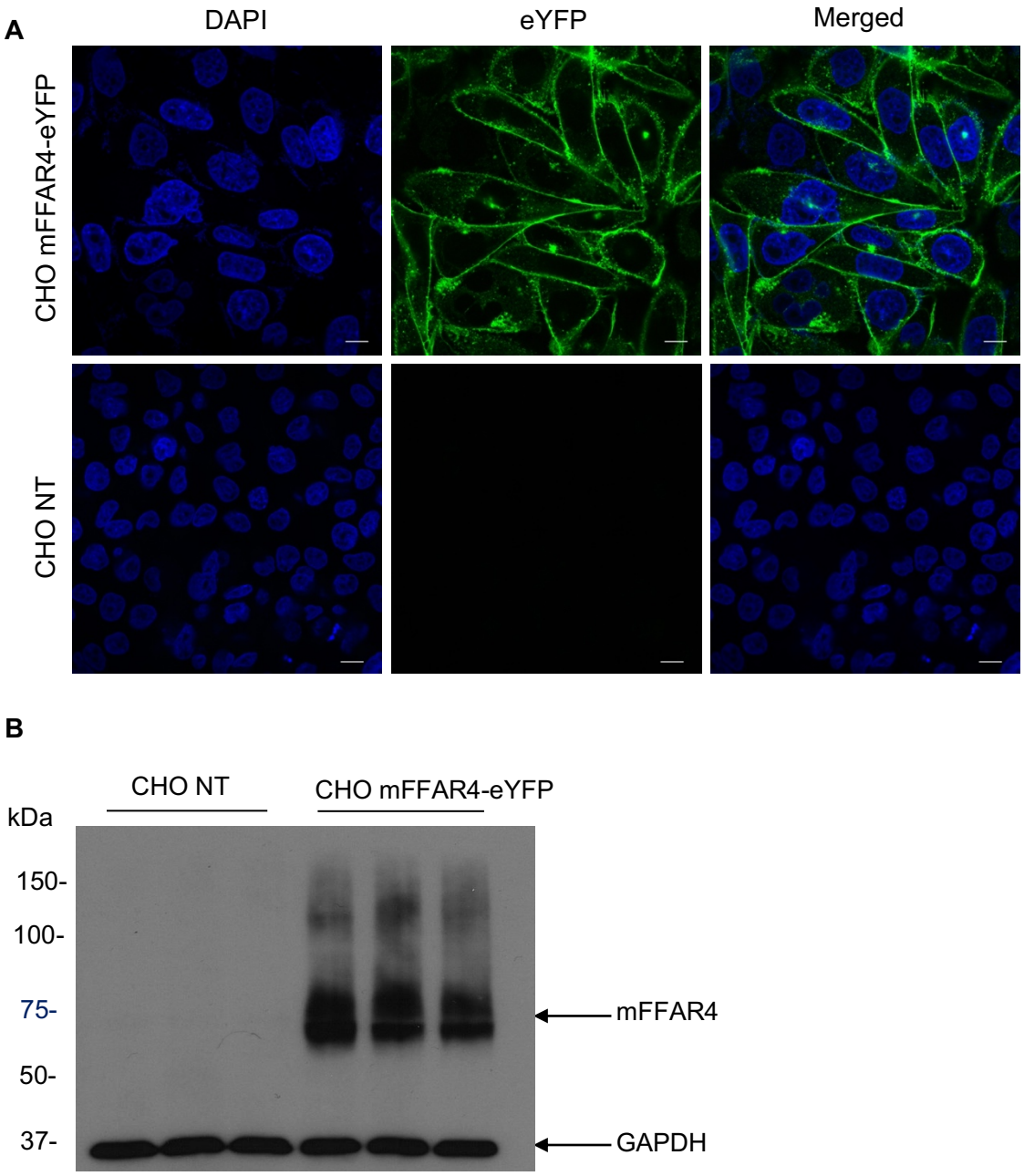


Figure 3-2: Murine FFAR4 receptor expression in transfected and not transfected cell lines. (A) The expression of the receptor was identified by immunohistochemistry studies. Representative images show mFFAR4-e-YFP (green) co-localised with cell nuclei (DAPI, blue). Scale bar represents 10 µm, n=3. **(B)** Western Blot analysis using phospho-specific antibodies against the sites Thr^{347} and Ser^{350} to detect the e-YFP tagged receptor. mFFAR4-eYFP was detected at the molecular mass of approximately 75 kDa. NT= non-transfected cells. Representative results are shown from one of three independent experiments.

3.3.2 mFFAR4 becomes phosphorylated after treatment with ligands

Previous studies have demonstrated that FFAR4 displays a response to naturally occurring fatty acid ligands, such as α-linoleic acid, although its effectiveness is relatively lower in comparison to synthetic FFAR4 ligands like TUG-891 (Hudson *et al.,* 2013). Hudson *et al.* (2013) have also verified that TUG-891 exhibits the highest potency as a stimulator of FFAR4. The selection of this particular ligand as a reference in the Western blot assay was based on its extensive characterization as the most well-studied ligand for FFAR4. This implies that the impact of each FFAR4 ligand will be assessed in relation to the effect of TUG-891. The ligands used in this study encompassed both commercially available ligands and newly developed ligands. Specifically, TUG-891 was identified as a highly effective ligand for FFAR4, while TUG-1197 (Prihandoko *et al.,* 2020) was found to selectively activate FFAR4. Additionally, GSK137647 was identified as a potent agonist for FFAR4 (Sparks *et al.,* 2014). Merck compound A (Oh *et al.,* 2014) is a highly effective and specific agonist for the FFAR4 receptor. The compounds developed by Caldan Therapeutics, referred to as "CDT," are innovative and were graciously supplied by the company. The confirmation of the ligands' responses was conducted by Caldan Therapeutics. In addition, FFAR4 Agonist II is an FFAR4 agonist that was derived from the patent US 20110313003 A1, as described by Shi *et al.* in 2012.

Western blot assays were conducted to assess FFAR4 ligands' ability to activate the receptor. The experimental procedure involved the stimulation of CHO cells expressing mFFAR4 with two different concentrations of ligands (10 μM and 1 μM) for a duration of 5 minutes. The aforementioned effects were observed through the utilisation of a phospho-specific antibody that specifically targets the Thr^{347}/Ser^{350} residues in mFFAR4. This phenomenon can be attributed to the findings of prior research, which have emphasised the role of these sites in modulating the phosphorylation of the FFAR4 receptor (Prihandoko *et al.,* 2016; Butcher *et al.,* 2014).

FFAR4 phosphorylation at these sites was found to be elevated in response to all tested ligands in comparison to the control condition (0.1% DMSO) (Figure 3-3A). This is similar to evidence reported by Euston in 2023. But it was not possible to determine which ligand stimulated the receptor more effectively than others. This may be attributed to the presence of significant variability, causing it challenging to make comparisons between ligands in cases where the distinction between 10 μM and 1 μM is negligible.

Interestingly, it was found that in the presence of Merck cpd A, the activity of FFAR4 increases by five-fold compared to untreated control (Figure 3-3). However, there is no

significant difference between the different concentrations of Merck cpd A using either 1 or 10 µM (P> 0.05). Unlike Merck cpd A, addition of CDT-347 only slightly increases phosphorylation of the receptor at 10 µM whereas 1 µM show half of the phosphorylation potential compared to untreated control. It is important to note that there was a signal in the vehicle-treated control which indicate a level of constitutive activity which can be inhibited by FFAR4 antagonist namely AH7614 as reported by Watterson *et al.* in 2017.

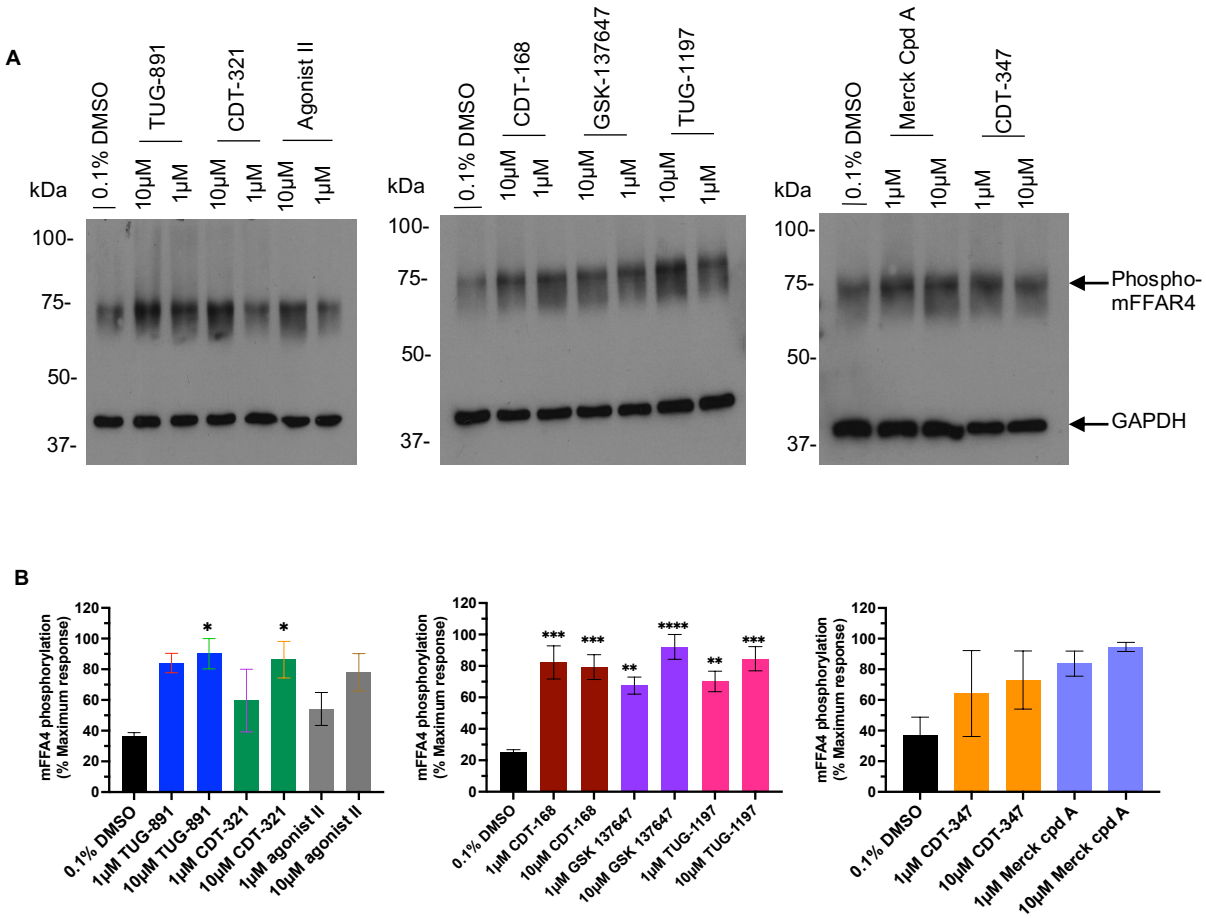


Figure 3-3: Murine FFAR4 becomes phosphorylated upon agonist stimulation. mFFAR4 becomes phosphorylated when CHO cells stably expressing the receptor were treated with the indicated concentrations of each ligand for 5-minutes **(A and B).** Phospho-specific antibodies were used against the sites Thr^{347} and Ser^{350} at a concentration of 1:2000 (WB) and GAPDH at 1:5000. Before analysis by autoradiography, ECL reagent was used at a concentration 1:5000 for a duration of 1-minute. All FFAR4 ligands were able to phosphorylate the receptor at both the 1 µM and the 10 µM concentration. 10 µg of Lysate from CHO mFFAR4 were used in this experiment. Images shown are representative of three independent experiments. **(B)** Analysis of Western blot gel is shown where data are normalised to % maximum response. Images shown are representative of three independent experiments. The statistical analysis employed in this study was a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test, with 0.1% DMSO serving as the control group. *=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001.

3.3.3 mFFAR4 coupling to ERK1/2 phosphorylation and activation

Activation of mFFAR4 can result in the coupling of the receptor to various G proteins and their downstream signalling pathways, including $G_{q/11}$ (Butcher *et al.,* 2014; Milligan *et al.,* 2015) and G_i- families (Engelstoft *et al.,* 2013). As FFAR4 induces phosphorylation of ERK1/2, mostly through Gq signalling routes, assessing the levels of activity of these MAPKs has been employed as an indicator of overall receptor stimulation (Hirasawa *et al.,* 2005; Briscoe *et al.,* 2006; Alvarez-Curto *et al.,* 2016). Herein, the aim was to measure the ERK1/2 phosphorylation by using both a pERK1/2 antibody and CisBio ERK1/2 phosphorylation assay kit. Firstly, a pERK1/2 antibody was used in a Western blot assay to assess the ability of FFAR4 to couple to these MAPKs. Herein, protein samples from CHO cells stably expressing mFFAR4-eYFP that had been stimulated for 5 minutes with either 1 μM and 10 μM of FFAR4 ligands were analysed (Figure 3-4). Interestingly, each of the tested ligands was able to couple to ERK1/2 phosphorylation, and this is also consistent with the finding of Hudson *et al.* (2013). However, it was difficult to assess the rank order of potency of these ligands (Figure 3-5B). With the knowledge that CDT-321, CDT-168 and CDT-347 came from the same source (Caldan Therapeutics) and were having similar pharmacological effects only CDT-347 was then used in subsequent studies.

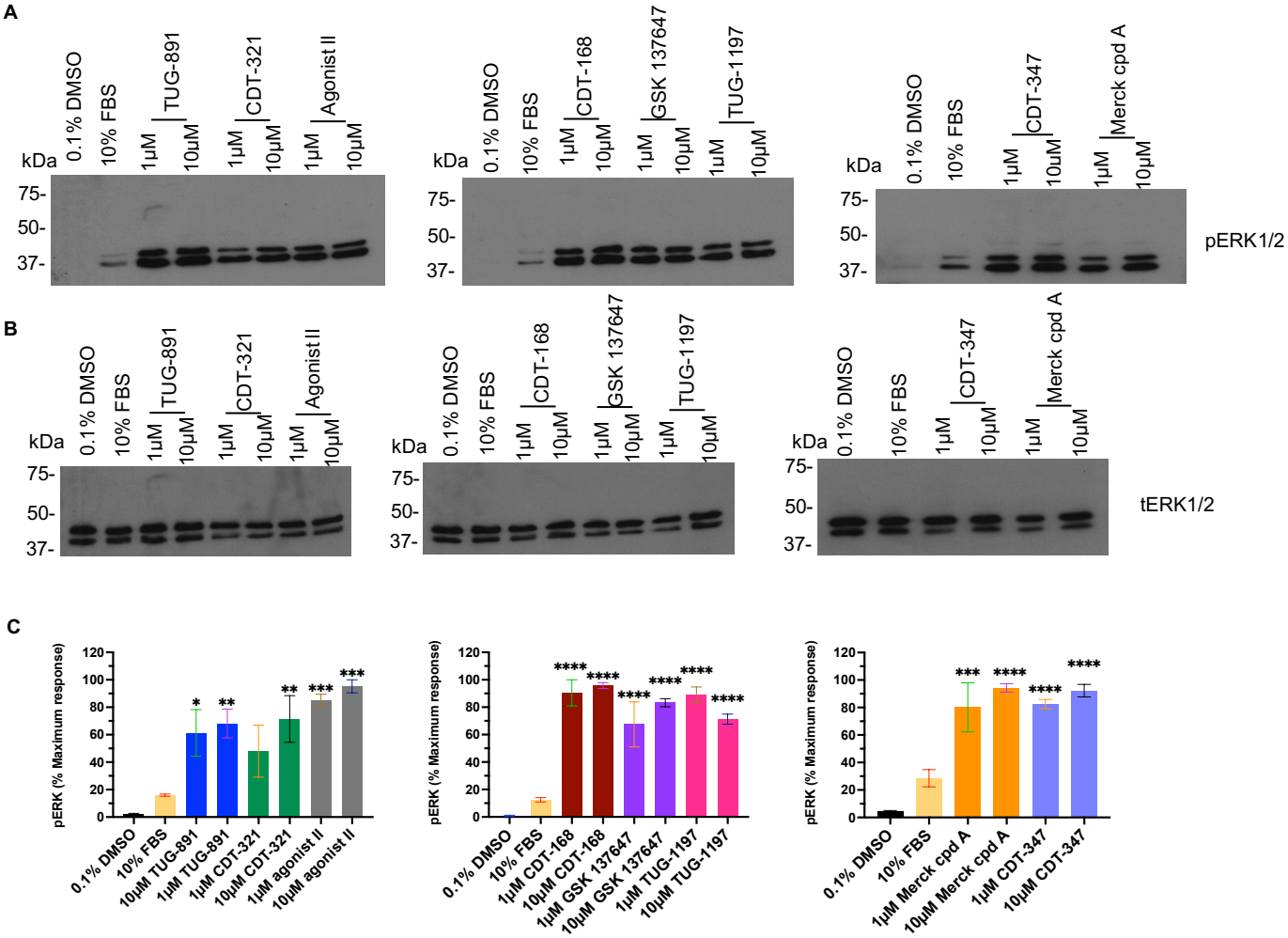


Figure 3-4: mFFAR4 can activate a phospho-ERK signalling pathway. CHO cells stably expressing mFFAR4 were grown in 6-well plates and then stimulated for 5-minutes with two concentrations of each ligand (10 μM and 1 μM). **(A)** A western blot analysis using p-ERK and **(B)** total ERK which was used as a control for protein loading, was shown to assess the ability of the receptor to couple to the downstream signalling pathway of ERK1/2 (MAP kinases). Both p-ERK and t-ERK was used at a concentration of 1:2000. Ultimately, all FFAR4 ligands were able to couple to the downstream signalling of ERK at both 10 μM and 1 μM concentrations. **(C)** Analysis of Western blot gel is shown where data normalised and the total response in percentage in each gel were calculated. Images shown are representative of three independents experiments. The statistical analysis employed in this study was a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test, with 10% FBS serving as the control group. *=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001.

With the awareness that a pool of ligands caused the activation of the FFAR4, these ligands were then assessed in a quantitative pERK1/2 HTRF assay, which measures the level of ERK1/2 phosphorylation on specific tyrosine (Tyr204) and threonine (Thr202) residues. The quantification of the optimal incubation time required for ligands to induce a maximal effect was performed in a pERK1/2 time course analysis (Figure 3-5). The peak response for all ligands was observed at the 5-minute mark, followed by a gradual decline until reaching basal levels by the 30-minute mark. It is intriguing to note that this was not the same decline

in response for one of the ligands, FFAR4 Agonist II, where it shows slower decline in response (Figure 3-5A). This ligand exhibited a slower attainment of basal level, indicating potential variations in signalling mechanisms for this particular ligand and another explanation is that this ligand could be slowly released from the receptor resulting in prolongation in the time it dissociates from the binding site of the receptor. In order to test this hypothesis, a binding assay could be performed. This is because a receptor binding assay is a method that enables comprehensive characterization of the interaction between a receptor and its ligands, including the intrinsic affinity of the ligands for the receptor, the rates at which they bind and dissociate, and the density of the receptor within tissues or cells (Bylund and Toews, 1993; Flanagan, 2016). However, performing this assay is beyond the focus of this thesis.

FFAR4 induces ERK1/2 phosphorylation primarily via G_q signalling routes (Alvarez-Curto *et al.,* 2016; Senatorov *et al.,* 2020), where the function of this signalling pathway was determined as a measure for overall stimulation of receptors and also helped in the evaluation of the signalling capacity of FFAR4 to pERK1/2 pathway. The quantification of potency (pEC_{50}) and efficacy ($\%_{Emax}$) of FFAR4 agonists was made possible by constructing concentration-response curves for the phosphorylation of ERK1/2. The reference ligand TUG-891, an FFAR4 agonist, was employed in this study. This is because TUG-891 is widely recognised as the "gold standard" synthetic agonist for FFAR4 (Hudson *et al.,* 2013). Typically, natural free fatty acids are not employed as a standard reference due to the presence of various long chain fatty acids that can activate the receptor and it is unknown which ligand is the genuine endogenous ligand for FFAR4. Moreover, according to Butcher *et al.* (2014), TUG-891 exhibits no discernible bias towards any specific stimulus when compared to endogenous ligands, making it an optimal choice as a reference ligand. In this study's assay, the potencies of all agonists examined were either comparable to or notably lower than that of TUG-891. Conversely, the efficacy of all agonists studied was not significantly different from that of TUG-891, as indicated in Table 3-1 ($P > 0.05$ for all compounds). However, the potency values (pEC_{50}) obtained in this study for all compounds differed from those reported in the literature. Specifically, the experimental pEC_{50} values obtained in this study were higher than those previously published (Hudson *et al.,* 2013; Oh *et al.,* 2014; Prihandoko *et al.,* 2020; Sparks *et al.,* 2014). Among the compounds tested, GSK137647A showed a slightly lower efficacy (99.5 ± 6.5) compared to TUG-891 (102.3 ± 5.8), though this difference is not statistically significant ($P > 0.05$). No phosphorylation of ERK1/2 was observed in CHO cells that were not transfected, thereby providing additional evidence that the phosphorylation of ERK1/2 induced by the agonist was in fact a result of

FFAR4 agonist activity (Figure 3-5C). Since CDT-347 was displayed similar potency as Merck cpd A and GSK137647A, this compound was excluded from further study.

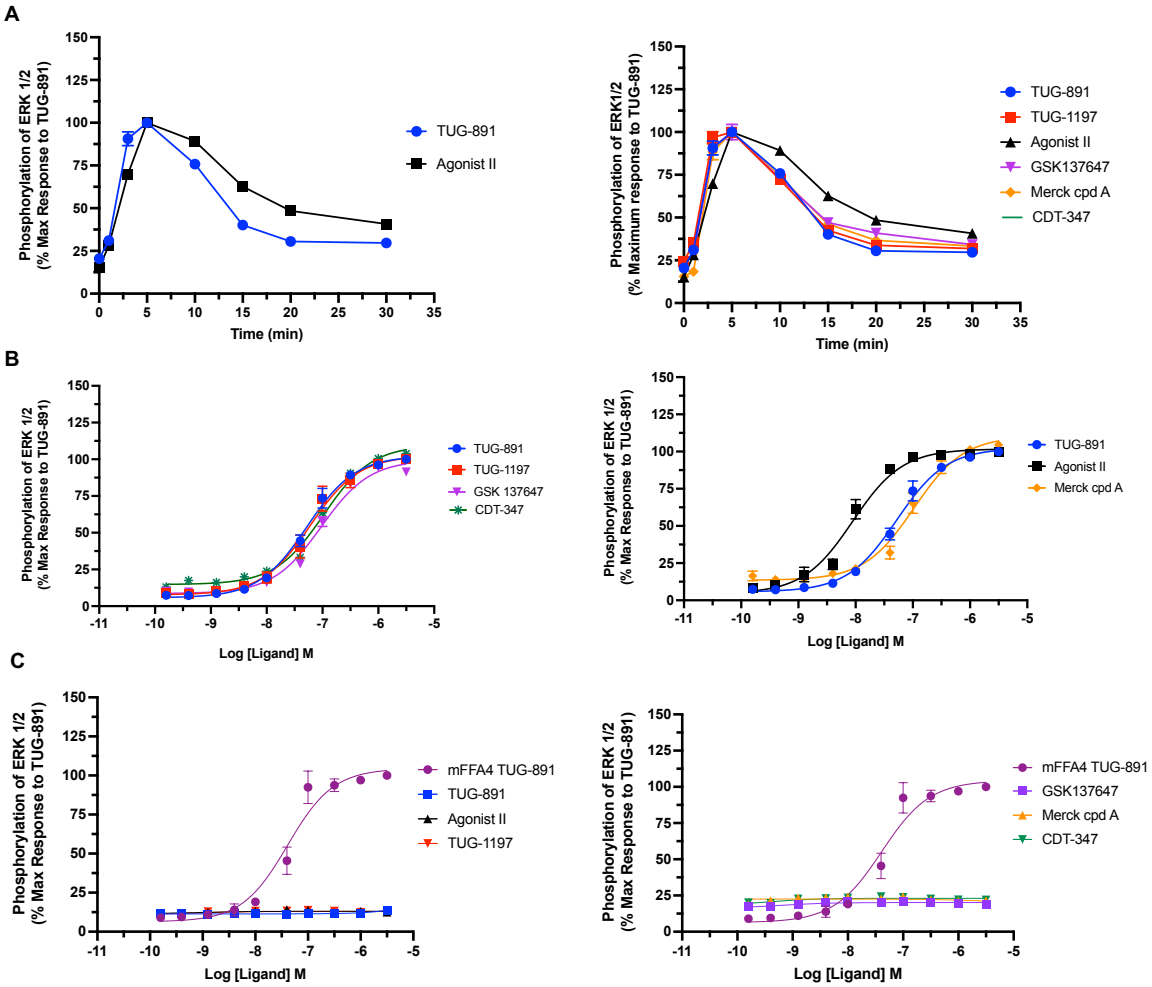


Figure 3-5: FFAR4 mediated phosphorylation of ERK1/2. A time course of pERK1/2 was performed in response to 10μM of ligands and showed the following: **(A)** ligands reached their maximal response at 5-minutes and this was then followed by a drop in the response except for FFAR4 agonist II which showed a prolonged reduction in the response; **(B)** A concentration response curve was generated by treating cells with a variety of concentrations for 5 minutes. **(C)** No response was detected in the non-transfected cell line. The data shown are means ± SEM of three independent experiments.

3.3.4 Exploring the mechanism(s) responsible for ERK activation

In addition, to test the signalling pathway of MAP kinases, the second objective was to assess if the response seen in the ERK1/2 assay was through the G_i pathway G protein. To determine this, the cells stably expressing the receptor were treated overnight with 200 ng/mL pertussis toxin (PTX), which can selectively block the G_i pathway, and the same concentrations of each ligand were used in order to compare the effects between the ones treated with PTX with the others that were not treated. Upon treatment, the ligands lost potency by almost a log fold-change (Table 3-1) with pEC_{50} of 7.3 ± 0.02 and 6.5 ± 0.06 (P> 0.9) for TUG-891 and TUG-891 treated with PTX respectively (Figure 3-6A). Moreover, the FFAR4 Agonist II had a pEC_{50} of 8.05 ± 0.08 and 7.3 ± 0.08 (P> 0.9) for non-treated and treated with PTX respectively (Figure 3-6A). These findings were also the same with TUG-1197 (Figure 3-6B) and Merck cpd A (Figure 3-6C) but with a slight drop in potency for both when treated with PTX, when the pEC_{50} for TUG-1197 was 7.2 ± 0.06 and 6.56 ± 0.07 (P> 0.7) and for Merck cpd A it was 7.0 ± 0.07 and 5.91 ± 0.14 (P> 0.8) for cells non-treated and the ones treated with PTX respectively. Lastly, GSK137647A had a pEC_{50} of 7.1 ± 0.05 and 6.42 ± 0.19 (P> 0.5) for non-treated and treated with PTX respectively (Figure 3-6D). The data, suggest that upon treatment with PTX most ligands have lost potency by almost a log fold-change in their pEC_{50} but these results are not significant (Table 3-1) suggesting that the phosphorylation of ERK1/2 is not dependent on G_i signalling. Furthermore, examining the effect of Gq inhibitor which will be explained in the next part might further indicate that the effect of FFAR4 on ERK1/2 phosphorylation is primarily dependent on Gq in its effect. Since TUG-1197 showed similar activity in comparison to other compounds, this compound was excluded from further study.

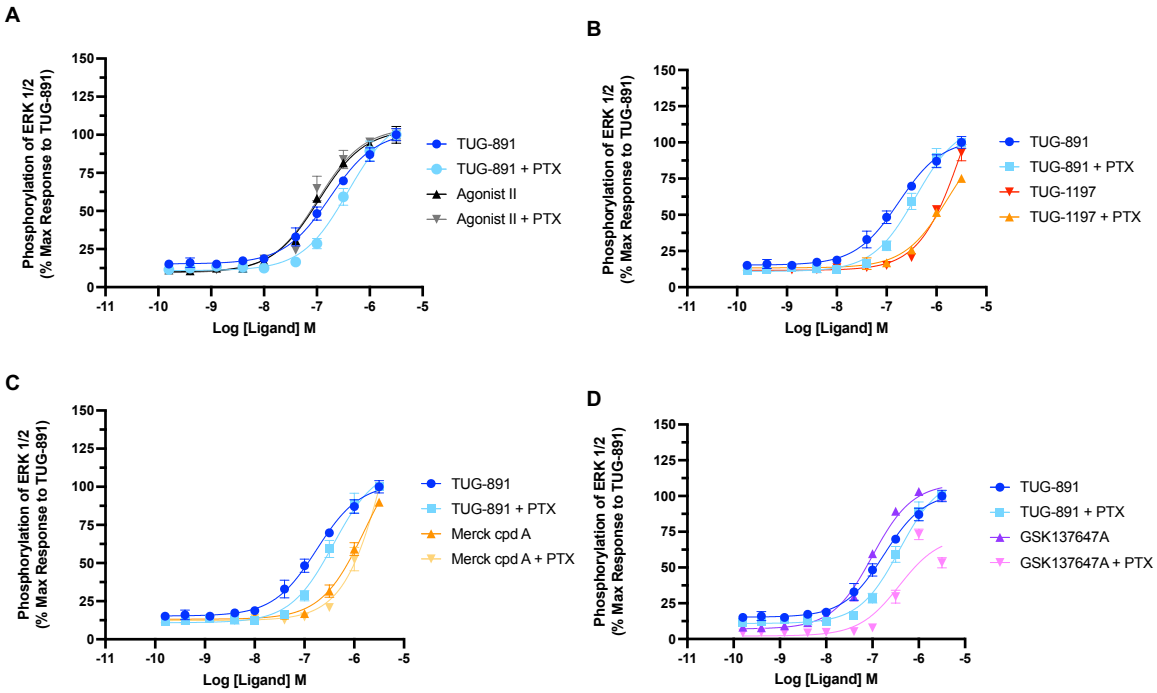


Figure 3-6: FFAR4 agonist promoted pERK1/2 phosphorylation in CHO cells stably expressing mFFAR4 was/was not affected by treatment with Pertussis toxin. Ligands have lost potency by almost a log fold-change in their pEC50. **(A)** Effect of PTX on TUG-891 and FFAR4 Agonist II. **(B)** Effect of PTX on TUG-1197; **(C)** Effect of PTX on Merck cpd A; **(D)** Effect of PTX on GSK137647A. The data shown are means ± SEM of three independent experiments.

FFAR4 Ligand	pEC_{50}		$\%E_{max}$ compared to TUG-891	
	Mean ± S.E.M	**p value***	**Mean ± S.E.M**	**p value**
TUG-891	7.3 ± 0.02	0.9 (ns)	102.3 ± 5.8	0.9 (ns)
TUG-891 + PTX	6.5 ± 0.06		105.9 ± 4.3	
TUG-1197	7.2 ± 0.06	0.7 (ns)	102.4 ± 3.3	0.9 (ns)
TUG-1197 + PTX	6.56 ± 0.07		75.1 ± 7.7	
Agonist II	8.05 ± 0.08	0.9 (ns)	101.7 ± 1.7	0.9 (ns)
Agonist II + PTX	7.3 ± 0.08		104.8 ± 1.9	
Merck cpd A	7.0 ± 0.07	0.8 (ns)	110.2 ± 4.6	0.9 (ns)
Merck cpd A + PTX	5.91 ± 0.14		85.4 ± 7.1	
GSK137647A	7.1 ± 0.05	0.5 (ns)	99.5 ± 6.5	0.3 (ns)
GSK137647A + PTX	6.42 ± 0.19		62.3 ± 8.8	
CDT-347	7.05 ± 0.06	Not tested	109.6 ± 12.4	-
CDT-347 + PTX	Not tested		-	

Table 3-1: The potencies and efficacies of FFAR4 agonists were assessed in the pERK1/2 assay and then subsequent treatment with a Gi inhibitor (PTX). The results represent the mean ± standard error of the mean (SEM) obtained from three independent experiments, with a sample size of three (n=3). The statistical analysis employed in this study was a one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons. Note: All untreated ligands showed no significant difference in pEC_{50} or $\%E_{max}$ compared to untreated TUG-891 ($P > 0.05$ for all comparisons). * p-value comparing the pEC_{50} of each ligand in the absence vs. presence of PTX. The notation "ns" was used to indicate non-significance ($P > 0.05$).

Secondly, to test whether the $G_{q/11}$ pathway is the primary mechanism by which FFAR4 phosphorylation of ERK occurs, cells were pre-treated with 1 µM of YM-254890 which is a Gq inhibitor. Co-crystallization of YM254890 (YM), a cyclic depsipeptide derived from bacteria, and its target protein G_q yielded the initial high-resolution configuration of the a G protein-inhibitor complex (Nishimura *et al.,* 2010). This experiment was performed to ascertain whether the activated receptor transmits signals via $G_{q/11}$ coupled pathways and to further investigate if the phosphorylation of MAP kinases is predominantly Gq coupled, as indicated previously by Alvarez-Curto *et al.* (2016). mFFAR4-eYFP CHO cells were preincubated with YM-254890 for a duration of 1 hour prior to the stimulation by ligands for 5-minutes (Figure 3-7). The $G_{q/11}$ inhibitor YM-254890 demonstrated a statistically significant inhibiting effect on the response to Agonist II ($P< 0.0002$; 95% reduction), TUG-891 ($P < 0.004$; 98% reduction), Merck cpd A ($P< 0.009$; 98% reduction), and GSK 137647A ($P< 0.005$; 98% reduction). This finding suggests that mFFAR4 is associated with $G_{q/11}$ signalling and further indicates that the pERK1/2 pathways regulated by mFFAR4 are predominantly coupled with G_q. This finding is consistent with Hudson *et al.* (2013) as they showed that YM-254890 is an effective G_q inhibitor that is significantly able to block FFAR4 phosphorylation mediated by ERK1/2 pathways.

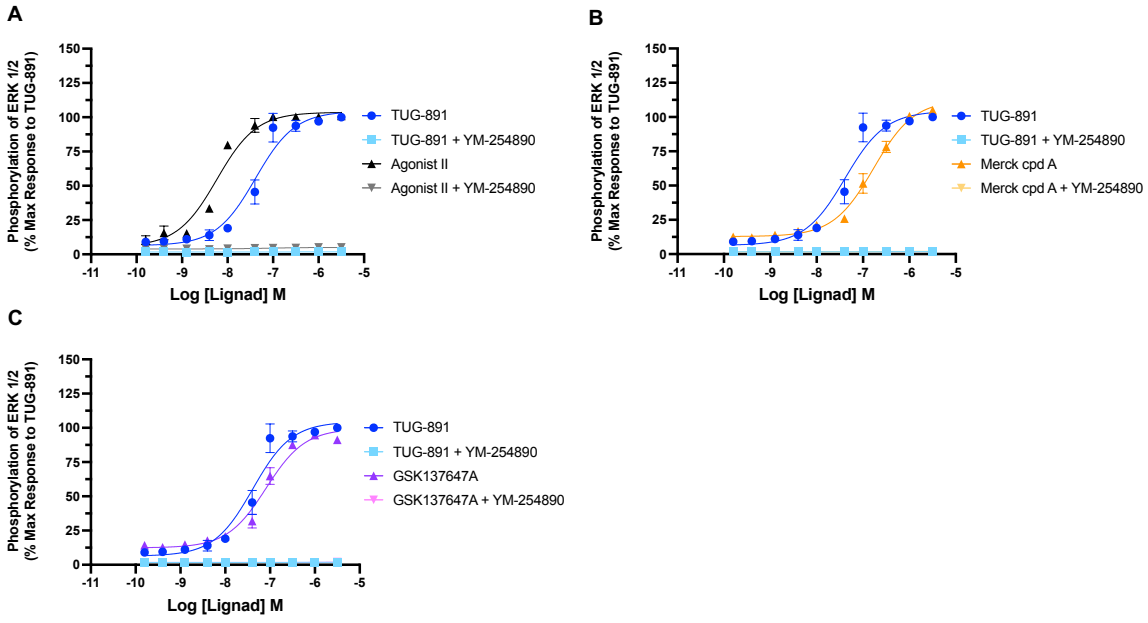


Figure 3-7: FFAR4 agonist promoted pERK1/2 phosphorylation in CHO cells stably expressing mFFAR4 was affected by treatment with YM-254890. Ligands had lost potency upon treatment with YM-254890. (**A)** a comparison between cells treated with TUG-891 and FFAR4 Agonist II ± YM-254890. **(B)** effect of YM-254890on Merck cpd A; **(C)** effect of YM-254890 on GSK137647A. The data shown are means ± SEM of three independent experiments.

FFAR4 Ligand	pEC_{50}		$\%E_{max}$ compared to TUG-891	
	Mean ± S.E.M	**p value***	**Mean ± S.E.M**	**p value****
TUG-891	7.38 ± 0.10	0.004	104.4 ± 4.3	-
TUG-891 + YM-254890	-		-	-
Agonist II	8.23 ± 0.09	0.0002	103.6 ± 2.7	0.6 (ns)
Agonist II + YM-254890	-		-	-
Merck cpd A	6.77 ± 0.05	0.009	112.4 ± 2.8	0.9 (ns)
Merck cpd A + YM-254890	-		-	-
GSK137647A	7.09 ± 0.07	0.005	99.4 ± 3.1	0.9 (ns)
GSK137647A + YM-254890	-		-	-

Table 3-2: The potencies and efficacies of FFAR4 agonists were assessed in G_q signalling pathway. Each compound + G_q inhibitor was compared against the reference compound (TUG-891). The results represent the mean ± standard error of the mean (SEM) obtained from three independent experiments, with a sample size of three (n=3). The statistical analysis employed in this study was a one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons. Note: Rows for YM-254890 treated conditions are empty for pEC_{50} and $\%E_{max}$ due to a 98% reduction in activity, making these measurements unfeasible. $\%E_{max}$ comparisons are made against untreated TUG-891 as the reference. The notation "ns" was used to indicate non-significance (P> 0.05). *p-value comparing the pEC_{50} of each ligand in the absence vs. presence of YM-254890. ** p-value comparing the $\%E_{max}$ of TUG-891 untreated with YM-254890 against the $\%E_{max}$ of each untreated ligand.

3.3.5 IP1 accumulation assay in mFFAR4 expressing CHO cells

As FFAR4 exhibits a preference for coupling with G_q proteins as shown before upon treatment with the compound YM-254890, another method for evaluating $G_{\alpha q/11}$ protein-dependent signalling involves the detection of inositol phosphate (IP1) accumulation, which is a by-product of the inositide signalling pathway. This was conducted on mFFAR4 CHO cell-line demonstrating G_q coupling abilities (Figure 3-8A) in comparison to non-transfected CHO cells (Figure 3-8B). Figure 3-8 presents a concentration-response curve illustrating the

IP1 accumulation in response to TUG-891, which serves as the reference ligand. This curve enables the evaluation of the potency (EC_{50}) and efficacy (E_{max}) of novel FFAR4 ligands in comparison to the reference ligand. In this experiment, the concentration response curve was used to assess the effects of FFAR4 agonists on IP1 accumulation in mFFAR4 CHO cells. The results showed that all novel ligands exhibited G_q coupling abilities, as evidenced by the detection of IP1 accumulation. This finding was compared to the response observed with TUG-891, a reference compound (Figure 3-8). When evaluating the response of ligands, it was observed that Merck cpd A exhibited comparable potency and efficacy to TUG-891 (Figure 3-8A). In contrast, Agonist II elicited a significantly more potent response (P value= 0.03) (Figure 3-8A). However, it was observed that GSK137647A did not achieve a maximum response in concentration response curves (Figure 3-8A). This can be attributed to the lower pEC_{50} values of this ligand, indicating that this compound was not effective activators of G_q coupled signalling. These results align with what was previously reported by Euston in 2023. Therefore, this suggest that GSK137647A is not completely dependent on G_q signalling pathway in its function on FFAR4.

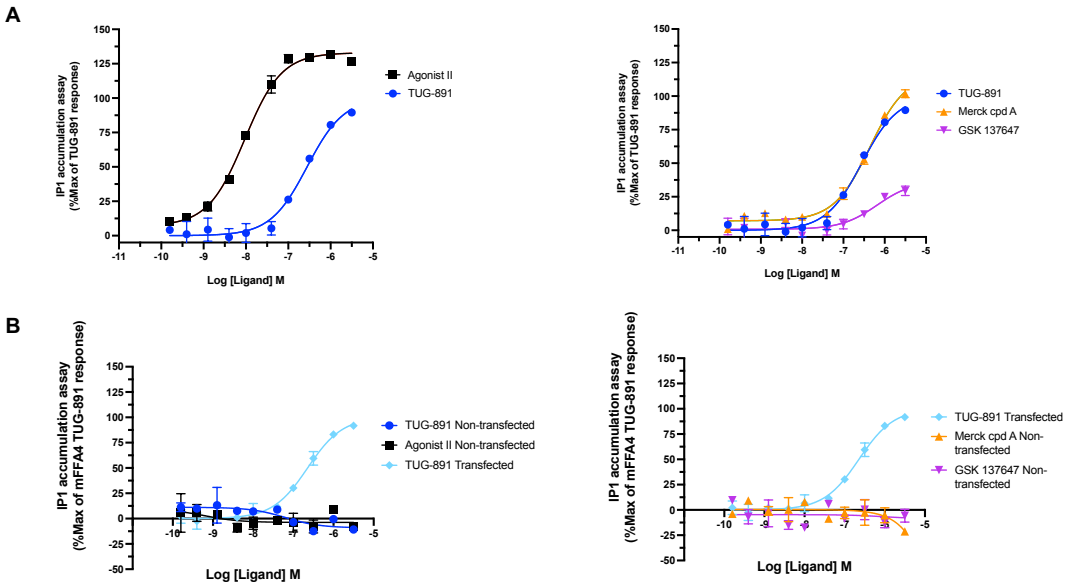


Figure 3-8: IP1 accumulation in CHO cells stably expressing mFFAR4 and non-transfected CHO cells. The accumulation of IP1 in cells was quantified after treatment with FFAR4 ligands. **(A)** Flp-In CHO cells stably expressing mFFAR4**. (B**) Non-transfected CHO cells following treatment with FFAR4 ligands. The reference ligand utilised in all experiments was TUG-891. The results represent the mean ± standard error of the mean (SEM) obtained from three independent experiments (n=3).

FFAR4 Ligand	pEC_{50}		$\%E_{max}$ compared to TUG-891	
	Mean ± S.E.M	p value	Mean ± S.E.M	p value
TUG-891	6.64 ± 0.07	-	100.0 ± 4.5	-
Agonist II	8.05 ± 0.05	0.03	132.0 ± 2.3	0.009
Merck cpd A	6.39 ± 0.07	0.9 (ns)	114.3 ± 5.4	0.9 (ns)
GSK137647A	6.4 ± 0.21	0.6 (ns)	37.74 ± 5.02	0.5 (ns)

Table 3-3: The potencies and efficacies of FFAR4 agonists were assessed in G_q signalling pathway. The results represent the mean ± standard error of the mean (SEM) obtained from three independent experiments, with a sample size of three (n=3). The statistical analysis employed in this study was a one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons. The notation "ns" was used to indicate non-significance. Note: The lower $\%E_{max}$ value for GSK137647A (37.74 ± 5.02) reflects that its dose-response curve only reached less than half the maximum response of TUG-891 within the tested concentration range. This suggests GSK137647A may be a partial agonist in this assay system.

3.3.6 cAMP assay in CHO cells stably expressing mFFAR4

In order to determine the potential of synthetic FFAR4 ligands to induce Gαs coupled signalling responses, Gαs signalling analyses were conducted by using cAMP assay. The test is built on the idea that endogenous cAMP might reduce the energy transfer between cAMP-labeled acceptor allophycyanin (XL665) and anti-cAMP antibodies labelled with europium cryptate, leading to a lower assay signal (Kühn and Gudermann, 1999). This assay was conducted on mFFAR4 CHO cell-lines demonstrating a decrease in Gαs coupling abilities (Figure 3-9A) in comparison to non-transfected CHO cells (Figure 3-9B). It was found that the application of various FFAR4 ligands to the cells resulted in less cAMP accumulation than the positive control group treated with forskolin, a direct activator of AC that induces cAMP production (Syrovatkina *et al.,* 2016) (Figure 3-9A). The outcomes observed in non-transfected cells were comparable to those observed in transfected cells, suggesting that mFFAR4 does not engage in signalling through Gαs coupled pathways upon activation by synthetic FFAR4 ligands (Figure 3-9B). However, FFAR4 agonist II was seen to exhibit some degree of Gαs coupling suggesting that this ligand may have partial function through the Gαs signalling pathway (P value= ns). When considered as a whole, except

FFAR4 agonist II, it seems that in this cellular system, the addition of these FFAR4 ligands did not trigger events in signalling via Gαs pathways.

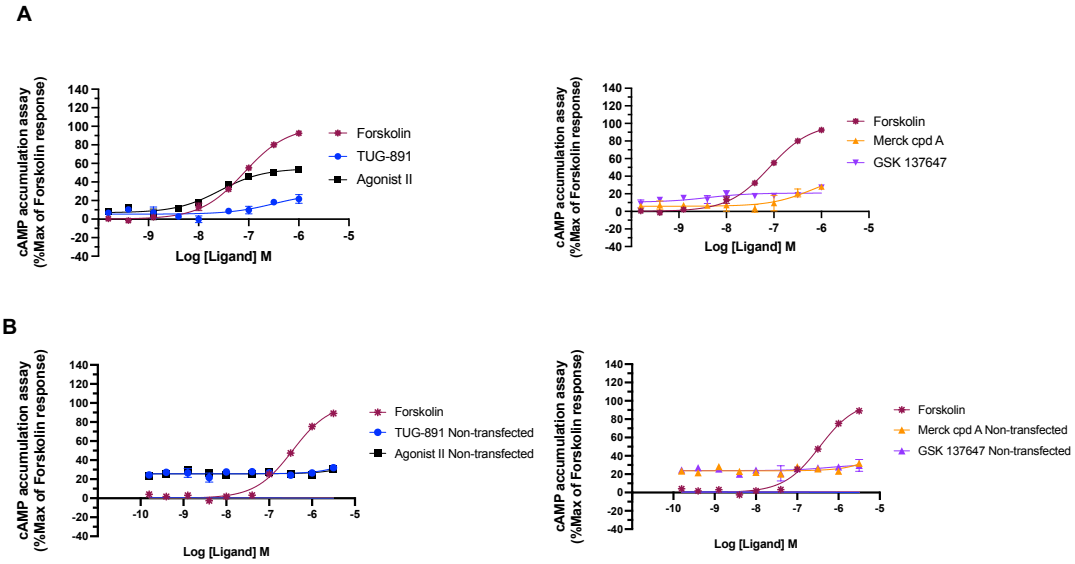


Figure 3-9: Assessment of the process of FFAR4 signalling through Gs-coupled pathways. The study involved the measurement of ligand-induced cAMP accumulation in two different cell types: **(A)** Flp-In CHO cells that were stably expressing mFFAR4, and **(B)** non-transfected CHO cells. This measurement was conducted after treating the cells with FFAR4 ligands. The cells were subjected to stimulation using an FFAR4 ligand at various concentrations over a duration of 1 hour and 45 minutes, resulting in the generation of a concentration-response curve. In all experiments, forskolin was employed as the reference ligand. The results presented in this study represent the mean ± standard error of the mean (SEM) obtained from three independent experiments, with a sample size (n) of three.

3.3.7 β-arrestin-2 recruitment assay

To evaluate the recruitment of β-arrestin-2 by mFFAR4 cells upon stimulation with FFAR4 ligands, BRET assays were conducted (Figure 3-10). When the receptor is active, this assay measures the recruitment of β-arrestin proteins to the activated GPCRs. It is useful for understanding how GPCR activation triggers downstream signalling pathways, especially those regulated by β-arrestin, which impact different cellular reactions and physiological processes (Alharbi *et al.,* 2022). All ligands exhibited the ability to induce recruitment of β-arrestin-2, thereby showing their capacity to couple with β-arrestin-2. Nevertheless, although the potencies of the compounds were comparable to that of TUG-891, the efficacies of the response exhibited variability, as indicated in Table 3-4. The effectiveness of response to

GSK137647A activation was found to be substantially reduced (P= 0.0001) compared to TUG-891. This suggests that this ligand may not effectively facilitate the coupling of mFFAR4 to β-arrestin-2. However, when compared to TUG-891, the effectiveness of FFAR4 Agonist II was shown to be significantly higher (P= 0.0001), whereas the efficacy of Merck cpd A had remained equivalent to the level of β-arrestin-2 recruitments of TUG-891.

In Figure 3-11, a summary is shown to compare novel FFAR4 ligands with their respective pharmacological assays. The comparison consists of TUG-891 with other FFAR4 ligands that are now available, namely FFAR4 agonist II, Merck cpd A, and GSK137647A, makes it abundantly evident that FFAR4 agonist II is the most powerful and efficient FFAR4 agonist that was evaluated in this thesis. The findings of this study have the potential to lead to the development of therapies that are more specifically focused and effective for diseases that are impacted by FFAR4 activation. These findings offer exciting implications for the advancement of our knowledge of FFAR4 signalling pathways.

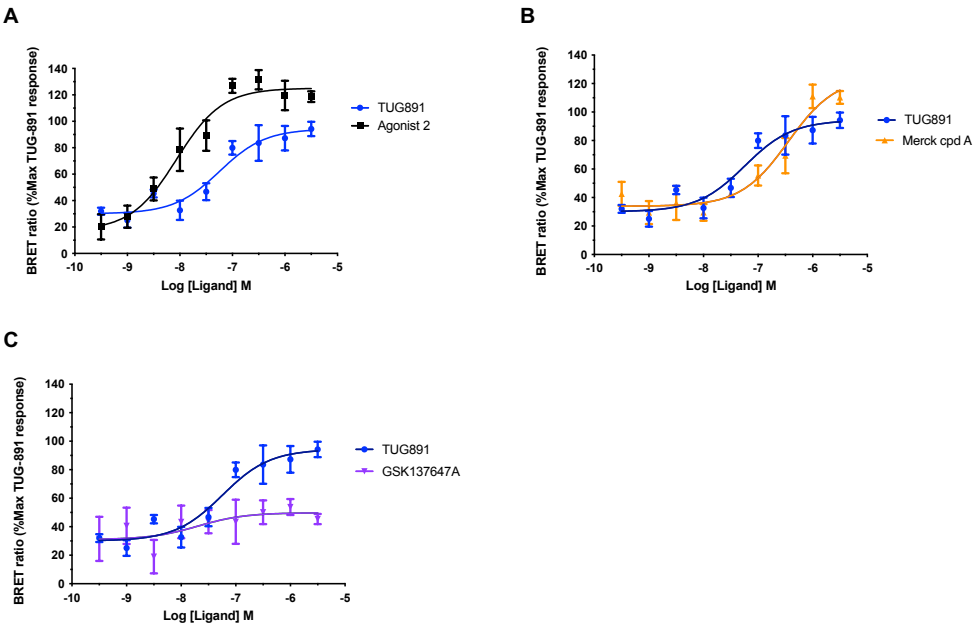


Figure 3-10: FFAR4 ligand stimulated β-arrestin-2 recruitment. The recruitment of β-arrestin-2 produced by ligands was quantified using a BRET assay in HEK293 cells that were stimulated to express mFFAR4. The cells were subjected to stimulation with FFAR4 ligands at various concentrations for a duration of 5 minutes in order to generate a concentration response curve. The data are presented in the form of a ratio, specifically the ratio between eYFP and R-luciferase interactions, with wavelengths of 535nm and 475nm. The results represent the mean ± standard error of the mean (SEM) derived from three independent experiments, with a sample size (n) of three.

FFAR4 Ligand	pEC_{50}		$\%E_{max}$ compared to TUG-891	
	Mean ± S.E.M	p value	Mean ± S.E.M	p value
TUG-891	7.2 ± 0.19	-	94.2 ± 4.3	-
Agonist II	8.1 ± 0.16	0.0001*	125.1 ± 8.4	0.009*
Merck cpd A	6.4 ± 0.19	0.6 (ns)	108.6 ± 4.7	0.8 (ns)
GSK137647A	6.2 ± 0.9	0.0001*	49.8 ± 6.4	0.0001*

Table 3-4: Illustration the potencies and efficacies of FFAR4 ligands as determined in a β-arrestin-2 recruitment assay. The results presented in this study are reported as the mean ± standard error of the mean (SEM) of three independent experiments, with a sample size (n) of three. The statistical analysis conducted in this study involved the use of one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons. P-values in the pEC_{50} column indicate statistical significance of the difference between each ligand's pEC_{50} and that of TUG-891. P-values in the $\%E_{max}$ column indicate statistical significance of the difference between each ligand's %$\%E_{max}$ and that of TUG-891. The notation used to indicate the significance levels was as follows: "ns" denoted non-significant results ($P > 0.05$), * denoted a significance level of $P < 0.01$.

3.4 Discussion

Despite being responsive to stimulation by the same ligands, many GPCRs are capable of generating signals that are limited to a particular cell type. Therefore, the observed variations in signalling could potentially be attributed to the varying degree to which distinct G protein-mediated and non-G protein pathways that a particular receptor can engage with in different cellular contexts. Significantly, this variation enhances the broad spectrum and functionality of naturally occurring ligands and their receptors (Thompson *et al.,* 2014), surpassing the prevailing perspective of "biased" ligands that exhibit a preference for engaging with one signalling pathway over another within a particular kind of cells (Luttrell *et al.,* 2015; Violin *et al.,* 2014). FFAR4 has the capacity to elicit various physiological outcomes in diverse cell types through the activation of specific pathways of signalling (Milligan *et al.,* 2015). For instance, FFAR4 has been found to facilitate an increase of calcium ions through the involvement of G_{q11} G proteins, thereby stimulating the secretion of incretin hormones like glucagon-like peptide-1 from cells in the gut (Hirasawa *et al.,* 2005). However, it has also been observed that this receptor can modulate the secretion of hormones which encourage satiety from gastrointestinal cells by activating G_i family G proteins that are sensitive to pertussis toxin (Engelstoft *et al.*, 2013). Furthermore, it has been discovered that the anti-

inflammatory properties of omega-3 fatty acids synthesized by macrophages are regulated by FFAR4. These outcomes are achieved through the involvement of non-G protein dependent pathways, which include the recruitment of β-arrestin-2 and subsequently occurring interactions between TAB1 and TAK1 (Oh *et al.*, 2010; Oh *et al.*, 2014). However, the progress in validating FFAR4 as a possible target for therapy has been impeded by the limited availability of ligands for this receptor. While it is possible to discover agonist ligands at FFAR4 that exhibit significant bias in preserving receptor configurations, enabling distinct interactions with G_{q11} and G_i G proteins or alternative pathways, the existing synthetic ligands for FFAR4 primarily consist of molecules that resemble fatty acids and feature carboxylic acid groups (Milligan *et al.,* 2015). Hence, the existing chemical diversity is presently inadequate to anticipate the effective targeting of unique signalling pathways with a notable level of selectivity in comparison to the naturally occurring ligands. Consequently, direct testing of this claim remains unfeasible.

In recent times, it has been demonstrated that the prevailing notion that GPCRs solely exert their detrimental impact by activating one or more members of the heterotrimeric G protein family is inaccurate (Lefkowitz, 2013). Therefore, the pharmacological impacts of newly discovered FFAR4 ligands and their potential for greater effectiveness compared to TUG-891 needs to be assessed. The objective of this chapter is to delineate the canonical pathways of signalling of various newly discovered FFAR4 ligands utilizing the cellular model, *Flp-In* mFFAR4 CHO cells, in order to provide a comprehensive understanding. The cell model employed in this study was chosen as it allows the efficient and quick development of stable cell lines expressing the FFAR4 protein from a Flp-In™ expression vector. Novel FFAR4 specific ligands were evaluated in pERK1/2 tests to investigate whether their action led to the activation of mFFAR4. Among these ligands TUG-891 was the best characterised FFAR4 ligand, however, it can activate FFAR1 receptor as well. For this reason, TUG-891 was used as a reference ligand and its effect compared with other ligands to identify any improved efficacy. The selection of these ligands was based on their demonstrated selectivity for activating FFAR4 in previous studies (Sparks *et al.*, 2014; Prihandoko *et al.*, 2020; Croze *et al.*, 2021). Prior to this work, the individual pharmacological characteristics of these FFAR4 agonists have been assessed in different papers. However, a thorough comparative investigation of their potency and effectiveness compared to TUG-891 has not been undertaken.

The ligand time course experiments revealed that the maximal responses were observed at 5 minutes for all ligands, which is consistent with the findings reported by (Hudson *et al.,*

2013). Frequently, the pERK1/2 responses associated with G protein signalling exhibit rapid kinetics, characterized by an initial peak response within 2.5 minutes of stimulation, followed by a swift decline in pERK levels. Subsequently, after 10 minutes of ligand treatment, the pERK levels reach a steady state, indicating a plateau in the response that occurs. The slower and sustained signals discussed in the literature are associated with pathways mediated by β-arrestin (Nobles *et al.,* 2011; Shenoy and Lefkowitz, 2005). However, the FFAR4 phosphorylation of ERK1/2 levels is not linked to arrestin recruitment as seen with the results obtained by the YM-254890, a specific G_q inhibitor. This is different from other GPCRs as arrestins are linked to ERK signalling (Eishingdrelo *et al.,* 2015). The study findings revealed an intriguing observation wherein FFAR4 Agonist II exhibited a slower return of ERK phosphorylation to basal levels compared to other ligands investigated. This suggests the possibility of distinct signalling mechanisms behind this specific ligand, potentially involving a gradual release from the receptor and a potential association with β-arrestin. Nevertheless, previous studies by Prihandoko *et al.* (2016) and Alvarez-Curto *et al.* (2016) do not provide any evidence regarding the involvement of β-arrestin-2 in pERK1/2 signalling by FFAR4. Furthermore, it was observed that FFAR4 Agonist II did not exhibit any signalling bias towards arrestin pathways (Euston, 2023). This indicates that the temporal profile of FFAR4 Agonist II should not significantly vary from that of the reference ligand, TUG-891. Hence, these findings indicate that FFAR4 Agonist II may not engage in signalling through β-arrestin-2 in assays that evaluate the phosphorylation of ERK1/2. Rather, it is likely to induce a more gradual homologous desensitization effect compared to other ligands that have been examined. This desensitization process can happen over a time span ranging from seconds to hours, as previously reported by Rajagopal and Shenoy, (2018) and Kelly *et al.* (2008).

Various pharmacological assays were employed to evaluate the coupling properties of mFFAR4 subsequent to stimulation by novel ligands. Since the assays used in this study were quantitative in nature, it was possible to characterize the effectiveness and outcomes of the ligands. In the experiments conducted to assess the activation of receptors and pERK1/2 signalling potential through pERK1/2 assays, the observed effectiveness values are different from the values stated in the literature. It is important to note that variations in the assay methodology employed to obtain results can contribute to these discrepancies (Leroy *et al.,* 2007). The variations in effectiveness primarily stem from disparities in the expression of receptors and subsequent receptor reserve. It is important to note here that the cells used in the experiments were containing e-YFP at the C-terminal tail which could alter the coupling to the downstream effectors. Furthermore, the manner in which the pathways are evaluated,

such as the selection of specific time points, can also have an influence on the effectiveness of ligands. This study found that none of the tested compounds showed significantly greater effectiveness than the TUG-891 reference ligand. However, it is noteworthy that all the compounds exhibited numerically higher effectiveness values compared to TUG-891, although these differences did not reach statistical significance. This observation implies that these ligands have the potential to be therapeutically effective and should be further investigated in *in vivo* experiments.

FFAR4 is widely recognised as a receptor that exhibits an exceptionally high degree of specificity in its interaction with members of the $G_{q/11}$ G protein subfamily (Hirasawa *et al.,* 2005). This interaction leads to an increase in intracellular calcium levels through the activation of phosphoinositidase phospholipase C, which subsequently generates inositol (1,4,5) trisphosphate. Consequently, the accumulation assay of IP1 was performed in response to ligands of FFAR4. The ligands TUG-891, Merck cpd A, and FFAR4 Agonist II demonstrated the ability to induce robust and effective responses resulting in the accumulation of IP1. However, GSK137647A did not exhibit this capability. This observation may indicate that ligands lacking a carboxylic group, such as GSK137647A, exhibit limited efficacy in inducing IP1 accumulation, potentially due to structural dissimilarities. According to Seger and Krebs (1995) and Yang *et al.* (2013) it is possible for signal amplification to take place due to the downstream occurrence of ERK1/2 phosphorylation following IP1 accumulation. This amplification may result in a rise in the number of stimulated substances at every phase of a signalling pathway, potentially explaining the observed variations in pharmacological values.

Furthermore, there is a lack of specific documentation regarding the coupling of FFAR4 to Gαs signalling pathways in different cell lines. The primary objective of previous studies was to examine the mechanism by which FFAR4 is selectively coupled to Gαi/o and Gαs proteins in specific tissues, such as pancreatic δ-cells, gastric ghrelin-secreting cells, and intestinal L-cells (Engelstoft *et al.*, 2013; Stone *et al.*, 2014; Tsukahara *et al.*, 2015; Moniri, 2016). Nevertheless, it has been observed that the coupling of G proteins can undergo alterations in response to ligand bias, as demonstrated by Hauser *et al.* (2022). In order to establish that the FFAR4 ligands under investigation did not elicit Gαs coupling, cAMP analyses were conducted. While FFAR4 ligands were found to not induce cAMP accumulation, previous studies indicated that FFAR4 is capable of coupling to Gαi pathways (Engelstoft *et al.,* 2013; Stone *et al.,* 2014). The previous studies have shown that FFAR4 signals through Gαi coupled pathways to decrease somatostatin production in the pancreas

during periods of high glucose levels (Stone *et al.*, 2014; Croze *et al.*, 2021) and stimulates the release of ghrelin from the stomach (Engelstoft *et al.,* 2013). However, it should be noted that these findings may be unique to certain tissues, as they were not reproduced in the cellular model used in this study. The study conducted by Okashah *et al.* (2019) utilized bioluminescence resonance energy transfer (BRET) assays to demonstrate that GPCRs that basically interact with Gαq G proteins may also exhibit promiscuous coupling with Gαi G proteins. However, it is important to note that certain functional assays, including the ones utilized in this chapter, may not be capable of detecting weak coupling patterns. This limitation may explain why Gαi coupling was not observed in the cell lines investigated in this study. Nevertheless, even though secondary couplings of this nature may be weak, they can still elicit physiological effects. The β2AR, which is coupled to the Gαs protein, has been found to also couple to Gαi proteins, leading to a decrease in cardiac contractility (Okashah *et al.,* 2019; Xiao *et al.,* 1995).

Canonical signalling through G proteins plays a crucial role in regulating the synthesis of second messengers. However, it is worth noting that significant amounts of intracellular adaptive proteins have the ability to engage with GPCRs either directly or as components of larger protein complexes. These interactions may alter downstream pathways of signalling and exert influence on various physiologic processes. The adaptor proteins that have been extensively researched are primarily from the arrestin family (Gurevich and Gurevich, 2015; Luttrell and Gesty-Palmer, 2010; Smith and Rajagopal, 2016). Regardless of their initial designation based on their ability to "arrest" and inhibit G protein-mediated signalling, it is evident that they can also positively influence numerous cellular processes in a GPCR-dependent manner (Gurevich and Gurevich, 2015; Luttrell and Gesty-Palmer, 2010; Smith and Rajagopal, 2016). This phenomenon arises due to their ability to restrict the intensity and duration of G protein-mediated signalling by attaching to GPCRs that are engaged by agonists. Consequently, this prevents concurrent association with heterotrimeric G proteins. It has been reported that FFAR4 predominantly couples with Gαq G proteins (Hirasawa *et al.,* 2005); however, FFAR4 also exhibits coupling with β-arrestin-2 (Alharbi *et al.,* 2022; Alvarez-Curto *et al.,* 2016). This study also examined the recruitment of β-arrestin-2 to mFFAR4 receptors, and the data obtained confirmed that the ligands were capable of promoting mFFAR4 β-arrestin-2 coupling. It is worth noting that there were no significant differences in ligands' effectiveness between TUG-891 and FFAR4 agonist II. However, previous reports have indicated that TUG-1197 and GSK137647A exhibit considerably lower efficacy, which suggests a weaker recruitment with β-arrestin-2 (Euston, 2023). The data presented in their study demonstrates a correlation between lower effectiveness and a

failure to achieve maximal responses in the IP1 and pERK1/2 assays. Collectively, the interaction between FFAR4 and arrestins is robust and contingent upon the presence of an agonist, aligning with the results documented in prior studies conducted by Butcher *et al.* (2014), Prihandoko *et al.* (2016), Hudson *et al.* (2013), and Hudson *et al.* (2014). This happen because the phosphorylation of two groups of serine and threonine residues situated in the intracellular carbon terminal tail of the receptor (Burns *et al.,* 2014; Butcher *et al.,* 2014; Prihandoko *et al.,* 2016) plays a significant role in this process.

Several studies have been conducted to examine the bias properties of FFAR4 ligands in order to evaluate the downstream coupling of FFAR4 with G proteins (Li *et al.,* 2015). The potential importance of this expression profile in different cell subtypes could be significant and could be utilized if specific FFAR4 ligands can be discovered that exhibit a preference to stimulate signalling through $G_{q/11}$ and G_i family G proteins. In the preliminary investigations on deorphanisation, Hirasawa *et al.* employed techniques to assess the internalisation of FFAR4 from the cellular surface in transfected cells. The internalization of FFAR4 generated by ligands is both strong and widespread, as observed in previous studies (Hudson *et al.,* 2013; Watson *et al.,* 2012). In the case of HEK293 cells, this internalization process is primarily mediated by interactions with an arrestin adapter protein (Alvarez-Curto *et al.*, 2016). The complete characterization of the non-canonical, non G protein-mediated signalling functions of an FFAR4/arrestin complex is still under consideration. In their study, Alvarez-Curto *et al.* found that arrestins do not play a significant role in the phosphorylation of ERK1/2 caused by FFAR4 in HEK293 cells that lack expression of Gαq plus Gα11 or β-arrestin-1 and β-arrestin-2. This finding contradicts the commonly held belief that arrestins are closely associated with the temporal regulation of GPCR-mediated ERK1/2 MAP kinase signalling. Furthermore, the most important function of arrestins in FFAR4 signalling in these cell settings was their more typical function of working to desensitize G protein-mediated signalling. This was because the deletion of arrestins led to Ca^{2+} 'spikes' being created continuously with further exposure to a ligand. This was the essential role that arrestins played in FFAR4 signalling. Recently, Li *et al.* (2015) provided a description of a series of FFAR4 agonists that exhibit a notable bias towards signalling through arrestin-mediated pathways, however the exact mechanism of these ligands has not been confirmed yet. While the bias of these compounds at FFAR4 has not been confirmed, it would be intriguing to explore whether FFAR4 ligands can exhibit a preference for one signalling pathway over another (Hudson *et al.,* 2014).

In a recent study, Euston (2023) provided evidence that FFAR4 Agonist II exhibits a bias towards IP1 accumulation, in contrast to TUG-891 which had previously been reported to lack stimulus bias (Butcher *et al.,* 2014). Significantly, the G proteins and non-G proteins routes, namely Gαq, Gαi, and β-arrestin-2 routes, have the potential to induce phosphorylation of ERK1/2 (Eishingdrelo, 2013). Yet, Alvarez-Curto *et al.* (2016) and Prihandoko *et al.* (2016) have reported that β-arrestin recruitment does not play a role in the activation of MAPK signalling and phosphorylation of ERK1/2 by FFAR4. It seems unlikely that there is a receptor bias between IP1 and pERK1/2 if they are equally downstream of receptor via the $G_{q/11}$ association. However, the data presented by Euston (2023) provides evidence of bias, indicating that there may indeed be distinct receptor activation processes at play within these routes. This phenomenon could potentially be attributed to the intricate nature of pERK1/2 signalling. Nevertheless, the FFAR4 Agonist II exhibits a bias towards IP1, which consequently leads to enhanced activation of the downstream G_q coupled signalling route. This bias is attributed to the ligand's ability to generate robust and effective responses, ultimately resulting in the accumulation of IP1. The G_q signalling route, in which IP1 is a constituent, results in an increase in intracellular calcium levels. This increase in calcium can give rise to various reactions in the body, including proliferation, division, contraction, exocytosis, and liver metabolic processes (Berridge, 2009). The preferential stimulation of responses in the body mediated by FFAR4 is potentially significant. It has been observed that the activation of FFAR4 leads to a rise in intracellular Ca^{+2} levels, which in turn promotes the secretion of GLP-1 from enteroendocrine cells. This pathway has been suggested to play a role in facilitating GLP-1 secretion (Hirasawa *et al.,* 2005; Tanaka *et al.,* 2008). Therefore, when taken together, all of these studies show that biased ligands like FFAR4 Agonist II, which promote G_q signalling route pathways, might have therapeutic value in fields like metabolic conditions; however, this is something that will need to be researched further.

The majority of molecules classified as FFAR4 ligands possess a carboxylate or bioisostere moiety, which has been proposed to engage in interactions with Arg^{99} of the FFAR4 within the orthosteric attaching pocket. The compound GSK137647, as described by Azevedo *et al.* (2016), represents the first nonacidic FFAR4 ligand and contains a sulfonamide group. The absence of structural variety in the synthesis of various FFAR4 ligands contributes to a deficiency in their selectivity, thereby posing challenges (Milligan *et al.,* 2017a). Studying FFAR4 presents certain obstacles due to its limited sequence conservation in comparison to the FFAR1 receptor. However, it is noteworthy that both receptors exhibit responsiveness to long chain free fatty acids. The development of synthetic agonists is complicated by the fact

that agonists targeting FFAR4 receptors may also have the ability to attach to FFAR1 receptors (Hara *et al.,* 2009). For example, the initial synthetic FFAR1 active agonist, GW9508 (4-{[(3-phenoxyphenyl)methyl]amino}benzenepropanoic acid), was promptly observed to also exhibit activation of FFAR4, albeit with significantly reduced effectiveness by a factor of approximately 100 (Briscoe *et al.,* 2006). According to an earlier study by Houthuijzen *et al.* (2017), it was found that TUG-891 exhibited activity at FFAR1, indicating that this particular ligand functions as a dual agonist for both FFAR4 and FFAR1. This finding is consistent with the results obtained from the mouse ortholog by Hudson *et al.* (2013). Previous studies by Oh *et al.* (2014) and Azevedo *et al.* (2016) have documented the high specificity of TUG-1197 and Merck cpd A as agonists for FFAR4. In previous studies, it has been documented that GSK137647A exhibits responsiveness at the FFAR1 receptor, while demonstrating a potency that is 50 times greater at the FFAR4 receptor (Sparks *et al.,* 2014). When working with systems such as animal models, which may express both FFAR4 and FFAR1, this is a crucial consideration to keep in mind.

To summarise, this chapter has provided evidence that FFAR4 ligands possess the capability to stimulate the mFFAR4 receptor, leading to the activation of Gαq/11 coupled signalling and the recruitment of β-arrestin-2. The characterization of synthetic ligands is of significant importance as it has the potential to elucidate the physiological impacts of FFAR4 in *ex vivo* and *in vivo* investigations. A variety of pharmacological assays were employed in this research to characterise several novel FFAR4 ligands, with the objective of evaluating their effectiveness in comparison to a reference ligand. Findings of this research confirmed that treatment with PTX demonstrates no significant impact on the ERK1/2 pathway, indicating that the activation of ERK1/2 is not reliant on the Gi pathway. However, data generated using the compound YM-254890 indicate that FFAR4 is predominantly associated with the Gq/11 pathway. Pharmacological assays confirmed that ligands have the ability to activate the FFAR4 and MAP kinases pathways to different extents. Among the compounds examined, FFAR4 Agonist II exhibited higher effectiveness, while Merck cpd A and GSK137647A demonstrated comparable or lower potency when compared to TUG-891, which served as the reference ligand. The FFAR4 Agonist II was determined to be a more effective ligand than TUG-891. FFAR4 Agonist II has the potential to serve as a valuable instrument for conducting *ex vivo* and *in vivo* investigations aimed at confirming FFAR4 as a viable therapeutic target in the context of metabolic disorders.

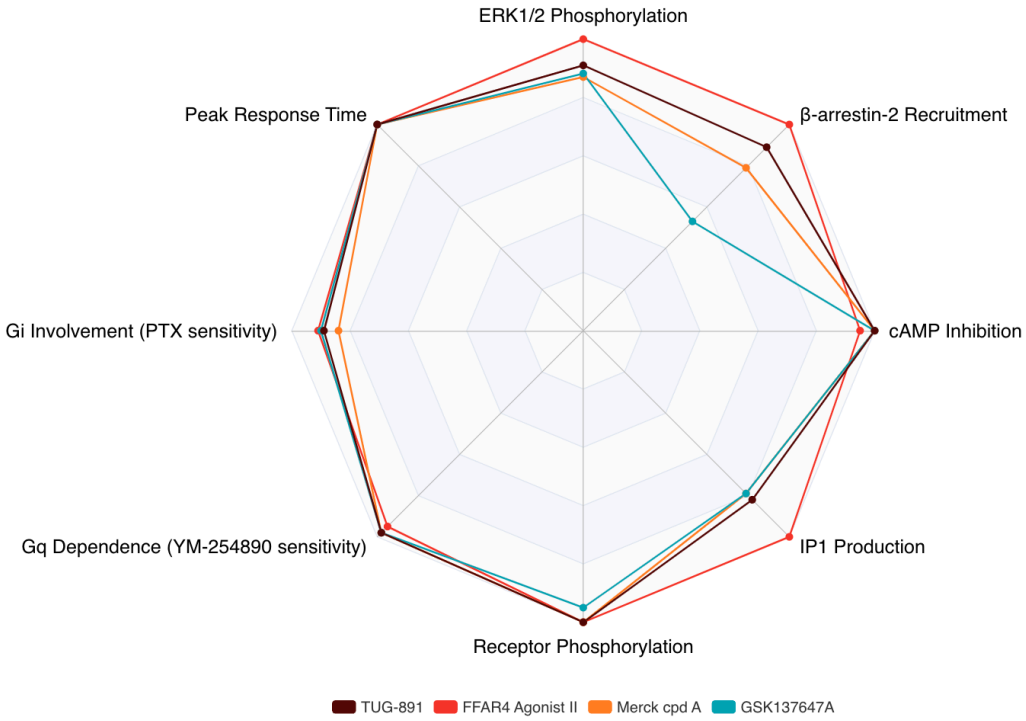


Figure 3-11: Radar chart comparing the FFAR4 ligands' pharmacological characteristics. The chart displays the comparative performance of TUG-891, FFAR4 Agonist II, Merck cpd A, and GSK137647A in relation to eight pharmacological parameters: Receptor Phosphorylation, ERK1/2 Phosphorylation, cAMP Inhibition, IP1 Production, β-arrestin-2 Recruitment, Gq Dependence (YM-254890 sensitivity), Gi Involvement (PTX sensitivity), and Peak Response Time. The data were obtained via many assays, including Western blot analysis, pERK1/2 HTRF assay, cAMP assay, IP1 accumulation assay, and BRET assay for β-arrestin-2 recruitment. The best-performing ligand was assigned a value of 100% for each parameter, while the values for the remaining ligands were calculated as relative percentages. For inhibitor studies with YM-254890 (Gq inhibitor) and PTX (pertussis toxin, Gi inhibitor), the percentage change in potency was used.

Chapter IV *Ex vivo* evaluation of FFAR4 function in the pancreas

4.1 Introduction

The pancreatic islets of Langerhans have a vital function in regulating metabolic balance by releasing several hormones in a coordinated manner. The endocrine system is an intricate structure of glands that play a crucial role in maintaining blood glucose levels and general metabolic well-being via complicated hormone production and interactions. These mechanisms have been clarified by recent developments in islet biology, which has improved our knowledge of both healthy physiology and pathological diseases like T2DM.

The hormones secreted by the islets of Langerhans play crucial roles in maintaining metabolic homeostasis. Insulin, produced by β-cells, is a key anabolic hormone primarily responsible for regulating glucose and lipid metabolism. Its secretion is strongly triggered by a rise in blood glucose levels after a meal, a process known as glucose-stimulated insulin secretion (GSIS) (Figure 4-1). When glucose levels increase, it enters β-cells via GLUT2 transporters, leading to increased ATP production. This closes ATP-sensitive potassium channels, causing membrane depolarization and subsequent calcium influx, ultimately triggering insulin release (Campbell and Newgard, 2021).

The efficacy of GSIS is enhanced by several factors. Incretin hormones, such as glucose-dependent insulinotropic polypeptide (GIP) and GLP-1, potentiate insulin secretion through cAMP-dependent pathways. These incretins, produced by the gastrointestinal tract in response to nutrient ingestion, bind to their respective receptors (GIPR and GLP-1R) on β-cells. Additionally, amino acids and fatty acids further augment insulin secretion.

Conversely, glucagon secretion by α-cells is regulated in a manner opposite to insulin. It is inhibited by elevations in glucose and insulin levels but stimulated by amino acids and fatty acids, whose levels rise during fasting. Recent evidence indicates that the endocrine activity of α-cells plays an important part in regulating the production of insulin in β-cells in response to glucose stimulation (Capozzi *et al.,* 2019; Zhu *et al.,* 2019). In addition to glucagon, α-cells also synthesise GLP-1 by an alternative metabolism of the proglucagon peptide precursor. Both hormones elevate cAMP levels in β-cells, hence controlling the release of insulin in response to nutrients. The main mechanism of this signalling is the interaction between these hormones and GLP-1R, with a secondary interaction occurring with the

glucagon receptor (GCGR) (Müller *et al.,* 2019). This differential regulation allows for appropriate hormonal responses to varying metabolic states.

Somatostatin, synthesized by δ-cells, acts as a paracrine inhibitor of both glucagon and insulin release, providing an additional layer of regulation within the islets. A distinctive paracrine relationship occurs via the production of Urocortin 3 (UCN3) by β-cells. UCN3 stimulates δ-cell function, leading to an increase in the production of somatostatin via activation of the corticotropin-releasing hormone receptor (CRHR2). The increase in somatostatin secretion suppresses the secretory function of β-cells, hence establishing a negative feedback mechanism that aids in regulating insulin release (Rorsman and Huising, 2018).

The metabolic effects of islet hormones align with the factors that influence their production in various physiological situations. This intricate interplay between nutrients, hormones, and cellular mechanisms ensures a finely tuned response to maintain energy balance in the body. It is imperative to comprehend the complex relationships between these signalling pathways in order to understand islet function in both healthy and diseased states, including diabetes mellitus, where disruptions in these pathways can result in metabolic dysregulation.

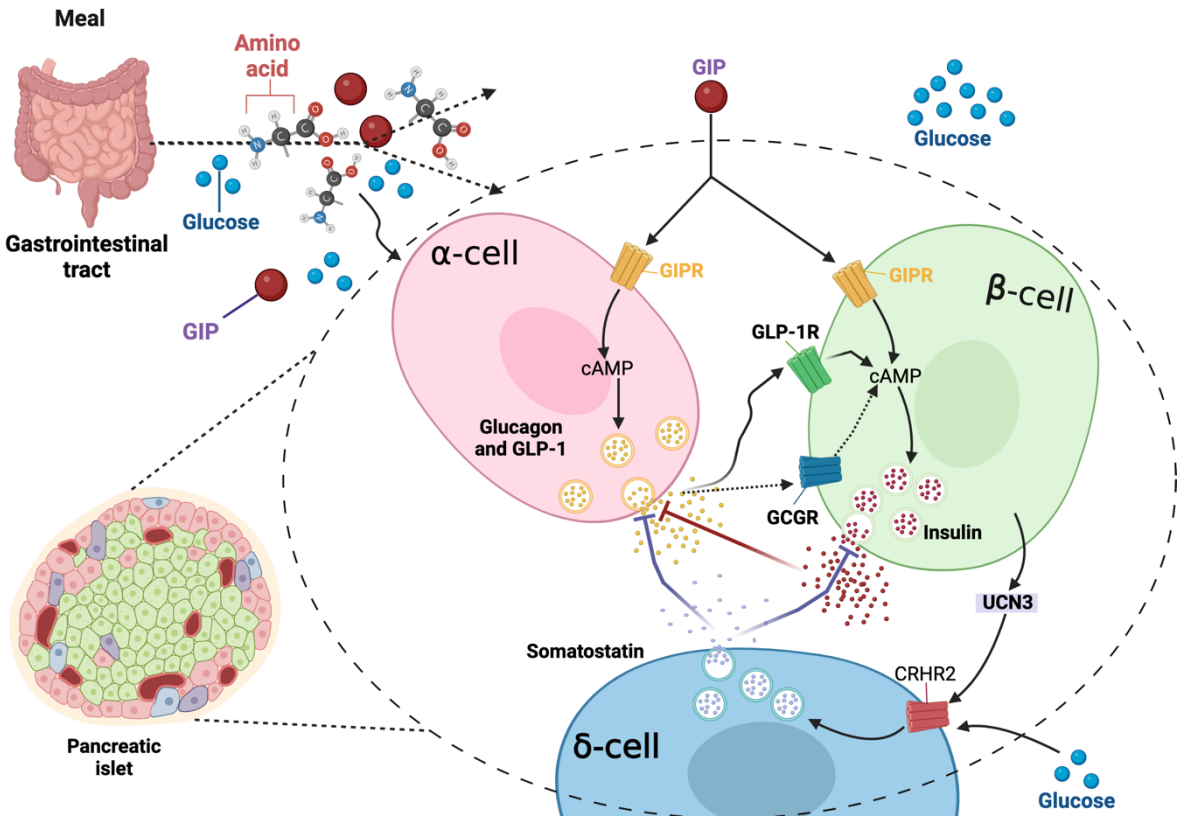


Figure 4-1: Islet hormone secretion and interactions in response to nutrient ingestion. Ingesting nutrients leads to an increase in the levels of glucose, amino acids, and incretin hormones (glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1)) in the bloodstream. GIP and GLP-1 interact with their respective receptors (GIPR, GLP-1R) on β-cells, which leads to an increase in glucose-stimulated insulin secretion (GSIS) via pathways that rely on cAMP. GIP further triggers glucagon release from α-cells in response to amino acids. α-cells secrete both glucagon and GLP-1, which stimulate the production of cAMP in β-cells, hence regulating the release of insulin. This predominantly occurs via binding to the GLP-1R and secondly through the glucagon receptor (GCGR, shown by a dashed arrow). Crucially, the release of glucagon often counteracts the effects of insulin, whereas insulin has a suppressive effect on glucagon secretion, resulting in a harmonious interaction. β-cells secrete Urocortin 3 (UCN3), which stimulates δ-cells to release somatostatin via CRHR2. This somatostatin then suppresses the production of β-cells, creating a negative feedback loop. Primary routes are shown by solid arrows, whereas secondary or less known interactions are represented by dashed arrows. Inhibitory arrows (⊣) show the suppressive effects of insulin on glucagon secretion and somatostatin on β-cell and α-cell secretion. The complex system of paracrine and endocrine signalling guarantees accurate control of glucose homeostasis in accordance with the body's nutritional condition.

The production of ligands within tissues and their resulting interactions with their related GPCRs play a crucial role in maintaining tissue homeostasis and normal physiological functions. A number of endogenous ligands of GPCR circuits have been identified within pancreatic islets. For example, the endogenous circuit involving GLP-1 and GLP-1R within pancreatic islets plays a crucial role regulating the release of insulin and islet regeneration (De León *et al.,* 2003; Salehi *et al.,* 2010; Smith *et al.,* 2014). The production of endogenous

acetylcholine by pancreatic α-cells in humans has been found to have an impact on the release of insulin (Molina *et al.,* 2014; Rodriguez-Diaz *et al.,* 2011). This impact is not limited to the stimulation of insulin-secreting β-cells through the activation of muscarinic acetylcholine receptors M3 and M5. It has also been observed that δ-cells are stimulated by acetylcholine through the activation of M1 receptors (Molina *et al.,* 2014; Rodriguez-Diaz *et al.,* 2011). The controlled release of somatostatin (SST) release from pancreatic δ-cells and its interaction with SST receptors in pancreatic β-cells through conditional and epigenetic processes play crucial roles in modulating islet homeostasis (Li *et al.,* 2017). Nevertheless, the precise mechanisms underlying the endogenous signalling pathways of various GPCRs in pancreatic islets, which play a crucial role in regulating glucose metabolism, remain unclear.

FFAR4 is among the top 20 genes identified by systems genetic analysis as being associated with the management of T2DM (Taneera *et al.,* 2012). The stimulation of FFAR4 has garnered growing attention in recent years due to its positive impact on glucose and energy homeostasis in preclinical studies (Liu *et al.,* 2015). The knockout of FFAR4 in mice is associated with obesity characterised by impaired glucose tolerance and the presence of fatty liver disease (Ichimura *et al.,* 2012). Additionally, it has been observed that FFAR4 activation can stimulate the development of fat cells (Hilgendorf *et al.,* 2019) and enhance the thermogenic activity of brown adipose tissue (Quesada-López *et al.,* 2016). Furthermore, FFAR4 stimulation has the ability to suppress the breakdown of fats in white adipose tissue (Husted *et al.,* 2020), regulate the intake of food (Auguste *et al.,* 2016), and influence the release of enteroendocrine hormones such as ghrelin (Lu *et al.*, 2012; Engelstoft *et al.*, 2013), glucagon-like peptide-1 (Hirasawa *et al.,* 2005), glucose-dependent insulinotropic polypeptide (Iwasaki *et al.,* 2015), cholecystokinin (Iwasaki *et al.,* 2015), and somatostatin (Egerod *et al.,* 2015). The studies conducted by Moran *et al.* (2014) and Zhang *et al.* (2017) demonstrated that FFAR4 signalling has the ability to enhance the release of insulin in cell lines that produce insulin through the process of intracellular calcium mobilisation. FFAR4 is expressed in various types of islet cells, including α, β, δ, and γ cells. Nevertheless, the tissue distribution of FFAR4 continues to be a subject of substantial debate, with the specific islet cell types that express this molecule remaining uncertain. Its stimulation has been shown to alleviate dysfunction and apoptosis in β-cells (Taneera *et al.,* 2012; Wang *et al.,* 2019), as well as influence the release of islet hormones (Croze *et al.,* 2021). The activation of FFAR4 has been found to increase glucose-stimulated insulin secretion (McCloskey *et al.,* 2020), enhance the release of glucagon (Wu *et al.,* 2021), prevent gastric somatostatin

release (Stone *et al.*, 2014), and stimulate pancreatic polypeptide (PP) release (Zhao *et al.,* 2020). The identification of FFAR4 expression patterns was accomplished through the utilisation of transcriptomic profiling and RT-PCR techniques. The results obtained from these analyses have suggested that FFAR4 is predominantly expressed in δ-cells, with comparatively lower levels of expression observed in α and β cells (Croze *et al.,* 2021; Taneera *et al.,* 2012). The confirmation of preferential expression of FFAR4 in islet δ-cells was achieved through the knock-in of a LacZ reporter into the FFAR4 locus in mice (Stone *et al.*, 2014). Nevertheless, the precise function of FFAR4 in the distinct pancreatic islets cell types and its overall impact on insulin and somatostatin release have yet to be determined.

Mouse models are fundamental tools for investigating processes in the body. Consequently, in order to examine the impact of FFAR4 on hormone secretion from the mouse pancreas, mouse models were employed. GenOway successfully generated a C57BL/6 mouse model with a HA-tagged FFAR4 protein (named FFAR4-WT-HA). This was achieved by introducing a HA tag sequence upstream of the STOP codon, which is situated in exon 3 of the FFAR4 gene. Nevertheless, the receptor's function should be unaffected since it continues to be expressed under the regulation of the intrinsic FFAR4 promoter. In a comparable manner, GenOway successfully created another mouse line featuring a mutant variant of the FFAR4 receptor that has a number of amino acids for agonist-induced phosphorylation altered to Ala (PD mouse line), based on the findings of our group's phosphorylation studies (Butcher *et al.,* 2011; Prihandoko *et al.,* 2016). A HA tag and six-point mutations in the C-terminal tail were introduced here. The mutations led to the generation of a mutant (ADAA-AAA) that was unable to undergo phosphorylation. This was achieved by substituting the serine and threonine residues in the C-terminus with alanine residues. Finally, a mouse lacking FFAR4 gene expression (FFAR4-KO) was generated using a method provided by Invitrogen. In this study, the gene encoding the FFAR4 receptor was replaced with a sequence able to encode β-galactosidase (β-gal), which also included a nuclear localization signal. This modification led to the creation of a constitutive knock-out animal model but with the potential to identify cells in which the FFAR4 promoter drives expression of β-gal. The nuclear localization signal ensures that the expressed β-gal enzyme is directed to the nucleus of the cells where the FFAR4 promoter is active (Figure 4-2).

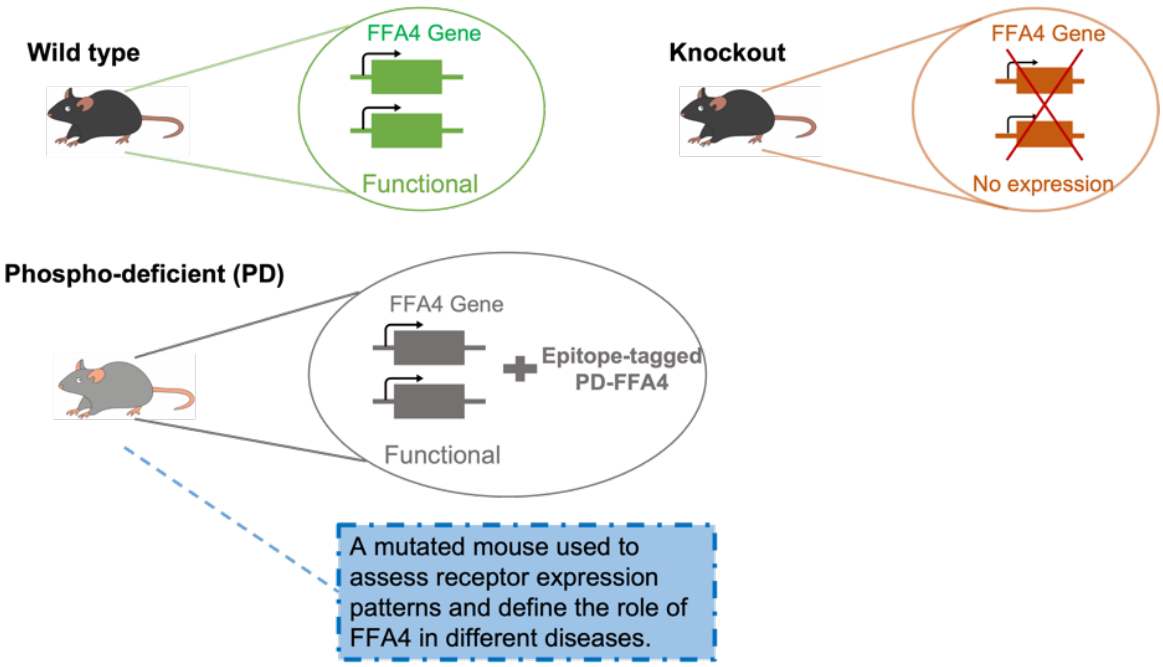


Figure 4-2: Genetically modified FFAR4 mouse lines. To explore the role of regulated phosphorylation of FFAR4, the wild type receptor was replaced with a variant in which all the hydroxy-amino acids in the C-terminal tail were replaced by Alanine (phospho-deficient, PD). PD-FFAR4-HA mice have a series of mutations from Threonine/Serine to Alanine in the C-terminal tail, resulting in the inability for these sites to become phosphorylated. In FFAR4-KO mice the gene coding for FFAR4 is replaced with β-galactosidase enzyme. Together with the wild type and FFAR4 knock-out littermates, a comprehensive understanding of the role of the phosphorylation of FFAR4 may be established by studies conducted using the PD-FFAR4-HA line.

The precise regulation of hormone release by the endocrine pancreas is crucial for the maintenance of glucose homeostasis. FFAR4 is of significant importance in the modulation of insulin and glucagon production, as well as the suppression of somatostatin release within the islets of Langerhans. However, a comprehensive understanding of the distinct contributions of α, β, and δ cells in mediating the insulinotropic and glucagonotropic effects induced by FFAR4 remains to be fully elucidated. The hypothesis of this study is based on the discovery that FFAR4 mRNA is substantially expressed in δ-cells that produce somatostatin. The theory suggests that the impact of activating FFAR4 leads to the stimulation of insulin and glucagon release by blocking the secretion of somatostatin.

4.2 Objectives

The objectives of this study were to:

- To define the cellular localization of FFAR4 in pancreatic islets.
- To investigate the functional impacts of FFAR4 expression in pancreatic islets.
- To assess the activity of FFAR4 ligands to stimulate the release of insulin.

4.3 Results

4.3.1 Gene expression of FFAR4 in mouse pancreatic islets

A. qPCR analysis

The expression of mFFAR4 has been documented in previous studies conducted on the mouse pancreas (Stone *et al.*, 2014; Croze *et al.*, 2021). To validate this observation, q-PCR was performed on cDNA samples derived from isolated mouse pancreatic islets. Experiments were conducted utilising FFAR4-WT-HA, FFAR4-KO and FFAR4-PD animal models. As anticipated, the presence of FFAR4 mRNA expression was validated in FFAR4-WT-HA and FFAR4-PD mice, while it was absent in mice lacking the FFAR4 receptor (Figure 4-3). This observation was consistent with previously reported studies showing that FFAR4 is expressed in the adult mouse pancreas (Stone *et al.*, 2014; Zhao *et al.*, 2020; Croze *et al.*, 2021).

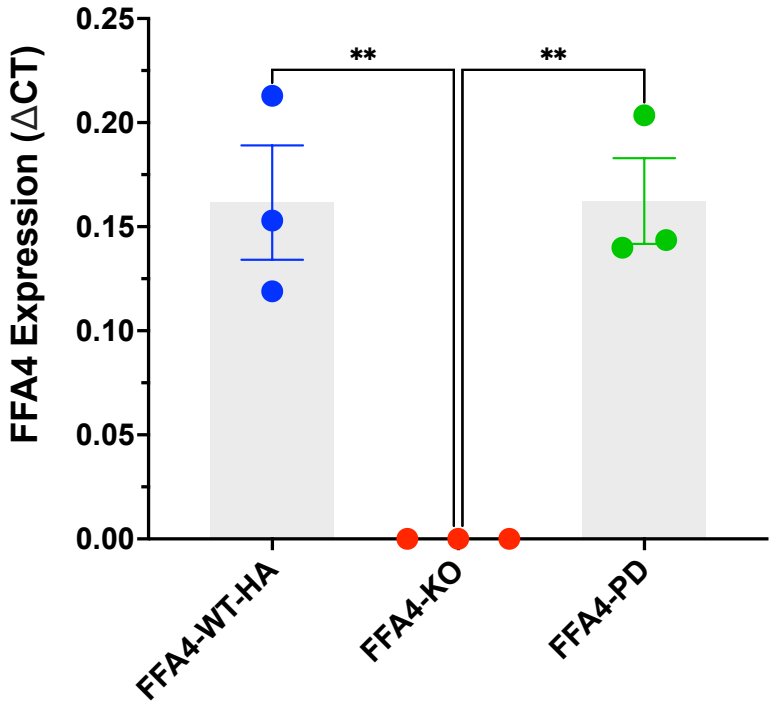


Figure 4-3: Expression of FFAR4 in mouse pancreas. Isolated pancreatic islets were obtained from FFAR4-WT-HA, FFAR4-KO, and FFAR4-PD. FFAR4 primers were used to conduct q-PCR analysis on cDNA. These results represent mean ± S.E.M of three independent experiments. The statistical analysis that was carried out consisted of repeated measures one-way analysis of variance (Dunnett's multiple comparisons), with the following significance levels **=P<0.01.

B. Immunohistochemistry staining using β-galactosidase antibody

To determine the distribution or expression of mFFAR4 in the pancreas, which was initially detected by qPCR, immunohistochemistry (IHC) was conducted on sections of mouse pancreas. For this study, an antibody specific to β-gal was employed to identify β-gal expression driven by the mFFAR4 promoter in tissues from the FFAR4-KO mice. Using the β-gal antibody provides two primary advantages:

I. Precise identification: β-gal is not normally expressed in mammalian systems but is commonly employed as a reporter gene in many transgenic mouse models.

II. Precise observation: By combining the β-gal antibody with a suitable secondary antibody and detection system, it becomes possible to observe cells in which the mFFAR4 promoter is active with great sensitivity.

In these experiments, paraffin-embedded pancreas sections recovered from FFAR4-WT-HA and FFAR4-KO animals were stained with β-gal antibody followed by an Alexa fluor 546

red antibody to the presence of β-gal is in FFAR4-KO animals, and by comparison with FFAR4-WT-HA, demonstrated a distinct staining pattern within specific pancreatic cells (Figure 4-4A). This pattern was absent from the FFAR4-WT-HA animals (Figure 4-4B). These stained cells comprised only a small subpopulation of the total pancreatic cells complement of each islet and were arranged peripherally within the islet mantle.

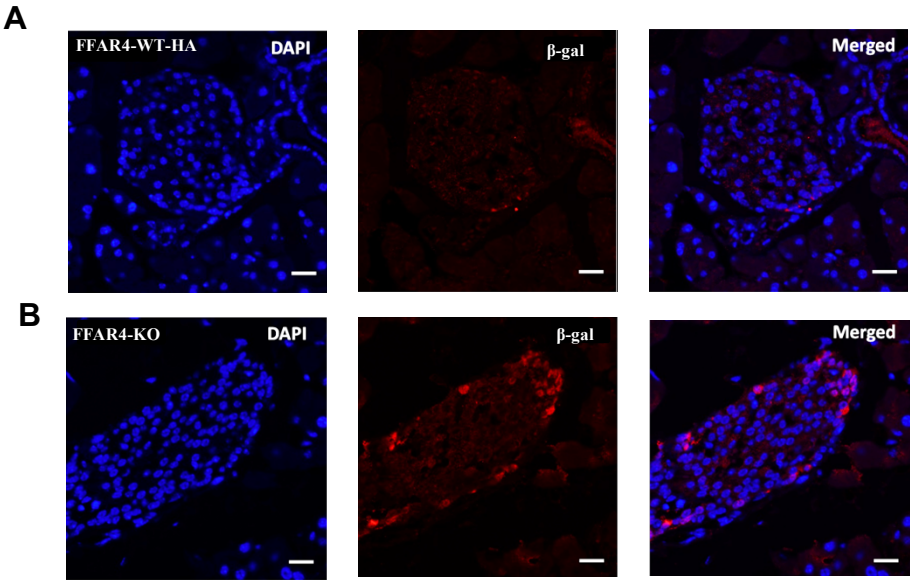


Figure 4-4: β-galactosidase (as a surrogate for FFAR4) is expressed in mouse peripheral cells in the islets of Langerhans. Pancreatic sections were subjected to immunohistochemistry processing and staining with β-galactosidase primary antibody prior to examination under a confocal microscope with a 40x objective **(A)** from WT and **(B)** from FFAR4-KO. The images show n=3 and the scale bar is 20 μm.

As this method specifically detected the expression of mFFAR4 in the pancreas, the objective was next to locate mFFAR4 in individual pancreatic cell types. Again, sections of mouse pancreas from the FFAR4-KO and FFAR4-WT-HA animals were dual-stained with antibodies to specific pancreatic hormones, namely insulin, glucagon and somatostatin with β-gal antibody to locate which pancreatic cell types express the receptor. These hormones were selected as they comprise a large percentage of pancreatic mass, 70%, 20% and 5%, respectively. The presence of β-gal was seen in a specific group of endocrine cells located at the periphery of the islets in the pancreas of FFAR4-KO mice (Figure 4-4). During the first part of this chapter, it was reported that the β-gal construct that was used to substitute

the FFAR4 gene in the knockout mice had a nuclear localization signal. This signal guides the β-gal enzyme to the nucleus of the cells. Thus, it came as no surprise that the staining for β-gal, which signifies the presence of cells with active FFAR4 promoter, was limited to the nuclei of the cells. Immunoreactivity to β-gal was detected predominantly in the nuclei of pancreatic δ-cells, cells expressing somatostatin (Figure 4-5A). Furthermore, a minority of glucagon positive cells (α-cells) had β-gal positive nuclei, suggesting that mFFAR4 is present in α-cells (Figure 4-5B). Comparatively low signal was observed in the nuclei of insulin positive cells, suggesting that mFFAR4 is less expressed in β-cells (Figure 4-5C). These findings aligned with the FACS-sorted α, β, and δ cell expression pattern (DiGruccio *et al.,* 2016) as well as prior research (Stone *et al.*, 2014; Adriaenssens *et al.*, 2016; Zhao *et al.*, 2020) demonstrating that islet δ-cells expressed FFAR4 more abundantly in the adult mouse pancreas.

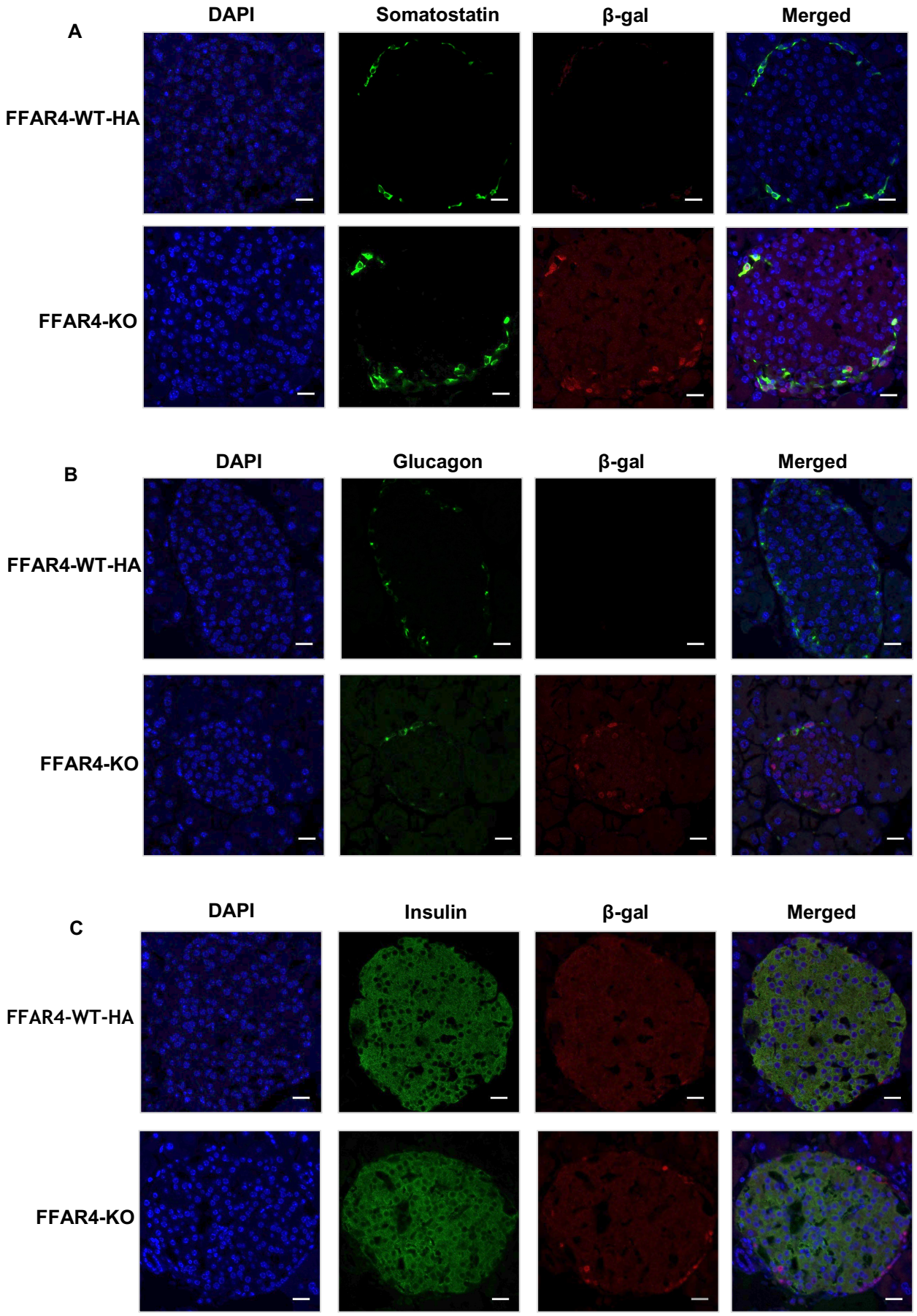


Figure 4-5: Locating mFFAR4 by β-galactosidase. β-galactosidase is mostly found in the delta cells of mouse islets of Langerhans. Immunofluorescence staining was performed on formalin-fixed, paraffin-embedded pancreatic sections obtained from FFAR4-WT-HA and FFAR4-KO animals. The staining was used to detect the presence of β-gal (red) and the three islet hormones somatostatin **(A),** glucagon **(B),** and insulin **(C),** which are shown in green colour. The nuclei were stained with DAPI (blue). The images were acquired using a confocal microscope with a 40x lens. The images represent n=3 with a 20 μm scale bar. β-gal= β-galactosidase.

4.3.2 Protein expression of FFAR4 in mouse pancreatic islets

A. Immunohistochemistry analysis

Quantifying gene expression using methods like qPCR against genes offers vital insights into the level of transcriptional activity shown by a certain gene (for example, β-gal) (Burn, 2012; Takahashi *et al.,* 2000). Nevertheless, it is crucial to recognise that the quantity of RNA identified does not consistently correspond to the extent of protein expression. The difference may be related to different regulatory processes that occur after transcription and translation, which affect the amount of protein present. For instance, the stability of mRNA, the efficiency of translation, and the rates of protein degradation may all influence the ultimate protein levels inside a cell. Hence, although qPCR is a potent technique for evaluating gene expression, it is crucial to supplement these results with protein expression measurement methods like immunohistochemistry or Western blotting to obtain a more detailed understanding of the biological system being studied.

In order to identify mFFAR4 expression, IHC was conducted using an HA antibody, as it has been previously validated by members of our research group as a reliable protein marker for the receptor of interest if they contain the HA-sequence (Barki *et al.,* 2022; Euston, 2023; Scarpa, 2022). An important characteristic of the FFAR4-WT-HA and FFAR4-PD-expressing animals is that the integrated C-terminal HA epitope tag enables highly precise identification of cells expressing the receptor using immunochemical methods (Bolognini *et al.,* 2019). For the purpose of identifying the most suitable primary antibody to detect mFFAR4-HA expression in mouse pancreatic slices, two distinct antibodies were utilised in the experiment: rat anti-HA and rabbit anti-HA antibodies. The goal was to identify the antibodies that would yield the most precise and strong signal, while reducing the chances of cross-species reactivity, given that the samples originated from mice. Through an evaluation of the IHC performance of these two antibodies, it would be possible to determine which antibody is most appropriate for the detection of mFFAR4-HA expression in mouse pancreatic slices. The sections of pancreas, which had a thickness of 3 µm, were stained using the specified two primary antibodies. Subsequently, secondary antibodies conjugated with Alexa-fluor 488, 546, 594, or 647 were applied, as illustrated in Figure 4-6. By using a variety of fluorescent dyes, it was possible to simultaneously detect several targets and evaluate the possibility of overlaps between the antibodies under investigation. The objective of this approach was to ascertain the primary antibody that exhibited the most favourable signal-to-noise ratio and minimal non-specific binding, thereby ensuring the most precise and reliable identification of mFFAR4-HA expression in the pancreatic tissue. Through a

comparison of the intensities and patterns of staining produced by each primary antibody, it would be possible to ascertain the most appropriate option for subsequent experiments.

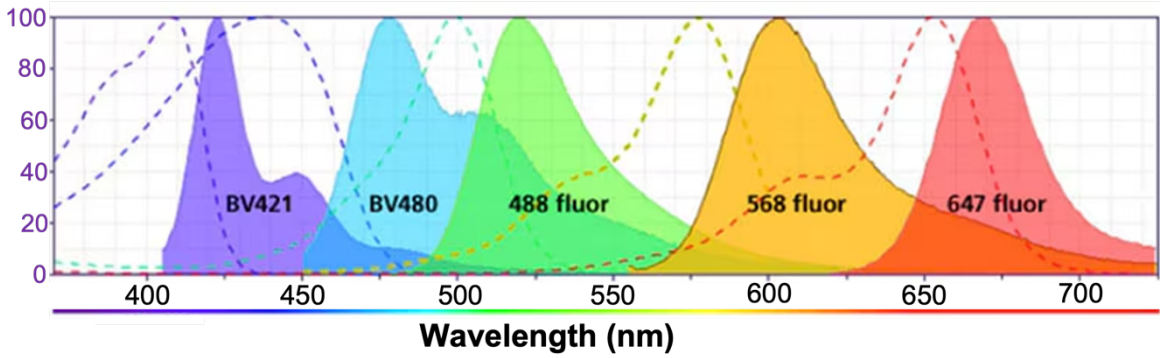


Figure 4-6: Diagrammatic representation of wavelength spectrum. The wavelength of light, which is proportional to frequency and energy, influences the observable colours. The colour spectrum encompasses a range from blue to red. In order to perform immunofluorescence, tissues or cells must be fixed and permeabilized. Immunostaining involves conjugating fluorophores to antibodies that target certain antigens, and then observing the resulting fluorescence signal by image microscopy. Image adopted from JIR Brilliant Violet™.

Upon analysing the images obtained from IHC experiments using the anti-HA antibody, it was not possible to confirm the expression of mFFAR4. This was due to the absence of any observed colocalization with anti-insulin, somatostatin, or glucagon antibodies. Representative images from somatostatin hormone are shown in Figure 4-7. Based on the observed lack of colocalization, it appears that anti-HA antibodies may not be an appropriate choice for detecting FFAR4-HA protein expression in pancreatic slices. It is possible that the low amount of HA-tagged FFAR4 protein in the mouse pancreas makes it difficult to detect the receptor expression using this approach.
In addition, prior research (Croze *et al.,* 2021; Stone *et al.,* 2014) indicates that FFAR4 is predominantly found in pancreatic δ-cells, which make up approximately 5% of the cells in the pancreatic islets. considering the low number of δ-cells in the islets, it becomes more challenging to detect FFAR4 expression using the HA antibody. This is because the signal from these cells might be overshadowed by the more abundant cell types like β-cells and α-cells, which are responsible for secreting insulin and glucagon.

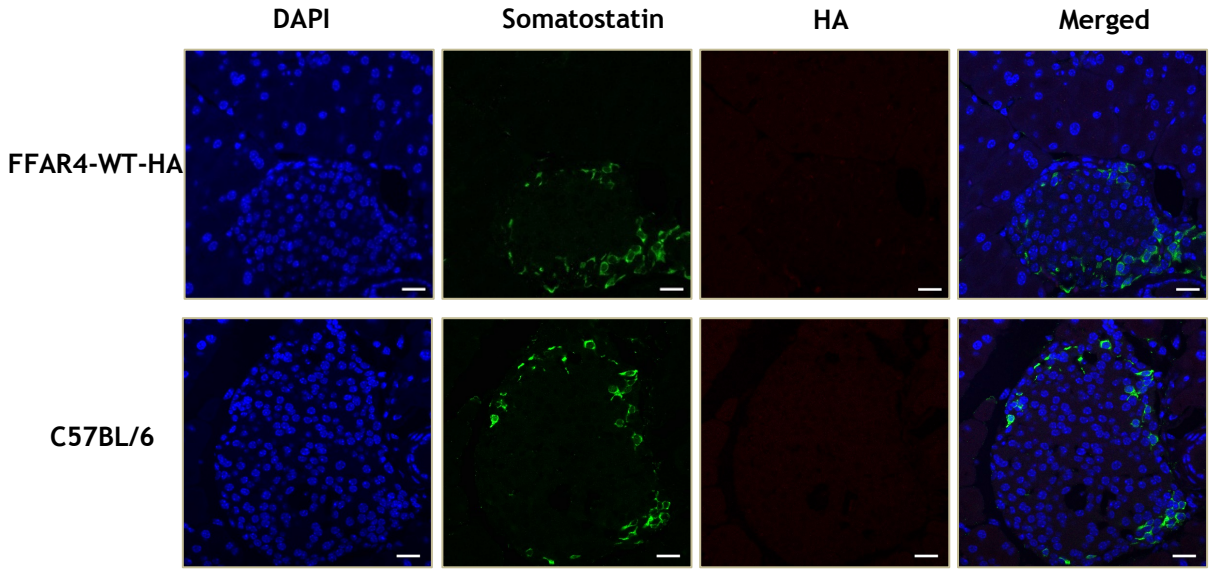


Figure 4-7: Locating mFFAR4 using anti rat-HA antibody. Immunofluorescence staining was performed on formalin-fixed, paraffin-embedded pancreatic sections obtained from FFAR4-WT-HA and C57BL/6 animals. The staining was used to detect the presence of HA (red, Alexa-fluor 647) and the somatostatin hormone which is shown in green. The nuclei were stained with DAPI (blue). The images were acquired using a confocal microscope with a 40x lens. The images represent n=3 with a 20 µm scale bar.

B. Immunoprecipitation studies

In order to conduct a more comprehensive analysis of the expression of mFFAR4, the receptor was immunoprecipitated from pancreatic tissue isolated from FFAR4-WT-HA and FFAR4-PD mice using the attached HA tag. Equivalent pull-down experiments were performed on tissue obtained from hFFAR2-DREADD-HA animals, serving as a positive control (Barki *et al.,* 2023). As described by Fritzwanker *et al.* (2023), a protease inhibitor cocktail was utilised in this experiment to prevent the possible degradation of proteins from FFAR4-WT-HA, FFAR4-PD and hFFAR2-DREADD-HA. Following SDS-PAGE, the immunoprecipitates obtained from these animals were analysed by immunoblotting with a HA antibody. The findings indicated that both hFFAR2-DREADD-HA and FFAR4-WT-HA were detected, predominantly around 45 kDa, with lesser quantities at 40 kDa (Figure 4-8). The hFFAR2-DREADD-HA showed a stronger band compared to FFAR4-WT-HA. However, FFAR4-PD, which has the HA tag at the C-terminal, was below the detection level in this analysis. The effective detection of hFFAR2-DREADD-HA was probably facilitated by strong expression of FFAR2 in pancreatic β-cells (Bolognini *et al.,* 2021; Pingitore *et al.,* 2019). Nevertheless, the absence of HA detection in FFAR4-WT-HA and FFAR4-PD

indicates that this technique might not be the most optimal for identifying mFFAR4 expression in the pancreas. As a result, the strategy involving the utilisation of an antibody targeting the β-gal gene was considered the best approach in identifying the expression of mFFAR4 in the pancreas.

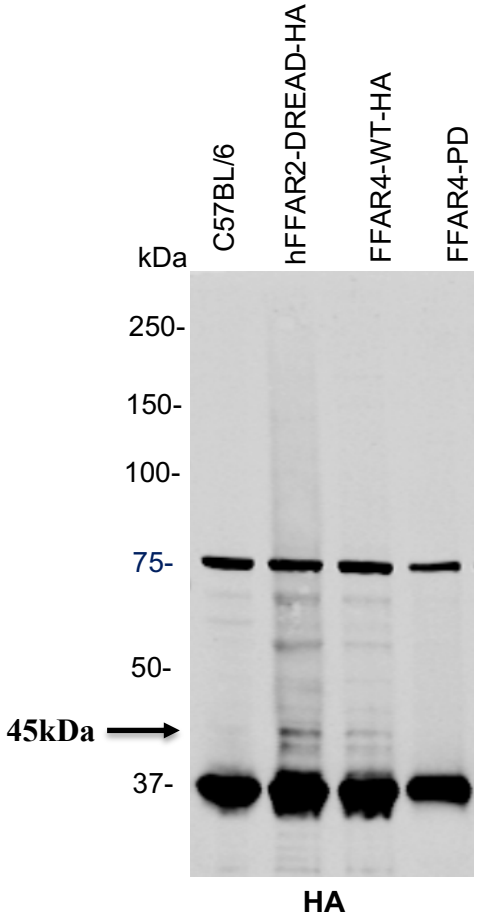


Figure 4-8: Immunoprecipitation of HA-tagged receptors from pancreatic tissue. Pancreatic tissue obtained from C57BL/6, hFFAR2-DREADD-HA, FFAR4-WT-HA, and FFAR4-PD mice was analysed. Preparations of lysates were made and then immunoprecipitation was performed using anti-HA monoclonal antibodies. The immunoprecipitated samples were then subjected to SDS-PAGE and immunoblotted to identify HA immunoreactivity. This image is representative of one experiment.

4.3.3 *Ex vivo* analysis of Insulin secretion from mice pancreatic islets

Initially, the hormone assay technique was validated by examining the responses of isolated mouse islets to glucose exposure. This was done by monitoring the ability of islets to secrete insulin when exposed to low glucose (3.3mM) or high glucose (16.7mM) over 1 hour. In this experiment, oxotremorine which is a muscarinic agonist with M3 activity was used as a positive control as it is known to facilitate insulin secretion from pancreatic β-cells (Rossi *et al.,* 2015). This experiment involved isolated islets from FFAR4-WT-HA, FFAR4-KO and

FFAR4-PD animals and the response was measured to glucose alone or in the presence of oxotremorine in both low glucose and high glucose. A brief layout of the treatment approach used in this assay in illustrated in Figure 4-9.

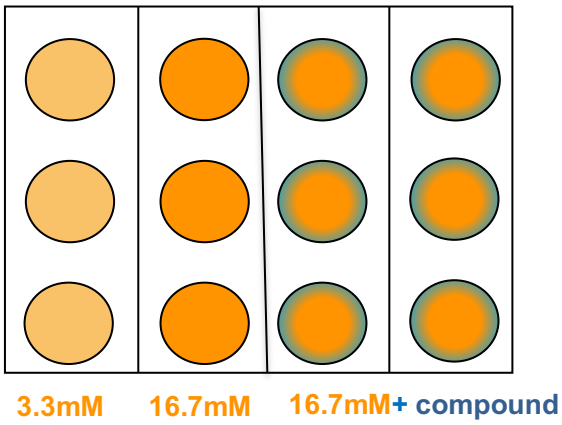


Figure 4-9: Illustration of the insulin secretion assay procedure. Isolated pancreatic islets (10 per well) were treated with 3.3 mM glucose (low glucose), 3.3 mM glucose with compounds, 16.7 mM glucose (high glucose), and 16.7 mM glucose with compounds. The islets were incubated for 1 hour and then both supernatant and islets were collected in separate tubes. An enzyme-linked immunosorbent assay (ELISA) kit was used to measure insulin levels and evaluate treatment effects on insulin release.

Data are presented as mean insulin secreted into the medium (ng/mL) (Figure 4-10A and D), amount of insulin released and then normalised to the total insulin content in each well (% total) (Figure 4-10B and E) and finally the amount of insulin stored in islets in each well (Figure 4-10C and F). In FFAR4-WT-HA animals, there was no difference when adding oxotremorine to low glucose ($P > 0.9$). On the other hand, it was observed that increasing the glucose concentration from 3.3 mM to 16.7 mM enhanced insulin release from isolated islets (Figure 4-10A). This is consistent with previous studies confirming the integrity of this islet preparation assay (Kong *et al.,* 2010; Rossi *et al.,* 2015). The administration of oxotremorine, in conjunction with a high glucose concentration substantially elevated the release of insulin. ($P < 0.0001$). This is also consistent with previous data from numerous studies (Gautam *et al.,* 2006; Kong *et al.,* 2010; Rossi *et al.,* 2015). Interestingly, the oxotremorine-induced enhancement of insulin secretion observed in FFAR4-WT-HA islets was significantly reduced in islets isolated from FFAR4-KO animals ($P = 0.0006$) when comparing the effect of 16.7 mM glucose + oxotremorine between the two genotypes (Figure 4-10A). This suggests that the full insulinotropic effect of oxotremorine depends on the presence of

FFAR4. When comparing the amount of insulin stored in islets of FFAR4-WT-HA and that of islets from FFAR4-KO animals, no statistically significant difference was observed (Figure 4-10C). This suggests that the absence of FFAR4 does not substantially alter the total insulin content in islets under these experimental conditions. Although data does not indicate any notable disparities in the amount of stored insulin between FFAR4-WT-HA and FFAR4-KO islets, it is crucial to acknowledge that the deletion of FFAR4 may impact other elements of β-cell function. Ichimura *et al.* (2012) found that when FFAR4 is genetically deleted in mice, it may cause obesity, which is often linked to alterations in insulin sensitivity. Considering the decrease in insulin secretion triggered by oxotremorine in FFAR4-KO islets, it is necessary to do more research on β-cell function. In order to achieve this objective, I used GLP-1 to examine the capacity of pancreatic β-cells to release insulin and to evaluate if there is any impairment in the functionality of these cells in FFAR4-KO mice. The GLP-1 experiment, and its outcomes will be thoroughly examined in section 4.3.4.

In FFAR4-PD animals, the effect of oxotremorine was significantly enhanced when compared to FFAR4-WT-HA ($P < 0.0001$) (Figure 4-10D). This suggests that the mechanism by which FFAR4 impacts on insulin release may be associated with FFAR4 phosphorylation since the islets isolated from the FFAR4-PD mutant mouse line responded to oxotremorine differently from the wild type islets. Unlike the data seen with FFAR4-KO animals, the amount of insulin stored in FFAR4-PD islets was the same as FFAR4-WT-HA animals (Figure 4-10F). This suggests that FFAR4 is playing a crucial role in the mechanism by which oxotremorine enhances insulin release from pancreatic β-cells.

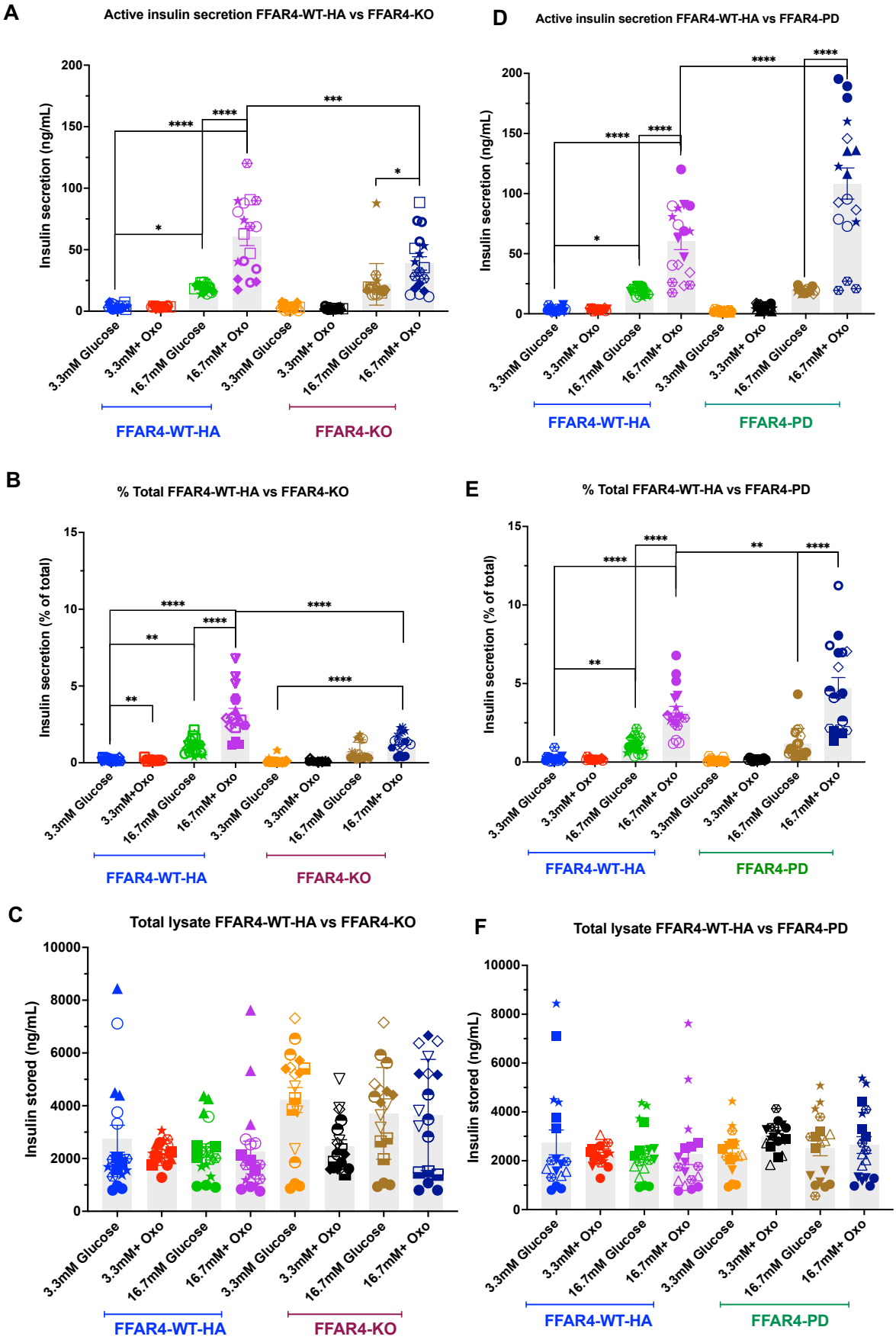


Figure 4-10: Analysis of insulin secretion from pancreatic islets of FFAR4-WT-HA, FFAR4-KO and FFAR4-PD mice. (A-C) Pancreatic islets isolated from FFAR4-WT-HA and FFAR4-KO, **(D-F)** Pancreatic islets isolated from FFAR4-WT-HA and FFAR4-PD. Islets from mice were incubated in KRB containing the specified concentrations of glucose for one hour at 37°C, with or without oxotremorine (100 µM). The total insulin content of each well (islets plus medium) was used to calculate the quantity of insulin released into the medium, which is represented by the % total. Each experiment was performed in triplicate and similar shapes in a column represents one experiment. The results are the mean ± S.E.M. of six separate studies (n=6). Statistical analysis was one-way analysis of variance (Geisser-Greenhouse multiple comparisons), with the following significance levels: *=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001.

4.3.4 Assessing the ability of pancreatic β-cells to release insulin

As the effect of oxotremorine on insulin release was significantly affected in islets from both FFAR4-KO and FFAR4-PD animals, the next studies tested the function of β-cells to secrete insulin and to assess whether this effect required the FFAR4 receptor. This assessment was made via two set of experiments. Firstly, by stimulating β-cells with GLP-1 which was predicted to have a direct effect on insulin release from pancreatic β-cells. Secondly, by potentially blocking the effect in wild type animals by acute provision of an FFAR4 antagonist.

GLP-1 is an incretin hormone that regulates insulin release via the GLP-1 receptor. It is produced by intestinal L-cells in response to glucose and other foods that are consumed. In the pancreas, this hormone inhibits the production of glucagon by α-cells and promotes the secretion of insulin by β-cells in a manner dependent on blood glucose concentration (Drucker, 2013; Marathe *et al.,* 2013). Following GLP-1 addition alongside high glucose by isolated pancreatic islets of FFAR4-WT-HA and FFAR4-KO mice, there was an increase in insulin secretion except for FFAR4-PD (Figure 4-11). Unlike oxotremorine, GLP-1 demonstrated an increase in insulin levels in isolated islets from FFAR4-KO mice (Figure 4-11A). When comparing the effect of GLP-1 between FFAR4-WT-HA and FFAR4-KO, it was observed that in the knockout animals, GLP-1 increased insulin levels significantly (P= 0.002), suggesting that there is no general lack of function in β-cells and the previously seen absence of oxotremorine effect (Figure 4-11A) was indeed affected by the elimination of the FFAR4 receptor. On the contrary, when GLP-1 was added with high glucose in isolated islets from FFAR4-PD animals there was no effect on insulin release, indicating a potential lack of a mechanism by which this hormone was acting in this mouse line. However, when compared to oxotremorine, the effect of GLP-1 was absent, while the effect that was previously seen with oxotremorine increased by a 2-fold difference in FFAR4-PD mice (Figure 4-11D) which suggest that both oxotremorine and GLP-1 mechanisms are substantially affected in the FFAR4-PD animals.

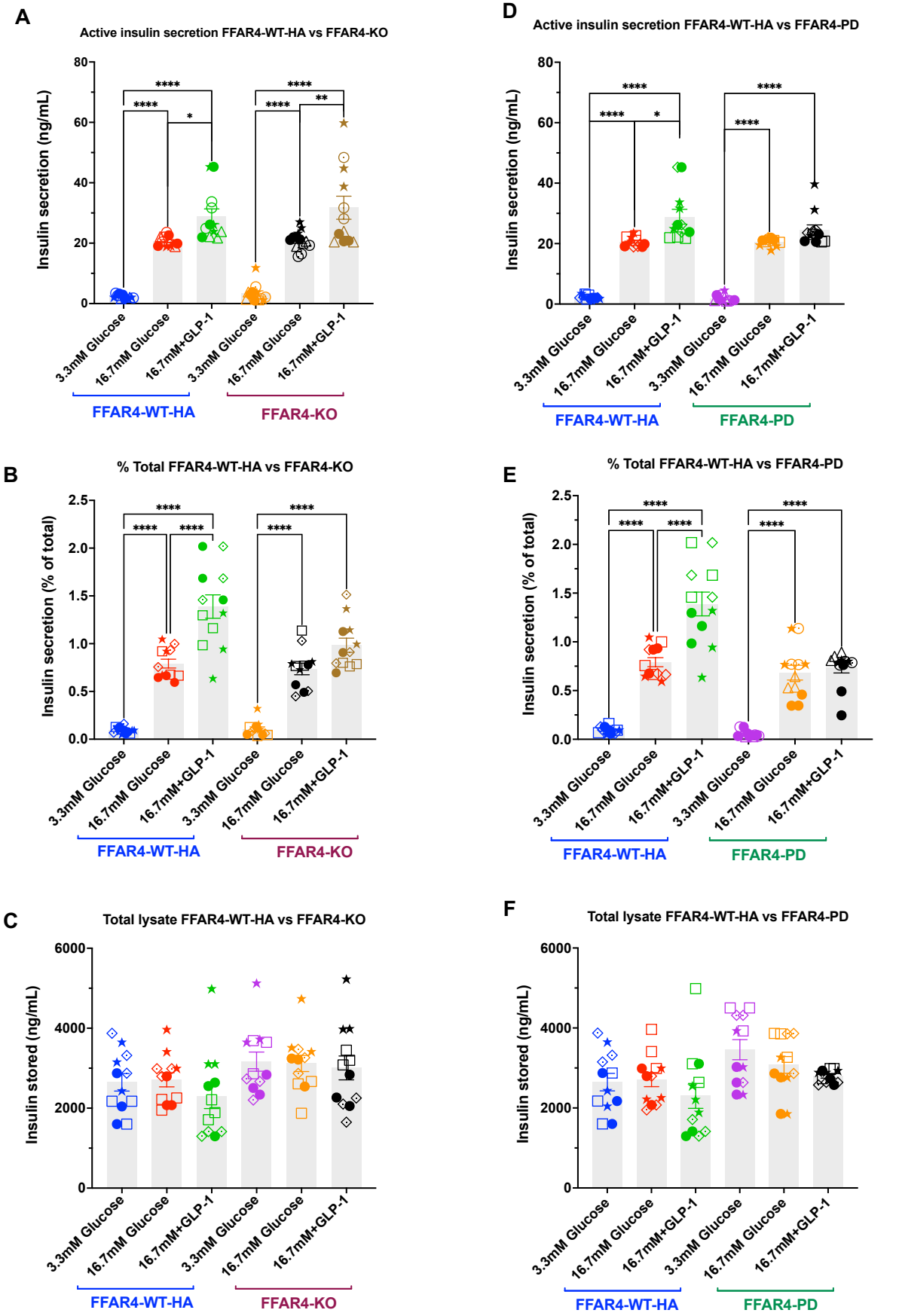


Figure 4-11: Analysis of GLP-1 effects on insulin secretion from pancreatic islets of FFAR4-WT-HA, FFAR4-KO and FFAR4-PD mice. (A-C) Pancreatic islets isolated from FFAR4-WT-HA and FFAR4-KO, **(D-F)** Pancreatic islets isolated from FFAR4-WT-HA and FFAR4-PD. Islets from mice were incubated in KRB solution containing the specified concentrations of glucose for one hour at 37°C, with or without GLP-1 (10 μM). The total insulin content of each well (islets plus medium) was used to calculate the quantity of insulin released into the medium, which is represented by the % total. Each experiment was performed in triplicate and similar shapes in a column represents one experiment, and the results are the mean ± S.E.M. of four separate studies (n=4). The statistical analysis that was carried out consisted of repeated measures one-way analysis of variance (Geisser-Greenhouse multiple comparisons), with the following significance levels: *=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001.

The second method used to test the role of FFAR4 in pancreatic β-cells was to block the effect of the receptor in wild type animals by acute provision of an FFAR4 antagonist. The effect of AH7614, a moderate affinity but apparently specific FFAR4 antagonist, was assessed on islets from FFAR4-WT-HA and FFAR4-KO. Pre-incubation of pancreatic islets with AH7614 for one hour resulted in insulin from FFAR4-WT-HA derived islets in response to 16.7 mM glucose and oxotremorine (P= 0.0001) (Figure 4-12A and B). The significance level (P = 0.0001) suggests that there is a statistically significant difference between this condition and the vehicle (16.7 mM glucose). Nevertheless, in the absence of a specific examination of oxotremorine alone, it is not possible to determine if AH7614 resulted in an increase, reduction, or no change in oxotremorine-induced insulin secretion. This was not expected as blocking the FFAR4 receptor in wild-type mice did not seem to have the same impact as in FFAR4-KO animals due to the unexpected rise in oxotremorine-stimulated insulin release reported in FFAR4-WT-HA islets when the FFAR4 antagonist AH7614 was present. Simultaneously, glucose-stimulated insulin secretion was not affected when exposed to TUG-891 after pre-incubation with AH7614 (P= 0.9) (Figure 4-12A and B). However, with addition of TUG-891 alongside oxotremorine, a significant drop in insulin release was observed in the presence on AH7614 (P= 0.0001) (Figure 4-12A and B). This suggests that TUG-891 blocked the action of oxotremorine at the M3 receptor site hence reducing its maximal response on insulin release from pancreatic β-cells. The rationale for this impact is described in pharmacology, an antagonistic response is one in which the combined impact of two drugs is less than the total of their individual effects when used individually (Salahudeen and Nishtala, 2017). In the context of an insulin experiment, an antagonistic response indicates that one of the ligands counteracts the other's influence on insulin release from pancreatic β-cells. There are a number of potential causes for antagonistic responses. The two ligands may be competing for the same region on the pancreatic β-cells' receptors. The combined effect of two ligands may be diminished because one of them may compete with the other for receptor binding. There is also the potential that the two ligands engage with separate signalling pathways inside the β-cells, which would result in opposite effects on insulin release when they are combined.

In FFAR4-KO animals, oxotremorine significantly promoted insulin release upon pre-incubation with AH7614 (P= 0.0001) (Figure 4-12D), suggesting that this ligand’s impact on insulin release is appeared to be regulated by FFAR4. This effect is similar the previously seen effect of oxotremorine in FFAR4-WT-HA (Figure 4-10A), suggesting that there is no lack of ability of β-cells to secrete insulin and oxotremorine is dependent on FFAR4 in its mechanism of insulin release. Simultaneously, when TUG-891 is combined with

oxotremorine in the presence of AH7614 there was no impact on oxotremorine effect (Figure 4-12D) which again suggests that oxotremorine is dependent on FFAR4 in its mechanism of insulin release.

Based on these findings, a qPCR was performed to assess the expression of M3 in the isolated pancreatic islets and validate the observations seen with both GLP-1 and AH7614. These were performed on cDNA samples derived from isolated mouse pancreatic islets of FFAR4-KO, FFAR4-WT-HA, and FFAR4-PD animals. Analysis of the M3 expression, showed no significant difference between FFAR4-WT-HA, FFAR4-KO and FFAR4-PD mice in terms of global M3 expression (Figure 4-12G).

When taken as a whole, the data that are provided in Figures 4-10, 4-11, and 4-12 offer compelling evidence that the FFAR4 receptor plays a significant role in modulating the release of insulin via parasympathetic pathways. This finding is an important advancement in the field, since it provides information on the particular pathways that are responsible for the influence that FFAR4 has on insulin secretion.

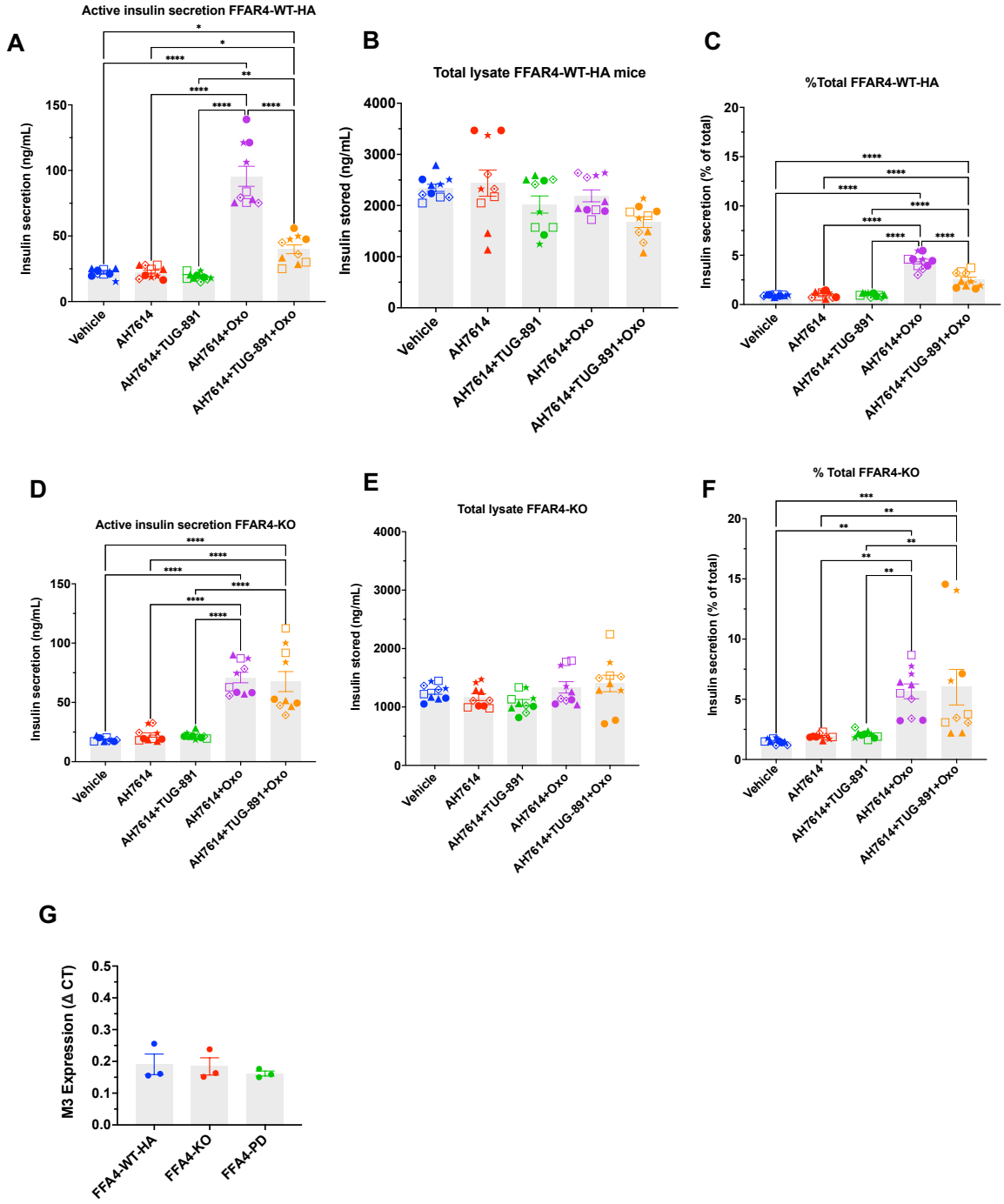


Figure 4-12: Analysis of AH7614 effect on insulin secretion from pancreatic islets of FFAR4-WT-HA and FFAR4-KO upon oxotremorine stimulation under 16.7mM glucose. (A-C) Pancreatic islets isolated from FFAR4-WT-HA. **(D-F)** Pancreatic islets isolated from FFAR4-KO. Islets from mice were pre-incubated in KRB solution containing AH7614 (30 μM) for one hour at 37°C. Islets then were treated with 10μM TUG-891 or 100μM Oxotremorine for one hour at 37°C. The total insulin content of each well (islets plus medium) was used to calculate the quantity of insulin released into the medium, which is represented by the % total. Each experiment was performed in duplicates and similar shapes in a column represents one experiment, and the results are the mean ± S.E.M. of five separate studies (n=5). (G) Expression of M3 in mouse pancreas using where q-PCR analysis on cDNA was performed usingM3 primers (n=3). Statistical analysis that was carried out consisted of repeated measures one-way analysis of variance (Geisser-Greenhouse multiple comparisons), with the following significance levels: *=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001.

4.3.5 Comparative Analysis of Mouse Weight in FFAR4 Mouse Models

Research on FFAR4 has shown that this receptor is essential for controlling metabolic processes and weight gain (Oh *et al.*, 2010; Ichimura *et al.*, 2012; Oh *et al.*, 2014). Animals lacking FFAR4 were used in these studies and provided new insight into its importance, especially in the context of obesity. The tendency to obesity in mice deficient in the FFAR4 receptor is consistent with the receptor's function in regulating energy balance and fat metabolism. Dysregulation of lipid metabolism and energy expenditure causes increased adiposity and weight gain when FFAR4 is absent. I assessed weight of mice from the FFAR4-WT-HA, FFAR4-KO and FFAR4-PD genotypes within the age range 12 weeks to 18 weeks, within the same age range as the mice used in insulin regulation assays. The data suggest a trend where male FFAR4-KO mice appeared to gain more weight compared to female FFAR4-KO mice (Figure 4-13), although statistical analysis is needed to confirm this observation. Similarly, FFAR4-KO animals of both sexes more weight compared with FFAR4-WT-HA and FFAR4-PD animals ($P< 0.0001$) (Figure 4-13). No apparent sex differences were observed in insulin secretion from *ex vivo* isolated islets. This weight gain trend in FFAR4-KO mice is consistent with the studies of Ichimura *et al.* (2012), which demonstrated that the genetic deletion of FFAR4 in mice resulted in obesity. Interestingly, there was no noticeable difference in weight observed between FFAR4-WT-HA and FFAR4-PD animals in both males and females. This indicates that FFAR4, despite its crucial involvement in metabolic processes and weight gain, may not be influenced by the phosphorylation status of FFAR4. The apparent weight gain in FFAR4-KO animals compared to FFAR4-WT-HA and FFAR4-PD animals emphasises the potential significance of FFAR4 in controlling energy balance and fat metabolism, regardless of its phosphorylation state.

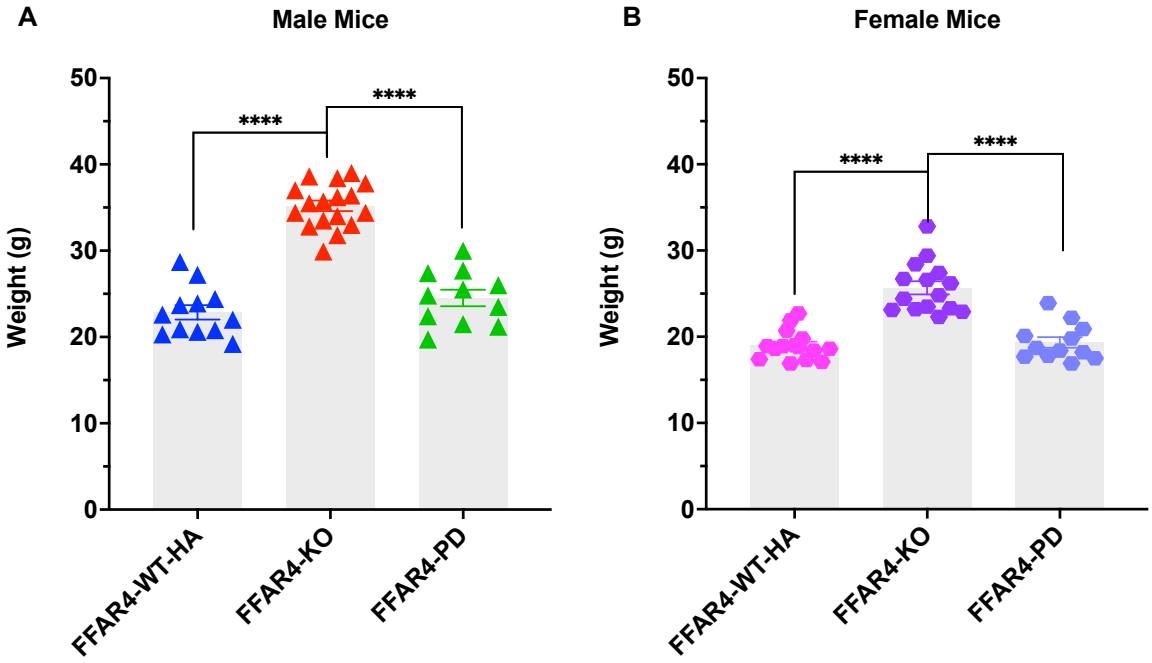


Figure 4-13: Analysis of weight of FFAR4-WT-HA and FFAR4-KO mice. (A) Male mice. **(B)** Female mice. The results are the mean ± S.E.M. of 12-17 separate studies (n=12-17). Statistical analysis used repeated measures of one-way analysis of variance (Geisser-Greenhouse multiple comparisons), with the significance level: ****=P<0.0001.

4.3.6 Effect of FFAR4 Ligands on Insulin Secretion under 3.3mM Glucose Conditions

Based on the observation in Figure 4-5 where FFAR4 is expressed in relatively few pancreatic β-cells, insulin assays were performed to determine whether mFFAR4 was functional in these cells and able to produce an increase in insulin secretion following the addition of FFAR4 agonists, namely TUG-891 and FFAR4 Agonist II. The effect of mFFAR4 on insulin release here were assessed following stimulation with agonist in the presence of either 3.3 mM or 16.7 mM glucose. Following addition of TUG-891 in the presence of 3.3 mM glucose to isolated pancreatic islets from FFAR4-WT-HA and FFAR4-KO, there was a significant reduction compared to the vehicle (3.3 mM glucose only) in FFAR4-WT-HA (P=0.007) (Figure 4-14B) whilst there was no reduction following treatment with TUG-891 in islets from FFAR4-KO animals (Figure 4-14B). This suggests the reduction seen in the FFAR4-WT-HA is a FFAR4 dependent effect. In comparison, islets from FFAR4-PD animals showed a significant increase in insulin secretion following stimulation with both 1 µM TUG-891 (P= 0.03) and 10 µM TUG-891 (P= 0.003) (Figure 4-14D). These data suggest that the FFAR4 variant expressed in this mutant mouse line

(FFAR4-PD) responds differently to TUG-891 compared to FFAR4-WT-HA mice islets, which may indicate alterations in receptor function or signalling pathways. Considering this, it is likely that the absence of FFAR4 receptor phosphorylation modifies the signalling pathway activated by TUG-891, resulting in the noted variations in insulin release. The mutant mouse line may have an altered response to TUG-891 compared to wild-type islets due to the presence of the FFAR4 receptor variant, which may disrupt the normal phosphorylation-dependent regulation of FFAR4 signalling.

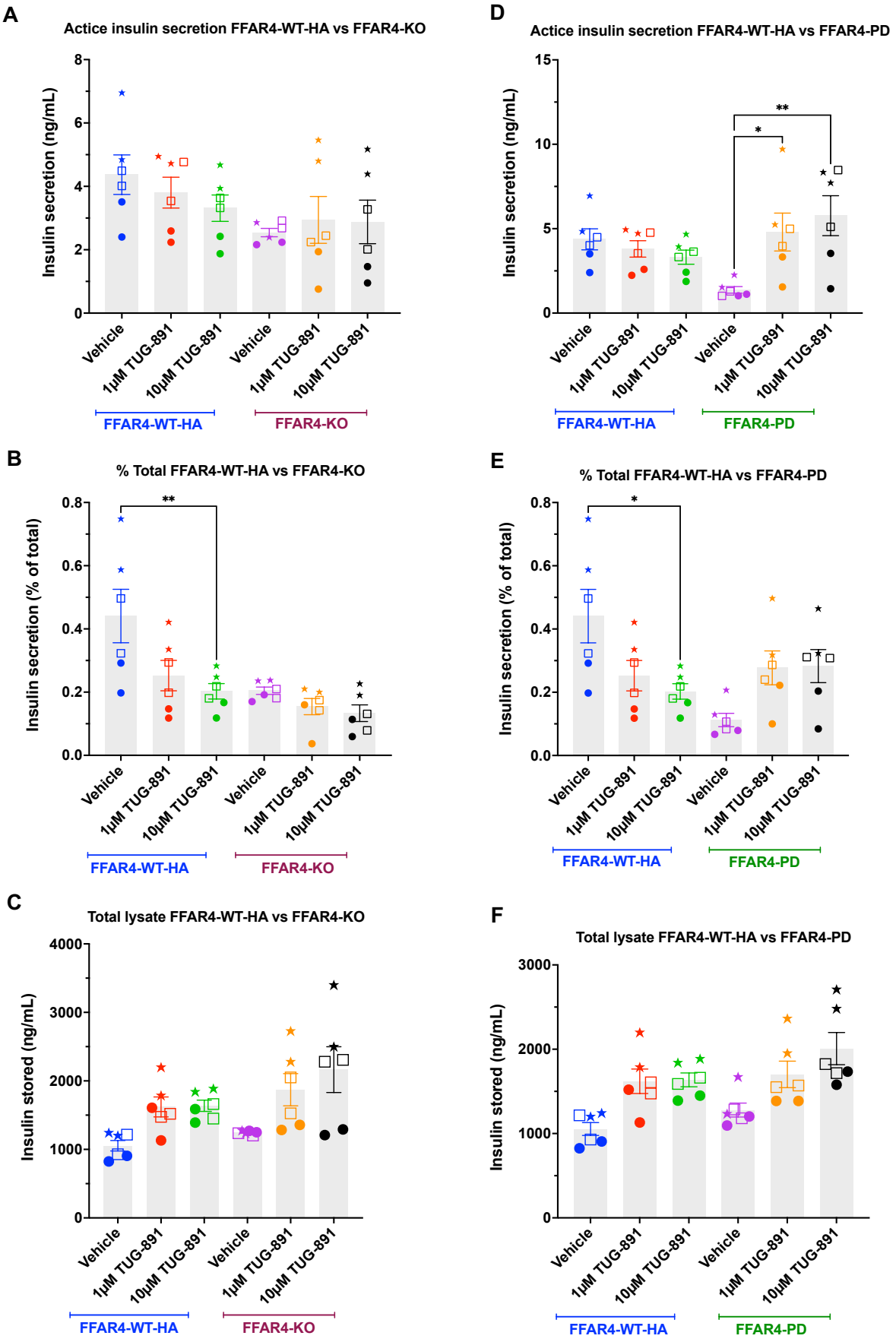


Figure 4-14: Insulin secretion assay following 1 μM and 10 μM TUG-981 treatment under 3.3mM glucose. (A-C) Pancreatic islets isolated from FFAR4-WT-HA and FFAR4-KO, **(D-F)** Pancreatic islets isolated from FFAR4-WT-HA and FFAR4-PD. Islets from mice were incubated in KRB solution containing the specified concentrations of glucose for one hour at 37°C, with or without TUG-891 at the indicated concentrations. The total insulin content of each well (islets plus medium) was used to calculate the quantity of insulin released into the medium, which is represented by the % total. Each experiment was performed in duplicates and similar shapes in a column represents one experiment, and the results are the mean ± S.E.M. of three separate studies (n=3). The statistical analysis that was carried out consisted of repeated measures one-way analysis of variance (Geisser-Greenhouse multiple comparisons), with the following significance levels: *=P<0.05; **=P<0.01.

To further understand the role of mFFAR4 in insulin release in the presence of 3.3 mM glucose, FFAR4 agonist II was next tested. The agonist was chosen because, according to its pharmacological profile, which was covered in Chapter Three, it was more effective and potent than TUG-891 (Table 3-1). Following the addition of FFAR4 agonist II to isolated islets from FFAR4-WT-HA and FFAR4-KO animals, there was no significant effect of this agonist on insulin release from those mouse lines when compared to the vehicle ($P > 0.05$) (Figure 4-15) which suggest that FFAR4 agonist II does not have an effect on insulin release from pancreatic β-cells. One possible explanation for the difference between the effects of TUG-891 and FFAR4 agonist II on the release of insulin from pancreatic β-cells is that TUG-891 has the ability to activate both FFAR4 and FFAR1 receptors. As previously demonstrated by Hudson *et al.* (2013), FFAR1 is known to promote insulin release from pancreatic β-cells.

In islets from FFAR4-PD, there was a significant increase in insulin release following 1 μM ($P = 0.0001$) and 10 μM ($P = 0.0004$) FFAR4 agonist II (Figure 4-15D). As for TUG-891, the response to FFAR4 agonist II in the mutant mouse line that expresses the variant FFAR4 receptor is different from that of wild-type islets. These data suggest that FFAR4 phosphorylation alters the response to agonist II in the FFAR4-PD variant compared to FFAR4-WT-HA islets. It is possible that the altered phosphorylation pathway that was found with TUG-891 in the PD mouse line might extend to additional FFAR4 agonists, such as FFAR4 agonist II.

In summary, the findings from the experiments involving TUG-891 and FFAR4 agonist II indicate that the mutant mouse line expressing a variant of the FFAR4 receptor might exhibit impaired phosphorylation of the receptor itself following agonist binding. Further investigation is necessary to better understand the activation and signalling pathways of FFAR4 receptors. This could shed light on potential changes in the receptor phosphorylation process when receptor modified variants are present. These findings align with the pharmacological characterization of FFAR4 ligands discussed in Chapter Three, emphasising the significance of receptor phosphorylation in mediating the downstream effects of FFAR4 activation. Based on the observed differences in the response to TUG-891 and FFAR4 agonist II in the mutant mouse line, it appears that the phosphorylation state of FFAR4 could be a key factor in determining the effects of agonist binding. This finding highlights the need for additional research into the signalling pathways and regulatory mechanisms that are involved.

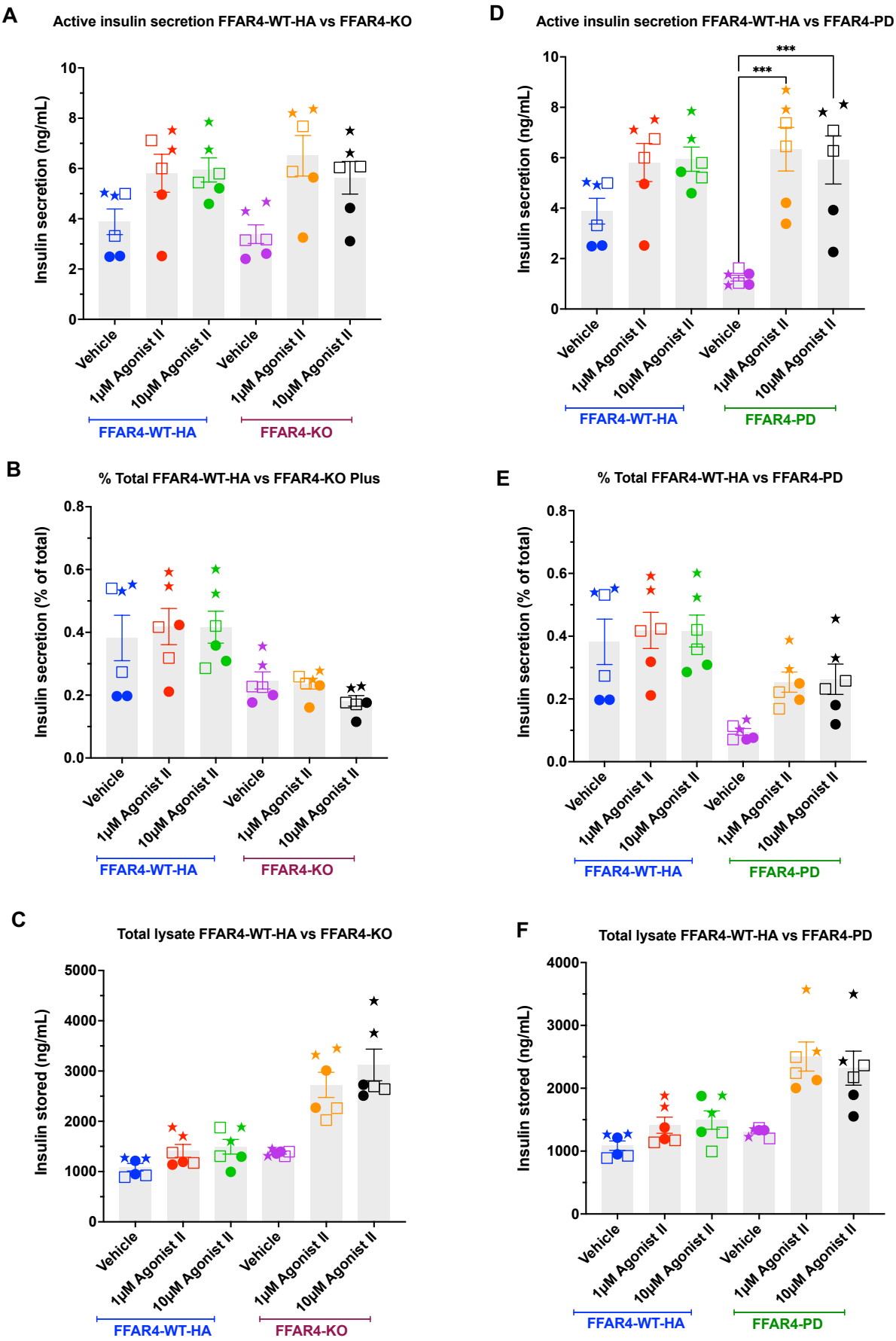


Figure 4-15: Insulin secretion in response to 1 μM and 10 μM FFAR4 agonist II treatment in the presence of 3.3mM glucose. (A-C) Pancreatic islets isolated from FFAR4-WT-HA and FFAR4-KO, **(D-F)** Pancreatic islets isolated from FFAR4-WT-HA and FFAR4-PD. Islets from mice were incubated in KRB solution containing the specified concentration of glucose for one hour at 37°C, with or without TUG-891 at the indicated concentrations. The total insulin content of each well (islets plus medium) was used to calculate the quantity of insulin released into the medium, which is represented by the % total. Each experiment was performed in duplicate and similar shapes in a column represents one experiment, and the results are the mean ± S.E.M. of three separate studies (n=3). The statistical analysis that was carried out consisted of repeated measures one-way analysis of variance (Geisser-Greenhouse multiple comparisons), with the following significance levels: **=P<0.01; ***=P<0.001; ****=P<0.0001.

4.3.7 Effect of FFAR4 Ligands on Insulin Secretion at elevated glucose concentration

Insulin secretion from islets of FFAR4-WT-HA mice was stimulated by 1 μM and 10 μM of TUG-891 in the presence of 16.7 mM glucose. TUG-891 significantly increased insulin secretion compared to vehicle (P= 0.02) (Figure 4-16A). The higher concentration of TUG-891 did not further enhance insulin secretion over that produced by 1 μM TUG-891 (Figure 4-16A). To test the selectivity of TUG-891 towards mFFAR4, isolated islets from FFAR4-KO animals were treated with the same concentrations of TUG-891 (Figure 4-16A). In FFAR4-KO islets, the potentiation of insulin secretion that was produced by TUG-891 in FFAR4-WT-HA islets was absent and the insulin content was not affected. This finding provides further evidence that mFFAR4 is necessary for the insulinotropic action of TUG-891. In comparison, FFAR4-PD animals showed a significant increase following stimulation with 1 μM TUG-891 (P= 0.04) (Figure 4-16D). Considering the fact that TUG-891 has been found to stimulate both FFAR4 and FFAR1 receptors, the rise in insulin secretion observed in FFAR4-PD animals indicates that the insulinotropic effect associated with TUG-891 in high glucose conditions might be mainly influenced by the activation of FFAR1, rather than FFAR4.

The FFAR4-PD animals do not have the necessary phosphorylation sites for FFAR4 receptor function. These sites include Thr^{347}, Thr^{349}, Ser^{350}, Ser^{357}, and Ser^{361} (Prihandoko *et al.,* 2016). The alteration in phosphorylation sites in FFAR4-PD mice may influence the receptor's response to TUG-891. While TUG-891 is known to activate both FFAR4 and FFAR1, with the latter known to enhance insulin release from pancreatic β-cells, the observed effects cannot be solely attributed to FFAR1 activation. If FFAR1 activation were the only factor, it would expect to see similar effects in FFAR4-KO mice, which presumably have normal FFAR1 levels. The difference in response between FFAR4-PD and FFAR4-KO mice suggests that the modified FFAR4 in FFAR4-PD mice may still play a role in mediating TUG-891's effects, albeit differently from FFAR4-WT-HA. Further investigation is needed to elucidate the specific mechanisms underlying these observations.

As indicated by the different response from that of wild-type islets in the presence of 3.3mM glucose (Figure 4-14), it appears that the FFAR4 receptor may experience altered phosphorylation in FFAR4-PD animals when TUG-891 binds to it. However, when exposed to high concentrations of glucose (16.7mM glucose), TUG-891 had the same impact in FFAR4-PD animals as it did in FFAR4-WT-HA animals. Although the FFAR4 receptor variant in the FFAR4-PD animals may have an effect on the receptor phosphorylation

process when TUG-891 binds to it under normal conditions, it appears that the receptor phosphorylation remains the same or is not affected by high glucose levels.

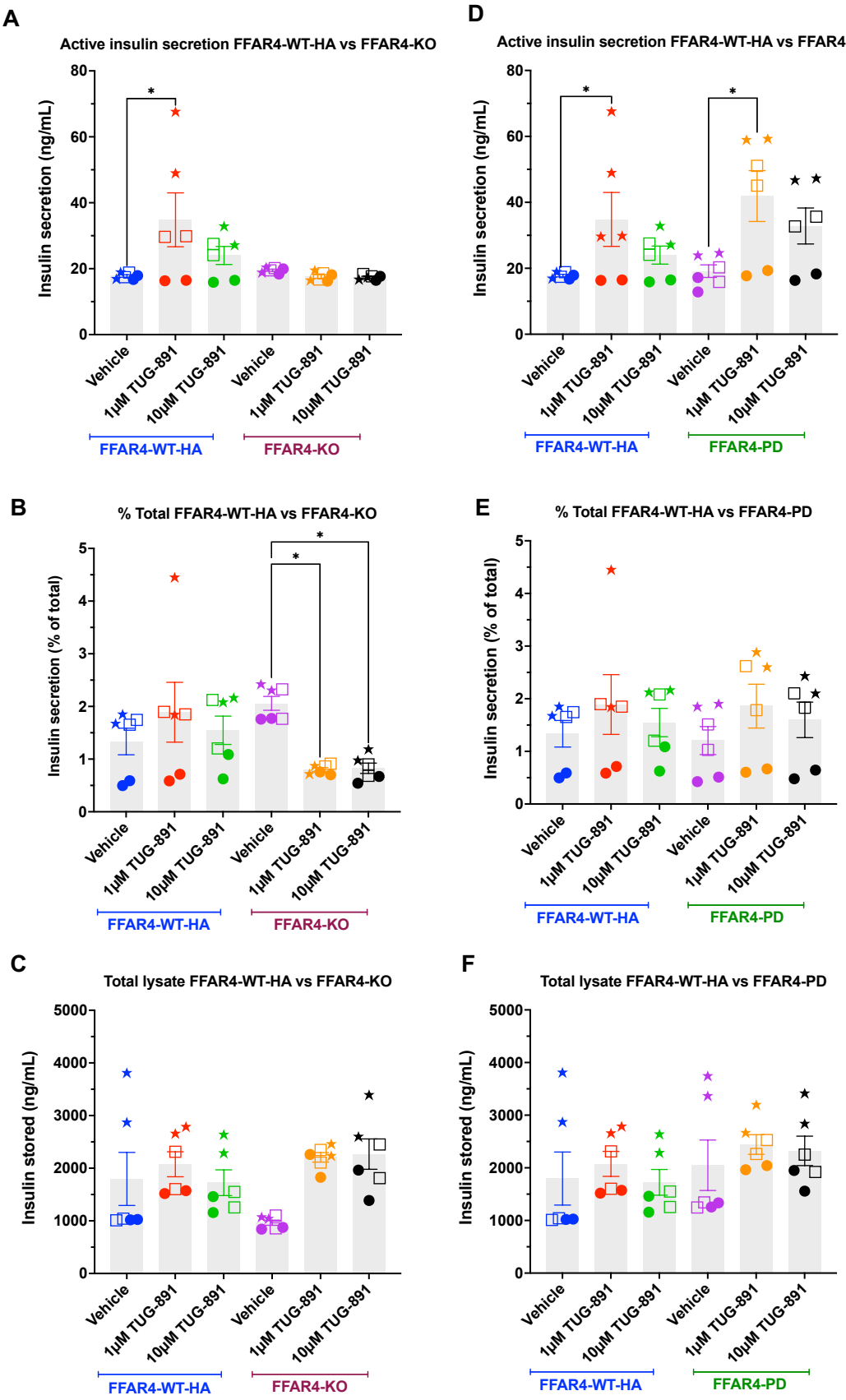


Figure 4-16: Insulin secretion upon treatment with 1 μM and 10 μM TUG-891 in the presence of 16.7mM glucose. (A-C) Pancreatic islets isolated from FFAR4-WT-HA and FFAR4-KO, **(D-F)** Pancreatic islets isolated from FFAR4-WT-HA and FFAR4-PD. Islets from mice were incubated in KRB solution containing the specified concentrations of glucose for one hour at 37°C, with or without TUG-891 at the indicated concentrations. The total insulin content of each well (islets plus medium) was used to calculate the quantity of insulin released into the medium, which is represented by the % total. Each experiment was performed in duplicates and similar shapes in a column represents one experiment, and the results are the mean ± S.E.M. of three separate studies (n=3). The statistical analysis that was carried out consisted of repeated measures one-way analysis of variance (Geisser-Greenhouse multiple comparisons), with the following significance level: *=P<0.05.

4.3.8 Effect of FFAR4 on somatostatin secretion

Considering the significant expression of mFFAR4 in pancreatic δ-cells, somatostatin assays aimed to evaluate the impact of mFFAR4 on somatostatin secretion. In this study, the responses of isolated murine islets to glucose stimuli were investigated, and the amount of somatostatin secreted by these islets was quantified. In FFAR4-WT-HA animals, an increase in glucose concentration from 3.3mM to 16.7mM significantly enhanced somatostatin secretion from isolated islets ($P= 0.0001$) (Figure 4-17A). To assess if mFFAR4 had a role in somatostatin release from the pancreas, the somatostatin secretion experiments were repeated in islets from FFAR4-KO animals. In these animals, the level of somatostatin was significantly increased, with a 2.5-fold increase (~150%) in somatostatin release when the glucose concentration was raised from 3.3mM to 16.7mM ($P< 0.0001$) (Figure 4-17A). It is worth noting that there was a significant difference in somatostatin release between FFAR4-WT-HA and FFAR4-KO islets under high glucose conditions (16.7mM). FFAR4-KO islets released a significantly higher amount of somatostatin compared to FFAR4-WT-HA islets ($P< 0.0001$). However, there was no difference in basal somatostatin secretion (3.3mM glucose) between the two groups (Figure 4-17A). It appears that the release of somatostatin is higher in FFAR4-KO islets compared to FFAR4-WT-HA islets when exposed to high glucose conditions. This suggests that the FFAR4 receptor might play a role in inhibiting somatostatin secretion. In the absence of FFAR4, the inhibitory action is lost, leading to increased somatostatin secretion. This finding suggests that FFAR4 may regulate somatostatin secretion from pancreatic δ-cells independently of ligand activation, presumably via constitutive activity or interactions with other cellular components.

In FFAR4-PD animals, a similar increase in somatostatin release to that of FFAR4-WT-HA was observed when the glucose concentration raised from 3.3mM to 16.7mM ($P= 0.0002$) (Figure 4-17D). Based on the results, it seems that the FFAR4-PD mutation has no effect on how somatostatin secretion reacts to variations in glucose concentration. Based on these results, it seems that the FFAR4-PD mutation may not have an effect on the regulation of somatostatin release in response to glucose stimulation.

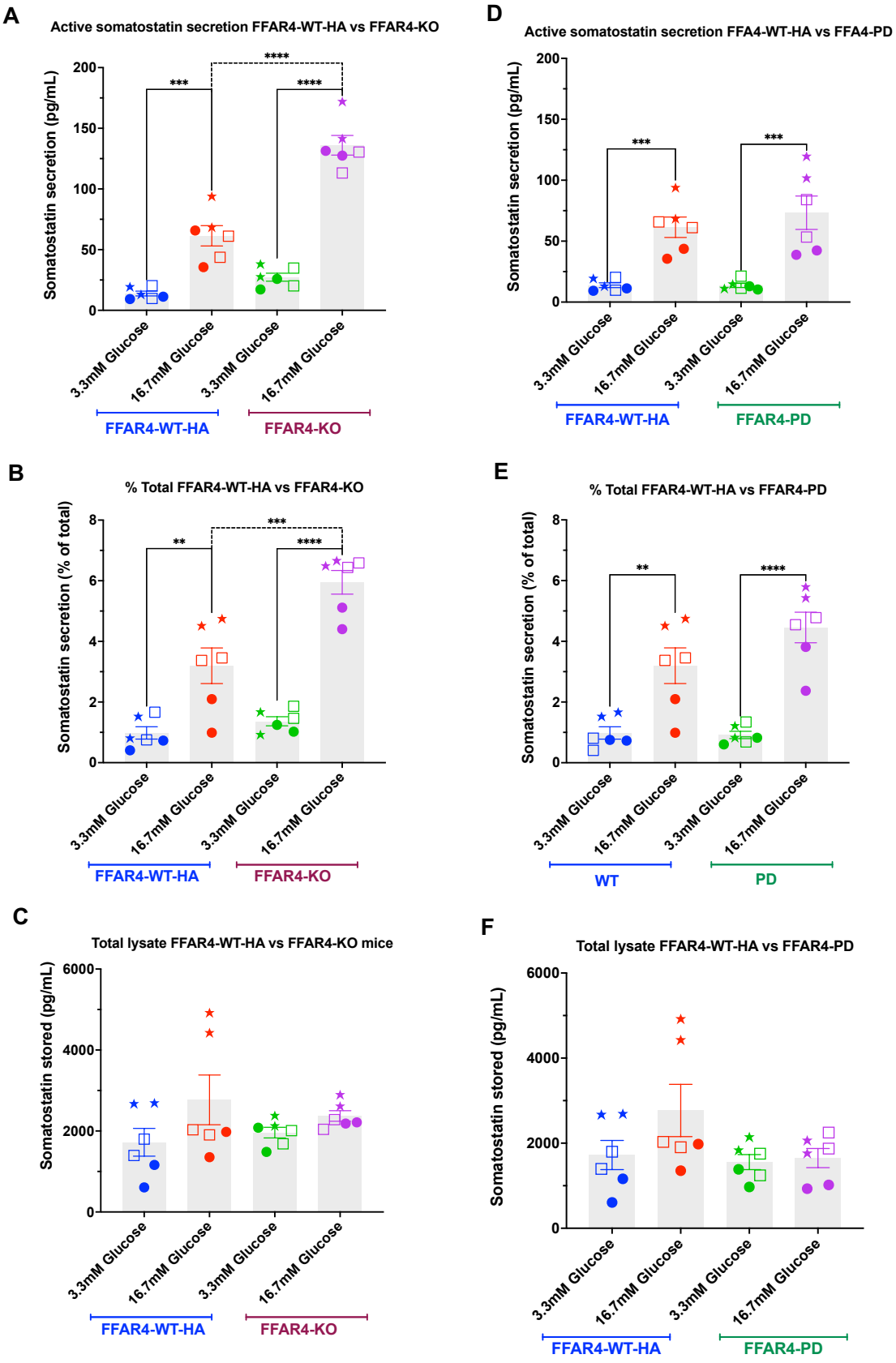


Figure 4-17: Somatostatin secretion in the presence of 3.3mM and 16.7mM glucose. (A-C) Pancreatic islets isolated from FFAR4-WT-HA and FFAR4-KO, **(D-F)** Pancreatic islets isolated from FFAR4-WT-HA and FFAR4-PD. Islets from mice were incubated in KRB solution containing the specified concentrations of glucose for one hour at 37°C at the indicated concentrations. The total somatostatin content of each well (islets plus medium) was used to calculate the quantity of somatostatin released into the medium, which is represented by the % total. Each experiment was performed in duplicates and similar shapes in a column represents one experiment, and the results are the mean ± S.E.M. of three separate studies (n=3). The statistical analysis that was carried out consisted of repeated measures one-way analysis of variance (Geisser-Greenhouse multiple comparisons), with the following significance level: **=P<0.01; ***=P<0.001; ****=P<0.0001.

Figure 4-18 presents a concise overview of the functions that FFAR4 plays in regulating pancreatic islet hormones. FFAR4 is essential in β-cells for enhancing the release of insulin induced by oxotremorine. Oxotremorine, a substance that activates the Muscarinic receptor M3 (M3R), relies on the presence of FFAR4 to effectively enhance the release of insulin. This indicates a possible connection between the signalling pathways of FFAR4 and M3R. The phosphorylation status of FFAR4 also seems to influence its impact on insulin secretion, suggesting an intricate regulatory mechanism. FFAR4 suppresses the release of somatostatin in δ-cells. This suppressive effect seems to be unrelated to ligand activation. The precise method by which FFAR4 inhibits somatostatin secretion is not completely understood, however it could involve the modulation of cAMP levels. The results emphasise the importance of FFAR4 in regulating the delicate balance of pancreatic hormone secretion, affecting the release of both insulin and somatostatin. However, further research is needed to fully understand the specific mechanisms behind these effects.

4.4 Discussion

Despite the fact that FFAR4 has been found colocalized with pancreatic hormones like somatostatin and insulin, there is still some debate about what exactly FFAR4 does in the pancreas due to contradictory research (Hirasawa *et al.*, 2005; Oh *et al.*, 2010; Paulsen *et al.*, 2014; Sundström *et al.*, 2017). According to Oh *et al.* (2010) and Patti *et al.* (2022), long-chain unsaturated fatty acids are known to bind to FFAR4, making it a promising therapeutic target for the treatment of diabetes. The purpose of this chapter was to determine whether or not mFFAR4 was functional in the pancreas and to study the impact of mFFAR4 agonists on insulin production. Three mouse models were used in the study: wild-type FFAR4 expressing hemagglutinin tag (FFAR4-WT-HA), FFAR4-KO and a phosphorylation deficient mouse (FFAR4-PD).

FFAR4 expression in the mouse pancreas was first verified through gene expression experiments, utilising both quantitative PCR (qPCR) and IHC with anti-β-galactosidase antibodies. Based on the qPCR analysis, it was found that FFAR4 mRNA is present in the pancreatic tissue, suggesting that the gene responsible for FFAR4 is actively being transcribed. For additional confirmation, IHC experiments were performed using anti-β-

galactosidase antibodies. Based on the IHC results, distinct staining patterns were observed in the pancreatic tissue, which provided confirmation of FFAR4 gene expression.

Despite substantial protocol optimisation, the attempts to detect FFAR4 protein expression in the mouse pancreas using IHC with HA antibodies did not yield any FFAR4 immunoreactivity. Several methods were utilised, such as utilising secondary antibodies with different labels (Alexa-fluor 488, 594, and 647), primary antibodies sourced from different animals (rat and rabbit), and employing both paraffin embedding and cryo-sectioning techniques for tissue preparation. Based on the observations, it appears that the FFAR4 protein expression in the mouse pancreas might be quite low, which poses a challenge in detecting it using the current IHC approach. There are several factors that could contribute to the low protein expression, potentially including post-transcriptional or post-translational regulation. These mechanisms may restrict the amount of FFAR4 protein present in the pancreatic tissue. Furthermore, the IHC protocol's sensitivity is limited, and the use of HA antibodies may also pose potential limitations in detecting FFAR4 protein expression. To address these challenges, researchers could investigate alternative approaches, like biotin amplification, to boost the signal of HA and enhance the sensitivity of FFAR4 protein detection in the mouse pancreas.

In this study, it was discovered that FFAR4 activity modulated the effect of oxotremorine on insulin secretion in high-glucose conditions, whereas previously the precise function of FFAR4 on muscarinic receptor within the pancreas was unknown. The study revealed that oxotremorine significantly increased insulin secretion in FFAR4-WT-HA mice under high glucose conditions ($P< 0.0001$). Notably, oxotremorine still caused a significant increase in insulin secretion in FFAR4-KO islets. However, this effect was significantly lower compared to the enhancement observed in FFAR4-WT-HA islets ($P= 0.0006$) when comparing the effect of 16.7mM glucose + oxotremorine between the two genotypes (Figure 4-10A). These findings indicate that the whole insulin-stimulating action of oxotremorine relies on the existence of FFAR4, while there is still some response shown even when FFAR4 is not present. This observation implies that in order for oxotremorine to exert its insulinotropic effect, the presence of the FFAR4 receptor is crucial. Oxotremorine increases insulin release via a signalling route that seems to be disrupted in these animals when FFAR4 is absent, suggesting that FFAR4 is an essential component of this process. At the same time, oxotremorine had a much stronger impact on insulin release in FFAR4-PD animals compared to FFAR4-WT-HA mice ($P< 0.0001$) (Figure 4-10D), indicating that FFAR4 phosphorylation is an important factor in controlling the insulin secretion generated by

oxotremorine. When taken as a whole, it is probable that the phosphorylation of FFAR4 acts as a regulatory mechanism to adjust the level of activity of the signalling pathway that is shared by M3 muscarinic receptors and FFAR4 receptors. It is possible that this regulating mechanism will be lost if FFAR4 phosphorylation is not present, which may result in an increased insulinotropic response to oxotremorine as seen when using FFAR4-PD mice.

The precise molecular processes involved in the interaction between FFAR4 and M3 muscarinic receptors in the control of insulin secretion have yet to be fully understood. Based on the study's results, it is possible to hypothesise that FFAR4 and M3 receptors may interact via a common signalling mechanism, presumably including GPCR signalling cascades. Activation of FFAR4 has been shown to initiate Gq/11-coupled signalling, resulting in the activation of PLC, elevation of intracellular calcium levels, and activation of PKC (Alvarez-Curto *et al.,* 2016; Burns *et al.,* 2014). Furthermore, it has been shown that M3 muscarinic receptors have the ability to initiate Gq/11-coupled signalling, leading to the activation of PLC and an elevation in intracellular calcium levels (Kan *et al.,* 2014). The confluence of these signalling pathways downstream of FFAR4 and M3 receptors may plausibly result in a synergistic impact on insulin production. The lack of FFAR4 or its inability to be phosphorylated may interfere with this cooperation, resulting in the observed changes in oxotremorine's capacity to stimulate insulin secretion.

Insulin release from pancreatic β-cells was stimulated by the incretin hormone GLP-1 to evaluate β-cell activity and the function of FFAR4 in insulin production. GLP-1 stimulated insulin secretion in both FFAR4-WT-HA and FFAR4-KO islets. The graph shows that the increase in insulin secretion in response to GLP-1 was similar between the two genotypes (P= 0.002) (Figure 4-11A). This confirms that the lack of response to oxotremorine in FFAR4-KO animals is directly linked to the loss of the FFAR4 receptor. GLP-1, on the other hand, had no impact on insulin release in FFAR4-PD islets, indicating a potential problem with the hormone's mechanism of action in this particular mouse strain.

In contrast to GLP-1, another approach included studying the effects on islets from FFAR4-WT-HA and FFAR4-KO mice to determine the impact of acutely administering an FFAR4 antagonist, AH7614, on oxotremorine-induced insulin production in these animals. When pancreatic islets were treated with AH7614 for one hour before being exposed to 16.7mM glucose stimulation, oxotremorine increased insulin release in FFAR4-WT-HA islets (P= 0.0001) (Figure 4-12A and B). However, without a direct comparison to oxotremorine alone in the same graph, it cannot be concluded whether AH7614 treatment resulted in a substantial increase in this effect. The unexpected augmentation in insulin secretion induced by

oxotremorine in the presence of the FFAR4 antagonist AH7614 in FFAR4-WT-HA islets indicates that the observed impact in FFAR4-KO mice cannot be reproduced in wild-type animals by only inhibiting the FFAR4 receptor. This finding suggests that the interaction between FFAR4 and M3 muscarinic receptor signalling in the control of insulin secretion is more intricate than first expected. This implies the presence of signalling crosstalk. FFAR4 and M3 muscarinic receptors may participate in intricate signalling interaction that regulates their impact on insulin production. Inhibiting FFAR4 with AH7614 has the potential to modify this communication between cells, resulting in an unexpected enhancement of insulin release produced by oxotremorine. This may include alterations in the equilibrium of downstream signalling pathways, such as GPCR signalling cascades, which eventually impact insulin secretion. Concurrently, the release of insulin in response to glucose stimulation was not altered when TUG-891 was administered after pre-incubation with AH7614 ($P= 0.9$) (Figure 4-12A and B). However, when TUG-891 was combined with oxotremorine, a significant decrease in insulin release was detected in the presence of AH7614 ($P= 0.0001$) (Figure 4-12A and B). These findings indicate that TUG-891 counteracted the activity of oxotremorine at the M3 receptor site, resulting in a decrease in its maximum effect on insulin release from pancreatic β-cells.

Previous studies have showed that FFAR4 mediates the release of insulin from pancreatic islets where TUG-891 potentiated glucose-stimulated insulin secretion (GSIS) in a concentration-dependent manner, with significant effects observed at glucose concentrations of 8mM and above (Stone *et al.*, 2014; Sundström *et al.*, 2017). Accordingly, the function of FFAR4 in insulin secretion was explored utilising the specific FFAR4 agonists TUG-891 and FFAR4 Agonist II. Both TUG-891 and FFAR4 Agonist II did not seem to stimulate insulin production at low glucose concentrations (3.3mM), but when used in FFAR4-PD islets, they demonstrated a notable rise in insulin secretion ($P< 0.05$). This indicates that the presence of the FFAR4 receptor variant may affect the phosphorylation effect of these FFAR4 agonists upon binding to the receptor. The impact of TUG-891 on insulin secretion when exposed to high glucose (16.7mM) was also investigated in this study. In FFAR4-WT-HA islets, TUG-891 considerably enhanced insulin production in comparison to vehicle ($P= 0.02$) (Figure 4-16A). There was no apparent distinction between 1 μM and 10 μM concentrations, suggesting that the impact was at its peak at 1 μM. The fact that this enhancement did not occur in FFAR4-KO islets provides further evidence that mFFAR4 is crucial to the insulinotropic effects of TUG-891. It seems that the altered phosphorylation process seen under low glucose conditions may be reversed or unaffected by high glucose levels, as TUG-891 considerably increased insulin production at 1 μM ($P= 0.04$) in the FFAR4-PD islets (Figure 4-16D). The results presented here are in agreement with those

obtained by Hauge *et al.* (2015), who investigated the effects of the FFAR4 agonist TUG-891 on the production of insulin in isolated mouse islets. It was discovered that at glucose concentrations of 12mM and 16mM, TUG-891 considerably enhanced GSIS (Hauge *et al.,* 2017).

The investigation on the impact of FFAR4 Agonist II on the release of insulin under high glucose (16.7mM) conditions was unable to be performed due to the restricted time available during the PhD project. Conducting this additional experiment would have presented valuable insights into how FFAR4 affects insulin secretion when exposed to this particular agonist in high blood sugar conditions. To further understand the potential therapeutic implications of targeting FFAR4 for the treatment of T2DM, future research should prioritise examining the effect of FFAR4 Agonist II on insulin release in the context of high glucose concentrations.

The novel insights into the complicated control of hormone release within the islets of Langerhans are provided by the study's results on the involvement of mFFAR4 in somatostatin secretion from pancreatic δ-cells. The production of insulin and glucagon by β-cells and α-cells, respectively, is greatly influenced by somatostatin, a peptide hormone released by δ-cells (Rorsman and Huising, 2018). The fact that glucose-stimulated somatostatin secretion is significantly elevated in FFAR4-WT-HA islets which in turn emphasises the role of glucose in controlling somatostatin release.

Nevertheless, the most notable observation is the somatostatin secretion increase of two to three times that which was observed in FFAR4-KO islets relative to FFAR4-WT-HA islets ($P< 0.0001$) (Figure 4-17A). This finding provides further evidence that FFAR4 may normally suppress somatostatin secretion. It would seem that the lack of FFAR4 in the knockout mouse eliminates this inhibitory function, which results in a substantial rise in the release of somatostatin. Since the enhanced somatostatin production was seen in response to glucose stimulation alone, it seems that this impact is unrelated to ligand activation. It is worth considering that FFAR4 could control somatostatin secretion not just via ligand-mediated activation, but also through baseline activity or interactions with other cellular components.

The observation that FFAR4-PD islets demonstrate a comparable surge in somatostatin secretion when exposed to elevated glucose levels as FFAR4-WT-HA islets ($P=0.0002$) (Figure 4-17D) implies that the phosphorylation status of FFAR4 does not have a significant impact on its ability to regulate somatostatin release. This is in contrast to the reported effects

of FFAR4 phosphorylation on insulin secretion, which showed that the FFAR4-PD mutation altered the responsiveness to FFAR4 agonists.

There is a complicated interaction between the numerous cell types in the islets of Langerhans, and FFAR4 may have many functions in controlling hormone release, as shown by its diverse effects on insulin and somatostatin production. The potential implications of the elevated somatostatin secretion observed in FFAR4-KO islets for the regulation of insulin and glucagon secretion are important to take into account, since somatostatin is recognised for its ability to inhibit the release of these hormones (Rorsman and Huising, 2018). To fully comprehend the physiological effects of the elevated somatostatin release seen in FFAR4-KO islets and to determine the exact mechanisms by which FFAR4 controls somatostatin secretion, more study is required. Finding out how FFAR4 interacts with other receptors and signalling pathways that control somatostatin secretion might also help unravel the complex system of factors that controls hormone release in the pancreatic islets. In summary, the study of somatostatin shows the involvement of mFFAR4 in the secretion of somatostatin from pancreatic δ-cells which reveal a new aspect of FFAR4 operation and underscore the criticality of incorporating intercellular interactions and signalling pathways into inquiries concerning the modulation of hormone discharge in the islets of Langerhans. These findings suggest potential new directions for investigation and could influence future efforts to create tailored treatments for metabolic diseases.

This study concludes with compelling evidence supporting the notion that FFAR4 regulates the secretion of insulin and somatostatin from pancreatic islets. These results show that FFAR4 phosphorylation is important for insulin secretion control and that FFAR4 affect somatostatin release apart from ligand activation. Instead of showing a major effect on insulin release, the results show that FFAR4 seems to control the oxotremorine response, indicating that FFAR4 regulates the muscarinic stimulation of insulin release. Furthermore, isolated islets from a mutant mouse line expressing an FFAR4 receptor variant that cannot be phosphorylated reacted differently to oxotremorine than wild type islets, suggesting that FFAR4 phosphorylation is related with the mechanism by which FFAR4 effects on insulin release. This chapter's data suggests that FFAR4 on δ-cells acts as a local regulator of muscarinic-induced insulin release from β-cells, since initial expression profiling of FFAR4 in the pancreas shows that FFAR4 is mainly expressed in pancreatic δ-cells with low expression in δ-cells (Figure 4-5). Further investigation is required in order to have a better understanding of the specific mechanisms that regulate the activation of FFAR4 and the

signalling pathways that are involved in the context of glucose homeostasis and metabolic health.

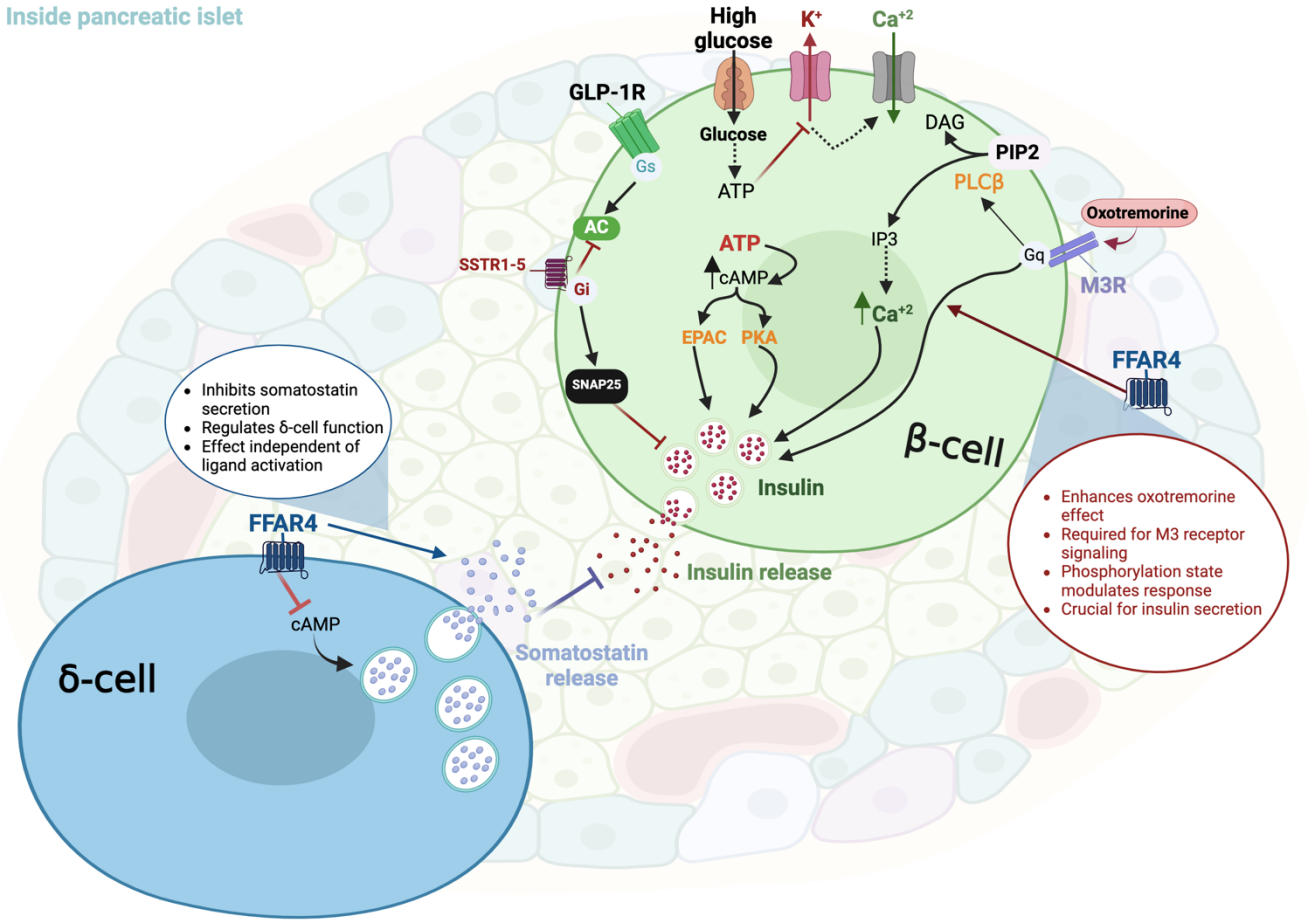


Figure 4-18: The mechanism by which FFAR4 regulate the secretion and development of pancreatic islet hormones. Glucose metabolism in β-cells stimulates ATP production, which in turn causes the closure of K^+ channels. This closure leads to the depolarisation of the cell, resulting in an influx of Ca^{2+} and insulin release. GPCRs have a role in regulating this process. Gq-coupled receptors, such as Muscarinic receptor M3 (M3R), stimulate phospholipase C (PLC), causing the breakdown of phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol trisphosphate (IP3). IP3 then triggers the release of Ca^{2+} which enhances the secretion of insulin in response to glucose. On the other hand, Gs-coupled receptors, like GLP-1R, elevate the levels of cAMP leading to the activation of protein kinase A (PKA) and exchange protein (EPAC). Conversely, Gi-coupled receptors, such as somatostatin receptors (SSTR1-5), inhibit the release of insulin by reducing the levels of cAMP. This can be via interacting with synaptosomal-associated protein 25 (SNAP-25). FFAR4 has a dual function. In β-cells, it enhances the release of insulin stimulated by oxotremorine through the Gq pathway. It is necessary for complete insulin secretion mediated by the M3 receptor, and its effect is influenced by its phosphorylation state. In δ-cells, it inhibits somatostatin secretion, potentially through modulation of cAMP levels, although the exact mechanism remains to be fully elucidated. This inhibitory effect on somatostatin secretion appears to be independent of ligand activation. Primary routes are shown by solid arrows, whereas secondary or less known interactions are represented by dashed arrows. Inhibitory arrows (⊣) indicate suppressive effects, including SNAP25 and somatostatin inhibition of β-cell secretion and ATP's closure of K^+ channels. This underscores the significant function of FFAR4 in regulating both insulin and somatostatin release. AC= Adenylyl Cyclase.

Chapter V Final discussion

5.1 The Therapeutic Potential of FFAR4 in Metabolic Diseases

It has been estimated by Hauser *et al.* (2017) that around 27% of all targeted treatments are based on GPCRs, which constitute a substantial number of pharmacological targets. One reason for this is the vast range of stimuli they may react to and the variety of physiological processes they are involved in (Nejat *et al.,* 2021; Zhang and Xie, 2012). As one of the G protein-coupled receptors (GPCRs) with unexplored therapeutic potential (Hirasawa *et al.*, 2005; Patti *et al.*, 2022), FFAR4 has garnered significant attention in metabolic disease research. This interest is largely driven by genetic evidence linking its dysfunction to the development of obesity and insulin resistance in both mice and humans (Ichimura *et al.,* 2012). The FFAR4 gene has a significant single-nucleotide polymorphism (SNP) that results in the R254H variation in the short isoform (or R270H in the long isoform). This variant has been linked to obesity in European populations (Ichimura *et al.,* 2012). Although early investigations indicated that the R270H variation had no function, further study shown that the R254H variant still had some level of function in Gq/11 and Gi/o signalling, although at a lower level, and maintained normal FFAR4-arrestin interactions (Vestmar *et al.,* 2016). The relationship between this polymorphism and metabolic outcomes has been shown to vary across various research and populations (Bonnefond *et al.,* 2015). It has been linked to elevated fasting glucose levels in several European groups, albeit not consistently with T2DM (Vestmar *et al.,* 2016). The prevalence of this genetic variation varies greatly across different groups, with a minor allele frequency (MAF) of around 3% among Europeans, while being less than 0.001% in Japanese populations (Milligan *et al.,* 2017b). Additional investigation is required to have a comprehensive understanding of the influence of this and other FFAR4 polymorphisms on metabolism and inflammation.

A number of studies have demonstrated that FFAR4 is an important regulator of glucose-stimulated insulin release and glucagon secretion, which makes it a promising target for developing a number of new antidiabetic medications (Croze *et al.,* 2021; McCloskey *et al.,* 2020; Moran *et al.,* 2014; Suckow *et al.,* 2014; Sundström *et al.,* 2017). Previous studies have demonstrated that the activation of FFAR4 by pancreatic β-cells enhances insulin secretion in response to glucose stimulation. This is achieved by modulating intracellular calcium levels and stimulating Gαq-coupled signalling pathways (Hirasawa *et al.,* 2005; Moran *et al.,* 2014). In addition, Suckow *et al.* (2014) found that FFAR4 agonists block the release of glucagon from pancreatic α-cells, which adds to their role in maintaining glucose homeostasis. The fact that FFAR4 promotes insulin sensitivity and reduces inflammation

provides evidence to its medicinal potential in T2DM. Improved insulin sensitivity, enhanced glucose absorption, and the reduction of pro-inflammatory cytokine production have all been linked to FFAR4 activation in adipose tissue (Oh *et al.,* 2010; Song *et al.,* 2017). In addition, research has shown that FFAR4 agonists may reduce inflammation in macrophages, which might mean that they could help with the persistent low-grade inflammation associated with obesity and T2DM (Oh *et al.,* 2014). Exploring FFAR4's functions in various tissues might lead to the discovery of new receptor therapeutic targets and the definition of previously unknown functions of FFAR4.

Two clinical trials have examined the impact of natural and synthetic FFAR4 agonists on metabolic processes. In an ongoing study (NCT03774095), investigates the effects of pine nut oil which include FFAR1/FFAR4 agonists, on glucose tolerance in healthy overweight or obese people are being investigated. In a study conducted by Christiansen *et al.* (2015), found that pinolenic acid, which is a significant component of pine nut oil, has the ability to improve glucose tolerance in mice. This natural compound acts as a dual agonist of FFAR1 and FFAR4 receptors. By studying the activation of FFAR1 and FFAR4, by these natural agonists, scientists have discovered that it has a positive effect on GLP-1 secretion from enteroendocrine cells and glucose-stimulated insulin secretion from pancreatic β-cells. This finding provides new possibilities for developing therapeutic approaches to tackle diabetes and obesity.

During a comprehensive clinical trial (NCT02444910), researchers examined the impact of KDT501, a synthetic FFAR4 agonist, on various metabolic characteristics in individuals with insulin resistance. A study conducted by Kern *et al.* (2017) found that KDT501 was well tolerated by nine individuals who were insulin-resistant and prediabetic. The treatment with this agonist resulted in a decrease in plasma triglycerides and TNF-α levels, while there was a significant increase in plasma adiponectin. It is possible that KDT501's capacity to increase β-adrenergic signalling and improve mitochondrial activity in adipocytes of subcutaneous white adipose tissue is responsible for these systemic effects (Finlin *et al.,* 2017). These clinical trials have revealed promising possibilities for using FFAR4 as a target for treating metabolic disorders. Additionally, they demonstrated valuable knowledge about the mechanisms that drive the positive impacts of FFAR4 agonists in humans.

5.2 Challenges in Translating FFAR4 Research to Clinical Trials

The potential of FFAR4 as a therapeutic target has not been fully recognised due to several factors. These include difficulties in determining the FFAR4 receptor function, pharmacokinetics, and pharmacodynamics, and problems with selectivity between FFAR4 and FFAR1. Selectivity concerns exist between FFAR4 and FFAR1 as both are structurally like each other and are activated by the same long chain free fatty acids. Because the two receptors are quite similar, especially in their ligand-binding domains, it has been difficult to design selective FFAR4 agonists (Hudson *et al.,* 2013). This problem with selectivity has confounded the interpretation of preclinical investigations and made it difficult to attribute physiological consequences to FFAR4 activation. Research into the receptor's roles and therapeutic potential has also been constrained by a lack of commercially available FFAR4 agonists.

5.3 Pharmacological Characterization of FFAR4 Ligands

By conducting pharmacological experiments on FFAR4 compounds, this thesis aimed to overcome these limitations by characterising ligand properties and directly comparing their potencies and efficacies to a reference ligand, TUG-891. There has been a lack of consistency in the published pharmacological data for FFAR4 ligands (Hudson *et al.*, 2013; Oh *et al.*, 2014; Sparks *et al.*, 2014). Even though prior research has evaluated a variety of agonists in a variety of methods. This study has offered vital insights into the signalling pathways connected to mFFAR4 by systematically assessing a number of agonists. The primary emphasis of this work has been on the Gαq G protein pathway.

This study's pharmacological data enabled the use of many quantitative tests to characterise the efficacy and consequences of novel ligands on mFFAR4. The results of these experiments revealed how these ligands stimulated mFFAR4's coupling capabilities by measuring receptor activation and pERK1/2 phosphorylation activity potential. The efficacy values that were actually observed, however, were different from what was claimed in the literature. The inconsistencies in the test methods used, changes in receptor expression and reserve, and variations in the presence of e-YFP at the C-terminal tail of the receptor, which might influence coupling to downstream effectors, are a possible explanation for these differences (Leroy *et al.,* 2007).

It is important to note that all the compounds that were evaluated had efficiency values that were higher than those of the reference ligand, TUG-891. As a result of this discovery, it

seems that these ligands have the potential to be useful in therapeutic settings, which calls for more research to be conducted in *in vivo* studies. These compounds' greater efficacy values as compared to TUG-891 suggest that they may be more powerful in activating mFFAR4 and its downstream signalling pathways, perhaps leading to better therapeutic effects.

5.4 The Role of FFAR4 in Insulin and Somatostatin Secretion

This thesis expanded on the pharmacological characterization of FFAR4 agonists by examining the function of FFAR4 in different physiological tissues using innovative mouse models. These models included FFAR4-WT-HA tagged mice, FFAR4-KO mice, and FFAR4 mice with PD mutations that disrupt phosphorylation-dependent signalling, such as β-arrestin pathways. These methods facilitated the assessment of the specific effects of FFAR4 agonists on mouse tissues, with a particular emphasis on the pancreas and its involvement in the release of insulin and somatostatin.

The results on the function of mFFAR4 in insulin release from pancreatic β-cells provide solid evidence for the receptor's significance in regulating glucose homeostasis. The finding that glucose-induced insulin release increased when FFAR4 agonists are present underscores the possibility of targeting FFAR4 as a therapy for T2DM. Remarkably, the phosphorylation status of FFAR4 seems to have a vital influence on controlling the receptor's response to agonists and its effect on insulin production, as seen by the modified responses reported in FFAR4-PD islets.

The absence of oxotremorine's impact on insulin release in FFAR4-KO mice provide convincing proof of the vital function of FFAR4 in regulating insulin secretion from pancreatic β-cells. The lack of oxotremorine action in FFAR4-KO islets suggests that FFAR4 is crucial in the signalling route by which M3 muscarinic receptors promote insulin release. This discovery implies that there may be a connection or communication between FFAR4 and M3 receptors, where the activation of FFAR4 is required for the complete activity of M3-mediated insulin secretion. The absence of oxotremorine's impact on FFAR4-KO mice may be linked to the interruption of this interaction, resulting in a dysfunction in the signalling pathway that typically enhances insulin secretion. The role of FFAR4 in insulin secretion is further supported by the results obtained from FFAR4-PD islets, which showed a considerably increased impact of oxotremorine on insulin release compared to

FFAR4-WT-HA. These findings indicate that the phosphorylation of FFAR4 is essential for regulating the insulin secretion generated by oxotremorine. FFAR4-PD mice, which lack the capacity of FFAR4 to be phosphorylated, seem to enhance the insulin-stimulating impact of oxotremorine. This suggests that phosphorylation of FFAR4 may function as a regulatory mechanism to adjust the level of M3-mediated insulin secretion. The results emphasise the important function of FFAR4 in the intricate control of insulin release and its potential as a target for therapy in metabolic diseases including T2DM.

Moreover, the examination of mFFAR4's involvement in somatostatin secretion from pancreatic δ-cells uncovered novel aspects of FFAR4's function and highlighted the significance of considering the interactions between various cell types and signalling pathways when investigating the control of hormone release in the islets of Langerhans. The increased somatostatin secretion seen in FFAR4-KO islets implies that FFAR4 have an inhibitory influence on somatostatin release in normal conditions, and this control seems to be unaffected by ligand activation. These discoveries provide new opportunities for further investigation and might potentially impact the development of specific treatments for metabolic diseases.

5.5 The Importance of *Ex Vivo* Studies in Understanding FFAR4 Function

To comprehend the intricate interactions between various tissues and organs in controlling glucose homeostasis, it is essential to employ mouse models to investigate the whole mouse response to external stimuli that cause the release of insulin or somatostatin. Although *in vitro* studies using cell lines like MIN6 have yielded vital knowledge on the signalling pathways and activities of FFAR4 (Stone *et al.,* 2014), they may not comprehensively depict the complex interactions and feedback mechanisms present *in vivo*.

When compared to cell-based experiments, *ex vivo* studies that make use of isolated mouse islets provide an ideal environment for investigating the effects of FFAR4 activation on insulin and somatostatin production in a situation that is more physiologically relevant. Isolated islets, although lacking the intricate interactions with other tissues and organs found in a whole-body system, still maintain the complex structure and cellular makeup of the endocrine pancreas when compared to *in vitro* cell lines. This enables a deeper understanding of the function of FFAR4 to regulate hormone secretion.

Mouse islets in isolation consist of the primary cell types responsible for maintaining glucose balance, namely β-cells, α-cells, and δ-cells. These cells release insulin, glucagon, and somatostatin, respectively. Researchers may use isolated islets to examine the impact of FFAR4 activation on the release of insulin and somatostatin, taking into account the intercommunication and local interactions between various cell types. This technique offers a deeper understanding of the specific regulatory processes that control hormone secretion inside the islets of Langerhans.

Furthermore, conducting *ex vivo* experiments with isolated islets enables the examination of the specific impacts of FFAR4 agonists and antagonists on insulin and somatostatin production, without the interference of systemic variables encountered in a whole-body context. This facilitates a more distinct comprehension of the precise signalling routes and methods by which FFAR4 influences hormone release in the endocrine pancreas.
The significance of investigating the response in *ex vivo* environment is demonstrated by the unanticipated rise in insulin release induced by oxotremorine that was detected in FFAR4-WT-HA islets when the FFAR4 antagonist AH7614 was present. This discovery emphasises the complicated interaction between FFAR4 and M3 muscarinic receptor signals in insulin secretion regulation, which may be missed in cell-based studies. The use of mice models enables the discovery of such undetectable interactions, resulting in a more complete knowledge of FFAR4's physiological activities.

Furthermore, a study of somatostatin secretion in FFAR4-KO islets indicated that the lack of FFAR4 causes a 2.5-fold increase (~150%) in somatostatin release when compared to FFAR4-WT-HA islets. This data implies that FFAR4 may have an inhibitory influence on somatostatin production under normal settings, which may not be obvious in cell-based studies. The use of mice models enables the discovery of such unique elements of FFAR4 activity, emphasising the need of taking into account the connections between various cell types and signalling pathways when researching hormone release control in the islets of Langerhans. The novel physiological PD mouse model may also identify receptor phosphorylation roles in other FFAR4-expressing tissues, such as the colon and adipose tissues, allowing for the identification of distinct phosphorylation dependent and independent routes, such as those described in the M1 and M3 muscarinic receptors using equivalent mouse models (Bradley *et al.,* 2016; Scarpa *et al.,* 2021).

The use of isolated islets derived from genetically engineered mouse models, namely FFAR4-KO and FFAR4-PD animals, improves the effectiveness of *ex vivo* research in understanding the function of FFAR4 in the production of insulin and somatostatin. Researchers could obtain useful insights into the unique effects of FFAR4 and its phosphorylation status on hormone release by comparing the responses of islets from these models to those from wild-type mice.

Overall, conducting experiments on isolated mouse islets outside of the living organism (*ex vivo* research) offers a robust method to examine the impact of FFAR4 activation on the release of insulin and somatostatin in an environment that closely mimics the natural physiological conditions. Although isolated islets may not completely replicate the intricacy of a whole-body system, they provide a more thorough comprehension of the specific regulatory mechanisms and signalling pathways by which FFAR4 influences hormone release in the endocrine pancreas. Adopting this method is crucial for progressing the understanding of the physiological significance of FFAR4 and its capacity as a therapeutic target for metabolic diseases. It will help direct the creation of focused treatments that can efficiently restore glucose balance.

In addition to static insulin release experiments, dynamic insulin secretion studies that make use of isolated islets in perfusion chambers have the potential to provide useful insights into the kinetics of insulin release. With this method, it is possible to identify small differences in insulin secretion patterns over a period of time that might remain undetected in static experiments. For instance, research conducted by Rossi *et al.* (2015) revealed a significant decrease in the sustained phase of insulin release in islets from mice with a knockin mutation in the M3 muscarinic receptor (M3R-KI). This emphasises the crucial role of this receptor in regulating insulin secretion over a period of time. Studying isolated islets in *ex vivo* experiments allows researchers to delve into the intricate details of insulin release and gain insights into the complex mechanisms that govern the regulation of insulin secretion by different receptors, such as FFAR4.

5.6 Limitations of *Ex Vivo* Studies

Although *ex vivo* investigations employing isolated mouse islets offer significant insights into the mechanism by which FFAR4 regulates the secretion of insulin and somatostatin, it is crucial to acknowledge the limitations associated with this methodology. The absence of complicated connections with other organs and tissues in isolated islets is a significant

restriction compared to whole-body systems. Influences from the neurological system and circulating substances such as hormones, as well as the pancreas's communication with other metabolically important tissues including skeletal muscle, adipose tissue, and the liver, may greatly alter the control of hormone production. Furthermore, it is possible that the procedure for isolating islets could induce stress in the cells, thereby modifying their behaviour and potentially compromising the outcomes achieved in *ex vivo* experiments. In addition, although studying islets in isolation permits research into certain signalling pathways and processes, this may not be representative of the whole range of adaptations and compensatory mechanisms that develop in live organisms in response to pharmacological or genetic treatments. Which in turn highlight a combination of *ex vivo* and *in vivo* studies to capture the whole-body mechanism in response to a stimulus to study the receptor's physiological functions and therapeutic potential.

5.7 Future Directions in FFAR4 Research

Despite the challenges that were faced throughout the process of characterising novel FFAR4 agonists, such as the low potency of some agonists and the limited signalling bias that was identified, the future of research that is based on FFAR4 remains promising. The finding of physiological responses to FFAR4 activation in the pancreas, together with the use of novel mice models such as the PD mouse, lays the foundation for future research into this receptor's activities and therapeutic potential.

Future research should concentrate on the creation of more effective and biased FFAR4 agonists, which may enable the receptor's positive effects, such as anti-inflammatory characteristics and the stimulation of insulin and GLP-1 secretion. Novel mouse models, including β-arrestin null FFAR4 mice, are capable of distinguishing between the effects of receptor phosphorylation and β-arrestin-2 recruitment, leading to a better understanding of FFAR4 signalling pathways.

To summarise, this thesis has made major advances to our knowledge of FFAR4 pharmacology and its involvement in metabolic control, notably insulin and somatostatin release from the mouse pancreas. The pharmacological characterisation of FFAR4 ligands, the use of novel animal models, and the discovery of physiological responses to FFAR4 activation provide the groundwork for future research targeted at generating safer and more effective treatments for metabolic diseases. As more powerful and biased agonists become

accessible, and with the continuous use of innovative *in vivo* models, FFAR4's potential for therapy may be fully realised, leading to better results for patients with T2DM and other metabolic disorders.

Future study might expand *in vivo* studies, such as those involving high-fat diets, to better understand how FFAR4 regulates metabolism and its therapeutic potential. Creating a mouse model that has FFAR4-DREADD (Designer Receptor Exclusively Activated by Designer Drugs) is one potentially useful strategy. DREADDs are specifically constructed GPCRs that help to know the exact regulation of receptor signalling in terms of both space and time by selective activation by otherwise inert ligands. This method has been effectively used to investigate the *in vivo* functioning of additional receptors, such FFAR2 and M1 receptor, by avoiding the compensatory processes often seen in KO mice. For example, researchers have used FFAR2-DREADD mice models to explore the receptor's function in white adipose tissue and immune cells, and M1-DREADD models to study the receptor's function in cognitive functions. In light of this, the physiological roles of the FFAR4 receptor may be better understood with the use of a novel FFAR4-DREADD mouse strain that allows researchers to selectively activate the receptor in certain tissues. Additionally, this method may avoid the compensating processes that are often seen in knockout mice, which may make it difficult to understand the data since adaptations are implemented to compensate the lack of a receptor. To reduce the possibility of developmental compensations, the FFAR4-DREADD model allows for acute manipulation of the receptor's activity in adult animals. Targeted treatments for metabolic disorders like T2DM may be guided by this innovative technology when used in conjunction with advanced FFAR4 agonists and other *in vivo* methods. It might greatly enhance our knowledge of FFAR4's function in metabolic regulation.

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