Evaluation of signalling and phosphorylation responses of the human histamine H_4 receptor (H_4R) and the human free fatty acid receptor 4 (FFA4)

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Abstract

The knowledge that G protein-coupled receptors (GPCRs) are regulated by phosphorylation in a process that results in the recruitment of arrestins, leading to receptor desensitisation is now well known. The histamine H_4 receptor (H_4R) and the free fatty acid receptor 4 (FFA4) are family A GPCRs that both have the ability to become phosphorylated in their third intracellular loops and C-terminal tails by kinases found in the cytosolic milieu of the cells and tissues they are expressed in.

Investigations into the phosphorylation status of the histamine H_4 receptor have revealed a receptor that is highly phosphorylated even in the basal state. The endogenous, full agonist for the human H_4R, histamine, induced a robust increase in receptor phosphorylation. However, the β-arrestin-biased agonist JNJ7777120 did not. Extending this study using mass spectrometry revealed the individual sites of phosphorylation. Histamine and JNJ7777120 also caused H_4R internalisation. Our data suggests a similar level of endocytosis induced by histamine and JNJ7777120 at 5 or 30 min stimulation. Thus, we show that JNJ7777120, while previously demonstrating its differing effects on H_4R signalling, also shows differences in the phosphorylation of the H_4R when compared to histamine.

Using the wild type FFA4 receptor and its phosphorylation-deficient mutants, we show the importance of phosphorylation in the recruitment of arrestin to the receptor as well as delineating G protein-dependent and independent downstream signalling pathways. Knowledge of the different signalling cascades and their mechanism of activation would be useful in the design of biased ligands for therapeutic benefits in order to develop safer and more efficacious drugs. The use of a FFA4 receptor which is phosphorylation-deficient and, therefore, couples to arrestin-3 in a reduced manner may be useful in proof-of-concept studies where the downstream signalling in a physiological setting is mediated by arrestin as opposed to G proteins. Further evidence of the importance of phosphorylation is provided by my work with the phosphomimetic FFA4 mutant receptor, which I show does not faithfully mimic phosphorylated serine and/or threonine residues resulting, in a reduced ability to couple to arrestin-3.

Publications

Papers

Bradley, S.J., **Riaz, S.A.,** and Tobin, A.B. (2014) Employing novel animal models in the design of clinically efficacious GPCR ligands. *Curr Opin Cell Biol*. 27 (100): 117-25

Abstracts

Riaz, S.A., Charlton, S.J., and Tobin, A.B. Regulation of the M_1 RASSL by Phosphorylation. *4th Focused Meeting Cell Signalling 23rd-24th April 2012. Poster Presentation.*

Riaz, S.A., Charlton, S.J., Butcher, A.J., and Tobin, A.B. Studies to Validate a Chemical Genetics Approach to Investigate the M1 Muscarinic Acetylcholine Receptor. *13th Annual Joint meeting of the Great Lakes GPCR Retreat 17th-19th October 2012. Poster Presentation.*

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"Verily, with every hardship comes ease"

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Abbreviations

AC	adenylyl cyclase
Ach	acetylcholine
AP-2	clathrin associated protein-2
ATP	adenosine 5'-triphosphate
Ca^{2+}	Calcium ion
cAMP	cyclic adenosine 3', 5'- monophosphate
CaS	calcium-sensing receptor
CCPs	clathrin-coated pits
CHO	chinese hamster ovary
CK	casein kinase
COX-2	cyclooxygenase-2
CRD	cysteine-rich domain
CREB	cAMP response element-binding protein
CRF_1	corticotropin-releasing factor receptor 1
CTC	cubic ternary complex model
DAG	1,2 diacylglycerol
ECL	extracellular loop
ERK	extracellular-signal-regulated kinases
ETC	extended ternary complex model
FAs	fatty acids
FFA1	free fatty acid receptor 1
FFA2	free fatty acid receptor 2
FFA3	free fatty acid receptor 3
FFA4	free fatty acid receptor 4
FFAs	free fatty acids
$GABA_B$	γ-aminobutyric acid receptor type B
GAPs	GTPase-accelerating proteins
GCGR	glucagon receptor
GDI	guanine nucleotide disassociation inhibitor
GDP	guanosine-5'-diphosphate
GEF	guanine nucleotide exchange factor
GIRK	G protein-regulated inward-rectifier K^+ channels
GPCRs	G-protein coupled receptors
GRKs	G protein coupled receptor kinases
GTP	guanosine-5'-triphosphate
H_1R	histamine H_1 receptor
H_2R	histamine H_2 receptor
H_3R	histamine H_3 receptor
H_4R	histamine H_4 receptor
HEK 293	human embryonic kidney 293
histamine	2-(4-imidazolyl)ethylamine dihydrochloride

ICL	intracellular loop
IL-6	interleukin-6
IP_3	inositol 1,4,5-triphosphate
JNJ7777120	1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine
kDa	kilodalton
KO	knockout
LCFAs	long chain fatty acids
LPS	lipopolysaccharide
mAchR	muscarinic acetylcholine receptor
MAPKs	mitogen-activated protein kinases
MCFAs	medium chain fatty acids
mGlu	metabotropic glutamate receptors
PH	pleckstrin homology
PI3K	phosphoinositide 3-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PKD1	protein kinase D1
PLC	phospholipase C
PLC-β	phospholipase C-β
PTX	pertussis toxin
RGS	regulators of G protein signalling
SCFAs	short chain fatty acids
T4L	T4 lysozyme
TAB1	TAK1-binding protein 1
TAK1	transforming growth factor beta-activated kinase 1
TLR4	toll-like receptor 4
TM	transmembrane
TNF	tumour necrosis factor
TUG-891	4-[(4-fluoro-4'-methyl[1,1'-biphenyl]-2-yl)methoxy]-benzenepropanoic acid
V_2R	vasopressin V_2 receptor
VEGF	vascular endothelial growth factor
VFTM	venus flytrap module

Chapter 1 | Introduction

1.1. G protein-coupled receptors

G-protein coupled receptors (GPCRs) are the largest superfamily of proteins in the human body and comprise ~2% of the human genome (Lagerström and Schiöth 2008). GPCRs can be activated by a variety of ligands including light, peptides, biogenic amines and hormones which result in the transduction of extracellular signals into internal responses. The most characterised intracellular GPCR coupling partners are the heterotrimeric G proteins but GPCRs also interact with other membrane associated and intracellular proteins. This flexible nature of GPCRs makes them therapeutically important drug targets with one-third of all prescribed drugs on the market targeting GPCRs (Overington *et al.,* 2006). Whilst the overall sequence homology of GPCRs varies considerably, this superfamily of proteins do share common characteristics including an extracellular *N*-terminus, seven transmembrane (TM) spanning α-helices connected with intra- and extracellular loops (ICL and ECL, respectively) and an intracellular *C*-terminal tail.

This thesis uses two particular GPCRs; the histamine H_4 receptor and the free fatty acid 4 receptor to investigate important regulatory mechanisms of GPCRs. We also explore the impact modulation of these key processes has on the behaviour of these receptors with regards to downstream signalling.

1.1.1. Classification of GPCRs

GPCRs fall into three major subfamilies which include receptors related to the rhodopsin receptor (class A), the Secretin and Adhesion families (class B) and Glutamate family, i.e. those receptors related to the metabotropic neurotransmitter receptors (class C) and the Frizzled/TAS2 family (Katritch *et al.,* 2013).

The largest GPCR family consisting of approximately 700 GPCRs in humans (Lundin *et al.,* 2003), family A GPCRs share an overall arrangement of their transmembrane domains, with TM3 being the centre most helix and the others organised sequentially in a counter clockwise manner when viewed from the extracellular surface. This was confirmed by the first GPCR X-ray structure to be solved; the inactive or dark-state of bovine rhodopsin (Palczewski, 2000) and subsequently confirmed by other studies

(Cherezov *et al.,* 2007; Rasmussen *et al.,* 2007; Jaakola *et al.,* 2008; Warne *et al.,* 2008). Overall sequence identity for the family A receptors is low, however, there are certain highly conserved key residues which play important roles in structural and/or functional integrity of receptors. One such residue, an arginine (Arg) in the Glu/Asp-Arg-Tyr (E/DRY) motif located between TM3 and ECL2 was confirmed upon crystallisation of inactive bovine rhodopsin (Palczewski 2000). It is conserved in 96% of family A GPCRs and forms a salt bridge with the neighbouring acidic side chain residue Asp or Glu (68% or 20%, respectively) (Vogel *et al.,* 2008). This salt bridge has been found in all inactive receptor structures thus far but is absent in the active-state β_2-adrenergic receptor-nanobody complex (Rasmussen *et al.,* 2011), the activated Adenosine A_{2A} receptor (A_{2A}AR) structures (Lebon *et al.,* 2011; Xu *et al.,* 2011) and in the crystal structure of the histamine H_1 receptor coupled with the antagonist doxepin (Shimamura *et al.,* 2011). In the active state of rhodopsin and the β_2-adrenergic receptor-G-protein structures, the salt bridge is broken as the conserved Arg residue has changed its rotamer conformation to interact with the C-terminal of the G_α-subunit (Scheerer *et al.,* 2008; Standfuss *et al.,* 2011; Choe *et al.,* 2011; Rasmussen *et al.,* 2011).

In rhodopsin, the highly conserved Arg of TM3 forms hydrogen bonds with a Glu residue in TM6 forming what is commonly referred to as the "ionic lock" which was thought to be important in keeping the receptor stable in its "inactive" conformation. The active state of rhodopsin shows conformation changes occurring in opsin, there is a break in the "ionic lock" which allows an outward tilt of TM6 at the cytoplasmic end of the receptor with subsequent rearrangements of TM5 and TM7 (Park *et al.,* 2008). Further evidence for the role of the "ionic lock" stabilising an inactive receptor conformation came when charge-neutralising mutations of the Asp in the β_2-adrenergic receptor and the α_{1b}-adrenoceptor caused increases of constitutive and basal activity of the receptors, respectively (Scheer *et al.,* 2000; Ballesteros *et al.,* 2001). However, the acidic residue in TM6 (i.e. Glu) is only conserved among 30% of GPCRs (Katritch *et al.,* 2013), and crystal structures have shown an absence of the "ionic lock" between the E/DRY motif and residues in TM6 without exhibiting aberrant basal activity suggesting the "ionic lock" is not an essential feature of the inactive receptor (Jaakola *et al.,* 2008; Warne *et al.,* 2008). Family A GPCRs also contain a disulphide bridge between two conserved cysteine residues in the second and third extracellular loops.

Microswitches are described as rotamer changes in highly conserved side chains between GPCRs (Nygaard *et al.,* 2009). These microswitches stabilise the overall movements of helices and are involved in priming the intracellular side of the GPCR for G-protein binding. The E/DRY motif is one such example of a microswitch. Another conserved residue and also thought to behave as a microswitch is the NPXXY motif where the Tyr is highly conserved in 92% of family A GPCRs (Katritch *et al.,* 2013). The NPXXY motif connects the TM helix VII and the cytoplasmic helix VIII. In inactive receptors, the conserved Tyr residue points towards helix I, II or VIII. In contrast, when receptors become activated the Tyr changes its rotamer conformation and points towards the middle axis of the 7TM bundle interacting with helices III and VI (Katritch *et al.,* 2013). The helix VIII of a large proportion of family A receptors is typically three turns in length with cysteines present at the C-terminal end of the helix. These cysteines act as palmitoylation sites and confer stability to the receptor. Indeed, disrupting helix VIII is seen to impair proper functioning of the receptor. Mutating the corresponding cysteine in the β_2-adrenergic receptor results in a non-palmitoylated form of the receptor with a diminished ability to stimulate adenyl cyclase (AC) activity (O'Dowd *et al.,* 1989). Helix VIII of rhodopsin has also been shown to interact with visual arrestin to cause the activation of the latter (Kirchberg *et al.,* 2011) suggesting an important role of the helix VIII in β-arrestin regulation and/or signalling.

Family A GPCRs have a remarkable feature whereby they bind a diverse range of ligands of various shapes, sizes and chemical properties. While all ligands have been observed to bind in a pocket on the extracellular side of the TM bundle, some ligands penetrate deeper within the pocket. Certain residues from TM3, TM6, and TM7 all contact the ligand in nearly all receptors creating consensus contacts with diverse ligands across family A receptors. In addition to residues which directly contact the ligand, it has been shown that water molecules to mediate indirect contacts between ligand and receptor (Lebon *et al.,* 2011; Jaakola *et al.,* 2008; Xu *et al.,* 2011).

Family B GPCRs, also referred to as the secretin family of GPCRs, include receptors for 15 peptide hormones such as secretin, glucagon and parathyroid hormone (Lagerström and Schiöth 2008). These receptors can be grouped into five subfamilies based on their physiological role and are important drug targets in many human diseases such as diabetes, obesity, and cancer amongst others (Hollenstein *et al.,* 2014). Structurally, these GPCRs consist of a large N-terminal extracellular domain and seven

typical transmembrane domains which are involved in signalling via G proteins. The large N-terminus of the receptors contain several cysteine residues postulated to form a network of disulphide bridges which play important roles in the stabilisation of the N-terminal domain (Unson *et al.,* 2002; Lagerström and Schiöth 2008). Whilst the structures of the extracellular domains have been published and well-studied by X-ray crystallography and NMR (Hollenstein *et al.,* 2014), it is only recently that structures of the transmembrane domains have been crystallised (Hollenstein *et al.,* 2013; Siu *et al.,* 2013). The structures of the human glucagon receptor (GCGR) and human corticotropin-releasing factor receptor 1 (CRF_1) show transmembrane domains consisting of seven membrane-spanning α-helices typical of a GPCR. However, the receptors both assume conformations that are more open at their extracellular sides and take the shape of the letter V when viewed from within the membrane (Hollenstein *et al.,* 2014) when compared to the structures of family A GPCRs. One arm of the V is composed of the extracellular halves of TM2-TM5 whereas the other arm comprises the extracellular portions of TM1, TM6, and TM7. The highly conserved GWGXP motif is also present in the TM4 in both these receptor structures and seems to play an important role by non-covalently linking the TM2, TM3, and TM4. The E/DRY and NPXXY motif are absent in family B GPCRs as is the palmitoylation site at the C-terminus (Gether, 2000).

Family C GPCRs are distinguished by a large extracellular domain and constitutive dimerisation. They include the metabotropic glutamate (mGlu) receptors, γ-aminobutyric acid type B ($GABA_B$) receptors, the calcium-sensing (CaS) receptor, the sweet and amino acid taste receptors, putative pheromone receptors and a number of orphan receptors. A distinct feature of family C GPCRs is the exceptionally large extracellular domain that contains a venus flytrap module (VFTM) and a cysteine rich domain (CRD, the $GABA_B$ receptor providing the only exception). The receptors contain 7TM domains but differ from the other GPCR families in that they only contain allosteric binding sites here, the orthosteric binding sites being contained in the VFTM. The second intracellular domain is also the largest for family C GPCRs, in contrast to rhodopsin like receptors where the third intracellular loop is the largest. However, in common with family A and B GPCRs, family C GPCRs do share the conserved cysteines which connect extracellular loops 2 and 3 via a disulphide bridge (Gether, 2000). The other unique characteristic of family C GPCRs is their propensity to form

homodimers (mGlu and CaS receptors) or heterodimers ($GABA_B$ receptor and T_1Rs) (Chun *et al.,* 2012).

The Frizzled/Smoothened receptor family consist of 10 Frizzled receptors (FZD1-10) and a Smoothened (SMO) receptor in mammals (Lagerström and Schiöth 2008). The FZD receptors bind a family of Wnt glycoproteins while the SMO receptor functions in a ligand-independent manner by interacting with Patched, a transmembrane protein which forms a complex with SMO and sonic hedgehog (SHH) (Bhanot *et al.,* 1996; Murone *et al.,* 1999; Lagerström and Schiöth 2008).

The human genome encodes for 25 functional Taste 2 receptors (T_2Rs) which can be divided into five different subgroups with the degree of sequence homology differing considerably (Conte *et al.,* 2002). This feature is thought to explain why a limited number of receptors can sense thousands of different bitter compounds on the tongue and palate epithelium (Fredriksson *et al.,* 2003). The vomeronasal 1 receptors (V_1Rs) are responsible for the detection of exogenous pheromones. Both these receptors have short N- and C-termini and lack the commonly conserved cysteine residues which link the two extracellular loops, the ligand binding pocket is thought to be buried in the 7TM domains (Kristiansen, 2004).

1.1.2. GPCR activation

The proposal by Langley in 1905 of a "receptive substance" marked the advent of the concept of chemical synaptic transmission. After work on adrenomedullary extracts, he proposed the "receptive substance" was the site of action of chemical mediators released from nerve stimulation (Langley, 1905). Paul Ehrlich, a German scientist, also developed a similar theory involving selective binding of toxins and nutritive substances to receptors. By 1907, Ehrlich had revised this theorem to include drugs binding to receptors he called "chemoreceptors" (Rubin, 2007). The word "receptor" was, however, only advocated by a small group of researchers during the first half of the 20th century including Ariens and Stephenson. Their work led to the discovery of partial agonists and the terms "efficacy" and "receptor reserve". The idea of receptors existing in a simple "on or off" state clashed with the new found knowledge that different drugs acted on the same receptor with different efficacies. Therefore, the idea of receptors occurring in different receptor conformations was put forward by Del Castillo and Katz (1957). It was suggested that acetylcholine (Ach) binding to nicotinic acetylcholine

receptors induced a conformational change in the receptor from the resting (R) to activated (R*) state resulting in channel opening (Del Castillo and Katz 1957). Leff (1995) improved upon this model when evidence was presented showing receptors in different conformations even in the absence of agonist binding. The two-state model states an agonist higher affinity for the R* and an inverse agonist has higher affinity for the R state (Leff, 1995). De Lean *et al* (1980) posited the ternary complex model after the finding that the initial step in GPCR signal transduction consisted of interactions between a GPCR and a G-protein (De Lean *et al.,* 1980). This model, however, did not account for every experimentally observable behaviour and so was extended to form the extended ternary complex (ETC) model (Samama *et al.,* 1993). This model proposed the receptor to exist in two functionally distinct states, the R (inactive) state and the R* (active) state. In the absence of agonist, the receptor exists as either active or inactive with the equilibrium between the two states determining the level of spontaneous or constitutive activity of the receptor. The efficacy of ligands is suggested to be their ability to alter the equilibrium between the R and R* states. The ETC model was further extended to take into account the ability of an inactive receptor to bind G-protein even though it could not signal and was called the cubic ternary complex (CTC) model (Weiss *et al.,* 1996). It is now known that GPCRs exist in different conformations upon agonist binding which can lead to distinct downstream signalling effectors becoming activated. The discovery that β-arrestin also signals after its recruitment to the activated receptor has added a further element of complexity to GPCR activation and signalling.

1.1.3. G-protein dependent signalling

Heterotrimeric G-proteins play crucial roles as molecular switches in signal transduction pathways mediated by GPCRs. G-proteins are typically composed of three subunits; α, β and γ, and their switching function depends on the ability of the α-subunit to cycle between an inactive conformation where it is bound to guanosine-5'-diphosphate (GDP) and ready to interact with a receptor, and an active conformation which is bound to guanosine-5'-triphosphate (GTP) and is able to modulate downstream effector proteins. In humans, there are 21 Gα subunits encoded by 16 genes, 6 Gβ subunits encoded by 5 genes and 12 Gγ subunits (Downes and Gautam 1999). Heterotrimeric G-proteins are divided into four main classes: $G\alpha_i$, $G\alpha_q$, $G\alpha_s$, and $G\alpha_{12}$ (Simon *et al.,* 1991). The GTPase domain is conserved amongst all members of the G-protein superfamily and hydrolyses GTP; it is also responsible for binding the Gβγ

dimer, GPCRs and effector proteins. A helical domain is unique to Gα subunits and is formed of a six α-helical bundle which creates a lid above the nucleotide binding pocket, thereby burying the nucleotides and allowing nucleotide exchange to be controlled by the activated receptor. Post-translational modifications help to regulate membrane localisation and protein-protein interactions of Gα subunits. All Gα are palmitoylated at the N terminus (with the exception of $G\alpha_t$), with $G\alpha_i$ proteins also being myristoylated at the N-terminus (Chen and Manning 2001; Smotrys and Linder 2004).

The Gβ subunit has a seven-bladed β-propeller structure composed of seven WD40 repeats (a tryptophan-aspartic acid sequence which repeats every 40 amino acids and forms small antiparallel β strands); the N terminus adopts an α-helical conformation forming a coiled-coil with the N terminus of Gγ whilst the C tail of Gγ binds to blades five and six of Gβ (Wall *et al.,* 1995; Sondek *et al.,* 1996). This interaction can only be broken under denaturing conditions (Schmidt *et al.,* 1992). All the Gγ subunits undergo isoprenylation of their C terminal with either an addition of farnesyl ($G\gamma_1$, $G\gamma_8$, $G\gamma_{11}$,) or geranylgeranyl (all others) moiety (Zhang and Casey 1996). The primary interaction between Gα and the Gβγ dimer occurs in a hydrophobic pocket of the GTPase domain of Gα (Oldham and Hamm 2008). There is, however, no evidence of interaction between the Gα and Gγ subunits, although the acylated N terminus of Gα and the C terminus of Gγ are in close proximity to each other. These interactions all increase the affinity between Gα and the Gβγ dimer.

In the inactive heterotrimeric state, GDP is bound to the Gα subunit and all three subunits are associated with one another. Receptor activation through agonist binding results in an increased affinity of the GPCR to the heterotrimeric G protein complex via conformational changes in the GPCR. This association results in the rapid exchange of GDP for GTP at the Gα subunit, therefore, the activated GPCR acts as a guanine nucleotide exchange factor (GEF). The receptor-G protein complex exists only transiently as binding of GTP to the Gα subunit serves to cause a conformational change in Gα leading to a loss of the hydrophobic pocket crucial in Gβγ binding promoting its release (Cabrera-Vera *et al.,* 2003a). No conformational changes occur in the Gβγ dimer and thus the Gα subunit acts as a negative regulator of Gβγ signalling (Lambright *et al.,* 1996). Intrinsic GTPase activity of the Gα subunits causes hydrolysis of GTP to GDP resulting in the termination of signalling and the re-association of Gβγ to Gα at the

inner surface of the plasma membrane. Whilst it is true that both Gα-GTP and Gβγ bind to and activate downstream effector proteins (Digby *et al.,* 2006), evidence exists in HEK cells transiently transfected with tagged G-protein subunits where some G proteins remain closely associated following receptor activation (Bünemann *et al.,* 2003)

The downstream targets of Gα have been well documented with AC being the first to be recognised (Sutherland and Rall 1958; Rall *et al.,* 1957). $G\alpha_s$ was isolated after it was found to being stimulating AC (Ross and Gilman 1977), causing AC to catalyse the breakdown of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). $G\alpha_i$ was identified soon after and is known to inhibit AC and, therefore, oppose the actions of $G\alpha_s$ (Smith and Limbird 1982; Hsia *et al.,* 1984; Hildebrandt and Birnbaumer 1983; Hildebrandt *et al.,* 1983). It is now known that AC is membrane bound and responds positively or negatively to a wide array of inputs including Gβγ and divalent cations (Sunahara and Taussig 2002; Hanoune and Defer 2001). Gα signalling is also involved in sensory transduction with G proteins such as $G\alpha_{gust}$ and $G\alpha_{olf}$ important in taste and odorant receptors, respectively (Buck, 2000; Margolskee, 2002). Gα signalling is also important in vision where $G\alpha_t$ regulates guanosine monophosphate (GMP)-gated Na^+/Ca^{2+} channels through cyclic GMP (cGMP) phosphodiesterase (Arshavsky *et al.,* 2002). G protein subunits of the $G\alpha_q$ class include $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{16,}$ and are all involved in stimulating the enzyme phospholipase C-β (PLC-β) (Rhee, 2001). PLC-β hydrolyses the phosphoester bond of the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate inositol 1,4,5 triphosphate (IP_3) and 1,2 diacylglycerol (DAG). IP_3 is involved in Ca^{2+} release from intracellular Ca^{2+} stores through binding to IP_3 receptors, and DAG has the ability to activate certain isoenzymes of the protein kinase C (PKC) family. The fourth class of G proteins contains $G\alpha_{12/13}$ proteins which regulate the small G protein RhoA via effectors which contain pleckstrin-homology (PH) and Dbl-homology (DH) domains characteristic of Rho-family GEFs (Worthylake *et al.,* 2000). Activated $G\alpha_{12/13}$ subunits stimulate a host of targets including PDZ-RhoGEF (Longenecker *et al.,* 2001; Fukuhara *et al.,* 1999), and leukaemia-associated RhoGEF (LARG) (Booden *et al.,* 2002; Vogt *et al.,* 2003). $G\alpha_{13}$ (but not $G\alpha_{12}$) stimulates p115RhoGEF activity (Kozasa *et al.,* 1998; Hart *et al.,* 1998).

Originally thought to only facilitate the coupling of Gαβγ heterotrimers to GPCRs and act as a Gα inhibitor due to its guanine nucleotide disassociation inhibitor (GDI)

activity, the Gβγ dimer is now known to have signalling properties in its own right. The first effector of Gβγ to be identified were the G protein-regulated inward-rectifier K^+ channels (GIRK or $K_{ir}3$ channels) (Logothetis *et al.,* 1987). Subsequent studies have also implicated Gβγ in binding to the N- and C-termini of GIRK1-4 (Lei *et al.,* 2000; Huang *et al.,* 1995; Huang *et al.,* 1997; Inanobe *et al.,* 1995; Kunkel and Peralta 1995; Doupnik *et al.,* 1996). Gβγ is also known to directly regulate neuronal N- and P/Q-type Ca^{2+} channels (Kammermeier *et al.,* 2000; Delmas *et al.,* 2000; Lü *et al.,* 2001). Kinases and small G proteins are also effectors of Gβγ, as activation of certain GPCRs gives rise to Gβγ-mediated stimulation of ERK1/2, JNK and p38 mitogen-activated protein kinases (MAPKs) (Yamauchi *et al.,* 1997; Coso *et al.,* 1996; Crespo *et al.,* 1994; Faure *et al.,* 1994). Various forms of AC is both positively and negatively regulated by Gβγ (Gao and Gilman 1991; Tang and Gilman 1991; Taussig *et al.,* 1994), which is not surprising given the great variation in the components of the Gβγ dimer. For example, in COS7 cells, $G\beta_1\gamma_2$ and $G\beta_5\gamma_2$ inhibit AC1 whereas AC2 is stimulated by $G\beta_1\gamma_2$ but inhibited by $G\beta_5\gamma_2$ (Bayewitch *et al.,* 1998). Cell type and tissue expression are also important factors in determining the specificity of Gβγ dimers and their effectors, for example, the predominant Gβγ heterodimer is $G\beta_1\gamma_1$, but the most common heterodimer in the brain is the $G\beta_1\gamma_2$ complex (Cabrera-Vera *et al.,* 2003b). Gβγ has been shown to activate PLC-β and PLC-*ε* (Boyer *et al.,* 1992; Wing *et al.,* 2001).

Originally, it was thought that the duration of heterotrimeric G protein signalling was controlled by two factors: the intrinsic GTPase rate of the Gα subunit and the action of effectors such as PLC-β (Berstein *et al.,* 1992). This, however, did not corroborate with the intrinsic GTPase activity of Gα, as it was observed that it occurred at a much slower rate *in vitro* than can account for the observed deactivation rates of G protein controlled processes (Cabrera-Vera *et al.,* 2003b). But new discoveries brought to light the existence of a new family of GTPase-accelerating proteins (GAPs) for Gα proteins dubbed the "regulators of G protein signalling" (RGS proteins). These proteins consist of ~120 amino acid RGS domain which interacts with the Gα switch regions to stabilise the transition state for GTP hydrolysis (Tesmer *et al.,* 1997; Berman *et al.,* 1996). This terminates Gα signalling and re-association of the Gαβγ heterotrimer, thereby preventing signalling mediated by the Gβγ dimer. RGS9-1, a member of the RGS protein family, stimulates the GTPase activity of $G\alpha_t$ after it has been activated by rhodopsin. This accounts for the normal rate of hydrolysis observed *in vivo* (Arshavsky

and Pugh 1998). RGS proteins have also been seen to antagonise downstream effectors such as PLC-β; RGS4 and GAIP both hasten the GTPase activity of $G\alpha_q$ and also interfere with the interaction between $G\alpha_q$ and PLC-β (Hepler *et al.,* 1997). RGS12 and RGS14 contain a GoLoco motif as well as the usual RGS domains which possess GAP activity against $G\alpha_{i/o}$ proteins. The GoLoco motif is a second RGS-$G\alpha_i$ interaction site which also possesses GDI activity which stabilises $G\alpha_{i1}$ in its GDP-bound form, and also decreases the rate of exchange of GDP for GTP upon activation of the G protein (Kimple *et al.,* 2001).

1.1.4. G-protein independent signalling

GPCRs undergo heterologous and homologous desensitisation which serves to negatively impact the efficiency of receptor-G protein coupling (Freedman and Lefkowitz 1996). Heterologous desensitisation is independent of agonist binding and occurs when second messenger-dependent PKA and PKC phosphorylates residues in intracellular domains of the receptor. On the other hand, homologous desensitisation is dependent on agonist occupation of the receptor and the binding of accessory proteins. G protein coupled receptor kinases (GRKs) phosphorylate the agonist-occupied receptor at serine and threonine residues in the third intracellular loop and C-tail thereby recruiting arrestin molecules from the cytosol. These arrestins are a small family of GPCR-binding proteins originally thought to be solely involved in GPCR desensitisation and internalisation (Ferguson, 2001). However, evidence has emerged that arrestins also play a role as signalling molecules after interacting with an activated GPCR. Unlike the transient GPCR-G protein interaction, GPCR-arrestin complexes can persist from minutes to hours (Charest *et al.,* 2005; Pfleger *et al.,* 2006). It is now known that certain catalytically active proteins bind arrestin and are recruited to agonist-occupied receptors. As arrestin binding uncouples GPCRs from G protein, arrestin-dependent and G protein-dependent signals should be mutually exclusive from one another at the level of the single receptor. Theoretically, arrestin binding should switch the GPCR between two distinct signalling states. This is evident when observing the well-studied ERK1/2 cascade as several receptors including the angiotensin AT_{1A}, lysophosphatidic acid (LPA), type I parathyroid hormone/PTH-related peptide (PTH_1), and β_2-adrenergic receptors (β_2AR) demonstrate that the onset of arrestin-dependent ERK1/2 activation overlaps with the diminishing of G protein signalling and persists as receptors internalise (Ahn *et al.,* 2004; Gesty-Palmer *et al.,* 2005; Gesty-Palmer *et al.,*

2006; Shenoy *et al.,* 2006). More recently, the histamine H_4R has also demonstrated this phenomena (Rosethorne and Charlton 2011). Similarly, as the GPCR-arrestin complex is isolated from the rest of the cytoplasm in endosomes arrestin-dependent signalling should be spatially discrete. Receptors which form stable receptor-arrestin complexes such as protease-activated receptor (PAR)-2, angiotensin AT_{1A}, vasopressin V_2, and neurokinin NK-1 receptors are known to activate ERK1/2 that accumulates in early endosomes and which is not transcriptionally competent (DeFea, 2000; DeFea *et al.,* 2000; Luttrell *et al.,* 2001; Tohgo *et al.,* 2003). This differs from ERK1/2 which is activated via the heterotrimeric G protein-mediated pathway and also from receptors such as the β_2-adrenergic receptor and LPA receptors which form transient arrestin-receptor complexes which can translocate to the nucleus and impact transcription (Gesty-Palmer *et al.,* 2005; Shenoy *et al.,* 2006). This is corroborated by a study which swaps the C terminus of the V_2 receptor for the C terminus of the β_2-adrenergic receptor and thereby turns the interaction with arrestin from stable to transient. Vasopressin activated ERK1/2 is shown to enter the nucleus and promote cell proliferation (Tohgo *et al.,* 2003).

As well as signalling via G proteins and arrestins, GPCRs are also known to signal via other cellular proteins. PDZ proteins may also influence the downstream signalling of a GPCR, its trafficking and its recycling. An example of a class A receptor which contains a PDZ-binding domain is the β_2-adrenergic receptor which associates with the Na^+/H^+ exchanger regulatory factor (NHERF) resulting in the promotion of receptor recycling and signalling via NHERF1 (Hall *et al.,* 1998; Cao *et al.,* 1999). More recently it has been shown that sorting nexin 27 (SNX27) contains a PDZ domain, which binds the β_2-adrenergic receptor and serves as an adapter, sorting the receptor into retromer tubules (Tempkin *et al*., 2011). Parathyroid hormone 1 receptor (PTH1R) is an example of a class B receptor which associates with NHERF1 and 2, an interaction which tethers the receptor at the cell membrane. The interaction also serves to switch G protein signalling, regulate ERK signalling, impart ligand bias and regulate receptor desensitisation (Romero *et al.,* 2011). The metabotropic glutamate 5 receptor (mGluR5) is an example of a family C GPCR which interacts with NHERF2, causing a prolonged duration of the receptor-mediated Ca^{2+} response (Paquet *et al.,* 2006).

1.1.5. GPCR regulation by phosphorylation

Upon stimulation by agonists, most GPCRs undergo phosphorylation which acts as a means of receptor regulation (Lefkowitz, 2004). Phosphorylation is a rapid process which causes the receptor to undergo uncoupling from its associated G-proteins and the recruitment of arrestin to the receptor (Lefkowitz and Shenoy 2005). This recruitment serves to arrest further signalling of the activated receptor, hence the name arrestin, and causes receptor desensitisation and internalisation. Many cell types express several receptors concurrently; prolonged stimulation of a single type of receptor has the ability to causes homologous or heterologous desensitisation. Homologous desensitisation is where prolonged activation of one type of receptor causes attenuated signalling of only that receptor. Heterologous desensitisation attenuates signalling from not only that receptor but also other type(s) of receptor present in the same cell. This is consistent with modifications to components downstream of the signal transduction pathways that are shared with other GPCRs. GPCR phosphorylation can involve more than one protein kinase mediating multi-site phosphorylation (Tobin *et al.,* 2008). Taking the β_2-adrenergic receptor as an example, we see that the receptor at low levels of ligand occupancy was shown to be phosphorylated by the second messenger activated PKA resulting in sites in the third intracellular loop and C-terminal tail becoming phosphorylated regardless of whether the receptor was occupied by agonist or not. At high agonist occupancy, the receptor was phosphorylated by members of the GRK family at sites distinct from the PKA sites (Hausdorff *et al.,* 1989; Seibold *et al.,* 2000; Tran *et al.,* 2004). Interestingly, it has been shown that PKA-mediated phosphorylation has caused G-protein switching from $G\alpha_s$ to $G\alpha_i$ (Daaka *et al.,* 1997; Martin *et al.,* 2004). This highlights the different kinases able to phosphorylate receptors and also the different outcomes of receptor phosphorylation such as G-protein switching.

The GRKs are grouped into three subfamilies based on sequence identity and gene structure (Premont *et al.,* 1999). The GRK1 subfamily consists of GRK1 and GRK7; the GRK2 subfamily consists of GRK2 and GRK3; and the GRK4 subfamily contains GRK4-6. GRK2, 3, 5, and 6 are ubiquitously expressed in mammalian tissues and GRK1, 4, and 7 are confined to specific organs. GRK1 and 7 are expressed in rods and cones, respectively. GRK4 is found in the cerebellum, kidney and testis (Sallese *et al.,* 1997; Virlon *et al.,* 1998; Sallese *et al.,* 2000). GRKs share a common architecture with a conserved central catalytic domain, an N-terminal domain and a variable-length C-

terminal domain (Ribas *et al.,* 2007). The N-terminal domain is thought to be important in receptor recognition, for intracellular membrane anchoring (Murga *et al.,* 1996), and also contains a regulator of G-protein signalling homology (RH) domain. The presence of this domain may explain the potential mechanism by which GRK2 and GRK3 might regulate GPCRs independently of receptor phosphorylation (Carman *et al.,* 1999; Sallese *et al.,* 2000; Dhami *et al.,* 2002; Willets *et al.,* 2005). GRK2 and GRK3 share a pleckstrin homology (PH) domain in their C-terminus which mediates PIP_2 and G-protein βγ-subunit-mediated translocation to the inner part of the plasma membrane (Premont and Gainetdinov 2007). GRK4, GRK5, and GRK6 lack this G-protein βγ-subunit binding domain, instead these kinases use direct PIP_2 and covalent lipid modifications with palmitate to localise close to the plasma membrane (Premont and Gainetdinov 2007).

The casein kinase (CK) family has also been implicated in GPRC phosphorylation and consists of two members, CK1 and CK2, which are structurally distinct (Tobin, 2002). The CK1 family is made up of seven members (CK1 α, β, $\gamma_{1\text{-}3}$, δ and ε) that are expressed in a wide range of tissues (Tobin, 2002). CK2 is found in a tetrameric complex consisting of two catalytic (alpha) subunits and two regulatory beta subunits (Litchfield, 2003). Inhibiting both CK1 and CK2 has been shown to reduce phosphorylation of the M_3 mAchR indicating a role for these kinases in phosphorylation of M_3 muscarinic receptors (Budd *et al.,* 2000; Torrecilla *et al.,* 2007).

As well as receptor desensitisation and G-protein switching, GPCR phosphorylation plays an important role in receptor internalisation. The non-visual arrestin proteins, arrestin-2 (β-arrestin1) and arrestin-3 (β-arrestin2) play crucial roles in this process as adapters and scaffolds. Arrestins rapidly bind activated/phosphorylated receptors and terminate G-protein signalling (Zhang *et al.,* 1999). Recently, the crystal structure of arrestin-2 bound to a GPCR phosphopeptide has been solved which gives clues as to the general molecular mechanism by which arrestins become activated. The crystal structure reveals the pronounced structural changes of the N- and C-domains of arrestin-2 which undergo a twist relative to each other around the central axis upon arrestin activation (Shukla *et al.,* 2013). Arrestin contains several loops implicated in aspects of activation and receptor interaction (Han *et al.,* 2001), including the "finger loop", the "middle loop", and the "lariat loop". In the crystal structure, the GPCR phosphopeptide is shown to interact with the finger loop and occlude the inactive conformation of the

finger loop which is known to discriminate active and inactive GPCRs (Hanson *et al.,* 2006). All three loops display conformational changes which are activation dependent (Shukla *et al.,* 2013). We also see a disruption of the polar core of arrestin-2 which is composed of five interacting charged residues by the phosphopeptide of the GPCR by displacing the carboxy terminus of the arrestin molecule. As the carboxy terminus contains the clathrin binding site, its displacement is likely an important contributor to the clathrin-mediated GPCR internalisation that occurs upon arrestin binding to activated receptors. This confirms previous studies which have shown the interaction of arrestin with clathrin and clathrin associated protein-2 (AP-2), two major proteins present in clathrin-coated pits (CCPs), which play important roles in GPCR endocytosis (Marchese *et al.,* 2003). This clathrin-dependent pathway also relies on the GTPase activity of membrane bound dynamin which is thought to pinch the clathrin-coated pits from the plasma membrane and facilitates vesicle formation (Moore *et al.,* 2007). Caveolae-dependent internalisation of GPCRs is a secondary mechanism which can be employed by the cell, which requires caveolin activity and cholesterols (Pierce *et al.,* 2002). Internalised GPCRs are transported to endosomes and are either recycled back to the plasma membrane or transported to lysosomes for degradation. Recycled receptors need to be dephosphorylated by the endosome-associated phosphatase 2A (Pitcher *et al.,* 1995), before being sent back to the plasma membrane in a process which relies on the activity of small GTPases including Rab11 or Rab4 that targets the receptor to the plasma membrane (Sorkin and von Zastrow 2009). Receptors fated to become degraded are ubiquitinated (covalent addition of an 8.5 kDa ubiquitin moiety to lysine residues on the GPCR) and are broken down by lysosomal action (Hanyaloglu and von Zastrow 2008).

GPCRs can signal to a plethora of downstream effectors, but in a certain physiological setting, these receptors will only influence a small complement of their entire signalling repertoire (Tobin *et al.,* 2008). How a receptor does this will be influenced by various factors including cell-type-specific expression of downstream signalling targets and the nature of the receptor-signalling complex (Bockaert *et al.,* 2004). What is also apparent from several studies involving various receptors is that site-specific phosphorylation may play an important role in determining specific signalling outcomes. Studies involving the β_2-adrenergic receptor demonstrated clearly how differential receptor phosphorylation by individual GRKs produced distinct conformations in the recruited β-

arrestin thereby regulating its functional activities (Nobles *et al.,* 2011). Comparisons of the M_3 mAchR phosphorylation profile when expressed in CHO cells and in cerebellar granule neurones demonstrates there are common sites of phosphorylation but more importantly, there are also sites that are specifically phosphorylated depending on cell type (Torrecilla *et al.,* 2007), demonstrating the M_3 mAchR is phosphorylated in a cell-type-specific manner. This receptor can be phosphorylated by several kinases including GRK2, GRK6, CK1α, and CK2 (Tobin *et al.,* 2008), however, disrupting CK2 phosphorylation of the receptor does not affect internalisation of the receptor nor does it uncouple the receptor from the ERK1/2 pathway but does enhance receptor signalling through the Jun-kinase pathway (Torrecilla *et al.,* 2007). There is now a growing body of evidence which suggests GPCR phosphorylation is a complex process involving various proteins which can phosphorylate the receptor at different sites resulting in different signalling outcomes (Tobin 2008; Butcher *et al.,* 2011; Kao *et al.,* 2011; Oppermann *et al.,* 1999; Pöll *et al.,* 2010; Zidar *et al.,* 2009). This would mean GPCR phosphorylation recruits different protein kinases in cell types that could tailor the signalling response of the receptor to suit a particular physiological role. The pattern of phosphorylation in these receptors may form a "phosphorylation barcode" which codes for regulation of certain receptor mediated pathways (Liggett, 2011).

1.1.6. Biased agonism or functional selectivity

The historical view of a GPCR acting as a bimodal switch which changes from an inactive to an active conformation upon agonist binding is now known to be a very simple interpretation of receptor activation. Recent studies have demonstrated different ligands acting at the same receptor can stabilise distinct conformations leading to diverse functional outcomes (Urban *et al.,* 2007; Stallaert *et al.,* 2011; Kenakin and Christopoulos 2012). This phenomenon has been called biased agonism, stimulus bias, ligand-directed signalling or functional selectivity (Kenakin 2011; Violin and Lefkowitz 2007; Urban *et al.,* 2007; Stallaert *et al.,* 2011). This concept relies on receptors being able to adopt multiple active state conformations resulting in conformationally driven preference for signalling proteins (Kenakin, 2007). The earliest example of a study identifying a biased agonist involved the serotonin type 2A and 2C receptors, different agonists of 5-$HT_{2A/2C}$ receptors would preferentially activate phospholipase A_2-mediated arachidonic acid release of PLC-mediated inositol phosphate (IP) accumulation (Berg *et al.,* 1998). Other studies exhibiting the pleiotropic nature of

ligands includes the β_2 adrenergic receptor (Azzi *et al.,* 2003; Baker *et al.,* 2003; Galandrin and Bouvier 2006) which showed agonists possessing dual and opposite efficacies toward different pathways. JNJ7777120 acting at the histamine H_4R also displayed dual efficacy, acting as an agonist in arrestin recruitment but an antagonist for G-protein activation (Rosethorne and Charlton 2011). As bias can vary from G-protein bias, to arrestin bias, to the differential coupling of G-protein subtypes there is potential to promote different functional and cellular responses. Thus biased agonism may be utilised in the development of more effective therapeutic agents by selectively activating a therapeutically beneficial pathway without unwanted side effects (Kenakin, 2012)

1.2. Histamine receptors

Histamine has long been known to be an important mediator in a variety of conditions, and plays a critical role in the immune system. Initially, the presence of two distinct types of histamine binding receptors were proposed (Ash and Schild 1966), based on evidence of known histaminergic ligands on different tissues. The discovery of the first histamine H_2 receptor antagonists corroborated the theory of two discrete receptor subtypes (Black *et al.,* 1972). The histamine H_3 receptor was first identified in 1983 in the rat brain (Arrang *et al.,* 1983), but was not widely accepted until its gene was cloned nearly twenty years later (Lovenberg *et al.,* 1999). The discovery of the H_3 receptor gene allowed several groups to identify a fourth histamine binding receptor, namely the histamine H_4 receptor (Nakamura *et al.,* 2000; Oda *et al.,* 2000; Liu *et al.,* 2001; Morse *et al.,* 2001; Nguyen *et al.,* 2001; Zhu *et al.,* 2001).

1.2.1. Classification and alternative splicing of the histamine receptors

There are four receptors which bind histamine, with all four falling into the family A of GPCRs. The histamine H_1 receptor (H_1R) is encoded by a single exon gene on the distal short arm of chromosome 3p25b and contains 487 amino acids (De Backer *et al.,* 1998). The H_2R is encoded by the intronless gene located on chromosome 5 and is composed of 359 amino acids (Murakami *et al.,* 1999). The H_3R gene consists of four exons spanning 5.5kb on chromosome 20. Several splice variants have been reported for the H_3R, Galizzi and colleagues cloned five splice variants which differed from the hH_3R comprised of 445 amino acid which had been described previously (Cogé, *et al.,* 2001; Lovenberg *et al.,* 1999). The splice variants presented either a deletion in the putative

TM2 of the receptor or a large deletion in third intracellular loop. The H_4R is encoded by a gene containing three exons separated by two introns located on chromosome 18q11.2. It shares relatively high sequence homology with the H_3R (37-43%, 58% in the transmembrane regions) and like the H_3R, the H_4R shows alternative splice variants. Two alternative splice variants have so far been reported (van Rijn *et al.,* 2008), the severely truncated $hH_4R_{(67)}$ consists of only the first 67 amino acids of the receptor and $hH_4R_{(302)}$ possesses an 88 amino acid deletion between TM2 and TM4. These splice variants interfere with the trafficking of the full length receptor to the cell membrane, thus reducing its signalling capacity. This phenomena has been observed in other receptors such as the α_{1A} adrenergic receptor (Cogé *et al.,* 1999), the dopamine D_{3nf} receptor (Elmhurst *et al.,* 2000), an alternatively spliced rat vasopressin V_2 receptor (Sarmiento *et al.,* 2004), and the rat H_3R (Bakker *et al.,* 2006).

1.2.2. Localisation of the histamine receptors

Histamine receptors have been reported in a wide range of tissues. The study of the distribution of the H_1R was greatly aided by the development of radioactive probes for the receptor recognition site. [3H]-mepyramine was the first radioligand which recognised the H_1R and was initially used in labelling H_1R in the longitudinal smooth muscle of guinea pig ilium (Hill *et al.,* 1977). It was subsequently used to identify the H_1R in various mammalian tissues including the human peripheral lung (Casale *et al.,* 1985), astrocytoma cells (Nakahata *et al.,* 1986), lymphocytes (Casale *et al.,* 1985; Cameron *et al.,* 1986), the retina (Sawai *et al.,* 1988) and the myometrium during pregnancy (Gonzalez *et al.,* 1994). Other radioligands have also been used effectively in the search for the H_1R, [^{3}H]-histamine has revealed the presence of the H_1R in mouse uterine horns (Gonzalez *et al.,* 1993). More recently, RT-PCR and northern blotting studies have shown mRNA encoding the H_1R is found throughout the human gastrointestinal system including the duodenum, ilium and rectum (Sander *et al.,* 2006), in the enteric nervous system specifically in the ganglion cells of the myenteric plexus (Sander *et al.,* 2006), and in cranial arteries (Jansen-Olesen *et al.,* 1997). Northern blot and western blot analysis has revealed expression of the human H_1R to be in the heart (atrium and ventricle) as well as the brain (cerebrum) (Matsuda *et al.,* 2004), and in mouse mast cells (Hofstra *et al.,* 2003). Immunohistochemical studies as well as utilising known H_1R antagonists have also shown the H_1R to be expressed in human intestinal muscle layers and cultured airway smooth muscle (Sander *et al.,* 2006; Hardy

et al., 1996). Positron emission topography (PET) scans were used to demonstrate the presence of H_1R in brain regions such as the cortex and thalamus (Mochizuki *et al.,* 2004).

Table 1.1| Characteristics of the Histamine Receptor Subtypes. Adapted from (Shahid *et al.,* 2009). Abbreviations: cAMP = cyclic adenosine monophosphate, cGMP = cyclic guanosine monophosphate, MAP = mitogen-activated protein, NF-κB = nuclear factor-κB, NOS = nitric oxide synthase, PLC = phospholipase C.

Characteristics	**Histamine H_1 receptor**	**Histamine H_2 receptor**	**Histamine H_3 receptor**	**Histamine H_4 receptor**
Receptor described, human gene cloned (years)	1966, 1993	1972, 1991	1983, 1999	1994, 2001
Receptor proteins in humans	487 amino acids, 56kDa	359 amino acids, 40kDa	445 amino acids, 70kDa; splice variants	390 amino acids, 37-43kDa
Receptor expression	Widespread, including neurons, smooth muscle and immune cells	Widespread, including gastric mucosa parietal cells, smooth muscle, and immune cells	High expression in histaminergic neurones, low expression elsewhere	High expression in bone marrow and peripheral hematopoietic cells, low expression elsewhere
G protein coupling	$G\alpha_{q/11}$	$G\alpha_s$	$G\alpha_{i/o}$	$G\alpha_{i/o}$
Activated intracellular signals	$Ca^{2+}\uparrow$, cGMP, NF-κB, PLC↑, phospholipase A2 and D, cAMP, NOS	cAMP↑, Ca^{2+}, PKC, c-fos, PLC	$Ca^{2+\uparrow}$, MAP kinase↑; ↓cAMP	$Ca^{2+}\uparrow$, MAP kinase↑; ↓ cAMP
Selective agonists	Histamine, dimethylhista-prodifen	Histamine, arpromidine	Histamine, R-α-methylhistamine	Histamine, 4-methylhistamine
Selective antagonists	Mepyramine, diphenhydramine	Ranitidine, Cimetidine	Thioperamide	Thioperamide

Investigating the expression of the H_2R using radioligands proved somewhat more challenging than for the H_1R, therefore, H_2R-mediated adenylate cyclase (AC) or cyclic AMP (cAMP) responses were used as markers for mapping the distribution of H_2R in different tissues. Initial studies monitored AC activity to reveal H_2R presence in human gastric mucosa and duodenal mucosa (Simon *et al.,* 1977). Investigating the mRNA of the H_2R reveals expression of the receptor in the human gastrointestinal tract and immune cells found in the gastrointestinal tract (Sander *et al.,* 2006), mast cells (Lippert *et al.,* 2004), the atria and ventricle in human heart tissue (Matsuda *et al.,* 2004), mouse mast cells (Hofstra *et al.,* 2003), and in the mouse olfactory bulb, testes and bone marrow (Regard *et al.,* 2008). Immunohistochemical and in situ hydridisation studies have revealed the presence of the H_2R in the human enteric nervous system, epithelial and smooth muscle cells associated with the gastrointestinal system (Sander *et al.,* 2006), and the testes (Albrecht *et al.,* 2005) as well as several brain regions such as the caudate, putamen nuclei and external layers of the cerebral cortex (Honrubia *et al.,* 2000).

Initial radioligand binding studies using [^{3}H]-R-α-methylhistamine focused on the expression of the H_3R in rat brain areas. Subsequent studies in mouse and human have shown the presence of the H_3R in the human cerebral cortex (Arrang *et al.,* 1988). These results were confirmed and added to using northern blot analysis and RT-PCR to show that the H_3R was expressed in this brain region as well as the thalamus, caudate nucleus, putamen, amygdala, substantia nigra, hippocampus, hypothalamus and cerebellum (Cogé *et al.,* 2001). The use of [^{125}I]-iodophenpropit demonstrated the presence of the H_3R in mouse brain regions such as the thalamus, hypothalamus, hippocampus, cerebellum, and brainstem (Jansen *et al.,* 2000), in situ hybridisation, immunohistochemistry, southern blotting and RT-PCR confirmed the presence of the H_3R in these brain regions as well as others such as the striatum, olfactory tubercle and substantia nigra (Chazot *et al.,* 2001; Rouleau *et al.,* 2004).

Tissue distribution of the H_4R has been profiled at the mRNA level for human and mouse extensively. Using RT-PCR, human tissues which have been shown to express the H_4R include peripheral blood mononuclear cells, bone marrow, lung, heart, kidney, liver, pancreas, skeletal muscle, leukocytes, prostate, thymus, small intestine, spleen, testes, and lymph nodes (Nakamura *et al.,* 2000; Liu *et al.,* 2001; Zhu *et al.,* 2001). Further studies have also shown the H_4R to be expressed throughout the gastrointestinal

tract (Sander *et al.,* 2006), mast cells, eosinophils, dendritic cells, cerebellum, and hippocampus (Cogé *et al.,* 2001; Ling *et al.,* 2004; Lippert *et al.,* 2004; Morse *et al.,* 2001; Nguyen *et al.,* 2001; Oda *et al.,* 2000; Sander *et al.,* 2006). Northern blot analysis and in situ hybridisation studies have revealed the presence of the H_4R in the mouse brain (hypothalamus and dentate gyrus) and mouse mast cells and eosinophils (Hofstra *et al.,* 2003; Zhu *et al.,* 2001).

1.2.3. Structural architecture of histamine receptors

All histamine receptors share the same basic structure as other family A GPCRs. Recently, the crystal structure of the H_1R-bacteriophage T4-lysozyme (T4L) complex with doxepin, a first generation H_1R antagonist, has been reported (Shimamura *et al.,* 2011). This study sheds light on the molecular basis of H_1R antagonist specificity against H_1R. In this study, T4L has been inserted into the third intracellular loop with the first 19 residues of the receptor being truncated; this modification of the receptor does not affect histamine binding. Adding T4L in place of the third intracellular loop makes the resulting protein more amenable to crystallisation as the T4L is a well-folded soluble protein that crystallises under many different conditions and makes T4L-T4L and T4L-GPCR interactions which increase crystal packing. The H_1R shares common motifs with other family A GPCRs including D(E)RY in helix III, CWxP in helix VI and NPxxY in helix VII along with a disulphide bridge connecting extracellular loop 2 (ECL2) with the extracellular end of helix III ($Cys100^{3.25}$ to Cys180; superscripts indicate residue number as per the Ballesteros-Weinstein scheme (Ballesteros and Weinstein 1995)). A conserved $Pro161^{4.59}$-induced kink is present in helix IV forms a tight i+3 helical turn and also allows the accommodation of a bulky Trp side chain at position 4.56 which seems to be important in ligand specificity of aminergic GPCRs as this position is occupied by a Ser residue in β_2-AR and D3R and mutations of this residue in guinea-pig from Trp to Ala, Met and Phe reduced the affinity against the antagonist pyrilamine (Wieland *et al.,* 1999). The ionic lock in the solved crystal structure does not form a salt bridge as observed in rhodopsin structures (Palczewski *et al.,* 2000), but instead the side chain of $Arg125^{3.50}$ adopts a new confomer and forms a hydrogen bond with $Gln416^{6.36}$ in helix VI. Doxepin is shown to bind in a pocket defined mainly by side chains of helices III, V, and VI. An $Asp107^{3.32}$, which is a strictly conserved residue in aminergic receptors, forms a salt bridge with the amine moiety of the ligand and has been reported as essential for binding H_1R ligands in

mutational studies (Ohta *et al.,* 1994; Nonaka *et al.,* 1998; Bruysters *et al.,* 2004). Doxepin seems to bind deeper in the binding pocket of the H_1R as compared to other ligands in the other non-rhodopsin GPCR structures, therefore, doxepin does not interact with ECL2 (Shimamura *et al.,* 2011). ECL2 is known to contribute to binding specificity of GPCR ligands due to its variable primary and tertiary structures (Peeters *et al.,* 2011). A novel feature also presented by the structure of the H_1R is an anion binding site at the entrance to the ligand-binding pocket, modelling using a phosphate ion was found to affect the binding of some ligands and the stability of H_1R (Shimamura *et al.,* 2011). The modelling also showed the phosphate ion was coordinated by residues unique to the H_1R; this coupled with knowledge that the tertiary amine of doxepin formed an ionic interaction with the phosphate ion indicates the phosphate ion may serve as a positive modulator of ligand binding. The affinity of histamine and pyrilamine to the receptors also increased in the presence of phosphate (Shimamura *et al.,* 2011). The crystal structure of the H_1R in complex with doxepin has revealed possible ways in which antagonists can interfere with the GPCR activation mechanism. A key molecular switch in GPCR activation is the $Trp^{6.48}$ of the highly conserved $CWxP^{6.50}$ motif, this motif has been shown to stabilise rhodopsin in its inactive dark state by a direct interaction with retinal. In the structure of the active-state $A_{2A}AR$, a small ligand-induced shift of $Trp^{6.48}$ leads to a large movement of the intracellular part of helix VI (Xu *et al.,* 2011) indicating $Trp^{6.48}$ participates in the activation-related conformational changes. This role of $Trp^{6.48}$ is not as obvious in other GPCRs however; for example, $Trp^{6.48}$ in the β_2-AR lacks direct ligand interactions with full or inverse agonists (Rasmussen, *et al.,* 2011). In the H_1R structure, doxepin doesn't make extensive hydrophobic interactions with $Trp^{6.48}$ rings indicating it could possibly stabilise the hydrophobic packing around helix VI (Shimamura *et al.,* 2011). Another important ligand-induced switch is thought to be activation-related contraction of an extracellular ligand-binding pocket (Jongejan and Leurs 2005). When bulky H_1R antagonists block both activation-related contraction of this pocket in concert with blocking of the $Trp^{6.48}$ switch, it is thought to be a very efficient mechanism whereby the H_1R is locked in an inactive conformation and helps explain the reduction of basal activity of H_1R upon H_1R antagonist binding (Bakker *et al.,* 2001).

Alignment of the transmembrane regions and extracellular loops of the histamine receptors reveals that the receptors are distantly related, with only the H_3R and H_4R

showing a relatively high degree of sequence similarity. Because crystal structures for the H_2R have not yet been solved, homology models, molecular docking, and molecular dynamics methods have been employed to investigate interactions between the H_2R and its ligands. Initial mutational studies using the canine H_2R found three residues important in histamine binding at the H_2R. Asp98$^{3.32}$ in the third transmembrane domain is thought to serve as a counter anion responsible for interacting with the cationic amine moiety of histamine. Mutating this residue to an Asn residue and effectively removing the negative charge abolished both binding of the H_2R antagonist [methyl-^{3}H]tiotidine and histamine induced increases in intracellular cAMP content (Gantz *et al.,* 1992). Mutation of Asp186$^{5.42}$ in the fifth transmembrane domain to Ala or Asn resulted in a loss of [methyl-^{3}H]tiotidine binding but did not affect histamine-mediated cAMP signalling. The Thr190$^{5.46}$ residue is important in establishing the kinetics of histamine binding but isn't essential for H_2R selectivity (Gantz *et al.,* 1992). Tyr250 has also been shown to be potentially important in the receptor-ligand complex as it has been shown to form hydrogen bonds between itself and guanidinium groups on some agonists (Sun *et al.,* 2011). Although there are several reports which suggest ECL2 contributes to ligand-specificity in aminergic GPCRs, there is evidence to suggest that this may not always be the case. Mutating several non-consecutive residues in the ECL2 of the H_2R was shown to not have any effect on interactions between agonists and the receptor (Preuss *et al.,* 2007), although the study does point out other ligands/residues may affect ECL2 contributions to ligand specificity.

The H_3R shares the highest sequence homology with the H_4R (37%) compared with the average sequence homology between the subtypes (20%) (Hough, 2001). Homology models suggest what is common for most if not all ligands binding at the H_3R is the interaction of the negatively charged carboxylic group of Asp114$^{3.32}$ in helix III with a protonated amine group of an antagonist (Axe *et al.,* 2006), and Glu206$^{5.46}$ in helix V. Mutational studies which replace the highly conserved Asp114$^{3.32}$ with an Asn or Glu results in no detectable agonist-mediated change in cAMP levels (Uveges, 2002). The aromatic ring of this compound also shows evidence of interacting with the aromatic rings of Tyr115$^{3.33}$ and Trp371$^{6.48}$ (Axe *et al.,* 2006). Alignment of the H_3R with the H_2R reveals Asp186$^{5.42}$ and Thr190$^{5.46}$ correspond to Ala202 and Glu206 of the H_2R, mutating these residues in the H_3R affects both the potency and affinity of histamine to the receptor (Uveges, 2002), indicating these residues in the fifth transmembrane

domain are important for ligand binding. The Ala202 and Glu206 are also equivalent to residues in some other biogenic amine binding GPCRs which are shown to interact with ligands (Strader *et al.,* 1989; Pollock *et al.,* 1992), this would demonstrate that the TM5 of these receptors is functionally conserved were it not for studies involving the α2A-adrenergic receptor where the residue corresponding to Ala202 which appears to not be involved in agonist binding (Wang *et al.,* 1991). Although there is little sequence homology between the H_1R, H_2R and the H_3R, the residues mentioned above align with residues shown previously to be important in H_1R, H_2R and other members of the biogenic amine GPCR family.

The H_4R was first identified at the turn of the century (Oda *et al.,* 2000) and was followed very quickly by four other research groups (Nakamura *et al.,* 2000; Liu *et al.,* 2001; Morse *et al.,* 2001; Zhu *et al.,* 2001). This first reported sequence differed from the sequences reported later at three positions. This included Val instead of Ala at position 138, Arg instead of His at position 206 and Arg instead of Gln at position 253. The Ala138 and His206 polymorphisms have been confirmed by single nucleotide polymorphism (SNP) analysis, and the occurrence of these alleles is higher than that of Val138 or Arg206 (Leurs *et al.,* 2009). A Cys to Ser mutation has also been found at position 284, and a frame shift at residue Leu379 both in the coding regions of the H_4R. it has also been reported that 37 SNPs have been identified in intron 1, 45 in intron 2 and 21 in the 3'-untranslated region (Leurs *et al.,* 2009), however, no functional comparisons of these various SNPs has yet been described. The H_4R, like the other histamine receptors, retains the consensus motifs identified for family A GPCRs including $Asn33^{1.50}$, $Asp61^{2.50}$, $Arg112^{3.50}$, $Trp140^{4.50}$, $Pro186^{5.50}$, $Pro318^{6.50}$, and $Pro355^{7.50}$. Molecular modelling and site-directed mutagenesis studies have proven useful tools in delineating the histamine binding site for the H_4R, $Asp94^{3.32}$ in the third intracellular domain, is thought to interact with the protonated amine group of histamine (Shin *et al.,* 2002; Schultes *et al.,* 2013). The $Glu182^{5.46}$ present in the fifth transmembrane domain is a negatively charged residue that has been revealed to play an important role in the binding of histamine at the H_4R based on site-directed mutagenesis and computational analysis (Shin *et al.,* 2002; Uveges, 2002; Schultes *et al.,* 2013), as it provides an anchor for the imidazole ring of histamine. The binding pocket of the imidazole ring is formed of $Glu182^{5.46}$, $Thr178^{5.42}$, and $Trp316^{6.48}$

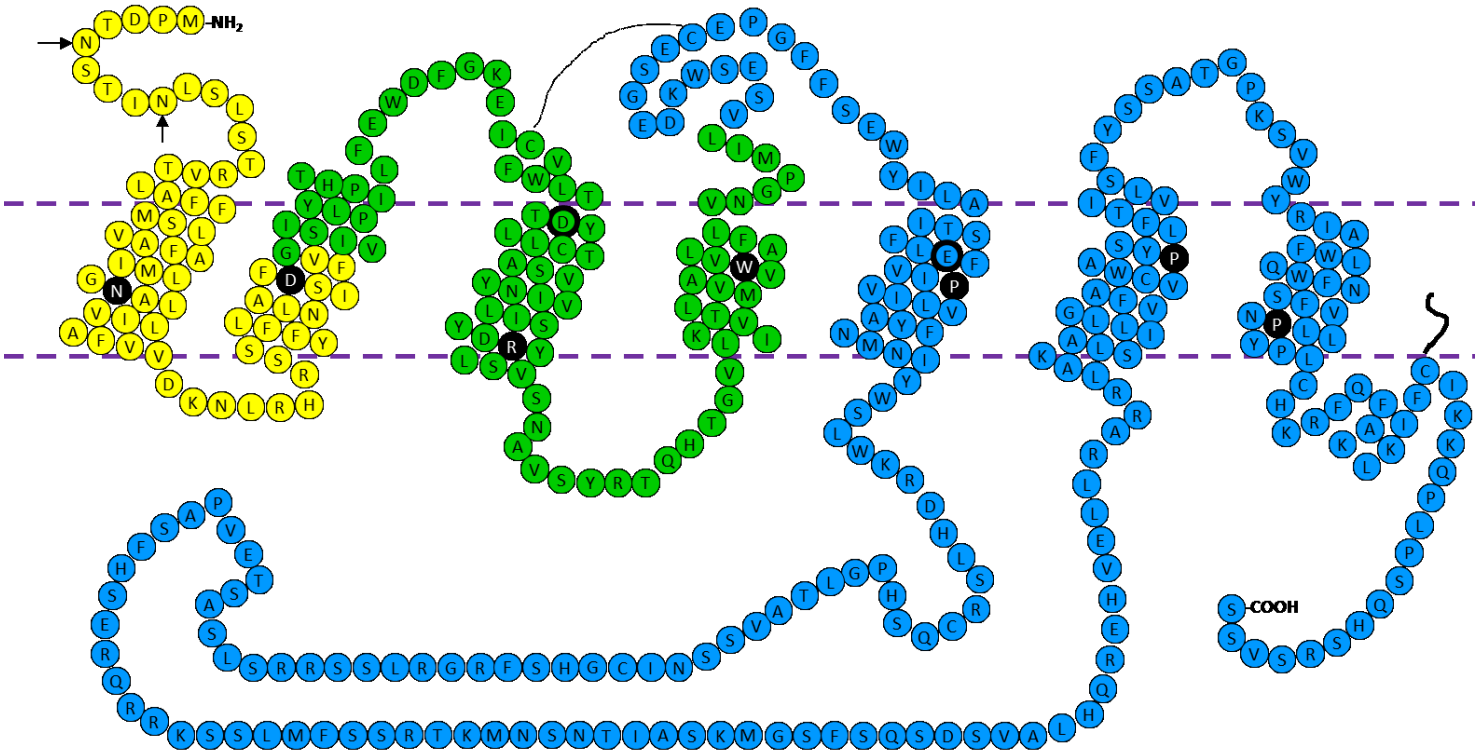


Figure 1.1| Snake plot of the human histamine H_4 receptor. The full length receptor is composed of 390 amino acids forming seven transmembrane helices, three extracellular and three intracellular loops and an extracellular N-terminal and an intracellular C-terminal tail. The grey line represents a disulphide bridge linking cysteines in the third transmembrane domain and second extracellular loop. Asp3.32 and Glu5.46 are marked with a bold border and play an important role in histamine binding. The H4R67 isoform conists of the first 67 N-terminal residues (marked in yellow), and the H4R302 isoform lacks the residues marked in green. The conserved residues in family A GPCRs are depicted as black circles. Two putative glycosylation sites (Asn5 and Asn9) are highlighted with arrows. A potential palmitoylation site (Cys374) at the C-terminal tail is suggested to be close to the membrane following membrane insertion of a putative attached palmitic acid. Adapted from (Leurs *et al.,* 2009).

forming a hydrogen bond with Trp316$^{6.48}$ allowing the latter residue to adopt an "active" rotamer conformation (Jongejan *et al.,* 2005; Jongejan *et al.,* 2008). Studies involving homology models of the H_3R and H_4R based on the crystal structure of the H_1R reveal all ligands share one preferable binding mode whereby the protonated –NH group of the ligand tightly interacts with Asp94$^{3.32}$ and the imidazole –NH group interacts with Glu182$^{5.46}$. The study also shows using molecular dynamics (MD) simulations that the H_4R bound with histamine shows strong conformational changes in TM3, TM5, TM6, and TM7 and also a conformational change of Tyr358$^{7.53}$. Conformational changes in TM7 are also an important characteristic of GPCR activation (Lebon, *et al.,* 2011; Xu *et al.,* 2011; Warne *et al.,* 2011; Standfuss *et al.,* 2011). Tyr358$^{7.53}$, which is part of the highly conserved NPxxY, has also previously been implicated in GPCR activation (Lebon, *et al.,* 2011; Xu *et al.,* 2011; Standfuss *et al.,* 2011). The H_4R has two potential glycosylation sites in its N-terminus (Asn5 and Asn9), and a Cys residue in helix VII which could potentially serve as a palmitoylation site (Leurs *et al.,* 2009). Also present, are two cysteine residues in TM3 and ECL2 which have the ability to form a disulphide bridge and thereby connecting and stabilising the third transmembrane domain and the second extracellular loop. These cysteine residues are known to be conserved among GPCRs and were shown in crystal structures reported previously (Palczewski 2000; Cherezov *et al.,* 2007; Warne *et al.,* 2008). This disulphide bridge is thought to confer structural constraint to extracellular loop 2 and could prove important in the involvement of ECL2 in histamine binding (Lim *et al.,* 2008). Detailed site-directed mutagenesis studies have also revealed the importance of Phe169, which is part of a Phe-Phe motif in ECL2 which when mutated into the corresponding residue in the mouse H_4R (Val171). This confers characteristics of agonist binding from the mouse orthologue to the human orthologue (Lim *et al.,* 2008), indicating a single amino acid is responsible for species differences observed with respect to agonist binding. It is suggested that the H_4R can exist in dimeric or oligomeric forms (van Rijn *et al.,* 2006; van Rijn *et al.,* 2008). Immunoblot analysis revealed the presence of three distinct species of the H_4R, with a low molecular weight species visible at 31 kDa likely representing the receptor monomer (van Rijn *et al.,* 2006; Leurs *et al.,* 2009). However, lower molecular weight species were not present in lymphocytes or brain membranes (van Rijn *et al.,* 2006; Connelly *et al.,* 2009). It is also important to note that the sizes of apparent dimeric structures of the H4R are not consistent with values ranging from 60-80 kDa in HEK 293 H_4R-transfected cells or in

other native tissue preparations such as lymphocytes (van Rijn *et al.,* 2006; Connelly *et al.,* 2009). Tissue-specific complements of H_4R isoforms or differential tissue-specific post-translational modifications such as glycosylation and palmitoylation could explain the observation of different molecular weights of the dimeric structures.

1.2.4. Downstream signalling of histamine receptors

Histamine receptors differ from each other with respect to their G-protein coupling profile, and therefore, activate different downstream signal transduction pathways. Upon ligand binding, the H_1R couples to $G\alpha_{q/11}$-proteins and activates phospholipase C (PLC) (Li *et al.,* 1995). Activated phospholipase C subsequently generates inositol-1, 4, 5-triphosphate (IP_3) and diacylglycerol (DAG) by hydrolysing a small pool of PtdIns(4, 5)P_2 (PIP_2) in the plasma membrane. IP_3 is able to mobilise calcium (Ca^{2+}) from internal Ca^{2+} stores by binding to the IP_3 receptor (IP_3R). Depletion of endoplasmic reticulum Ca^{2+} due to activation of the IP_3R leads to opening of store-operated calcium channels in the plasma membrane by interaction of the ER resident Ca^{2+} sensor, STIM with the calcium channel ORAI1. This results in calcium entry and refilling of intracellular stores (Smyth *et al.,* 2010). DAG is known to activate protein kinase C (PKC) (Hill *et al.,* 1997). Activation of the H_1R has also shown to potentiate forskolin-induced production of the second messenger cAMP (Leurs *et al.,* 1994). Currently there are two avenues by which this could occur; the first involves activation of specific AC isoforms by Ca^{2+}-activated calmodulin (Defer *et al.,* 2000), conversely it may involve Ca^{2+}-independent direct activation of ACs via the $G\beta\gamma$ subunits that disassociate from the $G\alpha$ subunits upon H_1R activation (Maruko *et al.,* 2005). The H_1R is also implicated in signalling via MAPKs. In bovine adrenal chromaffin cells, histamine was found to produce a H_1R-dependent increase in the phosphorylation levels of ERK1/2 (Cammarota *et al.,* 2003). Other studies also implicate the activated H_1R in MAPK signalling (Beermann *et al.,* 2014; Robinson and Dickenson 2001).

The H_2R interacts with both the $G\alpha_{q/11}$- and $G\alpha_s$-proteins, and therefore, couples to PLC and AC (Hill *et al.,* 1997). Consequently, histamine activation of the receptor initiates two biochemical pathways resulting in increases in intracellular cAMP or Ca^{2+}. The method by which $G\alpha_{q/11}$-proteins cause an increase in intracellular Ca^{2+} has been described above. The H_2R through its coupling to $G\alpha_s$-proteins stimulates AC causing an increase in intracellular cAMP levels due to breakdown of adenosine triphosphate

(ATP) (Haas *et al.,* 2008). Increases in intracellular cAMP levels lead to activation of protein kinase A (PKA) and the transcription factor CREB which are known to be key regulators of neuronal physiology and plasticity (Haas *et al.,* 2008). These biochemical pathways have been shown to be true in CHO cells expressing the canine H_2R (Fukushima *et al.,* 1996), in HEPA cells expressing the canine H_2R and primary canine parietal cells (Wang *et al.,* 1996), and in baculovirus-infected Sf9 insect cells expressing the H_2R (Kühn *et al.,* 1996). Heterologous expression of the H_2R in rat cardiomyocytes results in receptors interacting with both $G\alpha_{q/11}$- and $G\alpha_s$-proteins, in this system there is also evidence of increased cytosolic Ca^{2+} via phosphorylation of L-type Ca^{2+} channels by the $G\alpha_s$-cAMP-PKA pathway (Wellner-Kienitz *et al.,* 2003)

Studies involving the H_3R have shown the receptor mediates pertussis toxin-sensitive [^{35}S]-GTPγS binding in rat cerebral cortex membranes and in transfected cells (Clark and Hill 1996; Rouleau *et al.,* 2002; Bongers *et al.,* 2007). The human and rat H_3R expressed in Sf9 insect cells has been shown to couple equally well to $G_{i1,}$ $G_{i2,}$ $G_{i3,}$ and G_{o1} in studies measuring steady-state GTPase activity in H_3R/G-protein co-expression (Schnell *et al.,* 2010). Coupling of the receptor to $G\alpha_{i/o}$-proteins results in inhibition of AC activity, therefore negative coupling to cAMP accumulation as seen in SK-N-MC neuroblastoma cells recombinantly expressing the H_3R (Lovenberg *et al.,* 1999; Lovenberg *et al.,* 2000). The H_3R has also been implicated in the activation of MAPK pathways, COS-7 cells expressing the H_3R showed H_3R agonist-mediated MAPK phosphorylation which was pertussis toxin-sensitive (Drutel *et al.,* 2001). In native tissues, the H_3R has also been shown to mediate MAPK activation (Giovannini *et al.,* 2003), in rat hippocampal CA3 neurones H_3R-mediated MAPK activation is linked to memory improvement (Hill *et al.,* 1997). $G_{i/o}$-protein activation via the H_3R starts the cascade whereby activated phosphoinositide 3-kinase (PI3K) phosphorylates protein kinase B/Akt, which goes on to inhibit the action of glycogen synthase kinase 3-β, a tau kinase in the brain linked to diseases such as Alzheimer's (Bongers, *et al.,* 2007).

Like the structurally related H_3R, the H_4R couples to $G\alpha_{i/o}$-proteins, and inhibits forskolin-induced increases in cAMP (Oda *et al.,* 2000). The H_4R is also coupled to increases in intracellular Ca^{2+} through activation of PLC as shown in mast cells (Hofstra *et al.,* 2003). The best studied example of H_4R signalling in a native system is the human eosinophil. The H_4R mediates activities such as shape change, Ca^{2+} mobilisation and actin polymerisation that are processes which are sensitive to

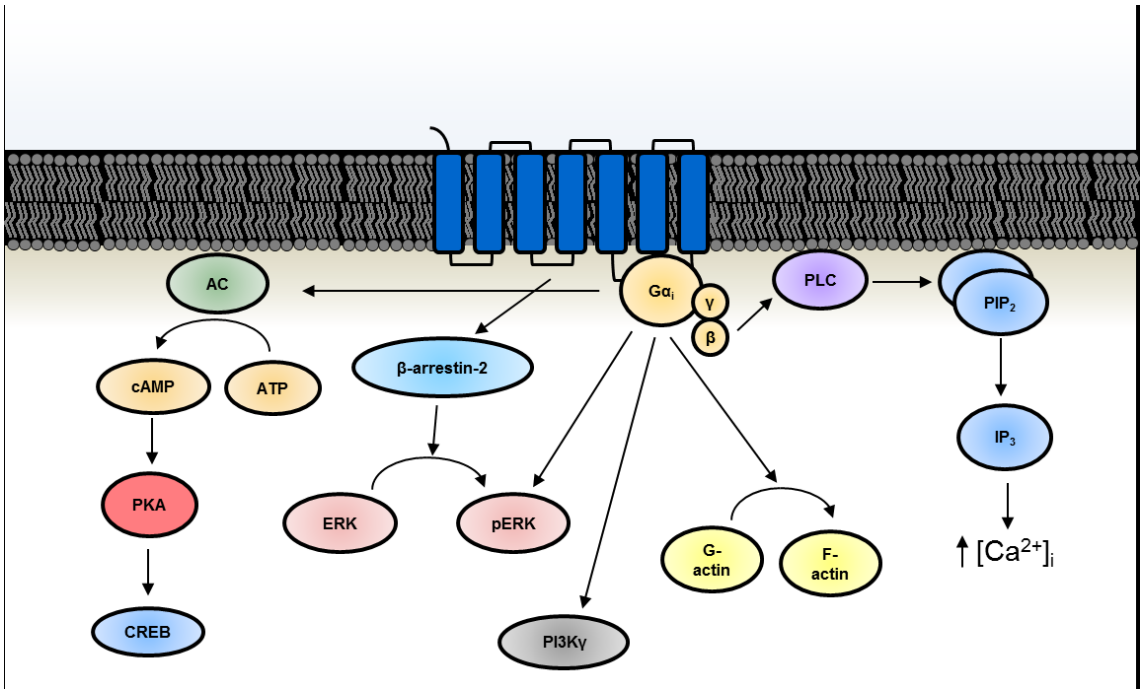


Figure 1.2| Overview of the signalling pathways for the histamine H_4 receptor in both endogenous and transfected cells. The histamine H_4R can signal constitutively, but can also be regulated by agonists, inverse agonists, and antagonists. The activated H_4R signals through $G\alpha_{i/o}$ proteins and/or β-arrestin and their downstream signalling pathways. Adapted from (Leurs *et al*., 2009).

pertussis toxin (Raible *et al.,* 1994). Mobilisation of Ca^{2+} in mouse bone marrow-derived mast cells is also sensitive to pertussis toxin treatment (Hofstra *et al.,* 2003). Pertussis toxin sensitivity only suggests the responses are mediated through the H_4R coupling to $G\alpha_i$- and/or $G\alpha_o$-proteins, however, myeloid cells express the $G\alpha_{i2}$ and $G\alpha_{i3}$-subunits but not $G\alpha_{i1}$-subunits or $G\alpha_o$-subunits (Birnbaumer 2007). This suggests that the H_4R predominantly couples to $G\alpha_{i2}$ and $G\alpha_{i3}$-subunits in these native systems. The conclusion that the H_4R is coupled to $G\alpha_{i/o}$-proteins is reinforced in transfected cell systems which show the H_4R mediated ligand-dependent inhibition of forskolin-induced cAMP accumulation (Nakamura *et al.,* 2000; Oda *et al.,* 2000; Liu *et al.,* 2001), is pertussis toxin sensitive (Oda *et al.,* 2000). Furthermore, detecting Ca^{2+} moblilisation in HEK293 cells mediated via a transfected H_4R requires the co-transfection of G15/16α subunits (Oda *et al.,* 2000), or a chimeric $G\alpha_q$ protein containing the last five C-terminal amino acids of $G\alpha_{i/o}$-proteins (Coward *et al.,* 1999; Panula *et al.,* 2015). In primary cells, the H_4R has been shown to mediate Ca^{2+} mobilisation. This has been shown primarily in cells of an immune lineage such as eosinophils (Raible *et al.,* 1994; Buckland *et al.,* 2003; Hofstra *et al.,* 2003), monocytes (Dijkstra *et al.,* 2007), and mast cells (Jemima *et al.,* 2014). Additionally, the H_4R has been shown to activate downstream kinases, for example, ERK (Rosethorne and Charlton 2011; Desai and Thurmond 2011; Karlstedt *et al.,* 2013), PI3K (and Akt) (Desai and Thurmond 2011; Ferreira *et al.,* 2012), and the transcription activating protein-1 (AP-1) (Gutzmer *et al.,* 2005; Gutzmer *et al.,* 2009; Mommert *et al.,* 2012).

1.2.5. Function of histamine receptors

H_1R knock-out (KO) mice exhibit immunological, metabolic, and behavioural state abnormalities such as the development of amygdaloid kindling (Hirai *et al.,* 2004), disrupted sleep/wake cycles (Huang *et al.,* 2006), disrupted control of energy metabolism, feeding rhythms and obesity (Masaki and Yoshimatsu 2006), and disrupted T-cell and antibody responses (Jutel *et al.,* 2001). These responses are similar to those observed in histidine decarboxylase (HDC)-KO mice (Parmentier *et al.,* 2002).

Activation of the H_2R causes an increase in the production of cAMP, protein kinase A and the transcription factor cAMP response element-binding protein; these proteins are known to be regulators of neuronal physiology and plasticity (Panula *et al.,* 2015). In the CNS, the H_2R can inhibit nerve cells upon activation (Haas and Bucher 1975).

Further, it can block long-lasting after-hyperpolarisation leading to a potentiation of excitation in rodent and human brains (Haas and Konnerth 1983; Haas and Panula 2003). H_2R KO mice have provided useful tools in delineating the full effects of the H_2R. These mice have shown deficits in long term potentiation (LTP) in the CA1 region of the hippocampus, and also showed impairment in studies which tested learning and memory processes where the frontal cortex, amygdala and hippocampus interact (Dai *et al.,* 2007). Knock out mice also displayed abnormalities in nociception, H_2R null mice caused antinociception and enhanced orexin A-induced antinociception in several assays including thermal, mechanical and chemical tests (Mobarakeh *et al.,* 2005). These mice also showed enhanced antinociceptive effects of morphine (Mobarakeh *et al.,* 2006). Th1 and Th2 cytokines, important in a host of immune responses, were upregulated in H_2R KO mice indicating the importance of the H_2R in regulating elements of the immune system (Jutel *et al.,* 2001; Teuscher *et al.,* 2004).

The H_3R was first identified as an autoreceptor and was found to be involved in the regulation of histamine synthesis and release in rat brain regions (Arrang *et al.,* 1983). H_3R KO mice display changes in phenotype which is associated with disturbed functions of the CNS. These mice displayed changes in behavioral states and reduced locomotion during dark phases, however, they maintained a normal circadian rhythm suggesting a role for histamine and the H_3R in arousal (Toyota *et al.,* 2002; Schneider *et al.,* 2014). H_3R KO mice have also implicated the receptor in negatively regulating food intake in rodents, reduced adiposity, and ameliorated hyperinsulinemia and hyperleptinemia (Toftegaard *et al.,* 2003; Yoshimoto *et al.,* 2006).

The function of the H_4R which was first described in detail was in mediating chemotaxis. This has been described in several immune cell types including mast cells which were shown to undergo chemotaxis upon histamine treatment, a phenotype which was abrogated in the presence of H_4R antagonists and in mast cells derived from H_4R KO mice (Hofstra *et al.,* 2003; Thurmond *et al.,* 2004). The H_4R expressed in eosinophils was shown to mediate chemotaxis, as well as cell shape change, actin polymerisation and induced adhesion molecule upregulation (Buckland *et al.,* 2003; Ling *et al.,* 2004). Other cells known to undergo H_4R-mediated chemotaxis/migration include dendritic cells which were seen undergoing H_4R-mediated migration *in vivo* in mice during an allergic response (Cowden *et al.,* 2010), Langerhans cells which were shown to undergo H_4R-mediated migration in an *ex vivo* assay (Gschwandtner *et al.,*

2010), and fibroblasts (Kohyama *et al.,* 2010). The H_4R has also been shown to be important in the release of cytokines from various cell types. It has long been known that histamine evokes IL-16 release from CD8+ T cells (Gantner *et al.,* 2002), a process which can be blocked by H_2R and H_4R antagonists. In human monocyte-derived dendritic cells and inflammatory dendritic epidermal cells, H_4R activation blocked IL-12p70 release (Gutzmer *et al.,* 2005; Dijkstra *et al.,* 2008), an important interleukin involved in the cytotoxic effects of CD8+ T cells. CCL2, a chemokine involved in recruitment of cells to sites of inflammation, was down-regulated in human monocytes, Langerhans cells, and inflammatory dendritic epidermal cells (Dijkstra *et al.,* 2007; Dijkstra *et al.,* 2008; Gschwandtner *et al.,* 2011; Gschwandtner *et al.,* 2012). Chemokine and cytokine production *in vivo* has been shown to be abrogated by the H_4R in a host of disease models. Th2 cytokines (IL-4, IL-5, and IL-13) have all been shown to be reduced in mouse models of asthma, allergic rhinitis, and allergic contact dermatitis upon H_4R antagonist treatment (Takahashi *et al.,* 2009; Cowden *et al.,* 2010; Seike *et al.,* 2010). A rat colitis model which was treated with H_4R antagonists including JNJ7777120 showed decreased levels of tissue TNFα (Varga *et al.,* 2005). Lipid mediators such as leukotriene B_4 and prostaglandin D_2, in models of pleurisy and peritonitis, were shown to be reduced when they were treated with H_4R antagonists (Takeshita *et al.,* 2003; Strakhova *et al.,* 2009). IL-17 is important in many immune regulatory functions with the most notable being the induction and mediation of pro-inflammatory responses. In Th17 cells, histamine and a selective H_4R agonist (4-methylhistamine) were shown to increase IL-17 secretion (Mommert *et al.,* 2012). Stimulation of whole blood with anti-CD3/CD28 and IL-23 in mice was seen to increase IL-17 production *in vitro*, however, H_4R KO mice and wild-type mice treated with H_4R antagonists showed a reduced IL-17 response (Cowden *et al.,* 2014). This reduction in IL-17 has also been seen in studies of animal models of asthma, dermatitis, and arthritis (Dunford *et al.,* 2006; Cowden *et al.,* 2010; Cowden *et al.,* 2014). Studies involving human and murine mast cells have also revealed histamine acting through the H_4R induces IL-6 production and increases lipopolysaccharide (LPS)-induced IL-6 production via ERK and PI3K activation (Desai and Thurmond 2011), degranulation and production of leukotrienes which can be blocked with the H_4R antagonist JNJ7777120 (Jemima *et al.,* 2014). Beyond immune responses, the H_4R has been implicated in neuronal responses but this remains controversial (Schneider *et al.,* 2014). A study investigating noradrenaline release and [^{35}S]-GTPγS binding in human, mouse

and guinea pig cortex showed that a H_4R antagonist failed to affect [^{35}S]-GTPγS binding and noradrenaline release whereas a H_3R antagonist increased binding and inhibited noradrenaline release (Feliszek *et al.,* 2015). Other studies have reported H_4R expression on neurones and studies have shown H_4R-induced hyperpolarisation in recordings from layer IV somatosensory cortex cells from mice using a H_4R specific agonist and antagonist (Connelly *et al.,* 2009). In a sub-set of skin-specific sensory neurones, Ca^{2+} increases were shown to be H_4R mediated (Panula *et al.,* 2015). Dual activation of H_1R and H_4R on sensory neurones which lead to excitation of histamine sensitive afferents have been postulated to be the basis of the sensation of itch (Rossbach *et al.,* 2011) and may underlie the nociceptive and antipruritic activities seen in animal models (Panula *et al.,* 2015). Immune cells of the brain, microglia, have recently been shown to express all four histamine receptors (Ferreira *et al.,* 2012). Microglia underwent migration and inhibited LPS-induced IL-1β production via the H_4R (Ferreira *et al.,* 2012), in another study histamine acting through the H_1R and H_4R mediated TNFα and IL-6 production (Dong *et al.,* 2014). Other functions where H_4R has been implicated include increased COX-2 expression, cell proliferation, and vascular endothelial growth factor (VEGF) production in COX-2 positive colon cancer cell lines HT29 and Caco-2 (Cianchi *et al.,* 2005). The H_4R has also been shown to play a key role in histamine-mediated biological processes such as cell proliferation and apoptosis in breast cancer (Medina *et al.,* 2011; Martinel Lamas *et al.,* 2015).

1.3. Free fatty acid receptors

Fatty acids (FAs) are nutritional components and metabolic intermediates involved in a wide range of cellular functions. FAs are carboxylic acids with an unbranched aliphatic tail and are categorised based on chain length and position of C=C double bonds (Dranse *et al.,* 2013). Long-chain FAs (LCFAs) contain more than 12 carbon atoms, medium-chain FAs (MCFAs) contain 6-12 carbon atoms and short-chain FAs (SCFAs) contain less than 6 carbon atoms (Ratnayake and Galli 2009; Tvrzicka *et al.,* 2011). LCFAs and MCFAs are usually derived from dietary sources but can come from adipose recycling and hepatic turnover of phospholipids, neutral fats and cholesterol esters (Tvrzicka *et al.,* 2011). SCFAs, on the other hand, are generated by anaerobic gut bacteria which ferment indigestible dietary carbohydrates and polysaccharides (Macfarlane and Macfarlane 2011). FAs have recently been shown to bind a group of GPCRs, the free FA 1-4 (FFA1-4) receptor family (Stoddart *et al.,* 2008; Davenport *et*

al., 2013). GPR84 which is still classified as an orphan receptor but is now considered to be the putative GPCR for medium chain free fatty acids (C9-C14), but is not activated by short or long chain fatty acids (Wang *et al.,* 2006). FFA1, FFA2, and FFA3 (previously known as GPR40, GPR43, and GPR41, respectively) were first identified as a group of closely related putative GPCRs encoded on chromosome 19q13.1 (Sawzdargo *et al.,* 1997). Using high-throughput screening methods these receptors were deorphanised as receptors for FFAs (Briscoe *et al.,* 2003; Brown *et al.,* 2003; Itoh *et al.,* 2003; Nilsson *et al.,* 2003). GPR120 does not share homology with the other members of the FFA receptor family but through the use of high-throughput screening was shown to bind and become activated by long chain FFAs and so was reclassified as FFA4 (Davenport *et al.,* 2013).

Two splice variants of the FFA4 receptor have been reported. These differ by the presence or absence of 16 amino acids in the third intracellular loop (Moore *et al.,* 2009), with the long isoform of the receptor only found in humans. The short isoform has been shown to couple to both G-protein-dependent and β-arrestin pathways, whereas the longer isoform only couples to the latter (Watson *et al.,* 2012). Currently, the expression profile of the longer isoform of FFA4 has been shown to only be expressed in the colon (Galindo *et al.,* 2012).

1.3.1. Localisation and physiological function of the free fatty acid receptors

The FFA1 receptor plays an important physiological role in the pancreas. FFAs have been shown to induce acute and chronic effects on the pancreas with some of these effects mediated by the FFA1 receptor and associated with glucose-stimulated insulin secretion (GSIS) (acute) and β-cell dysfunction (chronic) (Mancini and Poitout 2013). Silencing the FFA1 receptor via small interfering RNA (Itoh *et al.,* 2003; Shapiro *et al.,* 2005), antisense oligonucleotides (Salehi *et al.,* 2005), pharmacological inhibition (Briscoe *et al.,* 2006) or with the use of FFA1 KO mice (Steneberg *et al.,* 2005; Latour *et al.,* 2007; Lan *et al.,* 2008; Kebede *et al.,* 2008), results in a decrease in FFA potentiation of GSIS. Overexpression of the FFA1 receptor in normal and diabetic mice prevented hyperglycemia and improved glucose tolerance (Nagasumi *et al.,* 2009). The chronic effects of FFAs via FFA1 are more controversial. Conflicting data exists which suggests either FFA1 is involved in glucolipotoxicity leading to β-cell dysfunction and

demise (Steneberg *et al.,* 2005). Conversely, other studies have suggested FFA-induced hyperinsulinemia represents a process whereby the β-cell tries to compensate for insulin resistance with this process being compromised by FFA1 absence (Alquier and Poitout 2009; Mancini and Poitout 2013). Pancreatic α-cells are found within the islet and produce glucagon, a hormone which counteracts the effects of insulin and promotes liver glycogenolysis and gluconeogenesis. FFA1 has been shown to co-localise with glucagon in α-cells (Flodgren *et al.,* 2007), overexpression of FFA1 in a glucagon-secreting cell line promotes FFA-mediated glucagon production. In line with this, FFA1 knockdown in mouse islets caused a reduction in glucagon secretion in response to FFAs. This process requires PLC-mediated Ca^{2+} influx (Wang *et al.,* 2011). FFA1 KO mice also secrete lower amounts of glucagon compared to wild-type mice in response to increased circulating FFA levels (Lan *et al.,* 2008). However, it must be noted that these results conflict with other studies which do not detect FFA1 mRNA (Itoh *et al.,* 2003) or protein (Hirasawa *et al.,* 2008) co-localisation with glucagon containing cells in islets. There is also a lack of modulation of glucagon secretion by the FFA1-specific agonist TAK-875 in isolated rat and human islets (Yashiro *et al.,* 2012). The FFA1 has also been shown to be expressed in intestinal enteroendocrine cells and possibly glucagon-producing cells involved in glucose homeostasis (Latour *et al.,* 2007; Liou *et al.,* 2011; Edfalk *et al.,* 2008; Hara *et al.,* 2014a). Intestinal L and K cells which produce incretin hormones such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) (Edfalk *et al.,* 2008) also express the FFA1 receptor. It appears, therefore, that long chain FFAs may mediate insulin secretion both directly via stimulation of FFA1 expressed in β-cells and indirectly through release of incretins from L and K cells in the lumen of the large intestine, the production of which is mediated in part by stimulation of FFA1 (Hara *et al.,* 2014a).

The evidence currently available suggests the FFA2 plays a role in energy metabolism (Sleeth *et al.,* 2010), RT-PCR analysis has revealed FFA2 expression in white adipose tissues (WATs) and gastrointestinal tissues (Hong *et al.,* 2005). The intestines are involved in the secretion of gut hormones, appetite control, and absorption of nutrients (Batterham *et al.,* 2002; Davis *et al.,* 1998; Koda *et al.,* 2005). Enteroendocrine L-cells of the intestine secrete peptide YY (PYY) a hormone released by the ileum in response to feeding, and the proglucagon gene and thus produce both GLP-1 and GLP-2, immunohistochemical studies have shown the FFA2 to co-localise with these cells

(Karaki *et al.,* 2006). In mixed cultures derived from colon, SCFAs acting through FFA2 mediate GLP-1 secretion *in vitro* (Tolhurst *et al.,* 2012). qRT-PCR studies have shown FFA2 and FFA3 expression in GLP-1 secreting L-cells, here they cause elevated Ca^{2+} levels through $G\alpha_q$ signalling (Tolhurst *et al.,* 2012). FFA2 expression in WATs of mice fed on a high-fat diet is significantly upregulated compared to mice fed on normal chow (Hong *et al.,* 2005). In 3T3-L1 cells, SCFA treatment causes increases in FFA2 and the peroxisome proliferator-activated receptor-γ2 (PPARγ2) (a marker of mature adipocytes) mRNA expression. RNA interference to decrease FFA2 receptor expression inhibits adipogenesis in 3T3-L1 cells (Hong *et al.,* 2005). During feeding, SCFAs are produced by gut microbial fermentation of indigestible fibre and sugars. Studies which compare FFA2 KO mice with those over-expressing the adipose-specific FFA2 showed acetate strongly inhibits fat accumulation via insulin signalling suppression in WATs (Kimura *et al.,* 2013). This process was independent of cAMP inhibition in the cells; further work implicated the Gβγ subunits of the $G\alpha_{i/o}$ protein. FFA2 has shown to be involved in the regulation of immune responses upon SCFA stimulation (Maslowski *et al.,* 2009), with expression of FFA2 seen in neutrophils and eosinophils. Mice with a lack of gut microbiota and therefore a lack of SCFAs showed impairments in inflammatory responses, with increased recruitment of immune cells and inflammatory mediators in FFA2 KO animals with inflammatory conditions (Maslowski *et al.,* 2009). A dextran sulfate sodium treatment-induced model of colitis in FFA2 KO mice exhibited a potent inflammatory response which was absent in wild-type mice. This response was diminished upon acetate treatment in the wild-type mice but not in FFA2 KO mice (Masui *et al.,* 2013). In another inflammatory model, leukocyte migration to the intestine and cytokine release from these cells was reduced in FFA2 KO mice compared to wild-type mice and completely abolished in mice lacking any gut microbiota (Sina *et al.,* 2009).

The FFA3 receptor is known to bind SCFAs and its localisation in several cell types of cells in the intestine has been confirmed using western blotting and mRNA studies (Tazoe *et al.,* 2008; Tazoe *et al.,* 2009; Samuel *et al.,* 2008). Primary cell cultures derived from FFA3 KO mice show decreased levels of gut hormones such as PYY and GLP-1 (Samuel *et al.,* 2008). Of note, is the difference in phenotypes between male and female FFA3 KO mice. Male KO mice fed on a high fat diet showed a lower rate of energy expenditure and a lean body mass compared to females. Also, compared to

females, body fat mass and plasma leptin levels were elevated in males. These differences can be explained by the effect of sex hormones on metabolic regulation in the CNS and PNS and adipocyte tissue distribution (Bellahcene *et al.,* 2013), also gut-derived SCFAs could raise energy expenditure and aid in protecting against obesity by activating FFA3. Levels of IL-6 and neutrophil recruitment and infiltration in the colon was reduced in FFA3 KO mice, as was the frequencies of Th1 and Th7 cells (Kim *et al.,* 2013), indicating the FFA3 is important in the regulation of the immune system. FFA3 mRNA is abundantly expressed in the sympathetic ganglia (Kimura *et al.,* 2011), furthermore, FFA3 KO mice show a decrease in tyrosine hydroxylase protein and sympathetic nerve innervation suggesting a role for FFA3 in sympathetic nerve growth (Kimura *et al.,* 2011). Propranolol treatment has the effect of blocking propionate-induced elevations of heart rate and oxygen consumption, with *in vitro* studies demonstrating the FFA3 signalling mechanism occurs through the Gβγ–PLC β-3–ERK1/2 pathway (Kimura *et al.,* 2011). This results in synapsin 2-β phosphorylation indicating propionate-induced FFA3 activation promotes noradrenaline release from sympathetic neurones and the regulation of energy expenditure. But, it is interesting to note that the FFA3 suppresses energy expenditure in fasted states through the production of a ketone body (β-hydroxybutyrate) in the liver when food deprivation occurs. Through FFA3, this ketone body inhibits cAMP production and ERK1/2 phosphorylation, a process confirmed in sympathetic neurones and *in vivo* studies using mice (Kimura *et al.,* 2011; Inoue *et al.,* 2012).

The FFA4 receptor expression has been detected in adipocytes, human pancreatic islets, immune cells (particularly macrophages), lung, and enteroendocrine cells (Hirasawa *et al.,* 2005; Gotoh *et al.,* 2007; Tanaka *et al.,* 2008; Miyauchi *et al.,* 2009; Oh *et al.,* 2010; Taneera *et al.,* 2012). FFA4 gene expression has been shown to be upregulated in adipocyte tissue following a high fat diet, with FFA4 activation resulting in increased adipogenesis and glucose uptake (Cornall *et al.,* 2014; Hudson, *et al.,* 2013; Oh *et al.,* 2010). FFA4 KO mice fed a high fat diet have been shown to gain more fat mass than wild-type mice suggesting the FFA4 is protective against diet-induced obesity (Ichimura *et al.,* 2012). FFA4 KO mice also exhibit increased fasting glucose and impaired insulin responses in glucose tolerance testing (Oh *et al.,* 2010; Ichimura *et al.,* 2012). Phenotypes like increased insulin secretion, satiety, and improved glycemic control have been partially attributed to FFA4 mediated incretin release from

enteroendocrine cells (Offermanns, 2014). The pancreatic islets of diabetic and hyperglycemic individuals exhibit decreased FFA4 mRNA levels and the protective effects of a SCFA against induced cell apoptosis disappear in this scenario (Taneera *et al.,* 2012). FFA4 expression has also been reported in α-cells of the pancreas where it plays a role in the regulation of glucagon secretion (Suckow *et al.,* 2014). Recently, the FFA4 mediated systemic insulin sensitivity has been linked with FFA4-mediated anti-inflammatory effects on macrophages (Oh *et al.,* 2010). This study demonstrates elevated FFA4 expression in macrophages responding to obesity, but FFA4 activation leads to a decrease in pro-inflammatory gene expression in M1 macrophages and increased expression of M2 anti-inflammatory genes. There was also reduced macrophage infiltration of adipose tissues in FFA4 KO mice due to decreased chemotaxis (Oh *et al.,* 2010). It has been suggested that FFA4 acts through β-arrestin with TAB1 to inhibit LPS- and TNF-induced TAK1 stimulation, thereby attenuating toll-like receptor 4 (TLR4) and TNF-alpha inflammatory pathways (Oh *et al.,* 2010; Halder *et al.,* 2013; Li *et al.,* 2013).

1.3.2. Structural architecture of free fatty acid receptors

The FFA1 receptor is a typical family A GPCR; recently the crystal structure of the receptor bound with TAK875, an ago-allosteric modulator, has been solved (Srivastava *et al.,* 2014). This is the first FFA receptor to be crystallised and provides clues as to potential binding sites and important residues. TAK875 is seen to bind between transmembrane helices 3-5 with the ECL2 region providing the roof of the TAK875 binding cavity. Glu172 of the ECL2 forms a hydrogen bind with Arg258$^{7.35}$. This possibly represents an allosteric binding pocket in the receptor. There is evidence of a hairpin loop motif in the ECL2 and disulphide bond between TM3 (Cys79$^{3.25}$) and the carboxy portion of the ECL2 (Cys170). A β-sheet exists between the ECL2 hairpin and helices 4 and 5, which creates an inflexible region between Leu171 and Asp175 which forms a cap over the canonical binding site of the receptor. TAK-875 interacts with Arg183$^{5.39}$ and Arg258$^{7.35}$ as has been proposed by modelling studies (Sum *et al.,* 2007; Sum *et al.,* 2009), additionally two tyrosine residues (Tyr91$^{3.37}$ and Tyr240$^{6.51}$) are also implicated in ligand binding which have not been previously reported. Site-directed mutagenesis of key binding pocket residues Tyr91$^{3.37}$Ala, Arg183$^{5.39}$Ala, Asn244$^{6.55}$Ala and Arg258$^{7.35}$Ala has detrimental effects on the binding affinity of TAK-875. Point mutations of Arg183$^{5.39}$Ala, Arg258$^{7.35}$Ala, Tyr91$^{3.37}$Ala and Asn244$^{6.55}$Ala show

differential effects on the potencies of TAK-875 (a partial agonist) and γ-linoleic acid (full agonist), this coupled with the knowledge that Ca^{2+} flux assays point to a synergistic effects between TAK-875 and γ-linoleic acid and show positive cooperativity between the ligands suggests FFA1 possesses more than one ligand binding site (Srivastava *et al.,* 2014). Visual inspection of the receptor reveals the possibility of two extra binding pockets. One of these pockets is adjacent to TAK-875 and would allow a ligand to pass between TM4 and TM5; a conserved $Pro^{4.60}$ is absent replaced instead with a $Gly139^{4.58}$ near the equivalent position which may impart some flexibility to TM4. The third binding pocket is located at the more traditional GPCR orthosteric site between TM1 and TM7. It is interesting to note that it is currently unclear whether all three sites can be occupied at the same time and whether this would amplify the signal from the receptor (Srivastava *et al.,* 2014).

Although no crystal structure exists for the FFA4 receptor as of yet, a combination of molecular modelling, receptor mutagenesis and ligand SAR studies have been used to define important residues involved in ligand binding at the FFA4 receptor. The FFA4 receptor contains a putative glycosylation site (Asn21) in its N-terminal, and a conserved disulphide bridge connecting TM3 with ECL2. Two cysteine residues in the C-terminal tail of the receptor represent two potential palmitoylation sites. Previous work with the FFA1-3 receptors presents evidence of a pair of Arg residues near the top of the TM bundle (positions 5.39 and 7.35 according to Ballesteros and Weinstein numbering scheme) which have been shown to form ionic interactions with the carboxylate of endogenous fatty acids and synthetic ligands (Tikhonova *et al.,* 2007; Stoddart *et al.,* 2008). These residues are not conserved in the FFA4 receptor, however, several studies have implicated a single $Arg99^{2.64}$ as the critical residue interacting with carboxylate of ligands in the FFA4 receptor (Hudson *et al.,* 2013; Sun *et al.,* 2010; Watson *et al.,* 2012). A recent homology model using the FFA4-specific agonist TUG-891 revealed the presence of a hydrophobic ligand binding pocket at the receptor composed of $F115^{3.29}$, $F211^{5.42}$, $W277^{6.48}$, and $F304^{7.36}$, which when mutated to Ala residues eliminated responsiveness of TUG-891 at the FFA4 receptor as measured by a BRET based β-arrestin-2 assay (Hudson *et al.,* 2014). This study also used other, less potent agonists at the FFA4 receptor including the endogenous ligand α-linolenic acid (aLA). Interestingly, it was found the more potent the ligand, the more it was affected by logical mutations of the FFA4 receptor. This is consistent with the notion that more

potent ligands form more contacts with the receptor and is in line with previously reported observations of GW9608 compared with aLA at the FFA1 receptor (Sum *et al.,* 2007).

1.3.3. Downstream signalling of free fatty acid receptors

The primary signal transduction mechanism for the FFA1 receptor involves the receptor coupling to $G\alpha_{q/11}$ proteins (Itoh *et al.,* 2003) which go on to catalyse PLC-mediated hydrolysis of PIP_2 to IP_3 and DAG. IP_3 goes on to mobilise intracellular Ca^{2+} stores while DAG is implicated in PKC activation. This system is involved in glucose stimulated insulin secretion when the FFA1 receptor is found on pancreatic β-cells. Inhibition of this receptor, or of downstream PLC blocks potentiation of FFA-mediated GSIS *in vitro* (Shapiro *et al.,* 2005), with IP_3 generation in islets in response to FFAs demonstrated as being FFA1-dependent (Alquier *et al.,* 2009). It is important to note that insulin secretion from the pancreas is complex and FFA1-mediated elevations in intracellular Ca^{2+} comprise only a small role in the control of intracellular Ca^{2+} and subsequent insulin release (Mancini and Poitout 2013). Recently, the FFA1 receptor has also been implicated in the second phase of GSIS. This process is downstream of DAG and involves the activation of protein kinase D1 (PKD1) causing F-actin remodelling (Ferdaoussi *et al.,* 2012).

The FFA2 receptor was first reported to respond to FAs when FFA2 in transfected cells caused responses in Ca^{2+} assays upon acetate treatment (Brown *et al.,* 2003). With regards to G-protein coupling, the FFA2 receptor binds both $G\alpha_{q/11}$ as well as $G\alpha_{i/o}$ proteins (Le Poul *et al.,* 2003). The expression of the FFA2 receptor on immune cells and its ability to cause an increase in intracellular Ca^{2+} is thought to be important in the processes leading up to chemotaxis of these cells (Le Poul *et al.,* 2003). Signalling through the Gβγ subunit of the $G\alpha_{i/o}$ protein but not the $G\alpha_{q/11}$ proteins has also been implicated in the FFA2-mediated suppression of insulin signalling in adipocytes (Hara *et al.,* 2014b). The FFA3 receptor has been shown to activate the Ca^{2+} response in transfected CHO cells (Xiong *et al.,* 2004), as well as the phosphorylation of ERK1/2 and inhibition of cAMP production in a pertussis toxin-sensitive manner. This evidence suggests the FFA3 receptor is coupled to $G\alpha_{i/o}$ proteins.

Although the FFA1 and FFA4 receptors only share 10% homology (Hara *et al.,* 2014b), they share similar ligand profiles and both respond to medium- to long-chain fatty acids.

FFAs induced an increase in intracellular Ca^{2+} in HEK293 cells overexpressing the FFA4 receptor but failed to promote cAMP production (Hirasawa *et al.,* 2005; Hara *et al.,* 2009). FFAs acting at the FFA4 receptor caused the survival of serum-starved murine enteroendocrine STC-1 cells (Katsuma *et al.,* 2005a). This has been proposed to occur via two independent methods. The first includes a pathway mediated by PLC and ERK. The second involves a pathway mediated by PI3K. Akt is known to be a major downstream target of PI3K and FFA treatment of cells caused an activation of Akt which was blocked using PI3K inhibitors (Katsuma *et al.,* 2005a). As Akt plays a role in cell survival and/or proliferation, one can assume that the PI3K-mediated survival pathway is involved in Akt activation. The FFA4 receptor exists as a splice variant and is termed either long or short based on whether the receptor has an insertion of 16 amino acids in the third intracellular loop, these variants are not found in lower primates and rodents (Moore *et al.,* 2009). In studies comparing the short and long isoforms of the receptor in the same defined cell system (HEK 293 cells), it was demonstrated that the long isoform fails to activate G-proteins, but the ability to recruit arrestin and downstream internalisation and trafficking to lysosomal compartments remains intact. This finding has relevance for future studies as we now know arrestin plays a role in attenuating inflammatory pathways in immune cells (Oh *et al.,* 2010).

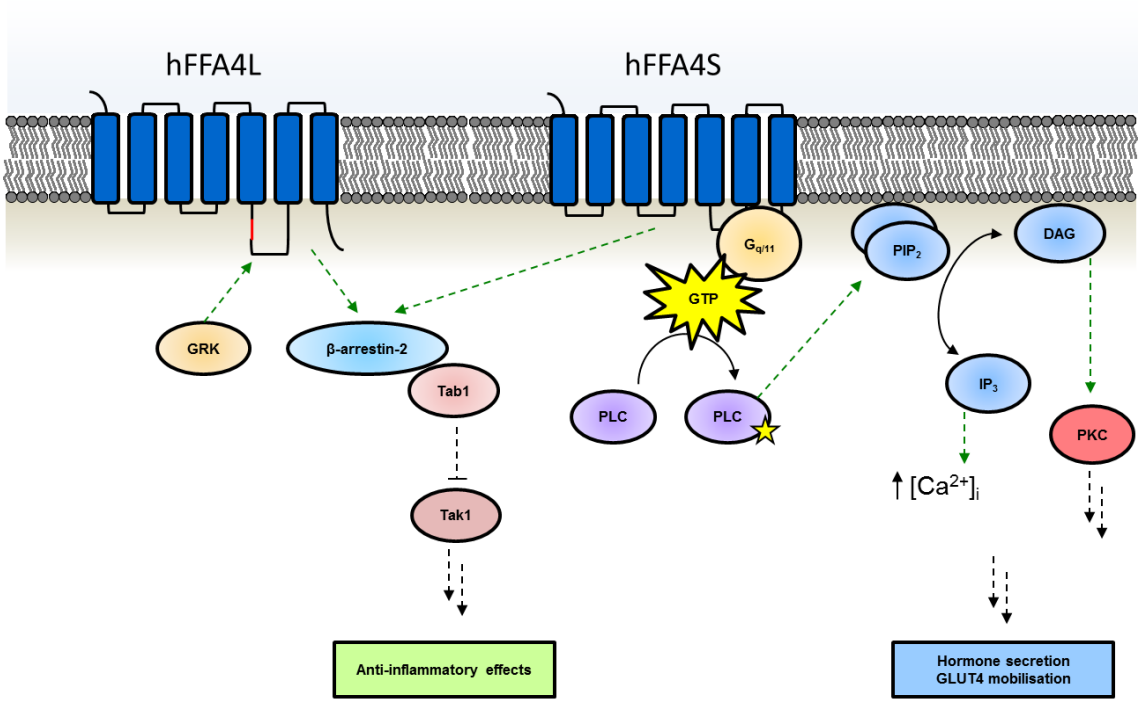


Figure 1.3| Overview of the signalling pathways for the human free fatty acid 4 receptor. The human free fatty acid 4 (FFA4) receptor exists in a long and short isoform (hFFA4L and hFFA4S, respectively). The long isoform contains an additional 16 amino acids in its third intracellular loop which uncouples the receptor from G-protein signalling, whereas both isoforms can interact with β-arrestin-2. Also shown are the downstream effectors of FFA4 signalling. Adapted from (Ulven and Christiansen 2015)

1.4. Thesis aims

Upon agonist stimulation, GPCRs undergo phosphorylation in a process resulting in the uncoupling of the receptor from its G-protein and the association of arrestins leading to desensitisation (Premont and Gainetdinov 2007; Ferguson, 2001). While there exists studies which investigate the phosphorylation of the H_1R and H_2R, studies into the phosphorylation and regulation of the H_4R are still lacking. Therefore, the initial aims of this thesis were to develop a cell line expressing the recombinant H_4R. Following the confirmation of the receptor, experiments were designed to look at the global, peptide, and individual amino acid level of the H_4R to investigate the phosphorylation profile of the *human* H_4R. Experiments were designed to compare the effects of the endogenous, full agonist, histamine, to the effects of a β-arrestin biased ligand, JNJ7777120 at the H_4R.

Like the H_4R, the FFA4 receptor is another family A GPCR which undergoes phosphorylation upon agonist stimulation (Butcher *et al.,* 2014). However, unlike the H_4R, the FFA4 is known to only be phosphorylated on 5 residues in its C-terminal tail and are responsible for the robust coupling of the receptor to arrestin-3 recruitment. The initial aims of Chapter 4 were to recapitulate these results. We then wanted to investigate the role the phosphorylation sites and by extension, arrestins played in the downstream signalling of the FFA4. Furthermore, I have used a novel $G\alpha_q$-protein inhibitor to investigate the role $G\alpha_q$-proteins play in the downstream signalling pathways.

In Chapter 5, I have attempted to utilise charge mutations at the 5 phosphorylation sites in the C-terminal tail of the FFA4 to further investigate the role phosphorylation plays in arrestin recruitment to the receptor. We also aim to explore the effects these mutations have in the kinetics of arrestin-3 recruitment to the receptor and internalisation of the receptor.

Finally, in Chapter 6, I have attempted to create a stable cell line expressing a recombinant *human* H_4R. To this end, I have tried to express the receptor in different cell types, and use different epitope tags in an effort to measure stable receptor expression. Finally, I aimed to create receptor chimeras using portions of the FFA4 receptor to try and increase stable expression of the H_4R and rescue arrestin-3 recruitment to the receptor.

Chapter 2 | Materials and Methods

2.1. Materials

2.1.1. Standard reagents, chemicals and consumables

General laboratory chemical and reagents were purchased from either Fisher Scientific (Loughborough, U.K.) or Sigma Aldrich (Poole, U.K.). Water used in the preparation of solutions was obtained from the ELGA Filtration System (ELGA Labwater, Marlow, U.K.). Water used for bacterial cell culture and molecular biology was sterilised by filtration and autoclaved at 121°C for 20 min. Mammalian cell culture reagents such as various cell culture media, phosphate buffered saline (PBS), Hanks' balanced salt solution (HBSS), foetal bovine calf serum (FBS), penicillin/streptomycin solution, hygromycin B and geneticin (G418) were purchased from Invitrogen (Paisley, U.K.). Glass coverslips and cell culture plastic consumables were purchased from VWR International (Lutterworth, U.K.). Agarose powder was purchased from Roche Diagnostics GmbH (Mannheim, Germany). RNase-, DNase-, pyrogens- and PCR (polymerase chain reaction) inhibitor-free microcentrifuge tubes (0.2 or 1.5 mL) were supplied by Scientific Laboratory Supplies Ltd (Nottingham, U.K.).

2.1.2. Specific reagents and assay kits

Complete EDTA-free protease inhibitors, PhosStop phosphatase inhibitor tablets, T4 DNA ligase, dNTPs for PCR (polymerase chain reaction), mini plasmid isolation kit, (Burgess Hill, U.K.). PfuUltra DNA polymerase was provided by Agilent Technologies LDA (Cheshire, U.K.). Plasmid Maxi Kits were from Qiagen (Venlo, Limburg, Netherlands). Nucleospin® Gel and PCR Clean-up kits were from Macherey-Nagal (Düren, Germany). Restriction enzymes and DNA ladders (1kb plus, 100bp-12kp range) were from New England Biolabs (Hitchin, U.K.). In-Fusion HD Cloning kit was purchased from Clontech (Saint-Germain-en-Laye, France). Lipofectamine 2000 tranfection reagent was provided by Invitrogen (Paisley, U.K.). SDS-PAGE equipment and reagents, Bradford reagent for protein quantification and pre-stained protein molecular size markers for immunoblotting studies were provided by Bio-Rad (Hemel Hempstead, U.K.). Phospho-ERK1/2 (Thr202/Tyr204) and Phospho-AKT (Ser473) HTRF kits were purchased from Cisbio (Codolet, France). Sequencing grade trypsin was obtained from Promega (Southampton, U.K.). Enhanced chemiluminescence

(ECL) films were purchased from GE Healthcare (Little Chalfont, U.K.) Enhanced chemiluminescence (ECL) reagent and polyvinylidene difluoride (PVDF) membrane were from Millipore (Watford, UK). Nitrocellulose membranes were supplied by Scientific Laboratory Supplies Ltd (Nottingham, U.K.). Vectashield containing DAPI for immunocytochemistry was purchased from Vector laboratories (Peterborough, UK). Coelentrazine h was from NanoLight Technology (Pinetop, AZ, USA). [^{32}P]-orthophosphate (0.900-1.100 Ci/mmol) was purchased from PerkinElmer (Waltham, Massachusetts, USA). GFP-Trap was from Chromotek (Planegg-Martinsried, Germany).

2.1.3. Bacterial strains

XL1-Blue Subcloning Grade Competent Cells are commercially available cells purchased from Agilent Technologies LDA (Cheshire, U.K.). These cells are commercially available and were used for all DNA transformation and sub-cloning procedures.

2.1.4. DNA plasmids and receptor constructs

The construct for the *human* histamine H_4 receptor was purchased from Origene (Rockville, MD, USA). FFA4 receptor constructs cloned into pcDNA3 also containing either a FLAG- and YFP-tag or YFP-tag alone were a kind gift from Dr Adrian Butcher (MRC Toxicology Unit, Leicester, U.K.).). Other custom DNA constructs were obtained from Eurofins Genomics (Ebersberg, Germany).

2.1.5. Mammalian cell lines

Human embryonic kidney 293 (HEK293) cells and WT Chinese hamster ovary cells (CHO-K1) were purchased from American Type Culture Collection (ATCC)-LGC standards (Teddington, Middlesex, U.K.). Cells stably transfected with the histamine H_4 receptor (U2OS-H_4) were a kind gift from Dr Steven Charlton (then at Novartis Institutes for Biomedical Research, Horsham, U.K.). WT U2OS (U2OS-wt) cells (a human osteosarcoma cell line) were a kind gift from Dr Nicoleta Moisoi (Department of Cell Physiology and Pharmacology, University of Leicester, Leicester, U.K.). Cells stably expressing the FFA4 receptor and its related mutants (FFA4-TSS, TSSS, and TSSST) were a kind gift from Dr Adrian Butcher (MRC Toxicology Unit, Leicester, U.K.).

2.1.6. Pharmacological agents

Histamine dihydrochloride and JNJ7777120 were purchased from Sigma Aldrich (Poole, U.K.). TUG891 was from Tocris Bioscience (Bristol, U.K.). UBO-QIC was obtained from Institut für Pharmazeutische Biologie, Universität Bonn, Germany.

2.1.7. Primers

All primers were purchased from Sigma Aldrich (Poole, U.K.).

2.1.8. Radioisotopes

[^{32}P]-orthophosphate (0.900-1.100 Ci/mmol was obtained from PerkinElmer (Waltham, Massachusetts, USA).

2.1.9. Antibodies

Rat monoclonal anti-HA (clone 3F10) antibody was obtained from Roche Applied Science (Burgess Hill, U.K.). Anti-EGFP antibody was purchased from Abcam (Cambridge, U.K.). Anti-histamine H_4 receptor polyclonal antibody was supplied by Insight Biotechnology Ltd (Middlesex, U.K.). Anti-alpha tubulin, anti-AKT and anti-phospho-AKT antibodies were from Santa Cruz Biotechnology (Heidelberg, Germany). Anti-ERK1/2, anti-phospho-ERK1/2, anti-CREB, and anti-phospho-CREB antibodies were purchased from Cell Signalling Technology (Danvers, MA, USA). Goat anti-rabbit, goat anti-mouse IgG, HRP-linked antibodies were purchased from Bio-Rad (Hemel Hempstead, U.K.). Donkey anti-goat IgG, HRP-linked antibody was from Millipore (Watford, U.K.). Anti-rat IgG HRP-linked antibody and mouse monoclonal ANTI-FLAG® M2 antibody was purchased from Sigma Aldrich (Poole, U.K.).

2.1.10. Specialised equipment

The ClarioStar microplate reader was obtained from BMG Labtech (Aylesbury, U.K.). The Beckman Coulter centrifuge was from Beckman Coulter Inc (High Wycombe, U.K.). The PCR thermal cycler and SpeedVac concentrator centrifuge was purchased from Eppendorf (Cambridge, U.K.). The confocal microscope was obtained from Leica Microsystems (Milton Keynes, U.K.).

2.2. Methods

2.2.1. Bacterial Cell Methods

2.2.1.1. Growth and Maintenance

Escherichia. coli (E.coli) cells were grown at 37°C in either Luria-Bertani (LB) broth (1% w/v tryptone, 0.5% w/v yeast extract, and 1% w/v NaCl) with shaking at 220rpm or on LB agar (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, and 1.5% w/v bacteriological agar) plates. Ampicillin (50 mg/ml) was used as appropriate.

2.2.1.2. Bacterial transformation

Plasmid DNA containing receptor sequences were transformed into XL-1 blue competent *(E.coli)* cells. Cells were thawed on ice for at least 30 min, 50 µl of the cells were then aliquoted into pre-chilled polypropylene round bottom falcon tubes. 0.5 µg of plasmid DNA or 5 µl of ligation reaction was added to the bacterial cells and left to incubate on ice for 30 min. The mixture was then heat-shocked at 42°C for 45 sec and immediately placed on ice for 2 min. The reaction mixture was adjusted to 500 µl with SOC (super optimal broth) medium (2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose). The cells were then incubated at 37°C with shaking at 220rpm for 1 hr after which 25-100 µl aliquots were spread onto 10 cm LB agar plates containing the appropriate antibiotics and incubated at 37°C overnight. The plates were then sealed with parafilm and stored inverted at 4°C.

2.2.2. Plasmid DNA preparation

Transformation of cells is often followed by antibiotic selection to ensure bacterial cells have taken up the plasmid of interest. Plasmid DNA was subsequently isolated using either miniprep or maxiprep scales using commercially available kits. Minipreps were conducted to obtain relatively small amounts of DNA for diagnostic purposes such as sequencing of the construct to ensure it contained the insert in the correct reading frame or the correct orientation in the plasmid. Maxipreps were conducted to obtain larger amounts of DNA for transfection into mammalian cells.

2.2.3. Minipreps

The high pure plasmid isolation kit from Roche was used to purify small amounts of plasmid DNA. 4 ml of bacterial suspension grown overnight at 37°C was pelleted by centrifugation (14,000 rpm; 1 min) and resuspended in 250 µl suspension buffer (50 mM Tris-HCl, 10 mM EDTA; pH 8.0) supplemented with RNase A (100 µg/mL) by pipetting. Addition of 250 µl lysis buffer (200 mM NaOH, 1% (w/v) SDS) and incubated at room temperature for 5 min lysed the cells. Pre-chilled binding buffer (4 M guanidine hydrochloride, 0.5 M potassium acetate; pH 4.2) was added and the tubes incubated for 5 min on ice to terminate lysis. Cell lysates were centrifuged (14,000 rpm; 10 min) and the supernatant loaded onto high pure filter tubes. The tubes were centrifuged (14,000 rpm; 1 min) and then washed twice with 700 µl wash buffer (20 mM NaCl, 2 mM Tris-HCl, 80% (v/v) ethanol; pH 7.5). The tube was centrifuged (14,000 rpm; 1 min) once more to get rid of any excess wash buffer/ethanol and bound DNA was eluted from the column by addition of 50 µl sterile water and centrifugation (14,000 rpm; 1 min) into sterile 1.5 mL Eppendorf tubes.

2.2.4. Maxipreps

The Qiagen plasmid maxi kit was used to purify large scale DNA samples from bacterial cultures. A 200 mL culture grown overnight at 37°C was centrifuged (10,000 rpm; 10 min; 4°C). The supernatant was decanted taking care not to disturb the pellet which was then resuspended in 10 mL of buffer P1 (resuspension buffer) supplemented with RNase. 10 mL of buffer P2 (lysis buffer) was added and mixed thoroughly and incubated at room temperature for 5 min. 10 mL pre-chilled buffer P3 (neutralisation buffer) was added to the lysed cells, mixed thoroughly and incubated on ice for 20 min. The lysed cell solution was then transferred to a 50 mL falcon tube and centrifuged (4,000 x g; 30 min; 4°C) until supernatant cleared. In the meantime, a QIAGEN-tip was equilibrated with 10 mL buffer QBT, and allowed to empty by gravity flow. The clear supernatant was then applied to the QIAGEN-(filter) tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was then washed twice with buffer QC (wash buffer) allowing buffer QC to move through the QIAGEN-tip by gravity flow. DNA was eluted with warmed buffer QF (elution buffer) into a sterile 50 mL falcon tube. DNA was precipitated by the addition of 10.5 mL (0.7 volumes) of room-temperature isopropanol and mixed followed by centrifugation (4,000 x g; 30 min; 4°C). The

supernatant was removed and the DNA pellet washed with 5 mL room-temperature 70% ethanol and centrifuged (4,000 x g; 15 min; 4°C). After carefully decanting the supernatant, the pellet was air-dried for up to 15 min and re-dissolved in a suitable volume of sterile TE buffer (10 mM Tris-HCl, 1mM EDTA; pH 8.0).

2.2.5. Plasmid DNA quantification

The concentration and purity of DNA samples was quantified using a NanoDrop spectrophotometer. Samples were loaded onto the machine which gave a read-out of DNA concentration, and also measured absorbance at two wavelengths (260 nm and 280 nm). DNA with a A_{260}/A_{280} ratio of between 1.75 and 1.90 was considered pure enough to continue with experiments, with a ratio of approximately 1.80 an indication of pure DNA.

2.2.6. Polymerase chain reactions (PCR)

PCRs were performed to either elongate a DNA fragment of interest in order to insert it into a plasmid, or to mutate one or several amino acids in a DNA fragment of interest which was already being expressed in the correct plasmid. Regardless of the purpose of the PCR, all reactions were performed in a total volume of 50 µl. The reaction mixture contained 1 µl of thermostable DNA polymerase PfuUltra (2,500 unit/mL), 5 µl of 10x PfuUltra reaction buffer, 1.25 µl (0.2 µg/µl) of each 5'- and 3'- primers, 1 µl of 10 mM dNTPs, 0.2 µg plasmid DNA template, 5 µl DMSO and an appropriate amount of H_2O. The reactions were carried out in an Eppendorf thermal cycler with a heated lid. The PCR protocol that was followed depended on the end goal of the PCR; **Table 2.2.6.1** shows the standard PCR protocol followed when elongating a DNA fragment of interest in order to insert it into an appropriate plasmid. **Table 2.2.6.2** shows the modified protocol when mutating a single or multiple amino acid residues in a plasmid. This modified PCR was followed by restriction digestion of the template DNA using DpnI. DpnI restricts methylated DNA and, therefore, cleaves template DNA to ensure only DNA containing mutations is transformed into *E.coli* in subsequent experiments.

Table 2.1| Standard PCR cycling parameters for amplifying receptor sequences

Step	**Number of Cycles**	**Temperature** (°C)	**Duration**
Denaturation	1	98	2 min
Denaturation	30	98	30 sec
Annealing		55	30 sec
Extension		72	2 min
Extension	1	72	5 min
Termination	1	4	Hold

Table 2.2| Modified PCR cycling parameters for mutating DNA sequences

Step	**Number of Cycles**	**Temperature** (°C)	**Duration**
Denaturation	1	98	2 min
Denaturation	18	98	30 sec
Annealing		55	30 sec
Extension		72	8 min 30 sec
Extension	1	72	5 min
Termination	1	4	Hold

2.2.7. Plasmid and DNA fragment restriction digest

To ensure cohesive ends in both the plasmid DNA and the purified PCR fragments, the appropriate restriction enzymes were used to digest the DNA. The reactions were carried out in a 50 µl reaction volume containing 2.5 µg of purified DNA, 5 µl of 10x enzyme-specific buffer, 10U enzyme (10U/µl), and an appropriate volume of H_2O. The mixture was incubated at 37°C for 1 hr. After digestion, the plasmid DNA was purified and treated with alkaline phosphatase in a reaction volume of 40 µl containing 2.5 µg DNA, 4 µl of 10x alkaline phosphatase buffer, 2 µl alkaline phosphatase (1U/µl) and an appropriate volume of H_2O at 37°C for 30 min. To stop the activity of alkaline phosphatase, EDTA (final concentration 20 mM) was added to each tube and incubated at 60°C for 15 min. After digestion and alkaline phosphatase treatment, target fragments were separated using agarose gel electrophoresis followed by DNA purification. Restriction digests were also performed on purified DNA for diagnostic purposes such as confirming the presence of an insert in a plasmid. These reactions were carried out in 20 µl containing 0.25 µg DNA, 2 µl of 10x enzyme-specific buffer, 1U enzyme (10U/µl) and an appropriate volume of H_2O. This digested DNA was also resolved using agarose gel electrophoresis.

2.2.8. DNA purification from agarose gel and solution

Agarose gel electrophoresis was used to resolve DNA fragments of interest such as intact PCR products, digested PCR products and plasmids. A 0.7% agarose gel was prepared by dissolving agarose powder in TAE buffer (Tris/Acetate/ EDTA: 40 mM Tris Acetate, 1 mM EDTA, pH 8.0) by heating in a microwave oven. Ethidium bromide was then added to the gel solution before it was poured into an appropriate casting tray containing a comb to form sample wells. After the gel was allowed to solidify at room temperature, it was inserted horizontally into a gel electrophoresis tank containing TAE buffer. DNA samples were combined with 6x gel loading dye (2.5% Ficoll-400, 11mM EDTA, 3.3mM Tris-HCl, 0.017% SDS, 0.015% bromophenol blue, pH 8.0) and loaded into sample wells. A 1.0 kb DNA ladder (0.5 µg/lane) was used to estimate the size of DNA fragments. The gel was run at 100 V for 60-90 min, removed from the tank and placed on a UV trans-illuminator and photographed using a digital camera.

2.2.9. DNA purification from agarose gel and solution

The Nucleospin® Gel and PCR Clean-up allowed the purification of DNA fragments from agarose gels and solutions containing impurities such as dNTPs, primers, and salts amongst other impurities.

Extracting DNA from the agarose gel involved using the UV-trans-illuminator to highlight the fragment of interest and then using a clean scalpel to excise the gel band and transfer it to a 1.5 mL tube. The weight of each gel fragment was calculated by weighing the tube with and without the gel fragment. 200 µl of binding buffer NTI was to every 100 mg of gel and incubated at 50°C for up to 10 min with occasional mixing to break up the gel fragment. A Nucleospin® Gel and PCR Clean-up column was placed into a collection tube and up to 700 µl of sample was loaded onto the column and centrifuged for 30 sec at 11,000 x g. The flow through was discarded and the remaining sample was loaded and the tube centrifuged again. The silica membrane of the column was washed twice with 700 µl wash buffer NT3 and centrifuged for 30 sec at 11,000 x g, this step was repeated once more to minimize salt carry-over. The silica membrane was then dried by centrifuging the column for 1 min at 11,000 x g. The column was placed in a clean 1.5 mL tube and the DNA eluted in a suitable volume of sterile H_2O.

2.2.10. DNA ligation

Digested DNA fragments containing the appropriate sticky ends were ligated into plasmid vector using T4 DNA ligase. A ratio of 1:7 vector to DNA insert was incubated for 5 hr at 25°C with 1 unit of T4 DNA ligase and 1 µl 10x ligase buffer in a final volume of 10 µl. 5 µl of this reaction was used to transform XL-1 blue competent cells described in **Section 2.2.2.**

2.2.11. DNA sequencing

DNA sequencing was performed by the "Protein and Nucleic Acid Chemistry Laboratory" (PNACL) (Centre for Core Biotechnology Services, University of Leicester, UK) using an Applied Biosystems 3730 automated capillary DNA sequencer. Sequences obtained were compared to the sequence available in the public domain (histamine H_4 receptor accession number *Q9H3N8, free fatty acid receptor 4 accession number* Q5NUL3). **Table 2.2.12.1** summarises the primers used to confirm the sequences by PNACL.

Table 2.3| Primers used to confirm the sequence of receptor constructs

Primer	**Sequence (5'-3')**
T7	AATACGACTCACTATAGGG
SP6	CATACGATTTAGGTGACACTATAG
BGHR	TAGAAGGCACAGTCGAGG

2.2.12. Cloning of the histamine H_4 receptor

cDNA encoding the untagged *human* histamine H_4 receptor was obtained from Origene (NCBI reference sequence NM_021624.3) and used as the template in subsequent PCRs. The sequence for the histamine H_4 receptor was amplified using PCR and inserted into a suitable vector which allowed the stable transfection and expression in mammalian cells.

Table 2.4 shows the primers used in adding a human influenza haemagglutinin (HA) epitope tag to the N-terminus of the histamine H_4 receptor. The 5'-end primer contains a BamHI restriction site, the HA epitope tag, and a portion of the histamine H_4 receptor sequence. The 3'-end primer contains an ECORV restriction site and a portion of the coding region of the histamine H_4 receptor sequence. The steps for the PCR are summarised in **Table 2.1**.

Table 2.4 shows the primers used in generation of the GFP-tagged histamine H_4 receptor construct in pcDNA3.1 which also contained a GFP protein fixed to the Cterminal tail and is therefore suitable for creating stable expressing mammalian cell lines. The primer contains a KpnI restriction site, and a part of the histamine H_4 receptor sequence. The opposite primer contains an EcoRV restriction site and a portion of the coding region of the histamine H_4 receptor sequence. The steps for the PCR are summarised in **Table 2.1**.

2.2.13. Creation of chimeric receptor cDNA

The histamine H_4 receptor was mutated to contain the N-terminus of the FFA4 receptor in place of the N-terminus of the histamine H_4 receptor. It was thought the N-terminus of the FFA4 receptor would behave as a signal peptide and shuttle the histamine H_4 receptor to the plasma membrane of the cell. To achieve this, the In-Fusion HD Cloning Kit was used. This kit allows the directional cloning of a fragment of DNA into a vector of choice. Primers were engineered which generated a 15 base pair overhang at the end of each DNA strand which allowed the kit enzymes to fuse these DNA fragments together.

Table 2.4 shows the primers used in amplifying the N-terminus of the FFA4 receptor. The steps for the PCR are summarised in **Table 2.1**.

Table 2.4 shows the primers used to amplify the vector DNA containing the histamine H_4 receptor minus its N-terminus. The primers also add a 15 base pair overhang consisting of segments of the N-terminus of the FFA4 receptor for subsequent cloning. The steps for the modified PCR are shown in **Table 2.2**.

The PCR products were then separated using agarose gel electrophoresis and the bands of interest excised and purified as described in **Section 2.2.9.** An In-Fusion cloning reaction was then set up (total volume 10 µl) containing 1 µl of purified PCR fragment (0.2 µg/µl), 1 µl of linearised vector (0.2 µg/µl), and 2 µl of 5 x In-Fusion HD Enzyme Premix and an appropriate volume of H_2O. The reactions were carried out in an Eppendorf thermal cycler with a heated lid for 15 min at 50°C. Following this incubation, the reaction mixture was placed on ice and 2 µl was used in the transformation procedure as described in **Section 2.2.2.**

Further mutants were created to replace helix VIII and the C-terminal tail of the H_4R with that of the FFA4 receptor. Constructs were created by Eurofins MWG Operon which contained a portion of the histamine H_4 receptor sequence missing its helix VIII and C-terminal tail. The sequence for the helix VIII and C-terminal tail of the FFA4 receptor was also synthesised. Furthermore, another construct was created which replaced all serine and threonine residues in the C-terminal tail of the FFA4 receptor to an aspartate residue which would act as a phosphomimetic.

Figure 2.1a shows the DNA sequence of one construct created by Eurofins MWG Operon. The construct consists of the histamine H_4 receptor sequence starting from an EcoRI restriction site present approximately 500 base pairs downstream of the start codon, the helix VIII and C-terminal tail of the FFA4 receptor and a KpnI restriction site.

Figure 2.1b shows the DNA sequence of another construct created by Eurofins MWG Operon. This construct is similar to the construct shown in **Figure 2.1a** except the FFA4 C-terminal tail now contains aspartic acid residues instead of the serine/threonine residues present in the wild-type receptor.

A vector containing the histamine H_4 receptor and the two constructs were digested using the appropriate restriction endonucleases and ligated together as described in

Section 2.2.10. The resulting chimeric receptor constructs were then used for bacterial transformation as described in **Section 2.2.2.**

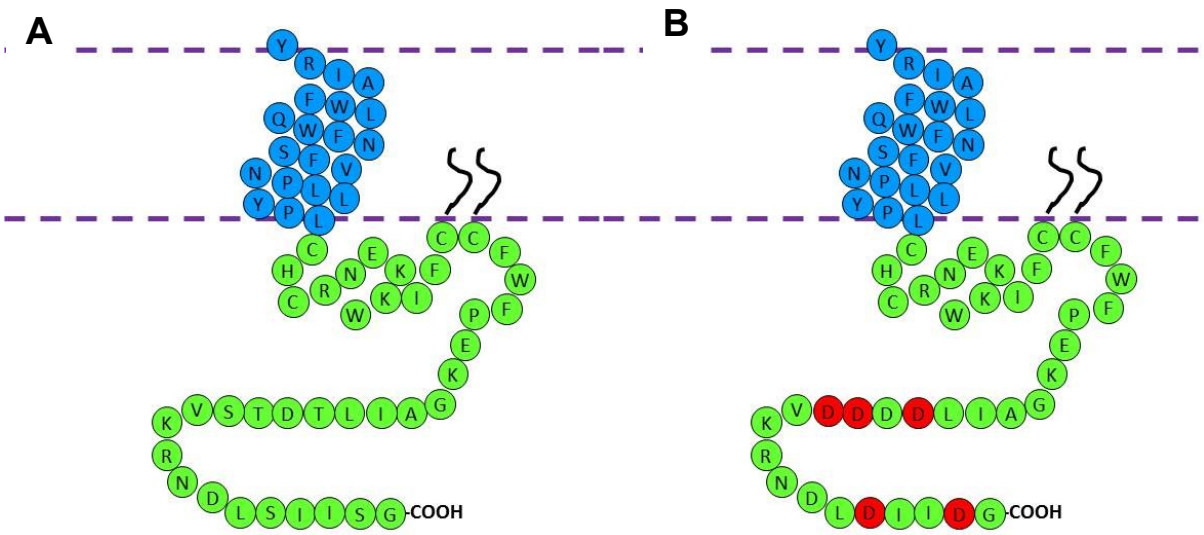


Figure 2.1| Snake plot of the chimeric histamine H_4 receptors.Residues pertaining to the histamine H_4 receptor TM7 are shown in blue, the Helix 8 and C-terminal tail of FFA4 is in green. Residues in red are the phosphomimetic residues in the chimera. (A) contains the wild-type FFA4 receptor sequence. (B) contains phophomimetics in place of the wild type serine/threonine residues.

2.2.14. Cloning of the FFA4 receptor containing phosphomimetic residues

cDNA encoding the untagged FFA4 receptor was used as the template in subsequent PCRs. The sequence of the FFA4 receptor was mutated using specific primers to replace residues in its C-terminal tail which were known to become phosphorylated upon agonist stimulation with aspartate residues which may act as phosphomimetic residues. The same residues were also mutated to alanine residues to create corresponding mutants which can no longer become phosphorylated upon agonist stimulation. The FFA4 receptor contains two clusters of serine and threonine residues termed cluster or module 1 and 2, respectively. Therefore, module 1 residues were mutated first and the resulting DNA construct was used as a template to mutate the residues of module 2 (**Figure 2.2**).

Table 2.4 shows the primers used to mutate the residues in the C-terminal tail of the FFA4 receptor to either aspartate or alanine residues. The 5'-end primer contains a portion of the FFA4 receptor followed by a region encoding the mutations and ending with more of the FFA4 receptor. The 3'-end primer is complementary to the 5'-end primer.

Table 2.4 shows the primers used to mutate the residues of module 2 to aspartate or alanine. Again, the 5'-end primer contains a portion of the FFA4 receptor followed by a region encoding the mutations and ending with more of the FFA4 receptor. The 3'-end primer is complementary to the 5'-end primer. The steps for the PCR are summarised in **Table 2.1**.

Following the PCR, the samples were digested with the restriction enzyme DpnI to cleave the (methylated) template DNA. The resulting DNA was then used for bacterial transformation as described in **Section 2.2.2.**

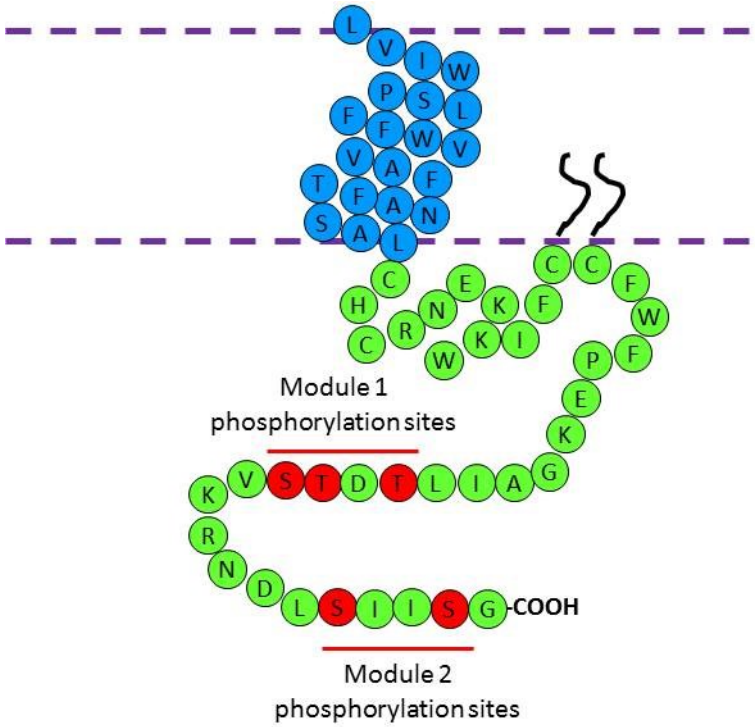


Figure 2.2| Schematic showing serine and threonine residues within the C-terminal tail of FFA4 receptor which are able to become phosphorylated. Residues downstream of the putative palmitoylated pair of cysteine residues undergo phosphorylation upon agonist stimulation. These residues are composed of five sites arranged in two clusters termed module 1 and module 2. Residues in green indicate TM7 of the FFA4 receptor, residues in green are the Helix 8 and C-terminal tail. Residues in red indicate the phosphorylation sites of the receptor.

Table 2.4| Complete list of primers used in this thesis. Sequences highlighted in red indicate restriction sites, sequences highlighted in green correspond to sequences for epitope tags, and sequences highlighted in blue indicate the receptor sequences.

Primer	**Sequence (5'-3')**
HA-H_4R Fwd	GATCGGATCCATGTACCCATACGATGTTCCAGATTACGCTCAG ATACTAATAGCACAATCAATTTATCAC
HA-H_4R Rev	CACAACACAGTCGGTCAGTATCTTCTTAAGATATCGATC
H_4R Fwd	GATCGGTACCATGCCAGATACTAATAGCACAATCAATTTATCA C
H_4R Rev	CACAACACAGTCGGTCAGTATCTTCTTAAGATATCGATC
FFA4 N-term Fwd	ATGTCCCCTGAATGCGCG
FFA4 N-term Rev	TGGTGCTGGCCGCGGTGGAGACAACC
FFA4/ H_4R Fwd	GCGGTGGAGACAACCTTTTTTATGTCCTTAGTAGCTTTTGCTAT AATGC
FFA4/ H_4R Rev	GGAGACCCAAGCTTGCCACCATGTCCCCTGAATGC
FFA4 Module 1 (S/T-D) Fwd	TTTTTTGCTGCTTCTGGTTCCCAGAAAAGGGAGCCATTTTAGAT GACGATGATGTCAAAAGAAATGACTTGTCGATTATTTCTGGC
FFA4 Module 1 (S/T-D) Rev	GCCAGAAATAATCGACAAGTCATTTCTTTTGACATCATCGTCAT CTAAAATGGCTCCCTTTTCTGGGAACCAGAAGCAGCAAAAAA
FFA4 Module 1(S/T-A) Fwd	GAAAAGGGAGCCATTTTAGCAGACGCAGCTGTCAAAAGAAAT GACTTG
FFA4 Module 1 (S/T-A) Rev	TAAAATGGCTCCCTTTTCAGCTGCGTCTGCCAAGTCATTTCTTT TGAC
FFA4 Module 2 (S/T-D) Fwd	TTTTTTGCTGCTTCTGGTTCCCAGAAAAGGGAGCCATTTTAGAT GACGATGATGTCAAAAGAAATGACTTGGACATTATTGACGGC
FFA4 Module 2 (S/T-D) Rev	GCCGTCAATAATGTCCAAGTCATTTCTTTTGACATCATCGTCAT CTAAAATGGCTCCCTTTTCTGGGAACCAGAAGCAGCAAAAAA
FFA4 Module 2 (S/T-A) Fwd	GTCAAAAGAAATGACTTGGCGATTATTGCTGGCGGTACCATGG TGAGC
FFA4 Module 2 (S/T-A) Rev	CAAGTCATTTCTTTTGACAGCAATAATCGC GCTCACCATGGTACCGCC

2.2.15. Mammalian cell culture

2.2.15.1. Cell counting and viability test

Cell numbers were determined using a haemocytometer observed under light microscope. Cell viability was measured using Trypan Blue (0.4%) exclusion.

2.2.15.2. Cell culture maintenance

U2OS cells stably expressing the histamine H_4 receptor were grown in McCoy's 5A supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 250 μg/ml geneticin (G418). Non-transfected U2OS cells were grown in the same medium minus the addition of G418. Cells stably expressing the FFA4 receptor and its associated phosphorylation-deficient mutants were grown in F-12 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 400 μg/ml hygromycin B. CHO FlpIn cells cells were grown in the same medium but without the addition of hygromycin B. HEK 293 cells were grown in Dulbeco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin. Cells were maintained in a 5% CO_2, 95% air, humidified incubator set at 37°C and passaged when confluent.

2.2.15.3. Generation of a stable cell line

CHO-K1 cells were seeded at 250, 000 cells/well in 6-well multi-dishes and allowed to adhere overnight at 37°C. Cell media was replaced with media containing no antibiotics and cells were then transfected with the appropriate *h*H_4R DNA using Lipofectamine 2000 according to manufacturer's instructions. Four hours later, media was replaced with complete media also containing 400 μg/ml of either G418 or hygromycin. Cells were continuously selected until total cell death was observed in non-transfected controls. Single colonies were picked, grown and screened for receptor expression using western blotting assay (**Section 2.2.16.4**). Where appropriate, cells were further selected using fluorescence-activated cell sorting (FACS).

2.2.15.4. Transient transfections

HEK 293 cells were seeded at 1 million cells/dish in a 10cm petri dish and allowed to adhere overnight at 37°C. Cell media was replaced with media containing no antibiotics and cells were then transfected with the appropriate DNA using

Lipofectamine 2000 according to manufacturer's instructions. Four hours later, media was replaced with complete media. The following day transfection efficiency was checked using fluorescence microscopy.

2.2.15.5. Fluorescence activated cell sorting (FACS)

Cells were analysed and sorted using a BD FACS AriaII-SORP (special order system) equipped with five lasers and an 85 μm nozzle.

2.2.16. Biochemical Assays

2.2.16.1. Whole cell lysate preparation

Cells expressing the appropriate receptor of interest were seeded at 400,000 cells/well in 6-well multi-dishes and left to adhere overnight at 37°C. Cells were serum starved for 24 hr then stimulated with a suitable ligand at 37°C. The reactions were terminated by aspiration of the media and addition of ice-cold lysis buffer (20 mM Tris, 3 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 0.5% Na deoxycholate, pH 7.4) containing phosphatase and protease inhibitors for at least 20 min on ice. Cell lysates were centrifuged (14,000 rpm for 10 min, 4°C), and the supernatant transferred to new tubes. The protein concentrations were determined by method of a Bradford protein assay (Bradford, 1976). Cleared cell lysates were stored at -20°C until required.

2.2.16.2. Membrane preparation

Cells expressing the receptor of interest were seeded at 400,000 cells/well in 6-well multi-dishes and left to adhere overnight at 37°C. Cells were serum starved for 24 hr followed by stimulation with the appropriate ligand at 37°C. Reactions were terminated by rapid aspiration of the media followed by collection of the cells using 1 mM EDTA in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, and 1.5 mM KH2PO4 at pH 7.4). Cells were collected in tubes and centrifuged (1000 rpm for 5 min, 4°C) to form pellets. Pellets were resuspended in 1 ml ice-cold TE buffer (10 mM Tris and 1 mM EDTA, pH 7.4) supplemented with protease and phosphatase inhibitors. The cell suspension was solubilised using a sonicator using two 5 second bursts separated by cooling on ice. The solubilised cell suspension was centrifuged (14,000 rpm for 1hr, 4°C) and the pellet was resuspended in TBS + 1% NP40 (50 mM Tris, 150 mM NaCl, and 1% Nonidet P-40, pH 7.4) on ice. The solubilised membranes were again

centrifuged (14,000 rpm for 10 min, 4°C) and the supernatant transferred to new tubes. The protein concentration was determined method of a Bradford protein assay. Membranes were stored at -80°C until required.

2.2.16.3. Immunoprecipitation assay

Cells expressing the receptor of interest were seeded at 400,000 cells/well in 6-well multi-dishes and left to adhere overnight at 37°C. Cells were serum starved for 24 hr followed by stimulation with the appropriate ligand at 37°C. Reactions were terminated by rapid aspiration of the media followed by harvest of the cells using a (ice-cold) modified lysis buffer (20 mM Tris, 3 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, pH 7.4) containing phosphatase and protease inhibitors for at least 20 min on ice. Cell lysates were cleared by centrifugation (14,000 rpm for 1hr, 4°C) and the supernatant transferred to fresh tubes. Equal volumes of immobilised eGFP antibody (GFP-Trap) was added to tubes and rotated for 1 hr at 4°C. The immobilised eGFP antibody was washed 3 x using appropriate volumes of lysis buffer. The final wash consisted of careful removal of the lysis buffer to leave only immobilised antibody in the tube. SDS-PAGE buffer (125mM Tris, 200mM dithiothreitol, 4% SDS, 20% glycerol, and 0.05% bromophenol blue, pH 6.8) was added to the samples and heated to 65°C for 3 min. The tubes were centrifuged and the SDS-PAGE buffer containing our protein of interest was transferred to fresh tubes. Samples were resolved on SDS-PAGE minigels immediately.

2.2.16.4. Protein quantification using the Bradford assay

Protein concentrations from cell lysates and membrane preparations were determined by measuring absorbance at 595 nm in a spectrophotometer. BSA standards (2000 μg/ml-25 μg/ml) were prepared and mixed with 1 ml of Bradford reagent. The spectrophotometer was blanked with 1:1000 dilution of appropriate buffer (1 ml volume) and 1 ml of Bradford reagent. Protein samples were diluted (1:1000) in appropriate buffer (1 ml volume) and 1 ml Bradford reagent was added to each sample. The absorbance of the protein samples was measured and the protein concentration determined by extrapolating their absorbance with the absorbance of the BSA standards.

2.2.16.5. Western blot

Samples were resuspended in equal volumes of 2 x SDS-PAGE loading buffer (125mM Tris, 200mM dithiothreitol, 4% SDS, 20% glycerol, and 0.05% bromophenol blue, pH 6.8). Samples were heated to 65°C for 4 min. An appropriate amount of protein was loaded onto SDS-PAGE minigels and run at 200 V for 45 min. Nitrocellulose membranes and minigels were equilibrated in semi-dry transfer buffer (25mM Tris, 192mM Glycine, 0.5% sodium dodecyl sulfate (SDS), 20% methanol) for 5 min prior to transfer using a semi-dry transfer cell (BIO-RAD). Transfer was performed for 1 hr at 25 V. Following the transfer of proteins, non-specific binding sites were blocked by incubation of the nitrocellulose membrane in 5% non-fat milk powder in TBS-Tween buffer (50 mM Tris, 150 mM NaCl, 0.1% v/v Tween-20) overnight at 4°C. Primary antibodies were diluted in 5% milk and incubated with the membrane (**See Table 2.5**) for 2 hr at room temperature. Membranes were then washed with 3 x 15 min washes with TBS-Tween and incubated with the relevant secondary antibody diluted in 5% milk for 1 hr at room temperature. Following this, membranes were washed 3 x 15 min washes with TBS-Tween and developed using ECL and exposure to Hyperfilm.

2.2.16.6. Immunocytochemistry

Cells were seeded onto poly-D-lysine coated 10 mm glass coverslips in 12-well multi-dishes and grown for 48 hr incubated at 37°C in a humidified air: 5% CO_2 atmosphere. Cells were then washed three times with warmed HBSS and either fixed immediately with 4% paraformaldehyde for 10 min at room temperature or incubated at 37°C in HBSS for 30 min to allow them to equilibrate to the new buffer. Cells were then stimulated with ligand for 5 min at 37°C, the reaction was terminated by rapid aspiration of the stimulation media. Cells were then fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were then washed 3 x 15 min with TBS-Triton® X-100 (20 mM Tris, 150 mM NaCl, 0.1% v/v Triton® X-100). Coverslips were mounted on microscope slides with Vectashield hardset anti-fade mounting medium with DAPI. Coverslips were sealed with nail varnish and stored in the dark at 4°C.

Table 2.5| Antibodies used for western blot analysis

Antibody	**Blocking reagent**	**Primary antibody**	**Secondary antibody**
H_4R	5% milk	1:1000 in 5% milk; 2 hr at 4°C	HRP-conjugated rabbit
HA	5% milk	1:2000 in 5% milk; 2 hr at 4°C	HRP-conjugated rat
FFA4-phosphospecific	5% milk	1:2000 in 5% milk; 2 hr at 4°C	HRP-conjugated rabbit
pAkt (pSer473)	5% milk	1:1000 in 5% milk; 2 hr at 4°C	HRP-conjugated rabbit
pAkt (pThr308)	5% milk	1:1000 in 5% milk; 2 hr at 4°C	HRP-conjugated rabbit
Akt	5% milk	1:2000 in 5% milk; 2 hr at 4°C	HRP-conjugated rabbit
pERK	5% milk	1:1000 in 5% milk; 2 hr at 4°C	HRP-conjugated rabbit
ERK	5% milk	1:2000 in 5% milk; 2 hr at 4°C	HRP-conjugated rabbit
pCREB	5% milk	1:1000 in 5% milk; 2 hr at 4°C	HRP-conjugated rabbit
CREB	5% milk	1:2000 in 5% milk; 2 hr at 4°C	HRP-conjugated rabbit
eGFP	5% milk	1:2000 in 5% milk; 2 hr at 4°C	HRP-conjugated mouse
β-Arrestin 2	5% milk	1:1000 in 5% milk; 2 hr at 4°C	HRP-conjugated mouse
α-tubulin	5% milk	1:2000 in 5% milk; 2 hr at 4°C	HRP-conjugated mouse

2.2.16.7. Cell surface endocytosis ELISA assay

HEK 293 cells were transiently transfected and plated out as described previously (**Section 2.2.16.6**). Cells were washed three times with HBSS (5.3 mM KCl, 0.44 mM KH_2PO_4, 4.1 mM $NaHCO_3$, 137 mM NaCl, 0.33 mM Na_2HPO_4, and 5.55 mM D-Glucose, pH 7.4) and then incubated in HBSS for 30 min at 37°C. Cells were further incubated at 37°C for 5 min after the addition of ligands. The reaction was terminated by rapid aspiration of the stimulation media and cells were fixed for 10 min with 4% paraformaldehyde at room temperature. Mouse anti-FLAG M2 monoclonal antibody was used to assess cell surface expression levels of FLAG-tagged FFA4 constructs. The primary antibody, diluted 1:2000, was incubated with the fixed cells overnight at 4°C, followed by extensive washing and the addition of the secondary horseradish peroxidase (HRP)-conjugated goat IgG anti-mouse antibody, diluted 1:2000, for 1 hr at room temperature. Following further extensive washing, total luminescence was measured three minutes after the addition of the Millipore Immobilon western chemiluminescent HRP substrate (ECL) using a ClarioStar plate reader (BMG-Labtech, Offenberg, Germany).

2.2.17. Receptor phosphorylation assays

2.2.17.1. *In vivo* labelling and receptor immunoprecipitation

Cells were seeded at 400,000 cells/well in 6-well multi-dishes and left overnight to adhere at 37°C. The following day, cells were washed three times in 1 ml of phosphate free Krebs buffer (10 mM HEPES, 118 mM NaCl, 4.69 mM KCl, 1.18 mM $MgSO_4.7H_2O$, 1.3 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11.7 mM glucose, pH 7.4) and then incubated with 100µCi/ml [^{32}P]orthophosphate (185MBq) for 1h at 37°C. Cells were stimulated with the appropriate agonist for 5 min at 37°C. The reaction was terminated by rapid aspiration of the buffer followed with addition of 300µl of ice-cold lysis buffer (20 mM Tris, 150 mM NaCl, 3 mM EDTA, 1% Nonidet P-40, and 0.5% Na deoxycholate, pH 7.4) for 15 min on ice. Lysed cells were collected in individual tubes and centrifuged (14000rpm for 10 min, 4°C). Immobilised eGFP antibody (GFP-Trap) was added to the samples which were incubated on a rotary shaker for 2 hr at 4°C. The immune-complexes were washed three times with lysis buffer before being re-suspended in 2xSDS-PAGE loading buffer (125 mM Tris, 200 mM dithiothreitol, 4% SDS, 20% glycerol, and 0.05% bromophenol blue, pH 6.8). The samples were heated

to 65°C for 3 min. Immune-complexes were resolved on 10% SDS-PAGE gels and electro-blotted onto nitrocellulose membranes using the semi-dry transfer method with transfer buffer (25 mM Tris, 190 mM glycine, 0.5% SDS, and 20% methanol). Receptor phosphorylation was detected by autoradiography. The samples were also analysed by western blot to check for consistency in sample loading.

2.2.17.2. Mass spectrometry and phosphorylation site identification

Cells were grown in expanded surface (Greiner) roller bottles until ~95% confluence and harvested using 1 mM EDTA in PBS (pH7.4). Cells were washed three times in 25 ml of complete Krebs buffer (10 mM HEPES, 118 mM NaCl, 4.69 mM KCl, 1.18 mM $MgSO_4.7H_2O$, 1.3 mM $CaCl_2$, 25 mM $NaHCO_3$, and 11.7 mM glucose, pH 7.4) and then incubated in 25 ml complete Krebs for 30 min at 37°C to allow cells to equilibrate in buffer. Cells were then stimulated with ligand for 5 min, the reaction was terminated by pelleting the cells and aspirating the stimulation buffer. The pellet was resuspended in 5 ml of ice-cold lysis buffer (20 mM Tris, 150 mM NaCl, 3 mM EDTA, 1% Nonidet P-40, and 0.5% Na deoxycholate, pH 7.4) supplemented with protease and phosphatase inhibitors on ice for at least 20 min. Lysed cells were aliquoted into 1.5 ml eppendorf tubes and centrifuged (14,000 rpm for 10 min, 4°C). The supernatant was transferred to a 15 ml falcon tube and immobilised eGFP antibody was added to the sample which was then incubated on a rotary shaker for 2 hr at 4°C. The immune complexes were washed three times for 10 min with ice-cold lysis buffer and receptors eluted with 100 µl of 2 x SDS-PAGE sample buffer. The purified receptors were resolved on a 10% SDS-PAGE gel, previous western blot analysis allowed us to estimate the location of the resolved receptors. The receptors were excised from the gel and cut into 2 mm squares. The gel squares were washed 3 x 15 min with 50 mM tetraethylammonium bromide (TEAB) and resuspended in 1 mM DTT (dissolved in 50 mM TEAB) and incubated for 30 min at 55°C. The DTT solution was discarded and the gel samples allowed to cool to room temperature. Gel fragments were resuspended in 100 mM iodoacetamide (dissolved in 50 mM TEAB) for 30 min at room temperature in the dark. Gel fragments were then washed 3 x 15 min with 50 mM TEAB containing 50% acetonitrile and 1 x 15 min 100% acetonitrile at room temperature. After removal of acetonitrile, the gel fragments were further dried using a speedvac centrifuge. Dry gel fragments were resuspended in 50 mM TEAB containing 1 µg sequencing grade trypsin and incubated overnight at 37°C. The following day, the supernatant was transferred to

fresh tubes and the gel fragments were washed 3 x 5 min with 50 mM TEAB containing 50% acetonitrile and 1 x 15 min 100% acetonitrile at room temperature. Supernatants after every wash were pooled and dried using a speedvac centrifuge and the gel fragments discarded.

The resulting peptides were further enriched for phosphopeptides. Peptides were resuspended in IMAC load/wash buffer (250 mM acetic acid, 30% (v/v) acetonitrile). PHOS-Select iron affinity gel resin was added to the resuspended peptides and incubated on a rotary shaker for 2 hr at room temperature. Dissolved peptides and resin were loaded onto fritted columns and washed three times with IMAC load/wash buffer (250 mM acetic acid, 30% (v/v) acetonitrile). The resulting unphosphorylated peptides from each wash were pooled into new tubes. Phosphorylated peptides were eluted from the resin by addition of IMAC elution buffer (400 mM ammonium hydroxide, 30% (v/v) acetonitrile). Both phosphorylated and unphosphorylated peptides were dried using a speedvac centrifuge and resuspended 0.1% Trifluoroacetic acid (TFA) and submitted to Protein and Nucleic Acid Chemistry Laboratory (PNACL) for analysis in LC MS/MS.

2.2.18. Cell signalling assays

2.2.18.1. FFA4/Arrestin-3 interaction assay

A BRET based assay was used to monitor interactions between the FFA4 receptor and arrestin-3. HEK 293 cells were transiently transfected with the appropriate DNA as described previously (**Section 2.2.15.4**). Briefly, cells were either transfected with the required receptor of FFA4 tagged with EYFP and with the *β*-arrestin-2–*Renilla* luciferase or with the *β*-arrestin-2–*Renilla* luciferase construct alone. Cells were seeded in an opaque 96-well multi-dish and left to adhere overnight at 37°C. Cells were washed three times with HBSS (5.3 mM KCl, 0.44 mM KH_2PO_4, 4.1 mM $NaHCO_3$, 137 mM NaCl, 0.33 mM Na_2HPO_4, and 5.55 mM D-Glucose, pH 7.4) and then incubated in HBSS for 30 min at 37°C. Coelenterazine H was added to each well to a final concentration of 2.5 μM and the cells were again placed at 37°C for 10 min in the dark before the addition of ligands. Cells were incubated at 37°C for a further 5 min before the BRET measurements were performed using a ClarioStar plate reader (BMG-Labtech, Offenberg, Germany). The BRET ratio was calculated as emission at 530 nm/emission at 485 nm. Net BRET was determined as the 530 nm/485 nm ratio of cells

co-expressing *Renilla* luciferase and EYFP minus the BRET ratio of cells only expressing the *Renilla* luciferase construct in the same experiment. This value was multiplied by 1000 to obtain mBRET units.

2.2.18.2. FFA4/Arrestin-2 interaction kinetics assay

HEK 293 cells were transiently transfected with the appropriate DNA as described previously (**Section 2.2.15.4**). Cells were seeded at a density of 10,000 cells/well in an opaque 96-well multi-dish and left to adhere overnight at 37°C. Cells were washed three times with HBSS (5.3 mM KCl, 0.44 mM KH_2PO_4, 4.1 mM $NaHCO_3$, 137 mM NaCl, 0.33 mM Na_2HPO_4, and 5.55 mM D-Glucose, pH 7.4) and then incubated in HBSS for 30 min at 37°C. Coelenterazine H was added to each well to a final concentration of 2.5 μM. Cells were placed at 37°C for 10 min in the dark. The ClarioStar plate reader was set to incubate the plate at 37°C and inject ligand into each well individually. The plate reader was also set up to measure and record every 6 sec for a total of 5 min.

2.2.18.3. Extracellular-signal regulated protein kinase 1/2 phosphorylation assay

Cells were seeded into transparent 96-well multi-dishes at a density of 20,000 cells/well and allowed to grow for 48 hr at 37°C. Cells were washed with serum-free medium and incubated at 37°C for a further 24 hr allowing FBS-stimulated pERK1/2 levels to subside. Cells were stimulated for 5 min at 37°C; where appropriate a $G\alpha_q$-protein inhibitor was used to assess the contribution of the $G\alpha_q$-protein in receptor mediated ERK1/2 phosphorylation. The reaction was terminated by rapid removal of the compounds and addition of 50 μl supplemented lysis buffer to each well. The lysates were agitated for at least 30 min at room temperature after which 8 μl of each lysate was transferred to a 384-well opaque Optiplate. 2 μl of premixed antibody solutions (v/v) of phospho-ERK1/2-cryptate antibody: phospho-ERK1/2-d2 antibody (1:1) was added to each well and the plate incubated at room temperature for 2 hr before fluorescence signal was measured using a ClarioStar plate reader.

2.2.18.4. Protein kinase B/Akt phosphorylation assay

Cells were seeded into transparent 96-well multi-dishes at a density of 20,000 cells/well and allowed to grow for 48 hr at 37°C. Cells were washed with serum-free medium and

incubated at 37°C for a further 24 hr allowing FBS-mediated pAkt levels to subside. Cells were stimulated for 5 min at 37°C; where appropriate a $G\alpha_q$-protein inhibitor was used to assess the contribution of the $G\alpha_q$-protein in receptor mediated Akt phosphorylation. The reaction was terminated by rapid removal of the compounds and addition of 50 µl supplemented lysis buffer to each well. The lysates were agitated for at least 30 min at room temperature after which 8 µl of each lysate was transferred to a 384-well opaque Optiplate. 2 µl of premixed antibody solutions (v/v) of phospho-AKT-cryptate antibody: phospho-AKT-d2 antibody (1:1) was added to each well and the plate incubated at room temperature for 4 hr before fluorescence signal was measured using a ClarioStar plate reader.

2.2.18.5. Data analysis and image rendering

Immunoblot band intensities were quantified using the ImageQuant and AlphaEase FC softwares. For concentration-response curves, relationships were analysed by non-linear regression using GraphPad Prism 5.0 software (San Diego, CA). To obtain rate constants, this software was also used with relationships being analysed by non-linear regression using a one phase association equation to obtain the rate constant. All data are expressed as mean ± SEM for the indicated number of experiments with statistical significance determined using a one-way analysis of variance (ANOVA) followed by Sidak's post hoc unless otherwise stated. For statistically significant data $P<0.05$ was accepted as being statistically significant.

Data related to immunocytochemistry were acquired using an LSM 510 laser-scanning confocal microscope (Zeiss, Germany).

Chapter 3 | Characterisation of the Histamine H_4 Receptor Phosphorylation Profile in U2OS Osteosarcoma Cells

3.1. Introduction

Upon agonist stimulation, GPCRs undergo phosphorylation in a process which uncouples the receptor from its cognate G-protein and drives G-protein independent signalling (Premont and Gainetdinov 2007; Tobin *et al.,* 2008). The histamine H_4 receptor (H_4R) was first discovered at the turn of the century by six independent groups who reported a GPCR which demonstrated high affinity to histamine (Liu *et al.,*, 2001; Morse *et al.,*, 2001; Nakamura *et al.,*, 2000; Nguyen *et al.,*, 2001; Oda *et al.,*, 2000; Zhu *et al.,*, 2001). It is now known that the H_4R is an emerging target for immune and inflammatory disorders (Leurs *et al.,* 2009).

Phosphorylation is central to receptor desensitisation after agonist stimulation (Lohse *et al.,* 1990a; Lohse *et al.,* 1990; Tobin 2008). Whilst phosphorylation of the H_1R and H_2R has been studied (Rodriguez-Pena *et al.,* 2000a; Kawakami *et al.,* 2003a; Iwata *et al.,* 2005a), studies into the phosphorylation and regulation of the H_4R are still lacking. This chapter aims to develop a cell line which expresses the H_4R at sufficient levels in order to uncover the phosphorylation status of the H_4R after stimulation with its endogenous agonist, histamine, and a reported biased agonist, JNJ7777120. We also aim to investigate the internalisation of this receptor when expressed in an osteosarcoma cell line after stimulation with these two drugs.

The H_4R couples to $G\alpha_{i/o}$ proteins and inhibits forskolin-induced cAMP and subsequently modulates transcription of genes regulated by the cAMP-responsive elements in cell lines recombinantly expressing the H_4R (Oda *et al.,* 2000; C Liu *et al.,* 2001; Zhu *et al.,* 2001). Stimulation of the receptor in HEK293 cells also elicits an increase in mitogen-activated protein kinase (MAPK) phosphorylation which is sensitive to pertussis toxin (Morse *et al.,* 2001), and intracellular Ca^{2+} release through IP_3 receptors via $G\alpha_{i/o}$ proteins and phospholipase C in mast cells (Hofstra *et al.,* 2003) or $G\alpha_{16}$ proteins which are only found in immune cells (Oda *et al.,* 2000; Morse *et al.,* 2001; Wilkie *et al.,* 1991).

JNJ7777120 has proven a useful tool in the study of the H_4R being that it was the first standard H_4R-specific antagonist (Seifert *et al.,* 2011). With respect to [^{35}S]-GTPγS binding in a U2OS osteosarcoma expression system, histamine acts as a full agonist whereas JNJ7777120 exhibits partial inverse agonism or neutral antagonist properties at the H_4R (Rosethorne and Charlton 2011). Histamine also stimulates β-arrestin binding to the receptor, which can serve as a signal-transducing protein stimulating G-protein-independent pathways such as ERK activation (Luttrell and Gesty-Palmer 2010). JNJ7777120 was found to behave as a partial agonist with respect to arrestin binding at the H_4R and was independent of receptor reserve and $G\alpha_{i/o}$ proteins (Rosethorne and Charlton 2011). In the same study, JNJ7777120 is also found to induce a prolonged ERK activation, whereas histamine only induced a transient activation. This has led to the H_4R joining other GPCRs demonstrating biased agonism, with JNJ7777120 being described as a biased agonist (Seifert *et al.,* 2011). Biased ligands such as these stabilise a unique active conformation of the receptor (Cescato *et al.,* 2010; Kao *et al.,* 2011; Pöll *et al.,* 2010). It has been hypothesised that these different conformations would allow kinases to differentially phosphorylate the receptor as different areas of the receptor may be exposed to the cytosol, and this may promote a different pattern of phosphorylation to encode specific signalling pathways (Butcher *et al.,* 2011; Nobles *et al.,* 2011; Tobin 2008; Zidar *et al.,* 2009).

With the knowledge that JNJ7777120 is a biased agonist and promotes a different signalling outcome when compared to the endogenous, full agonist histamine (Rosethorne and Charlton 2011), the aim of this chapter is to investigate the phosphorylation status of the human H_4R following stimulation with histamine versus JNJ7777120, to determine whether a biased agonist shows a distinct phosphorylation profile at this receptor.

3.2. Results

3.2.1. Using flow cytometry to enrich the population of cells expressing the *h*H_4-venus receptor construct

U2OS cells stably expressing a recombinant H_4R were a kind gift from Professor Steven J. Charlton (formerly of Novartis Pharmaceuticals). To confirm the expression of the *h*H_4-venus receptor construct in these cells, we used a combination of flow cytometry and fluorescence microscopy. These cells were chosen as they did not endogenously express any histamine receptors (Niforou *et al.,* 2008). A fluorescent tag was used in place of an epitope tag allowing non-invasive monitoring of protein expression. This fluorescent tag is called "venus" and is an improved version of eYFP as it is more stable, folds well and is tolerant of exposure to acidosis (Nagai *et al.,* 2002). U2OS cells stably expressing the *h*H_4-venus receptor construct were previously used to characterise the pharmacological properties of the H_4R (Rosethorne and Charlton 2011), this study shows that the H_4R couples to G-proteins in a [^{35}S]-GTPγS binding assay and stimulation of the receptor leads to ERK phosphorylation.

Flow cytometry and cell sorting were used as they allowed the generation of a homogenous population of cells which demonstrated relatively equal protein expression levels. All cells were visualised on a FSC/SSC dot plot. U2OS cells not transfected with the *h*H_4-venus receptor construct were used as a control. Gate P1 in **Figure 3.1A** shows typical U2OS cells, these cells were determined to be distinct from dead cells and cellular debris based on their size. These cells were also used to determine gate P2, as when U2OS cells expressing the *h*H_4-venus receptor construct were analysed we saw a distinct population of cells when they were excited by a 488 nm laser. Therefore, gate P2 represents U2OS cells which contain the *h*H_4-venus receptor construct (**Figure 3.1B**), this population of cells were collected by setting up emission collection filters 530 ± 30nm resulting in a heterogeneous population of cells expressing the receptor construct above threshold . This formed the enriched population of cells used in further experiments. Cells were cultured at 37°C and the enriched expression of the U2OS cells expressing the *h*H_4-venus receptor construct was assessed using fluorescent microscopy. **Figure 3.1C** represents cells before sorting and **Figure 3.1D** represents cells after the sorting step, showing an enriched population where more cells fluoresce due to the presence of the *h*H_4-venus receptor construct.

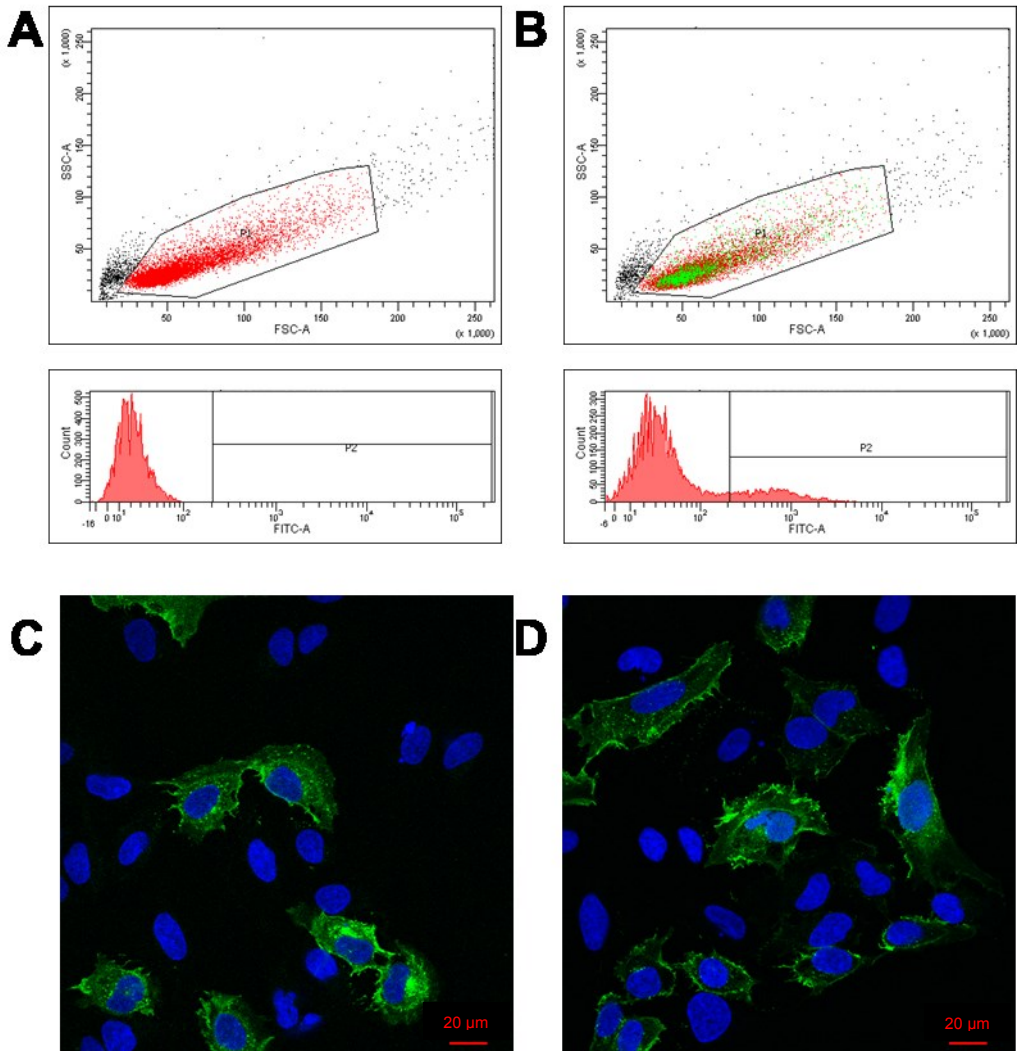


Figure 3.1| Development of a stably transfected histamine hH_4-venus cell line. U2OS cells stably expressing the hH_4-venus receptor construct were sorted by flow cytometry to create a more homogenous population of cells expressing the receptor. (A) Non-transfected U2OS cells were used to set the gating parameters to determine cells expressing fluorescence. (B) U2OS cells expressing the hH_4-venus receptor construct were then sorted using these gates to enrich the population of cells expressing the receptor construct. Approximately 17% of the initial number of cells analysed expressed the receptor construct above threshold level and were collected. (C) shows cells before the sorting step and (D) shows representative cells after cell sorting by flow cytometry showing a more homogenous population of cells expressing our receptor of interest.

3.2.2. Immunoprecipitation of the *h*H_4R-venus construct

Initial experiments were performed to establish whether the *h*H_4-venus receptor construct could be detected and subsequently immunoprecipitated using an immobilised anti-eGFP antibody (GFP-Trap) from the enriched population of U2OS cells expressing the receptor. U2OS cells expressing the receptor were resolved on minigels and probed with antisera specific to the *h*H_4R or eGFP. Immunoblots performed using either H_4R-specific antibodies or anti-eGFP antibodies identified a band at approximately 75 kDa (**Figure 3.2**). This is in line with the expected molecular weight of the *h*H_4-venus fusion protein as we know that the venus tag has a nominal molecular weight of 27 kDa (Hink *et al.,* 2000; Nagai *et al.,* 2002), and the *h*H_4R has a nominal molecular weight of 40 kDa and has been shown to migrate at molecular weights between 31-66 kDa when isolated from human spleen tissue (Leurs *et al.,* 2009). It is highly likely, therefore, that the bands shown here at 75 kDa represent the full length H_4R fused to the venus tag, as it is slightly above the predicted molecular weight of the fusion protein the additional mass could be accounted for by the addition of glycosylation and/or phosphorylation both of which are common post-translational modifications associated with GPCRs.

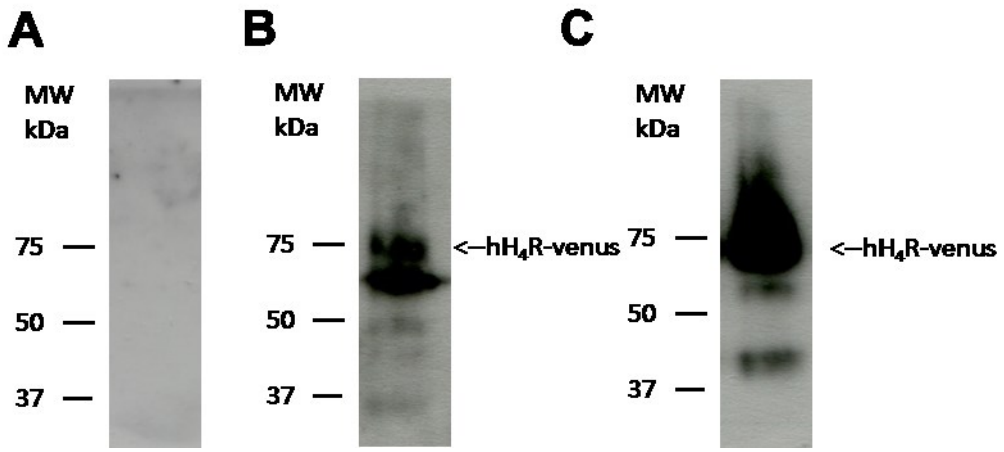


Figure 3.2| Immunoblot analysis of histamine hH_4-venus receptor expression in U2OS cells. Receptors were immuno-precipitated using an immobilised anti-eGFP antibody as described in *Materials and Methods*. Protein levels were detected using either *h*H_4-receptor- (B) or eGFP protein- specific antisera (C), (A) represents a non-transfected control probed with *h*H_4-receptor-specific antisera. Immunoblots represent one independent experiment.

3.2.3. ^{32}P Metabolic labelling reveals agonist promoted phosphorylation of the *h*H_4R

Many GPCRs are known to become rapidly phosphorylated in response to agonist occupation (Tobin, 2008; Tobin *et al.,* 2008). The effects of agonist addition on the phosphorylation status of the H_4R expressed in U2OS cells were investigated. To do this, cells were metabolically labelled with [^{32}P]-orthophosphate and stimulated with the endogenous ligand for the receptor, histamine (100 μM) for 5 min before being subjected to immunoprecipitation. Following SDS-PAGE and autoradiography, a band of approximately 75 kDa was observed to be increased in its phosphorylation in response to addition of the natural ligand (**Figure 3.3A**). The polypeptide at 75 kDa is consistent with this being the H_4R and is confirmed by western blotting analysis of the immunoprecipitated material using anti-eGFP antibodies (**Figure 3.3B**). Analysis of the data using densitometry shows that the H_4R phosphorylation was increased ~2.5 fold above basal upon stimulation with histamine (100 μM).

The small molecule, JNJ7777120, has previously been described as a biased agonist at the H_4R (Rosethorne and Charlton 2011). This study demonstrated that JNJ7777120 acts as a neutral antagonist in a [^{35}S]-GTPγS binding assay and a partial agonist in an arrestin recruitment assay. Furthermore, it is shown that JNJ7777120 promotes ERK phosphorylation in a manner which is distinct from histamine-mediated ERK phosphorylation. Stimulation of metabolically labelled U2OS cells expressing the venus-tagged human H_4R with JNJ7777120 may therefore be predicted to result in an increase in phosphorylation of the receptor which could be measured by ^{32}P incorporation. However, when the receptor was stimulated with JNJ7777120 (100 μM) for 5 min, no increase in phosphorylation of the polypeptide corresponding to the venus-tagged H_4R was observed (**Figure 3.4A**). This was confirmed by analysis of the data by densitometry which revealed no net increase in phosphorylation upon JNJ7777120 stimulation (**Figure 3.4C**).

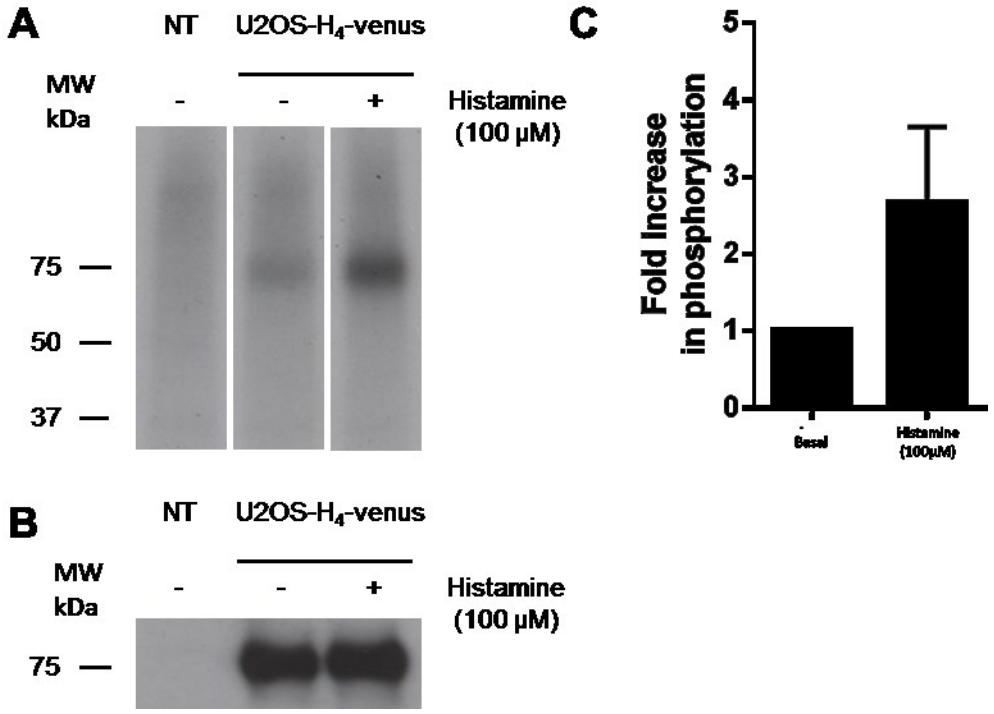


Figure 3.3| Global phosphorylation of histamine hH_4 receptor under basal conditions and in response to histamine stimulation. Cells were metabolically labelled with ^{32}P-orthophosphate and then stimulated with histamine (100 µM) for 5 min. Receptors were subjected to immunoprecipitation and resolved on SDS-PAGE minigels as described in *Material and Methods*. Phosphorylated receptors were detected by autoradiography (A), protein levels were detected using eGFP protein-specific antisera (B). Data are presented as fold increase over basal phosphorylation (C). Autoradiogram and immunoblot represents three independent experiments (n=3). Bar graph represents the mean ± S.E.M of the phosphorylation bands.

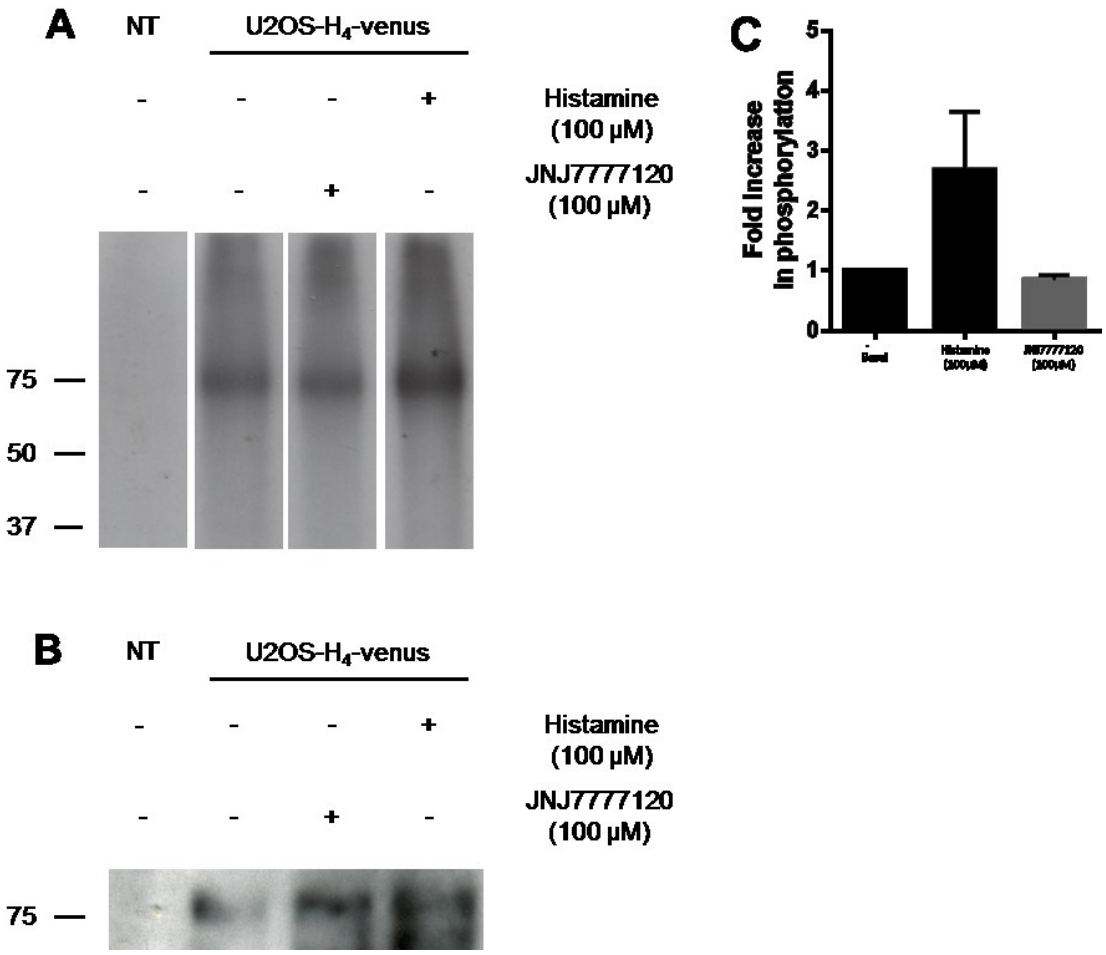


Figure 3.4| Global phosphorylation of histamine hH_4 receptor under basal conditions and in response to histamine and JNJ7777120 stimulation. Cells were metabolically labelled with ^{32}P-orthophosphate and then stimulated with histamine (100 µM) or JNJ7777120 (100 µM) for 5 min. Receptors were subjected to immunoprecipitation and resolved on SDS-PAGE minigels as described in *Material and Methods*. Phosphorylated receptors were detected by autoradiography (A), protein levels were detected using eGFP protein-specific antisera (B). Quantified data are presented as fold increase over basal phosphorylation (C). Autoradiogram and immunoblot represents three independent experiments (n=3). Bar graph represents the mean ± S.E.M of the phosphorylation bands.

3.2.4. Mass spectrometric analysis and identification of phosphorylation sites

Liquid chromatography couple to tandem mass spectrometry (LC-MS/MS) has become an increasingly attractive alternative over other well established techniques used for analysis of GPCR phosphorylation. The technique requires a relatively large (1 μg) amounts of purified receptor protein for each experiment but has advantages over [^{32}P]-metabolic labelling or 2D phosphopeptide mapping in that no radioactive label is required for identification of phosphorylation, and in addition, the exact location of phosphorylation sites can be determined (Ong and Mann 2005). Mass spectrometry has been used previously to successfully identify sites of phosphorylation on GPCRs (Nobles *et al.,* 2011). Here we use mass spectrometry to try and identify phosphorylated residues on the *h*H_4R under basal conditions, and when stimulated with the endogenous ligand histamine and a reportedly biased agonist JNJ7777120.

As relatively large amounts of receptor protein are required for these types of experiments, U2OS cells expressing the H_4R were grown in expanded surface rolling bottles. These vessels enable the production of large quantities of cells as 1 confluent rolling bottle has the equivalent surface area to 10 large flasks. Two confluent rolling bottles of U2OS cells were treated with vehicle or histamine (10 μM) or JNJ7777120 (10 μM) for 5 min and the H_4R was purified by immunoprecipitation using a commercially available immobilised anti-eGFP antibody coupled to agarose beads (available commercially as GFP-Trap). The immunoprecipitates were separated using SDS-PAGE and the region of the polyacrylamide gel corresponding to 75 kDa was extracted and processed for analysis by LC-MS/MS in order to see if H_4R peptides could be identified.

LC-MS/MS was carried out to reveal specific phosphorylation sites at the H_4R. This technique involves high performance liquid chromatography (HPLC) used to separate out peptides, which then enter the mass spectrometer after becoming ionised for individual analysis. In an LTQ orbitrap mass analyser which is used in our experiments, ions are trapped in an electrostatic field between an inner and outer electrode (Makarov, 2000). As the ions rotate around the inner electrode, they oscillate at a frequency which is characteristic of their mass/charge (m/z) ratio. The analyser detects and converts these oscillations into frequencies or wavelengths and then into

m/z ratios. Concurrently, peptides are held in a high pressure region in the mass spectrometer and peptides of interest are isolated. Energy is applied to these peptides resulting in the collision of the peptides with inert gas particles (usually N_2) and causing them to fragment. These fragmentations cause breakages of peptide bonds between random amino acids resulting in different sized peptide fragments being produced. The now fragmented peptides are sent to an adjacent low pressure region where the mass spectrometer measures their molecular weights. The location of the charge on the peptide before fragmentation is important in determining whether b ions of y ions will predominantly be produced. If the charge is located towards the carboxyl group of the peptide then y ions will predominantly be produced whereas if the charge is located toward the amine group of the peptide then b ions will predominate. Tryptic digestion of peptides results in peptides which end in the basic residues arginine or lysine. This means the charge of the peptide is predominantly found at the carboxyl region of the cleaved peptide resulting in more y ions being observed in our studies.

Figure 3.5B shows the spectra obtained after a fragmented peptide is detected by the mass spectrometer and shows the relative abundance of reporter ions. The related fragmentation table shows the total mass of the peptide fragment and observed b ions in blue and observed y ions in orange. **Figure 3.5B** shows the presence of a peptide which displays a double phosphorylation site. To determine the amino acid composition of the peptide fragment, the sequential masses of the fragmented peptide are subtracted from the total mass of the parent ion. From this, and the information known about the mass of amino acids, we can learn the amino acid structure of the peptide. Modifications such as phosphorylation add a further 80 Da to the mass of a phosphoacceptor residue such as serine, therefore, a phosphorylated serine will have a mass of the amino acid plus 80 Da.

Mass spectrometric analysis using LC-MS/MS revealed good coverage of both the third intracellular loop and the C-terminal tail of the *h*H_4R. This included a series of peptides which accounted for 22 of the 31 serine and threonine residues within these domains (**Figure 3.6A**). Experiments performed on the receptor which was not stimulated revealed that the *h*H_4R was basally phosphorylated at 13 separate serine or threonine residues, with 12 of these residues were located in the third intracellular loop and one phosphorylated residue found in the C-terminal tail (**Figure 3.5A**). This finding supports the ^{32}P metabolic labelling data presented earlier in this chapter which shows

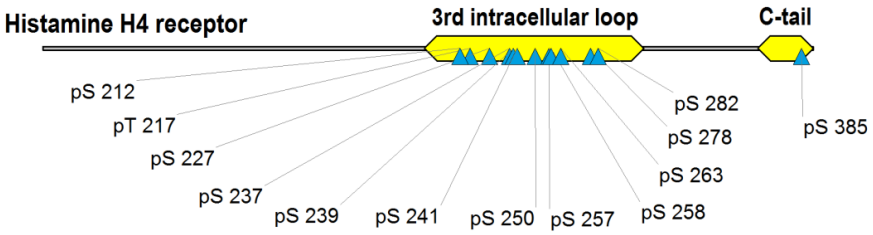
the H_4R to be phosphorylated in the absence of any agonist stimulation. **Figure 3.5B, C, D** show examples of MS/MS spectra of receptor phophopeptides where the positions of the phosphorylated residue can accurately be assigned. **Figure 3.6A, B** show a summary of the positions of the phosphorylated residues identified from the non-stimulated H_4R and the sequence of the tryptic phosphor-peptides which were generated. The observation that the H_4R is phosphorylated at multiple positions in the absence of agonist suggests the receptor is constitutively active. Previous studies have shown how some ligands behave as inverse agonists at the H_4R (Morse *et al.,* 2001; Rosethorne and Charlton 2011), more recently a study expressing receptors in Sf9 insect cells has demonstrated the importance of Phe169 and Ser179 found in the agonist binding site of the receptor in stabilising a ligand-free active state of the *h*H_4R (Wifling *et al.,* 2015).

The endogenous ligand for the *h*H_4R is histamine; based on our previous studies using ^{32}P metabolic labelling, we have determined that histamine induces a strong agonist-mediated increase in phosphorylation (**Figure 3.3A**). Extending this study, receptors were stimulated with histamine (10 μM) and subjected to immunoprecipitation and tryptic digestion before analysis by LC/MS/MS study. As observed in the experiments with the non-stimulated H_4R, there was good coverage of the third intracellular loop and the C-terminal tail which included peptides that accounted for 27 out of 31 serine or threonine residues present in these intracellular domains (**Figure 3.8A**). In total, 9 serine and threonine residues were found to be phosphorylated upon histamine stimulation (**Figure 3.7A**). This means that there is a decrease in the number of sites phosphorylated when compared to the receptor under basal conditions.

The biased agonist JNJ7777120 was used to stimulate the *h*H_4R, LC/MS/MS was used to identify the putative phosphorylation sites. The resulting peptides provided good coverage of the third intracellular loop and C-terminal tail (**Figure 3.10A**). 10 different residues were discovered to be phosphorylated upon JNJ7777120 stimulation from 10 separate peptide fragments which were observed in the study (**Figure 3.9A** and **Figure 3.10B**).

A summary table highlighting the changes in phosphorylation sites of the *h*H_4R upon basal, histamine-, and JNJ7777120-treated samples is shown to make clear the effect of both compounds on the phosphorylation sites of the receptor (**Table 3.1**).

A



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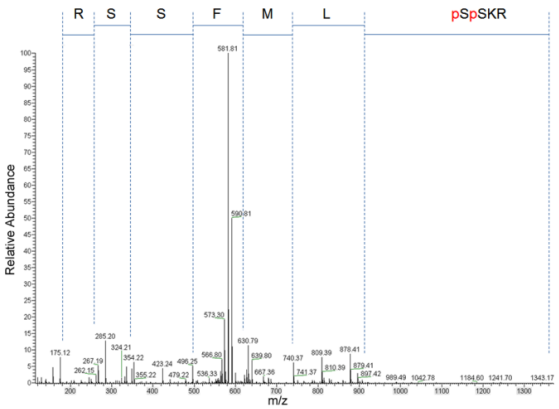
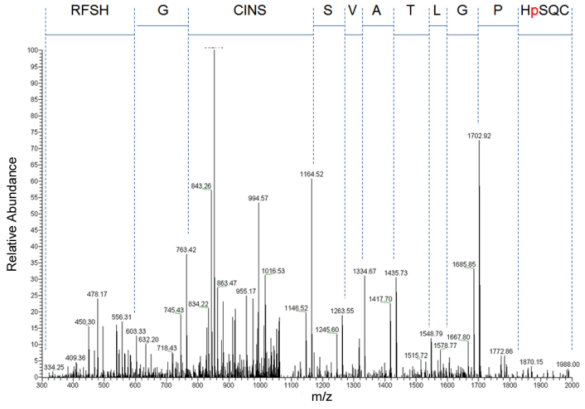


RKpSpSLMFSSR
(pSerine 257 & pSerine 258)

B IONS	SEQUENCE	Y IONS
157.1	R	1358.6
285.2	K	1202.5
452.2	pS	1074.4
619.2	pS	907.4
732.3	L	740.4
863.3	M	627.3
1010.4	F	496.3
1097.4	S	349.2
1184.5	S	262.2
1358.6	R	175.1

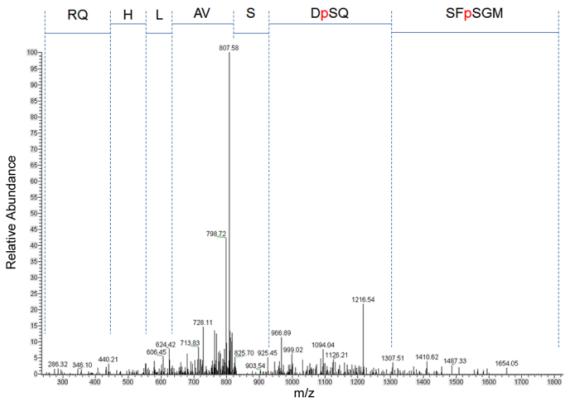
C



CQpSHPGLTAVSSNICGHSFR
(pSerine 212)

B IONS	SEQUENCE	Y IONS
144.0	C	2278.0
272.1	Q	2134.9
439.1	pS	2006.9
576.1	H	1839.9
673.2	P	1702.8
730.2	G	1605.8
843.3	L	1548.8
944.3	T	1435.7
1015.4	A	1334.6
1114.4	V	1263.6
1201.5	S	1164.5
1288.5	S	1077.5
1402.5	N	990.5
1515.6	I	876.4
1675.7	C	763.3
1732.7	G	603.3
1869.7	H	546.3
1956.8	S	409.2
2103.8	F	322.2
2278.0	R	175.1

D



MGpSFSQpSDSVALHQR
(pSerine 278 & pSerine 282)

B IONS	SEQUENCE	Y IONS
132.0	M	1809.7
189.1	G	1678.7
356.1	pS	1621.6
503.1	F	1454.6
590.2	S	1307.6
718.2	Q	1220.5
885.2	pS	1092.5
1000.3	D	925.5
1087.3	S	810.5
1186.4	V	732.4
1257.4	A	624.4
1370.5	L	553.3
1507.5	H	440.2
1635.6	Q	303.2
1809.7	R	175.1

Figure 3.5| Summary of phosphorylation sites of histamine hH_4 receptor under basal conditions determined by mass spectrometry. U2OS-cells stably expressing the histamine hH_4 receptor were grown in roller bottles and treated with vehicle before being harvested. Receptors were purified by immunoprecipitation and resolved on SDS-PAGE minigels. Receptor peptides were generated as described in *Material and Methods* and individual phosphorylation sites determined by mass spectrometry. Locations of the phosphorylation sites within the third intracellular loop and C-terminal tail of the receptor is indicated (A). Representative example spectra of five individual sites of phosphorylation are shown (B-D) along with associated fragmentation tables summarising the B- and Y- ions of the peptides observed.

A

```
  1  MPDTNSTINL SLSTRVTLAF FMSLVAFAIM LGNALVILAF VVDKNLRHRS
 51  SYFFLNLAIS DFFVGVISIP LYIPHTLFEW DFGKEICVFW LTTDYLLCTA
101  SVYNIVLISY DRYLSVSNAV SYRTQHTGVL KIVTLMVAVW VLAFLVNGPM
151  ILVSESWKDE GSECEPGFFS EWYILAITSF LEFVIPVILV AYFNMNIYWS
201  LWKRDHLSRC QSHPGLTAVS SNICGHSFRG RLSSRRSLSA STEVPASFHS
251  ERQRRKSSLM FSSRTKMNSN TIASKMGSFS QSDSVALHQR EHVELLRARR
301  LAKSLAILLG VFAVCWAPYS LFTIVLSFYS SATGPKSVWY RIAFWLQWFN
351  SFVNPLLYPL CHKRFQKAFL KIFCIKKQPL PSQHSRSVSS
```

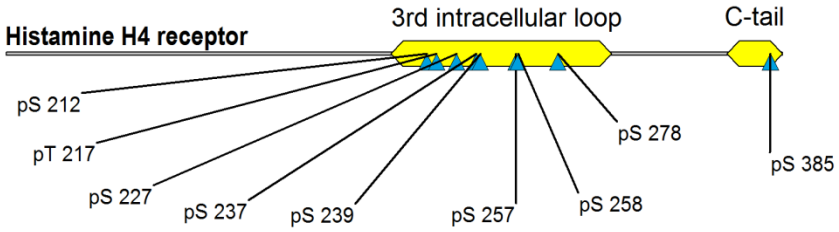
B

Summary of H_4R phospho-peptides

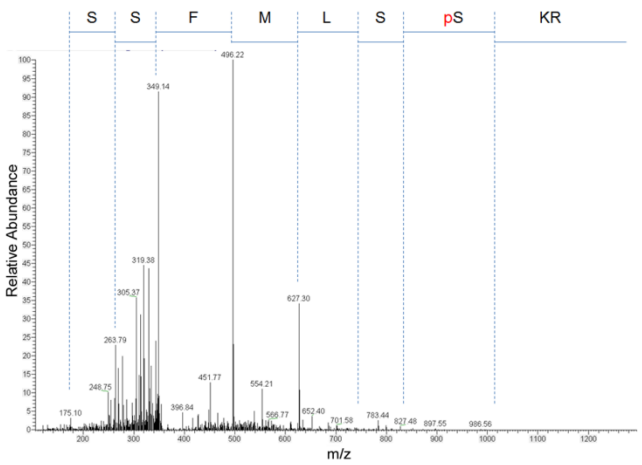
Peptide sequence	Mascot ion score	Residue number	Delta PPM	Number of times observed
CQpSHPGLTAVSSNICGHSFR	25.2	210-229	1.3	4
CQSHPGLpTAVSSNICGHSFR	-	210-229	-1.8	1
CQSHPGLTAVSSNICGHpSFR	-	210-229	0.51	1
pSLSASTEVPASFHSER	53	237-252	-2.2	5
RSLpSASTEVPASFHSER	32.9	236-252	-2.1	2
RSLSApSTEVPASFHSER	31.2	236-252	1.8	1
SLSASTEVPASFHpSER	22.8	237-252	-1.9	2
RKpSSLMFSSR	63.8	255-264	-2.5	31
RKSpSLMFSSR	56.3	255-264	-3.5	28
SSLMFSpSR	25.1	257-264	1.6	1
MGpSFSQSDSVALHQR	101.9	276-290	-1.5	19
MGSFSQpSDSVALHQR	6.1	276-290	1.1	1
QPLPSQHpSR	51	378-386	-1.4	3

Figure 3.6| Summary of phosphorylation sites of histamine hH_4 receptor under basal conditions determined by mass spectrometry. The primary sequence of the hH_4 receptor is shown (A), in *bold* are the third intracellular loop and C-terminal tail, *underlined* sections represent histamine hH_4 receptor peptides observed in mass spectrometry experiments and in *red* are amino acids identified as being phosphorylated. (B) represents a summary of the overall dataset. Results are representative of 5 independent experiments pooled together.

A



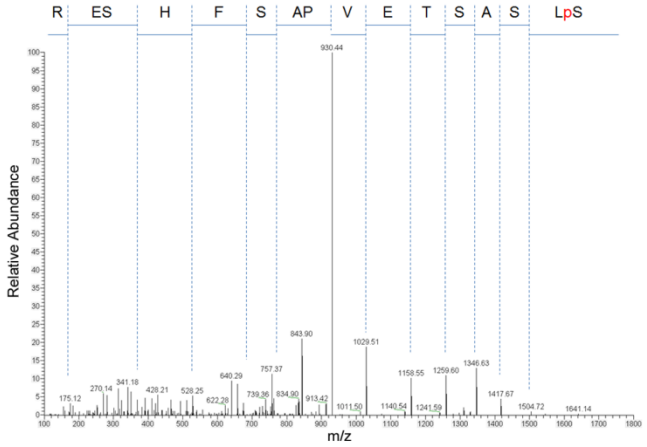
B



RKpSSLMFSSR
(pSerine 257)

B IONS	SEQUENCE	Y IONS
157.1	R	1278.6
285.2	K	1222.5
452.2	pS	994.4
539.2	S	827.4
652.3	L	740.4
783.4	M	627.3
930.4	F	496.3
1017.5	S	349.2
1104.5	S	262.2
1278.6	R	175.1

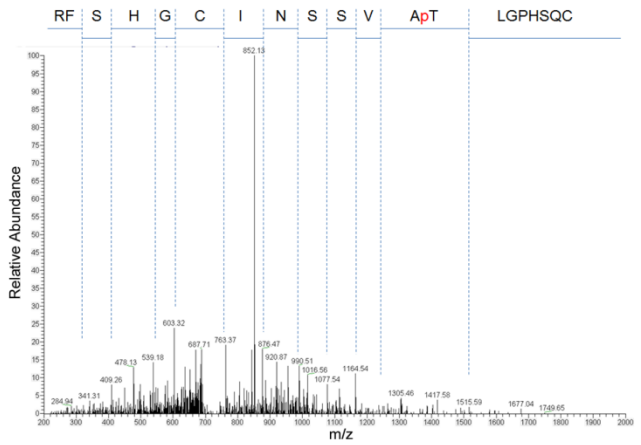
C



pSLSASTEVPASFHSER
(pSerine 237)

B IONS	SEQUENCE	Y IONS
168.0	pS	1784.8
281.1	L	1617.8
368.1	S	1504.7
439.2	A	1417.7
526.2	S	1346.6
627.2	T	1259.6
756.3	E	1158.6
855.3	V	1029.5
952.4	P	930.4
1023.4	A	833.4
1110.5	S	762.4
1257.5	F	675.3
1394.6	H	528.3
1481.6	S	391.2
1610.7	E	304.2
1784.8	R	175.1

D



CQSHSGLpTAVSSNICGHSFR
(pThreonine 217)

B IONS	SEQUENCE	Y IONS
161.0	C	2295.0
289.1	Q	2134.9
376.1	S	2006.9
513.2	H	1919.9
610.2	P	1782.8
667.3	G	1685.7
780.3	L	1628.7
961.4	pT	1515.6
1032.4	A	1334.6
1131.5	V	1263.6
1218.5	S	1164.5
1305.5	S	1077.5
1419.6	N	990.5
1532.7	I	876.4
1692.7	C	763.3
1749.7	G	603.3
1886.8	H	546.3
1973.8	S	409.2
2120.9	F	322.2
2295.0	R	175.1

Figure 3.7| Summary of phosphorylation sites of histamine hH_4 receptor upon agonist (histamine) stimulation determined by mass spectrometry. U2OS-cells stably expressing the histamine hH_4 receptor were grown in roller bottles and stimulated with the agonist histamine (10 μM) before being harvested. Receptors were purified by immunoprecipitation and resolved on SDS-PAGE minigels. Receptor peptides were generated as described in *Material and Methods* and individual phosphorylation sites determined by mass spectrometry. Locations of the phosphorylation sites within the third intracellular loop and C-terminal tail of the receptor is indicated (A). Representative example spectra of five individual sites of phosphorylation are shown (B-D) along with associated fragmentation tables summarising the B- and Y- ions of the peptides observed.

A

```
  1  MPDTNSTINL SLSTRVTLAF FMSLVAFAIM LGNALVILAF VVDKNLRHRS
 51  SYFFLNLAIS DFFVGVISIP LYIPHTLFEW DFGKEICVFW LTTDYLLCTA
101  SVYNIVLISY DRYLSVSNAV SYRTQHTGVL KIVTLMVAVW VLAFLVNGPM
151  ILVSESWKDE GSECEPGFFS EWYILAITSF LEFVIPVILV AYFNMNIYWS
201  LWKRDHLSRC QSHPGLTAVS SNICGHSFRG RLSSRRSLSA STEVPASFHS
251  ERQRRKSSLM FSSRTKMNSN TIASKMGSFS QSDSVALHQR EHVELLRARR
301  LAKSLAILLG VFAVCWAPYS LFTIVLSFYS SATGPKSVWY RIAFWLQWFN
351  SFVNPLLYPL CHKRFQKAFL KIFCIKKQPL PSQHSRSVSS
```

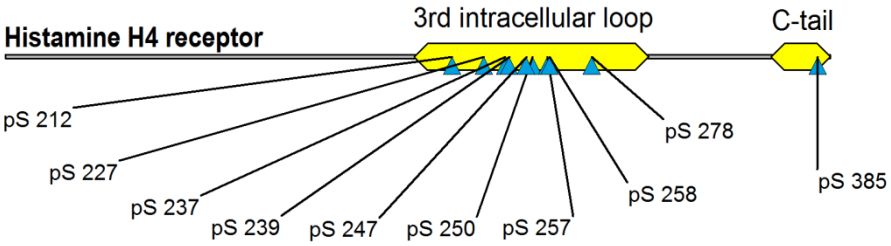
B

Summary of H_4R phospho-peptides

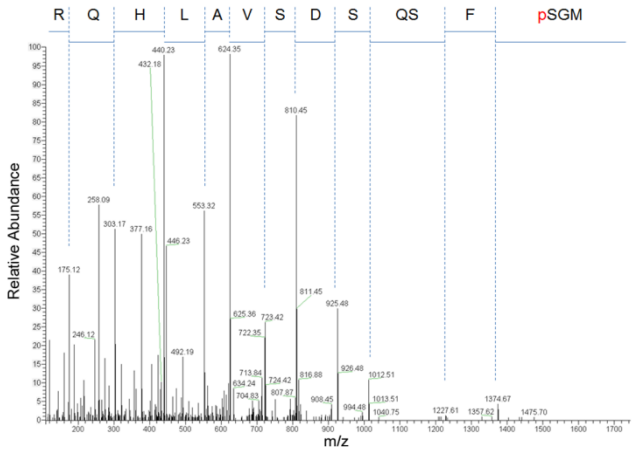
Peptide sequence	Mascot ion score	Residue number	Delta PPM	Number of times observed
CQpSHPGLTAVSSNICGHSFR	-	210-229	2.5	4
CQSHPGLpTAVSSNICGHSFR	46.9	210-229	0.31	2
CQSHPGLTAVSSNICGHpSFR	60.4	210-229	0.98	1
pSLSASTEVPASFHSER	85.1	237-252	0.98	11
RSLpSASTEVPASFHSER	-	236-252	1.1	1
RKpSSLMFSSR	41.8	255-264	0.73	1
RKSpSLMFSSR	40.7	255-264	-4.6	6
MGpSFSQSDSVALHQR	77.9	276-290	1.1	20
QPLPSQHpSR	24.3	378-386	0.18	1

Figure 3.8| Summary of phosphorylation sites of hH_4 receptor upon agonist (histamine) stimulation determined by mass spectrometry. The primary sequence of the hH_4 receptor is shown (A), in *bold* are the third intracellular loop and C-terminal tail, *underlined* sections represent histamine hH_4 receptor peptides observed in mass spectrometry experiments and in *red* are amino acids identified as being phosphorylated. (B) represents a summary of the overall dataset. Results are representative of 5 independent experiments pooled together.

A



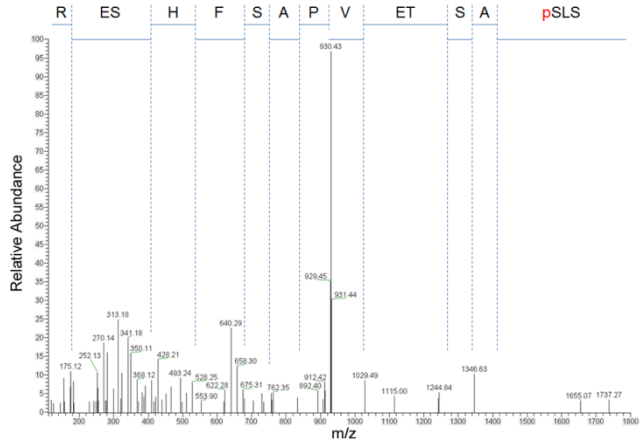
B



MGpSFSQSDSVALHQR
(pSerine 278)

B IONS	SEQUENCE	Y IONS
132.0	M	1729.7
189.1	G	1598.7
356.1	pS	1541.7
503.1	F	1374.7
590.2	S	1227.6
718.2	Q	1140.6
805.3	S	1012.5
920.3	D	925.5
1007.3	S	810.5
1106.4	V	723.4
1177.4	A	624.4
1290.5	L	553.3
1427.6	H	440.2
1555.6	Q	303.2
1729.7	R	175.1

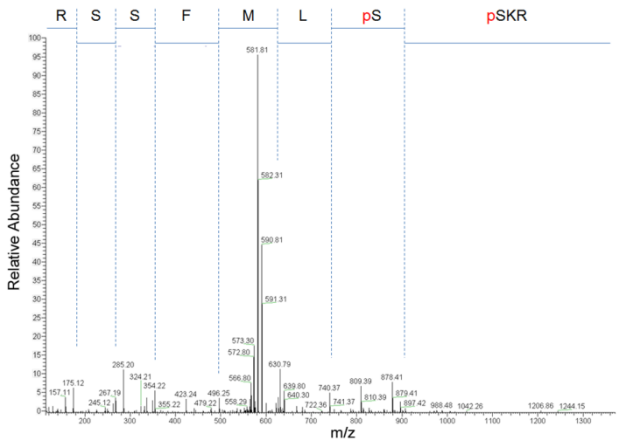
C



SLpSASTEVPASFHSEF
(pSerine 239)

B IONS	SEQUENCE	Y IONS
168.0	S	1784.8
281.1	L	1617.8
368.1	pS	1504.7
439.2	A	1417.7
526.2	S	1346.6
627.2	T	1259.6
756.3	E	1158.6
855.3	V	1029.5
952.4	P	930.4
1023.4	A	833.4
1110.5	S	762.4
1257.5	F	675.3
1394.6	H	528.3
1481.6	S	391.2
1610.7	E	304.2
1784.8	R	175.1

D



RKpSpSLMFSSR
(pSerine 257 & pSerine 25

B IONS	SEQUENCE	Y IONS
157.1	R	1358.6
285.2	K	1202.5
452.2	pS	1074.4
619.2	pS	907.4
732.3	L	740.4
863.3	M	627.3
1010.4	F	496.3
1097.4	S	349.2
1184.5	S	262.2
1358.6	R	175.1

Figure 3.9| Summary of phosphorylation sites of histamine hH_4 receptor upon agonist (JNJ7777120) stimulation determined by mass spectrometry. U2OS-cells stably expressing the histamine hH_4 receptor were grown in roller bottles and stimulated with the agonist JNJ7777120 (10 μM) before being harvested. Receptors were purified by immunoprecipitation and resolved on SDS-PAGE minigels. Receptor peptides were generated as described in *Material and Methods* and individual phosphorylation sites determined by mass spectrometry. Locations of the phosphorylation sites within the third intracellular loop and C-terminal tail of the receptor is indicated (A). Representative example spectra of five individual sites of phosphorylation are shown (B-D) along with associated fragmentation tables summarising the B- and Y- ions of the peptides observed.

A

```
  1  MPDTNSTINL SLSTRVTLAF FMSLVAFAIM LGNALVILAF VVDKNLRHRS
 51  SYFFLNLAIS DFFVGVISIP LYIPHTLFEW DFGKEICVFW LTTDYLLCTA
101  SVYNIVLISY DRYLSVSNAV SYRTQHTGVL KIVTLMVAVW VLAFLVNGPM
151  ILVSESWKDE GSECEPGFFS EWYILAITSF LEFVIPVILV AYFNMNIYWS
201  LWKRDHLSRC QSHPGLTAVS SNICGHSFRG RLSSRRSLSA STEVPASFHS
251  ERQRRKSSLM FSSRTKMNSN TIASKMGSFS QSDSVALHQR EHVELLRARR
301  LAKSLAILLG VFAVCWAPYS LFTIVLSFYS SATGPKSVWY RIAFWLQWFN
351  SFVNPLLYPL CHKRFQKAFL KIFCIKKQPL PSQHSRSVSS
```

B

Summary of H_4R phospho-peptides

Peptide sequence	Mascot ion score	Residue number	Delta PPM	Number of times observed
CQpSHPGLTAVSSNICGHSFR	25.2	210-229	1.3	4
CQSHPGLTAVSSNICGHpSFR	-	210-229	11	1
pSLSASTEVPASFHSER	23.4	237-252	2.1	5
RSLpSASTEVPASFHSER	56.8	236-252	-2.4	2
RSLSASTEVPApSFHSER	23.4	236-252	2.1	1
SLSASTEVPASFHpSER	22.8	237-252	-1.9	2
RKpSSLMFSSR	53.4	255-264	-3.0	31
RKSpSLMFSSR	56.4	255-264	-2.8	28
MGpSFSQSDSVALHQR	75.5	276-290	-3.0	19
QPLPSQHpSR	49.4	378-386	-2.7	3

Figure 3.10| Summary of phosphorylation sites of hH$_4$ receptor upon agonist (JNJ7777120) stimulation determined by mass spectrometry. The primary sequence of the hH$_4$ receptor is shown (A), in *bold* are the third intracellular loop and C-terminal tail, *underlined* sections represent histamine hH$_4$ receptor peptides observed in mass spectrometry experiments and in *red* are amino acids identified as being phosphorylated. (B) represents a summary of the overall dataset. Results are representative of 3 independent experiments pooled together.

Table 3.1| Summary table of phosphorylation sites observed of the *h*H_4 receptor upon basal or agonist stimulation as determined by mass spectrometry.

Phosphorylation site	Phosphorylated?		
	Basal	Histamine	JNJ7777120
pS 212	Yes	Yes	Yes
pT 217	Yes	Yes	
pS 227	Yes	Yes	Yes
pS 237	Yes	Yes	Yes
pS 239	Yes	Yes	Yes
pS 241	Yes	Not observed	Not observed
pS 247	Not observed	Not observed	Yes
pS 250	Yes	Not observed	Yes
pS 257	Yes	Yes	Yes
pS 258	Yes	Yes	Yes
pS 263	Yes	Not observed	Not observed
pS 278	Yes	Yes	Yes
pS 282	Yes	Not observed	Not observed
pS 385	Yes	Yes	Yes

3.2.5. Investigating the internalisation responses of the *h*H_4R in U2OS cells

It is widely accepted that GPCR activation and phosphorylation leads to the recruitment of arrestin to the activated receptor, this recruitment is central in receptor desensitisation and internalisation (Ferguson, 2001). GPCRs are regulated by β-arrestins, which not only desensitise G-protein signalling but also initiate G-protein-independent signalling (Lefkowitz and Shenoy 2005). This interaction of arrestin with GPCRs is believed to be a multi-step process which firstly involves a high affinity interaction of the phosphorylated form of the receptor with the phosphate sensor of the arrestin molecule (Gurevich and Gurevich 2006).

Histamine, the endogenous ligand for the *h*H_4R, promotes both [^{35}S]-GTPγS binding and arrestin recruitment to the receptor (Rosethorne and Charlton 2011), thereby acting as an agonist in both signalling readouts. In addition, data presented in this chapter shows that the H_4R becomes rapidly phosphorylated in response to histamine stimulation. JNJ7777120, however, has been shown to act as an neutral antagonist or inverse agonist with respect to [^{35}S]-GTPγS binding, but as a partial agonist in arrestin recruitment assays (Rosethorne and Charlton 2011; Seifert *et al.,* 2011). In this way, it can be described as a biased agonist capable of directing receptor signalling preferentially along one pathway. Here, the effects of histamine and JNJ7777120 on H_4R internalisation have been investigated. To do this, U2OS cells stably expressing the *h*H_4-venus receptor construct were stimulated with either histamine or JNJ7777120 for 5 or 30 minutes, immediately fixed and mounted on glass coverslips and observed using confocal microscopy. Under basal conditions, the venus-tagged *h*H_4R shows a predominantly plasma membrane and uniform localisation which appears as a "halo" at the boundaries of the cell (**Figure 3.11B**). Stimulation with histamine for 5 minutes does not appear to cause significant change to this distribution of the receptor and the "halo" is still visible (**Figure 3.11C**). However, after 30 minutes of stimulation, a more punctate membrane distribution and intracellular vesicle-like staining became apparent suggesting pronounced internalisation of the receptor (**Figure 3.11D**). Stimulation with JNJ7777120 follows a similar pattern of internalisation. After 5 minutes of stimulation, receptors are still visible at the cell surface, but stimulation for 30 minutes causes marked receptor internalisation (**Figure 3.11E** and **Figure 3.11F**).

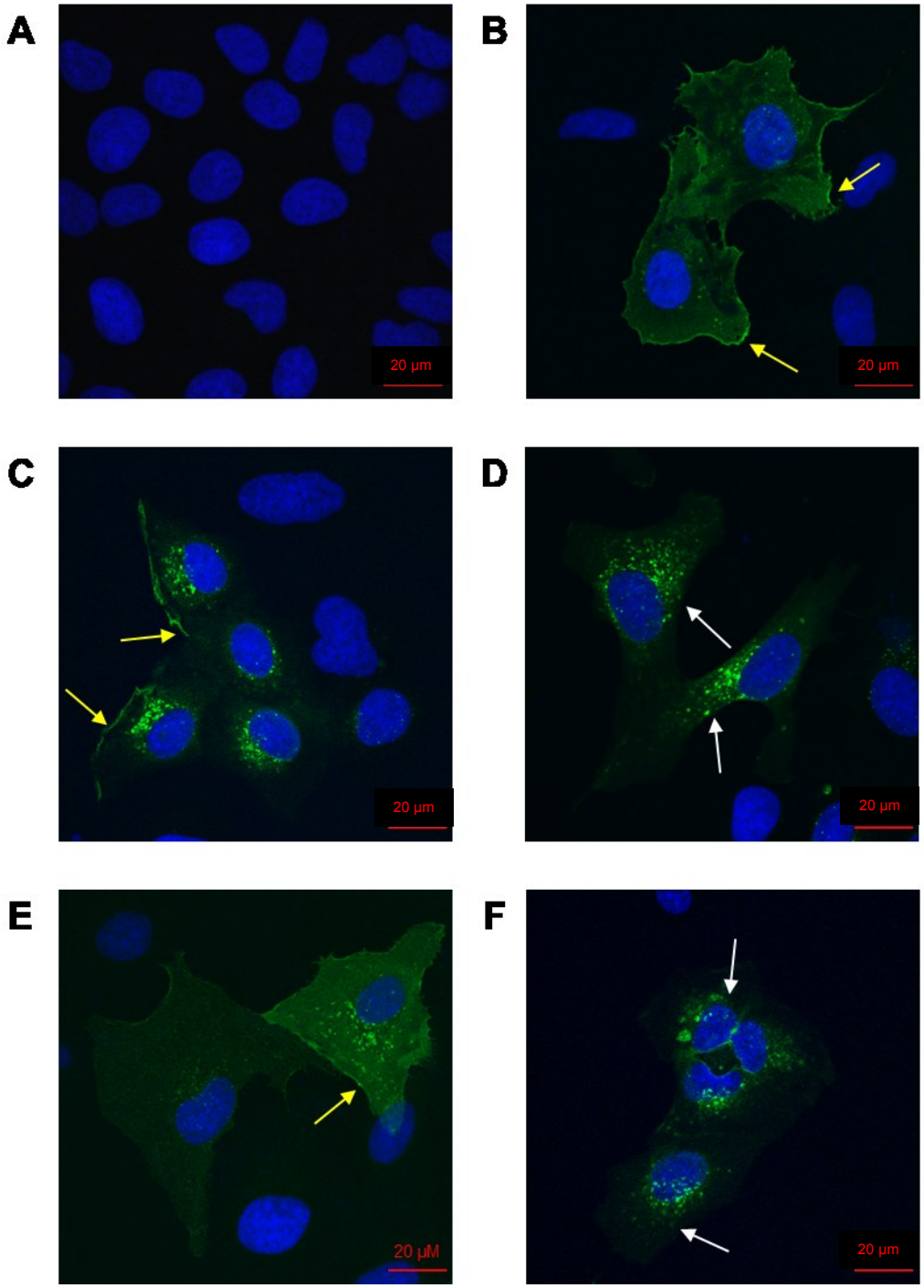


Figure 3.11| The histamine hH_4 receptor internalises upon agonist stimulation. Non-transfected U2OS cells or U2OS cells expressing the human hH_4 receptor were grown on poly-D-lysine coated coverslips and stimulated with vehicle, histamine (10 µM) or JNJ7777120 (10 µM) for 5 or 30 min and then fixed using 4% paraformaldehyde. Cells were mounted using hard-set mounting medium containing DAPI to stain the nucleus and viewed using confocal microscopy. (A) shows non-transfected cells. (B) cells expressing the human hH_4 receptor stimulated with vehicle. Cells were also stimulated with histamine (10 µM) for 5 min (C) or 30 min (D), or JNJ7777120 (10 µM) for 5 min (E) or 30 min (F). Yellow arrows indicate cell surface receptor, white arrows indicate internalised receptor. Scale bars, 20 µm. Images represent two independent experiments.

3.3. Discussion

In this present chapter, by looking at the global, peptide and individual amino acid level of the H_4R, we have investigated the phosphorylation profile of the *human* H_4R under basal conditions and after stimulation with the endogenous, full agonist histamine, and a β-arrestin biased ligand, JNJ7777120 demonstrating differential patterns of phosphorylation depending on which ligand stimulates the receptor. We also show that the H_4R is a functioning receptor that undergoes internalisation after an extended time of agonist stimulation. We have achieved this by employing ^{32}P-labelling of U2OS cells stably expressing the receptor, and mass spectrometry. Confocal microscopy is used to investigate the endocytosis of receptors from the plasma membrane.

GPCRs are highly regulated proteins, and the exposure of the receptor to agonist results in rapid desensitisation. One of the central mechanisms of this process is receptor phosphorylation (Lohse *et al.,* 1990; Lohse *et al.,* 1990; Ferguson, 2001; Tobin *et al.,* 2008; Tobin, 2008). Whilst phosphorylation of the H_1R and H_2R has been studied (Rodriguez-Pena *et al.,* 2000a; Kawakami *et al.,* 2003a; Iwata *et al.,* 2005a), studies into the phosphorylation and regulation of the H_4R are still lacking.

Several strategies can be employed to investigate GPCR phosphorylation including using phosphoacceptor site mutants, ^{32}P-labelling, phosphopeptide mapping, mass spectrometry and phosphospecific antibodies (Seibold *et al.,* 2000; Blaukat *et al.,* 2001; Torrecilla *et al.,* 2007; Butcher *et al.,* 2009; Butcher *et al.,* 2011; Chen *et al.,* 2013; Zindel *et al.,* 2015). ^{32}P-labelling provides evidence of the global phosphorylation events of the receptor upon ligand binding in a recombinant or native system. It cannot, however, identify single residues which have become phosphorylated. This is where mass spectrometry has proven a useful analytical tool in identifying the exact location of phosphorylation. Peptides with phosphorylated residues are 80 Da heavier when they contain one residue which has become phosphorylated compared with their original theoretical mass. This allows mass analysers to identify the amino acid sequence and exact location of phosphorylation. Numerous studies have employed this technique to identify sites of phosphorylation at several family A GPCRs including the β_2-adrenergic receptor, chemokine CXCR4, FFAR4, M_1 and M_3 mAchRs, and the μ-opioid receptors (Busillo *et al.,* 2010; Butcher *et al.,* 2011; Nobles *et al.,* 2011; Chen *et al.,* 2013; Butcher *et al.,* 2014). Mass spectrometry also allows one to ascertain the patterns of

phosphorylation or a "phosphorylation barcode" for each receptor, certainly there have been examples of different receptors undergoing distinct phosphorylation events in their third intracellular loops and C-terminal tails depending on the ligand used to stimulate these receptors (Butcher *et al.,* 2011; Nobles *et al.,* 2011). Nobles *et al.,* also showed that a biased ligand can induce a phosphorylation pattern which is distinct from an unbiased, full agonist (Nobles *et al.,* 2011). In this study, the β_2-adrenergic receptor was expressed in HEK 293 cells and stimulated with a biased agonist, carvedilol. This induced phosphorylation of two serine residues (Ser355 and Ser356) which were shown to be phosphorylated by GRK6. This contrasted with the phosphorylation events which occurred at the receptor after stimulation with an unbiased, full agonist, isoproterenol. Stimulation with isoproterenol resulted in phosphorylation of 13 different sites at the receptor. This result suggested that whereas isoproterenol stimulation recruited GRK2 and GRK6, carvedilol treatment only recruited GRK6 indicating biased ligands induce distinct receptor conformations and are able to recruit distinct GRKs.

U2OS cells were chosen as they did not endogenously express the histamine H_4 receptor. Initial experiments involved using flow cytometry to enrich the populations of cells stably expressing the venus-tagged H_4R. The nominal molecular mass of the H_4R is ~40 kDa and nominal the molecular weight of the venus tag was approximately ~27 kDa, in this study we see a polypeptide that runs at ~75 kDa on 10% SDS-PAGE gels. The higher molecular weight of the receptor may be explained by post-translational modifications such as palmitoylation, glycosylation, and phosphorylation. The H_4R has two potential glycosylation sites in its N-terminus and a potential palmitoylation site in its C-terminus.

After enriching the population of cells, ^{32}P-labelling experiments showed that the H_4R is phosphorylated under basal conditions, and shows a robust increase in phosphorylation upon stimulation with histamine. Histamine and JNJ7777120 have both been shown to recruit arrestin albeit with histamine acting as a full agonist, and JNJ7777120 acting as a partial agonist. Despite this, stimulation of the receptor with JNJ7777120 did not increase the global phosphorylation of the receptor. This study highlights the difference in phosphorylation events at the receptor depending on the ligand used to stimulate the receptor and goes some way in adding credence to the hypothesis that different ligands can cause differential patterns of phosphorylation (Butcher *et al.,* 2011; Nobles *et al.,* 2011).

The use of mass spectrometry has allowed us to highlight the complexity of GPCR phosphorylation; our studies have demonstrated that the H_4R is phosphorylated at 13 residues in its third intracellular loop and C-terminal tail. These residues are seen when the receptor is under basal conditions, and indicates that the H_4R has high basal constitutive activity, an observation corroborated by a recently published study (Wifling *et al.,* 2015). Interestingly, stimulation of the receptor with histamine or JNJ7777120 resulted in less phosphorylation sites being observed in our mass spectrometry studies. As our studies are not quantitative we cannot state whether this is the result of receptor dephosphorylation, a phenomenon described in previous studies from our lab using the M_3 mAchR stably expressed in CHO cells (Butcher *et al.,* 2011). The absence of an observed phosphorylated residue in a mass spectrometry experiment is not confirmation that the receptor is no longer phosphorylated at this particular site, only that the peptide was not observed. To extend these studies and be able to draw firm conclusions, growing cells in SILAC (stable isotope labelling by amino acids in cell culture) medium, and then performing mass spectrometry experiments with labelled receptors is an alternative method for investigating the phosphorylation pattern of the receptor as it provides a quantitative proteomics approach.

Biased ligands of GPCRs which prefer signalling through β-arrestin but not through a G-protein-mediated pathway have previously been shown to induce discrete conformational changes in β-arrestin which are distinct from conformations induced by unbiased agonists using an intramolecular BRET-based biosensor (Shukla *et al.,* 2008). That the β-arrestin-biased ligand, JNJ7777120, can potentially trigger a pattern of phosphorylation which is distinct from that obtained with histamine would be consistent with these findings. JNJ7777120 shows high affinity for the H_4R and has been used to elucidate the role of the H_4R in several allergic and inflammatory processes (Ling *et al.,* 2004; Thurmond *et al.,* 2004; Dunford *et al.,* 2006). The unique signalling mediated by JNJ7777120 suggests a potentially new avenue which to explore with regards to anti-inflammatory drugs (Rosethorne and Charlton 2011).

Previous studies have demonstrated that different patterns of phosphorylation have led to different signalling outcomes. Using the β_2-adrenergic receptor, a study successfully demonstrated that the kinetics of arrestin recruitment to the receptor was dependent on which GRK was involved in receptor phosphorylation (Nobles *et al.,* 2011). In studies which investigated the phosphorylation of the CCR7, GRK6-mediated phosphorylation

was shown to promote ERK1/2 activation, but concomitant GRK3 and GRK6 activity was needed to cause receptor internalisation and desensitisation (Zidar *et al.,* 2009). Furthermore, it was found that members of the GRK family can phosphorylate different sites on the CXCR4 leading to different signalling outcomes (Busillo *et al.,* 2010). The M_3 mAchR has been shown to be phosphorylated by several kinases (Torrecilla *et al.,* 2007; Luo *et al.,* 2008; Willets *et al.,* 2001; Tobin *et al.,* 1997). Phosphorylation by these kinases leads to different signalling outcomes for the receptor. Extension of these studies also found that agonists with varying efficacies to the M_3 mAchR could drive preferential receptor phosphorylation events (Butcher *et al.,* 2011). Our work with the H_4R may indicate this, and it may certainly be considered as consistent with this notion.

Receptor internalisation of GPCRs is generally dependent on receptor phosphorylation and the recruitment of arrestins to the receptor (Ferguson, 2001). The functional activity of the H_4R was examined by measuring ligand-induced internalisation. We show that the H_4R undergoes internalisation after treatment with histamine and JNJ7777120. That both ligands induce internalisation is not surprising as both have been shown to couple to arrestin in an agonist-mediated manner (Rosethorne and Charlton 2011). However, this study shows histamine acts as a full, agonist in an arrestin recruitment assay while JNJ7777120 acts as a partial agonist. Our data suggests the level of internalisation mediated by histamine and JNJ7777120 to be comparable after 5 and 30 min of agonist stimulation. This may suggest the partial recruitment of arrestin by JNJ7777120 to be enough to cause receptor internalisation. But further work is needed to derive more firm conclusions such as the use of a more quantifiable assay. One such assay would be to use an ELISA based assay and antibodies raised against an N-terminally tagged receptor. Extending these studies, phosphorylation-specific antibodies could also be raised to certain phosphorylated residues allowing us to focus on individual sites of phosphorylation. This would allow us to not only investigate recombinant expression systems but also primary cells and tissue to explore in detail the "phosphorylation barcode" hypothesis (Tobin *et al.,* 2008; Tobin 2008). Furthermore, a receptor can be mutated in such a way that it becomes inherently biased. This can be done by replacing phosphoacceptor sites in the third intracellular loop and C-terminal tail with residues which cannot become phosphorylated (usually alanine residues). This results in a receptor which is no longer able to efficiently recruit and/or signal through β-arrestin, but retains its ability to signal through G-proteins (Kong *et al.,* 2010; Poulin

et al., 2010). It may be beneficial to this study to further characterise the signalling response which is mediated by H_4R phosphorylation by creating phosphorylation-deficient mutants. Biasing a receptor in this way would allow the delineation of downstream pathways that may be known to give rise to adverse effects. Understanding the role of H_4R phosphorylation provides a basis for the design of H_4R ligands that may drive specific signalling outcomes (Rajagopal *et al.,* 2010). Like JNJ7777120, these drugs have been termed biased agonists and can potentially drive specific physiological outcomes by preferentially engaging a distinct subset of receptor signalling pathways. Indeed, JNJ7777120 has provided the basis for other biased ligands which preferentially couple to either $G\alpha_i$ proteins or β-arrestin (Nijmeijer *et al.,* 2013).

In conclusion, our work supports the hypothesis that one mechanism by which biased ligands may direct receptor signalling is their capacity to differentially drive receptor phosphorylation. We also show that the H_4R can become internalised upon binding of histamine and JNJ7777120.

Chapter 4 | Characterisation of the Free Fatty Acid 4 Receptor and Its Downstream Signalling

4.1. Introduction

GPCRs are highly regulated proteins, and the exposure of the receptor to agonist results in rapid desensitisation. A central mechanism in this process is receptor phosphorylation (Lohse, *et al.,* 1990; Lohse, *et al.,* 1990; Tobin *et al.,* 2008; Tobin, 2008). Whereas our results in the previous chapter show extensive phosphorylation throughout the third intracellular loop and C-terminal tail of the H_4R, the free fatty acid receptor 4 (FFA4) demonstrates phosphorylation of residues that are limited to the C-terminal tail. This chapter aims to delineate the importance of these phosphorylation sites and characterise the downstream signalling of FFA4 in a recombinant cell system.

Omega-3 polyunsaturated fatty acids (n-3 PUFAs) are prescribed for the prevention or secondary prevention of myocardial infarction and the treatment of hypertriglyceridemia (Davidson *et al.,* 2007; Hoy and Keating 2009). These n-3 PUFAs show greater potency at the FFA4 compared with other FAs tested at the receptor (Hirasawa *et al.,* 2005), possibly implicating this receptor in heart health.

The FFA4 receptor is a $G\alpha_{q/11}$-coupled receptor activated by long chain fatty acids (LCFAs). Studies have suggested FFA4 may be involved in protection from obesity and improved insulin sensitivity (Oh *et al.,* 2010; Ichimura *et al.,* 2012). These metabolic effects (insulin secretion, satiety and improved glycaemic control) have been linked, in part, to the FFA4-dependent incretin release from enteroendocrine cells, particularly GLP-1 (Cornall *et al.,* 2014; Hirasawa *et al.,* 2005). FFA4 expression is seen to increase in macrophages in response to obesity. Reduced pro-inflammatory gene expression in M1 macrophages and amplifications of anti-inflammatory genes in M2 macrophages is thought to be a result of FFA4 activation. Certainly, a decreased infiltration of macrophages into adipose tissues is seen in FFA4 KO mice due to decreased chemotaxis (Oh *et al.,* 2010). It is has been shown these anti-inflammatory effects are via arrestin signalling at the FFA4 receptor where arrestin-3 interacts with TAB1 which inhibits LPS- and TNF-α-induced TAK1 stimulation effectively blocking toll-like receptor 4 and the TNF-α inflammatory pathways (Oh *et al.,* 2010; Halder *et al.,* 2013; Li *et al.,* 2013).

The initial lack of FFA4-specific agonists hindered the full characterisation of the receptor, however, TUG-891 has been described as a potent and selective FFA4 agonist (Shimpukade *et al.,* 2012; Hudson *et al.,* 2013) and shows a nanomolar order potency to the receptor. TUG-891 has been shown to activate Ca^{2+} and ERK1/2 responses and arrestin recruitment. TUG-891 has also been implicated in glucose uptake in 3T3-L1 derived adipocytes, secretion of GLP-1 from STC-1 cells, and anti-inflammatory effects mediated by macrophages (Hudson *et al.,* 2013; Shimpukade *et al.,* 2012).

The FFA4 has only recently become deorphanised, but its function has been linked to several disease states such as obesity, type-2 diabetes, and inflammation-related diseases. This warrants further investigations of the receptor, and therefore, the aim of this chapter is to characterise the downstream signalling events which are mediated by the wild-type FFA4. We also aim to characterise the downstream signalling of phosphorylation-deficient mutants which have had sites of phosphorylation removed from the C-terminus of the receptor based on previous mass spectrometry studies. These mutations result in receptor mutants which should show decreases in coupling to arrestin and would, therefore, allow us to determine arrestin-mediated downstream signalling pathways. This information would be important in the design of new FFA4-specific ligands.

4.2. Results

4.2.1. Metabolic labelling of FFA4 and investigating global phosphorylation

The FFA4 receptor is known to become phosphorylated on 5 putative residues in its C-terminal tail (Butcher *et al.,*, 2014). It is known that removing these serine and threonine residues results in the receptor mutant (FFA4-TSSST/AAAAA) (**Figure 4.1A**) resulting in a receptor unable to become phosphorylated. Upon stimulation with the FFA4 receptor-specific agonist TUG-891, we see an enhanced phosphorylation of the FFA4-wt receptor as detected by increased incorporation of ^{32}P into a polypeptide with a molecular mass of ~45-50 kDa in CHO cells stably expressing the HA-tagged FFA4-wt receptor (**Figure 4.1B**). As predicted, there is no visible phosphorylation of the FFA4-TSSST/AAAAA mutant indicating a lack of ^{32}P incorporation into the polypeptide corresponding to this receptor isolated from CHO cells stably expressing the FFA4-TSSST/AAAAA receptor (**Figure 4.1B**). Total proteins levels for both receptors indicate equal loading (**Figure 4.1C**). This study suggests the five serine and threonine residues in the C-terminal tail of FFA4 are solely responsible for agonist-induced increases in phosphorylation at the receptor.

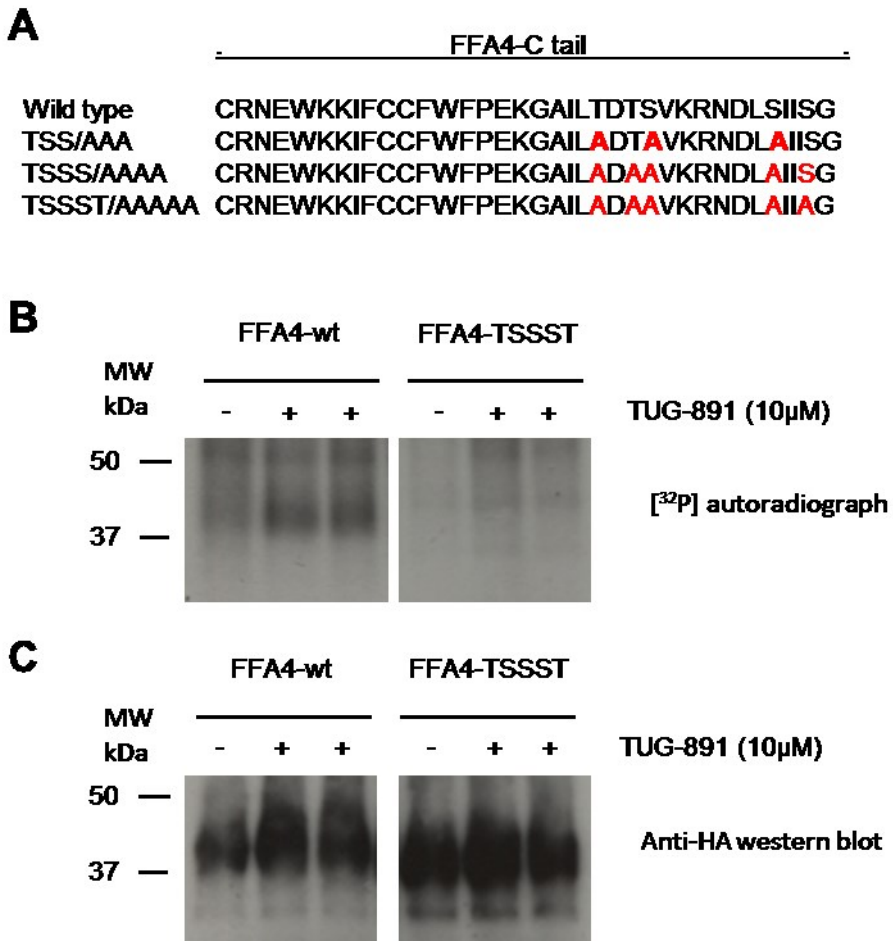


Figure 4.1| Global phosphorylation of FFA4 receptor under basal conditions and in response to agonist stimulation. Cells stably expressing C-terminally HA-tagged FFA4 were labelled with ^{32}P-orthophosphate and then stimulated with the FFA4-selective agonist TUG-891 (10 μM) or vehicle for 5 min. Receptors were purified by immunoprecipitation and resolved on SDS-PAGE minigels as described in *Material and Methods*. (A) shows the primary amino acid sequence of the C-terminal tail of FFA4, in *red* are the residues which are mutated to Ala to create the phosphorylation-deficient receptors. Phosphorylated FFA4-wt and total phosphorylation-deficient receptors were visualised by autoradiography (B), protein levels were detected using HA protein-specific antisera (C). Autoradiograph and immunoblot represents a single experiment.

4.2.2. Arrestin-3 interaction with the C-terminal tail of the FFA4

It is known that FFA4 interacts with arrestin-3 in an agonist-dependent manner (Hudson *et al.,* 2013). To investigate the impact of phosphorylation on this interaction, mutants were created which removed three, four or five key sites of agonist-mediated phosphorylation (TSS/AAA, TSSS/AAAA, and TSSST/AAAAA, respectively). When tested in a BRET-based arrestin-3 interaction assay, 5 min of TUG-891 (10 μM) stimulation resulted in a large increase in arrestin-3-RLuc/FFA4-eYFP BRET, increasing the basal signal from 18.41 ± 2.45 milli-BRET units to the TUG-891-stimulated value of 166.0 ± 8.26 milli-BRET units. Extension of these studies to test the phosphorylation deficient mutants demonstrated that the sequential removal of residues able to become phosphorylated produce clear reductions in BRET signal which correlated with the extent of residue removal (**Figure 4.2**). The FFA4-TSS/AAA response to the TUG-891 (10 μM) stimulated value was 58.78 ± 4.21% ($p < 0.01$) of wild type (**Figure 4.2B**), and for the FFA4-TSSS/AAAA mutant was 32.16 ± 6.62 % ($p < 0.001$) of wild type FFA4 (**Figure 4.2C**). Although, it is interesting to note that complete removal of the phosphorylation sites still results in a receptor mutant (FFA4-TSSST/AAAAA) which still demonstrates a BRET signal of 19.68 ± 3.36% ($p < 0.0001$) of the wild-type FFA4 receptor signal (**Figure 4.2D**). This highlights the importance of FFA4 phosphorylation in recruiting arrestin, an important protein in receptor desensitisation and downstream signalling, but also demonstrates other elements which play a role in recruitment of arrestin to the receptor. These experiments also demonstrated that the mutant receptors showed a changes in potency (for TUG-891 at the wild type FFA4, pEC_{50} = 7.09 ± 0.10, for FFA4-TSS/AAA, pEC_{50} = 7.43 ± 0.24, for FFA4-TSSS/AAAA, pEC_{50} = 5.39 ± 0.52, and for FFA4-TSSST/AAAAA, pEC_{50} = 6.26 ± 0.31) for arrestin-3 recruitment measured after 5 min in the BRET assay.

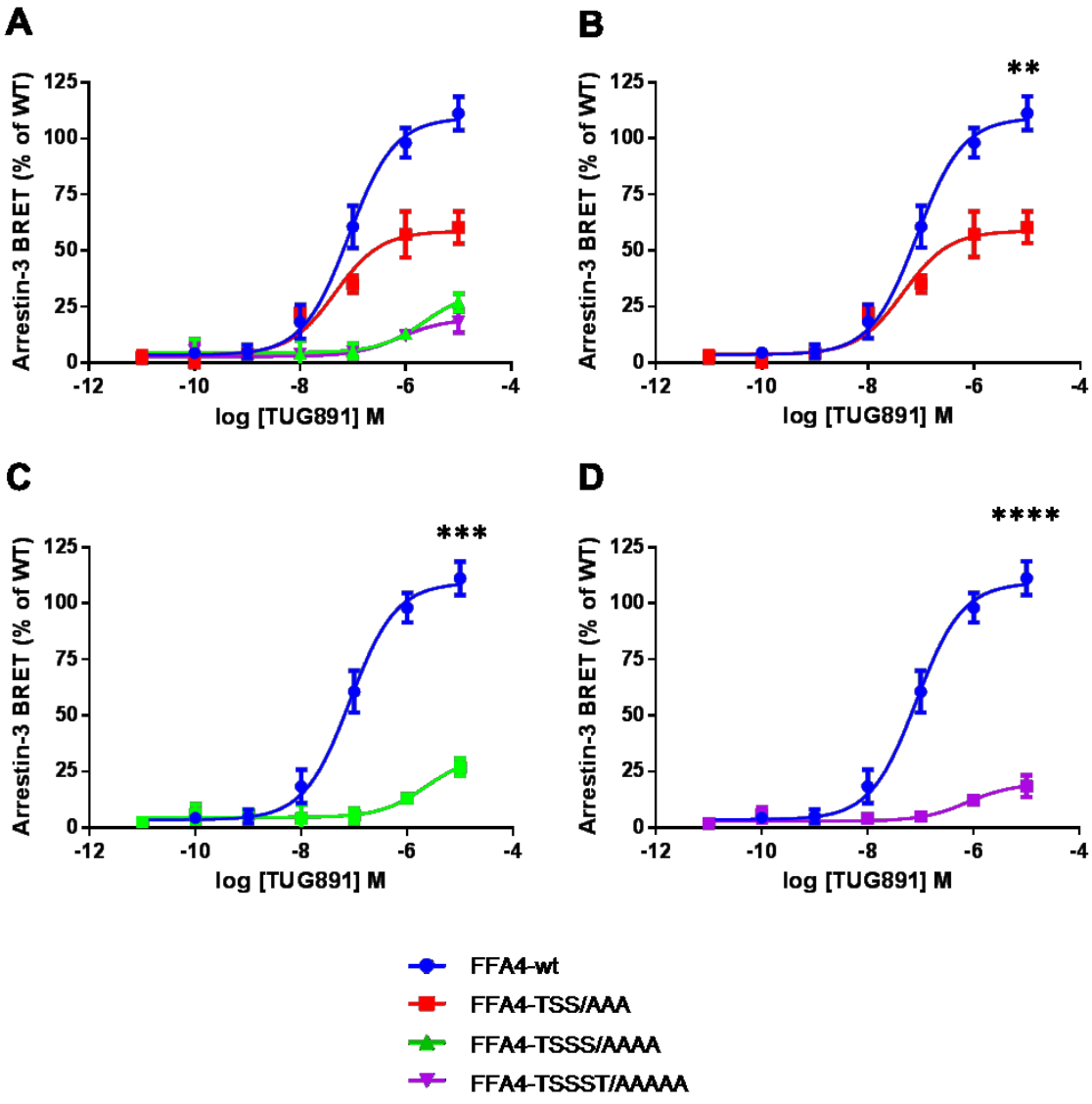


Figure 4.2| Residues involved in TUG-891-regulated phosphorylation in the C-terminal tail of FFA4 play an important but not exclusive role in agonist-induced FFA4/arrestin-3 interactions. HEK 293 cells were transiently transfected with DNA corresponding to the FFA4-wt receptor or mutations of FFA4 which eliminated three, four, or five key sites of agonist-mediated phosphorylation (TSS/AAA, TSSS/AAAA, and TSSST/AAAAA, respectively). These cells were then used in BRET-based arrestin-3 interaction studies in response to TUG-891 and compared with FFA4-wt. (A) the interactions of arrestin-3 are shown for wild-type FFA4 and the phosphorylation-deficient mutants. The interactions of arrestin-3 are then shown for the wild-type FFA4 and compared to each mutant individually (B-D). Data points represent the mean ± S.E.M of three independent experiments. **P <0.01, ***P <0.001, ****P <0.0001; as measured using a one way ANOVA with Sidak's multiple comparisons test.

4.2.3. Characterisation of a phosphospecific antibody against FFA4

A phosphospecific antibody was generated to a doubly phosphorylated peptide containing phosphates on residues corresponding to Thr347 and Ser350 of FFA4 (**Figure 4.3A**). This phosphopeptide was chosen based on previous mass spectrometry data which strongly suggested both these residues were phosphorylated concurrently and also because it was posited this double phosphopeptide would be highly immunogenic. The resulting antibody produced were purified and then tested on membrane preparations from cells stably expressing the FFA4-wt receptor or a phospho-deficient mutant (FFA4-TSS/AAA) which lacked the Thr347 and Ser350 residues in its C-terminal tail. **Figure 4.3B** (upper panel) shows that the antibody readily recognises the phosphorylated receptor upon TUG-891 treatment. Importantly, the band corresponding to phosphorylated FFA4 in immunoblots of stably expressing the FFA4-TSS/AAA receptor was absent. From **Figure 4.3B** (lower panel), we also see that FFA4 phosphorylation causes a slight increase in the molecular weight of the receptor upon TUG-891 treatment, this increase in molecular weight is not as significant in the lane corresponding to the FFA4-TSS/AAA receptor upon agonist treatment.

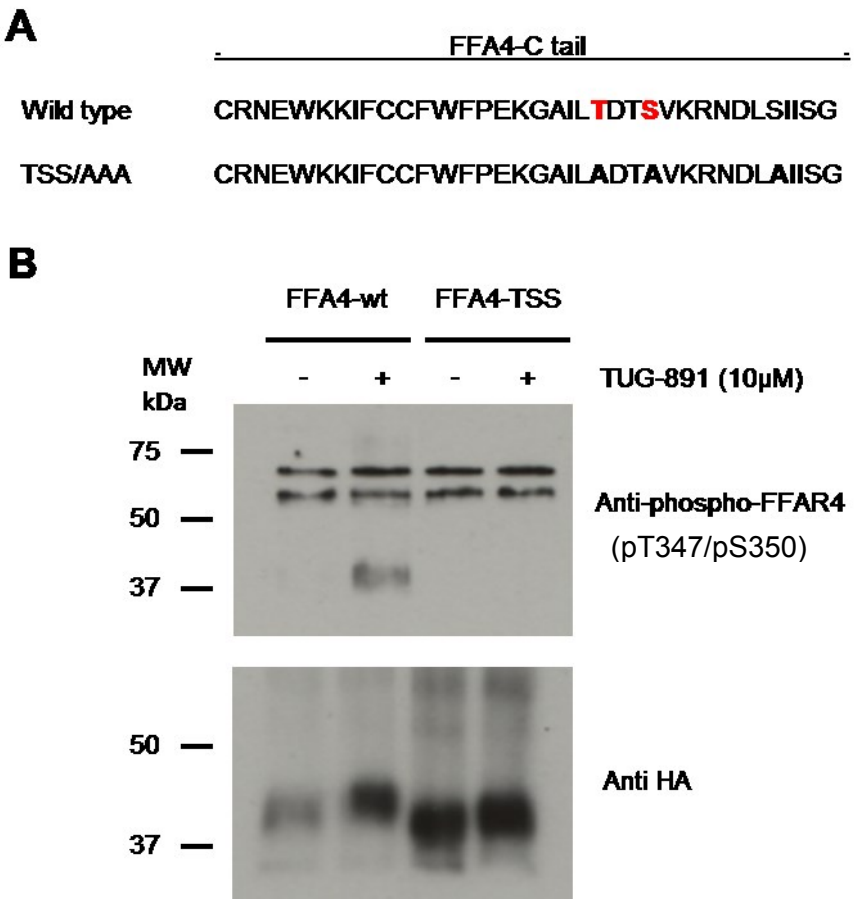


Figure 4.3| Use of an FFA4 phospho-specific antiserum. Membrane preparations from CHO cells stably expressing either the FFA4-wt receptor or the phospho-deficient mutant FFA4-TSS/AAA were run on minigels and probed with antiserum raised to identify Thr(P)347, Ser(P)350 within the C-terminal tail of the human FFA4 receptor. (A) indicates the C-terminal tail of FFA4, in *bold* are the residues which are mutated to Ala to create a semi-phosphorylation-deficient receptor, in *red* are the phosphorylated residues recognised by the antiserum. (B) Samples from the FFA4-wt and the FFA4-TSS/AAA receptors treated with vehicle or TUG-891 (10 μM) for 5 min were resolved and immunoblotted with the phosphospecific antibodies (*upper panel*). Loading controls (*B, lower panel*) represents the same samples immunoblotted to detect the HA-tag. Immunoblots represent a single experiment.

4.2.4. Kinetics of Akt and ERK phosphorylation mediated by FFA4

Akt (also known as Protein kinase B) is a serine-threonine protein kinase that plays important roles in several cellular processes including growth, proliferation, and apoptosis. GPCRs have long been known to modulate Akt function, one study which employed a recombinant system to express the M_1 and M_2 muscarinic receptors showed both receptors which are coupled to $G\alpha_q$-proteins and $G\alpha_i$-proteins, respectively, induced agonist-mediated activation of Akt (Murga *et al.,* 1998). This study went on to conclude that $G\alpha_i$-protein coupled receptors signal primarily through $\beta\gamma$ dimers, $G\alpha_q$-protein coupled receptor utilise both $\beta\gamma$–dependent and –independent mechanisms, the independent mechanism here being $G\alpha_q$-proteins. Phosphatidylinositol 3-kinase (PI3K) activation results in the generation of phosphatidylinositol 3, 4, 5-triphosphate (PIP3) at the plasma membrane and leads to recruitment of proteins which contain pleckstrin homology (PH) domains such as PDK1 (pyruvate dehydrogenase lipoamide kinase isozyme 1) and Akt. This recruitment leads to the phosphorylation of Thr308 and partial activation of Akt by PDK1 (Alessi *et al.,* 1997; Stokoe *et al.,* 1997). Further phosphorylation of Akt requires phosphorylation of its Ser473 by mTORC2 (mechanistic target of rapamycin complex 2) (Sarbassov *et al.,* 2005).

Akt activation has also been shown to be modulated by FFA4 such as in macrophage-mediated inflammatory processes; here Toll-like receptor 4 signalling via the Akt/JNK pathway causes an upregulation of COX-2 which goes on to convert arachidonic acid to prostaglandin H_2, a precursor to prostanoids that regulate inflammation (Li *et al.,* 2013). In this study, activated FFA4 coupled to arrestin-3 repressed the Akt/JNK signalling pathway (Li *et al.,* 2013), indicating FFA4 modulation of COX-2 expression. ERK1/2 phosphorylation has also been shown to be mediated by FFA4 (Suzuki *et al.,* 2008); this activation can be induced in either a G-protein-dependent or –independent manner (Shenoy *et al.,* 2006). It has been suggested the G-protein-independent aspect of this activation has a different time-course than the G-protein-dependent activation of ERK whereby the activation by the former is transient and the activation of ERK by the latter process is sustained and persists even after the receptor has internalised (Luttrell and Gesty-Palmer 2010). Investigating the kinetics of ERK activation would allow us to suggest how it is being activated via the FFA4 receptor, if this activation is rapid and transient then it would suggest $G\alpha_q$-proteins are likely responsible for ERK activation. If the response persists for extended periods of time then we can suggest an arrestin-

mediated signalling complex causes ERK activation. To this end, we performed a time-course experiment to investigate the kinetics of Akt and ERK phosphorylation by the FFA4 receptor. We saw clear, agonist-mediated Akt phosphorylation at Ser473 as measured by immunoblot analysis (**Figure 4.4A**). This increase peaked at 2 and 5 min of stimulation, after which phosphorylation of Akt was seen to decrease and return to basal levels after 45 min of stimulation. The time course observed for ERK phosphorylation was slightly different (**Figure 4.4B**); a clear, agonist-mediated increase of phosphorylation was observed and peaked at 5 min followed by a waning of the response. However, this response did not return to basal levels after 60 min of stimulation.

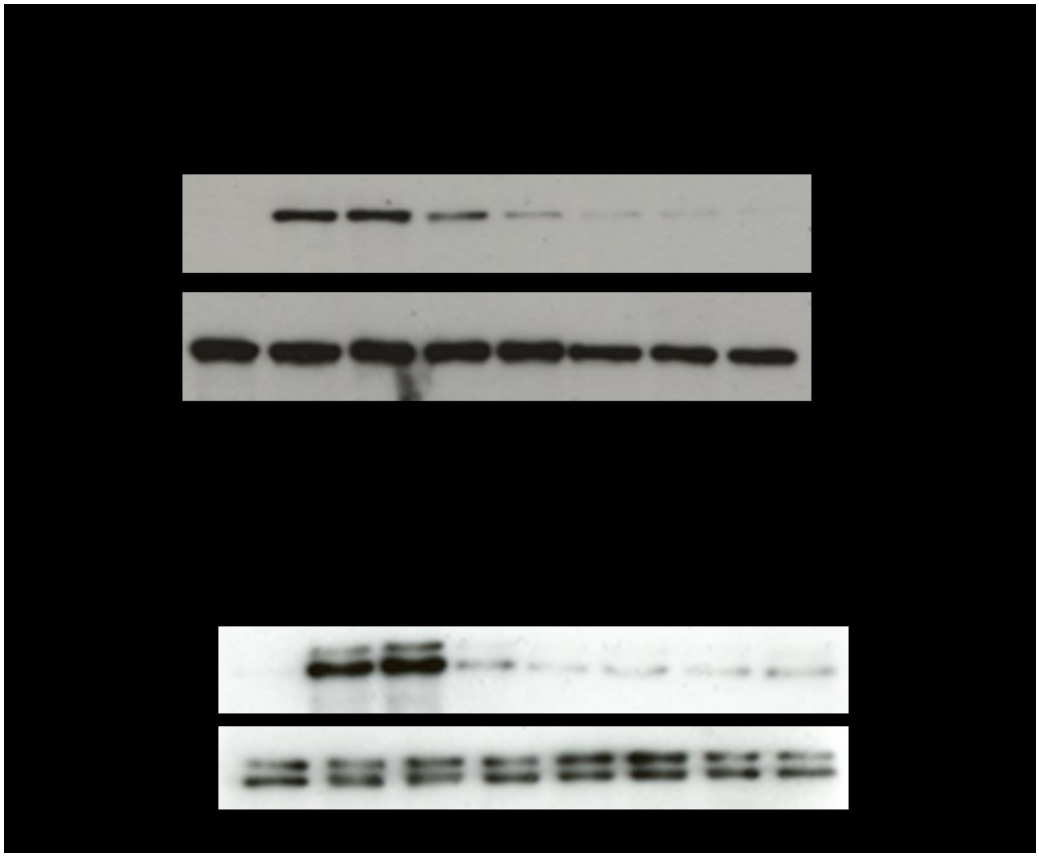


Figure 4.4| Kinetics of Akt and ERK phosphorylation. Cells stably expressing the FFA4-wt receptor were treated with TUG-891 (10 μM) for the times indicated. Cells were lysed and whole cell lysates were resolved on minigels as described in *Material and methods.* (A) Phosphorylation of Akt was identified using a phospho-specific antibody which detects phosphorylation on Serine473 (*A, lower panel*) shows the loading control representing the same samples immunoblotted to detect total Akt. (B) The phosphorylation of ERK was detected using a phospho-specific antibody (*B, lower panel*) shows total ERK protein. Immunoblots are representative of three independent experiments

4.2.5. Characterising the downstream Akt response of FFA4

It has been shown in previous studies, that arrestin interacts with the FFA4 in an agonist-dependent manner and that mutation of key serine and threonine residues in the C-terminal tail which are involved in agonist-mediated phosphorylation decreases arrestin recruitment to the receptor (**Figure 4.2**). Here, we have investigated whether Akt phosphorylation was mediated by FFA4 activation associated with G-protein-dependent or –independent signalling processes. CHO cells stably expressing the FFA4-wt receptor or FFA4 receptor mutants in which three, four, or five key sites of agonist-mediated phosphorylation have been changed to alanine (TSS/AAA, TSSS/AAAA, and TSSST/AAAAA, respectively) were treated with the FFA4-specific agonist TUG-891. A strong, agonist-mediated increase in phosphorylation of Akt at Ser473 was observed indicating that the kinase has become active. Treatment of the phosphorylation deficient mutants with TUG-891, results in an activation/phosphorylation of Akt, but to a lesser extent that the wild type FFA4 (**Figure 4.5A**).

University of Bonn-Gq inhibitor compound (UBO-QIC) is naturally derived agent extracted from *Ardisia crenatasims* (Fujioka *et al.,* 1988). It is a cyclic depsipeptide which has been shown to inhibit human platelet aggregation (Inamdar *et al.,* 2015), and has been successfully used to characterise signalling pathways downstream of $G\alpha_q$-protein coupled receptors (Jacobsen *et al.,* 2013; Bernard *et al.,*, 2014). UBO-QIC works by directly binding to $G\alpha_q$-proteins and inhibiting the release of GDP. Unpublished data from our laboratory showed that 1 μM pre-incubated for 30 min inhibited the signalling of a $G\alpha_q$-protein coupled receptor. Using an HTRF based assay; we have successfully measured changes in Akt phosphorylation at Ser473 upon TUG-891 stimulation of cells stably expressing the FFA4-wt receptor. This resulted in an increase in Akt phosphorylation which was statistically significant when compared to basal levels of Akt phosphorylation (**Figure 4.7C** and **Figure 4.7D**). Cells were subsequently pre-treated with UBO-QIC (1 μM), a $G\alpha_q$-protein inhibitor, for 30 min prior to TUG-891 stimulation. This stimulation had no impact on the ability of Akt to become activated in response to FFA4 (**Figure 4.7C** and **Figure 4.7D**), suggesting that Akt activation via the FFA4 receptor is through a mechanism which does not involve the G_q family of G-proteins.

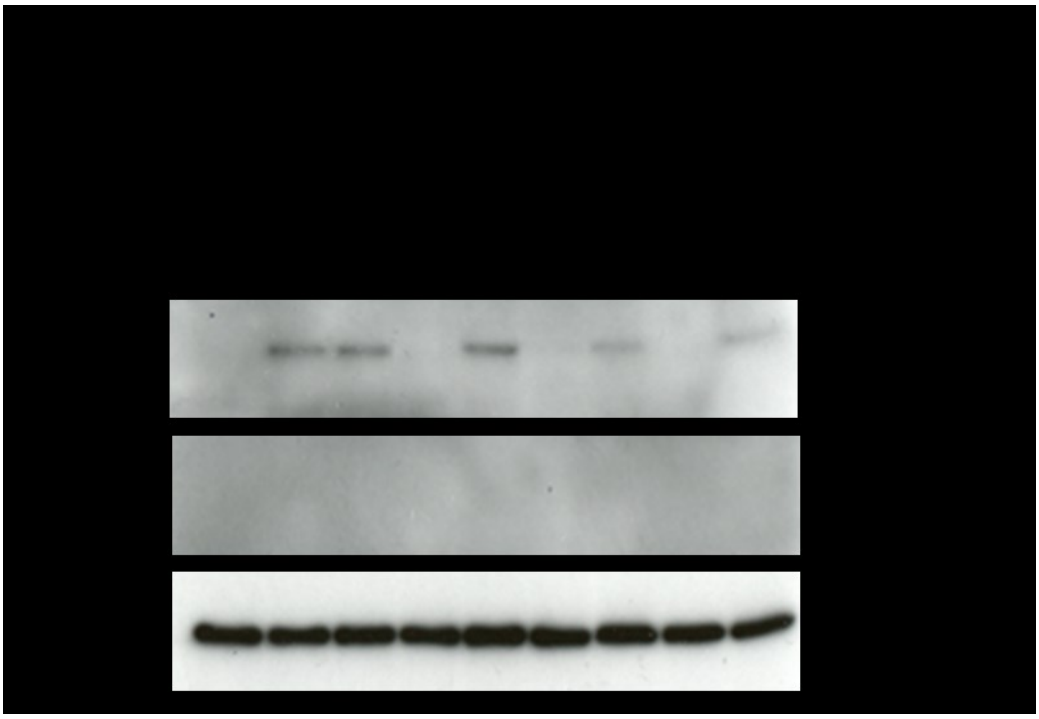


Figure 4.5| Residues involved in TUG-891-regulated phosphorylation in the C-terminal tail of FFA4 play an important but not exclusive role in agonist-induced Akt phosphorylation. CHO cells stably expressing the FFA4-wt receptor or FFA4 receptor mutants which eliminated three, four, or five key sites of agonist-mediated phosphorylation (TSS/AAA, TSSS/AAAA, and TSSST/AAAAA, respectively) were stimulated with TUG-891 (10 μM) and whole cell lysates resolved on minigels. Immunoblots were probed with phospho-specific antibodies against two phosphorylation sites present on Akt *(A, upper and middle panels*), total Akt was identified *(A, lower panel*) and was used as a loading control. Immunoblots are representative of three independent experiments.

4.2.6. Characterising the downstream ERK response of FFA4

The activation of ERK can be downstream of G-protein signalling or can be independent of G-protein signalling (Luttrell and Gesty-Palmer 2010). To test whether there is an arrestin component mediating a part of ERK activation, we have used phosphorylation-deficient mutants of FFA4 which are also deficient in their coupling to arrestin which were stably expressed in CHO cells. Immunoblotting analysis revealed a clear, agonist-mediated increase in ERK phosphorylation upon treatment of FFA4 expressing cells with TUG-891 over basal ERK phosphorylation (**Figure 4.6A**). The phosphorylation-deficient mutants exhibited a similar fold increase in basal ERK phosphorylation as assessed by immunoblot analysis (**Figure 4.6A**).

To further probe the mechanism of ERK activation via the FFA4 receptor, an HTRF assay was used which measured activation/phosphorylation of ERK. Using this assay, cells were stimulated for 5 min and a robust TUG-891-mediated increase in phosphorylated ERK was observed which was statistically significant when compared to basal levels (**Figure 4.7A** and **Figure 4.7B**). The use of the $G\alpha_q$-protein inhibitor, UBO-QIC (1 μM) for 30 min blocked this rise in agonist-mediated ERK phosphorylation to levels below those at basal in a statistically significant manner which suggests that the ERK phosphorylation response which results from FFA4 activation for 5 min is primarily mediated by the G_q family of G-proteins (**Figure 4.7A** and **Figure 4.7B**). To further investigate the ERK response in the FFA4-wt receptor as well as the phosphorylation-deficient mutants, the HTRF assay was utilised to perform concentration-response experiments using TUG-891 stimulation for 5 min to induce ERK phosphorylation. ERK phosphorylation is seen to increase in a concentration-dependent manner for all four receptors (**Figure 4.8A**). Interestingly, the greater the number of phosphorylation sites mutated in the C-tail of the FFA4 receptor, the greater the increase in the amount of p-ERK measured by the assay. Using the same concentrations of TUG-891, but this time pre-treating the cells with UBO-QIC (1 μM) for 30 min, results in a complete abolition of any response in ERK phosphorylation from the cells (**Figure 4.8B**).

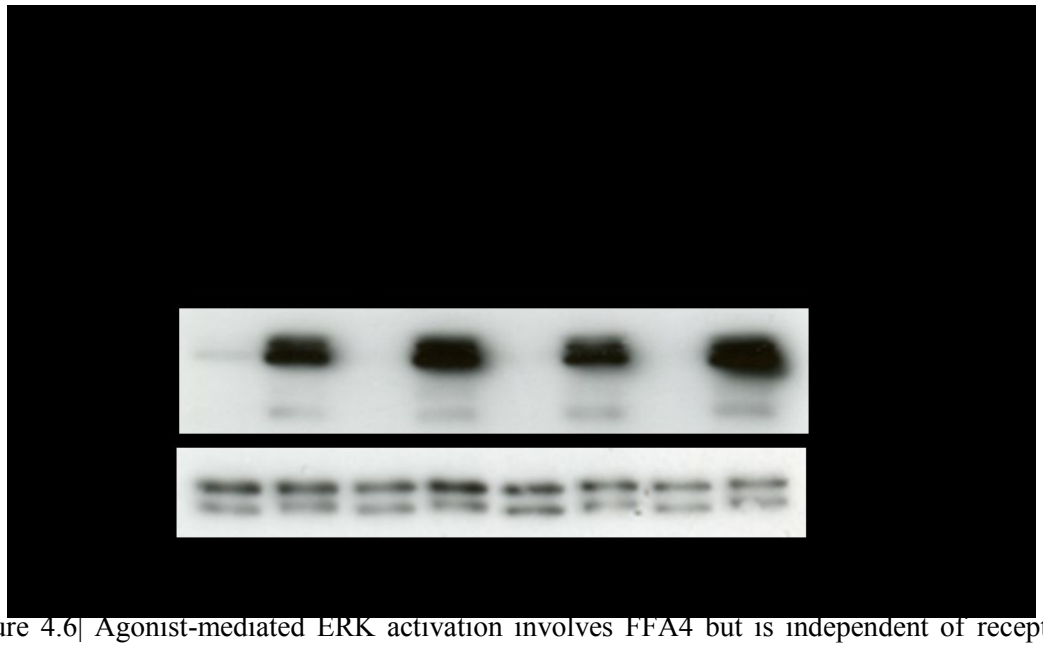


Figure 4.6| Agonist-mediated ERK activation involves FFA4 but is independent of receptor phosphorylation. CHO cells stably expressing the FFA4-wt receptor or FFA4 receptor mutants which eliminated three, four, or five key sites of agonist-mediated phosphorylation (TSS/AAA, TSSS/AAAA, and TSSST/AAAAA, respectively) were stimulated with TUG-891 (10 μM) and whole cell lysates resolved on minigels. Immunoblots were probed with phospho-specific antibodies against phosphorylation sites present on ERK *(A, upper panel*), total ERK was identified *(A, lower panel*) and was used as a loading control. Immunoblots are representative of three independent experiments.

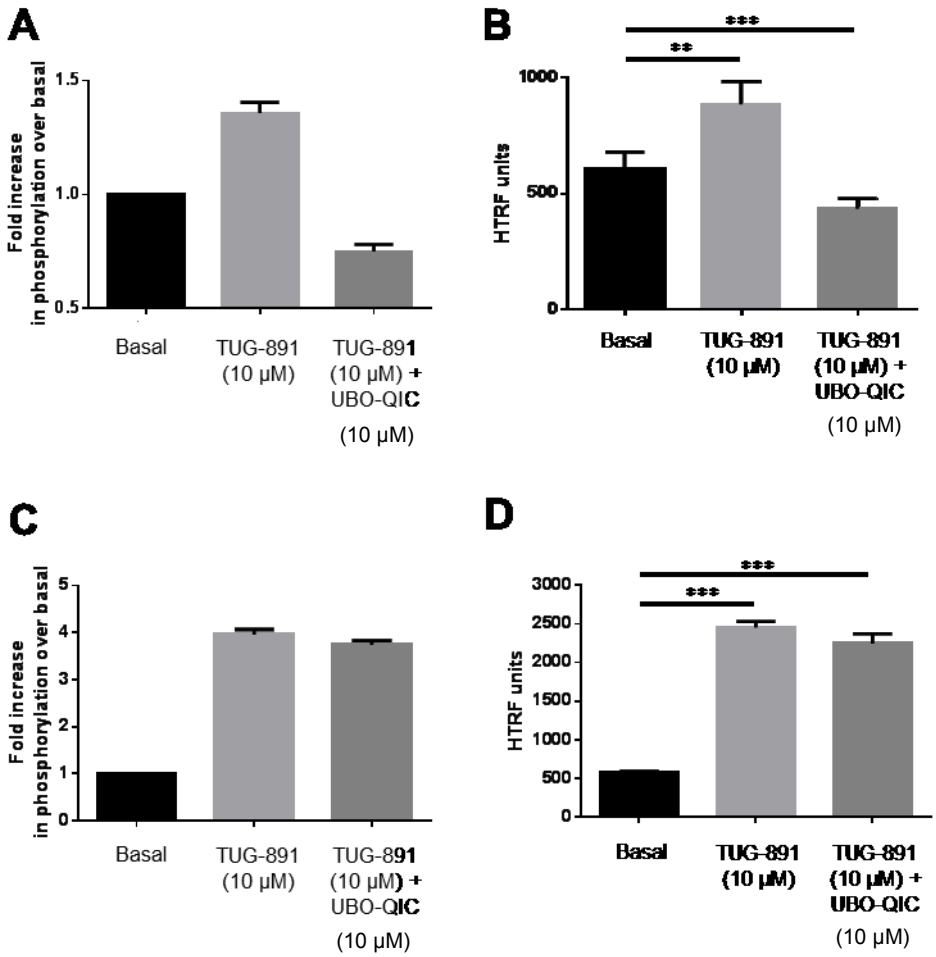


Figure 4.7| UBO-QIC, a Gαq-protein inhibitor blocks the activation of ERK but not Akt. Serum starved CHO cells stably expressing the FFA4-wt receptor were pre-incubated with UBO-QIC (10 µM) for 30 min prior to stimulation with TUG-891 (10 µM) for 5 min. Cells were lysed and cell lysates were assayed for phosphorylated ERK1/2 or phosphorylated Akt using HTRF assay kits. Fluorescence was measured using a ClarioStar plate reader. (A) shows fold increase of ERK phosphorylation over basal conditions upon stimulation with TUG-891 (10 µM) with or without pre-treatment of the cells with UBO-QIC (1 µM). (B) shows the raw data from (A). (C) shows fold increase of Akt phosphorylation over basal conditions upon stimulation with TUG-891 (10 µM) with or without pre-treatment of the cells with UBO-QIC (1 µM). (D) shows the raw data from (C). Graphs are representative of three independent experiments performed in duplicate. *Error bars* represent S.E.M.**P <0.01, ***P <0.001; as measured using a one way ANOVA with Sidak's multiple comparisons test.

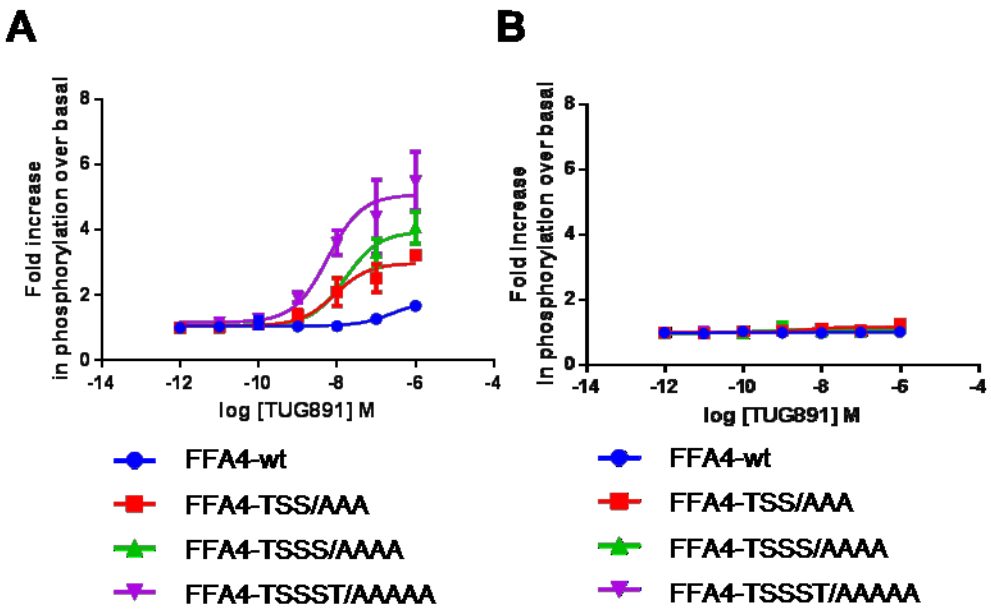


Figure 4.8| Mutations of key sites of agonist-regulated phosphorylation affects Gq-protein-mediated ERK activation. Serum starved CHO cells stably expressing the FFA4-wt receptor were pre-incubated with UBO-QIC (10 μM) or vehicle for 30 min prior to stimulation with TUG-891 for 5 min. Cells were lysed and cell lysates were mixed with the appropriate antibody solutions and incubated at room temperature for 2 hr. Fluorescence was measured using a ClarioStar plate reader. (A) shows concentration response curves for TUG-891 to invoke ERK1/2 phosphorylation with pre-treatment of the cells with vehicle. (B) shows concentration response curves for TUG-891 to invoke ERK1/2 phosphorylation with pre-treatment of the cells with UBO-QIC (10 μM). Graphs show the pooled results of at least three independent experiments. Error bars represent S.E.M.

4.2.7. Investigating potential interactions between arrestin-3 and Akt

Our previous results suggest a link between receptor phosphorylation, arrestin-3 recruitment to the FFA4 receptor and Akt phosphorylation (**Figure 4.5A**). Arrestins are versatile adapter and scaffold proteins that can form complexes with many signalling proteins trafficking signalling molecules into proximity with each other and the receptor (Pierce and Lefkowitz 2001). To investigate whether Akt activation via FFA4 occurs via direct interaction of Akt with arrestins, co-immunoprecipitation experiments were carried out on FFA4 expressing cells transiently transfected with a GFP-tagged arrestin-3 construct. Cells were stimulated with vehicle or TUG-891. The GFP-tagged arrestin-3 was immunoprecipitated using an immobilised anti-eGFP antibody coupled to agarose beads (GFP-Trap) and resolved using SDS-PAGE. Immunoblots were probed with anti-eGFP antibodies to confirm the successful isolation of the arrestin-3-eGFP fusion protein which is seen here with a molecular weight of approximately 70 kDa (**Figure 4.9A**). Probing these blots with an arrestin-3-specific antibody confirms successful immunoprecipitation of arrestin-3-eGFP fusion protein as bands corresponding to the protein at a molecular weight of approximately 70 kDa are observed (**Figure 4.9B**). Probing with an antibody which recognises total Akt protein does not reveal a band corresponding to Akt (**Figure 4.9C**); this suggests that although arrestin has been described as a scaffolding protein which forms complexes with sequential kinases resulting in their activation, this may not be true for Akt unless the interaction between Akt and arrestin is very transient or of such low affinity that it cannot be measured by this type of biochemical assay.

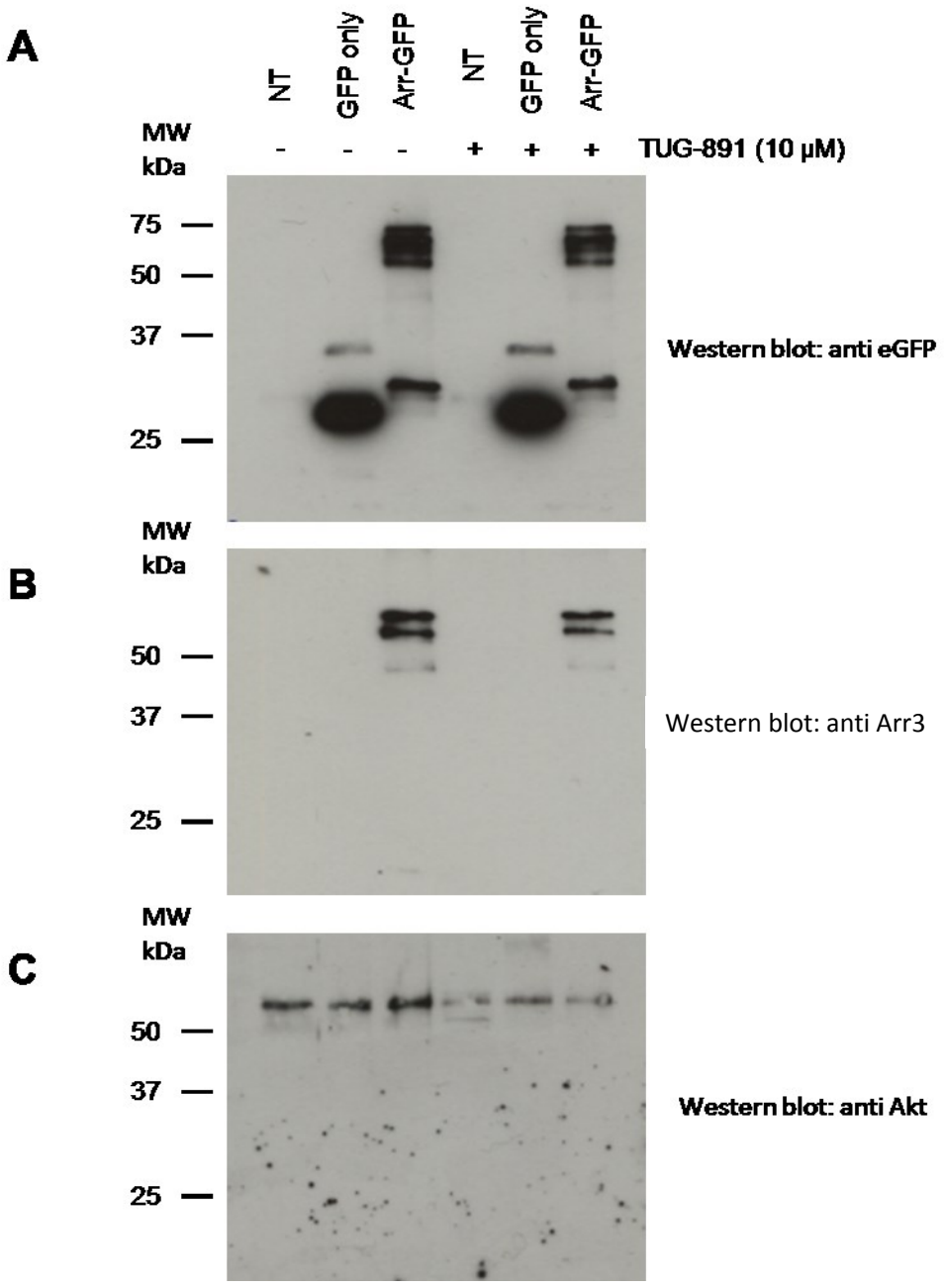


Figure 4.9| β-arrestin-2 does not interact directly with Akt. CHO cells stably expressing the FFA4-wt receptor were transiently transfected with either GFP only or a β-arrestin-2-GFP fusion protein as described in *Materials and Methods*. Cells were then stimulated with either vehicle or TUG-891 (10μM) for 5 min and immobilised eGFP antibody used to purify the GFP protein or the β-arrestin-2-GFP fusion protein by immunoprecipitation. Samples were resolved and immunoblotted with appropriate antibodies. (A) Samples were resolved and immunoblotted with antisera raised against eGFP, antisera raised against β-arrestin-2 (B), or Akt (C). Immunoblots are representative of two independent experiments.

4.2.8. Characterising the downstream CREB response of FFA4

Extracellular stimuli elicit changes in gene expression in cells by activation of intracellular protein kinases resulting in the phosphorylation of transcription factors within the nucleus, a well-studied example of this is cyclic AMP response element (CRE)-binding protein (CREB) (Shaywitz and Greenberg 1999). A host of protein kinases can phosphorylate CREB at Ser133, an important site which is required for CREB-mediated transcription (Shaywitz and Greenberg 1999), including Akt and PKC (Gonzalez *et al.,* 1989; Shaywitz and Greenberg 1999). To investigate whether FFA4 stimulation led to CREB phosphorylation, CHO cells stably expressing the FFA4-wt receptor were stimulated with TUG-891 to cause an increase in CREB phosphorylation at Ser133. This increase is similar to the increase in the phosphorylation-deficient mutants (**Figure 4.10A**). Therefore, we suggest that the activation of CREB is mediated by the FFA4 receptor and is another downstream signalling consequence of FFA4 activation.

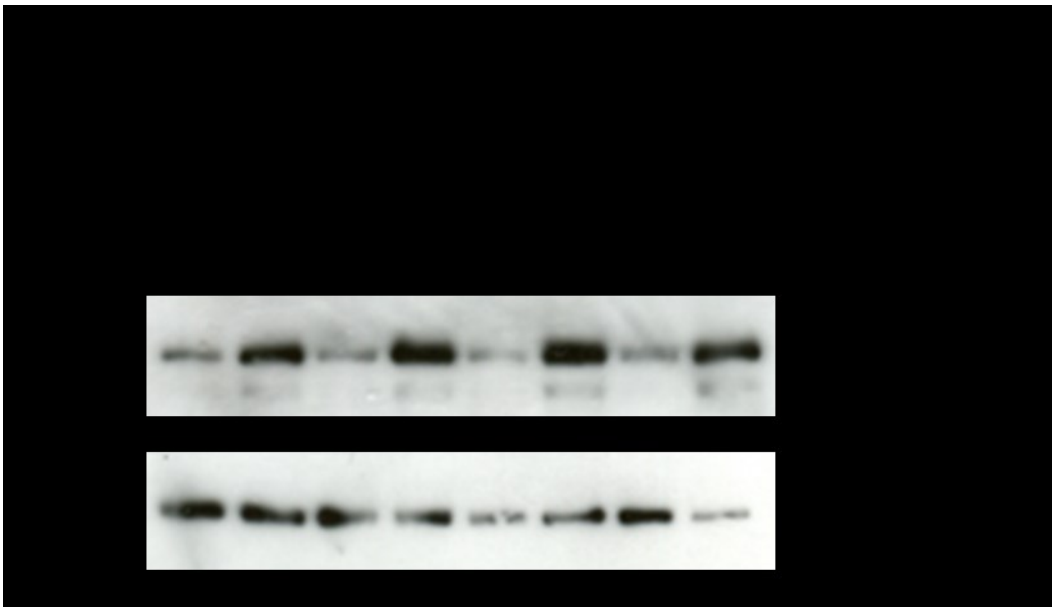


Figure 4.10| Agonist-mediated CREB activation involves FFA4 but is independent of receptor phosphorylation. CHO cells stably expressing the FFA4-wt receptor or FFA4 receptor mutants which eliminated three, four, or five key sites of agonist-mediated phosphorylation (TSS/AAA, TSSS/AAAA, and TSSST/AAAAA, respectively) were stimulated with TUG-891 (10 μM) and whole cell lysates resolved on minigels. Immunoblots were probed with phospho-specific antibodies against phosphorylation sites present on CREB *(A, upper panel*), total CREB was identified using an anti-CREB antibody *(*A, lower panel) and was used as a loading control. Immunoblots are representative of three independent experiments. Error bars represent S.E.M.

4.2.9. Investigating a GRK2 inhibtor at phosphorylation residues of the FFA4

Paroxetine is an FDA approved serotonin reuptake inhibitor usually used to treat depression and anxiety disorders (Roscoe *et al.,* 2005), which has recently been shown to inhibit GRK2 (Thal *et al.,* 2012; Schumacher *et al.,* 2015). In these studies, paroxetine inhibits or even reverses heart damage after myocardial infarction in mice (Schumacher *et al.,*, 2015) due to its inhibitory effects on GRK2 which is upregulated in this model. Using the previously described phosphospecific antibody, we wanted to know whether paroxetine pre-treatment of cells expressing the FFA4 receptor would decrease phosphorylation of two key residues in the C-terminal tail of the receptor. Pre-treatment with several concentrations of paroxetine (1-100 μM) for 30 min followed by a 5 min TUG-891 (10 μM) stimulation did not decrease the phosphorylation of Thr347 or Ser350 as detected by immunoblot analysis (**Figure 4.11**) one explanation of this may be that the TUG-891-stimulated phosphorylation at these residues is independent of GRK2 activity.

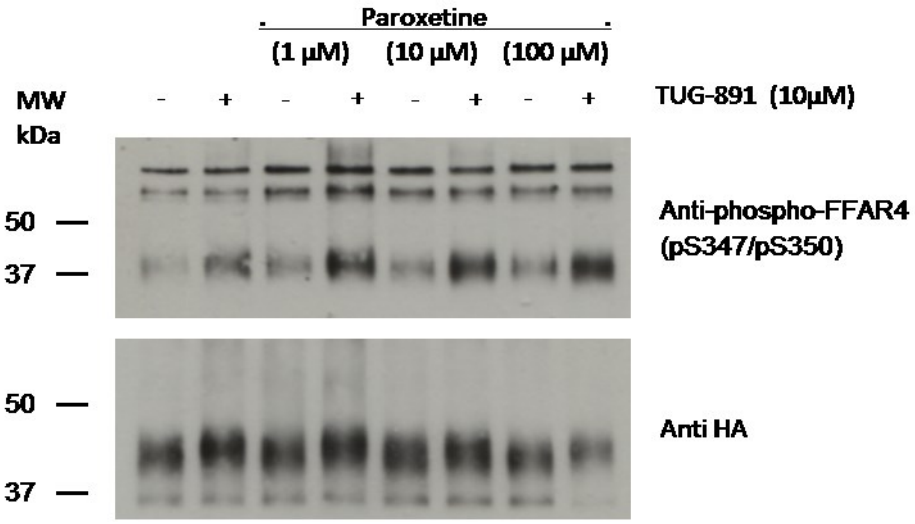


Figure 4.11| A GRK2 inhibitor does not decrease phosphorylation of two key sites in the C-terminal tail of FFA4. Membrane preparations from CHO cells stably expressing the FFA4-wt receptor were run on minigels and probed with antiserum raised to identify Thr(P)347, Ser(P)350 within the C-terminal tail of the human FFA4 receptor. Samples from the FFA4-wt receptors pre-treated with paroxetine at various concentrations for 30 min, treated with vehicle or TUG-891 (10µM) for 5 min then resolved and immunoblotted with the FFA4-phosphospecific antibodies (*upper panel*). Loading controls (*lower panel*) represents the same samples immunoblotted with an anti-HA antibody. Immunoblots represent three independent experiments.

4.3. Discussion

The deorphanisation of the FFA4 receptor (previously known as GPR120) led to it being added to the family of free fatty acid binding GPCRs which previously included FFA1-3 (Davenport *et al.,* 2013). It is now known that the FFA4 receptor binds medium- and long-chain free fatty acids and the human FFA4 receptor exists as a 361-residue short isoform and a long isoform. The long form differs from the short form by the addition of 16 amino acids in the third intracellular loop which renders the receptor unable to signal through $G\alpha_{q/11}$-proteins but preserves β-arrestin signaling (Watson *et al.,* 2012).

The FFA4 has been found to be expressed in a host of different cells and tissues including adipocytes, human pancreatic islets, immune cells (particularly macrophages), lung, and enteroendocrine cells (Hirasawa *et al.,* 2005; Gotoh *et al.,* 2007; Tanaka *et al.,* 2008; Miyauchi *et al.,* 2009; Oh *et al.,* 2010; Taneera *et al.,* 2012). It is known to have important roles in GLP-1 secretion from enteroendocrine cells (Hirasawa *et al.,* 2005), adipocyte differentiation (Miyauchi *et al.,* 2009), mediating anti-inflammatory pathways via macrophages (Oh *et al.,* 2010), and has even been reported to have a protective role in the liver and mediate increases in bone health (Koren *et al.,* 2014; Raptis *et al.,* 2014). Due to its importance in these processes it has led to the need to understand the biology of this receptor. Central to this has been the development of FFA4-specific agonists, as despite the distant phylogenetic relationship between FFA4 and FFA1, both of these receptors are activated by similar FFAs and early synthetic ligands showed activity at the other receptor (Ulven and Christiansen 2015). TUG-891 has been reported as a selective FFA4 agonist, and is known to bind to the orthosteric site of FFA4 (Shimpukade *et al.,* 2012; Hudson *et al.,* 2014). TUG-891 has been shown to cause GLP-1 secretion from STC-1 cells and murine enteroendocrine cells as well as increase uptake of glucose in 3T3-L1 adipocytes and inhibit TNF release from RAW264.7 macrophages (Ulven and Christiansen 2015).

A consequence of agonist stimulation of GPCRs is receptor phosphorylation (Ferguson, 2001; Tobin *et al.,* 2008; Tobin, 2008). The concept of functional selectivity or bias has traditionally referred to a ligands ability to preferentially direct signalling of receptors to one signalling pathway over another (Violin and Lefkowitz 2007). These ligands have been shown to also cause different patterns of phosphorylation at these receptors based

on the propensity of a ligand to induce a particular conformation in the receptor allowing kinases to access different parts of the GPCR (Nobles *et al.,* 2011; Butcher *et al.,* 2011). But a receptor can be mutated in such a way that it becomes inherently biased. An example of this is replacing phosphoacceptor residues in the third intracellular loop and C-terminal tail with residues which cannot become phosphorylated (usually to alanine residues). This results in a receptor which is no longer able to efficiently recruit and/or signal through β-arrestin, but signalling via G-proteins remains intact as has been shown with the M_3 mAChR in previous studies from our laboratory (Kong *et al.,* 2010; Poulin *et al.,* 2010). These GPCRs can serve as important tools for investigating downstream signalling pathways and the impact of these pathways on physiological processes. Here we show that stimulation with TUG-891 induces significant agonist-regulated increases in phosphorylation of the wild-type FFA4 receptor. Previous studies from our lab have used mass spectrometry to show that this increase in receptor phosphorylation is due to 5 putative sites of phosphorylation in the C-terminal tail of the receptor (Butcher *et al.,* 2014). Mutation of these residues to alanine (FFA4-TSSST/AAAAA) results in a receptor which shows no agonist-mediated increase in phosphorylation, is impaired in its ability to recruit arrestin, and could therefore be considered to be G-protein biased.

Further mutants were also created based on data from the aforementioned mass spectrometry studies resulting in mutants that had three or four phosphoacceptor sites mutated to alanine residues (FFA4-TSS/AAA and FFA4-TSSS/AAAA, respectively). The FFA4-wt receptor and the three phosphorylation-deficient mutants were then used in a BRET-based arrestin-3 recruitment assay. We show that sequential removal of the five phosphorylation sites in the C-terminal tail of FFA4 resulted in a progressive decrease in agonist-mediated recruitment of arrestin-3. This data supports the notion that bulk negative charge is perhaps responsible for mediating receptor/arrestin-3 interactions were it not for the redundant nature of phosphorylation at site Thr349 which seems not to contribute to arrestin-3 interaction. Thr349 is located within a module of phosphorylation made up of three sites (Thr347, Thr349, and Ser350), with it being highly possible only two sites are needed to effectively provide the contribution from this module. This module of phosphorylation seems to work concomitantly with phosphorylation of the remaining two serine residues in the C-tail (Ser357 and Ser360) which make up the second module to fully recruit arrestin-3. This suggests that it is the

precise location or pattern of phosphorylation present in the C-terminal tail and not simply bulk negative charge which mediates arrestin-3 recruitment to the FFA4 receptor. This corroborates other studies which show similar attributes regarding a phosphorylation pattern or barcode which induces specific signalling outcomes (Butcher *et al.,* 2011; Nobles *et al.,* 2011; Tobin *et al.,* 2008; Torrecilla *et al.,* 2007).

It is important to note that arrestin-3 recruitment does not seem to completely rely on phosphorylation of these sites alone, previous studies from our lab have shown "structural elements" in the form of acidic residues in the C-tail also contribute to arrestin-3 recruitment (Butcher *et al.,* 2014), and may be the reason why some arrestin-3 recruitment remains in the mutant which is completely phosphorylation-deficient. A recent x-ray crystal study involving a chimeric β_2-adrenergic receptor with a C-terminal peptide derived from the V2 vasopressin receptor binding with arrestin-2 has gone some way in helping understand the process by which receptors and arrestins interact (Shukla *et al.,* 2013). The study highlights the extensive contacts made through charge-charge interactions of the V2 vasopressin C-terminal peptide phosphates with the arginine and lysine side chains of arrestin-2. It has been suggested that these interactions determine the active conformation of arrestin, which in turn determines the signalling outcome of the receptor-arrestin complex (Nobles *et al.,* 2011; Shukla *et al.,* 2013). Therefore, it is quite possible that the structural elements of the FFA4 C-tail interact with arrestin and work in concert with the agonist-regulated phosphorylation sites to recruit arrestin and possibly cause a specific arrestin conformation, thereby mediating a specific signalling outcome of the FFA4-arrestin complex.

G-protein coupled receptor kinases play important roles in receptor phosphorylation and one particular member, GRK6, has been implicated in homologous phosphorylation of FFA4 (Burns *et al.,* 2014). This study utilised a HEK 293 cell model and siRNA knockdown of specific GRKs to show GRK6 but not GRK-2, -3, or -5 was responsible for the majority of docosahexaenoic acid (DHA)-mediated FFA4 phosphorylation. GPCR phosphorylation is a highly complex process and has been hypothesised to be tissue- and cell-type specific as well as ligand-specific, with the compliment of kinases available in the cytosolic milieu determining the sites of receptor phosphorylation (Butcher *et al.,* 2011; Tobin *et al.,* 2008; Tobin, 2008). A phosphorylation-specific antibody raised against the FFA4 receptor based on previous mass spectrometry data was used to probe the phosphorylation status of the receptor which was expressed in

CHO cells and stimulated with TUG-891. We show that the antibody is specific to the phosphorylated Thr347 and Ser350 residues by using a FFA4 receptor mutant which lacks these residues in its C-tail. TUG-891 stimulation of cells expressed in CHO cells shows an agonist-mediated increase in phosphorylation of the double phosphorylation site at Thr347/Ser350. To further understand which kinase may be responsible for this phosphorylation event, paroxetine was used as it has previously been reported as a GRK2 inhibitor (Schumacher *et al.,* 2015; Thal *et al.,* 2012). We show the double phosphorylation site of Thr347/Ser350 of FFA4 is unaffected by paroxetine and may indicate GRK2 is not involved in the phosphorylation of these residues. This would be in line with previously reported data (Burns *et al.,* 2014) and will form the foundations for further studies to investigate which GRKs are responsible for receptor phosphorylation in this setting using siRNA knockdown studies.

Signalling to extracellular-signal regulated kinases 1/2 has previously been shown to be mediated by both G-protein dependent and G-protein independent pathways (Luttrell and Gesty-Palmer 2010; Rosethorne and Charlton 2011; Shenoy *et al.,* 2006). Due to the differences in signalling via these pathways, the kinetics of ERK phosphorylation also differs. It is composed of a rapid and transient response which is associated with G-protein dependent signalling and a delayed response associated with G-protein independent signalling which is slower and more sustained (Luttrell and Gesty-Palmer 2010). We show that TUG-891 induces a rapid and transient increase in ERK phosphorylation which peaks at 5 min which could be attributed to signalling by $G\alpha_q$-proteins. There is also evidence of sustained ERK signalling up to 60 min after stimulation possibly indicating a population of ERK which is activated by G-protein independent mechanisms such as arrestins. Sequential removal of phosphoacceptor residues in the C-tail of FFA4 results in a receptor which shows a progressively reduced arrestin recruitment phenotype. Extending our studies, we used these mutants to show the rapid and transient increase in ERK phosphorylation which peaks at 5 min in response to TUG-891 stimulation does not seem to involve a β-arrestin mediated component. Rather, our data seem to show that reduced recruitment of β-arrestin to these receptors results in a receptor which is not desensitised as effectively as the wild-type FFA4 receptor and subsequently causes an increased ERK phosphorylation response as measured with an HTRF-based ERK phosphorylation assay. Why we do

not see a similar phenotype with our immunoblot studies needs to be investigated further but could be due to the limitations of this type of study.

Further evidence to support the important role of $G\alpha_q$ in the ERK phosphorylation which peaks at 5 min mediated by FFA4 is provided by the use of the compound UBO-QIC, a $G\alpha_q$-protein inhibitor which results in complete abrogation of agonist-mediated ERK phosphorylation. It is important to note that this data is not sufficient evidence to state that the sustained ERK activation seen at longer time points is not mediated by arrestins. Further work is needed to clarify the role of arrestins in ERK activation upon FFA4 stimulation at longer time points. This can be done by performing time-course experiments using the FFA4 receptor mutants which are known to exhibit a reduced arrestin recruitment/activation phenotype or by using UBO-QIC at longer time points. It is also known that subcellular destination of activated ERK1/2 may be different. G-protein-dependent ERK activation causes activated ERK to be shuttled to the nucleus, whereas ERK activated by an arrestin-dependent pathway stays mainly in the cytoplasm (Eishingdrelo and Kongsamut 2013). Monitoring the location of activated ERK would, therefore, give us a good indication as to how it was activated. Previous work has suggested FFA4 mediates a pERK response in a recombinant system which is mainly but not solely mediated by $G\alpha_{q/11}$-proteins (Hudson *et al.,* 2013), as well as a slow EGR-receptor-dependent pERK response in Caco-2 adenocarcinoma cells but not a rapid and transient pERK response (Mobraten *et al.,* 2013). Our work, in combination with these observations suggests that FFA4 may activate ERK through different pathways in different cell types.

The serine/threonine kinase Akt, also known as protein kinase B (PKB) is an important downstream target of growth factors, cytokines and other cellular stimuli (Manning and Cantley 2007). Akt exhibits full kinase activity when phosphorylated on two residues, a threonine residue (Thr308) in the activation loop and a serine residue (Ser473) in a hydrophobic domain contained within a carboxyl terminal regulatory domain (Bozulic and Hemmings 2009). Aberrations in Akt activation has been implicated in a variety of complex diseases such as type-2 diabetes and cancer. Recently, FFA4 has been added to the list of proteins which can regulate the function of Akt signalling. Studies have shown FFAs acting through FFA4 promote the activation of the PI3K pathway leading to anti-apoptotic effects in STC-1 cells (Katsuma *et al.,* 2005b). In macrophages, Toll-like receptor 4 signalling via Akt/JNK phosphorylation was suppressed by the FFA4-β-

arrestin 2 signalling complex (Li *et al.,* 2013). But FFA4-mediated Akt activation has also been reported to be regulated by the $G\alpha_q$-protein pathway of the receptor (Oh *et al.,* 2010). In an attempt to solve the conflicting results reported in the literature, we used a stably expressing wild-type FFA4 expressed in CHO cells. We show stimulation of the receptor with TUG-891 causes a rapid and transient increase in phosphorylation of Ser473 of Akt as assessed by western blot analysis, indicating activation of the protein (Sarbassov *et al.,* 2005). This phosphorylation event peaks at 5 min and returns to basal levels after 45 min of continued stimulation with TUG-891. Using phosphorylation-deficient FFA4 receptor mutants which we show have an impaired ability to recruit arrestin, we see a progressive decrease in the amount of Akt phosphorylated at Ser473, indicating β-arrestin plays a role in activating Akt which is in agreement with previous studies (Li *et al.,* 2013). Agonist stimulation of the fully phosphorylation-deficient mutant receptor is able to induce phosphorylation and hence activation of Akt, albeit at a significantly reduced level. This could be mediated by the ability of the phosphorylation-deficient receptor to still recruit arrestin albeit less effectively than the wild-type FFA4 receptor, or potentially via a $G\alpha_q$-protein-mediated component of Akt activation. To this end, a $G\alpha_q$-protein inhibitor was used to pre-treat cells expressing the wild-type FFA4 receptor prior to stimulation with TUG-891. Here, we demonstrate that the agonist-regulated increase in Akt phosphorylation is not mediated by FFA4 signalling through $G\alpha_q$-proteins as assessed in a HTRF-based assay. These data indicate FFA4-mediated Akt phosphorylation mainly occurs through a FFA4-β-arrestin signalling complex corroborating previous studies (Li *et al.,* 2013). It is important to note that like ERK activation, Akt activation via FFA4 could occur through different pathways in different cell types. It has become apparent that arrestins can act as agonist-regulated scaffolding proteins whereby the endocytosed receptor is physically linked to downstream signalling cascades (Pierce and Lefkowitz 2001). Using a co-immunoprecipitation study, we do not see a direct interaction between FFA4, arrestin and Akt. This could be due to the interaction between Akt and arrestin being very transient or of such low affinity that it cannot be measured by this type of biochemical assay. Interestingly, FFA4 mediated Akt phosphorylation only occurs at Ser473 and not Thr308. As mTor, as part of a signalling complex called mTORC2, is known to phosphorylate the serine residue of Akt (Sarbassov *et al.,* 2005; Manning and Cantley 2007), it could be possible that the FFA4-β-arrestin signalling complex activates the

mTORC2 complex which goes on to mediate Akt activation although further work is required to test this hypothesis.

Another downstream signalling consequence of FFA4 activation is the phosphorylation of cyclic AMP response element (CRE)-binding protein (CREB). This protein is a highly characterised stimulus-induced transcription factor which becomes phosphorylated at a particular residue (Ser133) and goes on to mediate the transcription of genes (Shaywitz and Greenberg 1999). CREB phosphorylation can occur in response to a wide range of stimuli and can become phosphorylated by several intracellular kinases (Shaywitz and Greenberg 1999). Incubation of human skeletal myotubes with either oleic acid or linoleic acid shows an enhanced pCREB response in these cells. These fatty acids can activate FFA1 and FFA4, however, the study fails to delineate which receptor is mediating this increase in CREB phosphorylation (Nardi *et al.,* 2014). It remains to be confirmed which receptor is present on skeletal myotubes, but we show that FFA4 stimulation can result in agonist-mediated increases in pCREB. Here, we show that the activation of CREB is a consequence of FFA4 receptor activation when expressed in CHO cells. However, we cannot distinguish whether this is a G-protein-dependent or independent response from our current data. Although the max response of CREB phosphorylation is no different between the wild-type FFA4 and phosphorylation-deficient mutants as assessed by western blot analysis, further work is required to make more conclusive judgements. The involvement of arrestin in CREB activation cannot be discounted as our phosphorylation-deficient mutant still couples to arrestin albeit less effectively. This residual arrestin recruitment may be enough to amplify downstream signals and induce the phosphorylation of CREB. Constructing concentration responses using the FFA4-specific agonist, TUG-891, at the wild-type FFA4 and phosphorylation-deficient mutants would allow us to calculate the potency of this ligand to induce changes in CREB phosphorylation between the receptors. Further work could also include the use of UBO-QIC as we have previously suggested this compound allows the delineation of G-protein-mediated responses.

In conclusion, we show that FFA4 signalling via $G\alpha_{q/11}$-proteins is not dependent on the phosphorylation of the receptor; however, arrestin-3 recruitment to the receptor is regulated by FFA4 phosphorylation. This observation may form the basis of future work, as ligands are emerging which show stimulus bias (Butcher *et al.,* 2011; Nobles *et al.,* 2011), these ligands bind receptors and cause distinct phosphorylation profiles or

"barcodes". One may use the findings of our work to suggest that different ligands may interact with the FFA4 receptor and promote different phosphorylation events which may result in ligands driving preferential signalling and physiological outcomes. As the FFA4 receptor is an emerging therapeutic target in type-2 diabetes and inflammatory conditions (Dranse *et al.,* 2013), designing ligands which show stimulus bias may be one strategy which improves clinical efficacy of these ligands and avoids adverse responses. The data in this study also highlights the downstream signalling of physiologically relevant effectors such as ERK, Akt and CREB which have been shown to be important in determining the effects of receptors in *in vivo* and *in vitro* studies.

Chapter 5 | Characterisation of a phosphomimetic mutant of the FFA4

5.1. Introduction

Receptor phosphorylation plays an important role in the regulation of GPCRs (Tobin, 2008; Tobin *et al.,* 2008). Our lab, and in collaboration with others, has particularly focused on receptor phosphorylation as a key mediator of both receptor regulation and downstream cellular signalling for several different GPCRs (Butcher *et al.,* 2009; Butcher *et al.,* 2011; Chen *et al.,* 2013; Kong *et al.,* 2010; Poulin *et al.,* 2010; Zindel *et al.,* 2015). Mutating residues to amino acids which are unable to become phosphorylated (usually serine/threonine to alanine mutations) is a technique employed to create biased receptors (Kong *et al.,* 2010; Poulin *et al.,* 2010). This can result in GPCRs which can no longer bind arrestin as efficiently as their wild type receptor counterparts and are powerful tools in studying GPCR functions *in vivo* (Butcher *et al.,* 2014).

The binding of β-arrestins to phosphorylated GPCRs is thought to involve two types of interaction between the arrestin molecule and the receptor (Gurevich and Gurevich 2006). The phosphate sensor of arrestin binds the phosphorylated residues in the C-terminal tail or third intracellular loop of the receptor, and a conformational sensor identifies the agonist-induced active conformation of the receptor core. Phosphomimetic residues can mimic phosphoacceptor residues due to their chemically similar nature, for example, an aspartate residue is chemically similar to phosphoserine. This property can be used to construct charge mutants and has previously been used to investigate the impact phosphorylation has on *in vitro* and *in vivo* signalling and physiological responses. A study using mice containing two Asp residues in place of two serine residues in the AMPA receptor GluA1 were central in implicating AMPA receptor phosphorylation in mechanisms underlying stress-induced pain transition (Li *et al.,* 2014). Charge mutants were also constructed for two major splice variants of the inositol 1, 4, 5-triphosphate receptors ($InsP_3R$) by substituting glutamate residues for serine residues in two functionally important phosphorylation sites at these variants of the $InsP_3R$. In this study, Ca^{2+} release was markedly enhanced compared with mutants

of the receptor unable to become phosphorylated indicating the importance of phosphorylation at these splice variants (Wagner *et al.,* 2004).

The bioluminescence resonance energy transfer (BRET) cell based assay is a valuable tool in shedding light on spatio-temporal dynamic of protein interactions in living cells, this assay allows us to focus on dynamic events occurring at our GPCR of interest upon ligand binding with regards to β-arrestin-2 recruitment. The BRET assay relies on the ratio of acceptor (yellow fluorescent protein (eYFP)) to donor (*Renilla* luciferase (RLuc)) being optimal to allow for obtaining appreciable BRET signals as well as in generating overall conclusions (Pfleger *et al.,* 2006). In BRET1 assays, RLuc catalyses oxidation of cell-permeable coelentrazine H, resulting in luminescence emission within the excitation wavelength of eYFP (Pfleger *et al.,* 2006). Fluorescence emission by eYFP only takes place if donor excitation occurs in close proximity of the acceptor (typically 100 Å). This property may be exploited to monitor interaction of different types of cellular proteins.

With the knowledge that phosphomimetic residues can help to further elucidate the importance of phosphorylation at proteins both *in vivo* and *in vitro* (Wagner *et al.,* 2004; Li *et al.,* 2014), the aim of this chapter is to create and investigate the role of the FFA4 receptor with charge mutations in its C-terminal tail. These mutations replace key residues known to be phosphorylated upon agonist stimulation and important in the recruitment of arrestin-3 to the receptor. By using a BRET-based assay, we aim to measure the total recruitment and recruitment kinetics of arrestin-3 to the phosphomimetic mutant receptor as compared to the wild type receptor and a phosphorylation-deficient mutant. We also aim to investigate the physiological consequence of arrestin recruitment by the receptor by observing receptor internalisation.

The data presented here show that the phosphomimetic mutant of the FFA4 receptor retains the ability to recruit arrestin-3; however, the maximum amount of recruited arrestin is lower. We also show the phosphomimetic mutant does not internalise as well as the wild type FFA4 receptor, perhaps showing a physiological effect of the reduced ability of the receptor to bind arrestin-3.

5.2. Results

5.2.1. BRET analysis for a new receptor construct

Initial studies sought to investigate the specificity of the interaction between the eYFP-tagged phosphomimetic mutant of FFA4 and arrestin-3-RLuc using BRET titration studies. It also served to allow us to identify the optimal ratios where we could see an appreciable BRET signal for future studies. Here, donor/acceptor ratios varied by progressively increasing the amount of acceptor constructs (FFA4-TSSST/DDDDD-eYFP) available for interaction for fixed amounts of donor (arrestin-3-RLuc) and treating receptors with a FFA4-specific agonist, TUG-891. Increasing amounts of FFA4-TSSST/DDDDD-eYFP efficiently increased energy transfer until reaching a plateau while stimulating receptors with TUG-891 (**Figure 5.1**), an observation consistent with the view that donor molecules (arrestin-3-RLuc) interact with acceptors (FFA4-TSSST/DDDDD-eYFP) until reaching saturation (Mercier *et al.,* 2002). We chose a ratio of acceptor/donor of 4:1 as it fell upon the asymptote in the graph, and also because previous studies had utilised this ratio (**Figure 4.2**) and (Butcher *et al.,* 2014).

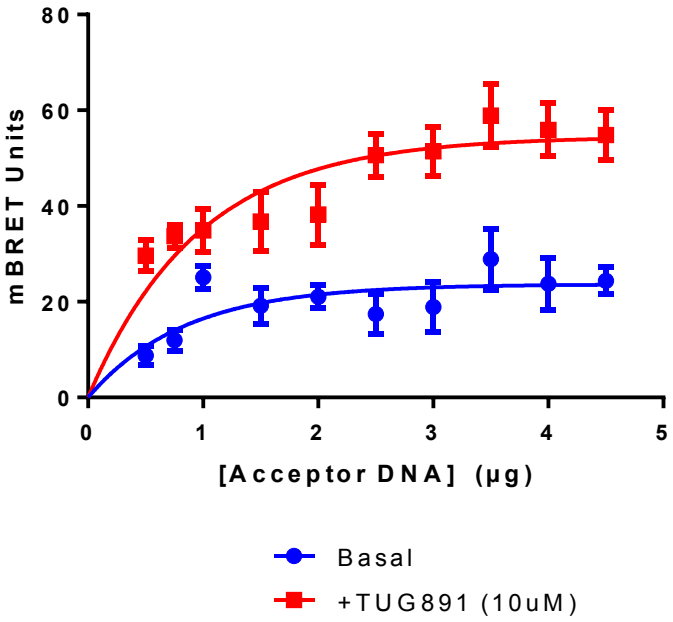


Figure 5.1| Increasing acceptor DNA concentrations leads to an increase in net BRET signal HEK293 cells were transfected with the FFA4-TSSST/DDDDD receptor construct tagged with eYFP and with the *β* -arrestin-2–*Renilla* luciferase or with the *β*-arrestin-2–*Renilla* luciferase construct alone as described in *Materials and Methods*. These cells were then used in a BRET-based arrestin-3 interaction study which comprised of stimulating cells with either vehicle or TUG-891 (10µM) for 5 min and reading the subsequent BRET signal using the ClarioStar plate reader. This graph is representative of three independent experiments performed in at least duplicate.

5.2.2. Arrestin-3 interaction with the C-terminal tail of the FFA4

Some GPCRs have eliminated the need for receptor phosphorylation to induce arrestin recruitment, instead negatively charged residues function as phosphomimetics (Mukherjee *et al.,* 2002; Galliera *et al.,* 2004). Our previous studies led us to the conclusion that phosphorylation was a key component in arrestin-3 interactions with the C-terminal tail of the FFA4 receptor (**Figure 4.2**). To test whether phosphomimetic residues had a similar phenotype to the wild type FFA4, mutants were created and tested in an arrestin-3-RLuc/FFA4-eYFP BRET assay. When tested in this assay format, 5 min of TUG-891 (10 μM) stimulation induced a robust increase in the BRET signal from 34.15 ± 1.68 milli-BRET units to the TUG-891-stimulated value of 126.8 ± 6.26 milli-BRET units for the FFA4 wild type receptor. Testing the receptor mutant which contained 5 aspartate residues in place of the serine/threonine residues (FFA4-TSSST/DDDDD) in the C-terminal tail, we see a decrease in the amplitude of the response induced by 5 min of TUG-891 (10 μM) stimulation to approximately 52.75 ± 9.11% milli-BRET units ($p < 0.01$) of wild type (**Figure 5.2**) indicating a decrease in efficacy for arrestin-3 recruitment to the phosphomimetic mutant receptor. Interestingly, the BRET signal is greater than the BRET signal from the receptor mutant which contains 5 alanine residues in its C-terminal tail which showed a response that was approximately 28.46 ± 3.67% milli-BRET units ($p < 0.001$) of wild type (**Figure 5.2**), indicating the phosphomimetic mutations still resulted in appreciable agonist-induced increases of BRET. These experiments also demonstrated that the mutant receptors showed a slight decrease in potency (for TUG-891 at the wild type FFA4, pEC_{50} = 7.16 ± 0.12, for FFA4-TSSST/DDDDD, pEC_{50} = 6.99 ± 0.55, and for FFA4-TSSST/AAAAA, pEC_{50} = 7.02 ± 0.21) for arrestin-3 recruitment measured after 5 min in the BRET assay.

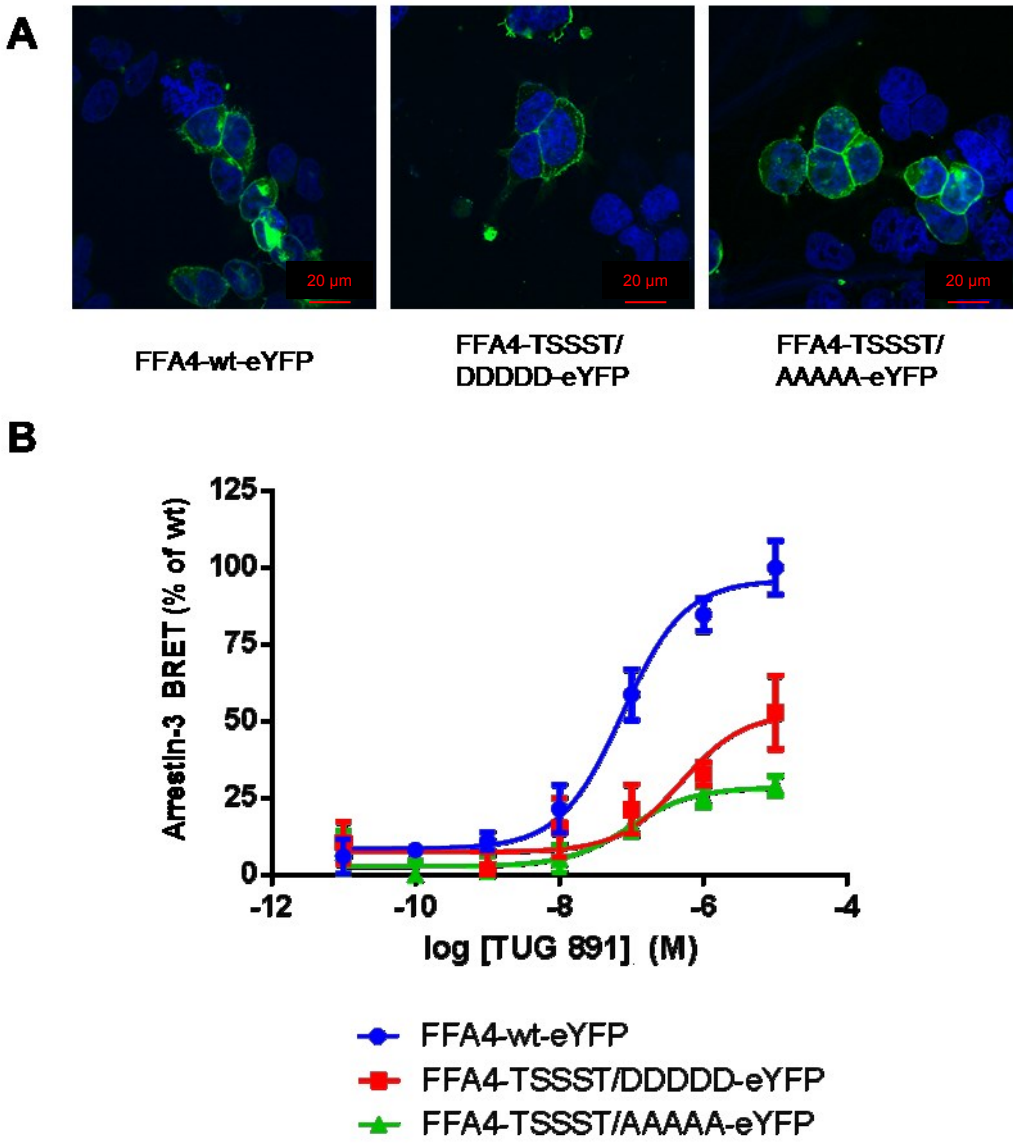


Figure 5.2| Mutating residues involved in TUG-891-regulated phosphorylation in the C-terminal tail of FFA4 to phosphomimetics does not emulate the wild-type receptor in agonist-induced FFA4/arrestin-3 interactions. HEK 293 cells were transiently transfected with DNA corresponding to the FFA4-wt receptor or mutations of FFA4 which replaced five sites of phosphorylation with phosphomimetic residues or eliminated the sites of agonist-mediated phosphorylation (TSSST/DDDDD, and TSSST/AAAAA, respectively). (A) shows representative images of transfected HEK 293 cells with the different constructs. (B) These cells were then used in BRET-based arrestin-3 interaction studies in response to TUG-891 and compared with FFA4-wt. Data points represent the mean ± S.E.M of three independent experiments performed at least in quadruplicate.

5.2.3. The kinetics of arrestin-3 interaction with the C-terminal tail of the FFA4 receptor

To further investigate the effect of replacing serine and threonine residues with aspartate residues in the C-terminal of FFA4, we tested the kinetics by which arrestin-3 was recruited to the receptor. In order to measure this, an equation which describes the association kinetics of the interaction between the FFA4 receptor and arrestin-3 was used. During each time interval, a certain fraction of the arrestin-3 became associated with the FFA4 receptor. As time advanced, fewer receptors were unoccupied so fewer arrestin-3 could associate and the curve levelled off. Analysing this curve by using an equation which measured one phase association allowed us to obtain a rate constant. These experiments again replicated the decrease in efficacy (for TUG-891 (10 μM) at the wild type FFA4, B_{max} = 0.58 ± 0.01 milli-BRET units, for FFA4-TSSST/DDDDD, B_{max} = 0.49 milli-BRET units ($p < 0.0001$), and for FFA4-TSSST/AAAAA, B_{max} = 0.48 milli-BRET units ($p < 0.0001$)) for arrestin-3 recruitment measured after 5 min in the BRET assay. We also show that the rate of association of arrestin to the mutant receptors is faster as measured by one phase association binding analysis and determining the rate constant, *K* (for TUG-891 (10 μM) at the wild type FFA4, K = 0.04 units s^{-1}, for FFA4-TSSST/DDDDD, K = 0.06 ± 0.01 units s^{-1}, and for FFA4-TSSST/AAAAA, K = 0.06 ± 0.01 units s^{-1}). However, the differences observed here were not statistically significant.

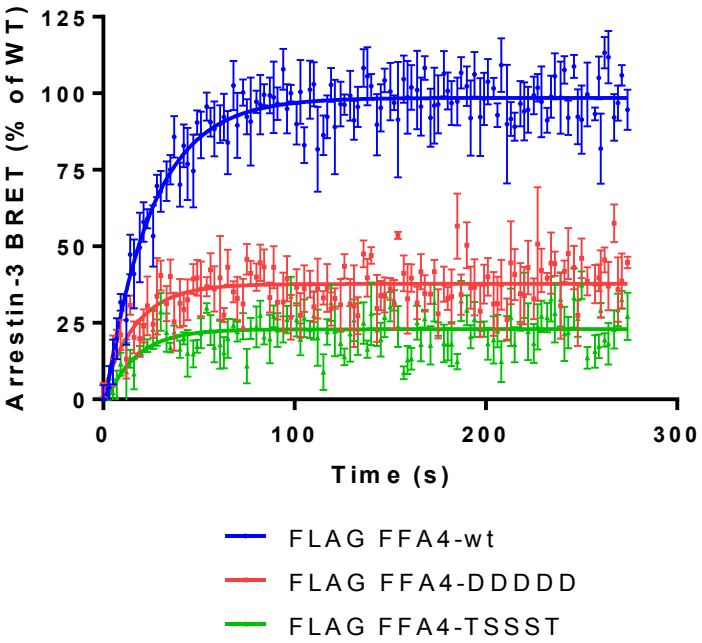


Figure 5.3| Mutating residues involved in TUG-891-regulated phosphorylation in the C-terminal tail of FFA4 to phosphomimetics slows agonist-induced FFA4/arrestin-3 interactions. HEK 293 cells were transiently transfected with DNA corresponding to the FFA4-wt receptor or mutations of FFA4 which replaced five sites of phosphorylation with phosphomimetic residues or eliminated the sites of agonist-mediated phosphorylation (TSSST/DDDDD, and TSSST/AAAAA, respectively). A modified BRET/arrestin-3 based assay was then performed to investigate the kinetics of arrestin-3 interaction with FFA4 and its mutants upon stimulation with TUG-891 (10 μM) over a period of 5 min. Data points represent the mean ± S.E.M of three independent experiments performed in at least triplicate.

5.2.4. FFA4 internalisation correlates with agonist-induced FFA4/arrestin-3 interactions

Receptor internalisation is an important component of GPCR signalling as it serves to sequester the signalling of activated receptors (Luttrell and Lefkowitz 2002). Arrestins play a central role in this process and agonist-mediated endocytosis is facilitated by these proteins for a host of GPCRs (Ferguson, 2001). To test the hypothesis that reduced arrestin-3 recruitment to the FFA4 mutants resulted in reduced internalisation of the receptor, a plate-based ELISA assay was performed. **Figure 5.4A** shows clear agonist-mediated internalisation of the FFA4-wt receptor (37.24 ± 8.33%) after 5 min stimulation with the FFA4-specific agonist TUG-891 (10 μM). The mutants receptors containing either aspartate or alanine mutations in their C-terminal tail as opposed to serine/threonine residues showed a smaller percentage of receptor internalisation after stimulation with the FFA4-specific agonist, TUG-891 (10 μM) (24.41 ± 9.97% and 21.89 ± 9.47%, respectively). These values are not statistically significant but there is a trend for the mutant receptors to be less susceptible to internalisation.

Immunocytochemistry was also utilised to investigate receptor internalisation. Cell surface FFA4-wt receptor expression is visualised as a cell expressing immunofluorescence at the boundaries of the cell when viewed by confocal microscopy as can be seen in **Figure 5.5A** highlighted by yellow arrows. Stimulation of the cells using TUG-891 (10 μM) for 5 min resulted in the disappearance of the immunofluorescence at the boundaries of the cell. Rather, the cells show punctate spheres indicating the internalised receptor in vesicles close to the cell surface highlighted by the white arrows that is distinguishable from eYFP-tagged immature receptor in the endoplasmic reticulum (**Figure 5.5B**). The receptor corresponding to the aspartate mutant (FFA4-TSSST/DDDDD) also shows cell surface expression which is somewhat diminished upon TUG-891 (10 μM) treatment (**Figure 5.5C** and **Figure 5.5D**), but not as efficiently when compared to the FFA4-wt receptor. The alanine receptor mutant (FFA4-TSSST/AAAAA) also shows a similar phenotype to the aspartate mutant in that there is reduced receptor internalisation upon TUG-891 (10 μM) stimulation (**Figure 5.5E** and **Figure 5.5F**).

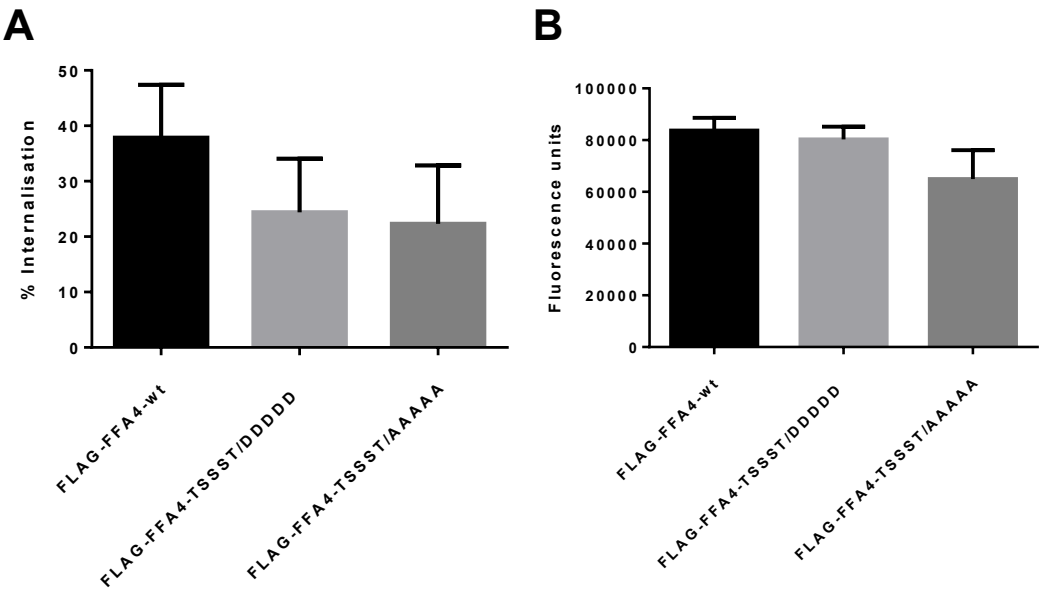


Figure 5.4| Receptor internalisation correlates with agonist-induced FFA4/arrestin-3 interactions. HEK 293 cells were transiently transfected with FFA4-wt receptor or mutations of FFA4 which replaced five sites of phosphorylation with phosphomimetic residues or eliminated the sites of agonist-mediated phosphorylation (TSSST/DDDDD, and TSSST/AAAAA, respectively). These cells were then used in a plate-based ELISA assay in response to stimulation with vehicle or TUG-891 (10 μM) for 5 min to determine internalisation of receptors. (A) shows the percentage of internalised receptors upon treatment with TUG-891 (10 μM) when compared to vehicle treatment alone. (B) shows the total amount of fluorescence as read by the ClarioStar indicating total amount of receptor present. Data points represent the pooled mean ± S.E.M of at least two independent experiments.

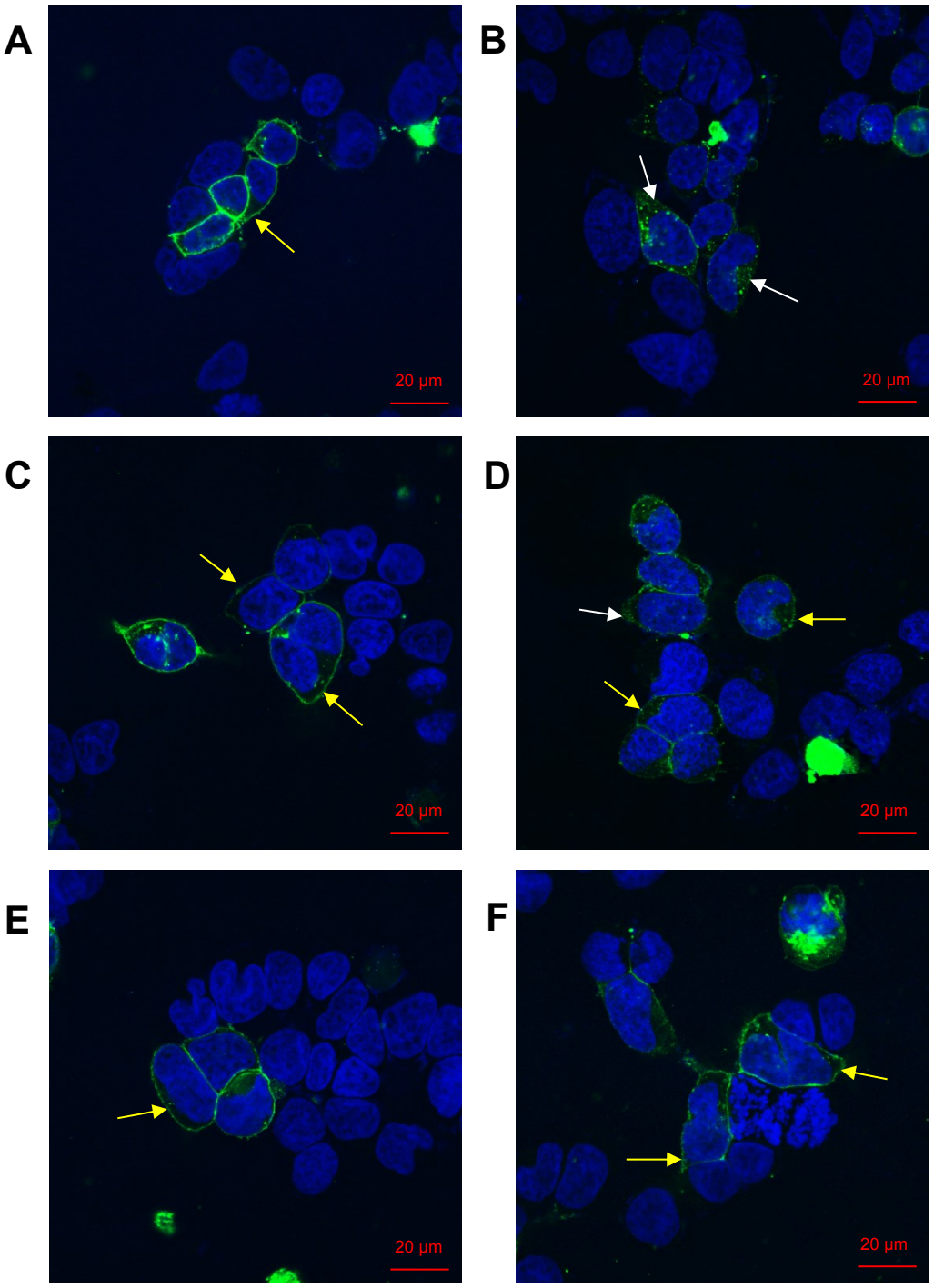

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Figure 5.5| Receptor internalisation correlates with agonist-induced FFA4/arrestin-3 interactions as determined by immunocytochemistry. HEK 293 cells were transiently transfected with DNA corresponding to the FFA4-wt receptor or mutations of FFA4 which replaced five sites of phosphorylation with phosphomimetic residues or eliminated the sites of agonist-mediated phosphorylation (TSSST/DDDDD, and TSSST/AAAAA, respectively). These cells were grown on poly-D-lysine coated coverslips and stimulated with vehicle or TUG-891 (10 μM) for 5 min and then fixed using 4% paraformaldehyde. Cells were mounted using hard-set mounting medium containing DAPI to stain the nucleus and viewed using confocal microscopy. Cells expressing the FFA4-wt receptors were stimulated with vehicle (A) or TUG-891 (10 μM) (B) for 5 min. Cells expressing the FFA4-TSSST/DDDDD were stimulated with vehicle (C) or TUG-891 (10 μM) (D) for 5 min. Cells expressing the FFA4-TSSST/AAAAA were stimulated with vehicle (E) or TUG-891 (10 μM) (F) for 5 min. Yellow arrows indicate cell surface receptor, white arrows indicate internalised receptor. Scale bars, 20 μm. Images represent two independent experiments.

5.3. Discussion

In this present chapter, we utilise charge mutations at the C-terminal tail of the FFA4 to demonstrate the importance of phosphorylation at this receptor. The FFA4 receptor has been shown in the previous chapter and from publications from our laboratory to be strongly coupled to arrestin-3 in a BRET based assay (Butcher *et al.,* 2014). We show that replacing 5 key phosphoacceptor sites in the C-terminal tail of FFA4 to phosphomimetic residues results in the reduction of potency and efficacy for the FFA4-specific agonist, TUG-891, to recruit arrestin-3 to the FFA4 in a BRET based assay. We also determine that this receptor mutant exhibits a faster association to arrestin-3 when compared with the wild type FFA4; however, this value is not statistically significant. By investigating internalisation of the FFA4, we suggest that reduced arrestin-3 recruitment to the FFA4 may result in the physiological consequence of decreased internalisation of the receptor upon TUG-891 stimulation.

For most GPCRs, agonist stimulation results in receptor phosphorylation and internalisation via the recruitment of arrestin (Violin and Lefkowitz 2007). The binding of arrestins to the phosphorylated GPCR involves two types of interactions; a conformational sensor recognises the agonist-induced active conformation of the receptor and a phosphate sensor interacts with phosphorylated residues in the third intracellular loop or carboxy tail of the GPCR (Gurevich and Gurevich 2006).

Recent studies using X-ray structure analysis have shed light on the GPCR-arrestin interaction. One study used a C-terminally truncated bovine arrestin-1 (rod arrestin) which exists naturally in a pre-activated state mimicking a splice variant called p44 (Kim *et al.,* 2013). This variant of arrestin had a higher affinity for light-activated phosphorylated rhodopsin than the non-truncated form of arrestin-1. An important difference between the structure of arrestin-1 and p44 is the state of the polar core, a buried hydrogen-bond network comprised of residues from the N- and C-domains and the C-tail of arrestin. This region contains Arg175, which is described in the study as a critical phosphate-sensor that interacts with residues on the lariat loop of arrestin. In the pre-activated form of arrestin-1, the gate loop which is a part of the lariat loop, undergoes a conformational change resulting in a cleft which exposes Arg175 and breaks nearly all hydrogen bonds to the residue. The exposed Arg175 is thought to contribute to the high affinity for phosphorylated receptor species by p44 (Schroder *et*

al., 2002). Key phosphate-binding residues also line this cleft and implicate it as a putative binding site for the phosphorylated receptor C-terminus (Kim *et al.,* 2013). Furthermore, the crystal structure of p44 also reveals that the flexible nature of the finger loop of arrestin is involved in tight binding to the active receptor as has been reported previously (Hanson *et al.,* 2006), and binding of arrestin-1 to an inactive but phosphorylated opsin molecule releases the finger loop from its restricted basal conformation. In addition to loop rearrangement, p44 demonstrates a significant rearrangement of the inter-domain regions which link the N- and C-domains. This rearrangement is largely due to changes in loop 17-18, which also contains the gate loop/lariat loop, and significantly affects the multiple hydrogen bonds that stabilise the interface between the N- and C-domains. Firstly, rearrangement of the gate loops breaks the polar core. Next, a short β-strand forms in the middle of the lariat loop of p44 which is stabilised by a hydrogen-bond network. The YKS(N)D(A) hydrogen-bond network which lies within the receptor-binding surface of arrestin-1 near the finger loop and loop 139 is broken due to the change in loop 17-18. These rearrangements in the inter-domain lead to a 20° rotation of the N- and C-domains of p44 relative to each other as compared to basal arrestin-1 (Kim *et al.,* 2013).

Using the pre-activated form of arrestin, p44, this study neatly demonstrates the steps leading to arrestin activation. Pre-binding of arrestin to the phosphorylated receptor displaces the C-tail/carboxy terminus of arrestin releasing the finger loop from its restricted basal state conformation. The YKS(N)D(A) inter-domain stabilised by a hydrogen-bond network is also disrupted, however, the position of the gate loop and the polar core remain as in basal arrestin-1. Full activation of arrestin and its tight binding to the receptor involves the active receptor being engaged by the finger and lariat loops, the phosphorylated C-terminus of the receptor binds within the cleft opened by movement of the gate loop. The increase in inter-domain flexibility and the 20° rotation of the N- and C-domains of arrestin are likely to facilitate a proper fitting of arrestin to the receptor and adoption of different binding modes of arrestin.

Another X-ray crystal structure analysis of the non-visual β-arrestin1 in complex with a fully phosphorylated carboxy-terminal peptide derived from the V_2 vasopressin receptor (V_2Rpp) further highlights the complex nature of arrestin binding to GPCRs (Shukla *et al.,* 2013). This study corroborates many aspects of the study involving p44, as there is also a 20° rotation of the N- and C-domains relative to one another. The V_2Rpp also

binds to the N-domain in a similar position occupied by the C-terminus of β-arrestin1 in an inactive state and makes extensive contacts through charge-charge interactions of V_2Rpp phosphates with β-arrestin1 arginine and lysine side chains. This study also highlights the activation-dependent conformational changes of the finger loop, middle loop and lariat loop. The V_2Rpp is shown to obstruct the inactive conformation of the finger loop which is known to be important for discriminating between active and inactive GPCRs (Hanson *et al.,* 2006), and may stabilise an extended conformation of this loop to facilitate contact with the receptor core.

Two major sets of intramolecular interactions are proposed to play a role in stabilising the inactive structure of arrestins: the three-element interaction and the polar core interaction. The three-element interaction consists of interactions between β-strand I, α-helix I and the carboxy terminus of arrestin (Vishnivetskiy *et al.,* 2000). Two well-conserved lysine residues (Lys10 and Lys11) of the β-strand I have previously been shown to "guide" phosphorylated residues of GPCRs toward the phosphate-sensitive trigger Arg175 and participate in the binding of phosphates in the active state of arrestin (Vishnivetskiy *et al.,* 2000). In this crystal structure, Lys10 and Lys11 are found to be binding phosphorylated residues (Ser363 and Ser357) of the V_2Rpp indicating their importance in arrestin activation and binding to the receptor. What is also observed in this study is the displacement of the carboxy terminus of arrestin with the C-tail of the V_2Rpp resulting in the disruption of the three-element interaction.

The polar core has been described previously when discussing the structure of p44. This study also implicated the polar core in arrestin activation as V_2Rpp binding resulted in disruption of the polar core. This is hypothesised to be due to an excess negative charge from the removal of an arginine residue located on the carboxy terminus and aspartate residues from the lariat loop. Interestingly, a lysine residue in the lariat loop shows interaction with a phosphorylated residue of the V_2Rpp. This residue may provide an additional mechanism which stimulates the rearrangement of the lariat loop and could help to stabilise β-arrestin-1 in an active conformation (Shukla *et al.,* 2013) indicating the importance of receptor phosphorylation in arrestin activation.

Recently, the significance of GPCR phosphorylation has been further demonstrated with the publication of a study which engineered phosphorylation sites into the C-terminal tail of the β_2-adrenergic receptor ($\beta2AR^{SSS}$) and showed increases in agonist-stimulated

phosphorylation and differences in arrestin-3 affinity and trafficking (Zindel *et al.,* 2015). In this study, three serine residues were introduced into the C-terminus of the β_2-adrenergic receptor 20 residues (residues 361-363) downstream from the palmitoylated Cys341 which was analogous to the V_2 vasopressin receptor which contains a similar serine cluster at the same distance from its putative palmitoylation site. Using ^{32}P metabolic labelling, it was shown that the serine cluster underwent agonist-induced increases in phosphorylation but did not affect coupling of the receptor to G-proteins. Further experiments showed that at least one or two serine residues in the cluster became phosphorylated. The increased phosphorylation events at $\beta2AR^{SSS}$ translated into an enhanced affinity for arrestin-3, as the mutant receptor showed faster association and slower disassociation rates from the arrestin molecule compared with the wild type β_2-adrenergic receptor. It is important to note that the location of the serine cluster was important in determining the increased affinity for arrestin as a mutant receptor which contained a serine cluster in the distal part of the C-terminus (residues 397-399) did not significantly change the disassociation kinetics when compared to the wild type receptor, suggesting the location of GPCR phosphorylation seems to be a critical in determining arrestin binding. Furthermore, it was found that the increased affinity for arrestin due to the addition of the serine cluster in the proximal part of the receptor resulted in a change in arrestin-3 trafficking. Stimulation of the wild type β_2-adrenergic receptor with agonist caused arrestin-3 re-distribution into the cytosol or near the plasma membrane. However, agonist stimulation of $\beta2AR^{SSS}$ resulted in arrestin-3 re-localisation to intracellular vesicles. This was shown to cause the $\beta2AR^{SSS}$ to internalise significantly more efficiently than the wild type receptor but had no effect on the receptors ability to be recycled back to the plasma membrane.

Interestingly, this study engineered sites of phosphorylation into the C-terminal tail of the β_2-adrenergic receptor based on the structure of the C-tail of the V_2 vasopressin receptor. X-ray crystal structure analysis has previously shown a fully phosphorylated carboxy-terminal peptide derived from the V_2 vasopressin receptor in complex with β-arrestin1 (Shukla *et al.,* 2013). An important observation from this study shows the binding of a well-conserved lysine residue (Lys10) on β-strand I of the arrestin molecule to phosphorylated Ser363. Incidentally, this serine residue makes up one of the residues in the cluster of serine residues added to the β_2-adrenergic receptor

indicating a potential mechanism whereby this cluster can alter the affinity of the mutant receptor to the arrestin molecule.

Usually, analysis of the effects of sites of phosphorylation involve site-directed mutagenesis of sites of interest, a strategy previously employed by our lab (Butcher *et al.,* 2011; Butcher *et al.,* 2014). In particular, serine or threonine residues are replaced by alanine residues and mutations of tyrosine residues to phenylalanine in transfected recombinantly expressed receptors prevent phosphorylation, and phenotypic changes are attributed to changes in the phosphorylation status of the receptor. Another strategy to investigate phosphorylation is to substitute phosphorylated residues with a residue which can mimic a phosphorylated amino acid (Tarrant and Cole 2009). Here, phospho-serine or phospho-threonine residues are replaced with aspartate or glutamate residues. Previous studies have successfully shown the use of mimetic residues faithfully mimic phosphorylated serine/threonine residues (Hao *et al.,* 1996; Potter and Hunter 1998). However, evidence for the introduction of mimetic residues in the third intracellular loop or C-tail of GPCRs is lacking. In this chapter, we present data related to an FFA4 receptor mutant which has five key phosphorylation sites in its C-terminal tail replaced with phosphomimetic (aspartate) residues. These sites have previously been shown to undergo agonist-stimulated phosphorylation and play an important role in arrestin-3 recruitment to the receptor (Butcher *et al.,* 2014).

Initial studies using our BRET based arrestin-3 recruitment assay need to include investigations into the ratio of donor/acceptor molecules; optimal ratios allow for obtaining appreciable BRET signals and are needed to generate overall conclusions. The level of energy transfer detected for a given concentration of donor should rise as the concentration of acceptor is increased until all donor molecules are engaged by acceptor. These energy curves should reach a plateau allowing saturation curves to be constructed. Following on from these initial studies, we then used the wild type FFA4, a phosphorylation-deficient FFA4 mutant, and the phosphomimetic FFA4 mutant in a BRET-based arrestin-3 recruitment assay. We show that the wild type FFA4 is strongly coupled to arrestin-3 in this assay format. The phosphorylation-deficient mutant shows a reduced agonist-mediated recruitment of arrestin-3, from previously studies (Butcher *et al.,* 2014), we know this is due to the loss of the phosphorylation sites from the C-terminal tail of the receptor. Replacing sites in the C-tail with phosphomimetic residues resulted in a receptor which recruited arrestin-3 in this assay format, however, this

recruitment was significantly reduced when compared with the wild type FFA4. Interestingly, the recruitment of arrestin-3 by the phosphomimetic mutant FFA4 was higher than the phosphorylation-deficient mutant. It is likely that the lack of abrogation of arrestin-3 recruitment by the phosphomimetic receptor is due to the negative charge which is imparted by the mimetic residues. This results in a receptor which has reduced affinity for the arrestin-3 molecule, and therefore, results in an overall decrease of total arrestin-3 recruitment as measured by this assay.

There are differences between a phosphorylated serine/threonine residue side chain and the side chains of acidic residues such as aspartate. In particular, the phosphate group, with its large hydrated shell owing in part to the large phosphate atom at its centre and an extra oxygen, and its negative charge of greater than 1 is distinct from the carboxyl side chains of aspartate and glutamate which only have a single negative charge and a smaller hydrated shell than phosphate (Tarrant and Cole 2009; Hunter, 2012). A protein-linked phosphate group can form salt bridges or hydrogen bonds either intra- or intermolecularly. In particular, the phosphate group is well matched for interaction with the guanidino group of arginine residues. This group is composed of a rigid, planar structure that can make direct hydrogen bonds to the doubly charged phosphate group at physiological pH. Due to a higher density of negative charge and a larger hydrated shell, phosphorylated amino acids form stronger and more stable hydrogen bonds and salt bridges than do aspartate or glutamate residues with arginine (Mandell *et al.,* 2007). Studies have highlighted the importance of a critical phosphate-sensor in arrestin, Arg175 (Gurevich and Benovic 1995; Y. J. Kim *et al.,* 2013) as well as other putative phosphorylation binding sites such as the well conserved lysine residues on β-strand-1 of arrestin (Shukla *et al.,* 2013) which help to form tight interactions with phosphorylated, active GPCRs. It is likely that replacing phosphorylated residues in FFA4 with phosphomimetics weakens the interactions between the receptor and arrestin resulting in a reduction in affinity and manifests itself as reduced total arrestin-3 recruitment in our BRET based assay.

Receptor phosphorylation is an important aspect of arrestin recruitment to the receptor (Ferguson, 2001). It was postulated that a receptor with phosphomimetic residues in place of serine and threonine residues would recruit arrestin quicker as the receptor would no longer need to become phosphorylated after activation. Here, we show data from a BRET based arrestin-3 recruitment assay which attempted to measure the rate of

recruitment to the activated receptor. We show in this assay format that the association of arrestin-3 and the phosphomimetic receptor is quicker when compared with wild type FFA4, giving credence to our hypothesis. However, the association of arrestin-3 to a completely phosphorylation-deficient mutant also seems to be faster than the wild type FFA4. This result is unexpected as previous studies from our lab have shown removal of sites of phosphorylation from the C-terminus of FFA4 results in a phosphorylation-deficient mutant which has a lower affinity for the arrestin-3 molecule and slows the rate of association to arrestin-3 (Butcher *et al.,* 2014). It is important to note that the values we have observed are not statistically significant, and the unanticipated results may be explained by the limitations of our assay. Whereas previous data from our lab has shown that a phosphorylation-deficient FFA4 shows a slower association to arrestin-3, this receptor was not a completely phosphorylation-deficient mutant like the receptor used in our study and showed only a partial reduction in affinity for arrestin-3. This translated into an association curve that had not plateaued after 5 min of stimulation and which eventually associated with arrestin-3 at levels comparable to the wild type FFA4 after an extended period of time (Butcher *et al.,* 2014). Our data for the phosphomimetic and phosphorylation-deficient mutants has clearly plateaued after 5 min of TUG-891 stimulation. Therefore, we hypothesise that even after extended periods of agonist stimulation we would not see levels of association comparable to wild type FFA4. Further work would entail measuring the levels of association for the wild type FFA4 as well as the phosphomimetic and phosphorylation-deficient mutants for extended time periods to confirm or refute thie hypothesis. We, therefore, have to surmise that the assay performed here is not sufficient to yield any meaningful data which we can use to accept or refute our original hypothesis with regards to the kinetics of arrestin association to the FFA4 and its mutants. The evidence from this study, as well as previous work from our laboratory (Butcher *et al.,* 2014), shows that the mutations to the receptor do result in altered rate constants but future work is needed to ascertain the true extent of these changes. A further limitation of the study is our inability to calculate association and disassociation constants (K_{on} and K_{off}, respectively) for the receptor-arrestin interaction. Further work would entail using several agonist concentrations to obtain an association constant and a modified assay which would allow the system to be perfused in order to obtain a disassociation constant.

Generally, GPCR regulation involves arrestin-mediated internalisation of the receptor (van Koppen and Jakobs 2004). Studies have shown that the FFA4 undergoes receptor internalisation upon agonist-stimulation after periods of as little as 5 min (Butcher *et al.,* 2014). This internalisation of the FFA4 is shown to rely on the ability of the receptor to successfully recruit β-arrestin, as sequential removal of five key phosphorylation sites in the C-terminal tail of the receptor resulted in a progressive reduction in agonist-mediated recruitment of arrestin-3. These phosphorylation-deficient mutants also showed a disruption in agonist-mediated receptor internalisation. Further evidence showing the importance of the C-terminal tail of FFA4 in receptor internalisation came from truncating the FFA4 to remove the C-terminal tail of the receptor which abrogated arrestin-3 recruitment and receptor internalisation. Here, we demonstrate a similar phenotype with regards to receptor internalisation. Using a plate-based ELISA assay measuring receptor internalisation as well as immunocytochemistry, we show a correlation between the ability of the FFA4 to recruit arrestin-3 and for the receptor to undergo agonist-mediated internalisation. Our studies show a reduction in efficacy of TUG-891 to recruit arrestin-3 to the phosphomimetic receptor which translates into a physiological response of reduced internalisation of the receptor after agonist-stimulation for 5 min when compared to wild type FFA4. We recapitulate data from previous studies (Butcher *et al.,* 2014) which demonstrated phosphorylation-deficient receptor mutants showed reduced receptor internalisation.

In conclusion, we show that mutation of five key residues in the C-terminal tail of the FFA4 to phosphomimetic residues does not faithfully mimic the actions of phosphorylated serine and threonine residues. This has been tested in a BRET-based arrestin-3 recruitment assay, and shows that the phosphomimetic mutant as well as a completely phosphorylation-deficient mutant exhibits a reduction in efficacy of a FFA4-specific agonist (TUG-891) to promote interactions with arrestin-3. We have attempted to delineate the kinetics of this process; however, further work is needed to derive any meaningful conclusions from the data. The data in this study also highlights a correlation between the ability of the FFA4 to interact with arrestin-3 and promote receptor internalisation corroborating previous studies from our lab.

Chapter 6 | Generating a testing tool for the expression of human histamine H_4 receptor constructs

6.1. Introduction

Mammalian cell systems are important tools used to overexpress recombinant proteins allowing the investigation of functional properties of these proteins such as downstream signalling responses. Expression in mammalian cells has the added benefit of conferring critical post-translational modifications to the recombinant proteins. Often, these post-translational modifications such as glycosylation and palmitoylation are required for proper folding and biological activity of the protein (Dalton and Barton 2014). Furthermore, mammalian cell systems allow for the stable transfection of a protein yielding a reusable resource which can be stored and cultured many times and still providing a consistent and reliable level of protein expression.

Chinese hamster ovary (CHO) cells and human embryonic kidney 293 (HEK 293) cells and their derivatives are commonly used. CHO cells provide a reliable cell line in culture and display an adaptable nature with regards to conditions these cells can grow in (Bandaranayake and Almo 2014). HEK 293 cells are also a common cell line utilised in the expression of proteins of interest, and may even represent a cell line which produces proteins which are a closer match to human proteins in terms of post-translational modifications and function (Walsh, 2010).

Fusion tags provide a tool to not only optimise protein folding and stability, but can also serve as a convenient means for purification of proteins (Dalton and Barton 2014). Epitope tags such as the polypeptide derived from the human influenza hemagglutinin (HA) molecule are well characterised and highly immune-reactive, can be exploited in co-immunoprecipitation studies and are easily detected via western blot analysis (Zhao *et al.,* 2013). Another strategy which can be employed to select cells which are expressing the protein of interest is to detect the fluorescence intensity of a co-expressed marker such as green fluorescent protein (GFP) (Mancia *et al.,* 2004).

The use of chimeric receptors composed of domains of various GPCRs has served as an important tool in the discovery of the structure, function, and biology of GPCRs (Yin *et al.,* 2004). Numerous examples exist in the literature which have used this strategy to

investigate issues such as the molecular nature of ligand binding domains and the properties of cytoplasmic domains in defining downstream signalling and receptor regulation/trafficking (Yin *et al.,* 2004). A classic example of this is the study which investigated the nature of GPCR resensitisation by using the β_2 adrenergic receptor (β_2AR), known to recycle and resensitise rapidly, and the vasopressin V2 receptor (V2R), known to recycle and resensitise slowly and the role of arrestin (Oakley *et al.,* 1999). This study created two chimeric receptors in which the C-terminal tails of the β_2AR and V2R were exchanged, one for the other, after the putative sites of palmitoylation resulting in two chimeric GPCRs indistinguishable from their wild-type counterparts with regards to ligand binding, receptor expression, and ability to activate adenylyl cyclase. It was found by using a GFP-tagged arrestin and confocal microscopy that the C-tails of the receptor conferred the properties of either fast or slow recycling to the plasma membrane (Oakley *et al.,* 1999). It was also found that the more stable the arrestin interaction with the receptor i.e. those receptors which resensitised and recycled to the membrane slowly also showed a decreased rate of receptor dephosphorylation. This study highlights the potential of chimeric receptors to alter the association of proteins, in this case arrestin, to the receptor and possibly the downstream signalling of GPCRs. The N-terminal of GPCRs remains largely unknown for receptors which do not employ their N-terminus domains to bind ligands such as secretin-like family of receptors, metabotropic glutamate-like receptors and some rhodopsin-like GPCRs that bind peptides. The importance of the N-terminus in the D_2 receptor was probed with regards to signalling and internalisation (Cho *et al.,* 2012). This study found that shortening of the N-terminus abrogated the correct expression of the receptor on the cell surface, increased receptor internalisation, and altered endocytosis of the receptor. In receptors such as the α1-adrenoceptor, which contains a large N-terminal region truncation led to increased receptor expression at the plasma membrane (Hague *et al.,* 2004). This indicates that the N-terminus of GPCRs control surface expression of the receptors, but it should be pointed out that these effects vary depending on the structural features of each receptor.

The histamine H_4 receptor (H_4R) plays an important role in various immune cell processes (Leurs *et al.,* 2009; Nijmeijer *et al.,* 2012). Its discovery was relatively recent compared with the other histamine receptors (Leurs *et al.,* 2009), and studies in recombinant cell lines particularly investigations into the phosphorylation status of the

receptor are lacking. To this end, the aim of this chapter was to create a stably expressing H_4R cell line. We also aimed to create and express chimeric receptors which could enhance the expression of the H_4R and also rescue the ability of the GPCR to recruit arrestin in a BRET-based arrestin-3 recruitment assay by using components of the FFA4 receptor which expressed very well and recruited arrestin-3 when stimulated with agonist.

By using western blot analysis, we show evidence of a polypeptide which corresponds to the correct molecular weight of HA-tagged H_4R in CHO cells. However, this is expression is not stable. We subsequently show what appears to be a clone expressing a GFP-tagged H_4R in CHO cells using flow cytometry, but upon further investigation the cells only express the GFP tag and the receptor is absent. The creation of chimeric receptors which use portions of the FFA4 receptor (N- and C-terminals of FFA4) did not enhance expression of the H_4R or rescue the ability of the receptor to recruit arrestin-3 in our BRET-based assay.

6.2. Results

6.2.1. Generation of stable cell line expressing a HA-tagged human histamine H_4 receptor

A stable CHO cell line expressing the human histamine H_4 receptor was generated by transfecting CHO cells with HA-tagged human H_4R DNA using Lipofectamine 2000 transfection reagent. Cells successfully transfected were selected for with 500 μg/ml of hygromycin B, cells which survived the antibiotic selection were plated at high dilutions to allow the formation of single colonies. These were picked and screened using immunoblotting analysis. **Figure 6.1** shows a representative blot showing a polypeptide at a molecular weight of ~43 kDa corresponding to the HA-tagged human H_4R.

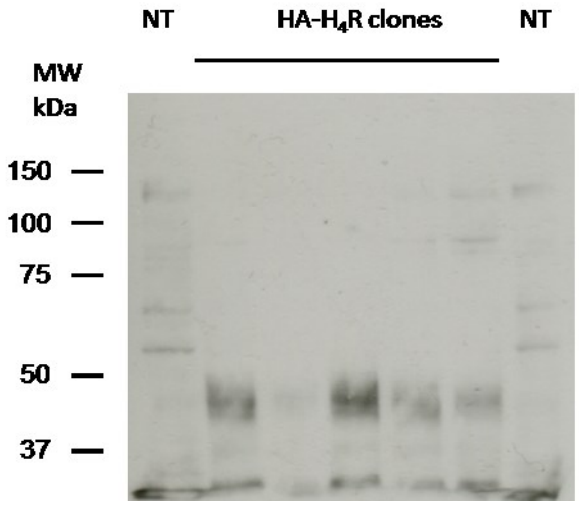
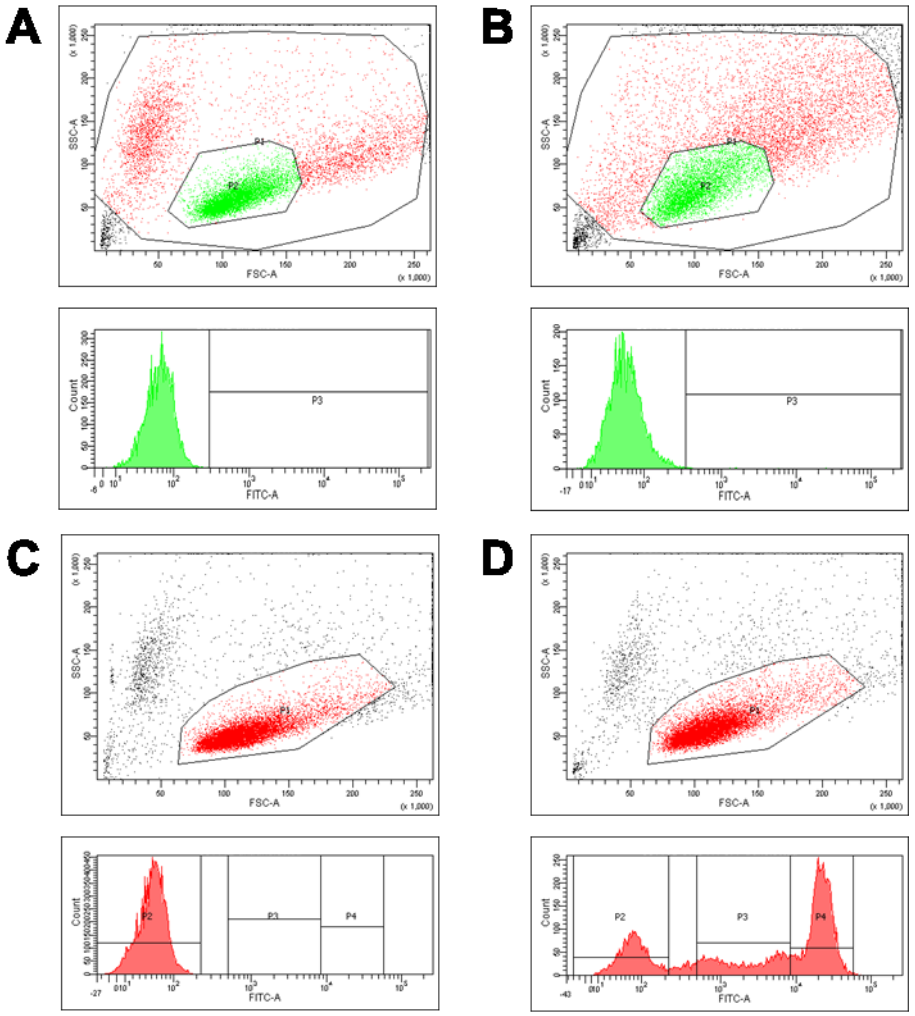


Figure 6.1| Immunoblot analysis of the histamine hH_4R expression in CHO cells. Receptors were immuno-precipitated using an immobilised anti-HA antibody as described in *Materials and Methods*. Protein levels were detected using HA protein-specific antisera. Immunoblots represent at least two independent experiments.

6.2.2. Generation of stable cell line expressing a eGFP-tagged human histamine H_4 receptor

A stable cell line expressing the human H_4R was generated by transfection of CHO cells with an eGFP-tagged human H_4R DNA construct using Lipofectamine 2000 transfection reagent. Transfected cells were selected for using 500 μg/ml of hygromycin B, with cells surviving this procedure plated at high dilutions to allow the formation of single colonies which were screened based on whether they fluoresced when viewed under a confocal microscope. Flow cytometry was used to further select for these cells and create a heterogeneously expressing cell population. Dead cells and cellular debris was omitted from the sorting step based on the approximate size of the cells. Non-transfected CHO cells were used to set gating parameters (**Figure 6.2A**). The eGFP tag was excited by a 488 nm laser with the emission collection filters 530/30 nm. Analysis revealed a population of cells which fluoresced when excited with the laser indicating the presence of the eGFP tag (**Figure 6.2B**). Further analysis using flow cytometry was used to enrich this population of cells which expressed the eGFP tag, which revealed the presence of two populations of cells (**Figure 6.2D**). These cells exhibited eGFP in relatively low (*h*H_4-GFP (+)) or high (*h*H_4-GFP (++)), fluorescence.

Using immunoblot analysis, cell lysates were from each cell population was probed with eGFP-specific protein antisera revealing a band corresponding to a polypeptide with a molecular weight of ~27 kDa. No band was seen at higher molecular weights which may correspond to the human H_4R and eGFP construct.


A
B
C
D
SSC-A
FSC-A
(x 1,000)
P1
P2
Count
FITC-A
P3
P4

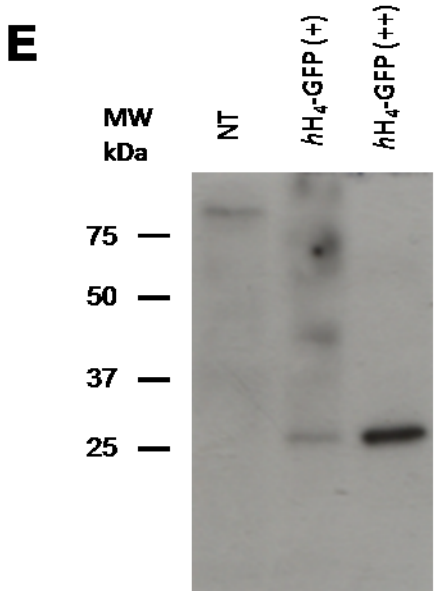

E
MW
kDa
NT
hH4-GFP (+)
hH4-GFP (++)
75
50
37
25

Figure 6.2| Analysis and sorting of a stably transfected histamine hH_4-eGFP cell line by flow cytometry. CHO cells thought to be stably expressing the hH_4-eGFP receptor construct were sorted by flow cytometry to create a homogenous population of cells expressing the receptor. (A) Non-transfected CHO cells were used to set the gating parameters to determine cells expressing fluorescence. (B) CHO cells thought to be expressing the hH_4-eGFP receptor construct were then sorted using these parameters to select for cells exhibiting eGFP fluorescence and therefore expressing the receptor construct. Approximately 0.6% of the initial number of cells analysed expressed the receptor construct at a satisfactory level and were selected for. The sorted cells were sorted again to enrich the population and create a homogenous population of cells expressing the receptor construct, (C) CHO cells were used to set the gating parameters of the new experiment. (D) Sorting cells revealed two populations of cells, P3 represents a population of cells exhibiting eGFP fluorescence in relatively low concentrations (hH_4-GFP (+)), *P4* represents a population of cells exhibiting eGFP fluorescence in a relatively high concentration (hH_4-GFP (++)). (E) immunoblots were performed on whole cell lysates, blots were probed with eGFP-specific protein antisera. Data represents one independent experiment.

6.2.3. Analysis of human histamine H_4 receptor expression in HEK293 cells

To investigate the interactions of the human H_4R with arrestin required the expression of the receptor in HEK293 cells. Cells were transfected with eYFP-tagged human H_4R DNA using Lipofectamine 2000 transfection reagent. Immunoblot analysis was used to confirm the presence of the tagged receptor in HEK293 cells when grown at 37°C (**Figure 6.3A**) or 30°C (**Figure 6.3B**). When the cells are grown at 30°C, we see evidence of the receptor by visualising a polypeptide with a molecular weight of ~70 kDA which is the expected molecular weight of the receptor (~43 kDA) plus the molecular weight of the eYFP-tag (~27 kDa), with this polypeptide absent when the cells are grown at 37°C.

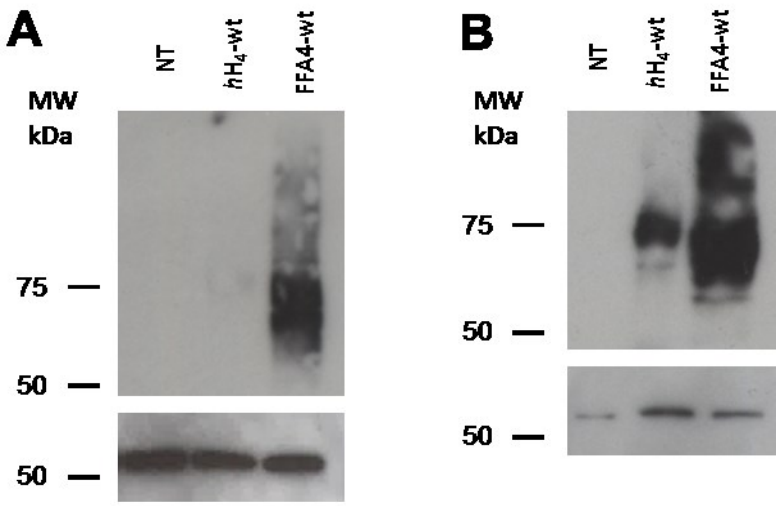


Figure 6.3| Immunoblot analysis of the histamine hH_4 receptor expression in HEK 293 cells. HEK 293 cells were transiently transfected with DNA corresponding to the histamine hH_4 receptor or the FFA4-wt receptor as described in *Materials and Methods*. Cells were grown either at 37°C (A) or 30°C (B) and whole cell lysates were prepared and samples resolved on SDS-PAGE minigels and probed with antisera raised against eGFP (*top panels*), loading controls were detected using antisera raised against α-tubulin. Immunoblots represent a single independent experiment.

6.2.4. Arrestin-3 interaction with the human histamine H_4 receptor

A BRET based assay in our lab has successfully shown the interaction of arrestin-3 with the FFA4 receptor. To investigate the interactions between arrestin-3 and the *h*H_4R in the same assay, cells were co-transfected with the eYFP-tagged human H_4R and the *β*-arrestin-2–*Renilla* luciferase constructs while grown at 30°C and 37°C. The FFA4 receptor was used as a positive control and when tested in the BRET-based arrestin-3 interaction assay, 5 min of TUG-891 (10 μM) stimulation resulted in a strong increase in arrestin-3-RLuc/FFA4-eYFP BRET, increasing the basal signal from 28.09 ± 5.50 milli-BRET units to the TUG-891-stimulated value of 175.20 ± 0.04 milli-BRET units. This was in contrast to the *h*H_4R, which did not show a difference between 5 min histamine (10 μM) stimulation compared with basal regardless of the temperature the cells were grown at (**Figure 6.4**).

Employing chimeric receptors which result in an altered C-terminal tail has been shown to successfully change the recruitment dynamics of arrestin to a GPCR (Oakley *et al.,* 1999; Pal *et al.,* 2013), and also change the downstream signalling of receptors (Mangmool *et al.,* 2010). N-terminal signal sequences are known to be involved in proper folding and translocation firstly to the endoplasmic reticulum and then to the plasma membrane (Schülein *et al.,* 2012; Rutz *et al.,* 2015). It is also known from previous studies (Butcher *et al.,* 2014; Hudson *et al.,* 2014), that the C-tail of the FFA4 receptor is very important in mediating interactions with arrestin-3. It was therefore hypothesised that creating chimeric H_4R which contained parts of the FFA4 receptor may rescue expression of the receptor, and also mediate interactions with arrestin-3. To this end, chimeric receptors were created which contained portions of the N-terminus (Met1-Thr45) or C-terminus including helix VIII (Asn333-Gly377) of FFA4 in place of the corresponding N- and C-termini of the histamine H_4 receptor. Chimeric receptors were also created which contained phosphomimetic mutations in the C-terminal tail of the receptor, when the receptor contained the FFA4 C-terminal tail. This was done as it was not certain whether the serine and threonine residues in the FFA4 C-tail would become phosphorylated when they were a part of the chimeric receptor. It was hypothesised that the phosphomimetic residues would overcome this issue. The FFA4 receptor was used as a positive control and when tested in the BRET-based arrestin-3 interaction assay, 5 min of TUG-891 (10 μM) stimulation resulted in a strong increase in arrestin-3-RLuc/FFA4-eYFP BRET, increasing the basal signal from 34.96 ± 4.67

milli-BRET units to the TUG-891-stimulated value of 224.50 ± 5.20 milli-BRET units. The chimeric receptors, however, did not show a histamine-mediated increase in BRET signal (**Figure 6.5A**). Immunoblot analysis revealed a band identifying a polypeptide with a molecular weight of ~70 kDa corresponding to the FFA4-wt receptor. As can be seen from the immunoblot, 10x more protein has been loaded into the lanes corresponding to the chimeric receptors compared to the lane containing lysates from cells expressing the FFA4-wt receptor (10 µg vs. 1 µg), however, the bands indicating the presence of the polypeptide with a molecular weight of ~70 kDa corresponding to the H_4R chimeras show a similar intensity to the band identified as the FFA4-wt receptor (**Figure 6.5B**).

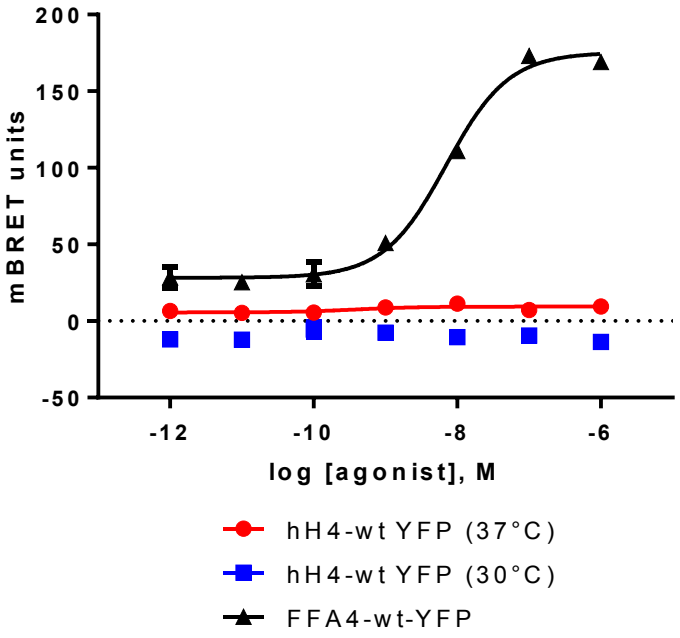


Figure 6.4| The histamine hH_4 receptor does not interact with arrestin-3 in this assay format. HEK 293 cells were transiently transfected with DNA corresponding to the histamine hH_4 receptor or the FFA4-wt receptor and grown at either 37°C or 30°C. These cells were then used in BRET-based arrestin-3 interaction studies in response to the appropriate ligand (histamine or TUG-891). Data points represent the mean ± S.E.M of three independent experiments.

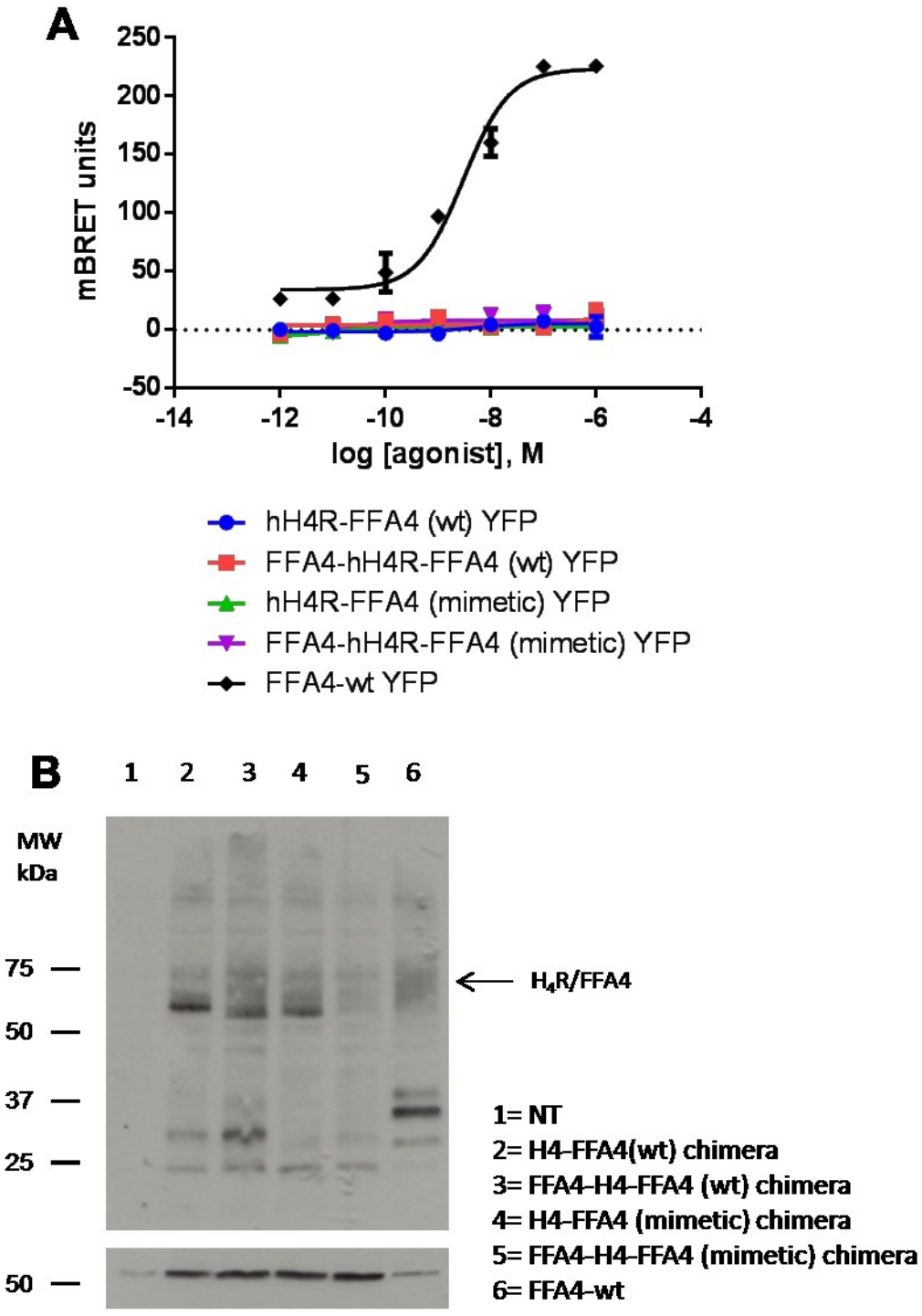

A
250
200
150
100
50
0
-50
mBRET units
-14
-12
-10
-8
-6
-4
log [agonist], M
hH4R-FFA4 (wt) YFP
FFA4-hH4R-FFA4 (wt) YFP
hH4R-FFA4 (mimetic) YFP
FFA4-hH4R-FFA4 (mimetic) YFP
FFA4-wt YFP
B
1
2
3
4
5
6
MW
kDa
75
50
37
25
50
H4R/FFA4
1= NT
2= H4-FFA4(wt) chimera
3= FFA4-H4-FFA4 (wt) chimera
4= H4-FFA4 (mimetic) chimera
5= FFA4-H4-FFA4 (mimetic) chimera
6= FFA4-wt

Figure 6.5| Chimeric H4 receptors employing portions of the FFA4 receptor do not rescue receptor arrestin-3 interactions or histamine hH4 receptor expression. HEK 293 cells were transiently transfected with DNA corresponding to the FFA4-wt receptor or chimeric receptors with portions of the FFA4 receptor added onto the histamine hH_4 receptor and grown at 30°C. These cells were then used in BRET-based arrestin-3 interaction studies in response to the appropriate ligand (histamine or TUG-891) (A). (B) Whole cell lysates were prepared and samples resolved on SDS-PAGE minigels and probed with antisera raised against eGFP (*top panel*), loading controls were detected using antisera raised against α-tubulin. Data points represent the mean ± S.E.M of two independent experiments. Immunoblots represent two independent experiments.

6.3. Discussion

The expression of functional membrane proteins is a multistep process. Details of individual steps are known, however, other aspects such as the precise role of ribosomes in membrane protein biogenesis or membrane protein folding and assembly in the lipid bilayer are lacking (Grisshammer, 2006a). A eukaryotic protein which is targeted to the plasma membrane is first directed to a protein-conducting channel in the endoplasmic reticulum (ER); the polypeptide is then released into the ER membrane where it folds into its native conformation. Quality control measures are in place that only allow correctly folded proteins to pass further into the Golgi apparatus (Ellgaard *et al.,* 1999). Proteins which fail to fold correctly are degraded (Tsai *et al.,* 2002). Membrane proteins are usually glycosylated, with modifications to glycan chains occurring on route to the plasma membrane.

The reasons why some membrane proteins are overexpressed easily in recombinant cell systems, but others are expressed poorly are not fully understood (Grisshammer and Tate 1995; Grisshammer, 2006b). It is known that this problem is not proportional to the number of transmembrane α-helices or size, but is related to the complexity of the membrane protein (Tate, 2001). It is likely that an interplay of several factors including; (in)stability of mRNA, folding of the nascent polypeptide chain in the ribosome and translocon, efficiency of membrane insertion, the role of post-translational modifications such as glycosylation in the folding process and the requirement of molecular chaperones to facilitate folding all dictate the level of functional protein expressed in a cell. For these proteins which fail to express easily in recombinant cell systems, different strategies can be implemented to try and increase expression levels.

The H_4R is an emerging drug target for immune and inflammatory disorders (Leurs *et al.,* 2009). To fully characterise the receptor including investigations into the phosphorylation status of the receptor which is central to receptor desensitisation (Lohse *et al.,* 1990a; Lohse *et al.,* 1990; Tobin 2008), a stable cell line expressing the H_4R needed to be created. Whilst phosphorylation of the H_1R and H_2R has been studied (Iwata *et al.,* 2005; Kawakami *et al.,* 2003; Rodriguez-Pena *et al.,* 2000), studies into the phosphorylation status of the H_4R are still lacking. Here we show data related to the generation of a stably transfected cell line expressing an N-terminally HA-tagged H_4R.

The vector chosen to host the DNA for the recombinant receptor construct was pcDNA3.1 which allowed the integration of the DNA into the host cell genome. After serial dilution of cells, single colonies were chosen using antibiotic selection and scaled up. Initial screening using western blot analysis revealed the presence of a polypeptide consistent with an N-terminally HA-tagged H_4R, however, subsequent screening showed the loss of the receptor construct. CHO cells were chosen as the cell system in which to express the H_4R as other GPCRs had been successfully transfected into these cells (data not shown), and there was no previous evidence of these cells endogenously expressing the H_4R. The HA-tag has been described as a well characterised and highly immune-reactive tag unlikely to interfere with the bioactivity and function of fusion partner proteins (Zhao *et al.,* 2013). Moreover, the HA-tag is routinely used in our laboratory to create stably expressing cell lines expressing the receptor of interest tagged with HA. Therefore, the inability to create a cell line stably expressing a HA-tagged receptor was unanticipated. It was postulated that the presence of the HA-tag on the N-terminal of the receptor may disrupt the proper folding and translocation of the protein to the plasma membrane suggesting a different strategy should be employed to create a stably expressing H_4R cell line.

Using fluorescent markers such as eGFP provides a non-invasive means of monitoring the dynamics of eGFP-tagged proteins *in vivo*, this has the added benefit of producing cells which can be FACs sorted based on the level of intracellular fluorescence of eGFP. For membrane proteins, addition of eGFP as a carboxyl-terminal fusion is useful in following the extent of protein expression and localisation (Dalton and Barton 2014). Here, we show data related to the generation of a stable cell line expressing the H_4R with an eGFP molecule fused to its carboxyl terminal. This strategy was employed in order to overcome any issues arising from using an N-terminal-located epitope tag. FACs sorting of cells yielded two populations of cells which were expanded and investigated for the expression of a receptor-eGFP fusion protein. We show eGFP alone is being translated by the cell, with the H_4R portion of the fusion protein not being translated. The literature shows that eGFP has the potential to impair protein biogenesis and block subcellular targeting signals (Yewdell *et al.,* 2011). This potentially results in destabilisation of the fusion protein due to improper folding and may lead to early degradation of the tagged protein. When there are issues surrounding the expression

and translocation of a protein, the potential effects of eGFP may exacerbate these issues and could explain the phenomenon we see in our study.

The use of reduced temperature cultivation (cold-shock) of mammalian cells has been proposed to enhance recombinant protein production in cells (Al-Fageeh *et al.,* 2006). Although it is important to note that the diversity of the cold-shock conditions employed, the expression systems used, and the consequential changes in the amount of recombinant protein expressed are considerable. We decided to culture HEK 293 cells which had been transiently transfected with DNA (H_4R tagged with eYFP) at a sub-physiological temperature (30°C) and investigate protein expression. Here we show data which indicates culturing cells at a sub-physiological temperature results in changes in recombinant protein expression. Sub-physiological temperature culturing of mammalian cells generally results in the elicitation of a cold-stress response resulting in global shutdown of protein synthesis by mechanisms which control mRNA translation (Phadtare *et al.,* 1999). The general mechanism of control is via the phosphorylation of initiation/elongation factors such as eIF2α, the phosphorylation of this molecule results in the decrease of cap-dependent mRNA translation (Webb and Proud 1997). Moreover, the 5'-untranslated region (UTR) can form stable secondary structures masking regions important in ribosomal attachment such as the Shine-Dalgarno sequence (Ermolenko and Makhatadze 2002). Internal ribosome entry sites (IRES) provide an additional control mechanism for translation. A recent study has implicated IRES regions in the enhanced translation of the mRNA of a cold-inducible mammalian protein, RBM3 (Sonna *et al.,* 2002). It is postulated that the IRES section of the 5'-UTR enhances mRNA translation upon cold-shock where normal regulatory mechanisms may be compromised by allowing the ribosome to become recruited to the start codon avoiding usual scanning mechanisms. It is important to note that extended periods of low temperatures cause the arrest of mammalian cell line proliferation in the G_1 phase of the cell cycle (Kaufmann *et al.,* 1999), and thus this strategy is probably not a viable alternative to creating stable cell lines.

Our lab utilises a BRET-based arrestin-3 recruitment assay, and has successfully shown the recruitment of arrestin-3 to FFA4 in this assay format (**Chapter 4**) and (Butcher *et al.,* 2014). As arrestin interaction is common for all GPCRs, and we now possessed evidence of H_4R expression in HEK 293 cells, we next attempted to demonstrate arrestin-3 recruitment to this receptor fusion protein. GPCR phosphorylation plays an

important role in arrestin recruitment and receptor desensitisation (Tobin 2008), and our previous work with the H_4R has shown the receptor undergoes histamine-mediated increases in phosphorylation (**Chapter 1**). Here we demonstrate that the H_4R does not recruit arrestin-3 in this assay format. This is not unexpected as other GPCRs in this assay have shown an inability to recruit arrestin-3 in this assay format (data not shown).

Chimeric GPCRs composed of domains of various receptors have provided useful tools to delineate the structure, function, and biology of GPCRs (Yin *et al.,* 2004). After agonist stimulation, receptors undergo desensitisation which is mediated by arrestins. The biochemical and kinetic properties of the cellular processes that govern resensitisation differ between receptors. Some internalised GPCRs can recycle rapidly back to the plasma membrane whereas others are retained inside the endosome and are recycled slowly or not at all. One elegant study highlights this by switching the C-terminal tails of two receptors with opposite recycling strategies (Oakley *et al.,* 1999). The β_2-adrenergic receptor (β_2AR) is known to recycle quickly back to the plasma membrane after activation in a β-arrestin-dependent manner resulting in normal receptor responsiveness. The V_2 vasopressin receptor (V_2R) in complex with β-arrestin is more stable and the receptor-arrestin complex persists in internalised endosomes and recycles slowly back to the plasma membrane. Switching the C-terminal tails of the β_2AR and the V_2R completely reverses their de-phosphorylation, recycling, and resensitisation kinetics (Oakley *et al.,* 1999). This phenomenon has also been described for the protease-activated receptor-2 (PAR_2) and neurokinin-1 receptor (NK1R) (Pal *et al.,* 2013). Other studies have used chimeric receptors to demonstrate the importance of the N-terminus on receptor expression and localisation. Studies involving the D_2 and D_3 dopamine receptors showed switching N-terminal regions of the two receptors also switched the cell surface expression of each receptor (Cho *et al.,* 2012). Here we present data showing the creation of chimeric receptors of the H_4R, with portions of the FFA4 fused to the N- or C-terminus or both of the H_4R. The FFA4 has been shown to express well in transient transfections (**Figure 5.5A, B**) and in a stable cell line (**Figure 4.1**). We postulated that these chimeric receptors may show expression comparable to the FFA4, and may also rescue arrestin-3 recruitment to the H_4R chimera in our BRET-based assay. Two chimeric receptors also contained phosphomimetic residues in their C-terminal tails in place of serine and threonine residues. It was thought that the C-tail of the FFA4 when fused to the H_4R may not become phosphorylated like the wild type

FFA4 which is known to be critical in arrestin-3 recruitment (**Chapter 4**) and (Butcher *et al.,* 2014). Therefore, phosphomimetic mutations were introduced into the C-tail of these chimeras to overcome this problem. Our results show that creating chimeric receptors does not rescue expression of the H_4R, nor does it rescue arrestin-3 recruitment to the same receptor. This is disappointing given the results from other studies which have used either chimeric receptors or have engineered in sequences to show proper receptor signalling and expression can be rescued (Cho *et al.,* 2012; Lindner *et al.,* 2009; Prado *et al.,* 2007).

In conclusion, we show that the expression of the H_4R remains problematic. We have used several strategies including the use of different epitope tags and chimeric receptors to try and rescue H_4R expression in a recombinant cell line. Transiently transfecting the receptor construct into mammalian cells and subsequently culturing the cells at a sub-physiological temperature shows promise in showing an increased H_4R expression profile in cells but further work is required to derive any meaningful conclusions. The data in this study highlights the potential problems one can face when setting up a recombinant cell line with a membrane protein such as GPCRs.

Chapter 7 | Final discussion

7.1. Discussion, critique and future work

7.1.1. Investigating the phosphorylation status of the histamine H_4 receptor

GPCRs can be activated by a host of different ligands ranging from photons of light to biogenic amines and free fatty acids resulting in the transduction of extracellular signals into internal responses. As their name suggests GPCRs couple to G-proteins as well as other membrane-associated and intracellular proteins. This flexible nature makes GPCRs important therapeutic drug targets with approximately one-third of all prescribed drugs on the market targeting GPCRs (Overington *et al.,* 2006). Several studies have demonstrated that receptors are not simply "on-off" switches, but rather adopt a range of conformations (Bockenhauer *et al.,* 2011; Kobilka and Deupi 2007). This conformational heterogeneity allows structurally different ligands inducing distinct conformational states, which results in the activation of distinct signalling pathways available to the receptor (Kobilka and Deupi 2007). GPCRs are highly regulated proteins; and the exposure of the receptor to agonist results in rapid desensitisation. One of the central mechanisms of this process is receptor phosphorylation (Lohse *et al.,* 1990; Lohse *et al.,* 1990; Tobin 2008). Taken together, one can hypothesise that different agonists causing distinct conformational states of a given receptor enables different kinases to phosphorylate the receptor on distinct sites in the third intracellular loop and C-terminal tail. This has been shown to be true for several GPCRs and their respective ligands (Busillo *et al.,* 2010; Butcher *et al.,* 2011; Nobles *et al.,* 2011; Tobin 2008; Tobin *et al.,* 2008).

An elegant study involving the β_2-adrenergic receptor demonstrates the ability of different ligands to cause the recruitment of different GRKs resulting in distinct receptor phosphorylation patterns and different physiological outcomes for the receptor (Nobles *et al.,* 2011). It was also demonstrated that the β-arrestin-biased ligand, carvedilol, induced a phosphorylation pattern distinct from that of an unbiased, full agonist, isoproterenol. This study, along with others, offers the opportunity of designing novel therapeutics with enhanced safety profiles which allow the activation of therapeutically beneficial signalling pathway(s) while abrogating the pathways which give rise to adverse effects (Boerrigter *et al.,* 2011; Boerrigter *et al.,* 2012).

Histamine receptors are composed of four distinct subtypes and represent prototypical family A GPCRs (Hill *et al.,* 1997; Leurs *et al.,* 2009). Among these, the histamine H_4 receptor (H_4R) is the most recently discovered and is an emerging drug target for immune and inflammatory disorders such as asthma and pruritus (Leurs *et al.,* 2009). An important tool for understanding the physiological role of the H_4R has been JNJ7777120, which displayed a high affinity for the receptor and is highly selective relative to other histamine receptors (Thurmond *et al.,* 2004). Using JNJ7777120 *in vivo* provided the first evidence that antagonism of the H_4R could have anti-inflammatory properties, whereby neutrophil influx in a mouse peritonitis model was reduced after pre-treatment with JNJ7777120 (Thurmond *et al.,* 2004). The use of JNJ7777120 has also implicated the H_4R in a variety of disease models such as asthma, dermatitis, pain, and pruritus (Thurmond, 2015). Interestingly, in these disease models JNJ7777120 is found to be acting as an antagonist, findings which are consistent with other distinct H_4R ligands and H_4R-deficient mice (Thurmond, 2015). Perhaps the best example of this is the role of the H_4R in mediating pruritic responses in mice (Dunford *et al.,* 2007). This study demonstrated that histamine-induced scratching in mice could be blocked by JNJ7777120 and was absent in H_4R-deficient mice. Additionally, other H_4R agonists also induced scratching which could be abrogated by JNJ7777120, but not in H_4R-deficient mice (Dunford *et al.,* 2007). It is important to note that these observed actions of JNJ7777120 are reported for the mouse receptor. Recent published data has shown that the mouse H_4R does not demonstrate constitutive activity and, therefore, JNJ7777120 primarily behaves as partial agonist at the mouse H_4R in Sf9 insect cells (Wifling *et al.,* 2015). This is somewhat surprising as it would contradict a variety of studies where JNJ7777120 has been found to be acting as an antagonist in *in vivo* studies and demonstrates the complexity of working with the H_4R. Although *in vivo* data for JNJ7777120 at the human H_4R is lacking, *in vitro* studies have demonstrated that JNJ7777120 functions as a β-arrestin-biased agonist (Rosethorne and Charlton 2011). This study establishes that JNJ7777120 is a biased agonist at the human H_4R when expressed in a U2OS cell expression system, acting as a neutral antagonist for [^{35}S]-GTPγS binding and a partial agonist for β-arrestin recruitment.

We extended this study in the first results chapter by investigating the effects of histamine and JNJ7777120 on the phosphorylation profile of the human H_4R and receptor internalisation in a U2OS cell expression system. By looking at the global

phosphorylation of the H_4R, our data indicated that histamine induced a robust increase in receptor phosphorylation; however, the β-arrestin-biased agonist JNJ7777120 did not. This is in line with other studies which have shown differences in receptor phosphorylation upon stimulation with a biased agonist versus an unbiased agonist (Nobles *et al.,* 2011). We further probed the phosphorylation status of the H_4R by looking at the peptide and individual amino acid level of the receptor using mass spectrometry. Here we show that the H_4R exhibits a highly phosphorylated basal state indicating that the receptor may be constitutively active and would be in line with recently reported data (Wifling *et al.,* 2015). Stimulation with either histamine or JNJ7777120 results in a reduction in the amount of phosphorylation sites observed from 13 observed sites under basal conditions to 9 and 10 sites after histamine and JNJ7777120 stimulation, respectively. It is important to note here that our mass spectrometry data are not quantitative. Also, the absence of an observed phosphorylation site in a mass spectrometry experiment is not confirmation that the receptor is no longer phosphorylated at this particular site, simply that the peptide was not observed. Taken together, it may be of benefit to this study to have used a quantitative method of mass spectrometry such as SILAC to investigate the phosphorylation profile of the H_4R. Extending these studies, phosphorylation-specific antibodies could also be raised to certain phosphorylated residues. This would allow us to focus on individual sites of phosphorylation, not only in recombinant expression systems but also in primary cells and tissue allowing us to explore in detail the "phosphorylation barcode" hypothesis (Tobin *et al.,* 2008; Tobin 2008). Furthermore, a receptor can be mutated in such a way that it becomes inherently biased. This can be done by replacing phosphoacceptor sites in the third intracellular loop and C-terminal tail with residues which cannot become phosphorylated (usually alanine residues). This results in a receptor which is no longer able to efficiently recruit and/or signal through β-arrestin, but retains its ability to signal through G-proteins (Kong *et al.,* 2010; Poulin *et al.,* 2010). It may be beneficial to this study to further characterise the signalling response which is mediated by H_4R phosphorylation by creating phosphorylation-deficient mutants. Biasing a receptor in this way would allow the delineation of downstream pathways that may be known to give rise to adverse effects. Understanding the role of H_4R phosphorylation provides a basis for the design of H_4R ligands that may drive specific signalling outcomes (Rajagopal *et al.,* 2010). Like JNJ7777120, these drugs have been termed biased agonists and can potentially drive specific physiological

outcomes by preferentially engaging a distinct subset of receptor signalling pathways. Indeed, JNJ7777120 has provided the basis for other biased ligands which preferentially couple to either $G\alpha_i$ proteins or β-arrestin (Nijmeijer *et al.,* 2013).

A consequence of receptor phosphorylation is the displacement of G-proteins with arrestin molecules and internalisation of the receptor (Ferguson, 2001). The functional activity of the H_4R was measured by investigations into its internalisation upon exposure to histamine or JNJ7777120. Histamine has been shown to act as a full agonist in arrestin recruitment to the H_4R, whereas JNJ7777120 has been described as a partial agonist (Rosethorne and Charlton 2011). Our data suggests histamine promotes similar levels of receptor internalisation after 5 and 30 min of stimulation when compared to JNJ7777120. One explanation which may account for our data is that the amount of arrestin recruited to the hH_4 receptor after JNJ7777120 stimulation is enough to cause receptor internalisation. Further recruitment of arrestin to the receptor, as caused by histamine stimulation does not cause an increase in either the total receptor internalisation or the speed of internalisation. This suggests partial recruitment of arrestin to the hH_4 receptor is enough to cause a measurable amount of receptor internalisation. Certainly, the study performed here would benefit from investigating histamine and JNJ7777120 stimulation of the H_4R at several time points using immunocytochemical analysis as well as other internalisation assays which might make the effects of JNJ7777120 exposure at the H_4R compared with histamine easier to quantify.

7.1.2. Characterisation of the FFA4 and its phosphorylation-deficient mutants

A major, novel finding in this thesis is the discovery that the H_4R becomes phosphorylated in response to agonist stimulation and that these sites of agonist-mediated phosphorylation can be mapped to its third intracellular loop and C-terminal tail. Whilst almost every GPCR has the potential to become phosphorylated as extensively as the H_4R in its third intracellular loop and C-tail, there exist receptors which do not demonstrate this phenomenon. The FFA4 is one such receptor, whereby agonist stimulation results in the phosphorylation of only 5 serine and threonine residues in the C-terminal tail of the receptor (Butcher *et al.,* 2014). Removal of these residues creates a receptor mutant which shows no observable difference in receptor phosphorylation when comparing basal versus agonist stimulation, a finding which has

been recapitulated in this thesis. Mutation of these 5 phosphorylation sites in the C-terminal tail to alanine results in a receptor which can be thought of as being inherently biased. Sequential removal of phosphorylation sites from the C-tail of the receptor results in a progressive reduction in the ability of the FFA4 to recruit arrestin-3. Indeed, the complete removal of phosphorylation sites to create a phosphorylation-deficient FFA4 mutant shows an impaired ability to recruit arrestin and could therefore be considered to be G-protein biased (Butcher *et al.,* 2014), data which we have recapitulated in this thesis. Removal of phosphorylation sites from the C-tail and subsequent impairment of arrestin-3 recruitment may support the notion that bulk negative charge as opposed to the specific pattern of phosphorylation is the primary mechanism which drives receptor/arrestin-3 interactions, however, the redundant nature of Thr349 phosphorylation which does not seem to contribute to arrestin-3 interactions opposes this idea.

This concept has been proposed previously from studies in our lab involving the M_3 mAchR (Butcher *et al.,* 2011) and for other receptors including the β_2-adrenergic receptor (Nobles *et al.,* 2011), CXCR4 receptor (Busillo *et al.,* 2010), thyrotropin-releasing hormone receptor (Gehret and Hinkle 2010; Hinkle *et al.,* 2012), and the somatostatin 2A receptor (Ghosh and Schonbrunn 2011). Taken together, this posits the theory that there is exist a "phosphorylation barcode" where specific sequences of phosphorylation are able to direct specific cell signalling outcomes resulting from receptor stimulation (Butcher *et al.,* 2011; Nobles *et al.,* 2011; Tobin *et al.,* 2008). As different cells or tissue systems may have a different complement of kinases which may be expressed at relatively different levels, it follows that receptors expressed in these systems may be phosphorylated differentially even when stimulated with the same ligand (Butcher *et al.,* 2011; Tobin *et al.,* 2008). It is known that different kinases phosphorylating different residues at a receptor can have substantially different effects on the downstream signalling outcomes for the receptor (Nobles *et al.,* 2011). The use of phosphorylation-specific antibodies can help to delineate the phosphorylation status of the GPCR of interest, and when used in concert with techniques such as siRNA knockdown of GRKs may allow one to state which GRKs may contribute differently to the processes of receptor desensitisation, endocytosis, and signalling (Butcher *et al.,* 2011; Kim *et al.,* 2005; Ren *et al.,* 2005). Whilst we have utilised a phosphorylation-specific antibody raised against a double phosphorylation event in the C-terminal tail of

the FFA4 and have preliminary data involving a GRK2 inhibitor, it would be beneficial to this study to investigate the expression and activity of the different GRKs in our recombinant cell system. This experiment, in concert with information obtained from our phosphorylation-specific antibody would allow a greater understanding of FFA4 phosphorylation and can be extended to cells and tissues where the receptor is endogenously expressed.

Expanding our work with the wild type FFA4 and its phosphorylation-deficient mutants has allowed us to investigate the downstream signalling of the receptor and attempt to define which processes may be G-protein-mediated and which may be β-arrestin-mediated. It is now known that GPCRs exist in multiple conformations induced by different ligands. Distinct conformations of receptors induced or stabilised by ligands may differentially recruit and interact with different cellular effectors to elicit specific signalling cascades. Knowledge of which signalling cascades can be elicited would be useful when designing biased ligands for therapeutic benefits in order to develop safer and more efficacious drugs.

With the realisation that GPCRs can mediate G-protein independent signalling via proteins such as β-arrestin, examining signalling to effectors such as ERK1/2 has become rather more complex. ERK 1/2 are important serine/threonine protein kinases which participate in the Ras-Raf-MEK-ERK signal transduction cascade which regulates a variety of cellular processes including proliferation and apoptosis (Roskoski 2012). ERK1/2 is a major cellular effector of many GPCRs (Eishingdrelo and Kongsamut 2013). It has been postulated that the time-course of ERK activation correlates with which molecule is involved in its activation (Luttrell and Gesty-Palmer 2010). ERK activation due to G-protein-dependent signalling has a rapid onset and wanes with desensitisation whereas G-protein-independent signalling has a slower onset and a sustained duration. Data from this thesis suggests that the ERK activation which peaks at 5 min that we observe after stimulation of the FFA4 and its phosphorylation-deficient mutants is largely G-protein-dependent. This is based on its rapid onset, and our work with a novel $G\alpha_q$ protein inhibitor which mostly abolishes the ERK signal. Interestingly, we also show that a reduced ability of the phosphorylation-deficient mutants of FFA4 to recruit arrestin translates to a physiological response of increased ERK activation as measured by an HTRF based assay, perhaps indicating increased G-protein signalling resulting from the reduced ability of the receptor to become

desensitised/internalised. The data also demonstrates a more sustained activation of ERK which persists after the intial peak seen at 5 min. Further work is needed to establish whether this sustained activation of ERK is result of G-protein-dependent or –independent activation. CREB (cyclic AMP response element (CRE)-binding protein) is a highly characterised stimulus-induced transcription factor (Shaywitz and Greenberg 1999). Here we show that FFA4 stimulation results in increases in the phosphorylation of CREB. However, our work does not allow us to conclusively state whether the activation of CREB is a G-protein-dependent or –independent response. Further work including the use of the novel $G\alpha_q$-protein inhibitor, UBO-QIC, would allow us to derive some definitive conclusions regarding the signalling pathway which leads to CREB activation in our cell system.

Another important downstream effector of FFA4 is Akt. Aberrations of this serine/threonine kinase have been implicated in a variety of complex disease states including type-2 diabetes and cancer. FFA4 signalling to Akt has been shown to be mediated by both G-proteins and arrestins (Li *et al.,* 2013; Oh *et al.,* 2010). We attempted to answer the seemingly conflicting reports in the literature regarding the activation of Akt by FFA4. The data presented in this thesis suggests that Akt is primarily activated by arrestin-dependent pathways upon FFA4 activation as receptor mutants previously shown to be impaired in their ability to recruit arrestin also show an impaired ability to activate Akt. Furthermore, a $G\alpha_q$-protein inhibitor does not appear to reduce Akt activation at the wild type FFA4. Extending these studies, we investigated the possibility that arrestin was directly interacting with Akt thus trafficking it to the plasma membrane where it would become activated. Arrestins are known to act as agonist-regulated scaffolding proteins where the endocytosed receptor is physically linked to downstream signalling cascades (Pierce and Lefkowitz 2001). However, we could not observe a direct interaction between FFA4, arrestin, and Akt. This could be due to the interaction between Akt and arrestin being very transient or of such low affinity that it cannot be measured by this type of biochemical assay. Bioluminescence resonance energy transfer (BRET)-based biosensors have been used extensively to study protein-protein interactions and intracellular signal transductions in live cells (Salahpour *et al.,* 2012). Indeed, this technique allows the investigations of cellular dynamic processes such as the modulation of the interaction between two proteins following a pharmacological treatment. It would be highly beneficial to this

study if we were to exploit this technology in order to ascertain the relationship between arrestins and Akt.

Understanding the role of G-protein-dependent and -independent signalling provides a basis for the rational design of FFA4 ligands showing a biased nature that may drive one signalling pathway preferentially over the other. Currently there is a lack of FFA4 specific ligands, but information from this thesis and the arrestin-3 uncoupled form of the FFA4 may be useful in proof of concept studies where the downstream signalling in a physiological setting are mediated by arrestin as opposed to G-proteins, and also as a model which allows easy identification of novel biased ligands. Work from this thesis suggests arrestin-3 recruitment to the FFA4 is regulated by phosphorylation and signalling via $G_{q/11}$-mediated pathways such as ERK activation is not dependent on the phosphorylation status of the receptor. This suggests that an FFA4 ligand which shows stimulus bias towards receptor phosphorylation/arrestin signalling would have a potentially different signalling outcome (activate Akt) from that of a ligand which shows G-protein bias (activate ERK) upon stimulation of the receptor at a certain time-point. Previous studies have demonstrated FFA4 signalling via arrestins is important in macrophages (Oh *et al.,* 2010). If one was to target inflammation then it seems advantageous to possess a ligand which will preferentially signal through arrestins, and in doing so may help to avoid stimulation of pathways which give rise to adverse effects. Ligands have been identified which cause a more subtle form of stimulus bias (Butcher *et al.,* 2011; Nobles *et al.,* 2011), these ligands have been shown to generate different "phosphorylation barcodes" which may stabilise different arrestin active conformations thus preferentially activating signalling and physiological outcomes. This may be important as continued study of the FFA4 has implicated it in a number of disease states and design of drugs showing stimulus bias may prove to be important in correct targeting of a disease and an improved clinical efficacy all the while avoiding or reducing adverse responses.

7.1.3. Investigations into a phosphomimetic form of FFA4

The importance of GPCR phosphorylation has been highlighted thus far in this thesis. Recent publications employing X-ray crystal structure analysis have further reinforced the significance of receptor phosphorylation particularly in the interaction of the receptor with arrestin. Previously we have demonstrated that the FFA4 is strongly

coupled to arrestin-3 in a BRET based assay. Here, five phosphorylation sites in the C-terminal tail of the receptor are primarily responsible for this interaction with arrestin. To extend our studies with the FFA4, we utilised charge mutations in the C-terminal tail of the receptor and replaced the five key phosphoacceptor sites with phosphomimetic residues. While there exists in the literature evidence of the use of phosphomimetics to investigate protein phosphorylation (Hao *et al.,* 1996; Li *et al.,* 2014; Wagner *et al.,* 2004), studies into the use of phosphomimetics to investigate GPCR phosphorylation is lacking.

Data from this thesis suggests phosphomimetic residues do not faithfully mimic phosphorylated serine and/or threonine residues located in the C-terminal tail of the FFA4. This is evidenced by the reduced ability of the phosphomimetic receptor to couple to arrestin-3 in a BRET based assay. However, the phosphomimetic FFA4 mutant does couple to arrestin-3 better than a completely phosphorylation-deficient mutant indicating the phosphomimetic residues are having a positive impact in this interaction. This is not entirely unexpected as the phosphate group with its large hydrated shell owing in part to the large phosphate atom at its centre and an extra oxygen, and its negative charge of greater than 1 is distinct from the carboxyl side chains of aspartate and glutamate which only have a single negative charge and a smaller hydrated shell than phosphate (Tarrant and Cole 2009; Hunter 2012). The protein-linked phosphate group is well matched for interaction with the guanidino group of arginine residues which is composed of a rigid, planar structure that can make direct hydrogen bonds to the doubly charged phosphate group at physiological pH. Due to a higher density of negative charge as well as a larger hydrated shell, phosphorylated amino acids form stronger and more stable hydrogen bonds and salt bridges than aspartate and glutamate residues with arginine (Mandell *et al.,* 2007). Arrestins contain a critical phosphate-sensor in the form of Arg175 (Gurevich and Benovic 1995; Kim *et al.,* 2013) and other putative phosphorylation binding sites such as the well conserved lysine residues on β-strand-1 of arrestin (Shukla *et al.,* 2013) which are involved in forming tight interactions with phosphorylated, active GPCRs. Our data suggests that replacement of phosphorylated residues with phosphomimetic residues in the C-terminal tail of FFA4 weakens the interactions between the receptor and arrestin resulting in a reduction in affinity and manifests itself as reduced total arrestin-3 recruitment in our BRET based assay.

It was hypothesised that phosphomimetic residues in place of serine or threonine residues in the C-terminal tail of FFA4 might result in the receptor recruiting arrestin more quickly as the receptor would no longer need to become phosphorylated following activation. Using a BRET based arrestin-3 recruitment assay, we attempted to prove this hypothesis. However, the data obtained was not statistically significant meaning we could not draw meaningful conclusions. An elegant study using the β_2-adrenergic receptor where extra sites of phosphorylation were engineered into the C-terminal tail of the receptor also measured the kinetics of receptor interaction with arrestin (Zindel *et al.,* 2015). In this study, FRET was used in an experimental set-up that was able to be perfused allowing the dynamics of arrestin-3 interaction with the receptor to be measured. Couple with the use of ligand at several concentrations, this allowed on- and off-rates to be measured thus providing robust kinetic constants of FRET experiments for arrestin-3 interaction with the receptors. It would be highly beneficial to our study to use this technique to investigate the FFA4 receptor and its mutants.

Arrestin-mediated receptor internalisation following exposure to agonist is an important aspect of the desensitisation process for GPCRs (van Koppen and Jakobs 2004). Previous studies have demonstrated FFA4 internalisation upon agonist stimulation (Butcher *et al.,* 2014). Removal of five key phosphorylation sites in the C-terminal tail of the receptor results in a reduction in potency and efficacy of the FFA4-specific TUG-891 to induce arrestin-3 recruitment to the receptor. This has the physiological consequence of an abrogation in FFA4 internalisation. In this work, we have recapitulated this data and have shown that a completely phosphorylation-deficient FFA4 mutant exhibits a diminished ability to recruit arrestin-3 and also become internalised. The phosphomimetic FFA4 mutant shows an increased ability to recruit arrestin-3 compared to the completely phosphorylation-deficient FFA4 mutant and this results in increased receptor internalisation.

This data gives credence to the hypothesis that it is the pattern of receptor phosphorylation i.e. its "barcode" which is the primary determinant in receptor signalling and not just bulk negative charge contributed by phosphorylation at the C-terminal tail of the receptor. This has been proposed for a number of other receptors (Busillo *et al.,* 2010; Butcher *et al.,* 2011; Nobles *et al.,* 2011). Our data suggests a reduction in receptor phosphorylation can have important consequences on the signalling of GPCRs, especially the FFA4 receptor. Evidence exists of ligands which

are thought to mediate agonist-dephosphorylation upon exposure to the receptor suggesting different ligands can have different impacts on receptor phosphorylation (Butcher *et al.,* 2011). That this reduced ability to recruit arrestin-3 correlates with a physiological response of abrogated receptor internalisation these studies confirm the importance of GPCR phosphorylation. A reduced internalisation phenotype would allow sustained signalling via the membrane-bound receptor. Taken together**,** our data suggests care is needed in the design of new FFA4 ligands and the ability of a ligand to affect phosphorylation is an important aspect of drug design.

Overall, this thesis provides the first evidence that the wild type *h*H_4 receptor undergoes robust agonist-mediated receptor phosphorylation when stimulated with its endogenous ligand, histamine. By using mass spectrometry, we have identified these residues as being located in the third intracellular loop and the C-terminal tail of the receptor. Using JNJ7777120, previously described as a β-arrestin-biased agonist at the receptor, we show this ligand also causes receptor phosphorylation. This data provides the first study into the phosphorylation status of the wild type *h*H_4 receptor and serves as the foundation to further work investigating the "phosphorylation barcode" of the receptor.

Furthermore, we confirmed that the FFA4 receptor undergoes agonist-mediated phosphorylation at 5 putative sites in its C-terminal tail. Sequential removal of these sites resulted in a progressive decrease in agonist-mediated recruitment of arrestin-3 to the receptor. We go on to suggest that this can have phsyiological consequences for the receptor, for example, an abrogation of receptor internalisation and can affect downstream signalling at the receptor.

Finally, we show using our phosphomimetic FFA4 mutant receptor, that phosphomimetic residues do not faithfully mimic phosphorylated serine and/or threonine residues resulting in a reduced ability of the mutant to couple to arrestin-3.

8.1. References

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