INTERACTIONS OF GPR54 AND GPR147 RECEPTORS WITH RF-AMIDE LIGANDS

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Declaration

I herewith declare that I solely carried out this work, except where acknowledgements are made by reference. No portion of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Megan Hendrikse

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Abstract

G protein-coupled receptors play a key role in cellular signaling by transducing extracellular signals via G proteins to elicit intracellular responses. Studies have provided evidence supporting the role of the GPCR GPR54 and its cognate peptide ligand, kisspeptin (an RFamide peptide), in the regulation of reproduction. Kisspeptin and GPR54 play a critical role in the control of the hypothalamic-pituitary-gonadal axis by regulating gonadotropin-releasing hormone secretion. Despite the physiological importance of GPR54/kisspeptin signalling, the GRP54 residues important for receptor activation and signalling have not been extensively investigated. Another hypothalamic peptide, gonadotropin inhibiting hormone (also known as RFamide-related peptide), which interacts with the GPCR GPR147, has been found to inhibit GnRH-induced gonadotropin release and is therefore also of importance in control of the HPG axis. As many of the RFamide and RFamide-related receptors and ligands can be promiscuous, there is the potential for cross-talk between the GPR54/kisspeptin and GRP147/RFRP systems (or other RFamides) which may be of importance in the regulation of reproduction. GPR54 chimeras and point mutants were constructed in order to investigate the residues important for kisspeptin binding and receptor activation. The data obtained indicate that the acidic residues within the extracellular loops of GPR54 contribute to cell surface receptor expression and play a role in receptor signalling. In order to investigate the interactions of kisspeptin/RFRP peptides at GPR147 and GPR54, binding and activation of these receptors was studied with a range of ligands and their analogs. In addition to RFRP and its analogs, kisspeptin and several kisspeptin analogs were found to act as agonists at GRP147. In contrast, of all the ligands tested, only kisspeptin was able to bind to GPR54 with high affinity and elicit a response, thus indicating that GPR54 has high specificity for kisspeptin in contrast to the more promiscuous GPR147. These data demonstrate the therapeutic potential of kisspeptin analogs, for the inhibition of gonadotropin secretion and treatment of sex steroid hormone disease. In addition, these data have identified ligand and receptor residues important for binding and activation of GRP54/GRP147 which may aid development of new analogs targeting these receptors and highlighted the importance of testing these analogs for receptor specificity.

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List of Abbreviations

ACTH	Adrenocorticotrophic hormone
AC	Adenylate cyclase
ARC	Arcuate
AVP	Arginine vasopressin
AVPV	Anteroventral periventricular
BIBP3226	(R)-N2-(diphenylacetyl)-N-[(4-hydroxyphenyl)-methyl]-argininamide
BSA	Bovine serum albumin
CCK	Cholecystokinin
CHO	Chinese hamster ovary
DAG	Diacyl glycerol
DMEM	Dulbecco's minimum essential medium
DMH	Dorsomedial hypothalamic area
DMN	Dorsomedial nucleus
Dyn	Dynorphin-A
ECL	Extracellular loop
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal–regulated kinase
FAK	Focal adhesion kinase
FCS	Fetal Calf Serum
FRET	Fluorescence Resonance Energy Transfer
FSH	Follicle-stimulating hormone
GABA	γ-amino-butyric acid
GAL1	Galanin receptor 1
GnIH	Gonadotropin inhibiting hormone
GnRH	Gonadotropin releasing hormone
GPCRs	G-protein coupled receptors
GRK2	G protein receptor kinase 2
HPG	Hypothalamic–pituitary–gonadal axis
ICL	Intracellular loop
IHH	Isolated hypogonadotropic hypogonadism
IP3	Inositol triphosphates
ir	Immunoreactive
Kir	Potassium channel
KNDy	Kisspeptin/NKB/dynorphin
KO	Knockout
KOR	Kappa opioid receptor
LB	Luria Broth
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
ME	Median eminence
MEF	Mouse embryonic fibroblast
MMP	Matrix-metalloproteases
MUA	Multiunit activity
NE	Norepinephrine

NF-Kb	Nuclear factor-kappa
NK3R	Neurokinin 3 receptor
NKA	Neurokinin A
NKB	Neurokinin B
NPAF	Neuropeptide AF
NPFF	Neuropeptide FF
NPSF	Neuropeptide SF
NPVF	Neuropeptide VF
NPY	Neuropeptide Y
OVX	Ovariectomized
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PI3K	Phosphatidylinositide 3-kinase
PIP2	Phosphatidylinositol bisphosphate
PLB	Passive lysis buffer
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
POA	Preoptic area
POMC	Proopiomelanocortin
PrRP	Prolactin-releasing peptide
QAE	Quaternized aminoethyl
QRFP	Pyroglutamylated RFamide peptide
RFRPs	RFamide related peptides
SAR	Structure activity relationships
SEM	Standard error of mean
SFM	Serum free media
TIMP-1	Tissue inhibitor of matrix metalloprotease-1
TM	Transmembrane
TMB	3,3',5,5'-Tetramethylbenzidine
TNF-α	Tumour necrosis factor alpha
TRPC	Transient receptor potential canonical

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1.1 Introduction

This review will focus on the RFamides, kisspeptin and gonadotropin inhibiting hormone (GnIH), their cognate receptors, GPR54 and GPR147, respectively, and the role they play in control of human reproduction and pubertal maturation and development. A special emphasis will also be placed on the structure-activity relationship (SAR) of these two ligand-receptor systems.

1.2 Literature Review

1.2.1 Introduction to G protein-coupled receptors

The G protein-coupled receptors (GPCRs) are a large and diverse family of membrane proteins consisting of seven transmembrane (TM) helices, an extracellular N-terminus and an intracellular C-terminus (Kroeze *et al.*, 2003). They are located in the plasma membrane and transduce external signals into cells through interactions with extracellular ligands and intracellular G proteins to initiate signalling cascades that allow cells to respond to changes within their environment. With over 800 members in the human genome, GPCRs represent the largest family of proteins involved in signal transduction across biological membranes. They are evolutionarily highly conserved (Strotmann *et al.*, 2011) and are expressed in most eukaryotic organisms. The molecular structure shared by all GPCRs consists of the seven TM domain composed of α-helices spanning the plasma membrane. The TM helices are connected by three extracellular (ECL) and three intracellular (ICL) loops (Salon *et al.*, 2011).

GPCRs in vertebrates are classically divided into five families on the basis of their sequence and structural similarity: rhodopsin-like (family A), secretin-like (family B), glutamate-like (family C), adhesion (Family D) and Frizzled/Taste (Family E)(Fredriksson *et al.*, 2003). The rhodopsin family is by far the largest and most diverse of these families, and members are characterized by conserved sequence motifs that imply shared structural features and activation mechanisms (Foord *et al.*, 2005). The rhodopsin-like GPCRs are the largest family and are activated by a diverse range of stimuli (ligands) which include hormones, neurotransmitters, and light.

All GPCRs transduce extracellular signals through interaction with intracellular G proteins, of which there are four families (G_s, $G_{i/o}$, $G_{q/11}$ and $G_{12/13}$) which are characterised by their coupling to different intracellular signalling pathways (Hurowitz *et al.*, 2000).

Over thirty years of cellular, biochemical, and biophysical investigations have provided a general overview of how the rhodopsin-like GPCRs function. However, these studies still lack a detailed description of the molecular events linking ligand binding to receptor activation. The first high-resolution structure, that of rhodopsin, was not solved until 2000 (Palczewski *et al.*, 2000), and another 7 years was needed for the determination of the first GPCRs bound to diffusible ligands such as hormones or neurotransmitters (Cherezov *et al.*, 2007, Rasmussen *et al.*, 2007. Crystallization of TM proteins is a difficult and complicated process which has limited the progress of determining high-resolution structures of GPCRs. Recent technological advances in engineering, producing and purifying membrane proteins, crystal formation, and X-ray diffraction has led to an explosion of GPCR structural biology work. Over the past

5 years, 47 structures representing 13 distinct GPCRs have been solved, in many cases with resolution of 3 Å or better (Audet and Bouvier, 2012).

The overall folding of the TM domain is highly conserved among all solved GPCR structures and was similar to that of rhodopsin, showing only slight differences in the relative orientation of the TM helices. More pronounced variances are found in the ECL domains, especially in the second ECL (ECL2) that displays receptor-specific folds (Rosenbaum *et al.*, 2007). The ECLs and N-terminus, with or without part of the TM helical region facing the extracellular space, are responsible for ligand recognition. This ligand-binding region is often situated within a cavity formed by the TM helices (Pin *et al.*, 2003). The ECL2 of many receptors form a compact helical shape adjacent to the TM bundle that, in conjunction with ECL1 and ECL3, allows soluble ligands to diffuse easily from the extracellular compartment toward the binding site inside of the receptor bundle (Rosenbaum *et al.*, 2007).

In contrast to the extracellular region that has evolved to recognize a very large number of diverse ligands, the intracellular region of GPCRs need only to identify and differentiate between a relatively small number of G proteins (Mirzadegan *et al.*, 2003).

1.2.2 Introduction to RFamide peptides

RFamides form a class of neuropeptides characterized by an arginine-phenylalanine-amide motif at their C-terminus. Members of the RFamide family are structurally-related peptides synthesised as precursor proteins, each encoded by a distinct gene. As a result, many of these structurally-related peptides are present in an individual species. Some of the RFamide precursors are

polyproteins, while others produce only a single ligand. Although members of this peptide family are grouped together based on an amidated C-terminal dipeptide, they differ in the length and sequence of their N-terminal extensions, which are important in conferring distinct binding characteristics and unique activities. Because of the structural similarities it is important to delineate the structure activity relationships (SARs) of ligands in order to circumvent ambiguity in assignment of peptide activity, and thus errors in delineating mechanisms associated with signalling and in the design of peptide analogs. Members of the RFamide family are predicted to share similarity in ligand–receptor interactions as a result of the high degree of structural and functional relatedness across species (Bass *et al.*, 2013).

In mammals, five genes encode the RFamide peptide family members and five genes encode their cognate GPCRs, as indicated in Figure 1 with peptide sequences of the RFamides displayed in Table 1.

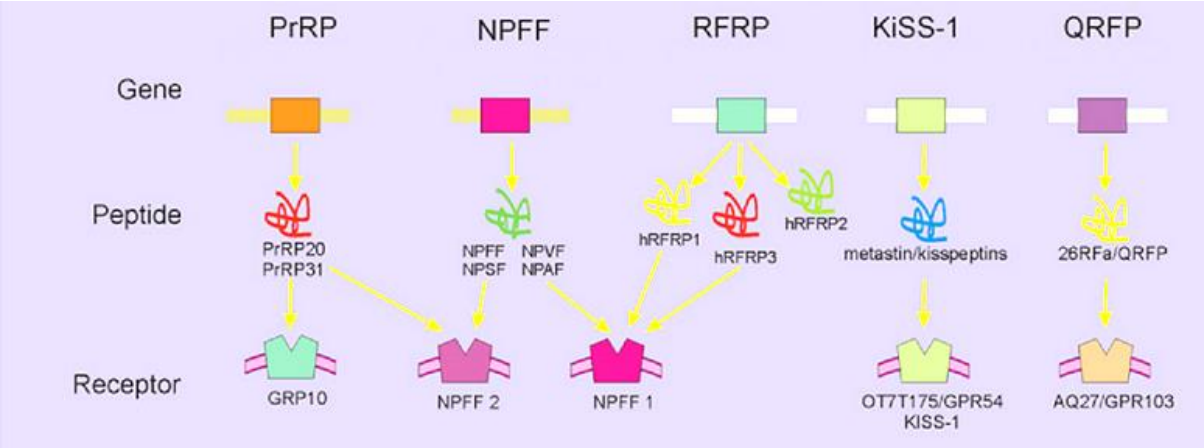

Figure 1: The mammalian RFamide family. The prolactin releasing peptide (PrRP) family, the family of NPFF (and related peptides NPAF, NPSF, and NPVF), human RFamide related peptides (hRFRPs), metastin/kisspeptins, and QRFP family. Specific receptors for each family of peptides have been identified, although in a number of instances cross-talk amongst these peptides and their receptors exists as indicated by arrows. Adapted from (Jhamandas and Goncharuk, 2013).

The RFamides include the prolactin-releasing peptide (PrRP) family (Hinuma *et al.*, 1998), the family of neuropeptide FF (NPFF; and related peptides neuropeptide AF (NPAF), neuropeptide SF (NPSF; RFRP1), and neuropeptide VF (NPVF; RFRP3) (Bonini *et al.*, 2000, Liu *et al.*, 2001), human RFamide related peptides (hRFRPs) (Hinuma *et al.*, 2000, Fukusumi *et al.*, 2001), metastin/kisspeptins (Ohtaki *et al.*, 2001), and pyroglutamylated RFamide peptide (QRFP) (26RFa) family (Chartrel *et al.*, 2003, Fukusumi *et al.*, 2003).

The RFamide peptides have been associated with the regulation of most of the major pituitary hormones such as prolactin, luteinising hormone (LH), follicle stimulating hormone (FSH), growth hormone, adrenocorticotrophic hormone (ACTH), oxytocin and arginine vasopressin (AVP), although this regulation may be exerted indirectly via other hypophysiotrophic regulatory hormones. They are able to influence endocrine functions such as maternal physiology, reproduction, growth, stress responses, diuresis and blood pressure regulation (Bechtold and Luckman, 2007, Osugi *et al.*, 2006).

All mammalian RFamide peptides have been found to be ligands of formerly orphan GPCRs. In some cases there is considerable overlap in the binding affinity between different RFamides and their receptors with some ligands having affinity for more than one receptor, since receptor selectivity appears to be determined by the few relatively conserved amino acids at the C-terminus (Yoshida *et al.*, 2003).

Table 1: Comparison of amino acid sequences of endogenous RFamide peptides in human (Findeisen *et al.*, 2011)

Family	Peptide	Sequence	Reference
NPFF	NPFF	SQAFLFQPQRF-NH_2	(Tsutsui *et al.*, 2000)
	NPAF	AGEGLNSQFWSLAAPQRF-NH_2	
RFRP	RFRP1 (NPSF)	MPHSFANLPLRF-NH_2	(Ubuka *et al.*, 2009a, Hinuma *et al.*, 2000)
	RFRP3 (NPVF)	VPNLPQRF-NH_2	
26RFa	43RFa (QRFP)	<EDEGSEATGFLPAAGEK-TSGPLGNLAEELNGYSRKKGGFSFRF-NH_2	(Bruzzone *et al.*, 2006, Chartrel *et al.*, 2003)
	26RFa	TSGPLGNLAEELNGYSRKKGGFSFRF-NH_2	
PrRP	PrRP31	SRTHR-HSMEIRTPDINPAWYASRGIRPVGRF-NH_2	(Hinuma *et al.*, 2000, Langmead *et al.*, 2000)
	PrRP20	TPDINPAWYASRGIRPVGRF-NH_2	
KISSPEPTIN	KP54	GTSLSPPPESSGSRQQPGLSAPHSRQI-PAPQGAVLVQREKDLPNYNWNSFGLRF-NH_2	(Kotani *et al.*, 2001, Ohtaki *et al.*, 2001)
	KP14	DLPNYNWNSFGLRF-NH_2	
	KP13	LPNYNWNSFGLRF-NH_2	
	KP10	YNWNSFGLRF-NH_2	

Pyroglutamic acid is shown as < E.

1.2.3 RFamides and their cognate receptors

The discovery of ligand-receptor systems for GPR54/kisspeptin and GPR147/RFRP3 followed the discovery of NPFF in mammals. NPFF is a member of the RFamide peptide family that is found in the central nervous system and in the periphery of several mammalian species including humans and was the first RFamide peptide to be identified in mammals (Panula *et al.*, 1996). The gene for NPFF has been cloned from human, bovine, rat, and mouse and is highly

conserved amongst these species (Hinuma *et al.*, 2000). An NPFF precursor protein encodes two proteins, NPFF and NPAF.

NPFF is able to mediate the antinociceptive effects of opioids (Chen *et al.*, 2006, Mouledous *et al.*, 2010) and subsequent studies revealed that NPFF may play an equally important role in the central processing of visceral autonomic signals related to feeding, generation of central cardiovascular responses, stress, and neuroendocrine regulation (Panula, 2009, Jhamandas and Goncharuk, 2013). Concentrations of NPFF and the NPFF receptors in the hypothalamus are amongst the highest in the brain (Bonini *et al.*, 2000, Liu *et al.*, 2001).

In 2000, Hinuma *et al*, analysed human genomic and cDNA sequences and identified a novel mammalian RFamide peptide gene which they named the RFamide-related peptide (RFRP) gene (Hinuma et al., 2000). Isolation of human, bovine, rat, and mouse RFRP cDNAs, demonstrated that the human and bovine RFRP gene encodes a RFRP precursor protein resulting in 2 peptides: RFRP1 (also known as RFSF), and RFRP3 (also known as NPVF). Whereas the rodent gene encodes only these two peptides, in humans and bovines, an additional related peptide (RFRP2) is also encoded by the RFRP precursor protein however, this peptide is not believed to be expressed *in vivo* (Yoshida *et al.*, 2003, Ukena *et al.*, 2003). The RFRPs share a similar C-terminal sequence with NPFF (see Table 1).

RFRP1 and RFRP3 are co-expressed in single neurons in the dorsomedial hypothalamus (DMH) (Fukusumi et al., 2001) and fibres containing RFRPs are distributed widely within the brain, including in the bed nucleus of the stria terminalis, septal areas, amygdala and hypothalamus (Fukusumi et al., 2006).

The DMH, where RFRP expressing neurons are localised, plays a pivotal role in the control of stress responses in neuroendocrine, autonomic and behavioural systems (Kaewwongse et al., 2011). RFRP1 acts in the hypothalamus to inhibit dopaminergic neuronal activity (Samson et al., 2003) and RFRP3 has emerged as important regulator of reproductive function (Yoshida et al., 2003, Murakami et al., 2008). Administration of RFRP1 to rats has been shown to facilitate prolactin release from the pituitary (Hinuma et al., 2000) and to attenuate morphine-induced analgesia (Liu et al., 2001). While RFRP3 has been shown to inhibit the GnRH system in mammals (Johnson et al., 2007, Clarke et al., 2008) and to increase food intake (Dockray, 2004).

In 2000, the putative receptors for NPFF/RFRPs were identified as GPR147 (also referred to as OT7TO22 or NPFF1 receptor) and GPR74 (also known as HLWAR77 or NPFF2 receptor) (Hinuma *et al.*, 2000, Bonini *et al.*, 2000, Elshourbagy *et al.*, 2000). GPR147 has a high affinity for many RFamide-related peptides, including the avian peptide LPLRFNH_2, NPFF, NPAF, RFRP1, RPRF2 and RFRP3 peptides (Bonini *et al.*, 2000, Elshourbagy *et al.*, 2000, Liu *et al.*, 2001). GPR74 has also been shown to bind NPFF and NPAF, RFRP1 and RFRP3 (Liu *et al.*, 2001, Fukusumi *et al.*, 2006).

For the purpose of this review, the terms GPR147 /GPR74 will be used to distinguish between the two receptors and RFRP1/RFRP3 to distinguish between the two RFRP peptides

1.2.3.1 Kisspeptin and GPR54

Kisspeptins are classified as neuropeptides. They are the endogenous ligand of the previously orphan GPCR, GPR54. They are peptide products of the KiSS-1

gene, which was first discovered as a metastasis suppressing gene of malignant melanoma cells (Lee *et al.*, 1996). Discovered in Hershey, USA, the gene was named after Hershey's chocolate kisses and the inclusion of SS in the name was scientifically indicative that the gene is a cancer suppressor sequence. The ligand was initially named metastin and is now referred to as kisspeptin to indicate the full length, 54 amino acid peptide. The KiSS-1 gene product consists of a 145 amino acid precursor protein, which is cleaved to produce a C-terminally amidated 54 amino-acid peptide, kisspeptin (KP54). This peptide has biological activity and is subsequently cleaved to produce the C-terminal cleavage fragments KP14, KP13, and KP10 which also possess biological activity. They are collectively referred to as kisspeptin/s (Figure 2).

Cleavage of the three C-terminal amino acids of kisspeptin by the matrix-metalloproteases 2 and 9 (MMP-2/9) renders the peptide inactive (Oakley *et al.*, 2009) and in 2001, Ohtaki *et al*. reported that the C-terminal KP10 was the minimal bioactive sequence of kisspeptins (Ohtaki *et al.*, 2001). High expression levels of GPR54 and kisspeptin are present in the placenta, with lower levels in the brain, particularly the hypothalamus and the pituitary. The preliminary interest in the kisspeptin system focused on its anti-metastatic activity and potential in cancer therapy. But, this focus changed in 2003, when several individuals with hypogonadotropic hypogonadism (HH) were identified as having mutations in the KISS1R gene (Seminara *et al.*, 2003, De Roux *et al.*, 2003). It has since been revealed in many species that kisspeptin, acting via GPR54, is responsible for the regulation of the reproductive axis and in particular puberty onset (Tena-Sempere, 2012).

In humans, KiSS-1 mRNA distribution showed high expression levels in placenta, pancreas, testis, liver, and small intestine (Lee *et al.*, 1996, Muir *et al.*, 2001, Ohtaki *et al.*, 2001). In addition, KiSS-1 mRNA expressing neurons within the hypothalamus have been identified by *in situ* hybridization in the infunidibular nucleus (Rometo *et al.*, 2007), which is the human equivalent of the arcuate nucleus in animals. KiSS-1 has also been found to be co-expressed by GnRH neurons (Kelly *et al.*, 2013). This will be discussed in greater detail in subsequent sections.

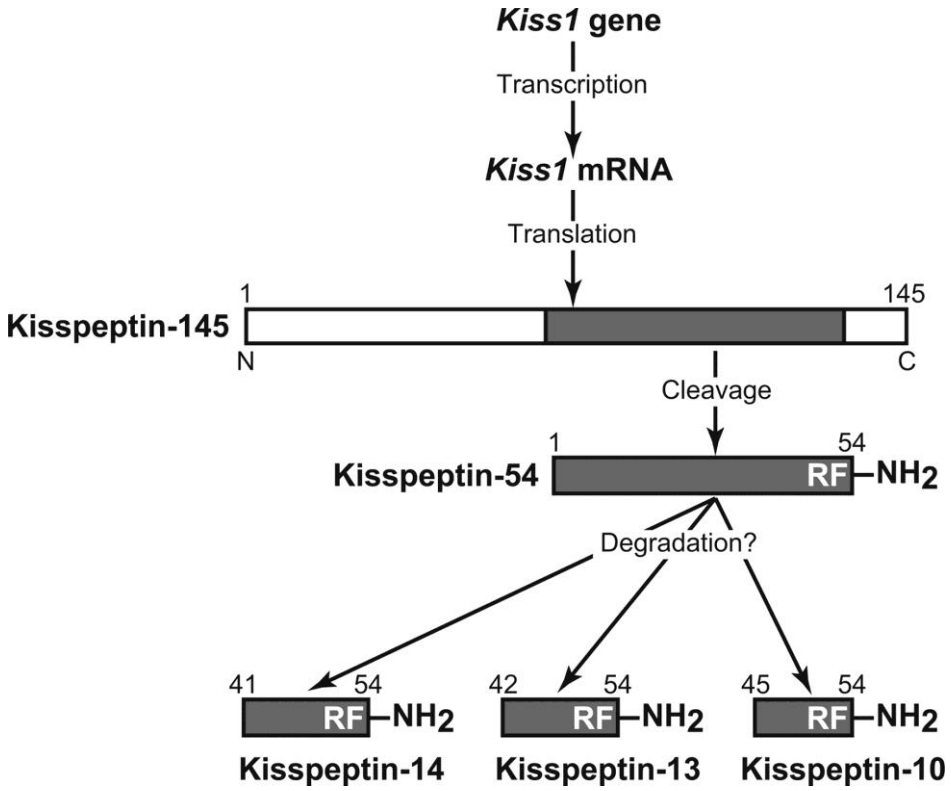

Figure 2: Kiss 1 cleavage products. *Kiss1* mRNA is transcribed from the *Kiss1* gene and translated to form a 145-amino-acid propeptide called kisspeptin-145. Shown are cleavage sites on the propeptide that lead to the production of the RF-amidated kisspeptin-54. Shorter peptides (such as kisspeptin-10, -13, and -14) share a common C-terminus and RF-amidated motif with kisspeptin-54. (Oakley *et al.*, 2009).

As mentioned above, kisspeptins are the endogenous ligands for a GPCR named GPR54 (also known as hOT7T175, AXOR12 or KISS1R) (Lee *et al.*, 1999b, Muir *et al.*, 2001). For the purpose of this review the name GPR54 will be used.

GPR54 was first cloned from rat brain tissue as an orphan receptor. The receptor with highest amino acid homology to the kisspeptin receptor is the galanin receptor (GAL1), with 45% homology, however galanin does not bind GPR54 (Lee *et al.*, 1999b). Rat and human kisspeptin receptors share 85% sequence identity, increasing to 98% in the transmembrane domains, whereas mouse and human share 82% homology. In 2001 kisspeptins were shown to be the endogenous ligands for GPR54 (Kotani *et al.*, 2001, Muir *et al.*, 2001, Ohtaki *et al.*, 2001). All kisspeptins can bind to GPR54, with KP10 having the highest potency (Kotani *et al.*, 2001).

In humans, GPR54 mRNA has been detected in the placenta, pituitary, pancreas, heart, skeletal muscle, kidney, liver, and also in regions of the brain (cerebral cortex, putamen, and medulla) and the spinal cord (Kotani *et al.*, 2001, Muir *et al.*, 2001, Ohtaki *et al.*, 2001, Clements *et al.*, 2001).

The major intracellular signalling system stimulated by GPR54 is the $G_{q/11}$ signal transduction pathway (Figure 3), which activates phospholipase C (PLC) to hydrolyse phosphatidylinositol bisphosphate (PIP2) to inositol trisphosphate (IP3) and diacylglycerol (DAG), which, in turn, lead to release of intracellular calcium from the endoplasmic reticulum, and activation of protein kinase C, respectively (Muir *et al.*, 2001, Becker *et al.*, 2005). Stimulation of GPR54-transfected cells with kisspeptin induces intracellular Ca^{2+} mobilization, indicating that GPR54 is coupled to a $G_{q/11}$ G protein pathway (Kotani *et al.*, 2001, Muir *et al.*, 2001,

Ohtaki *et al.*, 2001). GPR54 stimulation also induced the formation of IP3 through a PLC-dependent mechanism (Kotani *et al.*, 2001, Stafford *et al.*, 2002). Greater insight into the intracellular signalling pathways activated by GPR54 was derived from studies performed either in cell lines (HEK-293, CHO, B16-Bl6, COS-7, NIH-3T3, MDA-MB-35S, NPA) transfected with the receptor (Kotani *et al.*, 2001, Muir *et al.*, 2001, Ohtaki *et al.*, 2001, Becker *et al.*, 2005, Stafford *et al.*, 2002, Stathatos *et al.*, 2005) or in immortalized cell lines natively expressing the receptor (GT1-7, Human thyroid carcinoma ARO, AsPC-1, PANC-1) (Masui *et al.*, 2004, Stathatos *et al.*, 2005, Babwah *et al.*, 2012). Data from these studies indicated that GPR54 activation was associated with MAPK phosphorylation and activation of β-arrestin. Stimulation of the MAPK pathway by kisspeptin could be via ERK1/2 stimulation, stimulation of focal adhesion kinase, p38 kinase phosphorylation and PI3K/Akt activation (Kotani *et al.*, 2001, Ohtaki *et al.*, 2001, Becker *et al.*, 2005, Castellano *et al.*, 2006, Stathatos *et al.*, 2005). Knock-down of β-arrestin 2 was able to inhibit ERK1/2 pathway activation, which indicates coupling of the β-arrestin to the ERK pathway (Pampillo *et al.*, 2009). This was further demonstrated in studies using mouse embryonic fibroblast (MEF) cell lines derived from mouse strains lacking β-arrestin-1 and β-arrestin-2, and heterologously expressing GPR54. Both β-arrestin-1 and -2 combined with Gq/11 knock-out indicated that the Gq and β-arrestins pathways are simultaneously activated by KP10 (Szereszewski *et al.*, 2010). *In vivo* studies have shown that kisspeptin response declined after continuous stimulation (Messager *et al.*, 2005, Seminara *et al.*, 2006, Jayasena *et al.*, 2010). The reduced response is not due to intracellular depletion of GnRH (Messager *et al.*, 2005, Seminara *et al.*, 2006), but rather receptor desensitization and/or

internalization. Desensitization of GPR54 signalling was found to be mediated by serine/threonine kinase 2 (GRK2) and β-arrestin in HEK293-T cells (Pampillo *et al.*, 2009).

Kisspeptins act directly on GnRH neurons to induce a continual depolarization event and increase the rate of action potential firing (Han *et al.*, 2005, Quaynor *et al.*, 2007). Rapid recycling of GPR54 to the plasma membrane would ensure that non-desensitized receptors capable of sustaining a long-lasting response to stimulation would present at the cell surface. An intracellular GPR54 pool that could be dynamically recycled to the plasma membrane has been reported (Babwah *et al.*, 2012), although GPR54 at the cell surface may not have the potential to signal since long lasting depolarization in GnRH neurons is associated with a lack of response to further kisspeptin stimulation (Dumalska *et al.*, 2008, Liu *et al.*, 2008).

Heterologous expression of kisspeptin has also been shown to result in a reduction of cellular invasiveness which was associated with a reduction in activity of MMP-9 (Lee *et al.*, 1996). MMPs are a family of enzymes expressed in various human tumours and are thought to contribute to tumour invasiveness by degrading the extracellular matrix. Kisspeptin mediates its action on MMP through inhibition of NFkB translocation to the nucleus (Yan *et al.*, 2001, Takeda *et al.*, 2012).

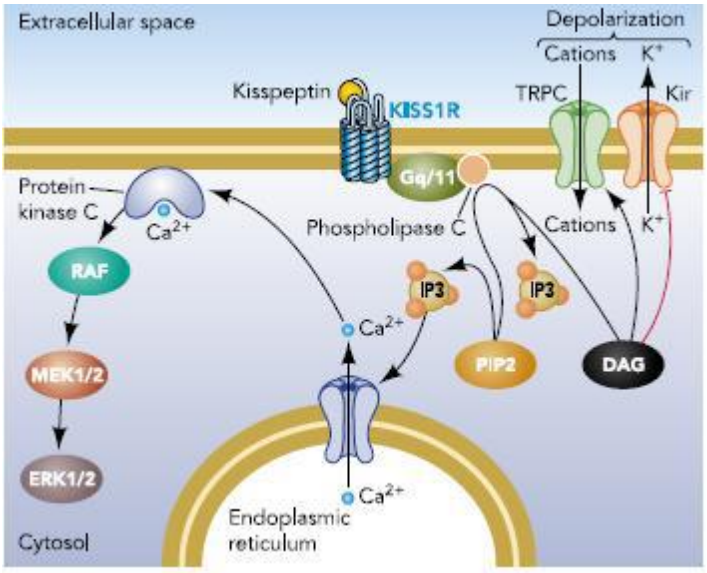

Figure 3: Summary of the mechanism of GPR54 activation. Kisspeptin binding to GPR54 activates the G-protein Gq/11 and phospholipase C to cleave phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol triphosphates (IP3) and diacylglycerol (DAG). IP3 causes intracellular $Ca2^{+}$ release from the endoplasmic reticulum, which activates protein kinase C and a kinase phosphorylation cascade (RAF, MEK1/2, and ERK1/2). GnRH depolarization is caused by activation of a nonselective cation channel (TRPC) and inhibition of an inwardly rectifying potassium channel (Kir) by DAG (D'anglemont De Tassigny and Colledge, 2010).

1.2.3.2 RFRP3 and GPR147

Tsutsui *et al* (2000) identified an RFamide related peptide that inhibited gonadotropin release in quail brains and termed it GnIH (Tsutsui *et al.*, 2000). Another group identified, using bioinformatics, a mammalian RFamide precursor protein in humans that was predicted to give rise to two mature RFamide peptides called RFRP1 (also known as NPSF) and RFRP3 (also known as NPVF or GnIH) (Hinuma *et al.*, 2000)). Ubuka *et al* (2009) have since confirmed human RFRP1 and RFRP3 as GnIH homologs (Ubuka *et al.*, 2009b). GPR147 preferentially binds RFRP1 and RFRP3, whereas GPR74 preferentially binds neuropeptides FF and AF (Bonini *et al.*, 2000).

The primary sequence of human GPR147 was compared with other known GPCRs and indicated that GPR147 shares approximately 30% homology with human neuropeptide Y receptor subtype 1, 2 and 4; 34% with human cholecystokinin (CCK) A receptor and 32% with human prolactin-releasing hormone receptor (Bonini *et al.*, 2000).

The GPR147 receptor activates the $G_{i/o}$ signal transduction pathway which results in a decrease in intracellular cAMP levels (Hinuma *et al.*, 2000, Bonini *et al.*, 2000) and both RFRP1 and RFRP3 have been shown to inhibit production of forskolin (FSK)-induced cAMP accumulation in Chinese hamster ovary (CHO) cells, expressing the human GPR147 receptor (Hinuma *et al.*, 2000).

Son *et al*. (2012) investigated the cell signalling pathway of the murine equivalent (mRFRP) and its possible interaction with GnRH signalling using a mouse gonadotrope cell line, LβT2 in which GPR147 expression had been confirmed by RT-PCR (Figure 4) (Son *et al.*, 2012). mRFRP inhibited GnRH-induced cAMP accumulation, indicating that mRFRP activated GPR147 functions to inhibit adenylyl cyclase (AC). mRFRP was also shown to inhibit GnRH-stimulated ERK phosphorylation and transcription of gonadotropin subunits. mRFRP however did not affect basal mRNA levels of gonadotropin subunits in the absence of GnRH stimulation. Inhibitors of the AC/cAMP/protein kinase A (PKA) pathway, were shown to inhibit GnRH-stimulated gonadotropin expression. However, an inhibitor of protein kinase C (PKC), did not inhibit GnRH-stimulated gonadotropin expression. Together these results indicate that mRFRP functions to inhibit GnRH-induced gonadotropin subunit gene transcriptions by inhibiting AC/cAMP/PKA-dependent ERK activation in gonadotrope cells (Son *et al.*, 2012).

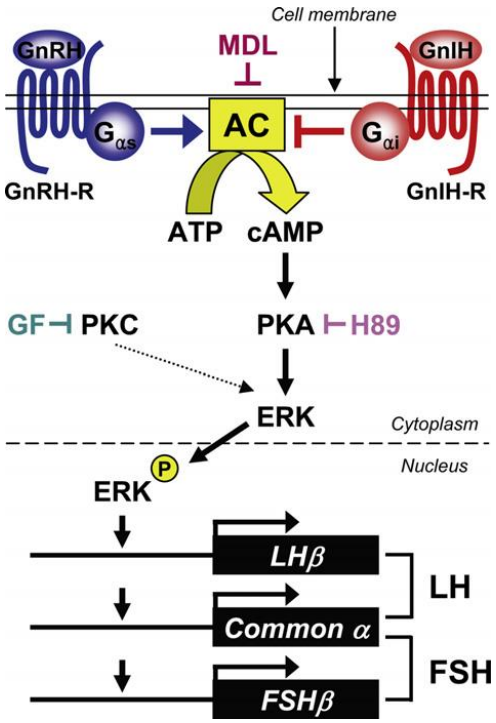

Figure 4: Model of the inhibitory mechanism of GnIH on GnRH-induced gonadotropin subunit, LHβ, FSHβ, and common α gene transcriptions. The inhibitors of adenylyl cyclase (AC)/cAMP/protein kinase A (PKA) pathway, MDL (inhibitor of AC) and H89 (inhibitor of PKA), effectively inhibited GnRH-stimulated gonadotropin expressions. On the contrary, the inhibitor of protein kinase C (PKC), GF, did not inhibit GnRH-stimulated gonadotropin expressions. Accordingly, mouse GnIH may inhibit GnRH-induced gonadotropin subunit gene transcriptions by inhibiting AC/cAMP/PKA-dependent ERK activation in LβT2 cells (Son *et al.*, 2012).

1.2.4 Neuroendocrinology of puberty and reproduction

Reproductive function is a finely regulated process that ensures continuation of the species. A variety of central and peripheral signals interact to control the coordinated action of the hypothalamus, pituitary, and gonads, which form the gonadotropic (HPG) axis. The hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) is a central mediator that carries these regulatory signals down to the pituitary level to execute the specific pattern of gonadotropin release (Fink, 2000).

1.2.4.1 Neuroendocrine control of reproduction

A variety of central and peripheral signals interact to control the coordinated action of the HPG axis (Fink, 2000). The function of this neurohormonal system depends on the interaction of three major groups of signals arising from:

- Hypothalamus - where a select group of scattered neurons synthesize and release GnRH
- Anterior pituitary - where gonadotropes secrete the gonadotropins, LH and FSH
- Gonads - generating gametes and are responsible for the synthesis and release of sex steroid and peptide hormones (Fink, 2000, Schwartz, 2000a).

These major components of the HPG axis interact via feed-forward loops where GnRH stimulates the secretion of gonadotropins which promote gonadal maturation and function (Figure 5). Other feedback regulatory loops function within this axis, to facilitate the homeostatic regulation of the system in different physiological conditions. The GnRH neurons are an important element of this system, and GnRH operates as the final output signal for the hypothalamic regulation of the downstream elements of the HPG axis. Pulsatile secretion of GnRH is critical for achieving and maintaining reproductive function (Belchetz *et al.*, 1978, Santoro *et al.*, 1986). The synchronized release of GnRH bursts is the result of the integral function of the GnRH pulse generator that encompasses GnRH neurons as well as other afferents and enables the pulsatile secretion of GnRH (Knobil, 1988). GnRH secretory patterns are not controlled solely by the intrinsic activity of GnRH neurons, but also through the contribution of additional

hypothalamic afferents (Maeda *et al.*, 2010). The reproductive axis goes through maturational and functional changes during fetal and postnatal development. This includes sexual differentiation of the brain and achieving reproductive capability at puberty. These processes display sexual dimorphism with detectable differences between males and females with regards to the development of reproductive brain circuits, the timing of puberty, and the function of the HPG axis in adulthood (Fink, 2000, Herbison, 2006, Schwartz, 2000a). Examples of these differences are the female-specific functional changes of the gonadotropic axis during the ovarian cycle, pregnancy, and lactation (Roa *et al.*, 2006, Schwartz, 2000b, Smith and Grove, 2002). Reproductive capability is inherently linked to other important functions, such as general health, immune/inflammatory state, and energy homeostasis (Fernandez-Fernandez *et al.*, 2006, Tena-Sempere, 2007, Tomaszewska-Zaremba and Herman, 2009), and is sensitive to an assortment of environmental triggers, such as photoperiods and stress conditions (Kinsey-Jones *et al.*, 2009, Li *et al.*, 2010, Simonneaux *et al.*, 2009). Thus reaching and maintenance of reproductive capability is dependent on appropriate interactions of a diversity of endogenous and exogenous regulators, including circulating hormones, neuropeptides, neurotransmitters, and metabolic products, which are partially able to impact on the GnRH pulse generator to mediate their regulatory actions (Fink, 2000, Herbison, 2006, Schwartz, 2000b). GnRH neurons are controlled by a variety of interacting trans-synaptic and glial inputs (Herbison, 2006, Ojeda *et al.*, 2006, Ojeda *et al.*, 2009). Glial cells are now recognized as a source of critical facilitatory signals for puberty onset and adult reproduction, such as glial-derived growth factors and glutamate, with stimulatory effects of GnRH release (Ojeda *et al.*, 2006, Ojeda *et al.*, 2009). Neuronal

afferents are crucial components in synchronizing triggering of pulsatile GnRH secretion (Herbison, 2006, Ojeda *et al.*, 2008). Recently, neuronal transmitters have been partially elucidated, with the recognition of the roles of glutamate and norepinephrine as major excitatory signals, and GABA and endogenous opioids as key inhibitory factors (Ojeda *et al.*, 2009), although GABA can also directly excite GnRH neurons under specific conditions (Herbison and Moenter, 2011), which illustrates the complex nature of the system.

The generation of GnRH pulses is therefore not controlled by the isolated action of single molecules but rather by the dynamic balance between excitatory and inhibitory signals (Christian and Moenter, 2010, Ojeda *et al.*, 2006).

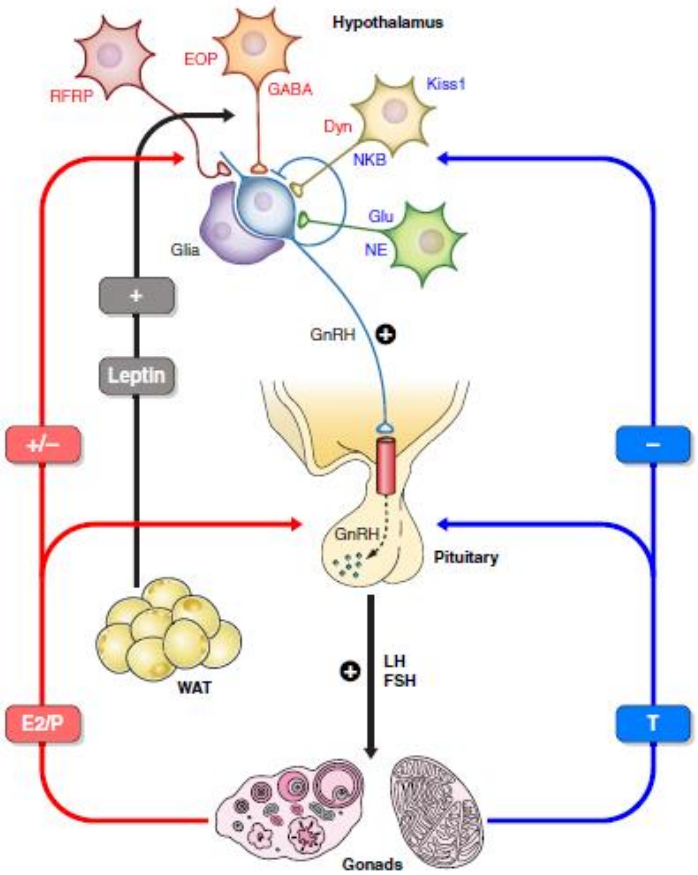

Figure 5: Neurobiology of the hypothalamic-pituitary-gonadal (HPG) axis. Graphic representation of the main elements of the neuroendocrine axis controlling reproduction: the HPG axis. Hypothalamic GnRH neurons, which receive trans-synaptic and glial inputs, release GnRH to the hypophysial portal blood system. GnRH dictates the pulsatile secretion of gonadotropins, LH and FSH that stimulate the maturation and regulate the function of the gonads; note that in the scheme, both the ovary and testis are presented. These major hormonal elements are connected via feed-forward and feedback regulatory loops. The function of the HPG axis is under the regulation of several peripheral signals that include gonadal steroids, responsible for feedback control: testicular testosterone (T) conducts inhibitory actions on GnRH/gonadotropin secretion (negative feedback), whereas ovarian steroids, mainly estradiol (E2) and progesterone (P), can carry out both negative- and positive-feedback actions depending on the stage of the ovarian cycle. Other peripheral regulators of the HPG axis are metabolic hormones; among those, the prominent stimulatory/permissive roles of leptin, produced by the white adipose tissue (WAT), are depicted. Some of the central transmitters involved in the control of the HPG axis are also shown: predominant inhibitory transmitters are depicted in red, whereas excitatory factors are labelled in blue. Among the excitatory signals to GnRH neurons, Kiss1 neurons are highlighted. Please note that to summarize presentation, discrimination between direct and indirect afferents to GnRH neurons is not made in the figure. Likewise, for sake of simplicity, some of the stimulatory and inhibitory signals to GnRH neurons are depicted in the same neurons; except for the Kiss1/NKB/Dyn neurons, this does not denote necessarily coexpression of these molecules in the same cells. Glu, glutamate; GABA, γ-aminobutyric acid; EOP, endogenous opioid peptides; NE, norepinephrine; NKB, neurokinin-B; Dyn, dynorphin; RFR, RF-related peptides (Pinilla *et al.*, 2012).

1.2.4.2 Role of Kisspeptin in puberty and reproduction

In 2003, two independent reports documented the presence of deletions and inactivating mutations of the *GPR54* gene in patients suffering familial or sporadic forms of idiopathic HH, a rare condition of impuberism, defective gonadotropin secretion, and infertility of central origin (De Roux *et al.*, 2003, Seminara *et al.*, 2003). The findings were the first to recognise the crucial roles of GPR54 and its ligands in the control of key aspects of reproductive function. These important findings in humans were strengthened by the report that mice engineered to lack functional GPR54 showed a very similar hypogonadotropic hypogonadism to that of affected patients (Funes *et al.*, 2003, Seminara *et al.*, 2003), a condition that was later confirmed in Kiss1 null mice (D'anglemont De Tassigny *et al.*, 2007, Lapatto *et al.*, 2007), even though Kiss1 KO animals appear to have a less severe reproductive impact than GPR54-deficient mice (Colledge, 2009).

The initial studies on patients with inactivating mutations and deletions of *GPR54* gene lead to many subsequent studies on the reproductive dimension of kisspeptins and began the characterization of the major physiological features of this system in the control of the HPG axis. Neuroendocrine characterization of humans with inactivating mutations/deletions of the *GPR54* gene evidenced that, despite their striking phenotype in terms of sexual immaturity, failure of gonadal function, and hypogonadotropism, the affected individuals retained their capacity to respond to exogenous GnRH (Seminara *et al.*, 2003). This feature excluded a primary defect at the pituitary level as cause for their HH, in contrast to previously reported cases of inactivating mutations of the GnRH receptor (De Roux *et al.*, 1997). Analyses of GPR54 null mice demonstrated that the hypothalamic content of GnRH was preserved (Seminara *et al.*, 2003). This observation ruled out the

possibility of a defective migration of GnRH neuron precursors from the olfactory placode, as had been proven the case for other monogenic forms of HH, such as those associated with mutations in *KAL1* or FGF receptor 1 (*FGFR1* or *KAL2*) genes (Albuisson *et al.*, 2005, Dode *et al.*, 2003, Shahjahan *et al.*, 2010). In spite of some phenotypic variability among mouse models, the conserved GnRH migration/content in the hypothalamus was later confirmed in KiSS-1 knockout mice (D'anglemont De Tassigny *et al.*, 2007).

The lack of obvious defects in GnRH neuronal migration, GnRH synthesis, or pituitary responsiveness to GnRH in individuals with impaired kisspeptin signalling implied that the kisspeptin/GPR54 system is a crucial, excitatory upstream regulator of GnRH neurons such that absence of kisspeptin signalling would result in suppressed GnRH secretion. This hypothesis was put forward before key findings such as the identification of kisspeptin neurons at the hypothalamus, the potent effects of kisspeptins on GnRH/ gonadotropin secretion, and the expression of GPR54 in GnRH neurons. These initial discoveries and hypothesis lead to the rapid progress of knowledge of the major features of the hypothalamic kisspeptin system as key regulator of the gonadotropic axis and have allowed the formation of an outline where kisspeptin neurons are upstream of GnRH neurons and act on them directly, with a role in switching on GnRH secretion at puberty (Figure 6).

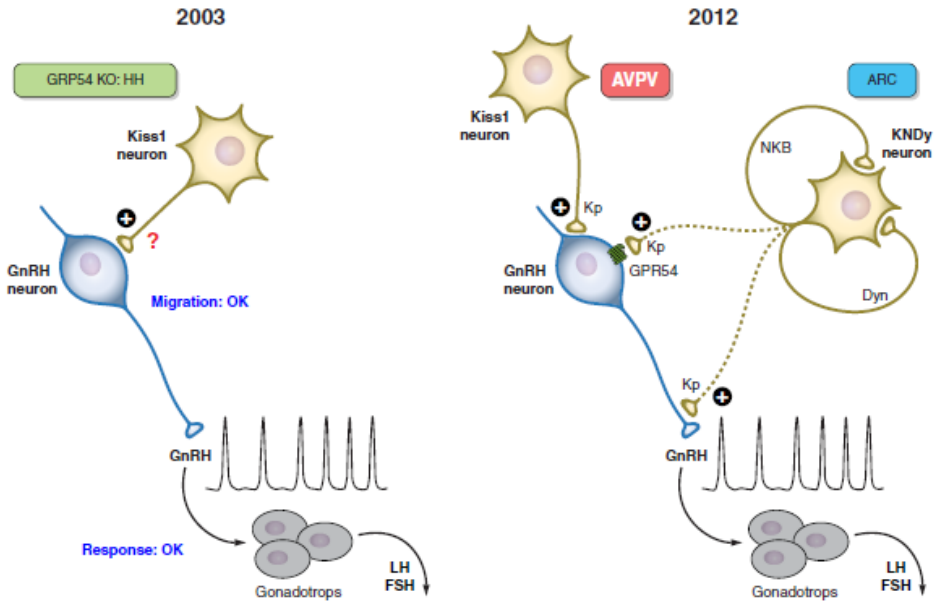

Figure 6: First hypothesis and current view for the central control of the HPG axis by the Kiss1 system. A tentative model is presented in the *left panel* for the original hypothesis on the roles of kisspeptins in the central control of the HPG axis, as delineated on the basis of initial findings in humans and mice with null mutations of the *GPR54* gene suffering hypogonadotropic hypogonadism (HH). Analyses in affected individuals revealed that HH was not due to defective pituitary responsiveness to GnRH, neither was it caused by lack of migration of GnRH neurons from the olfactory placode at early developmental stages. These observations indirectly pointed out that GPR54 signalling was essential for proper GnRH secretion. Yet, at that time, the existence of Kiss1 neurons and the expression of GPR54 in GnRH neurons remained unknown. In the *right panel*, a brief view of the current understanding of the kisspeptin neuronal networks governing GnRH secretion and, hence, reproductive function is depicted. Of important note, the neuroanatomical features of Kiss1 neurons schematically depicted here are taken from rodent data, as studies in these species have dominated the field. It is stressed that these characteristics may not fully represent the situation in other species, such as primates. Kp, Kisspeptins; KB, neurokinin-B; Dyn, dynorphin; KNDy, Kiss1/NKB/Dyn neuron; ARC, arcuate nucleus; AVPV, anteroventral periventricular nucleus (Pinilla *et al.*, 2012).

Chronic central administration of kisspeptin in prepubertal female rats advances the age of puberty (defined as the age of vaginal opening) (Navarro *et al*. 2004). Recent work by Pineda and colleagues using a newly developed kisspeptin antagonist, p234, shows that blocking kisspeptin action delays puberty in female rats (Pineda *et al*. 2010). The role of kisspeptin in puberty in the primate is supported by an increase in Kiss1 mRNA in the hypothalamus of pubertal monkeys compared to juvenile monkeys of both sexes (Shahab *et al*. 2005). In addition to kisspeptin, other neuropeptides have also been identified as potential

regulations of GnRH and/or kisspeptin neurons. Recent work has found that mutations in neurokinin B and its receptor (Neurokinin 3 receptor,NK3R) are also implicated in cases of isolated HH (Topaloglu *et al*. 2009). Interestingly, NKB expression is colocalized with kisspeptin in ARC kisspeptin neurons (Goodman *et al*. 2007). The role of body mass and leptin, the cytokine produced by white adipose tissue, in puberty and the control of the reproductive axis has been investigated for many years. It is known that low body mass is responsible for delayed puberty and oligo- or amenorrhoea. Furthermore, leptin and leptin receptor mutations have been found in human patients with disorders of puberty. A high proportion of kisspeptin neurons express leptin receptors (Smith *et al*. 2006), suggesting that leptin acts through kisspeptin neurons to influence the reproductive axis. Smith and colleagues also demonstrated that Kiss1 mRNA is reduced in the arcuate nucleus in the hypothalamus of mice deficient in leptin (ob/ob mice). A study by Navarro et al (Navarro *et al*. 2004) found that administration of kisspeptin could advance the age of puberty despite food deprivation, immunizing the rat against leptin, or in obese Zucher rats (a model of leptin resistance). These studies may indicate one therapeutic use of kisspeptin in the future.

1.2.4.3 Interactions of Kiss1 Neurons with RFRPs

The discovery that kisspeptins play a major role in the control of the HPG axis, has led to the re-examination of the proposed mechanisms and neuronal pathways whereby other central and peripheral signals regulate the GnRH/gonadotropin system. These studies will hopefully aid in the clarification of the hierarchy and interplay of kisspeptins and other relevant input signals controlling GnRH neurons.

Substantial attention has recently been dedicated to elucidating the interactions between kisspeptins and RFRPs as mammalian RFRPs have been shown to function as putative gonadotropin-inhibitory signals in several different mammalian species (Clarke *et al.*, 2009, Smith and Clarke, 2010, Tsutsui *et al.*, 2010). These studies indicate that RFRPs are an inhibitory signal for the HPG axis, probably acting at both central (likely on GnRH neurons) and pituitary levels. The structural and functional commonalities between kisspeptins and RFRP, since both belong to the superfamily of RFamide peptides and participate in the control of the HPG axis, although with opposing actions, made it appealing to suggest that it is the dynamic balance and interplay between these two sets of factors that drives the function of the reproductive system (Kriegsfeld *et al.*, 2006). However, the integral role of these peptides in the central control of reproduction has not been fully confirmed in mammals, in part because the extent and physiological relevance of RFRP effects on the gonadotropic axis appear to be less pronounced than those of kisspeptins. Nevertheless, the available evidence is compatible with an anatomical and functional interplay between the inhibitory and stimulatory pathways in the control of some aspects of reproduction in mammals. Morphological and expression data support the presumed interplay between kisspeptin and RFRP systems in different species. In sheep, studies have documented an inverse correlation between the expression levels of *KiSS-1* and *RFRP* mRNAs in the ARC and dorsomedial nucleus (DMN), respectively, during the breeding season, when the number of appositions to GnRH neurons also changes inversely showing increased number for kisspeptin neurons and a decreased number for RFRP neurons (Smith *et al.*, 2008). These observations suggest that kisspeptins and RFRP reciprocally interact to regulate GnRH

neuronal activity. Such interplay may involve not only direct, opposite actions at the level of GnRH neurons, but might also include reciprocal regulatory effects in terms of expression or biological actions. Rat studies have documented prominent expression of GPR147 in the AVPV, which might suggest a modulatory role of RFRP on kisspeptin neurons in this area (Quennell *et al.*, 2010). Functional electrophysiological analyses have also shown RFRP3 to inhibit the excitatory actions of kisspeptins on POMC neurons (Fu and Van Den Pol, 2010). Although the differences in the degree of absolute gonadotropin responses to kisspeptins and RFRPs have precluded direct analysis of their co-administration *in vivo*, combined injection of KP10 and an antagonist of GPR147 modestly increased the responses to KP-10 alone (Pineda *et al.*, 2010c). These data strongly suggest the possibility of interactions between kisspeptins and RFRP in the control of the HPG axis. The physiological relevance of the kisspeptin-RFRP interaction is not without controversy. Several reports have failed to show reciprocal changes between *KiSS-1* and *RFRP* expression. During the late follicular phase in the monkey or the pubertal period in the rat, both *KiSS-1* and *RFRP* are upregulated at the hypothalamus (Quennell *et al.*, 2010, Smith *et al.*, 2010).

While the role of kisspeptins in key reproductive phenomena, such as puberty onset or the preovulatory surge of gonadotropins, is well documented, the roles of RFRP pathways in those events appear to be much more modest, if any (Clarke *et al.*, 2009, Smith and Clarke, 2010). Big differences exist in the major sites of actions of kisspeptins and RFRP, as RFRP seems to function partially via inhibition of GnRH actions directly at the gonadotrope level (Clarke *et al.*, 2008, Pineda *et al.*, 2010b). Lastly, the role of RFRPs in mammalian reproduction

varies across species, with a more prominent role in seasonal breeders (Smith and Clarke, 2010). GnIH was first identified and plays a prominent regulatory role in birds, where the kisspeptin system has been apparently eliminated during evolution (Tsutsui *et al.*, 2010). The coevolution of both systems is thus under opposite forces, with a reciprocal equilibrium between the dominant roles of kisspeptins or GnIH/ RFRP, depending on the species and its reproductive strategy.

1.2.4.4 Potential interaction between kisspeptin and neurokinin B pathways

Of the various neuropeptides that possibly interact with kisspeptins in the control of the HPG axis, NKB has attracted most of the attention recently. NKB belongs to the family of tachykinins, which includes also neurokinin A (NKA), substance P, and hemokinin-1, as well as neuropeptides K and Y (Almeida *et al.*, 2004). Three tachykinin receptors have been identified to date: NK1R, NK2R, and NK3R. NKB preferentially activates NK3R, which is considered the canonical NKB receptor. The gene encoding NKB is named *TAC3* in humans and *Tac2* in rodents, whereas NK3R is encoded by the *TACR3*/*Tacr3* gene (Rance *et al.*, 2010). The roles of NKB and other tachykinins in the regulation of gonadotropin secretion had been previously addressed in various species (Rance *et al.*, 2010, Kalra *et al.*, 1991, Sandoval-Guzmán and Rance, 2004). Interest in the participation of NKB and NK3R in the control of the HPG axis has recently grown considerably, due mainly to two observations. Firstly the initial demonstration in sheep that the population of Kiss1 neurons located at the ARC actually coexpress NKB, as well as the endogenous opioid peptide, dynorphin (Dyn) (Goodman *et al.*, 2007); and secondly that inactivating mutations in *TAC3* and *TACR3* in humans are associated with HH (Topaloglu *et al.*, 2009), a phenotype similar to that

previously reported for inactivating GPR54 mutations. These observations highlighted the important physiological role of ARC neurons expressing kisspeptins and NKB, as well as other neuropeptides, such as Dyn, in the control of mammalian reproduction.

The physiological importance of NKB in the control of the HPG axis was highlighted when the association between inactivating mutations in *TAC3*/*TACR3* and human cases of HH was discovered (Topaloglu *et al.*, 2009). These associations renewed interest in previous functional studies that addressed the roles of NKB in the control of gonadotropin secretion, and lead to new investigations to elucidate the effects of NKB in the regulation of GnRH neurons and its potential interplay with kisspeptins and other cotransmitters, such as Dyn. The published data on the effects of NKB or the agonist of NK3R, senktide, on LH secretion has contradictory observations, with either null, inhibitory, or stimulatory actions being reported. Administration of an NK3R agonist, senktide, to ovariectomized (OVX) mice or rats, without hormonal replacement or very low estrogen substitution, resulted in inhibition of LH secretion (Kinsey-Jones *et al.*, 2009, Navarro *et al.*, 2009, Sandoval-Guzmán and Rance, 2004), an effect that seems at odds with the reported state of hypogonadotropism in humans with genetic inactivation of the NKB system. Additionally, NKB did not result in changes in LH secretion in adult male mice (Corander *et al.*, 2010), although a different group reported robust gonadotropin responses following icv administration of the NK3R agonist senktide in male mice (Navarro *et al.*, 2011). In agreement with the Navarro *et al*. 2011 study, evidence has been offered for the potent stimulatory effects of NKB and/or senktide on LH secretion in the adult ewe and juvenile monkey (Billings *et al.*, 2010, Ramaswamy *et al.*, 2010), with

the stimulatory effects in the ewe being detected during the follicular phase of the cycle. Robust LH releasing responses to icv injection of senktide in cyclic female rats and OVX rats with physiological supplementation of estradiol has also been reported (Navarro *et al.*, 2010). Pretreatment with a GnRH antagonist was able to abolish the observed senktide effect in monkeys (Ramaswamy *et al.*, 2010). Thus, under some physiological conditions, NKB signalling can exert potent stimulatory effects on GnRH secretion, thereby activating LH secretion. These reports indicate that the stimulatory actions of NKB are less conserved than those of kisspeptins and are most likely dependent on numerous physiological parameters, such as the sex steroid milieu and early brain differentiation, endogenous GnRH secretory activity, and developmental stage. Species differences may also contribute as indirectly supported by initial reports showing that, in contrast to humans, mice with inactivating mutations of *Tacr3* gene appeared to have preserved fertility (Siuciak *et al.*, 2007).

The additional presence of Dyn in neurons expressing kisspeptin and NKB (termed KNDy neurons) has encouraged functional analyses of the gonadotropic effects of this neuropeptide. Dyn is a member of the family of endogenous opioid peptides encoded by the prodynorphin gene that acts via the kappa opioid receptor (KOR) (Yen *et al.*, 1985). Dyn has been reported as an inhibitor of gonadotropin secretion (Goodman *et al.*, 2004, Yen *et al.*, 1985). Dyn has been shown to inhibit LH secretion in mice, and pulsatile LH secretion in goats (Navarro *et al.*, 2009, Wakabayashi *et al.*, 2010).

Studies performed in mice and goats suggest that NKB and Dyn may function as positive and negative modifiers of the pulsatile release of kisspeptins by KNDy neurons at the hypothalamic arcuate nucleus (ARC) (Navarro *et al.*, 2009,

Wakabayashi *et al.*, 2010). As suggested by this model, NKB and Dyn would act autosynaptically on ARC KNDy neurons, which in turn would project to and activate GnRH nerve terminals at the median eminence, using kisspeptin as output neuropeptide effector. Additional support to this autoregulatory mode of action stems from the observation that senktide, as agonist of NKB, can activate *KiSS-1*-expressing neurons in the ARC of female rats (Navarro *et al.*, 2011, Navarro *et al.*, 2010), but was unable to stimulate LH secretion in GPR54 knock-out mice (García-Galiano *et al.*, 2012). Further observations in the monkey suggest that the stimulatory actions of NKB on GnRH secretion take place upstream of GPR54 (Ramaswamy *et al.*, 2011). This hypothetical model combines a wide diversity of functional and anatomical observations, but further confirmation is required to determine its validity in different mammalian species and both sexes. Additional investigations are also required to determine if the actions of NKB and Dyn are conducted on the same neuron releasing the neuropeptides or upon adjacent and/or contralateral KNDy neurons in the ARC, as this neuronal population is interconnected (Ramaswamy *et al.*, 2011).

1.2.4.5 Distribution of Reproductive Neurons

GnRH neurons originate in the olfactory placode (Schwanzel-Fukuda and Pfaff, 1989, Wray *et al.*, 1989). From the placode, the cells migrate and colonize the basal forebrain in and around the preoptic area (POA) and the mediobasal hypothalamus (MBH) (Wray, 2002). GnRH is secreted into the hypophyseal portal system to stimulate gonadotropin release. The anatomical arrangement of the hypothalamus, median eminence (ME) and the pituitary gland allows for delicate control of the gonadotropes of the pituitary gland. The GnRH neurons project to the external secretory zone of the median eminence, where terminals are found

in close proximity to the primary capillary bed of the hypophyseal portal system (Page and Dovey-Hartman, 1984). GnRH neurons are surrounded by kisspeptin fibres (Irwig *et al.*, 2004) and express GPR54 mRNA (Han *et al.*, 2005).

Kisspeptin neurons are found in two distinct nuclei in the hypothalamus of the mammalian brain: the AVPV nucleus and the ARC nucleus (Navarro *et al.*, 2009, Mayer *et al.*, 2010). Kisspeptin neurons of the AVPV nucleus increase GnRH release, bringing about the onset of puberty (Mayer *et al.*, 2010). In females, kisspeptin neurons in the AVPV nucleus control the preovulatory surge of LH through activation by high concentrations of estrogens and progesterones (D'anglemont De Tassigny and Colledge, 2010). Kisspeptin neurons of the ARC nucleus typically modulate the pulsatile release of GnRH in males and females. Males do not have many kisspeptin neurons in the AVPV nucleus due to masculization of the AVPV area during neonatal development (D'anglemont De Tassigny and Colledge, 2010, Colledge, 2004).

In humans, the fully processed forms of RFRP homologs (RFRP1 and 3) were identified in an extract of hypothalamus. RFRP immunoreactive (RFRP-ir) neurons were observed in the dorsomedial hypothalamic area (DMH). RFRP-ir axon terminal like structures were observed in close proximity to GnRH neurons in the POA implying the regulation of GnRH neurons by RFRP. The receptor, GPR147, was found to be expressed in the hypothalamus. RFRP-ir axons also projected to the neurosecretory zone of the ME suggesting that RFRP might also directly regulate pituitary function. The expression of the human GPR147 mRNA in the pituitary supports this theory. RFRP3 homologs may thus regulate gonadotropin secretion by inhibiting GnRH neurons as well as directly acting on gonadotrope cells in the pituitary (Ubuka *et al.*, 2009a).

Distinct populations of *TAC3*-positive neurons have been recognized in the infundibular nucleus, anterior hypothalamic area, septal region, diagonal band of Broca, bed nucleus of the stria terminalis, amygdala, and neocortex (Chawla *et al.*, 1997, Pinto *et al.*, 2004). The expression of NKB has been demonstrated in a subset of Kisspeptin neurons in numerous species, with Kisspeptin/NKB/Dyn colocalization being a specific feature of the ARC/infundibular population of kisspeptin neurons, which has been renamed as KNDy to emphasize the potential usage of these three neuropeptides (Lehman *et al.*, 2010).

1.2.5 Other roles of kisspeptin

Cancer

The KiSS-1 gene that encodes kisspeptins was first discovered as an anti-metastasis gene in human melanoma cell lines (Becker *et al.*, 2005, Takino *et al.*, 2003). Dysregulation of tumour necrosis factor-alpha (TNFα) can induce cancer development and metastasis, via nuclear factor-kappa (NF-kB) activation. TNFα-induced NF-kB activation stimulates cellular mechanisms such as cell proliferation, migration, and invasion. Kisspeptin suppresses the migration and invasion of breast cancer cells by inhibiting TNFα-induced NF-kB pathway and RhoA activation (Lee *et al.*, 1996). KP10 also inhibits MMP-9 expression by reducing NF-kB binding to the MMP9 promoter, to cause an inhibition of cell migration (Yan *et al.*, 2001).

Pregnancy- Implantation and placentation

The kisspeptin system is implicated in other physiological systems, such as pregnancy. Similarities exist between the behaviour of invasive placental cells

and invasive cancer cells. Angiogenesis, a common feature of both implantation and cancer metastasis, is important in establishing the placenta. Kisspeptin acts as a vasoconstrictor, a critical phase in angiogenesis, in isolated coronary arteries (Mead *et al.*, 2007), suggestive of a role in the vascular system.

Kisspeptin and GPR54 are located at the fetal-maternal interface. Kisspeptins are highly expressed in the syncytiotrophoblast of the human placenta. The outer syncytiotrophoblasts lie adjacent to blood vessels allowing kisspeptin to move easily into the maternal blood. GPR54 has been identified in the villous and invasive extravillous human cytotrophoblasts (Janneau *et al.*, 2002, Bilban *et al.*, 2004). The spatial and temporal expression of kisspeptin and GPR54 in the human and rat are similar. Kisspeptin is expressed in the trophoblast giant cells of the rodent placenta, which mediate early invasion as they invade the spiral arteries and replace the endovasculature. These cells have the same functional phenotype as the human extravillous trophoblasts. As in the human, levels of KiSS-1 and its receptor gradually decline during placental maturation and are not detectable at embryonic day 18.5 in the rodent (Terao *et al.*, 2004).

Low levels of plasma kisspeptin can be found in males and non-pregnant females (Dhillo *et al.*, 2005). There is a drastic increase in plasma kisspeptin concentrations, with a 1000-fold increase in the first trimester of pregnancy, rising to a 10,000-fold increase in the third trimester (Horikoshi *et al.*, 2003). The high levels of kisspeptin/GPR54 mRNA in trophoblast cells during the first trimester in humans corresponds with the time of peak trophoblast invasion when regulation of this process is critical. *In vitro*, KP10 inhibits trophoblast migration and invasion (Bilban *et al.*, 2004) suggesting that kisspeptin plays a key role in controlling trophoblast invasion and regulating implantation and subsequent development.

1.2.6 Natural mutations of GPR54 results in HH and precocious puberty

Studies of patients with HH greatly increased our understanding of kisspeptin and revealed the connection between kisspeptin/GPR54 and puberty/reproduction. The frequency of mutations in the GPR54 (or KiSS-1) gene is low even for a rare disease such as HH (Cerrato *et al.*, 2006). The first reports of genetic inactivation of GPR54 in humans identified a 155 nucleotide deletion, eliminating the splicing acceptor site between intron 4 and exon 5, a homozygous L148S point mutation, and compound R331X and X399R point mutations of GPR54 in individuals with phenotypic HH (Seminara *et al.*, 2003, De Roux *et al.*, 2003). Other identified missense mutations of GPR54 in additional HH patients include; C223R and R297L substitutions (compound heterozygote) (Semple *et al.*, 2005), an insertion that changes the open-reading frame (Lanfranco *et al.*, 2005), a L102P mutation (Tenenbaum-Rakover *et al.*, 2007), and a combined insertion/deletion mutation at the 3' splicing acceptor site of exon 2 (Todman *et al.*, 2005), as well as additional rare variants of GPR54 in patients with late-onset HH (Cerrato *et al.*, 2006). A homozygous, loss-of-function mutation involving a pF272S substitution in the GPR54 peptide sequence has also been described in individuals with severe forms of familial HH of early onset (Nimri *et al.*, 2011). A schematic presentation of the major known inactivating mutations of GPR54 gene is depicted in Figure 7.

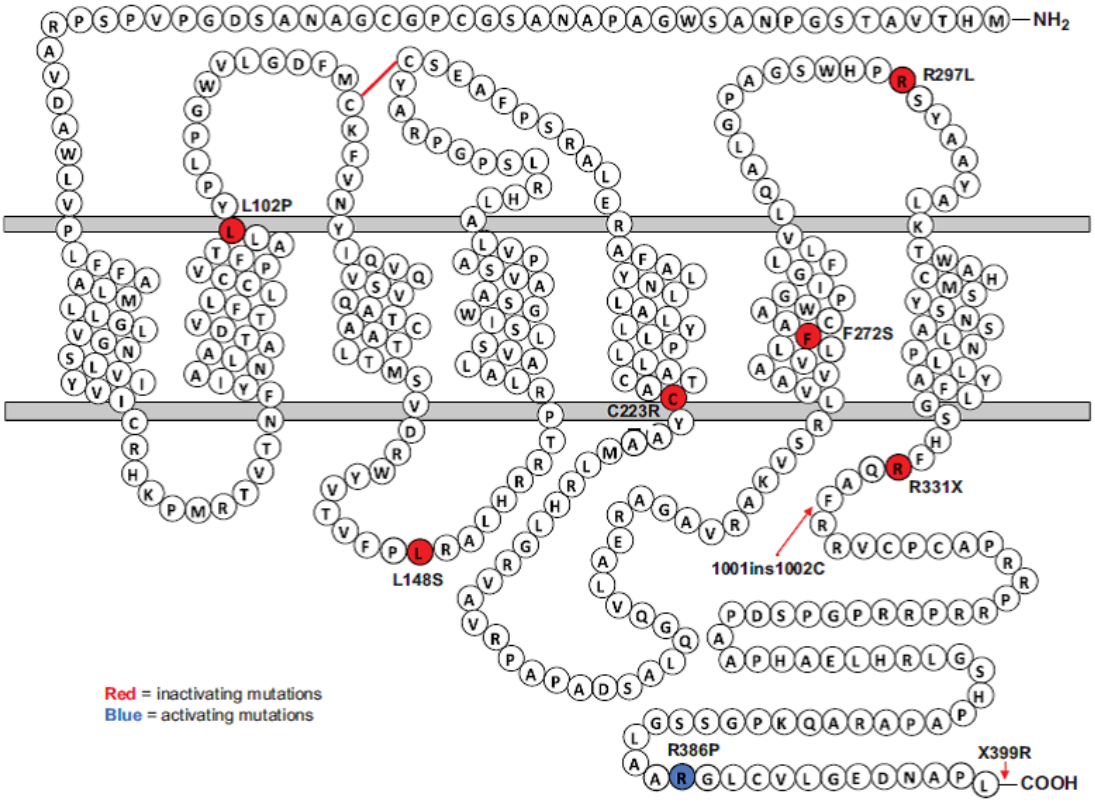

Figure 7: Schematic representation of disease-causing mutations of GPR54 gene reported thus far in humans. Red dots indicate the inactivating mutations with reference to the reported amino acid substitution. The predicted insertion (1001_1002insC) is indicated by a red arrow. The only activating mutation of GPR54 described so far in humans, as potential cause for precocious puberty is denoted as a blue dot. A 155-bp deletion, not displayed in the scheme, was originally reported by de Roux et al. in patients with HH (Pinilla *et al*., 2012).

Functional analyses of some of these mutations have been performed in heterologous cell systems. The effects of the mutations vary significantly, ranging from severe impairment of signalling (L148S) to mild or no impact on function (ins/del mutation at intron 2). Neuroendocrinological studies of individuals revealed that the L102P mutation resulted in a range of clinical manifestations ranging from mild to severe gonadotropin deficiency (Tenenbaum-Rakover *et al.*, 2007). This highlights the importance of the potential interactions between

genetic variability at GPR54 and other loci in defining the clinical phenotype associated with suppressed kisspeptin signalling.

Two different missense mutations of the KiSS-1 gene, resulting in P74S and H90D substitutions, have been identified in a cohort of children with central precocious puberty. Genetic and biochemical analyses of the mutants indicated that none of them affected the region encoding the active peptide core of kisspeptins; neither did they alter downstream signalling in cellular assays (Silveira *et al.*, 2010). The clinical phenotype was more prominent with the P74S mutation, identified in a homozygous state in a boy with onset of puberty at 1 year of age, and serum incubations from controls and affected individuals suggested a greater stability of KP54 caused by the P74S mutation. Further studies in patients with precocious puberty of central origin lead to the identification of the first case of an activating mutation of GPR54, an autosomal dominant R386P substitution which did not result in constitutive activity of the receptor but instead prolonged its downstream IP3 response upon stimulation, therefore suggesting decreased desensitization (Teles *et al.*, 2008).

1.2.7 Structure activity relationships (SAR)

To identify the most important residue/s of a ligand required to activate its cognate receptor, it is useful to identify the minimal necessary sequence. SAR is an approach designed to find relationships between chemical structure or structural-related properties and biological activity of studied compounds. Quantitative SARs (QSAR) represent an attempt to correlate structural or property descriptors of compounds with activities (Kontogiorgis *et al.*, 2005).These physicochemical descriptors, which include parameters to account

for hydrophobicity, topology, electronic properties, and steric effects, are determined empirically or by computational methods. The main principle of SAR is that the activity of molecules is reflected in their structure. Structure-affinity/activity studies help to characterize the relationship between ligand and receptor. Crucial residues of the ligand and receptor required for their interaction can be identified and distinguished from non-essential residues. Knowing the three-dimensional structure of biologically active ligands is important for the elucidation of structure-activity relationships and for the development of potent agonists and antagonists (Flohr *et al.*, 2002).

Within the neuropeptide family, the amidated C-terminal fragment of the ligand is essential for interaction with all receptors (Hinuma *et al.*, 2000). One structure-activity study was used to determine the importance of the C-terminal amino acids of NPFF in binding and agonistic activity. Affinities of NPFF analogs were tested toward NPFF receptors of the rat spinal cord and the human NPFF2 receptors transfected in CHO cells. Activity was also evaluated by their ability to inhibit AC in NPFF2 receptor transfected CHO cells. The authors found that substitutions of phenylalanine^{8} by a tyrosine, phenylglycine or homophenylalanine were deleterious for high affinity. Similarly, the replacement of Arginine^{7} by a lysine or *D*-Arginine induced a loss in affinity thus highlighting the importance of the amidated C-terminal fragment (Mazarguil *et al.*, 2001).

Having highly selective agonists are as important as specific receptor antagonists for functional investigations. The progress made in the development of agonists and antagonists for GPR54 and GPR147 is discussed in the following section.

1.2.7.1 GPR54/Kisspeptin Agonists

The discovery of GPR54 and its cognate ligands KP54, 14, 13, and 10, has led to structure-activity studies with several peptide and non-peptide ligands. The N-terminally truncated KP10 is the shortest, highly potent agonist and was the template structure for further structure-activity studies (Gutiérrez-Pascual *et al.*, 2009, Ohtaki *et al.*, 2001). Niida *et al*., employed D-amino acid scanning experiments of KP10, in which residues were systematically substituted with their D-isomer, showed that the five C-terminal residues are stereochemically critical for GPR54 activation (Niida *et al.*, 2006). Using similar experiments in which the residues of KP10 were systematically substituted with alanine, the importance of residues phenylalanine [6], Arginine[9], and phenylalanine [10] were highlighted due to a high loss of agonistic activity upon substitution (Niida *et al.*, 2006, Gutiérrez-Pascual *et al.*, 2009, Orsini *et al.*, 2007). This observation was corroborated by structural data, which show that these three residues lay on one face of the ligands structure and define a pharmacophore site for kisspeptin (Orsini *et al.*, 2007). Substitution of the C-terminal phenylalanine with tryptophan resulted in improved receptor activity (Orsini et al., 2007; Clements et al., 2001).

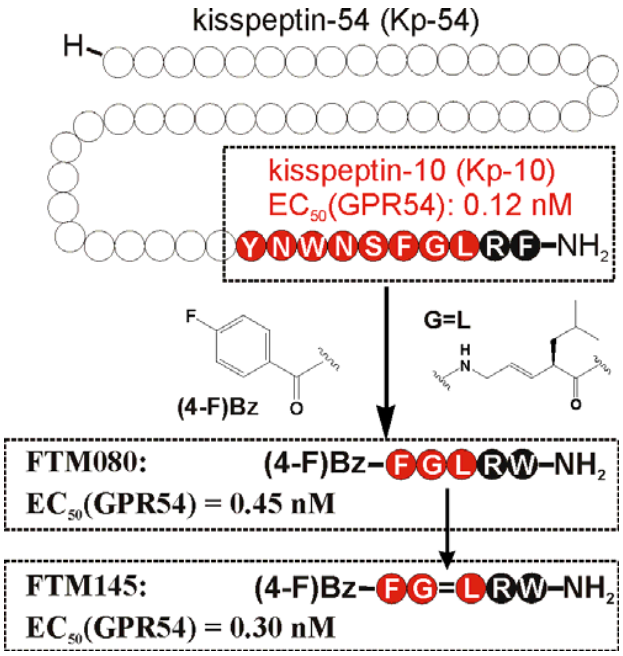

Figure 8: Sequences and bioactivity of down-sized agonists for the GPR54. Truncated KP10 was used as lead structure for developing FTM080 and FTM145. (4-F)Bz: 4-fluorobenzoyl (Oishi *et al.*, 2010).

Following these observations, Niida *et al*. developed so-called 'down-sized' analogs of KP10 (FM052a and FM053a) which retained KP10 like agonistic activity (Niida *et al.*, 2006). Further optimization of these pentapeptide-based C-terminal kisspeptin analogs resulted in a potent GPR54 agonist: H-Amb-Nal(2)-Gly-Leu-Arg-Trp-NH_2 (compound 34) (Tomita *et al.*, 2006). Further SAR and QSAR studies, performed on the N-terminal acyl groups of the pentapeptide agonists revealed that an aromatic acyl group and the inductive electronegative as well as small substituents at position 4 of the aromatic ring contributed to the agonistic activity and lead to the identification of the GPR54 activating agonist FTM080 (Figure 8), which is has a similar potency as KP10 (Tomita *et al.*, 2007). An (E)-alkene dipeptide isostere at the glycine-leucine site also was added to prevent proteinase degradation under biological conditions and the resulting agonist was called FTM145 (Figure 8) (Tomita *et al.*, 2008). Another KP10 analog

[*D*-Y] ^{1KP10} analog exhibited lower affinity for GPR54 but exhibited similar bioactivity *in vitro*. Peripheral administration of [*D*-Y] ^{1KP10} was shown to increase plasma LH and testosterone *in vivo* more potently than KP10 in mice and was thus suggested to be more resistant to proteolytic degradation compared to KP10. This hypothesis was supported by significantly increased total testosterone levels, measured 60 min after injection of 0.15 nmol [*D*-Y]^{1}KP10 while the same dose of KP10 had no significant effect (Curtis *et al.*, 2010). Comparisons of the binding activities and potencies of different KP10 analogs are presented in Table 2.

Table 2: Binding parameters (IC50) and potencies (EC50) of selected kisspeptin analogs (Findeisen *et al.*, 2011).

Compound	Sequence	EMax 100%	IC50 [nM]	EC50 [nM]	Reference
KP10	YNWNSFGLRFNH2	100.3 ± 7.3[a]	0.12	0.12	(Gutiérrez-Pascual *et al.*, 2009, Oishi *et al.*, 2010)
NF1	N**RNF**LRFNH2	NT	NT	800 ± 700	(Clements *et al.*, 2001)
Trp^{7}-NF1	N**RNF**LR**W**NH2	NT	NT	2100 ± 300	(Clements *et al.*, 2001)
Gly^{4},Trp^{7}-NF1	N**R**NGLR**W**NH2	NT	NT	200	(Clements *et al.*, 2001)
[*D*-Y]^{1}KP10	[*D*-Y]NWNSFGLRFNH2	NT	3.6 ± 0.3	NT	(Curtis *et al.*, 2010)
FM052a	**BisPy-Amb**-FGLR**W**NH2	88.9 ± 2.6[a]	NT	3.3	(Tomita *et al.*, 2006, Niida *et al.*, 2006)
FM053a	Gu-Amb-FGLRWNH2	93.7 ± 1.8[a]	NT	1.4	(Tomita *et al.*, 2006, Niida *et al.*, 2006)
Compound 34	H-**Amb-Nal(2)**-GLR**W**NH2	88.9 ± 0.4[a]	NT	0.82	(Tomita *et al.*, 2006)
FTM080	**(4-F)Bz**-FGLR**W**NH2	NT	0.71	0.45[b]	(Tomita *et al.*, 2007)
FTM145	**(4-F)Bz-F-G(ψ 1)**LR**W**NH2	NT	0.12	0.30[b]	(Tomita *et al.*, 2007)

Abbreviations: BisPy: bis[(2-pyridinyl)methyl]; Amb: 4-aminomethylbenzoic acid; Gu: guanidine; Nal(2): 3-(2-naphthyl)alanine; ψ 1: (*E*)-CH=CH-; (4-F)Bz: 4-fluorobenzoyl; [a] % activity are based on the relative maximum agonistic activity induced by 10 nM of the compounds (%). Maximum agonistic activity signal at 1 μM KP10 was used as reference (100%). [b] EC50 values represent the concentration required for 50% of the full agonistic activity induced by KP10 (1 μM). NT = not tested.

1.2.8 Therapeutic Potential of RFamides

The RFamide peptides represent a family with a large therapeutic potential since they are involved in several regulatory mechanisms related to energy homeostasis, reproduction, pain and behavioural processes like food intake, locomotion and stress response. However, due to their structural homology they are prone to interact with other GPCRs within this family. This is a major obstacle in investigations of the distinct roles for single RFamide peptides. Oishi *et al* (2010) provided an example of this when they showed that KP10 was able to interact with the NPFF receptors (Oishi *et al.*, 2010). Additionally, the NPFF receptors (GPR147 and GPR74) themselves both share high affinities to their endogenous ligands, NPFF and RFRP3 (Oishi *et al.*, 2010) and the GPR74 has been shown to also bind the neuropeptide Y (NPY) receptor 1 antagonist, BIBP3226 (Mollereau *et al.*, 2002). The ability of a few RFamide peptides to interact with multiple receptors drives the need to ensure that RFamide receptor selective agonists as well as antagonists are specific to a single receptor and do not cross react with other RFamide receptors.

Highly selective antagonists have important therapeutic applications and are excellent tools for investigating specific physiological effects mediated by individual receptors and their ligands.

1.2.8.1 Kisspeptin Antagonists

Roseweir *et al* (2009) created a highly potent peptide antagonist for GPR54 based on the substitution of leucine at position 8 of KP10 with *D*-Trp in combination with substitution of serine at position 5 with glycine (Roseweir *et al.*, 2009). This antagonist peptide 234 (p234), reduced pulsatile GnRH secretion in

female pubertal monkeys and inhibited the firing of GnRH neurons in the brain of the mouse. The inhibitory effect of p234 on LH release in rats and mice and the blocking of LH rise in castrated sheep, rats, and mice indicate that kisspeptin neurons mediate the negative feedback effect of sex steroids on gonadotropin secretion in mammals. Pineda *et al.*(2010) extended and refined the *in vitro* and *in vivo* testing of p234 through continuous infusion, and additionally modified the p234 with an N-terminal penetrating sequence to increase permeability through the blood-brain barrier and creates a path for new strategy for development of kisspeptin antagonists (Pineda *et al.*, 2010a).

A small molecule GPR54 antagonist with a 2-acylamino-4, 6- diphenylpyridine scaffold has also been reported (Kobayashi *et al.*, 2010a, Kobayashi *et al.*, 2010b). Kobayashi *et al*. (2010) identified an analog with a 2-furoyl group to be the most suitable of all tested 2-acylamino-4, 6-diphenylpyridines and their SAR studies led to compound 9l with an affinity of 3.7 nM in a GPR54 binding assay. The compound also displayed antagonistic activity in a cellular functional assay (Kobayashi *et al.*, 2010a). An optimized compound, 15a, displayed high affinity to human and rat GPR54 and displayed antagonistic activity and high brain permeability. Intravenous administration of 15a to castrated male rat curbed the plasma LH level, which could lead to the possibility of a small molecule kisspeptin antagonist as a novel drug for sex-hormone dependent diseases (Kobayashi *et al.*, 2010b).

1.2.8.2 Antagonists for GPR147

Several putative antagonists that target NPFF receptors (GPR147/GPR74) have been reported. These are compounds derived from endogenous NPFF ligands and included N-terminally truncated peptides such as desaminotyrosyl-

FLFQRFamide, dansyl-PQRamide and PFR (Tic) amide (Malin *et al.*, 1991, Tan *et al.*, 1999, Prokai *et al.*, 2001). However, most of them act as partial agonists with a low affinity which limits their use as pharmacological tools.

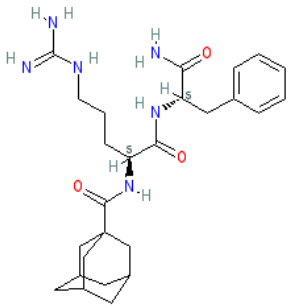

Figure 9: Structure of GPR147 antagonist, RF9 (Simonin *et al.*, 2006).

Simonin *et al* (2006) aimed to identify dipeptides that can act as NPFF receptor ligands which displayed affinity at NPFF receptors from rat spinal cord. Over 100 derivatives, produced by substitution of the phenyl ring of *N*-benzoyl RFamide or replacement with other heterocycles (e.g., indole or quinoline), or other nonaromatic lipophilic groups (tertiobutyl, cyclohexyl, or adamantane), were screened. RF9 and a modified dipeptide was identified as a potent and selective benzoyl-RFamide dipeptide antagonist for GPR147, RF2 was identified (Figure 9). RF9 dose-dependently reversed the inhibitory effect of RFRP3 on LH release with an EC_{50} of 4.7 ± 1.2 μM , thus confirming that this compound displays antagonist activity at GPR147 (Simonin *et al.*, 2006). Blockade of GPR147 by central administration of RF9 resulted in very robust LH and, to a lesser extent, FSH secretory responses in rats and mice. Distinct differences were detected, in terms of threshold doses and relative magnitude, between both gonadotropins. In

male rats, significant LH responses to RF9 were detected at doses of 1 nmol icv, with a maximal 8-fold increase over basal levels at doses of 20 nmol. In contrast, elevation of FSH levels was observed only after icv injection of maximal doses of RF9 (20 nmol), which accounted for a 65% increase over corresponding basal concentrations (Pineda *et al.*, 2010c).

1.3 Aims

There is intense ongoing research into kisspeptin and RFRP3 to further characterize the role that these two neuropeptides play in regulating puberty and reproduction. While this is important, it is equally important to try and discover how these ligands interact with their receptors and with other neuropeptide receptors especially for the purposes of designing novel agonists and antagonists targeting these receptors that may have therapeutic use. In particular, information facilitating the design of novel GPR147 ligands that are specific and do not cross react with other receptors would be especially useful. Although naturally-occurring human mutations have been identified in GPR54, there have been no classical mutagenesis studies on this receptor. Therefore, this thesis aimed to address these interactions in more detail. Furthermore, as there is much controversy surrounding the interplay between GPR54/kisspeptin and GPR147/RFRP3 pathways, the cross-reactivity of kisspeptin/RFRP3 analogs at these receptors has therefore been examined.

The specific objectives of this thesis were to investigate:

- The role of the N-terminus and extracellular loops of GPR54 in ligand binding and receptor activation by employing GalR2-GPR54 chimeras
- The role of the extracellular loop acidic residues of GPR54 in ligand binding and receptor activation
- The specificity of ligands for GPR147 by testing the affinity of different RFamide and RFamide related peptides
- SAR studies on RFRP3 analogs at GPR147
- Effects of kisspeptin analogs on GPR147

Chapter 2: Materials and Methods

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2.1 Materials

Dulbecco's minimum essential medium (DMEM), Optimem-1 and Trypsin powder were purchased from Gibco (Invitrogen). Fetal Calf Serum (FCS), Penicillin-Streptomycin and Med-199 were purchased from Highveld Biological (WhiteScience). Fugene HD and XtremeGene HP transfection reagents were purchased from Roche. Cell culture dishes and plates were purchased from Costar. Cell culture flasks were purchased from Corning. Matrigel Basement membrane matrix (Matrigel) was purchased from BD Biosciences. Electroporation cuvettes were purchased from Biorad.

The radiochemicals (Myo-[2-^{3}H (N)] inositol, Iodine-125 and Metastin 45-54, [^{125}I] ([^{125}I]-KP10) were purchased from Perkin Elmer. Scintillation fluid was from Zinsser Analytical. Quaternized aminoethyl (QAE) sephadex G25, 1x8-200 DOWEX-1 and Forskolin were purchased from Sigma. The Dual luciferase reporter assay kit and PureYield™ Plasmid Maxiprep system and Plasmid Maxi kit were purchased from Promega or Qiagen, respectively.

Kanamycin and ampicillin antibiotics were purchased from Sigma.

The GalR2-GPR54 chimera's (pEYFP) were a gift from Professor Chris Hague (University of Washington, USA). The acidic N-terminal FLAG tagged GPR54 mutants were prepared in pcDNA3.1(+) by GENEART (California, USA). The mouse GPR147 expression construct (in pcDNA3.1) was made by Dr Kevin Morgan (MRC, Human Reproduction Science Unit, Edinburgh). The pCRE-Luc and Renilla (pRL-TK) reporter plasmids were obtained from Clontech and Promega, respectively.

CCK was purchased from American Peptide Co. All other peptides used in this study were purchased from EZBiolab.

Monoclonal ANTI-FLAG® (Clone M2) antibody was purchased from Sigma. Anti-GFP (a mixture of clones 7.1 and 13.1) was purchased from Roche. Polyclonal goat anti-mouse HRP-conjugated secondary antibody was purchased from Santa Cruz. Goat anti-mouse Alexa 488-conjugated and donkey anti-mouse Alexa 488-conjugated secondary antibodies were purchased from Invitrogen. Donkey anti-mouse Cy3-conjugated secondary antibody was purchased from Jackson ImmunoResearch Laboratories, Inc. DAPI and Mowiol were purchased in-house from the UCT Confocal and Light Microscope Facility.

2.2 Methods

2.2.1 Preparation of competent bacteria

E.coli DH1α cells were streaked onto a Luria broth (LB) agar (170 mM NaCl, 1% tryptone, 0.5% yeast extract and 1.5% agar) plate and cultured overnight at 37˚C. Thereafter, a single colony was picked from the plate and inoculated into 5 ml sterile LB medium (170 mM NaCl, 1% tryptone and 0.5% yeast extract) and cultured overnight at 37˚C. This starter culture was then added to a 2L flask containing 500 ml of LB. The culture was shaken at 37˚C until it reached an OD_{600} of 0.45 nm after which it was centrifuged at 2000*g* in a fixed angle rotor centrifuge (J21,Beckmann) for 10 min at 4˚C. The supernatant was discarded and the pellet re-suspended in 100 ml ice cold 100 mM $MgCl_2$. After 30 min incubation on ice, cells were pelleted at 2000*g* (J21, Beckmann) for 10 min at

4˚C. Supernatant was discarded and pellet was re-suspended in 10 ml of 100 mM $CaCl_2$ with 15% glycerol. Aliquots of 100 µL were prepared in 2 ml tubes and snap frozen in liquid nitrogen before storage at -80˚C.

2.2.2 Bacterial Transformation

Plasmid DNA (1 ng) was added to 100 µl competent bacteria and incubated on ice for 30 minutes. Bacteria were then heat shocked for 30 seconds at 42°C and returned to ice for 2 minutes after which 900 µl LB media was added and incubated at 37°C for 60 minutes shaking at 250 rpm. Bacteria were then spread onto LB agar plates, containing appropriate selective antibiotic (100 mg/ml ampicillin or 50 mg/ ml kanamycin) and incubated overnight at 37°C.

2.2.3 Preparation of plasmid DNA

A single colony was inoculated into a starter culture of 10 ml fresh LB medium containing selective antibiotic (Ampicillin or Kanamycin at 100 mg/ml or 50 mg/ml, respectively) and grown for a period of 10 hours at 37°C with shaking at 250 rpm. This starter culture was diluted 1/1000 into 250 ml LB medium and cultured for 16 hours at 37°C with shaking at 250 rpm. Plasmid DNA was purified by using either PureYield™ Plasmid Maxiprep system or Plasmid Maxi kit, according to the manufacturer's instructions. Samples were eluted using TE Buffer (10 mM Tris-HCL (pH8), 1mM EDTA) or water and DNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Scientific).

2.2.4 Preparation of glycerol stocks of transformed bacteria

Glycerol stocks of transformed *E.coli* were prepared by adding 400 µl of 80% glycerol to 600 µl of bacterial culture that had been grown overnight at 37°C with

shaking at 250 rpm. Vials were inverted to mix the glycerol and stored at -80ºC. To recover the bacteria, a sterile inoculating loop was used to scrape the surface of the frozen culture, keeping the bacteria on dry ice. The bacteria were used to inoculate 250ml LB media and, after incubation for 16 hours at 37°C with shaking at 250 rpm, plasmid DNA was extracted as described in 2.2.3.

2.2.5 Agarose gel electrophoresis

Plasmid DNA and PCR products were separated by agarose gel electrophoresis at 120V using 1% agarose gels prepared in TAE buffer (40 mM Tris, 320 mM acetic acid, 1 mM EDTA, 10µg/ml ethidium bromide, pH7.2). Thereafter, gels were visualised under ultraviolet light using a transilluminator. Fragment size was determined using a 1kb DNA ladder (NEB) that was run as a size marker.

2.2.6 Maintenance of Cell cultures

COS-7 and HEK293-T cell lines were maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin and 100 IU/ml streptomycin. Cells were passaged twice-weekly employing enzymatic dispersal with trypsin. The medium was aspirated from the cells and washed with phosphate buffered saline (PBS; 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4 and 2mM KH_2PO_4, pH 7.4) before trypsin-EDTA (0.05% trypsin and 0.5 mM EDTA in PBS, pH 7.4) was added to each flask. Cells were incubated at 37ºC for 3-5 minutes, until detached. Trypsin was neutralized by the addition of growth medium. The cell suspension was then centrifuged at 600*g* for 2 minutes and the pellet re-suspended in an appropriate volume of medium. Cells were passaged in a 1:10 dilution or seeded in plates as needed for experiments. For experiments, HEK293-T cells were seeded in dishes treated with 1:30 dilution of Matrigel to aid cell attachment.

Flasks/dishes were then returned to the incubator at 37ºC in a humidified 5% CO_2 atmosphere.

2.2.7 Transfection techniques

2.2.7.1 Electroporation of COS-7 cells

COS-7 cells were plated into 15 cm diameter dishes and, at 80% confluence, cells were transfected. Cells were detached from the dishes by addition of trypsin-EDTA as described previously. Cell suspensions were pooled and collected via centrifugation at 600*g* for 2 minutes. Media was aspirated and the pellet gently re-suspended in 5 ml of Optimem-1 electroporation medium. The cells were collected by centrifugation at 600*g* for 2 minutes. The medium was aspirated and the pellet re-suspended in 0.7 ml Optimem-1 electroporation medium per dish. Thereafter, 0.7 ml of the cell suspension was added to each pre-chilled electroporation cuvette containing 10 µg DNA and mixed with gentle pipetting. Each cuvette was placed in the electroporation shocking chamber (Biorad Gene Pulsar II Electroporation System) and pulsed at 0.22 kV and 960 µF with an average time constant of 21ms. Cuvettes were then incubated for 10 minutes at room temperature after which cells were re-suspended in 25 ml cell culture medium per cuvette. Transfected cells were then seeded into 12-well plates at a density of $1x10^5$ cells/ 1ml for experiments as needed.

COS-7 cells were used for all radioactive experiments as they attach well and are not easily detached during the washing processes.

2.2.7.2 Chemical Transfection of HEK293-T cells

Fugene HD Transfection Reagent:

Cells were seeded at a density of $1x10^5$ cells/ml in 24-well plates or $5\ x10^4$ cells/ml in 48-well plates. The transfection medium was prepared as follows for each DNA construct:

2 µg DNA per 100 µl serum free medium (SFM; DMEM without supplements)

6 µl Fugene HD transfection reagent per 2 µg DNA

Samples were mixed by vortexing and incubated at room temperature for 30 minutes. 25 or 50 µl of the mixture was then added in a drop-wise manner to each well of 48-well or 24-well plates, respectively, swirling to ensure even distribution. The plates were then incubated at 37ºC in a humidified 5% CO_2 atmosphere for 48 hours. Transfection medium volumes were adjusted per experiment as required.

X-XtremeGene HP DNA Transfection Reagent:

Cells were seeded into 24-well plates at a density of $1x10^5$ cells/ml. The transfection medium was prepared as follows:

1 µg DNA per 100 µl SFM

2 µl X-XtremeGene HP DNA Transfection Reagent per 1 µg DNA

After mixing gently, the transfection mixture was incubated for 15 minutes at room temperature. 50 µl of the mixture was then added drop-wise to each well. Plates were swirled and incubated at 37ºC in a humidified 5% CO_2 atmosphere for 48 hours. Transfection medium volumes were adjusted per experiment as required.

2.2.8 Inositol-(1, 4, 5)-trisphosphate accumulation assays

COS-7 cells transfected by electroporation were seeded in 12-well plates as indicated in 2.2.7.1 and incubated for 24 hours at 37ºC. Thereafter, the medium was aspirated and cells were rinsed with 0.5 ml of IP3 media (Med199 supplemented with 2% FCS) for 15 minutes. Cells were then labelled with 0.5 ml/well of 2uCi/ml myo [2-^{3}H (N)]-inositol in IP3 media and incubated for 20 hours at 37ºC. After which cells were incubated with Buffer-I (140mM NaCl, 4mM KCl, 20mM HEPES, 8mM glucose, 0.1% bovine serum albumin (BSA), 1mM $MgCl_2$, 1mM $CaCl_2$ and 10mM LiCl) for 15 minutes at 37˚C. Peptides were diluted (0-1µM) in Buffer- I and added to the cells for 90 minutes at 37˚C. The media was then aspirated and 1 ml of 10 mM formic acid added to each well and incubated at 4˚C for 30 minutes to permeabilise the cells and release radiolabelled inositol phosphates.

Columns packed with Dowex 1x8-200 ion-exchange resin (Sigma) were washed sequentially with 3 ml 3M ammonium formate/0.1 M formic acid and 10 ml distilled water. The cell extracts were then added to the columns, followed by 10 ml distilled water and then 5 ml 0.1 M ammonium formate/0.1 M formic acid. Samples were then eluted from the columns in 1 ml 1M ammonium formate/0.1M formic acid. Scintillation vials were prepared with 2.6 ml scintillation fluid and 1 ml of elute was added to each vial and mixed well. Sample radioactivity was measured in a Tri-carb 2100TR liquid scintillation analyser (Packard).

2.2.9 Live cell imaging

Untransfected and WT-GPR54 and GPR54 chimeric receptor transfected HEK293-T cells were seeded on cover slips, coated with Matrigel. 48 hours post

transfection; cells were visualized using a ZEISS AXIOVERT 200M LSM 510 Meta confocal microscope at 100x magnification with the assistance of Susan Cooper and Prof Dirk Lang of the UCT Confocal and Light Microscope Facility.

2.2.10 Immunohistochemistry

Transfected WT-GPR54 and acidic mutant receptors and untransfected HEK293-T cells were seeded on cover slips, coated with Matrigel. 48 hours post transfection; cells were rinsed three times with 0.5 ml PBS. Thereafter, 500 µl of a 4% paraformaldehyde (PFA) solution was added to the cover slips to fix the cells, and incubated for 15 minutes at room temperature. After three 0.5 ml PBS rinses, cells were incubated for 30 minutes in 0.5ml blocking solution (10% FCS in PBS) followed by a 2 hour incubation with 50 µl primary antibody diluted in blocking solution. Primary antibody solution was then removed and cells were rinsed for 3 x 10 minutes in 0.5 ml PBS. The secondary antibody solution (50 µl) was added to the cells and incubated in the dark for 45 minutes. Cells were rinsed with 0.5 ml PBS and incubated with 1:2000 dilution of DAPI in PBS for 10 minutes and then rinsed again for 10 minutes with 0.5 ml PBS. Coverslips were then mounted on glass slides containing Mowiol mounting solution and stored in the dark. Cells were visualised using a ZEISS AXIOVERT 200M LSM 510 Meta confocal microscope with the assistance of Susan Cooper and Prof Dirk Lang of the UCT Confocal and Light Microscope Facility.

2.2.11 ELISA Assay

A modified ELISA, designed by Dr Claire Newton, University of Cape Town (unpublished), was performed to measure cell surface receptor expression. HEK293-T cells were seeded in matrigel-coated 48-well plates and transfected as

described in section 2.2.7.2. 48 hours post transfection, cells in each well were rinsed with 400 µl of PBS+ (PBS supplemented with 0.5mM $MgCl_2$ and 0.9mM $CaCl_2$) prewarmed to 37°C. Thereafter 150 µl of primary antibody (Monoclonal ANTI-FLAG® M2 or Anti-GFP 1:1000 diluted in DMEM supplemented with 10% FCS and pre-warmed to 37°C) was added to the cells and incubated for 2 hours at 37°C. Cells were then rinsed three times for 5 minutes with 400 µl PBS+ and incubated for 1 hour with 150 µl secondary antibody (goat anti-mouse HRP-conjugated 1:1000 dilution, diluted in DMEM supplemented with 10% FCS). Thereafter, three 400 µl PBS+ rinses were performed as before followed by a single PBS rinse. 150 µl of 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate (0.1mg/ml TMB stock in phosphate citrate buffer, pH5.0, supplemented with 0.2 µl/ml of 30% H_2O immediately before use) was added to the cells and incubated in the dark for 20 minutes at room temperature. The reaction was stopped by the addition of 150 µl 2M H_2SO_4 to the cells and the plates were placed on a rocker to mix. 150 µl of the supernatant was then transferred to a clear flat bottomed 96-well plate and absorbance was read at 450 nm using an Anthos 2001 spectrophotometer.

To measure total receptor expression, prior to addition of the primary antibody solution, cells were rinsed with PBS+ and 300 µl methanol was added to each well to permeabilize the cells. Cells were then incubated at -20°C for 10 minutes. Thereafter cells were rinsed three times for 10 minutes in 0.5 ml PBS+. 0.5 ml blocking solution (DMEM supplemented with 10% FCS and 5% BSA) was added to the wells and cells were incubated for 1 hour at 37°C. Cells were then rinsed once with 0.5 ml PBS+. The protocol was then continued from the addition of the

primary antibody solution as described for measurement of cell surface receptor expression.

2.2.12 Promega CRE-Luciferase Assay

HEK293-T cells were seeded in 24-well plates coated with matrigel. After 24 hours, cells were chemically transfected with GPR147 (483.5 ng/well), CRE-Luc reporter gene plasmid (483.5 ng/well) and Renilla plasmid DNA (33 ng/well). Plates were incubated for 24 hours post transfection in growth media, and then rinsed twice with 0.5 ml PBS+. Thereafter 1 ml HEPES-DMEM media (DMEM supplemented with 1% pen/strep, 2mM L-glutamine 100 IU/ml penicillin, 100 IU/ml streptomycin and 10mM HEPES) was added and the cells were grown for 24 hours. The media was then aspirated and replaced with 0.5 ml of peptide dilutions prepared in HEPES-DMEM. Plates were incubated for 6 hours at 37 °C after which they were transferred to ice and rinsed with 0.5 ml cold PBS+. Promega passive lysis buffer (PLB) was diluted according to the manufacturer's instructions and 20 µl was added to each well. Plates were placed on a shaker at high speed for 15 minutes at room temperature.

Lysates were diluted 1 in 4 with PLB and 20 µl was transferred to a white, flat bottomed 96 well plate. Luciferase activity was measured using a Dual luciferase reporter gene assay and luminescence was measured with a Lumat LB9501 luminometer.

2.2.13 Radiolabelling of peptides

A modified RFRP3 that includes a Tyrosine (Tyr-Val-Pro-Asn-Leu-Pro-Gln-Arg-Phe-NH_2) was radiolabelled with [I^{125}] using a variation of the Chloramine T

method. Briefly, 10 µl of peptide (1 µM stock) was re-suspended in 50 µl 0.5M phosphate buffer (pH7.4) and mixed with 1mCi carrier-free $Na^{125}I$ (100µCi/µl) . Thereafter, 10 µl chloramine T (2mg/ml) was added and the reaction was allowed to proceed for 10 seconds. Sodium metabisulfate (2mg/ml) was added immediately afterwards to terminate the reaction. The reaction was then loaded onto a QAE sephadex G25 size exclusion column and 1 ml fractions were eluted in a phosphate buffer containing BSA. 10 µl of the fractions were counted for 1 minute using a gamma counter (Berthold LB211) to obtain the elution profile and identify fractions containing the radiolabelled peptide.

2.2.14 Whole cell competitive radioligand binding assay

COS-7 cells were electroporated and seeded in 12-well plates as described above. 48 hours post-transfection media was aspirated and cells were incubated in 0.5 ml Binding media (DMEM supplemented with 10mM HEPES and 0.1% BSA) containing 100,000cpm/well or 50,000cpm/well $[I^{125}]$-RFRP3 or $[I^{125}]$-KP10, respectively, and increasing concentrations of non-radiolabelled peptide in the range of 0-1µM for 4 hours at 4°C. Cells were then rinsed twice with 1 ml cold PBS+. Thereafter, 0.5ml 0.1M NaOH was added to the cells and incubated at room temperature for 15 minutes in order to lyse the cells. Cell lysates were transferred to tubes and radioactivity measured (1min/sample) with a gamma counter (Berthold LB211).

2.2.15 Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM)**.** Statistically significant differences were determined by one-way analysis of variance or paired

student's *t*-test using Prism 5.0 software (GraphPad Software Inc., San Diego, CA) with $p<0.05$ considered significant.

Chapter 3: Role of N-terminus, ECL1-3 and their acidic residues in GPR54 expression, binding and activation

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3.1 Introduction

GPCRs are major drug targets. Therefore, much research has been devoted to the elucidation of the function and three-dimensional structure of this large family of membrane proteins. GPCRs have seven conserved transmembrane (TM) α-helices which are connected by three extracellular (ECL) and three intracellular (ICL) loops. They also have an extracellular N-terminus and intracellular C-terminus. The ECLs, N-termini, and/or part of the TM α-helical region facing the extracellular space are responsible for ligand recognition, whereas the ICL and C-termini recognize the specific G protein partner required for signal transmission within the cell (Palczewski and Orban, 2013).

Identifying the structural and biochemical requirements of integral domains of individual GPCRs assists modelling of the inactive and active receptor confirmations, an important factor both for targeted therapeutics and fundamental receptor biology. Various strategies are used to gain insight into the structure and function of GPCRs, especially since structural information is still relatively limited for many GPCRs. Techniques used to elucidate the structural determinants of ligand binding and functional activation of receptors include: site-directed mutagenesis (SDM), chimeric molecule construction, identification and characterization of naturally occurring mutations that alter receptor function, use of antibodies directed against receptor epitopes, use of synthetic peptides that are homologous to parts of the receptor sequence, and affinity labelling and other chemical labelling methods including cysteine modification studies (Kristiansen, 2004). GPCR crystallization is an important technique that can be used to elucidate GPCR structure but it is extremely challenging. GPCRs are unstable outside the cell membrane and they are known to adopt many conformational

states (Rosenbaum *et al.*, 2009). The relatively unstructured loops add to the conformational diversity of GPCRs. The combination of fragility and flexibility is a major hurdle which has limited the progress of high-resolution structures. However, with developments in engineering, producing and purifying membrane proteins, crystal formation, and X-ray diffraction has improved the elucidation of GPCR structures. Thus far, 47 structures representing 13 distinct GPCRs have been solved, in many cases with resolution of 3 Å or better (Audet and Bouvier, 2012).

Mutagenesis is another principal technique for examining GPCR function and activation. Through changes to single residues or entire receptor domains followed by structure-activity relationship (SAR) studies, insights into which residues are involved in ligand binding and/or receptor activation can be gathered (Beukers and Ijzerman, 2005, Martinelli and Tuccinardi, 2008, Heilker *et al.*, 2009). SDM combined with bioinformatics techniques utilizing the large number of protein sequences in the GPCR family helped bypass the lack of available structural data before 2008 (Conner *et al.*, 2010). Of the GPCR structures that have been elucidated since 2008, SDM and SAR studies have been used to validate these structures and have also provided information on receptors whose structures have yet to be elucidated.

The choice residue for substitution in SDM studies has been predominantly alanine, by either individual targeted substitutions or as part of a less-targeted alanine scan. Alanine is often chosen due to its small size and lack of reactive functional groups which results in no or minor influence on the protein backbone (Ahn *et al.*, 2009). Alternatively, conservative amino acid substitutions can be made in which residues are substituted with other chemically-similar residues,

theoretically resulting in relatively minor structural/biochemical changes or alternatively, non-conservative amino acid substitutions of amino acids with different properties can be introduced leading to more profound changes.

Another method used in structure/function studies is the creation of chimeric receptors. Chimeric GPCRs can provide insights into ligand binding factors, G protein, and effector coupling, and/or sites necessary for receptor internalization and trafficking. Usually, homologous domains are substituted in receptors within the same GPCR family. This aims to minimize the disruption of the basic receptor structure. Some limitations of the chimeric receptor approach may result in chimeric receptors not functioning because of problems in folding and/or targeting. Nonetheless, the use of chimeras is an effective tool to elucidate new insights into structure/function relationships within the superfamily of GPCRs (Yin *et al.*, 2004).

Glutamate and aspartate are often involved in receptor signalling or binding sites. Aspartates and glutamates are negatively charged and polar, and usually are located on the surface of proteins, exposed to an aqueous environment. Aspartate and glutamate can form ionic bonds (electrostatic bonds) with the positive charge on the basic amino acids (histidine, lysine, and arginine) of interacting ligands/signalling molecules. Aspartate has a shorter side chain than glutamate giving it more rigidity within protein structures. These characteristics mean that aspartate is most often involved in ligand binding or signalling sites (Betts and Russell, 2003).

As discussed in the Literature Review, the kisspeptin/GPR54 system was recognized as a regulator of reproduction when two groups found that mutations

in GPR54 caused delayed or absent puberty (De Roux *et al.*, 2003, Seminara *et al.*, 2003). Various studies have subsequently emerged investigating the effect of kisspeptin on GnRH secretion and the onset of puberty. The GPR54 protein shares 45% homology with the galanin family of receptors but does not bind either galanin or galanin-like peptide (Lee *et al.*, 1999a). A mutagenesis study on the GalR2 subtype identified four residues namely, histidine^{252} and histidine^{253} located in TM6 and phenylalanine^{264} and tyrosine^{271} in the ECL3, to be of great significance for ligand binding. The N-terminal tail of GalR2 was also shown to contribute to ligand binding, and the selective binding of the Gal(2–11) peptide includes interaction with the isoleucine^{256} residue located at the top of TM6 (Lundstrom *et al.*, 2007).

Kisspeptin has great therapeutic potential. However, to date, studies have been focused on developing kisspeptin agonists/antagonists for therapeutic use and on investigation of the naturally occurring mutations on GPR54 and little is currently known about the mechanism of binding of kisspeptin to GPR54, information which would aid further development of drugs targeting this receptor. Studies performed on GPR54 receptor mutations can provide information on the specific residues involved in the binding of kisspeptins and the types of inter-molecular bonds involved. In this chapter, chimeras and SDM studies will be employed to investigate residues important for GPR54 binding and activation.

3.1.1 Aims

- Determine whether the N-terminus and extracellular loops of GPR54 are important for receptor expression, ligand binding and receptor activation
- Examine the role of acidic residues of the GPR54 extracellular loops in ligand binding and receptor activation

3.2 Results

3.2.1 Chimeras of GPR54 and Galanin 2 receptor

Chimeric substitution is a useful tool to identify important regions for binding and activity of GPCRs. In this chapter, chimeric GPR54/galanin receptor 2 (GalR2) constructs have been utilised to examine the residues important for kisspeptin/GPR54 binding. The extracellular loops (ECL) of GPR54, namely the N-terminus and ECLs1, 2 and 3, were independently replaced with corresponding sequences from GalR2 (Figure 10). The four resulting chimeric GPR54/GalR2 constructs (N-terminus-GalR2-GPR54, ECL1-GalR2-GPR54, ECL2-GalR2-GPR54 and ECL3-GalR2-GPR54) were a gift from Professor Chris Hague of the University of Washington (Appendix I). The wild-type (WT) human (h) GPR54 was cloned into pEGFP, (to add a C-terminal GFP tag) and the GPR54/GalR2 chimeras were cloned into pEYFP (to add a C-terminal YFP tag).

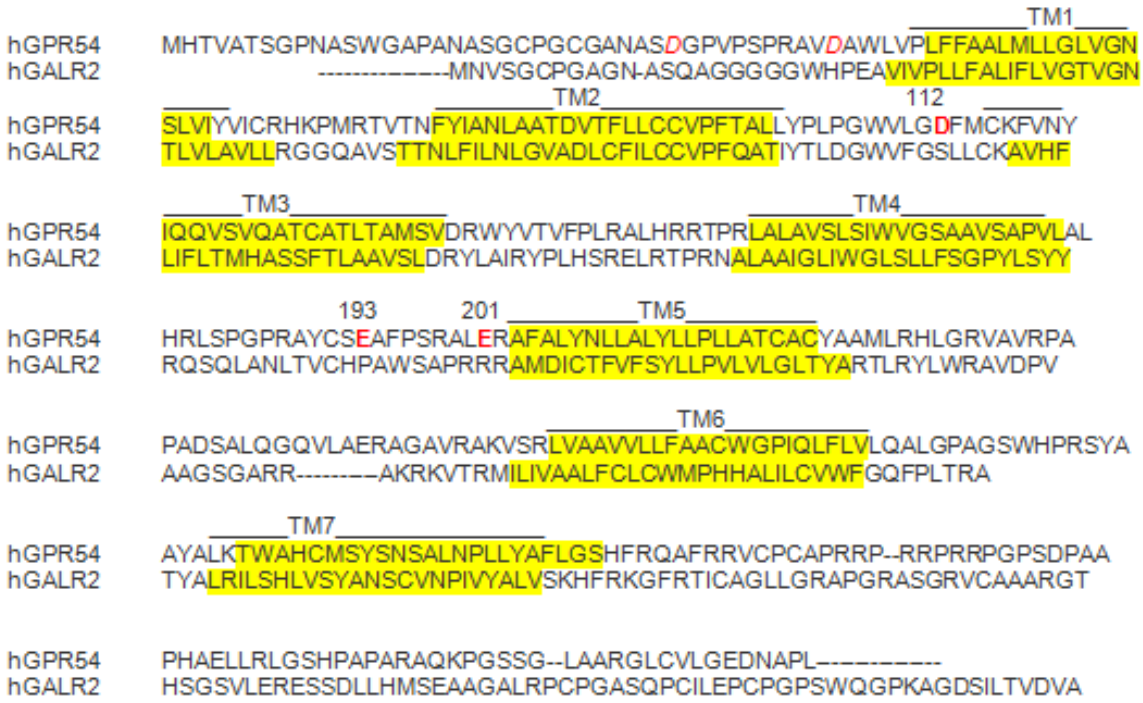


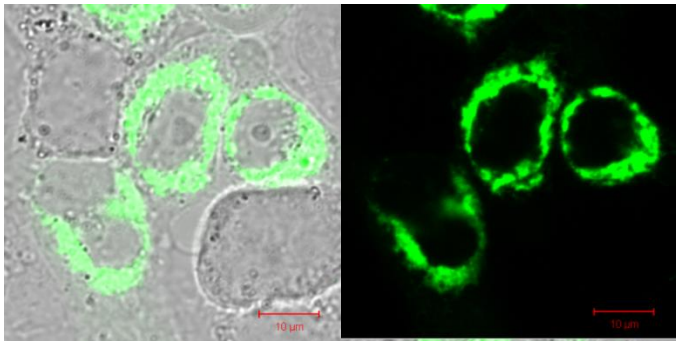
Figure 10: Amino acid sequence alignment of human GPR54 and Galanin receptor subtype 2. Sequences were obtained from the National Center for Biotechnology Information. (GPR54-GenBank: AAK83235.1) (GalR2-GenBank: EAW89364.1) Yellow denotes transmembrane domains. – indicates gaps in alignment, Acidic amino acids in extracellular domains of GPR54 are denoted in red text. Amino acids of GPR54 mutated to alanine are denoted in bold red text. The N-terminal region is situated up-stream of TM1; ECL1 is situated between TM2 and TM3, ECL2 between TM4 and TM5, and ECL3 between TM6 and TM7.

3.2.1.1 Expression of GPR54/GalR2 receptor chimeras

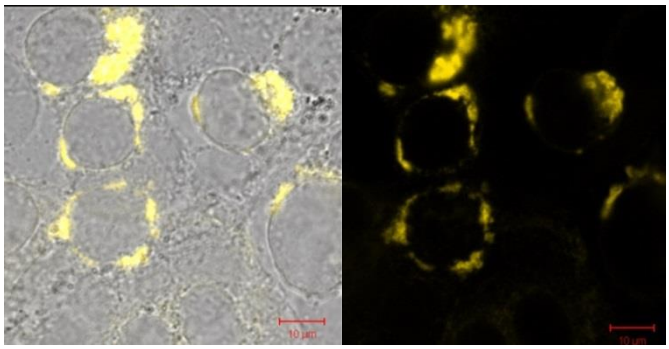
Initially the expression of the chimeric GPR54/GalR2 receptors was examined in order to ensure that substitution of the chimeric residues did not affect receptor expression. WT-hGPR54-GFP and GPR54/GalR2 chimeras were transiently transfected into HEK293-T cells. The receptor expression and localisation was then determined using live-cell confocal microscopy (Figure 11). The confocal microscopy analysis revealed that WT GPR54-GFP and all of the GPR54/GalR2-YFP receptors were localized to the plasma membrane to some degree and their localization patterns were indistinguishable suggesting that the chimeric substitutions did not cause trafficking alterations of GPR54. Untransfected

HEK293-T cells served as the negative control with no observable fluorescence in the untransfected cells. A limitation of the confocal microscopy is that under this experimental design, a quantitative analysis of receptor expression could not be performed.

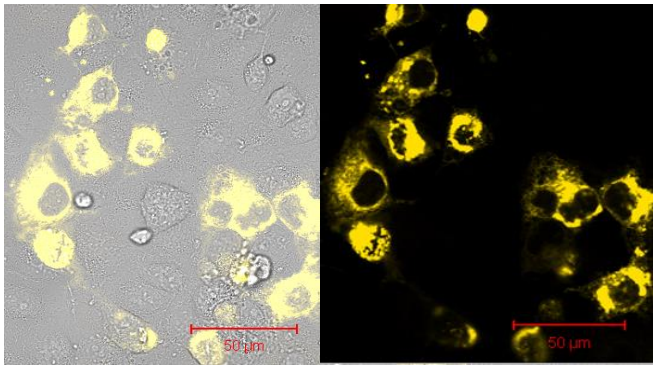
(a)



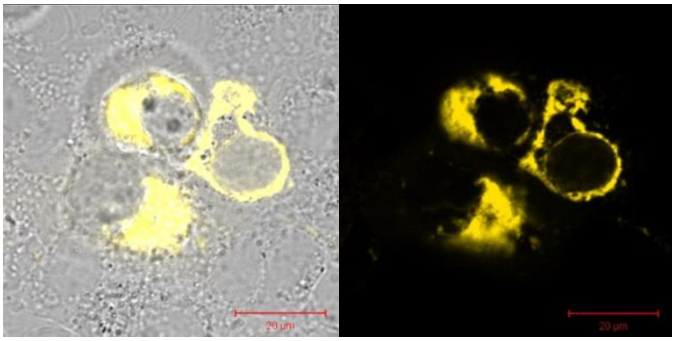
(b)



(c)



(d)



(e)

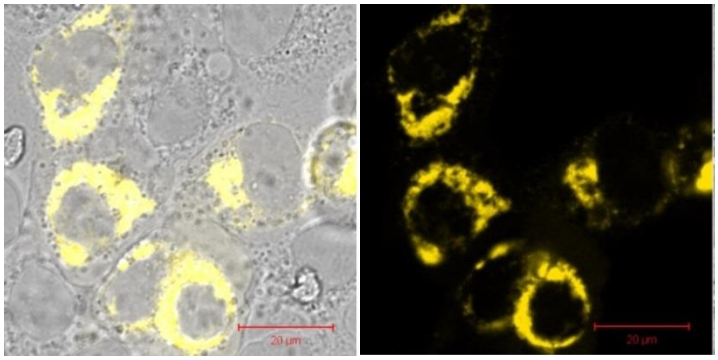


Figure 11: Live-cell confocal imaging of WT-GPR54-GFP and GPR54/GalR2-YFP chimeras. HEK293-T cells were transfected with (a) WT-GPR54-GFP (b) N-terminus-GalR2-GPR54-YFP (c) ECL1-GalR2-GPR54-YFP (d) ECL2-GalR2-GPR54-YFP or (e) ECL3-GalR2-GPR54-YFP receptors. 48 hours post transfection cells were visualized on a confocal microscope. Left panel represents a merge of fluorescence and differential interference contrast (DIC) images. Right panel is fluorescence only. Images representative of n=3.

Although the four chimeric receptors appeared to be expressed well and localised to the plasma membrane, no comment could be made about the relative levels of receptor expression. In order to obtain a quantitative measurement of receptor expression levels a whole cell ELISA was performed to measure the total receptor expression in the cell. Due to the WT-GPR54-GFP and chimeric-YFP receptors having C-terminal GFP and YFP tags, permeabilization of the cells was

required in order to allow antibody access. Total receptor expression within the cell and not cell surface receptor expression was thus measured. As antibodies raised against full-length GFP can also detect YFP and other GFP variants, an anti-GFP antibody was used for measurement of all receptors. There was no significant difference in total receptor expression between the WT-GPR54-GFP and the GPR54/GalR2-YFP chimeric receptors ($p>0.05$; Figure 12) indicating that the chimeras do not affect protein expression. Untransfected cells had no measureable absorbance at 450nm. Although total receptor expression is not different between the WT GPR54 and chimeric receptors, it should be noted that no conclusions can be drawn about their relative levels of cell surface expression which is critical for receptor binding and activation.

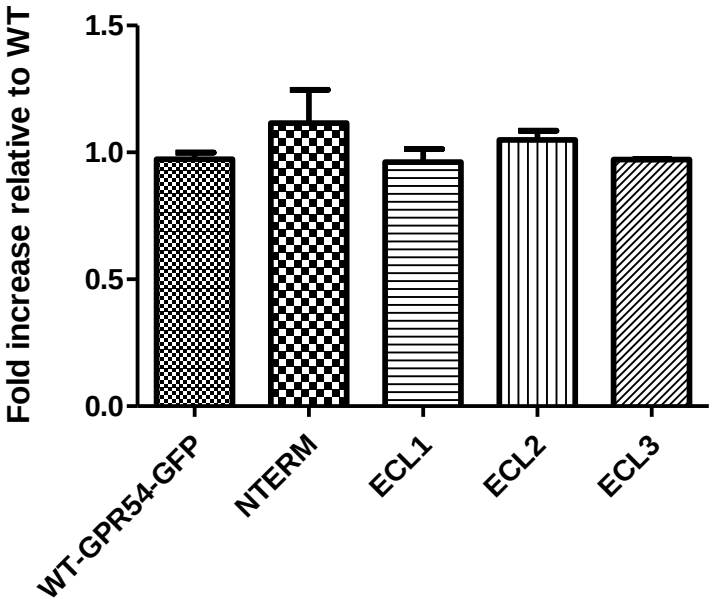


Figure 12: Relative total cellular expression of WT-GPR54-GFP and GPR54/GalR2-YFP chimeras. HEK293-T cells were transfected with WT-hGPR54-GFP, N-terminus-GalR2-GPR54-YFP (NTERM), and ECL1-GalR2-GPR54-YFP (ECL1), ECL2-GalR2-GPR54-YFP (ECL2) or ECL3-GalR2-GPR54-YFP (ECL3) receptors. 48 hours post transfection cells were permeabilzed with methanol and ELISA performed. Absorbance was read at 450 nm. Data are presented as fold increase relative to expression of WT GPR54-GFP. Representative graph of N=3 and P>0.05 for comparison of chimeric receptors and WT GPR54. The standard error for ECL3 was (0.001856) which is too small to be indicated and seen on the plot.

3.2.1.2 Expression and affinity of GPR54-GALR2 receptor chimeras

The interaction of ligands with their binding site on their cognate receptors can be characterized in terms of their binding affinity. Ligands that bind with high affinity are as a result of strong intermolecular force between the ligand and its receptor, while low-affinity ligands binding involve less intermolecular force between the ligand and its receptor. Whole-cell radioligand competition binding assays were performed, in which [^{125}I]-KP10 was competed with increasing concentrations of unlabelled KP10, in order to determine the affinity of KP10 for the WT GPR54 and GPR54/Gal2 chimeric receptors. Half maximal inhibitory concentration for each receptor, known as the IC_{50} (the concentration of competing ligand, which displaces 50% of the specific binding of the radioligand in a competition assay) was determined and used as a measure of relative affinity of KP10 for each receptor.

As whole cell binding assays were performed, it was also possible to compare levels of radioligand binding in the absence of competing ligand in order to measure relative receptor binding sites on the cell surface.

An initial radioligand competition binding experiment was performed with COS-7 cells transfected with WT-GPR54 and WT-GPR54-GFP to validate the assay and compare the affinity of tagged and untagged WT-GPR54 (Figure 13). COS-7 cells were used as they are more robust and would retain cell numbers better than HEK293-T under these experimental conditions. The IC_{50} value for the WT-GPR54 (2 nM) and WT-GPR54-GFP (1.5 nM) receptor were not significantly different ($p>0.05$), indicating that the GFP tag did not alter the ligand affinity at GPR54. The level of [^{125}I]-KP10 binding in the absence of competing ligand (0) was also not significantly different ($p>0.05$) indicating that the expression levels of

WT-GPR54 and WT-GPR54-GFP were also similar and that the GFP tag does not affect receptor expression. It should be noted that some non-specific radioligand binding was measured (indicated by residual radioactivity after dissociation of receptor-bound radioligand with high concentrations of unlabelled ligand). This is a common phenomenon observed with radiolabelled KP10 and results from non-specific binding of the peptide to the plasticware used in the assay.

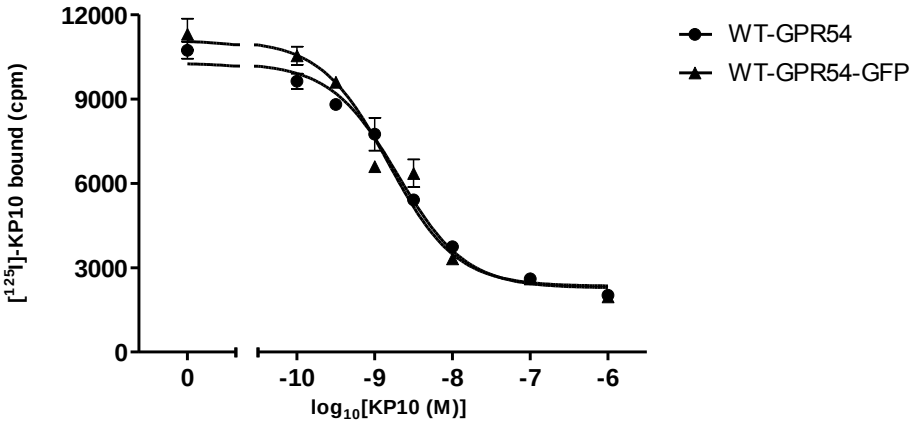
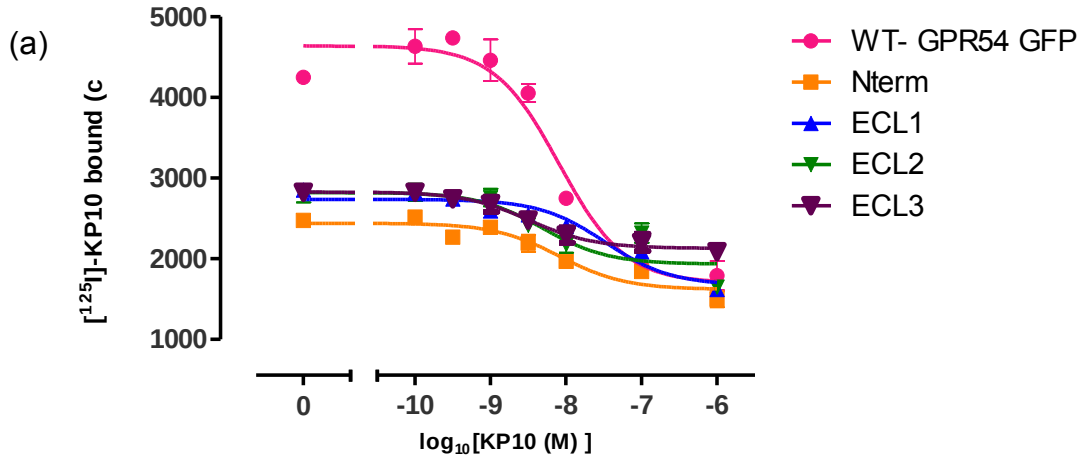


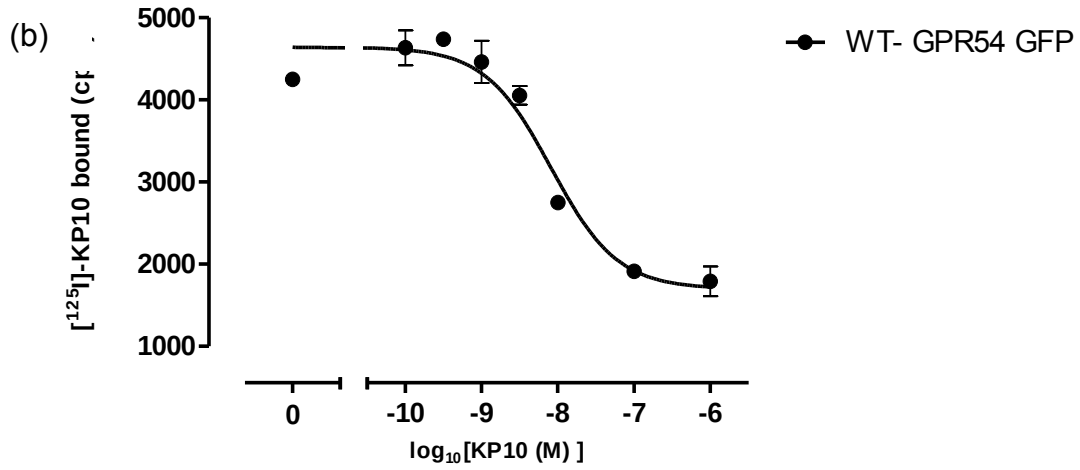
Figure 13: Radioligand competition binding assay with WT-GPR54 and WT-GPR54-GFP. WT- GPR54 and WT-GPR54-GFP transfected COS-7 cells were incubated with 50,000cpm/well [^{125}I] KP10 in the absence (0) and presence of increasing concentrations of unlabelled KP10 for 4 hours at 4℃. before washing and measurement of bound radioligand. N=3.

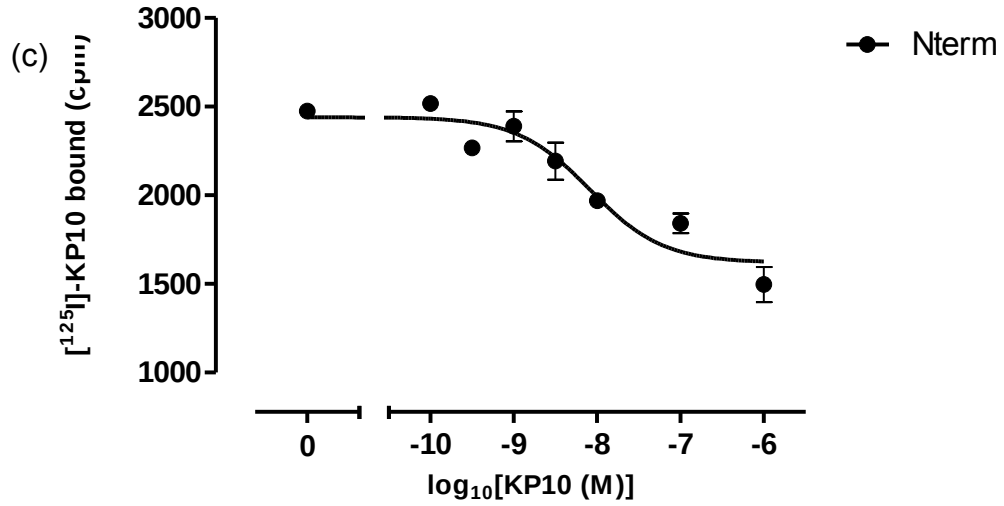
To test the expression of, and KP10 binding affinity at, the GPR54/GalR2 chimeric receptors, COS-7 cells were transfected with WT-GPR54-GFP, or the GPR54/GalR2 chimeric receptors and radioligand competition binding assays were performed.

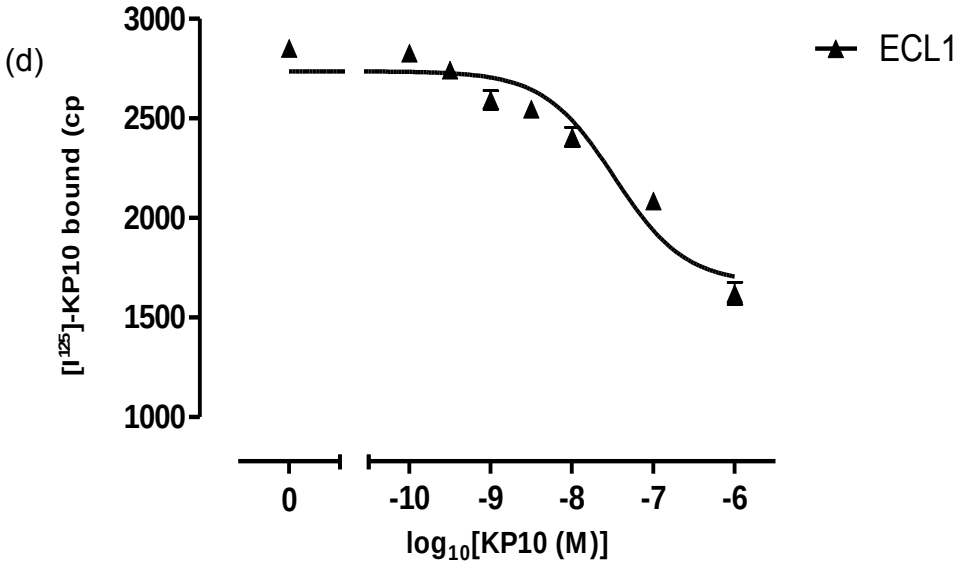
At the WT-GPR54 the IC_{50} measured for KP10 was 9nM (Figure 14), indicating a high affinity of KP10 for this receptor and consistent with the data presented in Figure 13. There were some differences in affinities (IC_{50}) between the receptors.

For example the IC_{50} of the ECL2 chimera was 2 fold less than WT-GPR54-GFP and the ECL1 chimera had a 3 fold higher IC_{50}. However, these differences were not significant and the affinity of KP10 for all of the GPR54/GalR2 chimeric receptors was not significantly different from its affinity for WT-GPR54-GFP ($p>0.05$; Table 3, Figure 14). However, in the absence of unlabelled KP10, the binding of [^{125}I]-KP10 was approximately 50% lower for the GPR54/GlaR2 chimeras than for WT-GPR54-GFP as indicated by the reduced B0 values (Table 3, Figure 14), although the BO values measured for each of the chimeras were not significantly different from each other ($p>0.05$). As they have no difference in KP10 affinity, this reduction in [^{125}I]-KP10 binding must reflect a reduced cell surface expression of the chimeric receptors in these cells.




(b)
WT- GPR54 GFP
[125I]-KP10 bound (cp
5000
4000
3000
2000
1000
0
-10
-9
-8
-7
-6
log10[KP10 (M)]


(c)
Nterm
[125I]-KP10 bound (cpm)
3000
2500
2000
1500
1000
0
-10
-9
-8
-7
-6
log10[KP10 (M)]


(d)
ECL1
[I125]-KP10 bound (cp
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2500
2000
1500
1000
0
-10
-9
-8
-7
-6
log10[KP10 (M)]

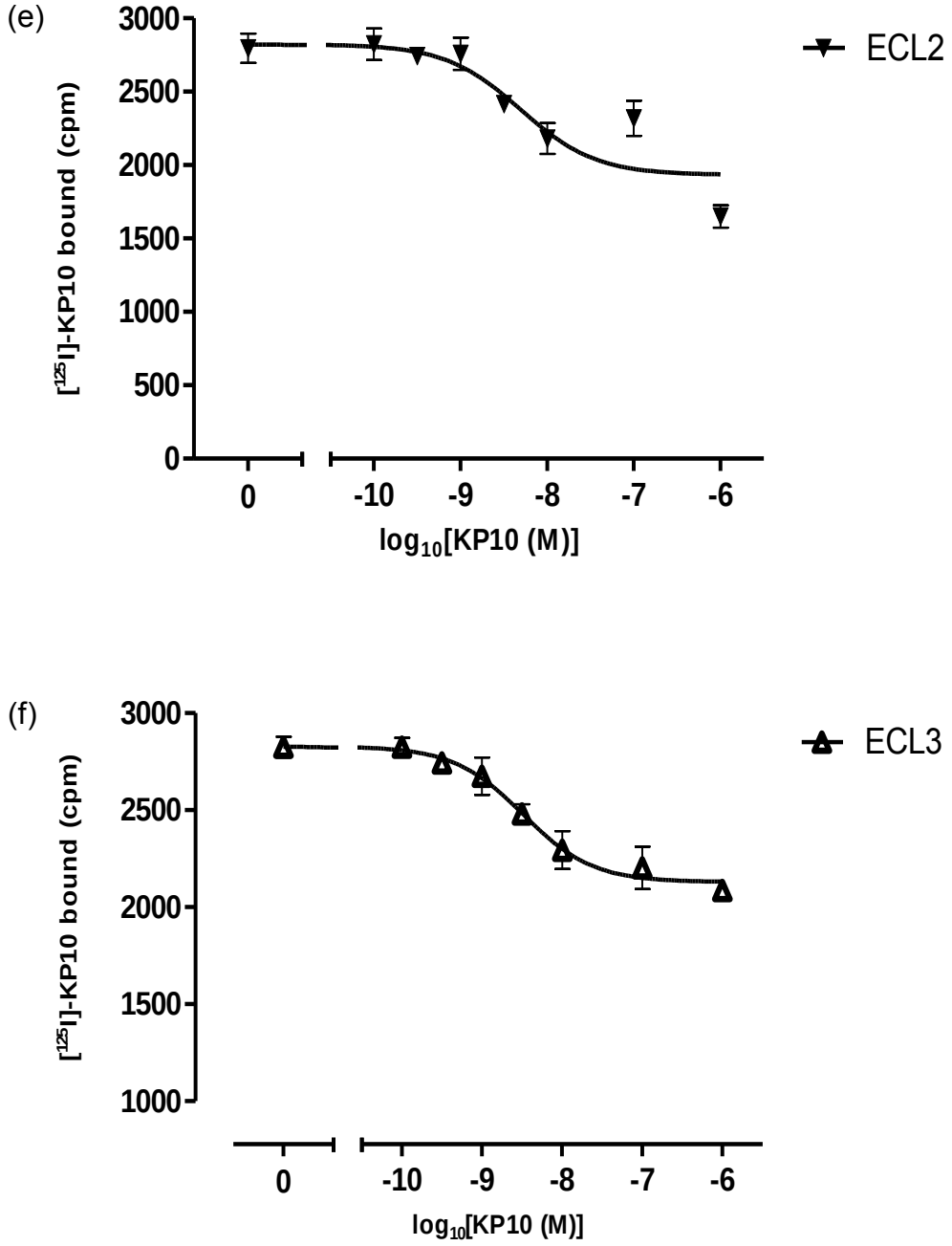


Figure 14: Radioligand competition binding assay with WT-GPR54 GPR54/GalR2-YFP chimeras. COS-7 cells were transfected with WT-GPR54-GFP or GPR54/GalR2-YFP chimeric receptors. 48 hours later whole cell radioligand competition binding was performed. 50,000cpm/well [^{125}I]-KP10 was incubated with cells in the absence (0) and presence of increasing concentrations of unlabelled KP10 for 4 hours at 4℃, before washing and measurement of bound radioligand. (a) All receptors. (b) WT-GPR54-GFP only (c) N-terminus-GalR2-GPR54-YFP chimera (Nterm) only (d) ECL1-GalR2-GPR54-YFP chimera (ECL1) only (e) ECL2-GalR2-GPR54-YFP chimera (ECL2) only (f) ECL3-GalR2-GPR54-YFP chimera (ECL3) only. N=3.

Table 3: IC_{50} and B0 values for GPR54/GalR2 chimeras (* p<0.05 for comparison with WT-GPR54-GFP, N=3)

RECEPTOR	IC_{50} ± SEM (nM)	B0 ± SEM (cpm)
WT-GPR54-GFP	9.17 ± 0.84	4023 ± 634
Nterm CHIMERA	7.53 ± 3.53	1860 ± 302*
ECL1 CHIMERA	21.69 ± 6.67	1955 ± 403*
ECL2 CHIMERA	3.92 ± 1.94	2167 ± 349*
ECL3 CHIMERA	9.58 ± 6.54	1904 ± 464*

In summary, taken together these data suggest that the N-terminal and ECL GPR54-GalR2 chimeras affect GPR54 cell surface expression based on the reduced B0 values (Figure 14,Table 3). However, the affinity of the chimera receptors is similar to that of WT-GPR54-GFP.

3.2.1.3 Activation of GPR54/GalR2 receptor chimeras

The GPR54/GalR2 receptor chimeras had similar affinity for KP10 as the WT-GPR54. We next tested whether the KP10 was able to activate the chimeric receptors by determining inositol phosphate (IP) production in cells expressing these receptors in response to KP10 stimulation. Briefly, COS-7 cells transfected with WT-GPR54-GFP or each of the receptor chimeras and loaded with radiolabelled myo-inositol were stimulated with increasing concentrations of KP10. Thereafter total IPs were extracted and purified. As myo-inositol is incorporated into IP by the cells, measurements of the level of radioactivity of the collected samples allowed quantification of the IP produced.

E_{Max} refers to the maximal response produced by a ligand. A comparison of the E_{Max} for KP10 stimulation of IP in cells expressing WT-GPR54-GFP and the GPR54/GalR2-YFP chimeras shows that KP10 stimulated the highest IP release in cells expressing WT-GPR54-GFP (Figure 15, Table 4). The N-terminus-GalR2-GPR54-YFP chimera showed the highest KP10 stimulated IP release of all of the

chimeric receptors with an E_{max} 86% of the WT-GPR54-GFP, compared to 56 % for the ECL1-GalR2-GPR54-YFP chimera, 38% for the ECL2-GalR2-GPR54-YFP chimera and 60% for the ECL3-GalR2-GPR54-YFP chimera.

EC_{50} refers to the concentration of ligand that produces 50% of the maximal response and can provide information about the ligands potency. KP10 had higher potency at the WT-GPR54-GFP receptor than at any of the GPR54/Gal2R-YFP chimeric receptors with an EC_{50} of 15 nM (Figure 15, Table 4). At the chimeric receptors, KP10 had the highest potency for the N-terminus-GalR2-GPR54-YFP chimera with only a 9-fold reduction in potency when compared to WT-GPR54-GFP. At the ECL1-GalR2-GPR54-YFP, ECl2-GalR2-GPR54-YFP and ECL3-GalR2-GPR54-YFP chimeras KP10 displayed a 23-, 31- and a 12-fold reduction in potency, respectively, compared to the WT-GPR54-GFP. Therefore, this data suggests that the ECLs of GPR54 are important for receptor activation while the N-terminus may be less important.

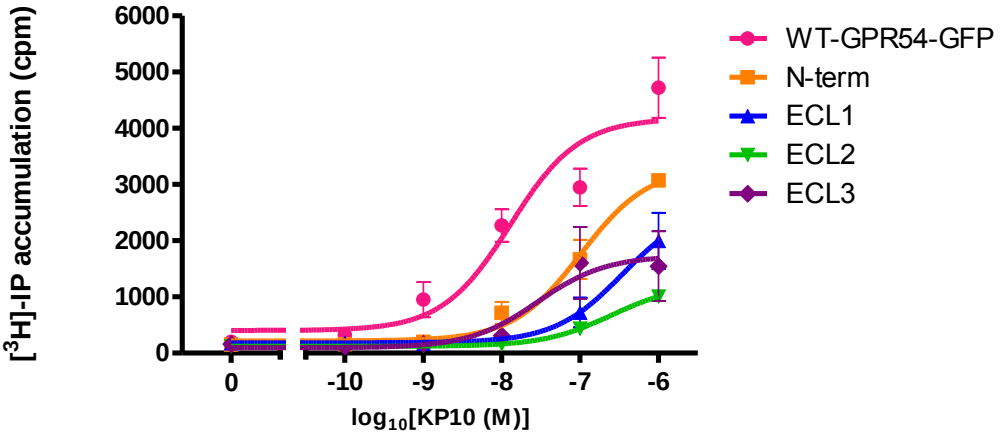


Figure 15: IP accumulation assay with WT-GPR54 GPR54/GalR2-YFP chimeras. COS-7 cells transfected with GPR54-GFP, N-terminus-GalR2-GPR54-YFP chimera (N-term), ECL1-GalR2-GPR54-YFP chimera (ECL1), ECL2-GalR2-GPR54-YFP chimera (ECL2) or ECL3-GalR2-GPR54-YFP chimera were stimulated with increasing concentrations of KP10 and assayed for total radioactive inositol phosphate accumulation. N=3.

Table 4: EC_{50} and E_{max} values for GPR54-GAL2 receptor chimeras (* p<0.05 for comparison with WT-GPR54-GFP, N=3)

Receptor	EC_{50} ± SEM (nM)	E_{max} ± SEM (cpm)
WT-GPR54-GFP	14.68 ± 1.26	4175 ± 41
Nterm chimera	135.33 ± 10.16*	3592 ± 310
ECL1 chimera	331.9 ± 9.45*	2366 ± 284*
ECL2 chimera	458.5 ± 3.092*	1566 ± 168*
ECL3 chimera	176.09 ± 76.42*	2527 ± 653*

3.2.2 Role of acidic mutations in the ECL domain of GPR54

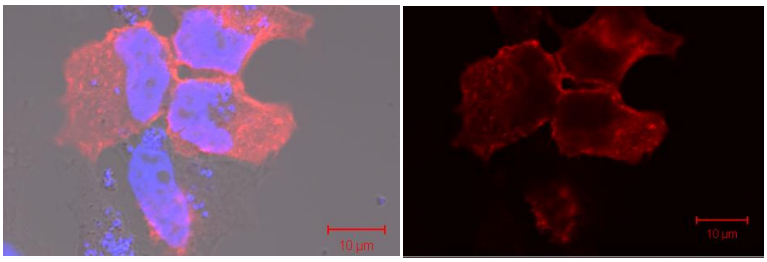
The chimeric receptor studies highlighted the importance of the ECL regions for GPR54 cell surface expression and receptor activation. The next step was to test to role of acidic (aspartate (D) and glutamate (E)) residues within these regions for GPR54 receptor expression, affinity and activation. There are 5 acidic residues located on the N-terminus and ECLs of GPR54: D31 and D41 (N-terminus), D112 (ECL1) and E193 and E201 (ECl2). Having shown that the N-

terminus was not critical for receptor activation, the aspartate residues in position 31 and 41 of GPR54 were not mutated while the other acidic residues were mutated to alanine. GPR54 mutants containing these point mutations ('acidic mutants') were constructed in pcDNA 3.1 and were tagged at their N-termini with a FLAG epitope tag. For comparison, WT GPR54 was also tagged at the N-terminus with a FLAG epitope tag.

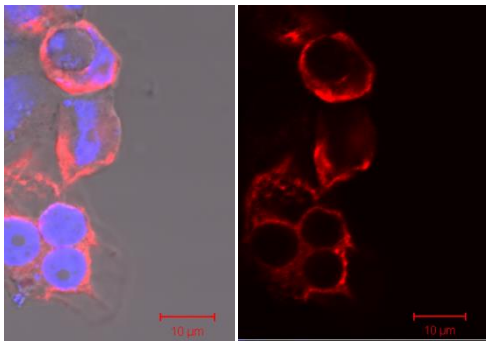
3.2.2.1 Expression of N-terminal FLAG-tagged GPR54 D112A, E193A and E201A receptor mutants

As the WT-GPR54 and D112A, E193A, and E201A mutants were FLAG tagged, this allowed for analysis of receptor localisation/expression by immuohistochemical analysis and ELISA. Briefly, for immunohistochemical analysis, HEK293-T cells transfected with the WT and mutant receptors were fixed 48 hours post transfection, cells were incubated with anti-FLAG antibodies and viewed under a confocal microscope. There was no difference in the cellular localisation of the three GPR54 mutants when compared to the WT-GPR54 indicating that these amino acid substitutions did not appear to affect the cellular location of the receptor (Figure 16).

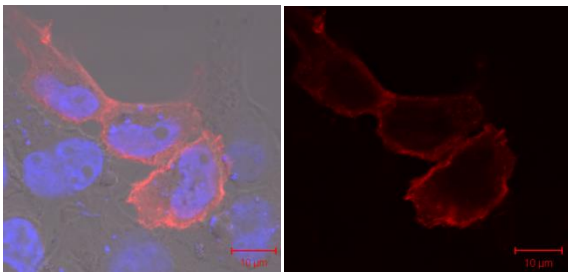
(a)



(b)



(c)



(d)

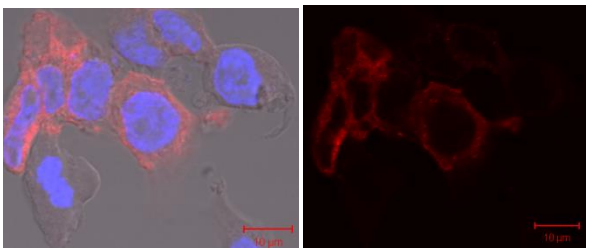


Figure 16: Cellular location of WT GPR54 and acidic mutants. HEK293-T cells transfected with (a) WT (b) D112A. (c) E193A or (d) E201A. GPR54 receptors were fixed and receptors labelled with anti-FLAG primary and Alexa 488-conjugated secondary antibodies (red). Cell nuclei were labelled with DAPI (blue) Scale bar is 10µm. Left panel: merged image showing distribution of transfected receptors (red), cell nuclei (blue and DIC imaging of cells. Right panel: image showing distribution of transfected receptors (red) only. Images representative of n=3.

As these acidic GPR54 mutants were FLAG-tagged at their extracellular N-termini, this enabled the measurement of cell surface receptor expression of non-lysed cells using an ELISA to give a quantitative determination of cell surface relative receptor expression (Figure 17). There was no statistically significant difference in cell surface receptor expression between the WT receptor and any of the acidic mutants ($p>0.05$). The alanine substitutions at residues 112, 193 and 201 therefore do not have an effect of GPR54 cell surface receptor expression.

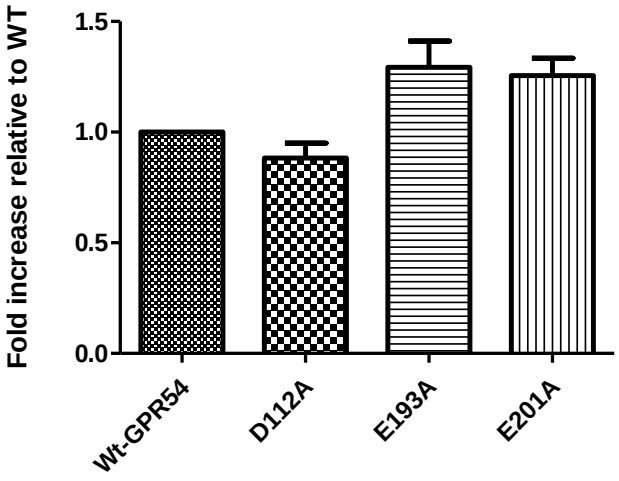


Figure 17**: Relative expression of WT GPR54, D112A, E193A and E201A.** HEK293-T cells were transfected with WT-hGPR54 and acidic mutation receptors. 48 hours post transfection cells and ELISA performed on intact cells. Absorbance was read at 450 nm. Data was normalized against WT-GPR54 and represented as fold increase relative to expression of WT GPR54. N=3 and $p>0.05$ for comparison of mutants and WT GPR54.

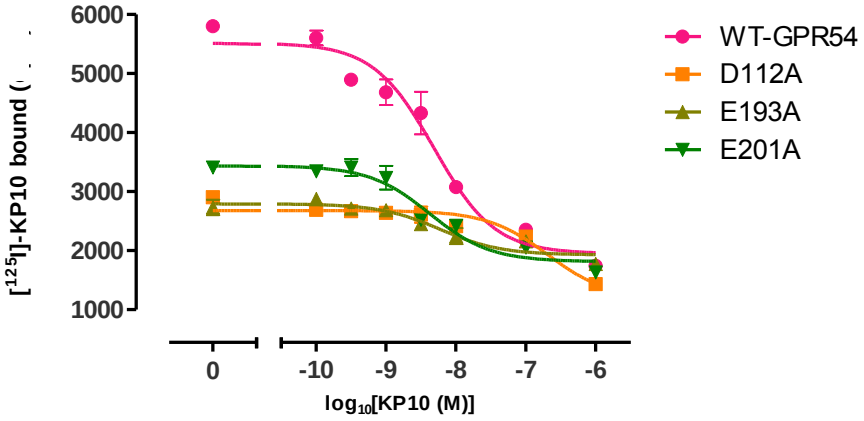
3.2.2.2 Ligand binding to WT GPR54 and of D112A, E193A and E201A receptor mutants

Having established that the acidic mutants do not affect receptor cell surface expression, their effects on ligand binding were evaluated. Whole-cell radioligand

competition binding assays were performed on transfected COS-7 cells expressing WT GPR54 and the GPR54 acidic mutants.

Interestingly, the level of [^{125}I]-KP10 binding measured in the absence of unlabelled ligand (B0) for the acidic mutants was 50 % lower than for the WT-GPR54 (Figure 18, Table 5). However, the affinity of KP-10 for WT-GPR54 was similar to previous data presented in this thesis (Figure 17, Figure 11 and Figure 14) with an IC_{50} of 5nM. The affinities of KP10 for the GPR54 mutant receptors with acidic residues of ECL3 substituted with alanine, namely E193A and E201A, were similar to that measured for WT-GPR54 (Figure 18), Table 5). However, there was about 30-fold reduction in the IC50 of KP10 for the ECL1 acidic mutant, D112A, compared to WT-GPR54 (as IC_{50} is inversely proportional to affinity). However, it should be noted that as the affinity of KP10 for the D112A mutant was reduced, it was not possible to fully define the dose response curve and the IC_{50} value presented has been obtained by extrapolation of the data.

(a)


6000
5000
4000
3000
2000
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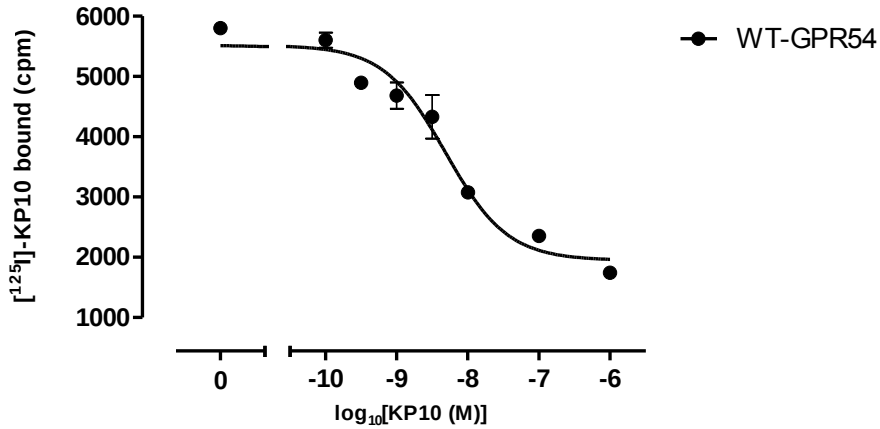
[125I]-KP10 bound (
0
-10
-9
-8
-7
-6
log10[KP10 (M)]
WT-GPR54
D112A
E193A
E201A

(b)


6000
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2000
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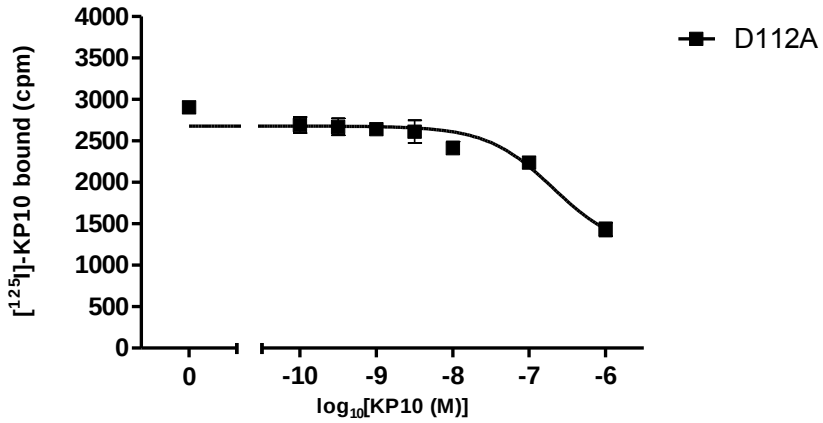
[125I]-KP10 bound (cpm)
0
-10
-9
-8
-7
-6
log10[KP10 (M)]
WT-GPR54

(c)


4000
3500
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500
0
[125I]-KP10 bound (cpm)
0
-10
-9
-8
-7
-6
log10[KP10 (M)]
D112A

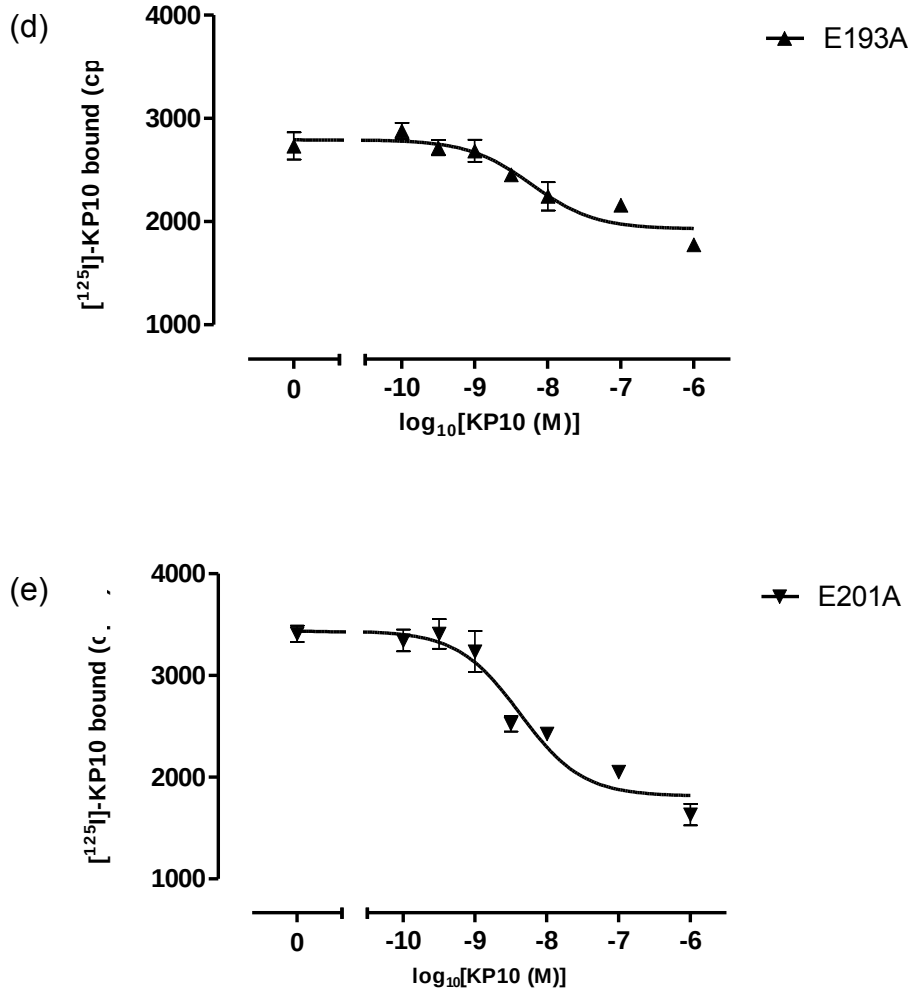

Figure 18: Radioligand competition binding assay with WT-GPR54 and GPR54 acidic mutants. COS-7 cells were transfected with FLAG-tagged WT-GPR54 or GPR54 acidic mutants. 48 hours later whole cell radioligand competition binding was performed. 50,000cpm/well [^{125}I]-KP10 was incubated with cells in the absence (0) and presence of increasing concentrations of unlabelled KP10 for 4 hours at 4℃, before washing and measurement of bound radioligand. (a) All receptors. (b) WT-GPR54 only (c) D112A mutant GPR54 only (d) E193A mutant GPR54 only (e) E201A mutant GPR54 only. N=3.

Table 5: IC_{50} and B0 values for GPR54 and GPR54 acidic mutants (* p<0.05 for comparison with WT-GPR54, N=3)

Receptor	IC_{50} ± SEM (nM)	B0 ± SEM (cpm)
WT-GPR54	5.33 ± 0.98	4683±818.9
GPR54-D112A	159.0 ± 32.62*	1985 ± 368.0*
GPR54-E193A	4.12 ± 1.85	2040 ± 417.5*
GPR54-E201A	7.11 ± 1.98	2372 ± 543.7*

3.2.2.3 Activation of D112A, E193A and E201A GPR54 receptor mutants

Inositol phosphate accumulation assays were performed on COS-7 cells transfected with WT-GPR54, and D112A, E193A and E201 GPR54 mutant receptors in order to establish the effects of the mutations on receptor activation.

KP10 was most potent at the WT-GPR54 receptor, and produced a robust IP response (Figure 19, Table 6). There was a 15-fold decrease in the potency of KP10 for the D112A mutant, a 32-fold decrease for the E193A mutant and an 8-fold decrease for the E201A mutant GPR54 receptors compared to WT-GPR54 (Figure 19, Table 6). As the affinity of KP10 for the E193A and E201A mutants was similar to the WT-GPR54, this suggests that these acidic mutant receptors cannot be activated as easily as the WT- GPR54 receptor by KP10. The efficacy, E_{Max}, of all of the acidic mutant receptors was similar to WT-GPR54.

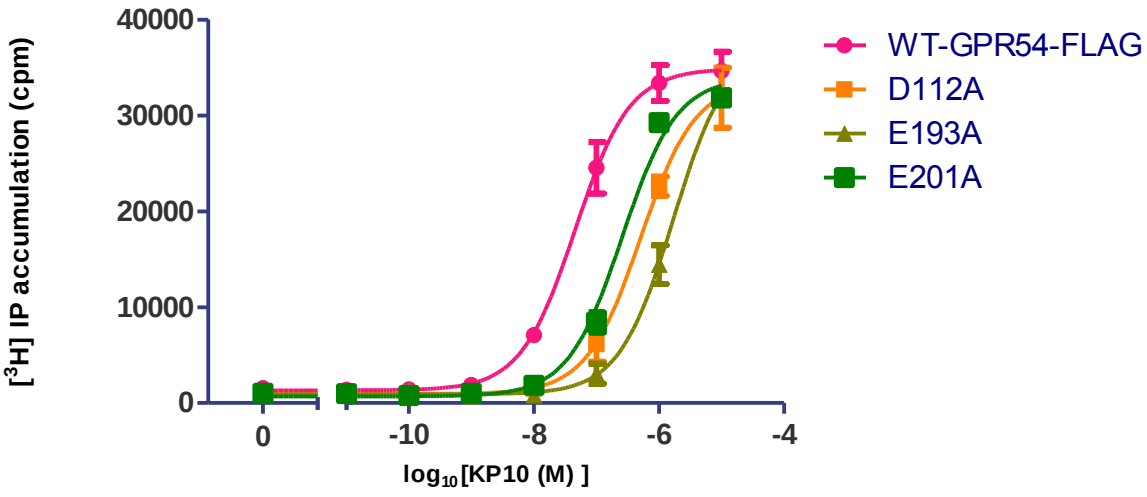

Figure 19: IP accumulation assay with WT-GPR54 and acidic mutants. COS-7 cells transfected with WT-GPR54, GPR54-D112A, GPR54-E193A and GPR54-E201A were stimulated with increasing concentrations of KP10 and assayed for total radioactive inositol phosphate accumulation. N=3.

Table 6: EC_{50} and E_{max} values for WT-GPR54 and acidic mutants (* p<0.05 for comparison with WT-GPR54, N=3)

Receptor	EC_{50} ± SEM (nM)	E_{max} ± SEM (cpm)
WT-GPR54	41.82 ± 7.87	37605 ± 8661
GPR54-D112A	635.9 ± 92.44*	35327 ± 7248
GPR54-E193A	1353 ± 258.6*	39679 ± 8459
GPR54-E201A	353.4 ± 79.55*	36359 ± 8086

3.3 Discussion

The aim of this study was to examine the role of the extracellular domains and acidic residues within the extracellular loops of GPR54 in receptor expression, ligand-binding affinity and receptor activation. All of the GRP54/GalR2 N-terminal and ECL chimeric receptors had expression and localisation indistinguishable from that of WT-GPR54 when viewed with the confocal microscope (Figure 11) indicating that the chimeric substitutions did not fully impair receptor trafficking to the cell membrane. Total receptor expression analysis by ELISA also showed no significant difference in receptor expression of WT-GPR54 and the chimeric receptors but it was not possible to distinguish between intracellular receptors and those present at the cell surface (Figure 12). However, in the binding assays (Figure 14), cell surface expression of all of the GPR54/GalR2 chimeras appeared to be reduced by 50 % compared to WT-GPR54 as indicated by the reduced B0 values. Therefore, the chimera substitutions do not impair receptor expression but do appear to have some effect on trafficking of the receptors to the plasma membrane.

The affinity of KP-10 (IC_{50}) for the GPR54/Gal2R chimeric receptors, although slightly altered were not significantly different from WT-GPR54. The similar affinity between WT-GPR54 and the chimeric receptors suggests that these domains are not essential for KP10 binding to GPR54.

None of the chimeric receptors were able to mimic the robust IP response of WT-GPR54 to stimulation by KP10 and the potency (EC_{50}) of KP10 at all of the chimeric receptors was significantly lower than that for WT-GPR54 (Figure 15). The N-terminal chimera had higher potency than the ECL chimeras and was also

able to stimulate an IP response of 86% of that seen at the WT-GPR54, indicating that the role of the N-terminal region in receptor activation is less critical that the ECL regions. KP10 had the most reduced potency and efficacy (E_{max}) at the ECL 2 chimera. The ECL2 region of GPR54 could thus play a critical role in receptor activation. ECL3 and ECL1 of GPR54 could also be important for receptor activation as a 12-fold and 23-fold decrease in KP10 potency, respectively, was observed for these chimeric receptors. The ECLs of GPCRs have high degrees of variability that differ in length and sequence. The roles of the extracellular residues of GPCRs have not been as well defined compared with residues in transmembrane helices. ECL2 has been the focus of research, because the x-ray structure of bovine rhodopsin revealed that ECL2 projects into the binding crevice within the transmembrane bundle. It has also been found to be directly involved in ligand binding and may contribute to G protein subtype selectivity (Shi and Javitch, 2004, Bokoch *et al.*, 2010). ECL1 has also been shown to contribute to receptor activation for the A_{2B}AR (Peeters *et al.*, 2011). Consistent with the research cited, the ECL2, and to a lesser extent ECL3 and ECL1, of GPR54 appears to influence receptor activation.

The chimera data indicated that the N-terminus was less important that the ECLs for receptor signalling. In contrast, the N-terminal tail of GalR2 has been shown to be involved in ligand binding, possibly through forming a disulfide bond with ECL3 (Lundstrom *et al.*, 2007). As the N-terminal region of GPR54 appeared to be less important than the ECL regions for receptor activation, only the ECL regions were studied in more detail.

There are five acidic residues on the extracellular domains of GPR54. The three acidic residues located on the ECLs of GPR54 D112, D139 and E193 were

substituted with alanine, to form the following receptor mutants, D112A, D139A and E193A. A qualitative confocal analysis of the expression pattern of these GPR54 acidic mutants (Figure 16) and a quantitative ELISA measuring receptor cell surface expression (Figure 17) showed that all three acidic mutants were expressed at the cell surface and that there was no significant difference in their localisation or expression level when compared to the WT-GPR54. These mutations therefore did not affect receptor expression of trafficking to the cell surface.

As with the chimeric receptors, binding assays using the acidic mutant receptors indicated that receptor expression (B0) was reduced by approximately 50% when compared to WT-GPR54 (Figure 18). KP10 had a similar affinity for the E193A and E201A mutant receptors compared to WT-GPR54 therefore the observed reduction in B0 was not an indirect result of reduced radioligand affinity. At the D112A mutant receptor, KP10 showed a significant 30-fold decrease in affinity. The acidic residues on ECL2 namely E193A and E201A are therefore not critical for ligand binding while the ECL1 acidic residue, D112A, may contribute to ligand binding. Additional mutational studies of this residue would aid in clarifying the effect of this acidic residue in position 112 on GPR54 binding.

In a receptor activation assay, KP10 had a 15-fold lower potency at the D112A mutant compared to WT-GPR54 reflecting the decreased affinity of KP10 for this mutant (Figure 19). It is interesting to note that there was only an 8-fold decrease in potency of KP10 for the E201A mutant receptor compared to the large 32-fold decrease observed for the E193A mutant receptor (Figure 19). Both these mutant receptors are located on ECL2 and although neither had altered KP10 affinity, they have different effects on receptor activation. Despite the differing effects of

the mutations on KP10 potency, all the acidic mutant receptors were able to elicit a maximum IP response similar to that of WT-GPR54. It is possible that the reduced receptor expression could contribute, in part, to the decrease in KP10 potency observed for the acidic mutant receptors due to the presence of 'spare receptors'. However, as the presence of spare receptors would result in the observed KP10 potency for the WT GPR54 being higher than its observed affinity, there is no evidence for spare receptors in this system. While the reduced potency of KP10 for the D112A mutant can be attributed to a reduced binding affinity at this mutant, for the E193A and E201A mutant receptors, which had similar KP10 affinity as WT-GPR54, the reduced potency of KP10 could indicate that these residues play a role in receptor activation. This would be consistent with the ECL2 chimera receptor findings further highlighting the importance of this domain for receptor activation.

The function of extracellular charged residues that are highly conserved throughout a subfamily of peptide GPCRs has been investigated using the vasopressin receptor 1a (V1aR). It was found that Asp112 (ECL1) in the V1aR was largely conserved throughout the neurohypophysial hormone receptor family. Several amino acid substitutions, [D112A]V1aR, [D112E]V1aR, and [D112K]V1aR had little effect on receptor function but introduction of R112 (i.e. replacement of an acidic reside with a basic residue), resulted in impaired agonist binding and signalling (Hawtin *et al.*, 2006). [The D112A]V1aR had similar ligand affinity but a 3-fold reduction in ligand potency compared to the WT receptor. This data is similar to the data obtained for D112A mutant of GPR54. In ECL2 of the V1aR, a [D204A]V1aR substitution had a marked effect on receptor function, with a decrease in ligand affinity, and impaired ligand potency being

observed (Hawtin *et al.*, 2006). Studies performed on human neuropeptide Y receptor also indicated that acidic residues namely, D194A, D200A, D205A and D287A, impaired ligand binding (Walker *et al.*, 1994) further highlighting the importance of these acidic residues in GPCR function.

In summary, these data indicate that the ECLs of GPR54 and acidic residues contained within them contribute to cell surface receptor expression (as measured in binding assays) and play a role in receptor signalling. The potential for ECL1 to be involved in ligand binding exists as it was the only chimera to show a slight decrease in KP10 affinity when compared to WT-GPR54. The D112A receptor mutant, located on ECL1 also appears to be involved ligand binding as it too had lower affinity when compared to WT-GPR54. The ECL2 chimera implicates this domain as having a critical role in receptor activation and both the acidic mutants of ECL2 corroborate this theory as they too had reduced KP10 potency.

A more comprehensive mutagenesis study should now be undertaken to investigate the role of other residues in the ECLs as well as additional mutations of the acidic residues to establish which amino acids can improve or abolish GPR54 receptor expression and binding. Further structure-function studies would expand the current knowledge of GPR54 and KP10 interactions and greatly aid in the design of new drugs with increased affinity and selectivity, thereby potentially decreasing the occurrence of off-target side-effects and potentially reducing the therapeutic doses required. This would be of great importance for GPR54 as its implications in reproductive development puberty and pregnancy make it an important target for design of novel therapeutics.

Chapter 4: GPR147 interactions with select RFamides and RFRP3 analogs

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4.1 Introduction

RFamide peptides are biologically active peptides with an RFamide motif at their C terminus and can be found in the animal kingdom from invertebrates to mammals. The members of the RFamide peptide family have rich therapeutic potential as they are involved in multiple regulatory systems. Many of the 'orphan' GPCRs have been found to be the receptors for mammalian RFamides. GPR54 and its cognate ligand kisspeptin are one example. Cases of extensive overlap exist in the binding affinity between different RFamides and their receptors. This is believed to be because receptor selectivity of these compounds seems to be determined primarily by a few relatively conserved amino acids at the C-terminus of the ligand (Yoshida *et al.*, 2003). This ligand promiscuity is a major stumbling block in investigations of the distinct roles for single RFamide peptides.

The NPFF receptors (GPR147/GPR74) have a number of endogenous ligands, including NPFF/NPAF (derived from the $NPFF_A$ precursor protein) and RFRP3/RFRP1 (derived from the $NPFF_B$ precursor protein). In addition to these cognate ligands, a number of other neuropeptides show considerable affinity and agonistic activity at these receptors (GPR147 and GPR74) (Engström *et al.*, 2003). The promiscuity of these two receptors is not restricted to RFamide peptides, because they also interact moderately with NPY, a RYamide (Bonini *et al.*, 2000, Engström *et al.*, 2003). KP10, the cognate ligand for GPR54, has also been described to target the NPFF receptors with nanomolar affinity (Oishi *et al.*, 2010). In addition, GPR147/GPR74 themselves share high affinities to their endogenous ligands (Oishi *et al.*, 2010). Due to the promiscuity of the endogenous ligands, the development of selective agonists and antagonists is

required in order to explore the distinct mechanism of the diverse pharmacological effects of these peptides and receptors.

Many SAR studies have been performed on the NPFF receptors and interactions with their ligands. Interactions of endogenous RFamide peptide hormones, NPFF, NPAF, RFRP3, and RFRP1 with GPR147 and GPR74 have been investigated. Systematic substitutions were made using NPFF as a template to determine the importance for particular residues for receptor activation. NPFF is able to activate GPR147 but additional peptide modifications result in decreased potency. A SAR study has also been performed using bovine RFRP3. Endogenous bovine RFRP3 is composed of 28 amino acids. This is 10 amino acids longer than endogenous rat RFRP3 (Ukena *et al.*, 2002), and 20 amino acids longer than human RFRP3 which is just eight amino acids long. Yoshida *et al*.(2003) investigated the effects of truncated bovine RFRP3 with regards to binding affinity and receptor activation (Table 7). Binding affinity was retained for all the peptides tested at GPR147 and activation was only impaired for a terminal tetrapeptide (PQRFNH2). In contrast, these modified bovine RFRP3 peptides were able to bind but not activate GPR74 (Yoshida *et al.*, 2003).

Yoshida et al.(2003) also found that three amino acid residues, namely PNL in RFRP3 and LFQ in NPFF, at the N terminus of PQRF-NH_2 played important roles for receptor specificity determination of the ligands, but also for stimulation of full receptor activation. Of these three amino acid residues, proline (P) and leucine (L) of RFRP3 were found to be especially important for the interaction of this ligand with GPR147. Their data further indicated that *in vitro*, short RFRP3 peptides (minimally PNLPQRF-NH_2) exhibited interactions with GPR147 and GPR74 as effectively as the full-length endogenous bovine RFRP3 (Yoshida *et*

al., 2003). Important residues such as the proline, leucine and PQRF-NH_2, are conserved between human, bovine, rat and mouse species of the ligand (Hinuma *et al.*, 2000, Fukusumi *et al.*, 2001), whereas the N-terminal portion shows diversity in both length and sequence between species.

Table 7: Comparison of RFRP3 interactions with GPR147 and GPR74 by competitive binding and cAMP-production-inhibitory assays (Yoshida *et al.*, 2003)

Peptide	Sequence	GPR147		GPR74	
		Binding IC_{50} (nM)	cAMP EC_{50} (nM)	Binding IC_{50} (nM)	cAMP EC_{50} (nM)
hRFRP3-31	SAGATANLPLRSGRNMEVSLVRRVPNLPQRF-NH_2	1.3	4.8	16	780
hRFRP3-28	ATANLPLRSGRNMEVSLVRRVPNLPQRF-NH_2	1.1	5.1	14	520
bRFRP3-28	AMAHLPLRLGKNREDSLSRWVPNLPQRF-NH_2	1.6	6.4	7.0	290
hRFRP3-17	NMEVSLVRRVPNLPQRF-NH_2	3.4	6.8	18	730
hRFRP3-8	VPNLPQRF-NH_2	1.2	4.1	150	>1000
hRFRP3-7	PNLPQRF-NH_2	0.62	3.0	110	>1000
hRFRP3-6	NLPQRF-NH_2	2.6	28	230	>1000
hRFRP3-5	LPQRF-NH_2	2.1	51	76	>1000
hRFRP3-4/ NPFF4	PQRF-NH_2	15	>1000	26	>1000

In the studies described in this Chapter , the effects of different RFamide and RFamide-related peptides on GPR147 receptor binding and activation were investigated. SAR studies were also performed on human RFRP3 to further characterize important residues for binding an activation of GPR147. As discussed in the Literature Review, like GPR54, GPR147 (but not GPR74) has been implicated in modulation of GnRH activity and therefore has a potentially important role in the regulation of reproduction and fertility. Therefore, this study was focussed on the GPR147 receptor.

4.1.1 Aims

- To investigate the binding affinities of selected RFamides at the GPR147 receptor in order to establish a ligand selectivity profile for this receptor
- To investigate the SAR of different modified RFRP3 analogs with respect to their binding and activation of the GPR147 receptor in order to identify key residues required for these processes

4.2 Results

4.2.1 Iodination of RFRP3 and competitive binding assays of GPR147 with selected RFamide peptides

The chloramine T method was used to radiolabel a modified form of human RFRP3 which had a tyrosine reside added to the N-terminal in order to permit iodination (Tyr-hRFRP3). The resulting [^{125}I]-hRFRP3 was purified using size exclusion chromatography and 1 ml fractions were collected and radioactivity measured in order to identify fractions containing the radiolabelled peptide (Figure 20). Fractions with the highest radioactivity (cpm) were then tested on COS-7 cells transiently, expressing murine GPR147 in order to identify fractions containing the highest concentration of peptide (determined as the fold difference between the level of binding measured in the absence (B0) and presence of 1μM RFRP3 (NSB) to use in subsequent binding experiments (Figure 21). From these data, fractions 15-18 were used in subsequent competitive binding assays. This procedure was also used for all subsequent iodination's in order to obtain the fraction/s with the greatest displacement potential.

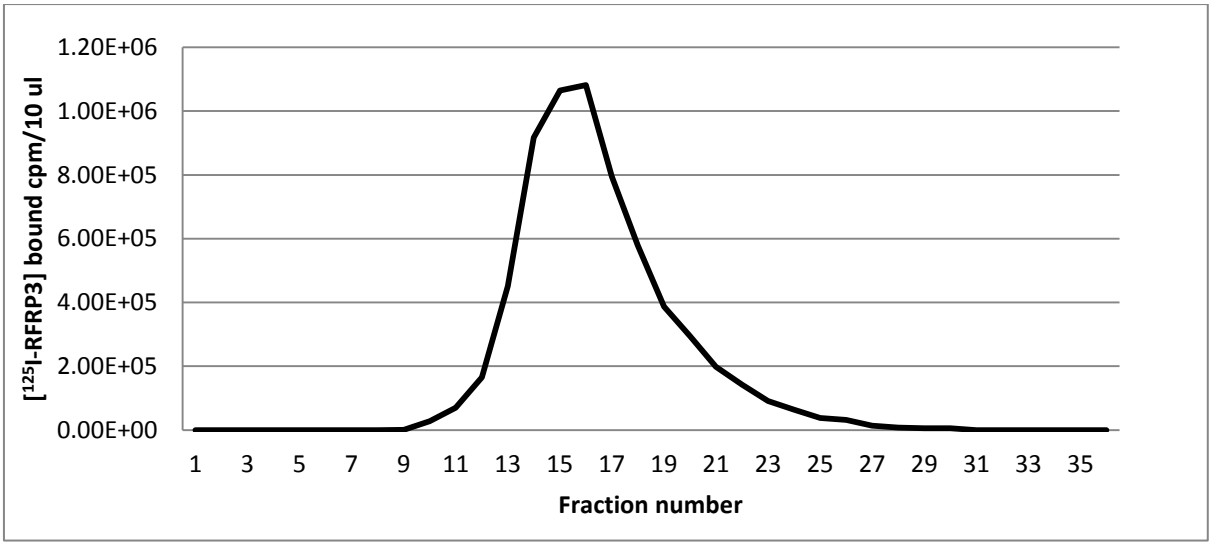

Figure 20: Typical elution profile for hRFRP3 iodination. 1 ml fractions were collected after which radioactivity of 10 µl samples was measured on a gamma counter.

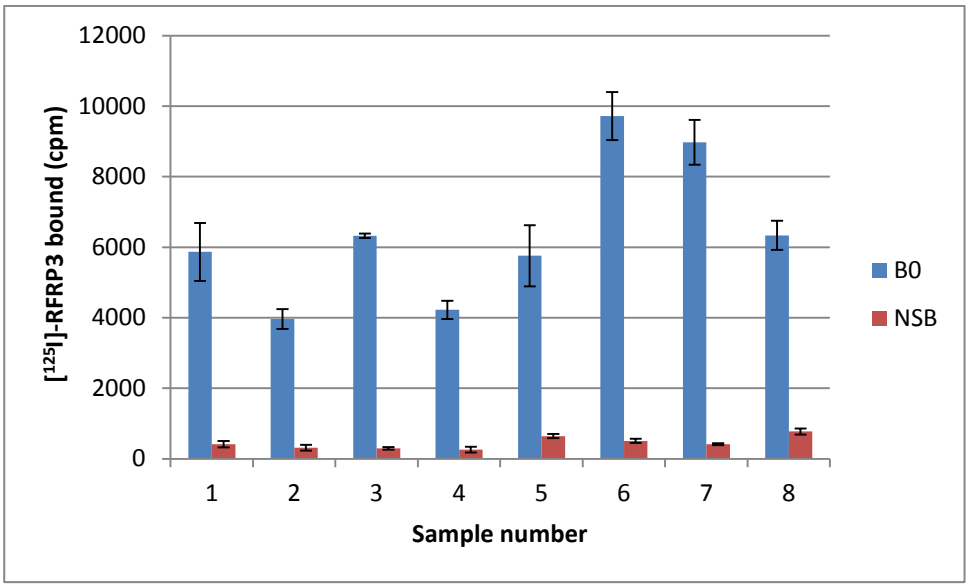

Figure 21: Typical displacement data for [^{125}I]-RFRP3 fractions. Fractions with the highest level of radioactivity were evaluated for their ability to be displaced by 1 µM RFRP3. Sample 1-fraction 12-13; Sample 2- fraction 9-11; sample 3- fraction 14; sample 4- fraction 21; sample 5-fraction 22; sample 6- 15-16; sample 7- fraction 17-18 and sample 8-fraction 19-20.

Table 8: Peptide sequence of RFamides

Peptide	Sequence
hRFRP3	VPNLPQRF-NH_2
[Ile8]hRFRP1	MPHSFANKPIRF-NH_2
hRFRP2	SAGATANLPLRS-NH_2
CCK	NY(SO3H)MGWMDF-NH_2
hPPY(3-36)	IKPEAPGEDASPEELNRYYASLRHYLNLVTRQRY-NH_2
KP10	YNWNSFGLRF-NH_2
hNPY	TPSKPDNPGEDAPAEDEDMARYYSALRHYINLITRQRQRY-NH_2

Several RFamide and RFamide-related peptides (Table 8) were then tested in radioligand competition binding studies with [125I]-RFRP3 in order to investigate their affinity for GPR147. In all cases, the level of radioligand binding in the absence of competing peptide (B0) was not significantly different ($p>0.05$), indicating that GPR147 receptor expression was similar, and the number of receptors available to the ligands was the same (Table 9). The endogenous GPR147 ligands, RFRP1 and RFRP3, had similar binding affinities (Figure 22, Table 9). KP10 had 3-fold lower affinity for GPR147 than RFRP1 and RFRP3 (Figure 22; Table 9) and was also able to fully displace [125I]-RFRP3, indicating that these peptides are interacting with the same population of receptors. RFRP2, PPY, CCK and NPY were not able to displace the bound [125I]-RFRP3 at the concentrations tested and determination of IC50 values was not possible. Therefore, no further studies were performed using these RFamide-like peptides due to their low binding affinities.

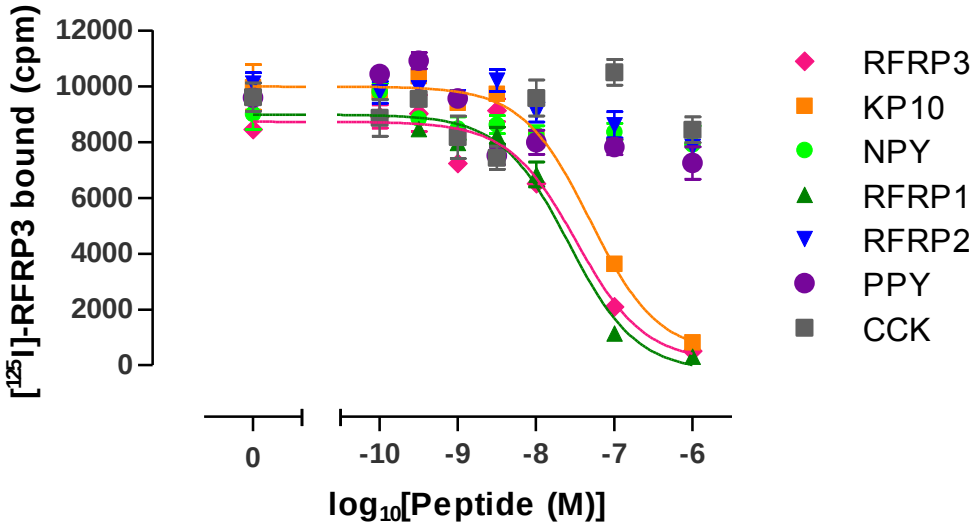

Figure 22: Radioligand competition binding assay for WT-GPR147 with different RFamides. COS-7 cells were transfected with WT-GPR147. 48 hours later whole cell radioligand competition binding was performed. 100,000cpm/well [^{125}I]-RFRP3 was incubated with cells in the absence (0) and presence of increasing concentrations of unlabelled peptide for 4 hours at 4℃, before washing and measurement of bound radioligand. N=3.

Table 9: IC_{50} and B0 values for GPR147 with different RFamides (* p<0.05 for comparison with RFRP3, N=3).

Peptide	IC_{50} ± SEM (nM)	B0 ±SEM (cpm)
RFRP3	14.61 ± 9.08	8067 ± 1382
KP10	51.00 ± 17.63	8956 ± 1590
RFRP1	12.63 ± 6.75	8390 ± 1545
RFRP2	ND	10052 ± 129
PPY	ND	9727 ± 710
CCK	ND	9612 ± 504
NPY	ND	9119 ± 1218

ND=not displaced

In summary, although RFamides share similar peptide characteristics, not all peptides have high affinities for other receptors within the RFamide family. KP10 displayed 3-fold lower affinity for GPR147 than RFRP3/RFRP1 and none of the other peptides tested were able to significantly displace [^{125}I]-RFRP3 from the receptor. Further SAR affinity and activity studies with kisspeptin/kisspeptin analogs and the GPR147 receptor were subsequently investigated and are described in Chapter 5.

4.2.2 SAR of RFRP3 analogs

4.2.2.1 Binding affinity of RFRP3 analogs at GPR147

Peptide analogs of RFRP3, developed as potential agonist or antagonists for the GPR147 receptor, were tested for their ability to bind GPR147. Radioligand competition binding experiments with [^{125}I]-RFRP3 were again used to measure affinities of the different analogs. The seven analogs tested (Table 10) included peptide 251 (a short acetylated RFRP3), peptide 252 (with a *D*-tryptophan substitution at position 6), peptide 253 (with an asparagine substitution at position 6), peptide 254 (with a *D*-alanine substitution at position 6), peptide 255 (with an alanine substitution at position 3), peptide 256 (with an alanine substitution at position 6) and peptide 257 (with alanine substitutions at positions 3 and 6).

Table 10: Sequence alignment of RFRP3 analogs. Changes to the endogenous sequence are indicated in bold.

Peptide name/amino acid position	1	2	3	4	5	6	7	8	
RFRP3	V	P	N	L	P	Q	R	F	NH2
251	**Ac**		N	L	P	Q	R	F	NH2
252	V	P	N	L	P	**(*D*)-W**	R	F	NH2
253	V	P	N	L	P	**N**	R	F	NH2
254	V	P	N	L	P	**(*D*)-A**	R	F	NH2
255	V	P	**A**	L	P	Q	R	F	NH2
256	V	P	N	L	P	**A**	R	F	NH2
257	V	P	**A**	L	P	**A**	R	F	NH2

Ac=acetylated

The endogenous ligand, RFRP3 fully displaced the radioligand binding and had an affinity (IC_{50}) of 5.14 nM (Figure 23, Table 11). All of the modified analogs tested displayed some binding impairment. Some of the analogs had moderate decreases in affinity such as peptide 251, the truncated RFRP3, which had 8-fold lower affinity than RFRP3 and peptide 255 (Ala^3 RFRP3) which displayed 10-fold lower affinity than RFRP3. The alanine substitution in position 6 (peptide 256) resulted in a 30-fold reduction of affinity. The substitution of *D*-Tryptophan or asparagine into position 6 (peptides 252 and 253) reduced binding affinity by greater than 100-fold compared to RFRP3. The double alanine substitution (pep 257) also had a 75-fold reduction in affinity. The severe reduction in binding affinity of peptides 252 and 257 meant that full displacement could not be achieved and IC_{50} values were calculated by extrapolation of the data. In the case of peptide 253 an accurate curve could not be fitted, preventing calculation of IC_{50}. The *D*-alanine substitution in position 6 (peptide 254) had the most severe effect on ligand affinity, and this ligand was unable to significantly displace the radiolabelled [^{125}I]-RFRP3. Taken together these results indicate that glycine in position 6 plays an important role in ligand binding and changes to asparagine

in position 3 also affects ligand binding but not as significantly as changes to glycine.

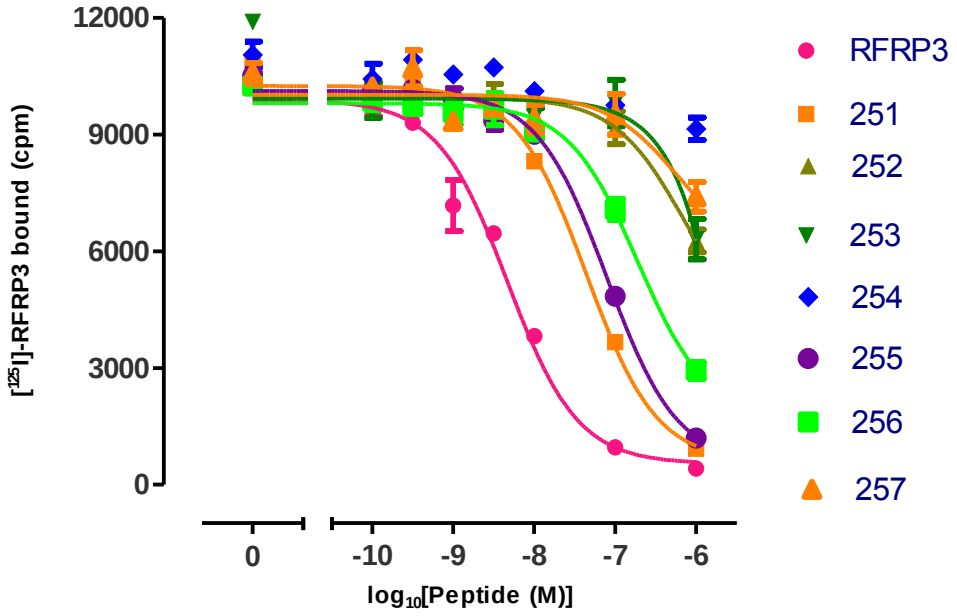

Figure 23: Radioligand competition binding assay with WT-GPR147 and RFRP3 analogs. COS-7 cells were transfected with WT-GPR147. 48 hours later whole cell radioligand competition binding was performed. 100,000cpm/well [125I]-RFRP3 was incubated with cells in the absence (0) and presence of increasing concentrations of unlabelled peptide for 4 hours at 4°C, before washing and measurement of bound radioligand. N=3.

Table 11: IC_{50} and B0 values of RFRP3 analogs. (* p<0.05 for comparison with RFRP3, N=3)

Peptide		IC_{50} ± SEM (nM)	B0 ± SEM (cpm)
RFRP3		5.14 ± 0.23	6098 ± 1959
251	Ac-3-8-RFRP3	39.45 ± 3.74*	6128 ± 2125
252	*D*-W^6-RFRP3	999.3 ± 674.5*	6363 ± 1847
253	N^6-RFRP3	ND	6399 ± 1891
254	*D*-A^6-RFRP3	137.9 ± 126.2*	6590 ± 2141
255	A^3-RFRP3	55.35 ± 16.2*	6396 ± 1920
256	A^6-RFRP3	173 ± 10.6*	6396 ± 1826
257	A^3A^6-RFRP3	384.2 ± 190.8*	6746 ± 1785

ND= not displaced; Ac=acetylated

4.2.2.2 Activation of GPR147 by RFRP3 analogs

GPR147 couples to the $G_{i/o}$ family of G proteins which, when activated, inhibit AC stimulated cAMP accumulation. Therefore, in order to measure receptor activation by the RFRP3 analogs, CRE-luciferase assays were used. AC was directly stimulated with forskolin (FSK), and the inhibition of FSK-induced cAMP production by the analogs was measured using a CRE-luciferase reporter (a luciferase gene under the control of a cAMP response element). Briefly, transfected HEK293-T cells were stimulated with 1μM FSK and 1μM peptide for five hours. Thereafter, CRE-luciferase luminescence was measured. To normalise for differences in transfection efficiencies and cell number, the CRE-luciferase signal was normalised against the luminescence measured for a co-expressed *Renilla* luciferase (constitutively expressed).

RFRP3 stimulation elicited a significant reduction in FSK induced cAMP production (Figure 24). Peptides 251, 253, 255 and 256 also significantly reduced cAMP production to a level similar to that seen for the control RFRP3. However, peptides 252, 254 and 257 did not decrease FSK cAMP production and thus did not appear to activate the receptor, probably due to their low binding affinity. These results indicate that glutamine in position 6 is not critical for receptor activation as some substitutions are tolerated in this position. The alanine substitution in position 3 (peptide 255) also did not impair receptor signalling indicating that this asparagine also does not play a significant role in receptor activation. The double substitution with alanine in positions 3 and 6 (peptide 257) did result in reduced receptor activation. Therefore, although these residues are

not essential for receptor activation on their own, in combination, the disruption in binding affinity of this peptide results in decreased receptor activation.

In summary, these data show that the N terminus of the peptide is not essential for receptor binding or activation as peptide 251 displayed only moderately reduced affinity and was also capable to activate GPR147. Glutamine in position 6 of hRFRP3 appears to be important for receptor binding as some substitutions at this position severely reduced binding affinity (peptides 252, 253 and 254). When alanine was substituted at positions 3 or 6 only a small impairment in binding affinity was observed, however the analog containing both substitutions was severely impaired. Interestingly, although peptide 253 had severely reduced binding affinity it was still able to activate the receptor to a similar level as RFRP3.

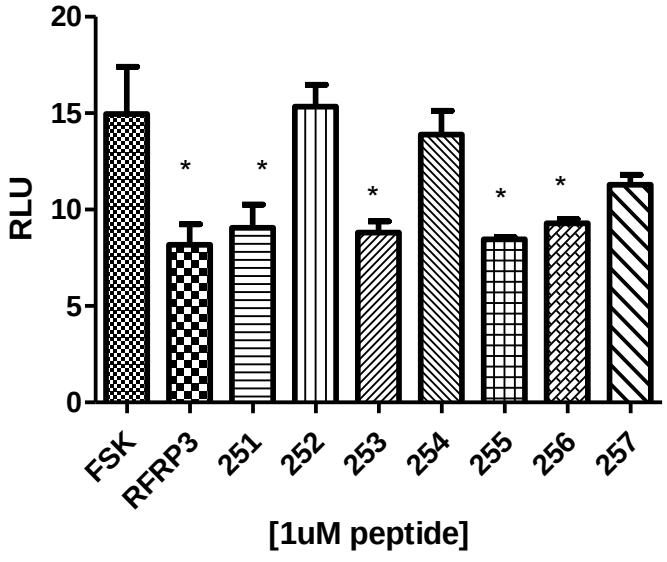

Figure 24: Forskolin inhibition assay of GPR147 with RFRP3 analogs. CRE-Luc assay was performed on GPR147 transfected HEK293-T cells incubated for 5 hours with 1 µM forskolin (FSK) and 1 µM peptide. Cell lysates were read on a luminometer and normalized with Renilla firefly. * $p<0.05$. RLU= relative luciferase units. FSK= Forskolin. N=3.

4.3 Discussion

The aim of this chapter was to investigate the binding affinity of select RFamides at GPR147 and to characterize novel RFRP3 analogs with regards to binding and activation of GPR147 in order to identify characteristics that determine the binding affinity and/or activity at this receptor.

The RFamide peptides share many features amongst themselves and with other members of the neuropeptide family. Therefore, it is possible that these short peptides may be recognized by more than one neuropeptide receptor. Some RFamides have been shown to have affinity and agonistic activity to the preferred receptors for NPFF family members, namely GPR147 and GPR74 (Engström *et al.*, 2003). These two receptors may not be limited to interacting with RFamide peptides, since there is some evidence that they may also interact with orexin and CCK peptides, whose function of food intake regulation overlaps with that of RFamides (Engström *et al.*, 2003, Bonini *et al.*, 2000). However, the affinity and activity of these peptides appears to be more prominent at GPR74 than GPR147 although these two receptors are classified together as NPFF receptors. In order to determine the ligand specificity of GPR147 the binding of several different neuropeptides whose receptors shared 30 % homology with GPR147 was examined in this study.

The IC_{50} values obtained for RFRP1 and 3 at GPR147 in this study were comparable with those obtained in published reports (Yoshida *et al.*, 2003, Bonini *et al.*, 2000, Mollereau *et al.*, 2002). The RFamide and RFamide-related peptides, NPY, CCK and PPY displayed little/no displacement/low affinity for the

GPR147 receptor in this study. Previous studies have also reported that NPY did not recognize GPR147 (Mollereau *et al.*, 2002).

In contrast to RFRP1 and RFRP3, RFRP2 did not displace [^{125}I]-RFRP3 from the GPR147 receptor indicating little/no affinity of this ligand for this receptor. In rodents only two RFRPs are encoded, namely RFRP1 and RFRP3 while the RFRP2 sequence has been lost (Ubuka *et al.*, 2012). This absence of RFRP2 in the rodent preproprotein sequence indicates that RFRP2 most likely has no significant function in mammals. In humans it has been also previously been shown that RFRP2 does not exhibit any activity at GRP147 when compared to RFRP1 and RFRP3 (Yoshida *et al.*, 2003), thus confirming the findings of this study that RFRP2 does not interact with GPR147.

KP10 was found to have high affinity for the GPR147 receptor. This is consistent with results obtained by Oishi *et al.*(2010) who found that KP10 exhibited potent binding and activation of GPR147 and GPR74 (Oishi *et al.*, 2010). Sequence alignment of RFRP3 and KP10 show that the asparagine (position 3 RFRP3) and the serine (position 4 KP10) are weakly similar whilst the leucine (position 4 RFRP3) and phenylalanine (position 5 KP10) share strong hydrophobic properties. These shared properties could contribute to KP10 interactions with GPR147 since these residues were found to be critical for RFRP3 interaction with GPR147 (Yoshida *et al.*, 2003) The relationship between kisspeptin and GPR147 will be explored further in subsequent chapters.

Modified RFRP3 analogs were then examined in order to identify residues that could play a role in binding and receptor activation. A truncated RFRP3 (peptide 251) retained moderate affinity (7-fold less than RFRP3) and was able to activate

GPR147. It has been previously reported the minimum sequence needed to bind and activate the GPR147 receptor was the seven-amino acid length C-terminal peptide of RFRP-3 (hRFRP3: PNLPQRF-NH2) (Yoshida *et al.*, 2003). However, the present study demonstrates that a shorter acetylated six-amino acid peptide (peptide 251) is also able to bind and activate this receptor.

Amino acid orientation within peptide ligands is important for receptor binding. Peptides 252 and 254 have D-isomers replacing the glutamine in position 6 of RFRP3. These peptides could not displace the radiolabelled RFRP3 and thus displayed low binding affinity and were unable to activate the GPR147 receptor. It is possible that these D-isomers restrict the ligands access to the binding site and result in poor affinity. Interestingly, an asparagine substitution at position 6 (peptide 253) also showed low affinity but was still able to activate the receptor significantly, suggesting that, once bound, this ligand may be able to induce receptor activation more effectively/potently than RFRP3 or that there is a high degree of 'spare receptors' in this system meaning that only relatively small concentrations of ligand are required to produce a large response. Further examination of the receptor-ligand interactions of this analog would be required before strong conclusions could be drawn. For example, it would be pertinent to perform receptor signalling assays with a range of peptide concentrations in order to determine the potency of this analog. Substitution of an alanine in position 6 (peptide 256) also resulted in a reduction in ligand affinity. However this peptide was still able to significantly activate the GPR147 receptor. The SAR studies performed on hRFRP3 thus revealed that the Gln in position 6 was important for receptor affinity but not for receptor activation. Thus the amide functional group

present in glutamine appear to represent an important group in the interaction of this ligand with GPR147.

Another analog, in which asparagine in position 3 was replaced (peptide 255), showed a 10-fold reduction in affinity compared to RFRP3. However, this analog was still able to activate the receptor; again indicating that asparagine in position 3 of RFP3 plays a greater role in ligand binding than receptor activation. A double substitution analog with alanine in positions 3 and 6 of RFRP3 (peptide 257) resulted in further decreased affinity compared to the analogs with the equivalent single alanine substitutions (peptides 255 and 256) indicating an additive effect of these substitutions. Unlike the analogs with equivalent single alanine substitutions, this doubly substituted analog was unable to decrease FSK induced cAMP production, most likely due to its more severe disruption in ligand binding.

Studies performed on bovine and human RFRP3 have suggested that three amino acid residues in position 2, 3 and 4 (PNL), in combination with the last four residues of RFRP3 (PQRF-NH_2) are important for determining receptor specificities as well as receptor activation. The authors further found that the proline (P) and leucine (L) of the PNL group were more critical for interaction with GPR147 (Yoshida *et al.*, 2003). The data in the present study have indicated a strong role of glutamine 6 and to a lesser extent, asparagine 3 in ligand binding to GPR147, but not receptor activation. Further SAR studies on RFRP3 utilising other analogs in which different residues have been substituted will help elucidate the residues critical for ligand binding and receptor activation.

While the potential for cross-talk and ligand promiscuity exists within the RFamide receptor family, we have found that GPR147 has relatively high ligand-specificity.

Aside from its cognate ligands (RFRP3 and RFRP1), only KP10 bound the GPR147 receptor with high affinity

The GPR147/RFRP3 system is emerging as an important player in reproduction. Greater understanding into ligand receptor interactions will aid in unravelling pathways activated by RFRP3/GPR147 and contribute to a greater understanding of receptor function and the data presented in the present study represent an important step on the path towards this goal.

Chapter 5: GPR147 interactions with selected kisspeptin analogs

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5.1 Introduction

Gonadotropin secretion is regulated by neuropeptides upstream of GnRH in the hypothalamic-pituitary-gonadal (HPG) axis. One such neuropeptide is kisspeptin, a member of the RFamide family of neuropeptides (Oakley *et al.*, 2009). It has been found that stimulation of their cognate receptor, GPR54, by kisspeptins promotes the release of gonadotropin-releasing hormone (GnRH) from GnRH neurons, which, in turn, increases the plasma level of gonadotropins and initiates the onset of puberty (Dhillo *et al.*, 2005, Shahab *et al.*, 2005).

In 2000, Tsutsui *et al* identified an RFamide related peptide that inhibited gonadotropin secretion in quail brains and termed it gonadotropin-inhibitory hormone (GnIH) (Tsutsui *et al.*, 2000). Another group later identified a mammalian RFamide precursor protein in humans using bioinformatics, that was predicted to give rise to two mature RFamide peptide GnIH homologs, RFRP1 (also known as NPSF) and RFRP3 (also known as NPVF) (Hinuma *et al.*, 2000). The precursor protein also encodes another related peptide with an RS-amide C-terminus (RFRP2), although further biochemical characterisation has revealed that RFRP2 is not produced *in vivo* (Yoshida *et al.*, 2003, Ukena *et al.*, 2003). GPR147 and GPR74 (which couple to the $G_{i/o}$ family of G proteins) have been identified as the cognate receptors for the RFRPs.

As discussed earlier, RFRPs and KP10 appear to have opposing actions on the regulation of the HPG axis and there is evidence of interplay between these two signalling systems. The RFRP peptides share characteristics with many other members of the neuropeptide family, including the kisspeptins, thus these peptides have the potential to be recognized by/recognize each other's receptors.

Indeed, Lyubimov *et al*. reported moderate activation of GPR74 by endogenous KP-13 and KP-14 (Lyubimov *et al.*, 2010) and in GPR147 expressing CHO cells, KP-54 and KP-10 both activated GPR147, leading to a reduction in FSK induced cAMP production (Oishi *et al.*, 2010).

In the present study, the relationship between kisspeptin analogs and the RFRP receptor GPR147 will be studied.

5.1.1 Aims

- To investigate the binding and activation of GPR147 by kisspeptin analogs in order to try to better elucidate the mechanism of interplay between these two important regulators of reproduction, and to identify analogs with the potential to acts as novel GRP54/GRP147 regulators that could be used as tools to aid further examination of these important signalling pathways and/or used for development of novel therapeutic interventions targeting these receptors.

5.2 Results

Kisspeptin has previously been shown to bind GPR147 (Oishi *et al.*, 2010) and the data presented in Chapter 4 of this thesis is consistent with those observations. In pursuit of kisspeptin antagonists, Roseweir *et al.(*2009) designed and tested several KP10 analogs. Of the analogs tested, peptide 234 was identified as the most potent antagonist for the GPR54 receptor (Roseweir *et al.*, 2009). However, during the development of this antagonist, several other kisspeptin analogs were produced. In the present study, a selection of these

analogs that had antagonistic potential (unpublished) (Table 12) were chosen and their ability to bind and activate the GPR147 receptor was investigated.

Table 12: Amino acid sequence of KP10 analogs

Peptide	0	1	2	3	4	5	6	7	8	9	10	
KP10		Y	N	W	N	S	F	G	L	R	F	NH2
234	**Ac**	***D*-A**	N	W	N	**G**	F	G	***D*-W**	R	F	NH2
349	***D*-A**	Y	N	W	***D*-A**	**Sar**	F	**Sar**	***D*-W**	R	F	NH2
356	***D*-A**	Y	N	W	N	**G**	F	G	***D*-W**	**K**	F	NH2
351	***D*-A**	Y	N	**A**	N	**Sar**	F	**Sar**	***D*-W**	R	F	NH2
352	***D*-A**	Y	N	W	N	**G**	F	G	***D*-W**	R	F	NH2
353	***D*-A**	Y	N	W	N	**Sar**	F	**Sar**	***D*-W**	R	F	NH2

Ac = acetylated, Sar = sarcosine

5.2.1 Binding affinity of kisspeptin analogs at GPR147

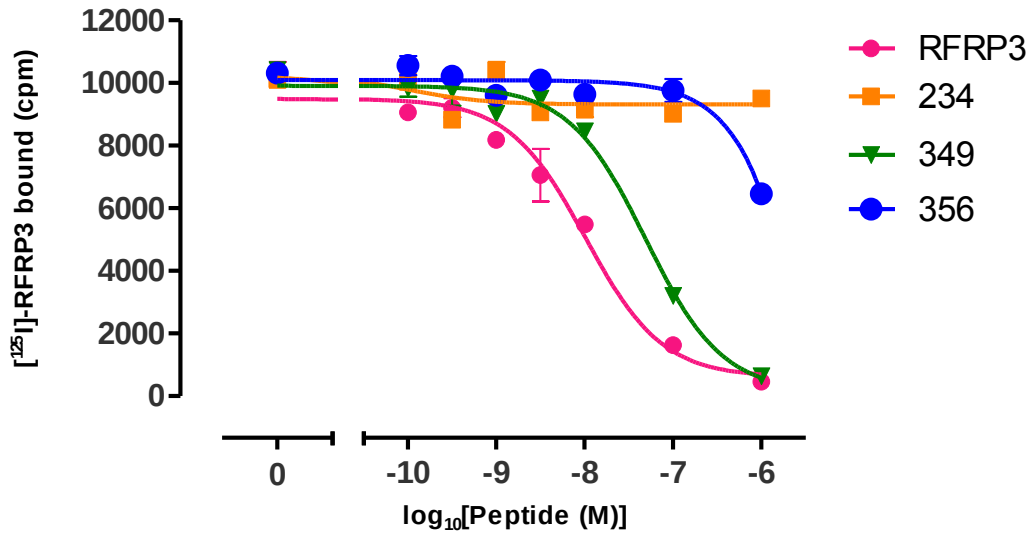
The binding affinity of the peptide 234 antagonist and other KP10 analogs were assessed using whole-cell radioligand competition binding assays. Briefly, GPR147 transfected COS-7 cells were incubated with [^{125}I]-RFRP3 in the absence or presence of increasing concentration of unlabelled test peptide.

RFRP3 served as the control and was able to maximally displace [^{125}I]-RFRP3 with an IC_{50} of 4 nM (Figure 25, Table 13). The receptor expression B0, was similar in all cases and served as a baseline from which displacement could be compared for the different analogs (Figure 25). In agreement with the data presented in Chapter 4, KP10 was able to fully displace [^{125}I]-RFRP3 binding, albeit with lower affinity than RFRP3. However, the potent kisspeptin antagonist, peptide 234, was not able to displace [^{125}I]-RFRP3 indicating low/no affinity of this peptide for GPR147 (Figure 25a, Table 13). Peptide 356 also had very low affinity for GPR147 (700-fold less than RFRP3). However, it was found that peptide 349

had only 8-fold lower affinity for the GPR147 receptor than RFRP3 and a similar affinity to KP10.

Additional analogs based on peptide 349 were subsequently tested (Figure 25b, Table 13). These peptides all had the addition of a *D*-Ala in position 0 and a *D*-Trp substitution at position 8 of KP10. These analogs all displayed affinity for GPR147 and were able to displace [^{125}I]-RFRP3 (Figure 25b, Table 13). Peptide 351 had 30-fold lower affinity for GPR147 than RFRP3 and was the only analog to have a lower affinity than KP10 (16-fold). Peptides 352 and 353 both had a higher affinity (approx. 5-fold) for GPR147 than KP10 and had only slightly reduced (peptide 352) or similar (peptide 353) affinities as RFRP3.

(a)



(b)

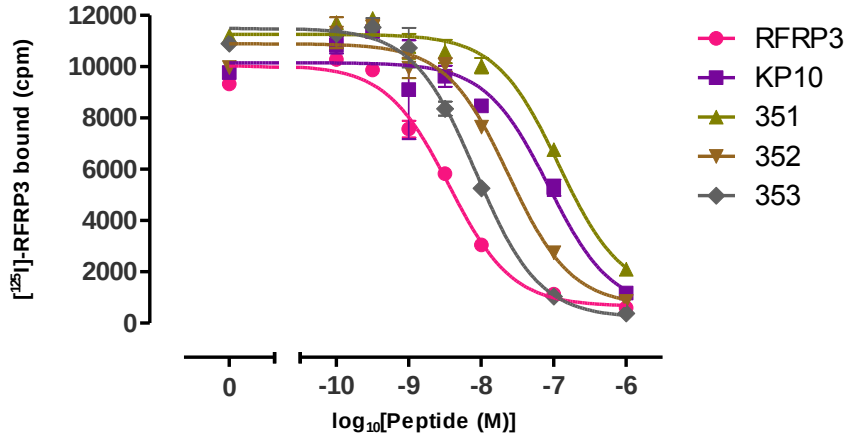


Figure 25: **Radioligand competition binding assay with WT-GPR147 and kisspeptin analogs**. COS-7 cells were transfected with WT-GPR147. 48 hours later whole cell radioligand competition binding was performed. 100,000cpm/well [125I]-RFRP3 was incubated with cells in the absence (0) and presence of increasing concentrations of unlabelled peptide for 4 hours at 4℃, before washing and measurement of bound radioligand. (a) Binding affinity of select KP10 analogs. (b) Binding affinity of select KP10 analogs based on pep 349 substitutions. n=3.

Table 13: IC_{50} and B0 values of kisspeptin analogs. (* p<0.05 for comparison with RFRP3, N=3)

Peptide	IC_{50} ± SEM (nM)
RFRP3	4.06 ± 1.44
KP10	53.72 ± 12.69*
234	ND
349	46.47 ± 18.97*
356	4103 ± 4102*
351	93.76 ± 11.04*
352	13.31 ± 3.86
353	6.96 ± 0.90

ND= no displacement

5.2.2 Activation of GPR147 by kisspeptin analogs

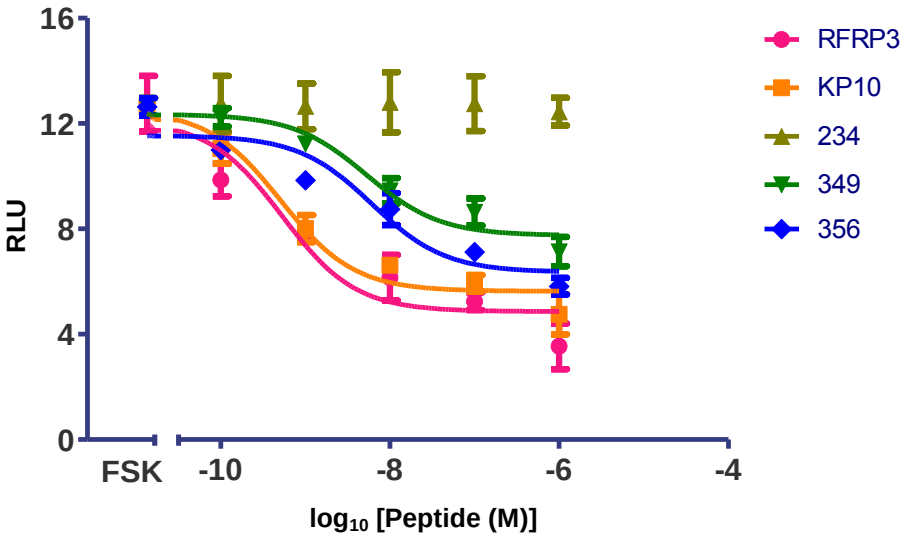
The kisspeptin analogs were then subjected to CRE-luciferase assays in order to measure their ability to simulate activation of GPR147. Briefly, GPR147 transfected HEK293-T cells were stimulated with 1μM FSK and increasing concentration of peptide for five hours. Thereafter, CRE-luciferase luminescence was measured and was normalised against Renilla-luciferase signal.

RFRP3 served as the positive control and it was able to significantly reduce FSK-induced cAMP production (Figure 26, Table 14). KP10 was also able to inhibit FSK-induced cAMP production to a similar level as RFRP3 (Figure 26a, Table 14a) but with slightly reduced (2-fold) potency. Peptide 234 had no effect on receptor activation, which can be attributed to its low binding affinity. Peptide 356, which also had severely reduced binding affinity, was able to activate the receptor, albeit with 15-fold lower potency than RFRP3 but a similar maximal response/efficacy (E_{max}). Peptide 349 was 10-fold less potent than RFRP3 and although the maximal effect seen with this peptide was lower than for RFRP3, this difference was not significant ($p>0.05$).

Of the peptides tested that were based on peptide 349 design, peptide 351 had 6-fold reduction in potency and 2-fold reduction in Emax compared to RFRP3 (Figure 26b,Table 14) and there was no significant difference in the potency or efficacy of peptides 352 and 353 for GRP147 compared to RFRP3 ($p>0.05$).

In summary, some KP analogs are able to act as agonists of GPR147. The kisspeptin antagonist peptide 234 was found to have no affinity or activity at GPR147. This is important as peptide 234 therefore represents a selective GPR54-specific antagonist. Conversely, peptides 352 and 353 appear to be potent agonists at GPR147, having high binding affinity and activity.

(a)



(b)

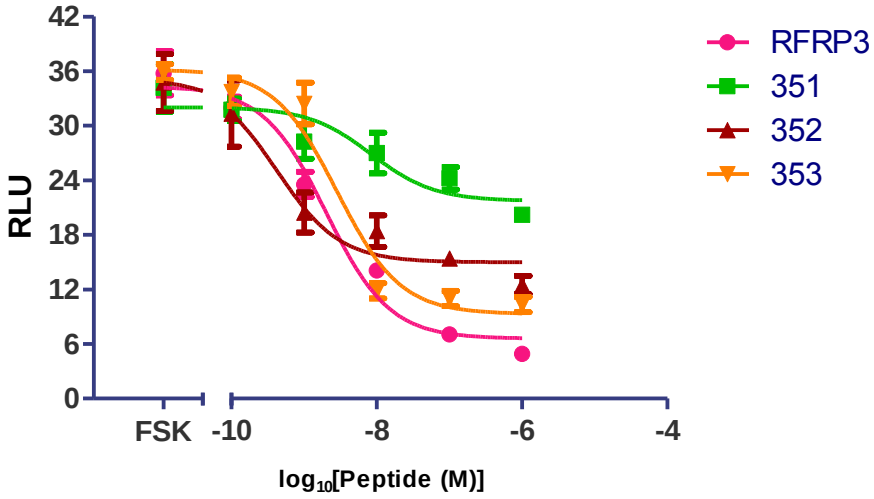


Figure 26: Forskolin inhibition assay of GPR147 with (a) kisspeptin analogs and (b) kisspeptin analogs based on peptide 349 substitutions. GRP147 transfected HEK293-T cells were incubated 1μM FSK with or without increasing peptide concentration for 5 hours. Cells were lysed then assayed for luciferase activity and normalised with *Renilla* firefly. RLU= relative luciferase units. N=3.

Table 14: EC_{50} and E_{max} values for WT-GPR147 with kisspeptin/ kisspeptin analogs (* p<0.05 for comparison with WT-GPR54, N=3)

Peptide	EC_{50} ±SEM	EMax (RLU) ±SEM
RFRP3	0.74 ± 0.65	6.76 ± 1.88
KP10	0.47 ± 0.03	5.63 ± 0.25
234	NI	NI
356	4.43 ± 0.85	6.36 ± 0.39
349	2.63 ± 0.35	7.75 ± 0.27
351	8.61 ± 0.96*	17.59 ± 1.40
352	1.61 ± 1.24	12.40 ± 0.86
353	1.51 ± 0.70	13.75 ± 0.50

NI=no inhibition

5.3 Discussion

The aim of this study was to investigate the ability of kisspeptin analogs to bind and activate the GPR147 receptor. The endogenous kisspeptins have been shown to interact with GPR147 and GPR74 (Oishi *et al.*, 2010, Lyubimov *et al.*, 2010). The data presented here concurs with these finding as KP10 was found to have a high affinity and potency at GPR147. The kisspeptin antagonist, peptide 234, however, had low affinity and was not able to activate the GPR147 receptor. The ability of synthetic analogs to antagonize/activate only their target receptors (GPR54 in this case) is an important characteristic for the development of research tools or potential therapeutic agents.

Roseweir *et al*. (2009) designed multiple kisspeptin analogs in an attempt to identify novel kisspeptin antagonists. In this study we performed binding and activity assays at GPR147 on previously untested kisspeptin analogs to determine if they had agonist/antagonist activity at this receptor. Kisspeptin analogs were selected on their antagonistic potential (Unpublished, Roseweir, 2009).

Peptide 356 has a lysine substituted for the arginine of the C-terminal of KP10. This modification resulted in a significant reduction in affinity (800-fold) for GPR147, possibly due to the disruption of the RF-NH_2 functionality. This is comparable to data for which substitution of the RF-NH_2 arginine NPFF with a lysine residue induced a 720-fold decrease in affinity at the GPR74 receptor (Mazarguil *et al.*, 2001). In general, the potency of the analogs tested reflected their binding affinity at the receptor. The exception was peptide 356 which although it had severely reduced binding affinity, had similar potency for

activation of GPR147 as the analogs with higher affinity. This suggests that this receptor, although impaired in its binding to the receptor, may be more effective at activating it once bound, and that the RF-amide functionality is not important for receptor activation. Peptide 351 had similar affinity as KP10 for GPR147 but almost 20-fold lower potency. This peptide differs from the other analogs as the tryptophan in position 3 of KP10 is replaced with an alanine. These data therefore indicate that this tryptophan of KP10 is important for GPR147 activation but not binding.

Peptide 352 and 353 were both found to be potent GPR147 agonists with similar affinities/potencies, therefore suggesting that the sarcosine/glycine substitutions at positions 5 and 7 of KP10 have similar effects and do not affect binding/receptor activation and may actually serve to increase the affinity of these ligands. Both peptides 352 and 353 displayed high affinity and potency at GPR147. Indeed, the affinity of these analogs for GPR147 was 5-fold greater than that of KP10 or the related peptide, peptide 349. Analog, 349, displayed similar affinity but slightly lower potency than KP10 at GPR147. In contrast to peptide 349, the 352 and 353 analogs do not contain an amino acid substitution in position 4 of KP10. These data therefore suggest that asparagine in position 4 of KP10 could be important for GPR147 receptor activation.

In summary, this study has confirmed that KP10 has high affinity and potency at GPR147 and has identified several kisspeptin analogs that function as potent agonists at GPR147. The information gained is valuable for validating the receptor specificity of these compounds as a pre-requisite for the development of these analogs as research/therapeutic tools. In addition, the SAR information

obtained may aid in the development of other similar analogs and help to further our understanding of the ligand-receptor interactions of this important receptor.

Chapter 6: GPR54 interactions with selected neuropeptides and kisspeptin analogs

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6.1 Introduction

Kisspeptin is a member of the RFamide neuropeptide family which has recently been discovered to play an important role in the hormonal control of reproduction, a finding that has been credited as one of the most important findings in the last decade in the field of reproductive biology. Studies have provided evidence that administering kisspeptin either centrally, via the ventricles of the brain, or peripherally via blood vessels, leads to a dose dependent increase in the gonadotropins LH and FSH. These studies have been conducted in numerous different species, including humans (Dhillo *et al.*, 2005).

As mentioned earlier, kisspeptin-GPR54 and RFRP3-GPR147 systems are known to regulate GnRH release in a positive and negative manner, respectively. RFRP3 acts in an opposite manner to kisspeptin to decrease plasma gonadotropin levels in mammals *in vivo* (Kriegsfeld *et al.*, 2010, Clarke *et al.*, 2012).

As emphasised earlier, the promiscuity of certain RFamide peptides is high and this drives the need to develop receptor selective agonists and antagonists. We have shown that KP10/KP10 analogs are able to bind to and activate GPR147 (Chapter 4 and 5). In this chapter reciprocal studies have been performed in which the binding affinity and potency of various RFamide, RFRP3 analogs and kisspeptin analogs at the GPR54 receptor have been studied.

6.1.1 Aim

- To investigate the binding affinity and potency (as measured by inositol phosphate stimulation) of selected RFamides, KP10 analogs and RFRP3 analogs at the GPR54 receptor, in order to determine a ligand-selectivity profile for this receptor and to determine SAR data which may aid in the development of novel ligands targeting this important receptor.

6.2 Results

In Chapters 4 and 5 the ability of several RFamides, RFRP3 analogs and KP10 analogs to bind to and activate the GPR147 receptor was examined. In this Chapter, the ability of the same ligands to interact with the GPR54 receptor has been investigated.

6.2.1 Binding affinity and activation of GPR54 by selected RFamides

The binding affinity of RFRP3, NPY and PPY (Table 8) at GPR54 was examined in radioligand competition binding assays. Briefly, GPR54 transfected COS-7 cells were incubated with labelled [^{125}I]-KP10 in the absence or presence of increasing concentration of unlabelled peptide and the level of bound radioligand measured.

KP10 was able to fully displace [125I]-KP10 binding (as expected some degree of NSB of [125I]-KP10 was observed) with an IC50 of 2 nM (Figure 27). As expected, the level of [125I]-KP10 binding in the absence of competing ligand (BO) was similar for all ligands tested (Figure 27). None of the peptides tested, namely RFRP3, NPY and PPY, were able to displace [125I]-KP10, indicating that these peptides have very low/no affinity for the GPR54 receptor.

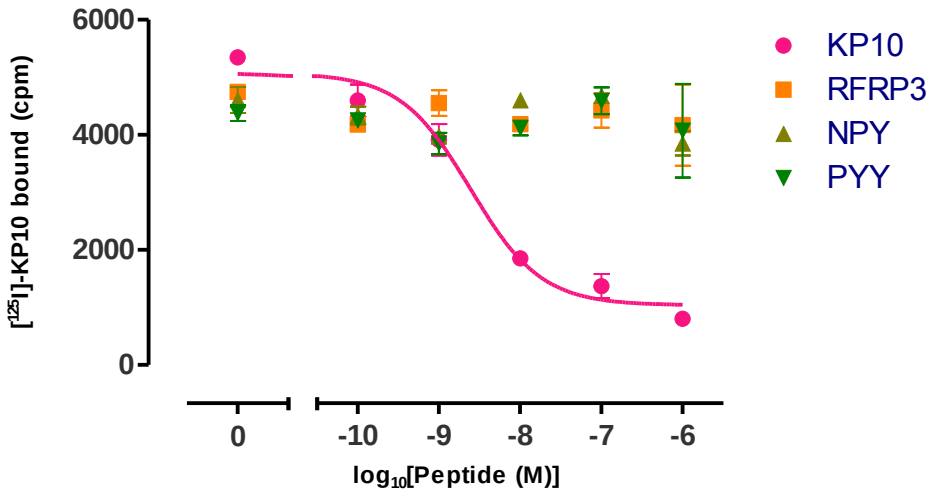

Figure 27: Radioligand competition binding assay with WT-GPR54 and select RFamides. COS-7 cells were transfected with FLAG-tagged WT-GPR54. 48 hours later whole cell radioligand competition binding was performed. 50,000cpm/well [^{125}I]-KP10 was incubated with cells in the absence (0) and presence of increasing concentrations of unlabelled peptide for 4 hours at 4℃, before washing and measurement of bound radioligand. N=3

KP10 was able to stimulate a robust IP response with an EC_{50} 39nM (Figure 28). However, none of the RFamides tested (RFRP3, PPY and NPY) elicited an IP response, reflecting their lack of affinity for this receptor.

In summary, only the KP10 peptide had high affinity and could activate the GPR54 receptor and the other RFamides tested had very low or no affinity and could not activate GPR54.

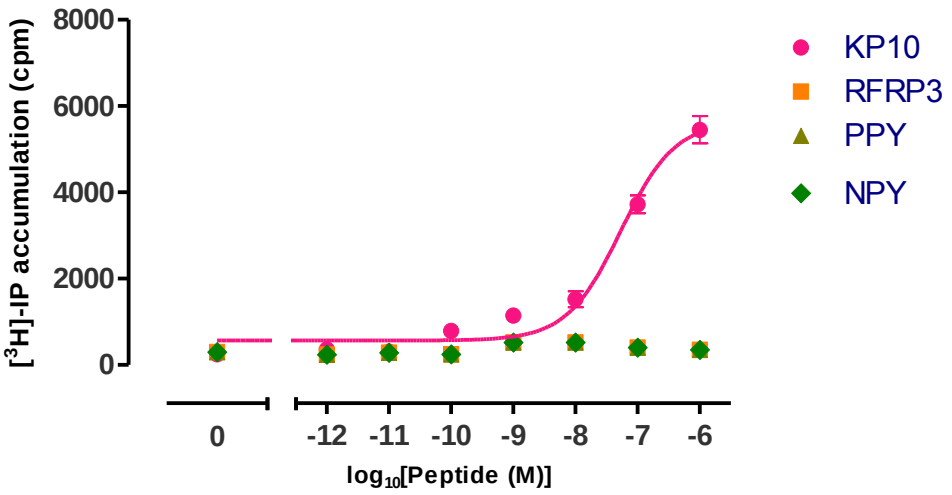

Figure 28: IP accumulation assay with WT-GPR54 and RFamides. COS-7 cells transfected with WT-GPR54 were stimulated with increasing concentrations of peptide and assayed for total radioactive inositol phosphate accumulation. N=3

6.2.2 Binding affinity and activation of GPR54 by kisspeptin analogs

In Chapter 5 it was shown that several KP10 analogs were able to bind to and activate GPR147. In this Chapter, the affinity and activity of these KP10 analogs (Table 12) at GPR54 has been examined in order to determine if there is any cross reactivity between the two receptors. These KP10 analogs have not been tested previously at GPR54 and were designed in our laboratory as part of a series of peptides attempting to identify kisspeptin antagonists.

The affinity of the peptides for GPR54 was determined using radioligand competition binding assays. Briefly, GPR54 transfected COS-7 cells were incubated with labelled [^{125}I]-KP10 in the absence or presence of increasing concentration of unlabelled peptide and the level of bound radioligand measured.

The positive control, KP10, was able to displace [^{125}I]-KP10 (in agreement with data in Figure 27) with an IC_{50} of 2nM (Figure 29). However, all of the KP10 analogs tested, showed very poor displacement with IC_{50} values >300nM-815nM indicating that all the KP10 analogs tested had lower affinity for the GPR54 receptor compared to KP10.

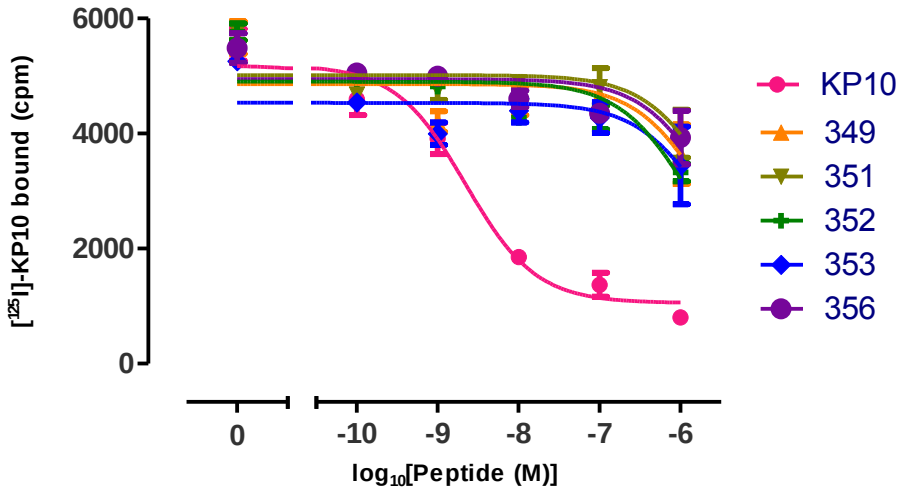

Figure 29: Radioligand competition binding assay with WT-GPR54 and kisspeptin analogs. COS-7 cells were transfected with FLAG-tagged WT-GPR54. 48 hours later whole cell radioligand competition binding was performed. 50,000cpm/well [^{125}I]-KP10 was incubated with cells in the absence (0) and presence of increasing concentrations of unlabelled peptide for 4 hours at 4℃, before washing and measurement of bound radioligand. N=3

KP10 served as the positive control and was able to elicit a robust IP response (in agreement with Figure 28) with an EC_{50} of 56nM (Figure 30). However, none of the KP10 analogs tested elicited an IP response, reflecting their inability to bind to this receptor.

In summary, although KP10 has high affinity for GPR54, the KP10 analogs tested are unable to bind to/activate this receptor.

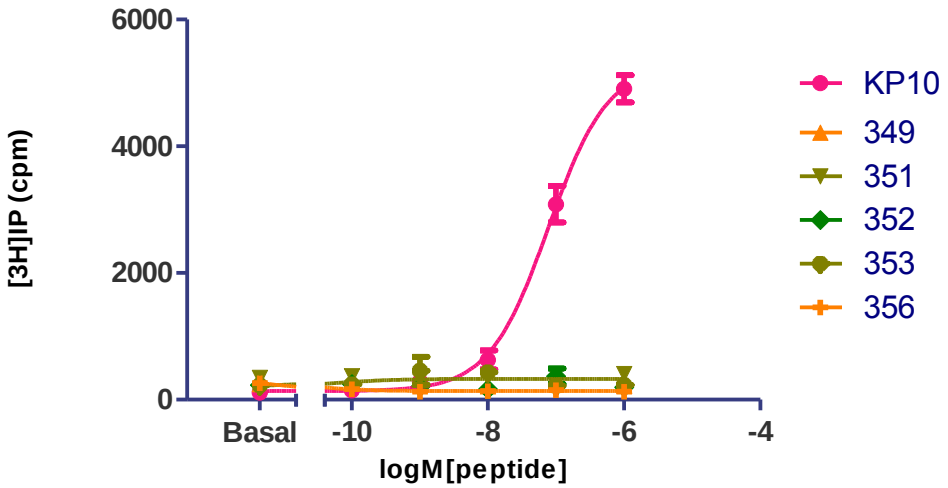

Figure 30: IP accumulation assay with WT-GPR54 and kisspeptin analogs. COS-7 cells transfected with WT-GPR54 were stimulated with increasing concentrations of KP10 and assayed for total radioactive inositol phosphate accumulation. N=3.

6.2.3 Binding affinity and activation of GPR54 by RFRP3 analogs

For completion, despite the observation that RFRP3 did not bind nor activate GPR54 (Figure 27 and Figure 28), RFRP3 analogs (Table 10) were tested for their ability to bind and activate the GPR54 receptor. As some KP10 analogs were found to be able to bind and activate the GPR147 receptor (Chapter 5), we were interested to determine if there was a reciprocal relationship between the ligands.

Single dose binding assays were used to screen the analogs for binding affinity. Briefly, GPR54 transfected COS-7 cells were incubated with [^{125}I]-KP10 in the absence or presence of a single high concentration (1μM) of unlabelled test peptide and the level of bound [^{125}I]-KP10 measured.

1μM KP10 was able to displace [^{125}I]-KP10, 4-fold (Figure 31). However, no displacement was observed with any of the RFRP3 analogs tested, indicative of

low/no affinity of these peptides for GPR54. As no response was seen with the high concentrations (1μM) of peptides used in this preliminary experiment, it was not appropriate to perform full dose-response analyses with these peptides.

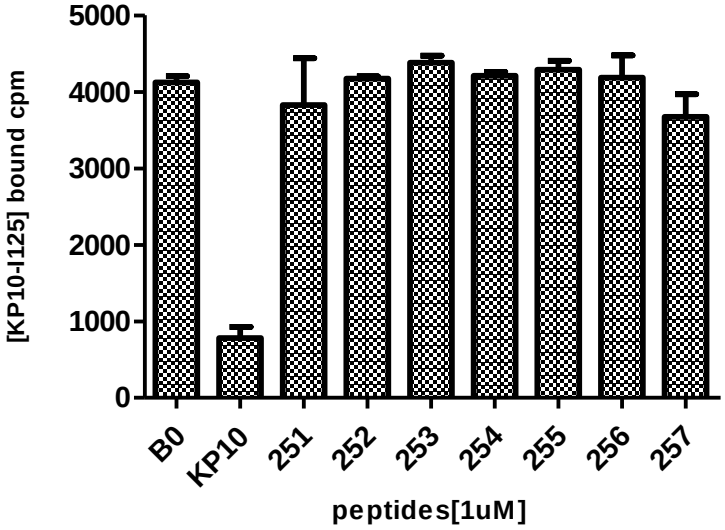

Figure 31: Radioligand competition binding assay with WT-GPR54 and select RFamides. COS-7 cells were transfected with FLAG-tagged WT-GPR54. 48 hours later whole cell radioligand competition binding was performed. 50,000cpm/well [^{125}I]-KP10 was incubated with cells in the absence (0) and presence of 1μM of unlabelled peptide for 4 hours at 4℃, before washing and measurement of bound radioligand. N=3

.

To confirm that none of the RFRP3 analogs could activate GPR54, single dose IP assays were performed. Briefly, COS-7 cells transfected with WT-GPR54, and loaded with [^{3}H]-myo inositol, were stimulated with a single high concentration (1μM) of peptide. Thereafter total inositol phosphates were extracted, purified and radioactivity was counted.

KP10 elicited robust IP production with a 40-fold increase compared to basal levels measured in the absence of peptide (Figure 32). However, none of the RFRP3 analogs tested stimulated IP production above basal levels, confirming that these ligands cannot activate the GPR54 receptor.

In summary, the RFRP3 analogs tested do not bind or activate the GPR54 receptor.

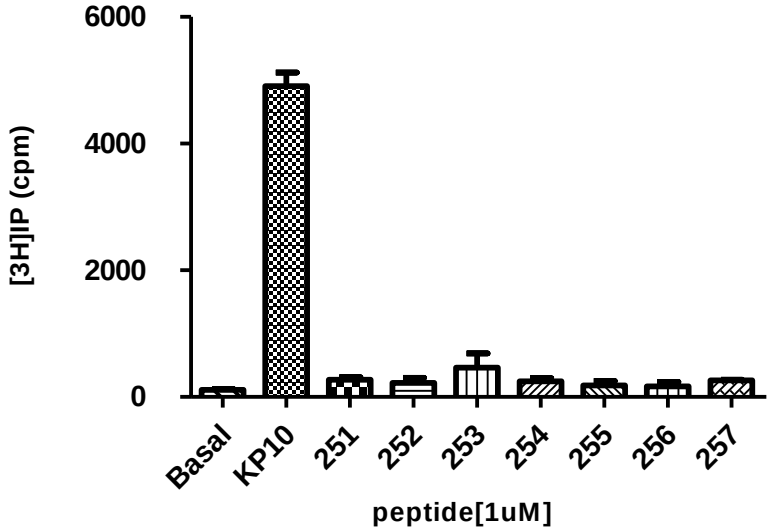

Figure 32: IP accumulation assay with WT-GPR54 and RFRP3 analogs. COS-7 cells transfected with WT-GPR54 were stimulated with increasing concentrations of peptide and assayed for total radioactive inositol phosphate accumulation. N=3.

6.3 Discussion

The aim of the studies described in this Chapter was to examine the ligand-specificity of GPR54.

In contrast to GPR147, which was able to bind/be activated by KP10/KP10 analogues in addition to its cognate ligands, none of the RFamides examined, namely NPY, RFRP3, CCK and PPY, had measureable affinity or potency at GPR54 (Figure 27, Figure 28). Other NPFF ligands have been previously examined for their activity at GPR54, including NPSF and NPFF, and were also not recognized by this receptor (Lyubimov *et al.*, 2010). The galanin receptor, GAL1, has 45% homology to GPR54 but its cognate ligand, galanin, is also unable to bind GPR54 (Lee et al., 1999).

Several of the KP10 analogs tested at GPR147 proved to be potent agonists for this receptor (Chapter 5). The activity of these analogs at the GPR54 receptor was therefore examined for comparison. The KP10 analogs tested had low affinity for GPR54 with affinities greater than 150-fold lower than KP10 (Figure 29) and thus no activity at this receptor (Figure 30). The combination of amino acid substitutions therefore appears to significantly disrupt binding to GRP54.

Several studies have attempted to identify the residues required to activate GPR54. Alanine and D-amino acid scans revealed that the five C-terminal amino acids of KP10 (phenylalanine^{6}-phenylalanine^{10}) are important for agonist activity (Niida et al., 2006). Analysis of KP13 identified the pharmacophore site of the kisspeptins as Phe^{9}, arginine^{12}, and phenylalanine^{13} (Orsini et al., 2007), with the peptide forming a helicoid conformation from asparagine^{7} to phenylalanine^{13}, which corresponds to phenylalanine^{6}-phenylalanine^{10} of KP10. The RF moiety, of

the RFamide ligands is evolutionarily conserved, indicating it has an essential role in receptor binding, supported by the impaired binding of the KP10 analog peptide 356 (which has a lysine^{9} modification, disrupting the RFamide moiety) to GPR54 and GPR147 in the present study.

Roseweir et al, 2009 identified that the five N-terminal amino acids of KP10 are required for receptor activation with serine^{5} and leucine^{8} critical for agonist activity (Roseweir et al., 2009). These authors also identified the potent kisspeptin antagonist, peptide 234 (ac [(D)-A] NWNGFG [(D)-W] $RFNH_2$) that contained seven residues conserved from KP10 and has high affinity for GPR54 (Roseweir *et al*., 2009). The KP10 analogs examined in the present study (peptides 349 and 351-353) and which were found to have poor affinity for GPR54 also contain the D-tryptophan^{8} substitution of peptide 234, indicating that this substitution does not disrupt binding affinity. The low binding affinity of these peptides at GPR54 could thus be attributed to their additional substitutions which may disrupt the binding pharmacophore of KP10. In addition to their other modifications, all of these analogs contain a modified N-terminus when compared to peptide 234, with a D-alanine substitution at position 0 of KP10 and no substitution at position 1 of KP10. The only different between peptide 352 and peptide 243 is this modified N-terminus, indicating that this modification is responsible for the loss of GPR54 binding affinity of this group of peptides, and highlighting the importance of the N terminal region of the peptide in receptor interaction.

The activity of RFRP3 analogs at GPR54 was also examined. However, like RFRP3, the RFRP3 analogs were unable to bind/activate GPR54 (Figure 31, Figure 32). RFRP3 and KP10 share the RFamide moiety and residues with

similar amino acids properties namely serine and phenylalanine (position 3 and 4 of KP10) and asparagine and leucine (position 3 and 4 of RFRP3). It is possible that the binding site of GPR147 is not as stringent as that of GPR54 which is why KP10 is able to bind GPR147 but RFRP3 cannot bind GPR54. Certainly, it appears that different ligand-receptor inactions are involved in the binding of ligand to these two receptors.

Taken together, these results suggest that the molecular recognition by GPR54 seems to be highly stringent and kisspeptin–selective unlike GPR147 which can be activated by RFamides. These data serve to highlight the importance of testing any new analogs for RFamide receptors against other RFamide receptor species before development as research/therapeutic agents.

Chapter 7: Conclusions

This study has highlighted the importance of the ECL domains of GPR54 in receptor activation by kisspeptin, with the ECL2 domain playing the most critical role. The acidic residues on the ECL2 domain of GPR54 (E193 and E201) are of particular importance for receptor activation (Chapter 3) and it is postulated that these acidic residues may be involved in interaction with the basic arginine at the C-terminus of kisspeptin. Modification of the ECL domains also caused a reduction in cell surface expression of GPR54, most likely through a disruption of the correct folding of the receptor protein causing it to be retained in the endoplasmic reticulum by the cellular quality control machinery. As some degree of cell surface expression was measured, the degree of misfolding is not likely to be too severe.

This study has also demonstrated that an acetylated six-amino acid peptide (pep 251) was the minimum sequence required to bind and activate GPR147 (Chapter 4) and thus contains the critical pharmacophore for this ligand. The SAR studies performed on hRFRP3 revealed that the glutamine in position 6 was important for GPR147 affinity but not for activation of this receptor. Four RFRP3 analogs were also identified as potent GPR147 agonists, namely peptide 251, 253 (Asn^{6}-RFRP3), 255 (Ala^{3}-RFRP3) and 257 ($Ala^{3, 6}$-RFRP3).

In addition to RFRP3 and its analogs, KP10 and several KP10 analogs (peptides 349, 351 and 352) were also identified as potent agonists at GPR147 (Chapter 5), indicating some promiscuity of this receptor. This cross-talk between different ligands can add complications when designing ligands/drugs for research or

therapeutic use and could lead to unexpected off-target side-effects and these data serve to highlight the importance of determining receptor selectivity profiles when developing novel analogs for RFamide receptors.

In some circumstances, ligand cross reactivity can be an advantage when the actions of ligand at two different receptors have an additive effect or can mediate each other. This study and others have demonstrated that KP10, an agonist at GPR54, also acts as an agonist at GPR147. This is interesting as these two receptors have opposing actions in the control of the HPG axis, but may reflect the evolution of an important control mechanism for this system to ensure that hormone secretion can be finely balanced.

Unlike GPR147, the ligand specificity of GPR54 was shown to be highly stringent, and only KP10 was also able to bind/activate this receptor (Chapter 6). SAR study of the differences between the structure of KP10, the antagonist peptide 234 and the KP10 analogs that displayed agonist activity at GPR147, has further highlighted residues important for interaction with/activation of GPR54 and/or GPR147. This information will be extremely valuable for the future development of analogs targeting these receptors.

In summary, the data presented in this thesis provide a valuable insight into the ligand-receptor interactions involved in the GRP54/kisspeptin and GPR147/RFRP systems which not only serves to increase our understanding of how these important systems function and inter-pay in the control of reproduction, but also has provided information valuable for the design of novel compounds targeting one or both of these systems.

Chapter 8: Appendices

Appendix I: GPR54-GalR2 Chimeras

The chimeric GPR54 constructs were a gift from Professor Chris Hague of the University of Washington. The chimeras were constructed using hGPR54 in pEYFP vector that had a C-terminal YFP tag. For the N-terminus chimera (Nterm) base pairs 1-29 were removed from hGPR54 and replaced with the base pairs 1-72 (aa M1-E24) from human galanin receptor 2 (hGalR2). For the ECL1 construct base pairs 309-357 were removed and replaced with base pairs 254-297 (aa IYTLDGWVGFCLLCK). ECL2, base pairs 540-600 were removed and replaced with base pairs 490-556 (aa RQSQLANLTVCHPAWSPRRR). Finally for the ECL3, base pairs 852-897 were removed and replaced with base pairs 783-816 (aa GQFPLTRATYA). A diagnostic digest using KPNI and BamHI was used for to verify the insertion of the constructs.

Appendix II: Cloning GPR147 cDNA

The GPR147 construct was made by Dr Kevin Morgan, HRSU, MRC, Edinburgh.

PCR primers for mouse GPR147 were:

Exon 1 F 5’ ggtaccaagctt / atggaggcggaaccctcccagcctc 3’

Exon 1 R 5’ cggtgataaggttgtccacgagggttgtgggca 3’

Exon1 -Exon 2 F 5’ acaaccttatcaccg / gttggccttttgacaatgccaca 3’

Exon2 R 5’ ctctccacggcaatggccaccagt 3’

Exon 2- Exon 3 F 5’ cattgccgtggagag / gttccgctgcatcgtacaccct 3’

Exon3 R 5’ ctcgagggatcc / tcaaatgttccaggctgggatagt 3’

Individual GPR147 exons were amplified using mouse genomic DNA, Herculase II high fidelity DNA polymerase, which does not add a 3’ overhang to amplified DNA (Stratagene, UK) and modified exon-specific primers (boundaries are denoted by /). Amplified exon DNA was purified by agarose gel electrophoresis, excision, centrifugal elution (Millipore, USA) and ethanol precipitation. Coding exons one and two were joined together in a second PCR reaction using exon 1F and exon 2R primers. The product of this reaction was mixed with amplified exon 3 DNA and joined together in a PCR reaction using exon 1F and exon 3R primers. The full length open reading frame cDNA fragment was purified following agarose gel electrophoresis as described above and digested with Hind III and Bam HI (Promega, UK). The digested DNA was purified by phenol/chloroform extraction and precipitated with a mixture of ammonium

acetate/ethanol/glycogen (Roche, UK). A proportion of the purified DNA was ligated into Hind III- Bam H I digested pcDNA3.1 using T4 DNA ligase and used to transform competent E.coli Top10 (Invitrogen, UK). Cloned plasmid DNA containing insert was subjected to automated DNA sequencing in both directions using T7 and BGH reverse primers to confirm the sequence of mouse gpr147 cDNA.

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