The structure and function of the human ghrelin receptor

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ABSTRACT

The peptide hormone, ghrelin, exerts its physiological effects through a G-protein-coupled receptor called the ghrelin-R. The ghrelin-R displays a high degree of constitutive activity, signalling through the inositol phosphate pathway in the absence of bound agonist.

TMs III and VI have been reported to be central to the activation of Family A GPCRs, with interactions between the two helices stabilising the ground state. During activation conformational rearrangements result in these interactions being broken, with new contacts forming and stabilising the active state. Investigation of the ghrelin-R constitutive activity gives an insight into the mechanisms involved in receptor activation. In this study the role of specific individual residues in the ghrelin-R has been investigated and the effect of disrupting or introducing intramolecular interactions was addressed. Site-directed mutagenesis and functional assays revealed that ghrelin-R constitutive activity can be increased and decreased with mutation of residues within the TM domains, specifically TMs III, VI and VII.

The extracellular loops have been found to be involved in ligand binding and activation in a number of Family A GPCRs. The residues within ECL2 of the ghrelin-R were systematically mutated to alanine to determine their role. In particular, one residue, Asn196, was identified as being critical in ghrelin-R function and may be forming stabilising interactions which maintain ghrelin-R constitutive activity.

The data presented in this thesis provide an insight into the structure and function of the ghrelin-R and the underlying molecular mechanisms of ghrelin-R constitutive activity.

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ABBREVIATIONS

Throughout this thesis, abbreviations are used as recommended by the Journal of Biological Chemistry. In addition, the following abbreviations have been used:

5-HT	5-hydroxytryptamine
7TM	seven transmembrane
$A_{2A}R$	A_{2A} adenosine receptor
AC	adenylyl cyclase
ACTH	adrenocorticotrophin hormone
AgRP	Agouti-related peptide
ARC	arcuate nucleus
$AT_{1a}R$	Angiotensin II type 1a receptor
AVP	[arginine^8]vasopressin
BRET	bioluminescence resonance energy transfer
bRho	bovine rhodopsin
BSA	bovine serum albumin
C5aR	C5a anaphylatoxin chemotactic receptor
CAM	constitutively active mutation
cAMP	cyclic 3', 5'-adenosine monophosphate
CART	cocaine and amphetamine-regulated transcript
CB_1 receptor	cannabinoid receptor 1
CB_2 receptor	cannabinoid receptor 2
CRE	cAMP responsive element
CREB	cAMP responsive element binding protein
D_1R	dopamine receptor 1
D_2R	dopamine receptor 2
D_4R	dopamine receptor 4
DAG	1,2-diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
ECL	extracellular loop
ELISA	enzyme-linked immunosorbent assay
ERK1/2	extracellular regulated protein kinase 1/2
FBS	foetal bovine serum
FFAR	free fatty acid receptor
FRET	fluorescence resonance energy transfer
FSHR	follicle-stimulating hormone receptor
GABA	γ-aminobutyric acid
$GABA_bR$	GABAb receptor
GAP	GTPase activating protein
GED	GTPase effector domain
GEF	guanine nucleotide exchange factor
GFP	green-fluorescent protein
GH	growth hormone
ghrelin-R	ghrelin receptor
GHRH-R	growth hormone-releasing hormone receptor
GHS	growth hormone secretagogue
GHS-R1a	growth hormone secretagogue receptor 1a (ghrelin-R)
GHS-R1b	growth hormone secretagogue receptor 1b

GnRHR	gonadotropin-releasing hormone receptor
GOAT	ghrelin *O*-acyltransferase
GPCR	G-protein-coupled receptor
G-protein	guanine nucleotide binding protein
GRK	G-protein-coupled receptor kinase
GαCT	C-terminal synthetic peptide of transducin
$G\alpha_t$	transducin
H_1R	histamine receptor 1
H_2R	histamine receptor 2
H_4R	histamine receptor 4
HA	Haemagglutinin
HEK	human embryonic kidney
HRP	horse radish peroxidase
ICL	Intracellular loop
InsP	inositol phosphate
$InsP_2$	inositol diphosphate
$InsP_3$	inositol 1,4,5-trisphosphate
LB	luria broth
LHR	luteinising hormone receptor
mAChR	muscarinic acetylcholine receptor
MAP	mitogen activated protein
MC4R	melanocortin 4 receptor
MSH	melanocyte-stimulating hormone
NPY	neuropeptide Y
NT-R	neurotensin receptor
OPD	*O*-phenlyenediamine dihydrochloride
OR	opioid receptor
P2Y1	purinoceptor P2Y1
P2Y6	purinoceptor P2Y6
PAF	platelet activating factor
PAGE	polyacrylimide-gel-electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PDL	poly-D-lysine
PEI	polyethylenimine
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
POMC	proopiomelanocortin
PTHR	parathyroid hormone receptor
PWS	Prader-Willi syndrome
RAMP	receptor activity-modifying protein
RGS	regulators of G-protein specificity
rho	rho small GTPase
Rho	rhodopsin
RTK	receptor tyrosine kinase
SDS	sodium dodecyl-sulphate
SP-analogue	[D-Arg^1, D-Phe^5, D-$Trp^{7,9}$, Leu^{11}]-Substance P
SST	somatostatin
T4L	T4 lysozyme
TBE	tris-borate-EDTA buffer

TBS	tris-buffered saline
TM	transmembrane
TRHR	thyrotropin releasing hormone receptor
TSHR	thyroid-stimulating hormone receptor
UBD	ubiquitin binding domain
$V_{1a}R$	vasopressin 1a receptor
V_2R	vasopressin 2 receptor
VPAC	vasoactive intestinal polypeptide/pituitary adenylate cyclase activating peptide
WT	wild-type
$\alpha_{1a}AR$	α_{1a} adrenoceptor
$\alpha_{1b}AR$	α_{1b} adrenoceptor
α_2AR	α_2 adrenergic receptor
β_1AR	β_1 adrenergic receptor
β_2AR	β_2 adrenergic receptor

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CHAPTER 1.

INTRODUCTION

1.1 G-Protein-Coupled Receptors

G-protein-coupled receptors (GPCRs) are seven transmembrane (TM) helix proteins responsible for the majority of signal transduction that occurs across cell membranes. Members of the GPCR superfamily are activated by a broad range of ligands from biogenic amines, peptides, glycoproteins, lipids, nucleotides and ions to proteases and, in the case of the opsins, light (Kolakowski, 1994). With approximately 2 % of genes within the human genome encoding GPCRs (more than 800 identified), they comprise the largest family of proteins (Fredriksson *et al.*, 2003; Klco *et al.*, 2005; Perez *et al.*, 2005). The diverse role of GPCRs in regulating physiological processes makes the receptor superfamily a major pharmaceutical target; almost 60 % of approved drugs target GPCRs (Muller, 2000). In addition, mutations that occur within the receptors are responsible for many human diseases, increasing the importance of GPCR research to the pharmaceutical industry.

1.1.1 GPCR Structure and Classification

GPCRs have a common molecular architecture, consisting of seven membrane-spanning α-helices, connected by alternating intracellular loops (ICLs) and extracellular loops (ECLs), with an extracellular amino terminus (N-terminus) and an intracellular carboxyl terminus (C-terminus) (Fig 1.1). Resolved to 2.8 Å, by X-ray crystallography in the year 2000, the crystal structure of ground-state, retinal bound, bovine rhodopsin (bRho) advanced understanding of the complex structure of GPCRs. The crystal structure confirmed the presence of the 7TM domains and identified an intracellular amphipathic eighth helix (HelixVIII), lying almost perpendicular to TMVII, and parallel to the plasma membrane. HelixVIII follows the conserved NPxxY motif at the C-terminal end of TMVII and ends at palmitoylated double cysteine residues, Cys322 and Cys323 (Palczewski *et al.*, 2000) (Fig 1.2). This first crystal structure, along with subsequent advances and refinements, identified the helices as being irregular, with a number displaying tilts relative to the membrane, kinked architecture, or a mixture of α- and 3_{10} helices (Palczewski *et al.*, 2000; Okada *et al.*, 2001a; Riek *et al.*, 2001; Teller *et al.*, 2001; Krebs *et al.*, 2003).

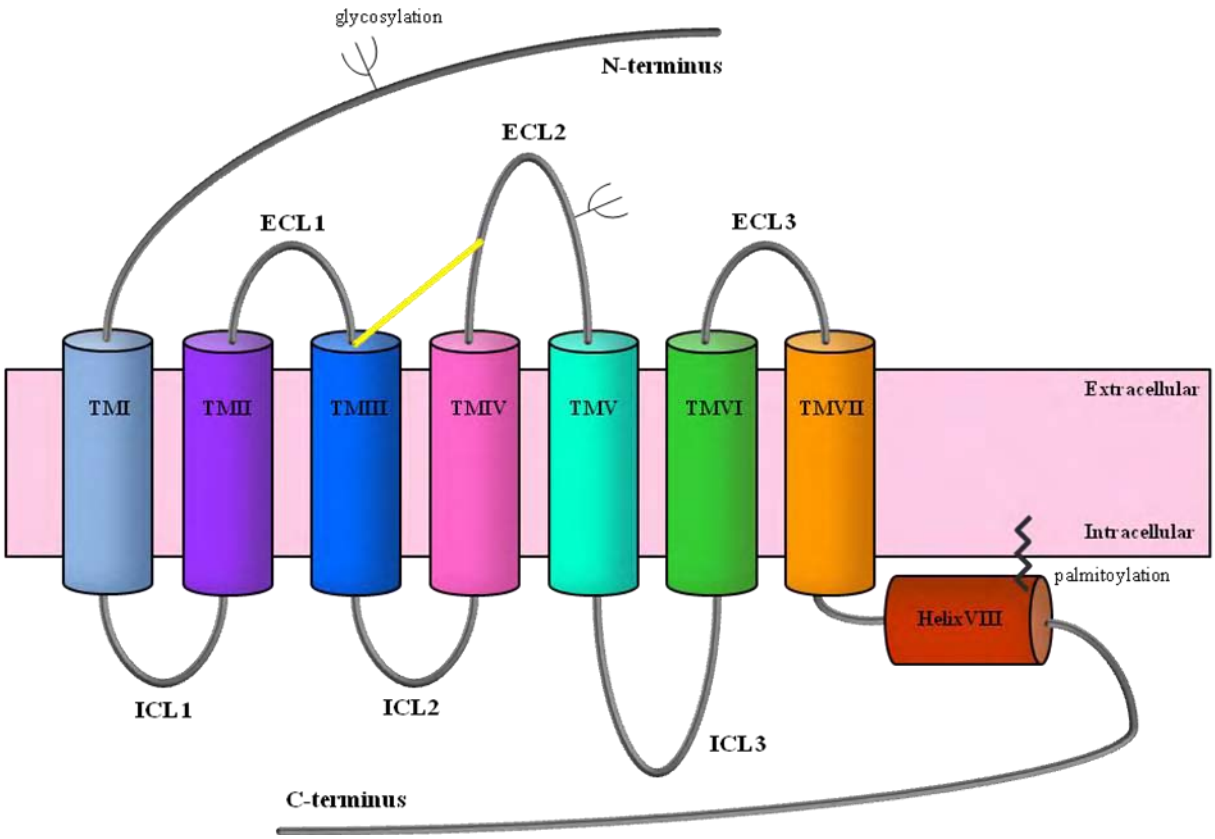


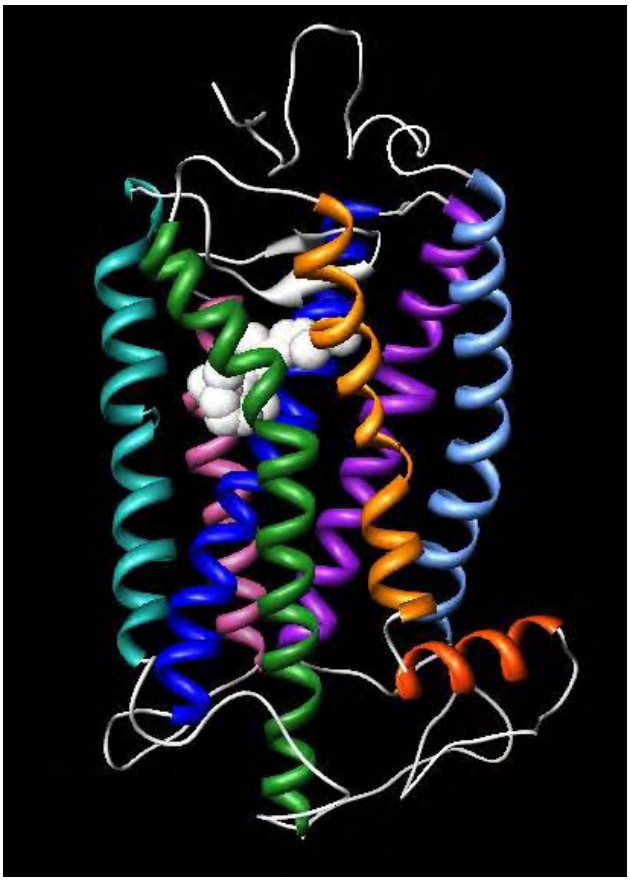
Figure 1.1 Schematic representation of a typical Family A GPCR: The 7TM helical domains of the receptor are connected by alternating intracellular and extracellular loops. Glycosylation sites are often found at the N-terminus and within the ECLs. An intramolecular disulphide bond is found between TMIII and ECL2. The HelixVIII region is frequently palmitoylated at conserved cysteine residues, anchoring the receptor C-terminus to the membrane, and essentially creating a fourth intracellular loop.

The TM helices are bent or tilted in relation to each other and to the plasma membrane. TMI, TMIV, TMVI and TMVII have been shown to be bent due to the presence of α-helix-disrupting proline residues, with TMVI having a significant "kink" at Pro267$^{(6.50)}$ in Rho, but all of the helices having a certain amount of curving (Palczewski *et al.*, 2000; Krebs *et al.*, 2003). Glycine, serine, threonine and cysteine have also been shown to induce irregularities within helices as a result of their hydrogen bonding capabilities (Teller *et al.*, 2001). Each helix was determined to be at a different plane within the plasma membrane. TMIV, TMVI and TMVII were observed to be almost parallel to the membrane plane, whereas TMs I, II, V and particularly III, are tilted in relation to the plasma membrane (Krebs *et al.*, 2003) (Fig 1.2).

Crystallization of GPCRs has proved difficult due to the flexibility of GPCRs and the negative effect that the detergents, used in purification processes, have on receptor stability within the membrane. The high availability of bRho, from rod cells, contributed to the crystallization of this Family A GPCR (Filipek *et al.*, 2003). Since the crystal structure of bRho was determined in the year 2000, a number of Family A GPCR crystal structures have been resolved. The structure of the β_2 adrenergic receptor (β_2AR) (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007), the A_{2A} adenosine receptor (A_{2A}R) (Jaakola *et al.*, 2008), the β_1 adrenergic receptor (β_1AR) (Warne *et al.*, 2008), the squid rhodopsin (Murakami *et al.*, 2008), and bovine opsin (Park *et al.*, 2008; Scheerer *et al.*, 2008) have all been determined in recent years, substantially enhancing knowledge of Family A GPCRs.

The structure of the β_2AR was resolved to 3.4/3.7 Å, bound to an inverse agonist, carazolol, and in complex with a Fab5 antibody fragment, replacing ICL3 (Rasmussen *et al.*, 2007). In the same year, the crystal structure of a β_2AR-T4 lysozyme complex, also bound to carazolol, was resolved to 2.4 Å (Cherezov *et al.*, 2007). The structures revealed a number of key differences to the bRho structure, principally, weaker interactions at the cytoplasmic ends of TMIII and TMVI, possibly explaining the constitutive activity of the β_2AR and giving more detailed insights into the structural basis of ligand-independent activation. Despite being bound to an inverse agonist, the cytoplasmic segments of the β_2AR have a more open arrangement than that seen for Rho. The ionic lock, found between Arg$^{3.50}$ and Glu$^{6.30}$ at the cytoplasmic ends of TMs III and VI in Rho (Palczewski *et al.*, 2000), and suggested from site-directed mutagenesis studies to occur in a number of Family A GPCRs (Ballesteros *et al.*, 2001; Greasley *et al.*, 2002; Shapiro *et al.*, 2002; Springael *et al.*, 2007) is not present in the β_2AR crystal structures (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007). The β_2AR structures

(a)



(b)

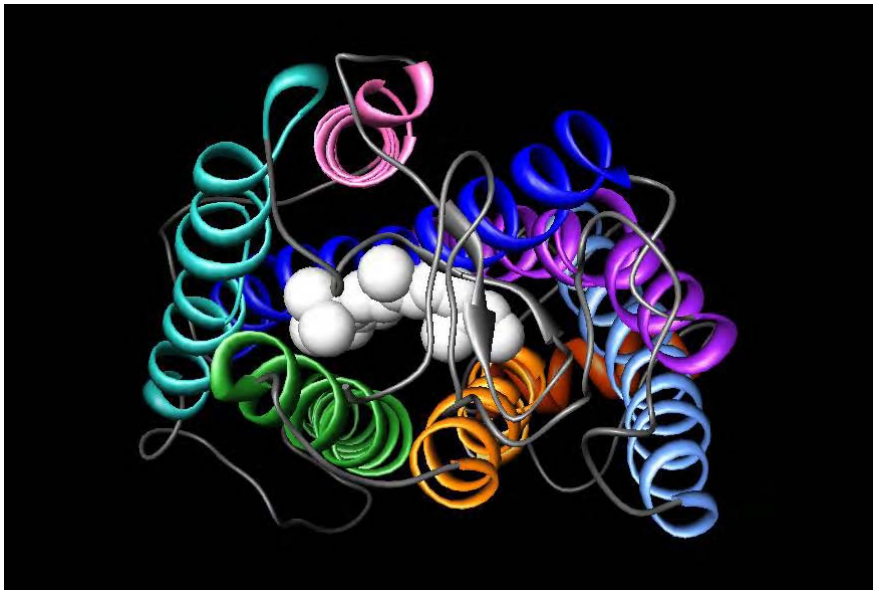


Figure 1.2 Crystal structure of bRho resolved to 2.8 Å: The 7TM domains and HelixVIII of bRho viewed (a) through the membrane and (b) from the extracellular side. The anticlockwise arrangement of the helices can be seen and retinal is represented within the binding pocket (white). TMI (**light blue**), TMII (**purple**), TMIII (**blue**), TMIV (**pink**), TMV (**turquoise**), TMVI (**green**), TMVII (**orange**) and HelixVIII (**brown**) (Palczewski *et al.*, 2000).

more closely resemble that of opsin, in which $Arg^{3.50}$ and $Glu^{6.30}$ form new stabilising interactions with TMs V and VI (Park *et al.*, 2008).

Despite a common structural topology, GPCRs within the superfamily share very little overall sequence similarity. The TM domains of GPCRs display the greatest sequence homology (Kobilka, 2007), whereas both the ICL and ECL regions have low homology, even amongst receptors from the same receptor family (Lawson *et al.*, 2004). The N-terminal domains have been shown to have the greatest sequence diversity (Kobilka, 2007). Based on receptor sequence homology, and the presence of various conserved residues and motifs, GPCRs can be divided into three major (Family A, Family B and Family C) and three minor (Families D, E and F) subfamilies, which encompass all vertebrate and invertebrate receptors (Kolakowski, 1994). Human GPCRs can be further subdivided into five classes; rhodopsin-like (class A), secretin-like (class B), glutamate (class C), adhesion and frizzled/taste2 (Fredriksson *et al.*, 2003).

Family A (rhodopsin/β_2-adrenergic receptor-like) is the largest GPCR subfamily, representing approximately 90 % of all GPCRs. Given the size of the family and the diversity of endogenous ligands, it is not surprising that there is low overall sequence homology amongst members of Family A. There are however a number of highly conserved residues and motifs which provide important structural and functional roles. These include two cysteine residues, located at the top of TMIII and within ECL2, that form a stabilising intramolecular disulphide bond; the D/ERY motif at the cytoplasmic end of TMIII, that is important for maintaining the ground state and for receptor activation and the NPxxY motif in TMVII, which is involved in a number of intramolecular interactions, both in the inactive (R) and active (R*) state. A residue numbering system was introduced by Ballesteros and Weinstein to enable direct comparison between members of Family A. The most conserved residue in each helix was assigned the number 50 and each helix numbered 1-7. Amino acids are then numbered according to their position within the helix, relative to the most conserved residue. For example, the arginine of the D/ERY motif in TMIII is the most conserved residue within this helix and is therefore numbered $Arg^{3.50}$. The aspartate/glutamate to the N-terminal side of $Arg^{3.50}$ is $Asp/Glu^{3.49}$, and the tyrosine to the C-terminal side is $Tyr^{3.51}$ (Ballesteros *et al.*, 1992) (conserved residues discussed further in Chapter 3).

Family B receptors are the glucagon-like/secretin-like receptors and are characterised by a large N-terminus, compared to Family A GPCRs. The N-termini contain a number of cysteine

residues that form intramolecular disulphide bonds and the N-terminus is extensively involved in ligand binding in this receptor class. Family C receptors are the metabotropic neurotransmitter-related receptors and generally have a larger N-terminus than either the Family A or B receptors (~600 amino acids). The large N-terminal domains of Family C receptors have been implicated in dimerisation, which is essential for Family C GPCR function (section 1.7). There are members of all three families that contain the two highly conserved cysteine residues at the top of TMIII and ECL2, that form the disulphide bond, but apart from this common feature there are no other conserved residues within all three families (Gether, 2000).

The three minor GPCR subfamilies are Family D (Ste2 receptors) and Family E (Ste3 receptors), which are the yeast pheromone receptors and Family F receptors which are present only in the amoeba *Dictyostelium discoideum* (Gether, 2000).

1.2 G-proteins

GPCRs signal via membrane-associated, heterotrimeric guanine nucleotide binding proteins (G-proteins) which consist of an α-, β- and γ-subunit. G-proteins can be grouped into four main subtypes, $G_{q/11}$, G_i, G_s and $G_{12/13}$ depending on the α-subunit present (Wess, 1997). There are sixteen α-subunits, five β-subunits and fourteen γ-subunits currently identified (Milligan *et al.*, 2006).

All G-proteins are activated by their GPCRs in essentially the same way despite the diversity of G-protein subtypes provided by the variety of subunits. After a ligand binds to a GPCR the conformational changes that occur within the receptor allow the α-subunit of the G-protein to couple to the GPCR. The conformational changes increase the affinity of the receptor for the G-protein and the rearrangement of the cytoplasmic ends of TMs III and VI allow the C-terminal end of the G-protein to couple to the receptor. It is known that the sites of G-protein interaction are located within ICL2, ICL3, the C-terminal domain and within a G-protein binding pocket located between the TM helices (Hill-Eubanks *et al.*, 1996). Activation of the GPCR exposes key residues to the G-protein that are otherwise protected from interaction with the G-protein in the inactive receptor state. The crystal structure of opsin in the G-protein interacting conformation was resolved using the opsin protein and 11 amino acids that made up a synthetic peptide derived from the C-terminus of the Gα subunit of transducin (GαCT). The crystal structure shows the interaction between $Arg^{3.50}$ of the D/ERY motif and the tip of

GαCT (Scheerer *et al.*, 2008). It has been noted that some GPCRs pre-couple to their target G-proteins and therefore do not require the same agonist-induced conformational rearrangements suggesting that other mechanisms must be involved (Zezula *et al.*, 2008). Despite the common G-protein binding and activation mechanisms, the specific interactions vary between receptors and G-protein subtypes, explaining why GPCRs can only activate a limited subset of G-proteins, and thus dictates receptor-G-protein specificity (Wess, 1997). Residue 5.58 in TMV is one residue that has been found to be involved in the selectivity of receptor-G-protein interactions. Located at the cytoplasmic interface of TMV and ICL3, 5.58 can dictate G-protein subtype interactions depending on the amino acid located at this locus (discussed further in Chapter 4) (Biebermann *et al.*, 1998; Erlenbach *et al.*, 1998).

The coupling of a G-protein to its GPCR causes the exchange of GDP, bound to the G-protein α-subunit, for GTP, thus activating the G-protein. This, in turn, allows the α-GTP complex to dissociate from the GPCR and from the G-protein βγ-subunits. The α-GTP and the βγ-subunits are then free to interact with their specific target proteins and initiate down-stream signalling cascades (Fig 1.3). The intrinsic GTPase activity of the α-subunit leads to hydrolysis of the GTP to GDP and allows re-association of the α- and the βγ-subunits at the plasma membrane (Rens-Domiano *et al.*, 1995). Although the Gα subunit possesses high GTPase activity, a family of GTPase activating proteins (GAPs) called regulators of G-protein signalling (RGS) proteins act as regulators of signalling by increasing the GTPase activity of the Gα subunit (Ross *et al.*, 2000) (Fig 1.3).

1.3 GPCR activation and intracellular signalling

Ligand binding to GPCRs results in the activation of the receptor and the initiation of subsequent intracellular signalling cascades. GPCR activation involves the disruption of the stabilising interactions that maintain the receptor in the R state in the absence of bound agonist. Conformational changes occurring within the helical bundle after agonist binding, result in the formation of the R* conformation, which is capable of binding G-proteins. A common mechanism is believed to occur in all GPCRs, despite profound differences in agonists and in the G-proteins that are targeted. It is now accepted that activation of Family A GPCRs requires the inward movement of the extracellular end of TMVI, towards TMIII, resulting in the movement of the cytoplasmic end of TMVI away from TMIII, and thus revealing a G-protein binding cavity. This movement has been demonstrated using spin

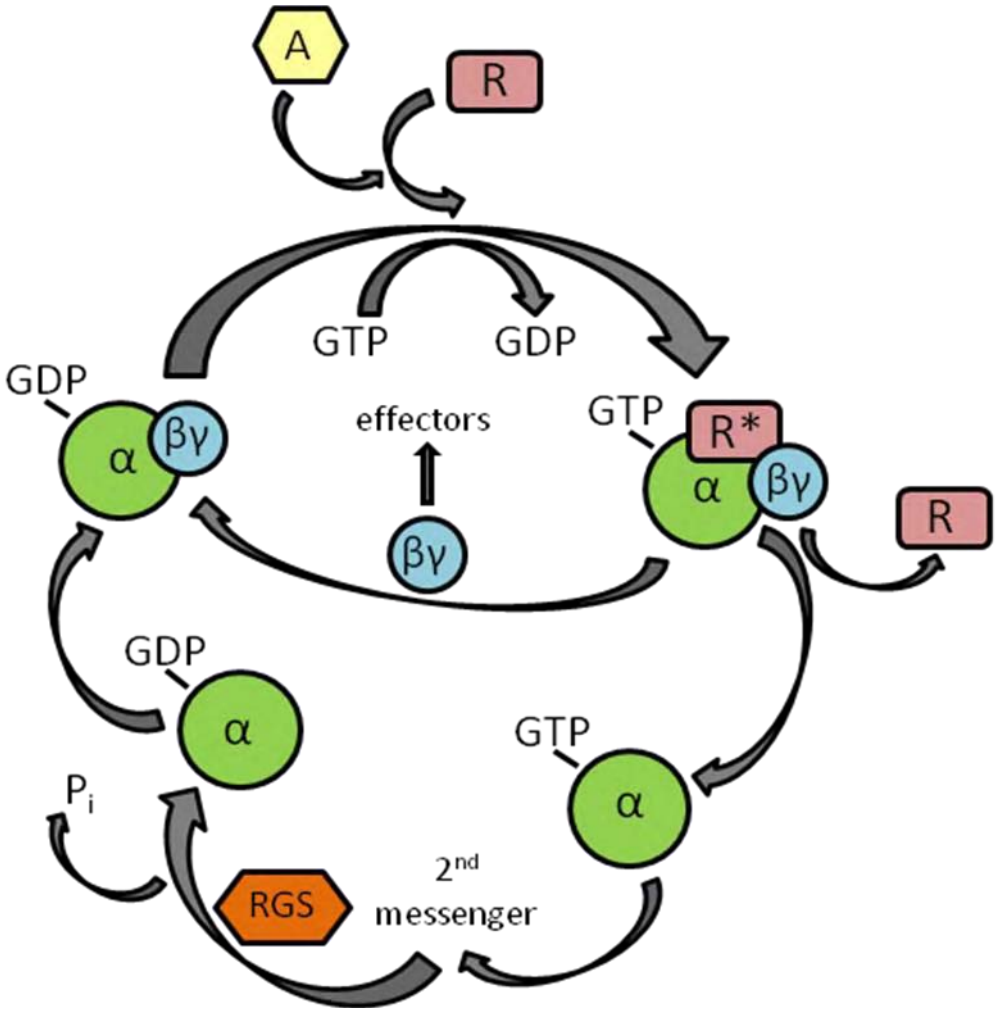


Figure 1.3 The G-protein cycle: Activation of a GPCR (**R**) by agonist (**A**) leads to association of a heterotrimeric G-protein. GDP is exchanged for GTP at the α-subunit to produce an active G-protein. The βγ-subunits dissociate from the α-subunit and all dissociate from the receptor. The α-GTP and βγ-subunits are then able to activate or inhibit target effector molecules leading to signal transduction via second messengers within the cell. The intrinsic GTPase activity of the α-subunit, accelerated by a GAP such as an RGS protein, hydrolyses GTP to GDP with the release of inorganic phosphate. The α- and βγ-subunits are then able to re-associate (Image adapted from Rens-Domiano *et al.*, 1995; Milligan *et al.*, 2006).

labelling (Farrens *et al.*, 1996; Hubbell *et al.*, 2003), fluorescent probes (Gether *et al.*, 1997; Jensen *et al.*, 2000), cysteine cross-linking (Ward *et al.*, 2003; Han *et al.*, 2005) and metal-ion site engineering (Elling *et al.*, 1997; Elling *et al.*, 2006) and is termed the 'global toggle switch' (Elling *et al.*, 2006; Schwartz *et al.*, 2006).

1.3.1 Key residues and motifs in Family A GPCRs

Key activation motifs and residues are highly conserved throughout Family A GPCRs. The 'global toggle switch' supports the movement of TMVI in a "see-saw-like" manner around a conserved TM proline ($Pro^{6.50}$) allowing the association of G-proteins and other signalling molecules (Elling *et al.*, 2006). $Trp^{6.48}$ of the CWxP motif found in TMVI is highly conserved (71 %) in Family A GPCRs (Mirzadegan *et al.*, 2003). $Trp^{6.48}$ is thought to act as a rotamer toggle switch, modulating the proline-induced kink within TMVI. Upon receptor activation $Trp^{6.48}$ undergoes a conformational rearrangement, releasing interactions with TMVII and forming new interactions with TMs III and V. The altered conformation of $Trp^{6.48}$ and the neighbouring $Cys^{6.47}$ allows for a decrease in the bend-angle of the proline-induced kink, straightening TMVI and moving the cytoplasmic end of TMVI away from TMIII (Shi *et al.*, 2002b; Ruprecht *et al.*, 2004; Schwartz *et al.*, 2006)

The highly conserved D/ERY motif at the bottom of TMIII has been shown to play a significant role in maintaining the ground state of GPCRs (Sheikh *et al.*, 1996). The arginine ($Arg^{3.50}$) residue of the D/ERY motif is highly conserved (96 %) in Family A GPCRs (Mirzadegan *et al.*, 2003). $Arg^{3.50}$ forms a salt bridge interaction with the neighbouring aspartate/glutamate ($Asp/Glu^{3.49}$) residue. Protonation of the aspartate/glutamate residue found at position 3.49 results in a disruption of the ionic interaction with $Arg^{3.50}$ (Arnis *et al.*, 1994: Scheer et al., 1996). The interaction is broken upon receptor activation, with both $Glu^{3.49}$ and $Arg^{3.50}$ forming new contacts (Park *et al.*, 2008). A network of hydrogen bonds known as the "ionic lock" has been proposed to form between the D/ERY motif in TMIII and residues within TMVI in certain members of the GPCR superfamily. This ionic interaction acts to constrain TMIII and TMVI at their cytoplasmic ends, maintaining the inactive state of the receptor in the absence of an agonist. Mutagenesis studies carried out on the β_2AR demonstrated that $Arg^{3.50}$ not only interacts with $Asp^{3.49}$, but also with a residue at the intracellular face of TMVI, $Glu^{6.30}$, via an interhelical ionic bond (Ballesteros *et al.*, 2001). It has been predicted, for some GPCRs, that the inactive receptor is constrained by interactions between the $Asp/Glu^{3.49}$, $Arg^{3.50}$ and $Glu^{6.30}$ along with other TMVI residues, and that activation involves the breaking of these interactions. Mutation of these critical residues can

lead to conformational changes that allow the active receptor conformation to be adopted in the absence of agonist, thus inducing constitutive activity (Alewijnse *et al.*, 2000; Ballesteros *et al.*, 2001). The identification of constitutively active mutations (CAMs) and mutations that decrease constitutive activity has aided investigation of the intramolecular interactions involved in activation of GPCRs. The crystal structure of bRho demonstrates the presence of the ionic lock interaction between TMs III and VI (Palczewski *et al.*, 2000) however, in all of the other Family A GPCR crystal structures resolved to date, the $Arg^{3.50}$ and $Glu^{6.30}$ side chains are too far apart for the interaction to occur (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Jaakola *et al.*, 2008; Warne *et al.*, 2008) (Fig 1.4). However all of these structures were obtained from GPCRs occupied by inverse agonists, which might affect an 'ionic lock-like' interaction. There is the additional complication of the T4L and Fab fragment in the β_2AR crystal structures, which may have affected interactions at the cytoplasmic ends of the TM helices allowing residual constitutive activity in the presence of the inverse agonist, carazolol (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Audet *et al.*, 2008) or may disrupt intramolecular interactions by themselves interacting with TM residues (Rosenbaum *et al.*, 2007). In the β_2AR (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007), the A_{2A}R (Jaakola *et al.*, 2008) and the β_1AR (Warne *et al.*, 2008), other interactions have been found to occur which stabilise the inactive receptor conformation. These interactions are discussed further in Chapter 4.

Other interactions are known to stabilise the R* state of Family A GPCRs. An interaction occurs between $Asn^{7.49}$ of the NPxxY motif and $Asp^{2.50}$ in TMII, upon receptor activation. $Asn^{1.50}$, $Asp^{2.50}$ and $Asn^{7.49}$ have been shown to participate in a network of hydrogen bonds with water molecules in Family A GPCRs. In the 5 hydroxytryptamine receptor 2A ($5HT_{2A}$R), as well as other Family A receptors, a hydrogen bond network between $Tyr^{7.53}$, $Asn^{1.50}$, $Asp^{2.50}$ and $Asn^{7.49}$ acts to stabilise the TMVII orientation (Konvicka *et al.*, 1998). $Asn^{7.49}$ appears to act as an on/off switch, changing conformation after agonist binding, stabilising the R* state (Govaerts *et al.*, 2001; Urizar *et al.*, 2005) (Chapter 4).

1.3.2 Models of receptor activation

Pharmacological models have been proposed to explain the signalling of GPCRs after ligand binding. Based on the laws of mass action a number of models have been described. Initially, the two-state model was proposed for receptor activation, whereby receptors exist in dynamic equilibrium between the R and R* states (Clark, 1937).

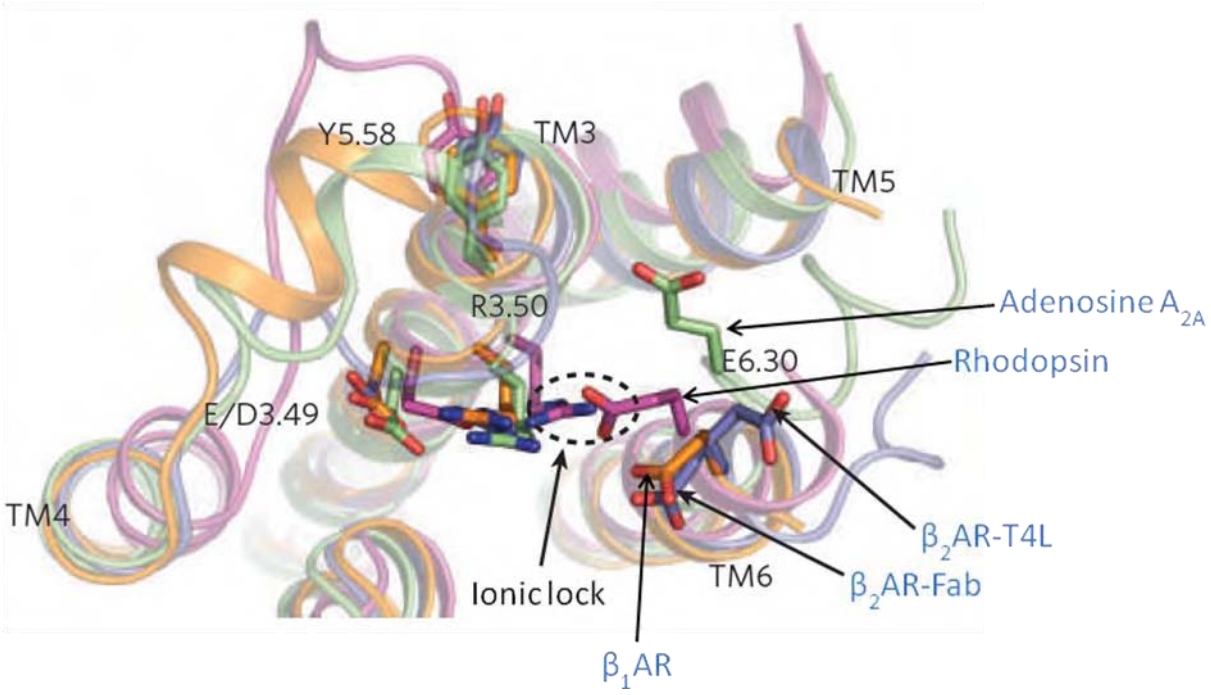


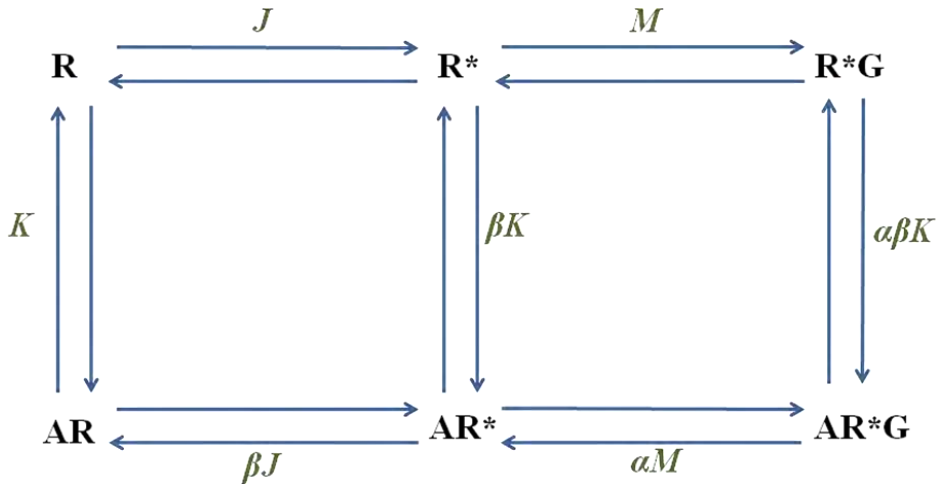
Figure 1.4 Crystal structure of Rho, the β_2AR-T4L and β_2AR-Fab, the β_1AR and the A_{2A}R: In the Rho crystal structure $Arg^{3.50}$ and $Glu^{6.30}$ are close enough to interact, producing the ionic lock and stabilising the inactive receptor conformation by maintaining the cytoplasmic ends of TMs III and VI in close proximity, preventing G-protein coupling. In the other crystal structures, $Arg^{3.50}$ and $Glu^{6.30}$ are too far apart for an ionic interaction to occur. **Green** is the A_{2A}R, **pink** is Rho, **blue** is the β_2AR and **orange** is the β_1AR. (Image taken from Rosenbaum *et al.*, 2009)

The actions of agonists and inverse agonists stabilise the R* and R receptor conformations, respectively. The ternary complex model is an extension of the two-state model and proposes equilibrium between R and inactive agonist-bound receptor (AR), agonist-bound receptor coupled to G-protein (ARG) and the receptor coupled to G-protein (RG) (De Lean *et al.*, 1980). With the discovery of naturally constitutively active receptors and CAMs, this model had to be adapted, producing the extended ternary complex (ETC) model (Samama *et al.*, 1993) and the cubic ternary complex (CTC) model (Weiss *et al.*, 1996) both of which take into account the ligand-independent activation of receptors and subsequent activation of associated G-proteins (Fig 1.5).

The ETC model describes the action of agonists, antagonists and inverse agonists on receptor activation, and takes into account the constitutive activity observed for a number of GPCRs (Fig 1.5 (a)). Six receptor species are proposed to exist at equilibrium; R, R*, R*G, AR, AR* and AR*G. The equilibrium constant, *J*, determines the equilibrium between R and R*. βJ defines the allosteric effect of the ligand (A) on *J* and the ability of A to activate the receptor. The constant, *M*, defines the affinity of R* for the G-protein (G), and αM the affinity of the activated ligand-bound (AR*) receptor for G. The equilibrium constant *K* is the affinity of the ligand for the receptor, in the active state (βK) and the active G-protein-bound state ($\alpha\beta K$). Agonists have a high affinity for R* and therefore exhibit positive efficacy and increase both α and β, increasing the population of R* due to a shift in equilibrium. Classical antagonists do not alter the equilibrium, being neutral, and therefore do not influence the populations of R or R*. Inverse agonists, presenting negative efficacy, have a higher affinity for R and decrease α and β, favouring the inactive conformations. The ETC does not however take into account the coupling of G-proteins to inactive receptors. The CTC model was proposed to include inactive GPCRs coupling to G-proteins, which has since been proven experimentally (Monczor *et al.*, 2003) (Fig 1.5 (b)).

A limitation of the CTC model is that it assumes that a receptor can exist in two states with respect to the activation of G-proteins; active or inactive. In fact, GPCRs appear to demonstrate much more complex behaviour, existing in a possibly limitless number of conformations. These theories lead to the proposal of the probabilistic model, whereby receptor conformations are influenced, not only by the type of ligand, but by the efficacy of the ligands. Therefore, different receptor conformations are more or less likely to be adopted, depending on the binding of ligands, the ligand efficacy and the binding of G-proteins, as well as other influencing factors such as accessory proteins and GPCR oligomers (Kenakin, 1995).

(a)



(b)

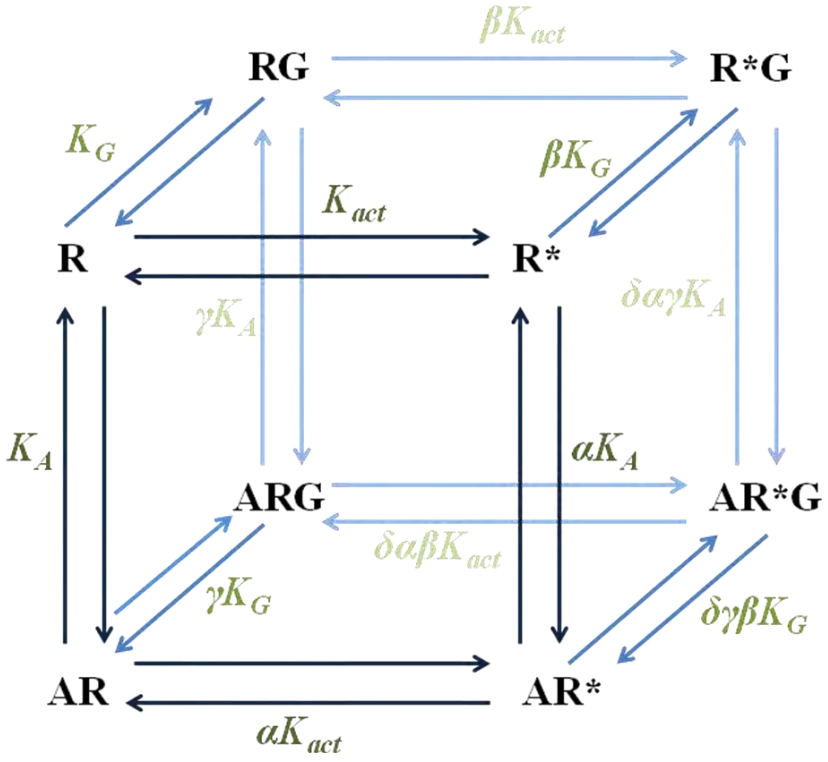


Figure 1.5 Extended ternary complex model and cubic ternary complex model of receptor activation: (a) The ETC model shows the equilibrium between six receptor species, R, R*, AR*, AR, R*G and AR*G. *K* represents the association constant of the receptor and ligand, *J* represents the equilibrium constant between the active and inactive receptor states and *M* represents the constant between the active states and G-protein-coupled states. (b) The CTC model builds on the ETC model to incorporate the ability of an inactive receptor to couple to a G-protein (Images adapted from Weiss *et al.*, 1996).

1.3.3 Classical signalling pathways

Activation of G-proteins results in the initiation of intracellular signalling cascades. Three classical signalling cascades have been identified for members of the GPCR superfamily. These involve the activation or inhibition of adenylyl cyclase (AC) and the activation of phospholipase C (PLC) (Fig 1.6).

1.3.3.1 The adenylyl cyclase signalling pathway

The $G\alpha_s$ and $G\alpha_i$ G-proteins couple to AC and act to stimulate or inhibit AC respectively. AC regulates the intracellular concentrations of cyclic 3', 5'-adenosine monophosphate (cAMP) as the activation of AC induces the synthesis of cAMP from ATP within the cell. cAMP, the second messenger, then activates cAMP-dependent protein kinases, specifically protein kinase A (PKA). The protein kinases catalyse the phosphorylation of serine or threonine residues on other target proteins to activate or inhibit enzymes, transcription factors or ion channels and thus initiate a response within the cell (Rang, 2003). $G\alpha_i$ activation results in inhibition of AC and a subsequent reduction in PKA activity.

1.3.3.2 The phospholipase C signalling pathway

The $G\alpha_q$ and $G\alpha_{11}$ G-proteins activate PLCβ which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2) to the second messengers inositol 1,4,5-trisphosphate ($InsP_3$) and 1,2-diacylglycerol (DAG). $InsP_3$ acts to modulate intracellular calcium concentrations, primarily by its effect on the $InsP_3$ receptor, but also on plasma membrane and endoplasmic reticulum calcium channels. Ca^{2+} binds to the intracellular calcium receptor, calmodulin, producing a complex that is then able to activate the calmodulin pathway, bringing about cellular responses (Means *et al.*, 1980). Increased intracellular calcium causes protein kinase C (PKC) to accumulate at the plasma membrane. DAG then activates PKC, which results in the initiation of further signalling cascades by its effect on target proteins, enzymes and transcription factors.

1.3.4 Non-classical/G-protein-independent signalling pathways

As well as the G-protein-dependent pathways, GPCRs can activate G-protein-independent pathways such as the mitogen activated protein (MAP) kinase pathway and the small GTPases. The activation of alternative signalling cascades raises the question of the name 'GPCR' and has prompted the use of the term 7TM receptor in a number of instances to incorporate G-protein-independent signalling.

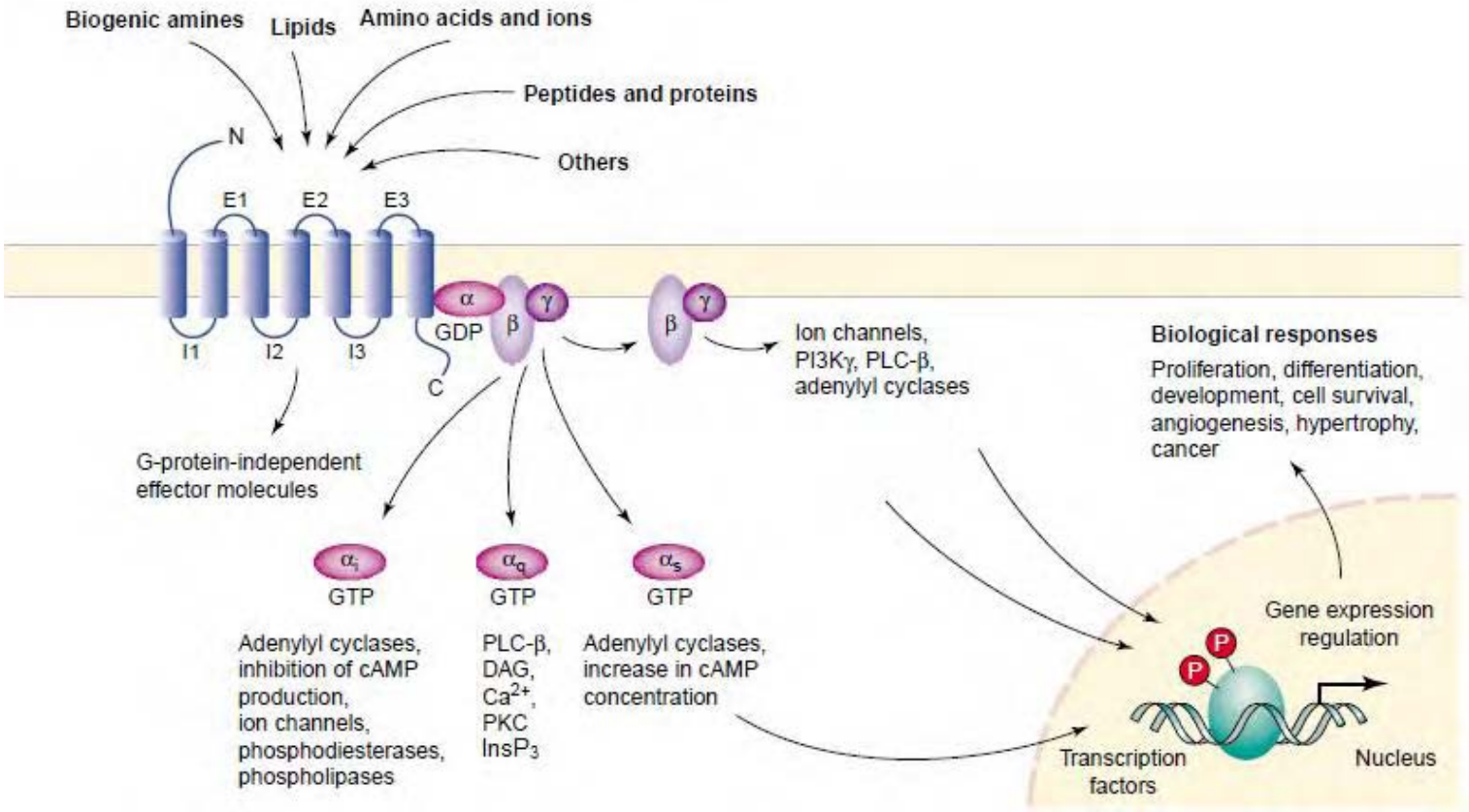


Figure 1.6 Intracellular signalling through GPCRs: G-proteins are activated by GPCRs after ligand binding resulting in the initiation of intracellular signalling cascades. The Gα subunit of the G-protein dictates the signalling pathway. $G\alpha_s$ and $G\alpha_i$ activate or inhibit AC respectively, leading to increases/decreases in the cAMP concentration within the cell. $G\alpha_{q/11}$ G-proteins activate PLCβ which in turn promotes the hydrolysis of PIP_2 to DAG and $InsP_3$. $InsP_3$ causes a release of Ca^{2+} from intracellular stores and DAG activates PKC. The second messenger signalling cascades result in activation or inhibition of enzymes and transcription factors which regulate gene transcription and result in various biological responses (Image adapted from Marinissen *et al.*, 2001).

1.3.4.1 Activation of MAP kinase pathways

Many GPCRs have been shown to interact with and activate members of the MAP kinase family. The three major pathways involve extracellular signal-related kinase 1/2 (ERK1/2), Jun-kinase and p38 (Luttrell *et al.*, 1999). The interactions can involve typical G-protein second messenger production pathways, transactivation of receptor tyrosine kinases (RTKs) or an interaction of the receptor with other proteins, such as β-arrestin (Gurevich *et al.*, 2006; Charest *et al.*, 2007). It has been found that RTKs can be 'transactivated' by GPCRs, where activation of the GPCR initiates tyrosine phosphorylation of RTKs and results in activation of ERK1/2 (Daub et al., 1997). β-arrestin was shown to be important in G-protein-independent angiotensin II-stimulated ERK1/2 activation, through the angiotensin II type 1 receptor (AT_1R) (Seta *et al.*, 2002). β-arrestin may act by initiating the assembly of the proteins involved in the MAP kinase signalling cascade into intracellular compartments, therefore directing the GPCR signalling pathways away from the G-protein-mediated production of second messengers and enhancing the efficiency of signalling to other pathways (Pierce *et al.*, 2001).

1.3.4.2 Activation of small G-proteins

GPCRs are also known to activate rho family GTPases which are small, monomeric G-proteins (Bhattacharya *et al.*, 2004). The rho family are regulators of a number of important processes that occur in all eukaryotic cells. The rho G-proteins are themselves regulated by signals that originate from many classes of receptors present at the cell surface and by other regulatory proteins (Kjoller *et al.*, 1999). Many of the GPCRs that can activate rho-dependant pathways are coupled to $G\alpha_q$ (Sah *et al.*, 2000). However $G\alpha_q$ does not interact with rho or the rho guanine nucleotide-exchange factors (GEFs) directly (Kjoller *et al.*, 1999; Sah *et al.*, 2000). $G\alpha_{12/13}$ G-proteins interact with rhoA directly, and many GPCRs that can couple to $G\alpha_{12/13}$ also couple to $G\alpha_q$ suggesting a mechanism by which $G\alpha_q$-coupled receptors can activate rhoA (Meyer *et al.*, 1999). Roles for rho, related to GPCRs, include inhibition of internalisation of the m1 muscarinic acetylcholine receptor (mAChR), and the m2 mAChR (Sah *et al.*, 2000).

1.4 Constitutive GPCR activity

Receptors exist in a dynamic equilibrium between the R and the R* states in the absence of ligands. This is known as the two-state activation model (De Lean *et al.*, 1980; Leff, 1995) (section 1.3.2). In receptors that display a high degree of constitutive activity, the bias is

toward the R* state resulting in receptor activation in the absence of bound agonist. Consequently, constitutively active receptors are able to adopt a structure that can activate G-proteins spontaneously (Parnot *et al.*, 2002).

Following the cloning of GPCRs, it was observed that recombinant expression in cultured cells at high levels could result in increased basal signalling. Following this, constitutively active receptors were identified after the observations that CAMs of GPCRs resulted in agonist-independent activation. By introducing CAMs into GPCRs, the interactions that constrain the inactive receptor can be abolished. CAMs can therefore be used to investigate the intramolecular interactions that dictate the active or inactive state of the receptor (Parnot *et al.*, 2002). After the evidence from CAMs and the development of more sensitive assays, able to detect agonist-independent activity even when at low levels, it became apparent that a number of wild-type (WT) GPCRs from Families A, B and C, display significant levels of constitutive activity (Seifert *et al.*, 2002; Smit *et al.*, 2007).

The β_2AR has been extensively studied and found to demonstrate a significant level of constitutive activity (Gotze *et al.*, 1994) which has been determined to be of importance to the physiological functioning of the receptor (Seifert *et al.*, 2002). WT constitutively active receptors have a broad number of physiological roles and could potentially act as "signalling set points" to counteract inhibitory mechanisms (Holst *et al.*, 2004b). It is however often difficult to determine and accurately assess constitutive activity *in vitro* as over-expression of receptors often results in a detectable increase in basal signalling that is not measurable in native systems (Holst *et al.*, 2004b). In contrast however, it is difficult to ensure total absence of endogenous agonists in native systems, and therefore, these systems do not necessarily make measuring GPCR constitutive activity easier than recombinant systems. Constitutive activity can also be particularly hard to detect and measure in certain cellular systems due to the inverse agonistic allosteric effects of sodium ions on receptor function (Milligan, 2003).

Many disease states are caused by naturally occurring CAMs within GPCRs. These may either be gain-of-function mutations or deleterious mutations, resulting in inhibition of cellular processes (Parnot *et al.*, 2002). In the thyroid-stimulating hormone receptor (TSHR), CAMs (D619G and A623I) were identified as being responsible for hyperthyroidism (Parma *et al.*, 1993), and a CAM in Rho (G90D) was found in people suffering from congenital night blindness (Rao *et al.*, 1994). In WT constitutively active receptors, mutations can result in a loss of basal signalling. A naturally occurring alanine to glutamate (A204E) mutation in the

ghrelin receptor (ghrelin-R) produces a functional receptor able to bind ghrelin and signal, but which lacks any constitutive activity resulting in short stature (Pantel *et al.*, 2006) or obesity (Wang *et al.*, 2004), again illustrating the physiological importance of the ligand-independent signalling.

1.5 Ligand classes and binding

The diversity of GPCRs is dictated by the wide range of endogenous ligands that are able to activate members of this receptor superfamily and require GPCRs to mediate physiological processes. GPCR ligands can be divided into five basic subtypes and each of these ligand classes bind to their receptors in a different manner (Fig 1.7); (i) Small biogenic amines (such as acetylcholine) and nucleotides bind to sites located deep within the TM helical bundle; (ii) peptide ligands bind to both the hydrophobic core created by the TM helices and to the extracellular domains; (iii) the small ligands Ca^{2+}, glutamate and neurotransmitters such as γ-aminobutyric acid (GABA) bind to the N-terminus and cause conformational rearrangements which allow the N-terminal domain to interact with the TM domains and ECLs, resulting in receptor activation; (iv) large ligands such as the glycoprotein hormones are known to bind to the extracellular regions, specifically the N-terminus; (v) protease ligands, such as thrombin, bind to the N-terminal domain and cleave it, allowing the truncated N-terminus to interact with the TM domains and with the ECLs. In this manner, the N-terminus functions as a 'tethered ligand'.

The binding sites of Family A GPCRs vary considerably with the type of ligand that activates the receptor, although the ECLs, N-terminus and the extracellular ends of the TM helices can all participate in formation of the binding cavity. Since the βAR (which bind diffusible ligands) crystal structures were resolved, various comparisons have been made between these and the binding site of Rho, which forms a covalent bond with 11-*cis* retinal within the binding pocket. In Rho (and opsin) a β-hairpin is formed by ECL2 which projects into the ligand binding crevice and forms a closed 'cap-like' structure, along with the N-terminus, protecting retinal from the extracellular environment (Palczewski *et al.*, 2000). In contrast, ECL2 of the β_1AR and β_2AR contains a short α-helical section stabilised by a second disulphide bond, and the loop adopts a more exposed conformation, allowing the diffusion of ligands into, and out of, the binding pocket (Cherezov *et al.*, 2007).

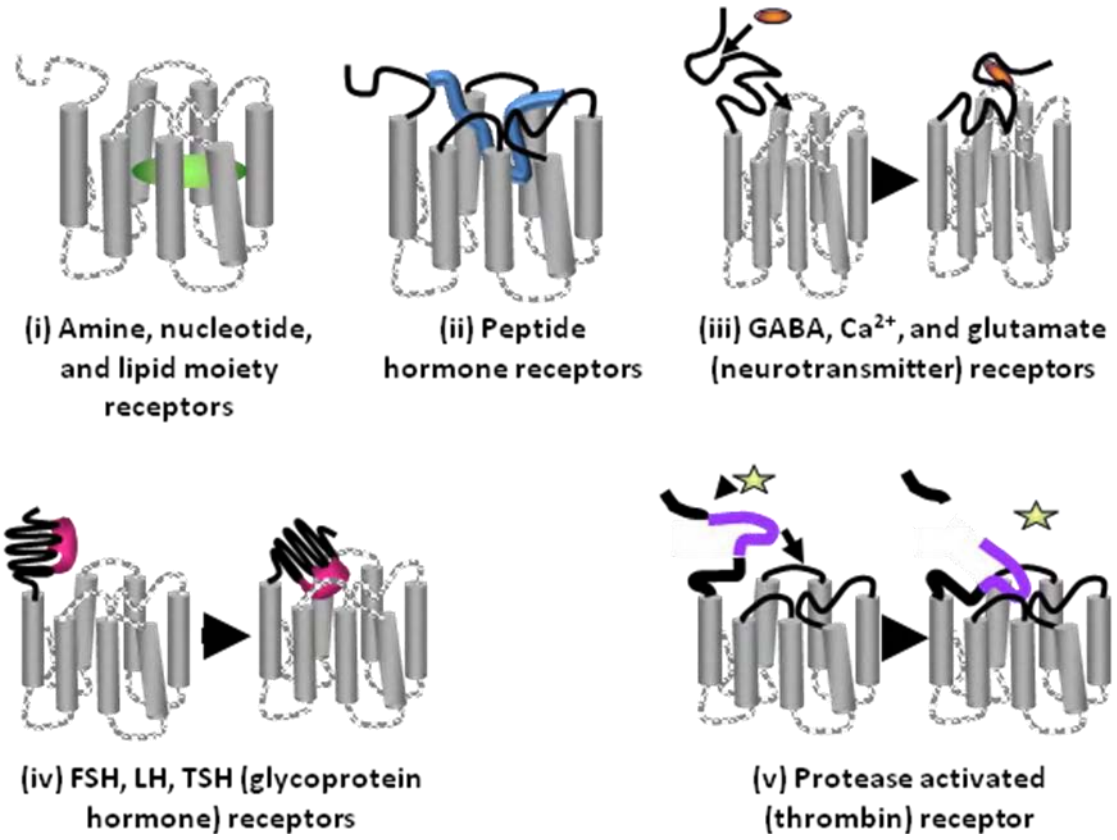


Figure 1.7 Representation of ligand binding to GPCRs: (i) Binding of amine ligands within the TM helices, (ii) peptide binding sites are found both within the TM helices, and within the extracellular regions, (iii) small neurotransmitters and ions bind to the N-terminus, (iv) glycoproteins bind to the characteristically large N-terminal domain and (v) the action of the protease on the thrombin receptor, where the N-terminal domain is cleaved and subsequently acts as a ligand for the receptor (Image adapted from Gether, 2000).

Both TM residues and residues within ECL2 make contacts with the ligands in the crystal structures (Palczewski *et al.*, 2000; Okada *et al.*, 2004; Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Warne *et al.*, 2008) although retinal binds deeper than the other ligands within the TM domains (Rosenbaum *et al.*, 2009). Retinal is covalently linked to $Lys^{7.43}$ via a protonated Schiff base (Kuhne, 1977; Palczewski *et al.*, 2000) with the carboxyl group of $Glu^{3.28}$, located at the extracellular interface of TMIII, being the counterion for this interaction (Sakmar *et al.*, 1989; Cohen *et al.*, 1992). Retinal is unique amongst GPCR ligands in that it acts as an endogenous inverse agonist when in the 11-*cis* conformation. Absorption of a photon results in isomerisation of 11-*cis* retinal to all-*trans* retinal. This allows for structural rearrangements of the TM helices of Rho and therefore receptor activation (Wald, 1968; Okada *et al.*, 2004). In the biogenic amine receptors a conserved aspartate at position 3.32 is required for agonist binding (Strader *et al.*, 1988; Porter *et al.*, 1996; Kristiansen *et al.*, 2000). The carboxyl group of $Asp^{3.32}$ participates in ionic bonding with the positive ion of the amino group of the agonists (Nagatomo *et al.*, 2000). In peptide GPCRs, interactions with ligands vary depending on the ligand in question as there is great variation in peptide size. Studies have implicated the ECLs and TM domains in binding and it is proposed that similar interactions occur in the peptide receptors as in the biogenic amine GPCRs (Fong *et al.*, 1995; Perlman *et al.*, 1997a; Hawtin *et al.*, 2006).

1.5.1 Agonists

Classical agonists are ligands which activate GPCRs and result in the activation of G-proteins and subsequent intracellular signalling cascades. Agonists act by altering the R↔R* equilibrium, due to their higher affinity for R*, and thus stabilise the R* state (Milligan *et al.*, 1995). Agonists can be classed as either full or partial agonists, depending on their intrinsic efficacy. Agonists may differ in their ability to stabilise different active conformations of GPCR, and each conformation may have varying abilities to activate, or different affinities for, G-proteins (Archer-Lahlou *et al.*, 2005). The vast majority of endogenous GPCR ligands display agonistic properties, resulting in activation of the target receptor and of downstream signalling cascades, inducing a physiological response.

1.5.2 Antagonists

Antagonists do not stabilize either the R or the R* state, but instead block the activity of either agonists or inverse agonists at GPCRs. Consequently, antagonist binding to constitutively active members of the GPCR superfamily does not result in a reduction in basal signalling, but instead prevents the action of agonists, thus inhibiting further activation. GPCR

antagonists (and inverse agonists, see section 1.5.3) are one of the largest classes of pharmaceutical drugs currently in use, and present a broad spectrum of therapeutic uses (Baker, 2008).

1.5.3 Inverse agonists

Inverse agonism is a property of a number of GPCR ligands. Many ligands that were initially thought to be antagonists of GPCRs actually act as inverse agonists, but this is often apparent only in receptor systems that naturally have a high level of basal signalling (Milligan *et al.*, 1995; Kenakin, 2004). Identified in the δ-opioid receptor (δ-OR), constitutive activity could be reduced by some antagonists that were found to have negative intrinsic efficacy (Costa *et al.*, 1989). Likewise, constitutive activity was identified in β_2AR signalling pathway and it was revealed that some antagonists of this system actually acted to reduce the basal signalling of the receptor (Gotze *et al.*, 1994). Inverse agonists display negative efficacy, being 'active' in their ability to suppress the constitutive activity of a GPCR (Costa *et al.*, 2005). There appear to be two mechanisms by which inverse agonists act; (i) by stabilising the inactive receptor, having higher affinity for the R-state, and so reduce the basal signalling of the receptor; (ii) by inactivating the receptor by forming an inactive inverse agonist-receptor complex (Strange, 2002). Inverse agonists may bind to GPCRs in a manner that prevents spontaneous receptor activation, binding to the receptor deep within the TM domain and thus restricting the movement of the top of TMVI and TMVII towards TMIII (Bond *et al.*, 1995; Holst *et al.*, 2006b). 11-*cis* retinal acts as an endogenous inverse agonist of Rho when bound, but not activated by a photon, by preventing the rotation of $Trp^{6.48}$ and in doing so, maintains the Rho ground-state (Matsuyama *et al.*, 2010). The melanocortin 4 receptor (MC4R) is a GPCR that is regulated *in vivo* by the endogenous inverse agonist, agouti-related peptide (AgRP) (Haskell-Luevano *et al.*, 2001; Adan *et al.*, 2003). The endogenous agonist in this system is melanocyte-stimulating hormone (MSH) and together with agouti (an antagonist) and AgRP, they act to control the activity of the MC4R (Adan, 2006).

It has been proposed that some ligands may act as agonists or inverse agonists for different signalling pathways activated by the same GPCR (Westphal *et al.*, 1996). Some β_2AR inverse agonists have been found to recruit β-arrestin and activate ERK signalling cascades, suggesting that a ligand can be an inverse agonist through one signalling pathway and behave as an agonist in another (Azzi *et al.*, 2003). There is the also the potential for protean agonist activity, where a ligand acts as either a classical agonist or an inverse agonist depending on the level of constitutive activity of the target receptor (Kenakin, 2001).

Inverse agonists are potentially invaluable therapeutic agents for the treatment of diseases that are caused by constitutively active mutations in GPCRs such as some forms of retinitis pigmentosa and hyperthyroidism (Smit *et al.*, 2007). Some viral infections and autoimmune disorders can lead to constitutive GPCR pathology, which may then be a target for inverse agonists as treatment (de Ligt *et al.*, 2000; Rosenkilde *et al.*, 2000; Kenakin, 2001). Inverse agonists may also hold some potential as adjuvant treatments for certain types of cancer where GPCR constitutive activity has been detected and which often result in increased cell proliferation (Mitsuhashi *et al.*, 1987; Kenakin, 2001).

1.6 Allosteric modulation

Allosteric ligand binding sites are distinct from the orthosteric binding sites of the endogenous ligands, enabling binding of allosteric modulators at the same time as the orthosteric agents (Monod *et al.*, 1963; Conn *et al.*, 2009). The main feature of allosteric modulators is that they increase or decrease the affinity of the endogenous or exogenous orthosteric ligands for the orthosteric binding site (Wess, 2005). An allosteric ligand can therefore have a positive or negative effect with respect to the ligand that binds the orthosteric site (Christopoulos *et al.*, 2002).

One well documented, non-selective, allosteric modulator of GPCR function is the monovalent cation, Na^+. Sodium acts to neutralise the charge on the highly conserved aspartate ($Asp^{2.50}$) in TMII. The change in charge can result in alterations to both the orthosteric ligand binding site and to the domains involved in G-protein coupling, thus affecting ligand binding affinity and receptor activation (Limbird, 1984; Horstman *et al.*, 1990; Neve *et al.*, 1991; Ceresa *et al.*, 1994). Na^+ therefore acts as an allosteric inverse agonist at a number of GPCRs, reducing agonist affinity, potentially increasing antagonist affinity and stabilising the R state (Fraser *et al.*, 1989; Horstman *et al.*, 1990; Seifert *et al.*, 2002).

Other metal ions may also function as allosteric modulators of GPCRs. Zn^{2+} has been shown to negatively affect the binding of antagonists of the dopamine D4 receptor (D_4R), decreasing antagonist affinity (Schetz *et al.*, 1997; Schetz *et al.*, 1999).

The potential therapeutic advantages of allosteric modulators of GPCR activity are aided by the observation that their effects are normally related to the enhancement or detriment of

endogenous ligand activity and are therefore saturable and less likely to induce adverse reactions in the event of overdose (Birdsall *et al.*, 1996; Christopoulos *et al.*, 2002). In relation to this, the effect of most allosteric modulators would only be observed in the presence of the orthosteric ligand, allowing physiological regulation of the drug's effects. In addition, the greater sequence variation in the non-orthosteric binding sites promises greater subtype selectivity of drugs that act as allosteric modulators (Birdsall *et al.*, 1996; Christopoulos *et al.*, 2002).

1.7 GPCR oligomerisation

Although previously considered to function as monomers, it is now accepted that some, if not all, GPCRs act as dimers or higher order oligomers *in vivo*. Studies carried out by Maggio *et al*. in the early 1990s used chimeric receptors of TMI-V and TMVI-VII of the α_2-adrenergic receptor (α_2AR) and the m3 mAChR and demonstrated that these chimeric receptors were dysfunctional when expressed alone. Co-transfection of the chimeras resulted in binding of both adrenergic and muscarinic ligands, and a certain degree of signalling (E_{max} 40-50 % of WT) after stimulation with a muscarinic agonist (Maggio *et al.*, 1993). The results of this early study demonstrated the possibility that GPCRs may be capable of forming interactions with each other and that these interactions can produce functional receptors from otherwise inactive monomers (Maggio *et al.*, 1993). The first clear evidence of dimer formation in native systems was for the Family C $GABA_b$ receptors, $GABA_b$R1 and $GABA_b$R2. These receptors function as a heterodimer to produce a functional $GABA_b$ receptor. It was found that expression of the $GABA_b$R1 alone did not produce functional activity *in vitro*. After the identification of a homologous receptor, the $GABA_b$R2, which was also dysfunctional when transfected alone, heterologous expression in cell lines demonstrated that the pharmacology exhibited by the combination of $GABA_b$R1 and $GABA_b$R2 was similar to that reported for $GABA_b$ receptors in the brain. It was further demonstrated that the two receptors are coexpressed in neurons and concluded that both subtypes are required to produce a functional $GABA_b$ receptor (Jones *et al.*, 1998), with the $GABA_b$R1 binding to ligands, and the $GABA_b$R2 coupling to, and activating, G-proteins (Galvez *et al.*, 2001).

Biochemical techniques involving immunoprecipitation of differentially tagged receptors have been used to demonstrate that a number of GPCRs are capable of forming oligomeric complexes *in vitro* (Devi, 2001; Angers *et al.*, 2002). Coimmunoprecipitation of GPCRs to demonstrate dimer formation does however present problems due to unspecific aggregation of

GPCRs after cell lysis (Angers *et al.*, 2002). The biophysical techniques of fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) eliminated some of the problems associated with coimmunoprecipitation studies. FRET and BRET can be carried out in living cells, avoiding unspecific aggregation, and dimerisation of GPCRs can be studied in a natural environment (Angers *et al.*, 2002). Both FRET and BRET are used to measure the non-radiative transfer of energy between molecules that are less than 100 Å apart by the use of energy donors and acceptors. In FRET a fluorescent molecule, usually cyan-fluorescent protein, and in BRET the enzyme luciferase, act as energy donors. The energy acceptor is usually green-fluorescent protein (GFP) (Devi, 2001; Rios *et al.*, 2001). The first GPCR that was shown to form homodimers *in vitro* using BRET techniques was the β_2AR (Angers *et al.*, 2002). Since the identification of β_2AR dimers, a number of GPCRs have now been shown to form homodimers including the m3 mAChR (Zeng *et al.*, 1999b), the dopamine D2 receptor (D_2R) (Ng *et al.*, 1996) and the gonadotropin-releasing hormone receptor (GnRHR) (Cornea *et al.*, 2001). GPCR heterodimers have been shown between the $A_{2A}R$ and the metabotropic glutamate receptor 5 (Ferre *et al.*, 2002) the β_1AR and β_2AR (Mercier *et al.*, 2002) and the δ-OR and κ-OR (Ramsay *et al.*, 2002) amongst others.

GPCR dimers may be synthesised in the endoplasmic reticulum or at the cell surface, either independent of agonists, or upon agonist stimulation. The β_2AR (Hebert *et al.*, 1996) and the somatostatin (SST) receptors (Rocheville *et al.*, 2000a; Rocheville *et al.*, 2000b) have been found to dimerise after agonist stimulation, with the agonist acting to stabilise, and sometimes increase, dimer formation within the cell membrane. Studies on other receptors, such as the yeast α-mating factor receptor, Ste2p (Overton *et al.*, 2000) the δ-OR (Cvejic *et al.*, 1997) and the D1 dopamine receptor (D_1R) (George *et al.*, 1998) have demonstrated that agonist stimulation has no effect on the level of dimer formation at the cell surface, suggesting that the dimers are already formed before transport to the cell surface. In some cases, receptor dimerisation may be necessary for correct receptor folding and transportation. The $GABA_bR1$ has been shown have low cell-surface expression when transfected alone in COS-7 cells. The $GABA_bR2$ heterodimer with $GABA_bR1$ aids cell-surface delivery (Sullivan *et al.*, 2000).

Another factor influencing the formation of GPCR dimers is the presence of G-proteins. G-protein coupling has been found to be a factor influencing the formation and maintenance of dimers of the δ- and μ-ORs (Fan *et al.*, 2005; Law *et al.*, 2005) the SST receptors; SST_2R and

SST_5R (Grant *et al.*, 2010) and the neuropeptide Y1 and Y2 receptors (Parker *et al.*, 2007; Parker *et al.*, 2008).

GPCR dimer formation has been found to alter receptor pharmacology *in vitro*. GPCRs exhibit a number of functions that cannot be explained by any theory other than that of dimerisation; ligand binding characteristics after the expression of two GPCRs *in vitro* are sometimes unexpected and not simply a combination of the individual receptor characteristics, such as the $GABA_b$ receptor pharmacology (Gouldson *et al.*, 2000). Heterodimerisation *in vitro* can lead to the production of novel ligand binding characteristics. The $GABA_b$ receptor is an example of a receptor whose pharmacology is defined by dimerisation (Jones *et al.*, 1998). Co-expression of μ- and δ-ORs affected a number of pharmacological properties. A novel binding site was created which had distinct pharmacological characteristics to the two receptors when expressed individually (George *et al.*, 2000) and treatment with ligands selective for one subtype increased binding and signalling through the other receptor subtype (Gomes *et al.*, 2000). Altered sensitivity to the pertussis toxin, a $G\alpha_i$ inhibitor, indicated that the μ/δ-OR heterodimer had altered G-protein coupling (George *et al.*, 2000). A heterodimer of the growth hormone secretagogue 1a receptor (GHS-R1a, also known as the ghrelin-R) and a splice variant of the receptor, the GHS-R1b, which is truncated and only consists of only five TM domains, has been found to regulate ghrelin-R function. Coimmunoprecipitation and BRET studies indicated that ghrelin-R/GHS-R1b heterodimers are produced and that overexpression of GHS-R1b leads to a decrease in the cell surface expression of the ghrelin-R, and a resultant decrease in signalling without an apparent change in the affinity of ghrelin for the ghrelin-R (Leung *et al.*, 2007).

There is also substantial data supporting the formation of dimers between GPCRs and other proteins. Accessory proteins, known as the receptor activity-modifying proteins (RAMPs) are known to form heterodimers with a number of Family B GPCRs. The RAMPs dictate the pharmacology of the receptors and therefore specify the ligand-binding characteristics by combining different RAMPs with the same GPCR (Juaneda *et al.*, 2000). For example, the RAMP that is present within a cell will determine whether adrenomedullin or calcitonin gene-related peptide receptors are produced on forming a dimer with the calcitonin receptor-like receptor (Hay *et al.*, 2004b). There is evidence that the RAMPs associate with other GPCRs such as the calcitonin receptor (Hay *et al.*, 2004a), the glucagon receptor, the parathyroid hormone receptor (PTHR) and the vasoactive intestinal polypeptide/pituitary adenylyl cyclase activating peptide receptor (Christopoulos *et al.*, 2003).

Despite the increasing evidence of dimer and oligomer formation *in vivo* and *in vitro*, it has been demonstrated that a single GPCR is sufficient to activate G-proteins. Lipid nanodiscs containing Rho monomers were found to be able to fully activate transducin ($G\alpha_t$) (Bayburt *et al.*, 2007) and the same result was found for the β_2AR in $G\alpha_s$ activation (Whorton *et al.*, 2007).

1.8 Post-translational modifications

Post-translational modifications have been shown to be important, if not essential, for receptor structure and function, influencing cell-surface expression, ligand binding, intracellular signalling and receptor regulation. There are four main types of post-translational modification of GPCRs; phosphorylation, glycosylation, palmitoylation and ubiquitination.

1.8.1 Phosphorylation

Phosphorylation occurs on specific serine, threonine and tyrosine residues of the intracellular domains of GPCRs. Phosphorylation modulates receptor signalling by allowing the association of the arrestin family of proteins and subsequent downregulation of the receptors (section 1.9).

1.8.2 Glycosylation

Glycosylation is the most common post-translational modification of GPCRs where carbohydrate moieties are added to asparagine residues (*N*-linked) or serine/threonine residues (*O*-linked) within the extracellular domain. Most GPCRs have putative *N*-linked glycosylation sites, which occur at the consensus sequence NxS/T, where 'x' is any amino acid except proline (Kornfeld *et al.*, 1985). The presence and utility of consensus sequences implies that glycosylation is functionally significant (Hawtin *et al.*, 2001a). Studies have shown that glycosylation can play a role in trafficking and cell-surface expression in the vasopressin 1a receptor (V_{1a}R) (Hawtin *et al.*, 2001a), the β_2AR (Rands *et al.*, 1990), the AT_{1A}R (Deslauriers *et al.*, 1999), the β_1AR (He *et al.*, 2002) and the follicle-stimulating hormone receptor (FSHR) (Davis *et al.*, 1995). Glycosylation has been shown to be important for agonist binding in the SST receptor (Christopoulos *et al.*, 2003) and the luteinizing hormone receptor (LHR) (Zhang *et al.*, 1991) and in signal transduction in Rho (Kaushal *et al.*, 1994).

O-linked glycosylation is more rare in GPCRs than *N*-linked glycosylation, but is not unreported; the vasopressin V_2 receptor (V_2R) has *O*-linked glycosylation modifications at most serine and threonine residues within the N-terminus (Sadeghi *et al.*, 1999) and the β_1AR has recently been shown to be *O*-glycosylated at the N-teminus during transportation (Hakalahti *et al.*, 2010). Other GPCRs that have been reported to be modified by *O*-linked carbohydrate moieties are the κ-OR (Li *et al.*, 2007) the chemokine receptor, CCR5 (Bannert *et al.*, 2001) and the bradykinin B2 receptor (Michineau *et al.*, 2006).

1.8.3 Palmitoylation

Palmitoylation is a modification involving the formation of a thioester bond between cysteine residues and a fatty acyl chain. Palmitoylation can occur on cysteine residues within the C-terminal tail of GPCRs (O'Brien *et al.*, 1984). The 16-carbon fatty acid, palmitic acid, can be reversibly, covalently attached to cysteine residues of the protein whilst it is retained in the Golgi (Hawtin *et al.*, 2001b). Palmitoylation and de-palmitoylation has also been observed to occur in an agonist-dependent manner (Hawtin *et al.*, 2001b; Qanbar *et al.*, 2003). These palmitic acid chains, being hydrophobic, are able to embed in the plasma membrane upon receptor expression at the cell surface. This creates a fourth intracellular loop structure, incorporating HelixVIII, that has structural and functional significance in some GPCRs (Hawtin *et al.*, 2001b). In the β_2AR crystal structure, the palmitic acid on Cys341 at the C-terminal end of HelixVIII was clearly resolved (Cherezov *et al.*, 2007). Studies have shown that palmitoylation regulates many functions of GPCRs including G-protein coupling (O'Dowd *et al.*, 1989; Horstmeyer *et al.*, 1996; Okamoto *et al.*, 1997), cell-surface expression (van Koppen *et al.*, 1991; Sadeghi *et al.*, 1997; Petaja-Repo *et al.*, 2006) and desensitisation and downregulation (Hawtin *et al.*, 2001b).

1.8.4 Ubiquitination

Ubiquitination of GPCRs has been found to be important for lysosomal sorting of internalised receptors (Marchese *et al.*, 2001; Shenoy *et al.*, 2001). However, ubiquitination is rarely a prerequisite for internalisation itself (Marchese *et al.*, 2001; Shenoy *et al.*, 2001). Ubiquitin is a 76 amino acid protein which binds to lysine residues on the intracellular domains of GPCRs and is recognised by the ubiquitin binding domains (UBDs) of proteins of the endocytic pathways (Hicke *et al.*, 2003). The proteins containing UBDs direct the ubiquitinylated GPCRs through different endosomal pathways, thus dictating the outcome of receptor internalisation (Hicke *et al.*, 2005).

1.9 GPCR regulation

GPCRs are extensively regulated by a number of cellular processes. This may be (i) to attenuate signalling in the presence of a specific ligand, (ii) to desensitise the cell to external signals or (iii) to up-regulate signalling through specific receptors by an increase in transcription. The attenuation of GPCR response to agonists involves three processes, namely desensitisation, sequestration and downregulation (Fig 1.8).

Desensitisation of GPCRs involves the uncoupling of receptors from their G-proteins. In order to attenuate signalling from activated GPCRs, a group of protein kinases called the G-protein-coupled receptor kinases (GRKs) phosphorylate active GPCRs on their cytoplasmic serine and threonine residues (Freedman *et al.*, 1996). After phosphorylation, β-arrestins are recruited to the GRK-phosphorylated receptors, resulting in inhibition of G-protein coupling (Lohse *et al.*, 1990; Shenoy *et al.*, 2003). The GRK phosphorylation acts to increase the affinity of the receptor for the arrestins. This form of desensitisation is known as homologous desensitisation as it only occurs at activated GPCRs and results in attenuation of agonist-mediated signalling. Homologous desensitisation does not always involve receptor endocytosis, as the steric hindrance of β-arrestin binding to the GPCR prevents G-protein coupling (Reiter *et al.*, 2006). Other protein kinases are able to phosphorylate serine and threonine residues in order to promote desensitisation. These include casein kinase, PKA and PKC. PKA and PKC can phosphorylate GPCRs independent of agonist stimulation, producing heterologous desensitisation of GPCRs (Benovic *et al.*, 1985).

Reducing the cell-surface expression of GPCRs by means of phosphorylation and endocytosis can reduce receptor activity quickly. This process of **sequestration** is characterised by a decrease in cell-surface binding sites and is a form of desensitisation, easily reversible upon dephosphorylation of the receptors and re-sensitisation by recycling back to the cell surface (Pierce *et al.*, 2001; von Zastrow, 2001). After the binding of β-arrestins during homologous desensitisation, β-arrestin binds simultaneously to the receptor and to a clathrin adaptor protein, AP-2. Clathrin is recruited to AP-2, facilitating the sequestration of the GPCRs into clathrin-coated pits (Krupnick *et al.*, 1997; Laporte *et al.*, 2002). The clathrin triskelions assemble spontaneously, creating an invagination of the membrane. Dynamin and other proteins assemble at the neck of the vesicle bud and promote the pinching off of the vesicle and its release into the cytoplasm. After internalisation, receptors are either rapidly

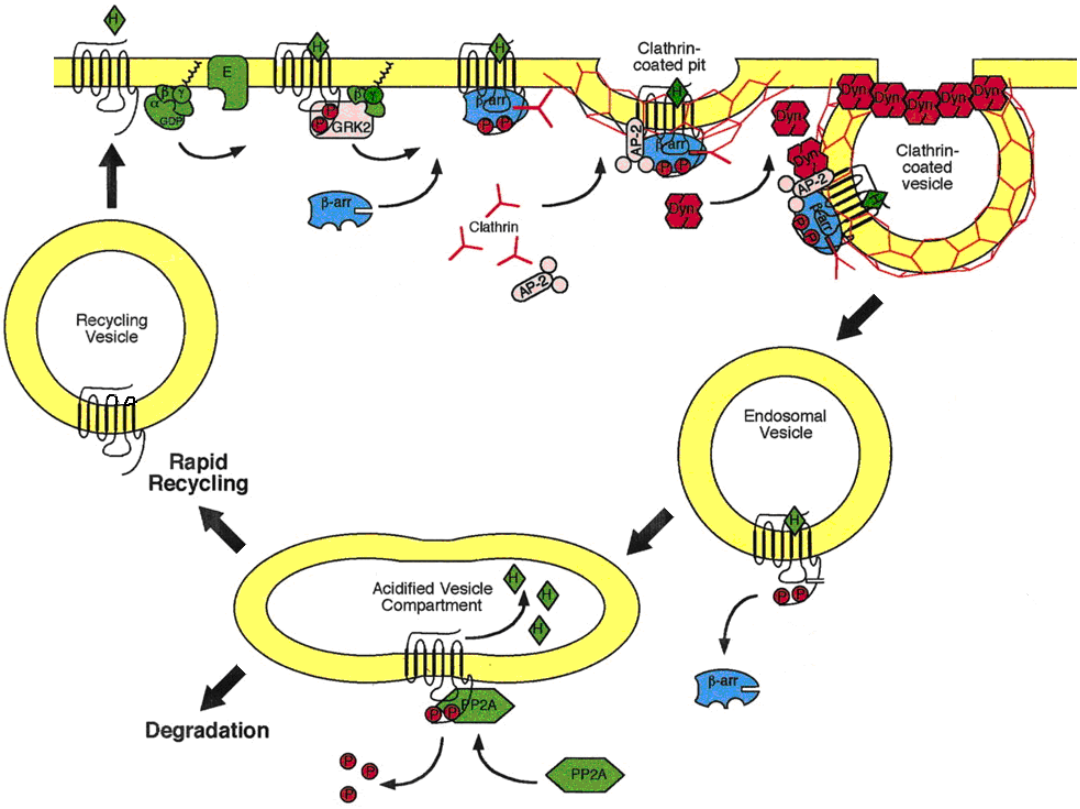


Figure 1.8 Schematic of GPCR desensitisation, internalisation and recycling: Desensitisation of a receptor occurs when an agonist-bound GPCR is phosphorylated by the GRKs. β-arrestin is then recruited to the phosphorylated receptor and receptor signalling is terminated. β-arrestin acts as an adaptor protein for the clathrin proteins which cause the membrane to bud. Dynamin allows membrane fission, creating an endocytic vesicle and allowing sequestration of the receptors. Depending on the receptor, different pathways are followed after internalisation (Luttrell *et al.*, 1999).

recycled, degraded, or retained in endosomes, resulting in attenuation of signalling without the complete termination of activity. Some GPCRs are capable of activating non-G-protein-dependent signalling pathways, such as MAP kinase from cytosolic endosomes (Daaka *et al.*, 1998).

The route taken by internalised GPCRs is dependent on sorting sequences contained within the cytoplasmic domain of the receptor and, for some GPCRs, the presence of ubiquitin, however these mechanisms are not well understood (Jean-Alphonse *et al.*, 2010). Cells can be resensitised after dephosphorylation of the GPCR in acidic endosomal vesicles and subsequent recycling back to the plasma membrane. **Downregulation** of receptors involves the process of permanently removing receptors from the cell surface and the trafficking of them to lysosomes for proteolytic degradation. This process is characterised by a change in receptor number in cell lysates, demonstrating that all cellular membranes have reduced receptor number (von Zastrow, 2001).

GPCRs may also be regulated at the G-protein level. RGS proteins are a class of 20 proteins that can accelerate the GTPase activity of the Gα-subunit by acting as GAPs, and in doing so, modulate G-protein activity. G-proteins can also be modulated by an increase in proteolysis, decreasing the ratio of G-proteins to GPCRs, potentially making the G-proteins a rate-limiting factor (Hepler, 1999; Schulte *et al.*, 2007).

GPCRs may also be regulated at the level of transcription. The oxytocin receptor (OTR) is one example of a receptor that demonstrates increases in cell-surface expression *in vivo* in response to external stimuli. The level of OTR expression in the uterus is low in early pregnancy, yet increases ~300-fold to peak during early labour (Fuchs *et al.*, 1984; Kimura *et al.*, 1996). Receptor regulation at the transcriptional level may act to regulate specific physiological functions, as with the OTR (Kimura *et al.*, 1996), or may be to maintain homeostasis after a pronounced reduction in receptor cell-surface expression after downregulation.

1.10 Ghrelin: an acylated peptide hormone

Ghrelin is a 28 amino acid peptide hormone that is released, predominantly, from secretory granules contained within the X/A-like endocrine cells of the submucosal layer of the stomach (Kojima *et al.*, 2001; Holst *et al.*, 2004b). In addition to the stomach, ghrelin mRNA has been

found in the brain (Korbonits *et al.*, 2001), small intestine, pancreas, heart, lungs and elsewhere (Gnanapavan *et al.*, 2002). Ghrelin is a unique peptide hormone in that it has an *n*-octanoic acid modification on Ser3, which is essential for its activity (Fig 1.9 (b)).

1.10.1 The discovery of ghrelin

Ghrelin is the first known biologically active peptide to be acylated (Kojima *et al.*, 2001). Ghrelin is an example of an endogenous ligand discovered by reverse pharmacology after the discovery of its GPCR (Kojima *et al.*, 2005). mRNA for the receptor for ghrelin, the ghrelin-R (also known as the GHS-R1a) (section 1.11), was found to be expressed in the pituitary and hypothalamus of the brain where it is activated by the synthetic peptide or non-peptide ligands, the growth hormone secretagogues (GHSs) (Howard *et al.*, 1996). These synthetic ligands were capable of stimulating growth hormone (GH) secretion *in vivo* (Bowers *et al.*, 1984) and were believed to mimic the activity of a natural hormone (Howard *et al.*, 1996). CHO cells were stably transfected to express the rat ghrelin-R and were treated with extracts from a variety of tissues to establish which of these could effectively stimulate the receptor. The results were unexpected, showing that treatment with stomach extracts resulted in high activity of the receptors. Purification of the extracts lead to the isolation of a peptide named ghrelin, based on the Proto-Indo-European word root, "ghre", which means "grow" (Kojima *et al.*, 1999). Sequence analysis determined the 28 amino acids of ghrelin and identified Ser3 as having a hydrophobic modification. Mass spectrometry identified an octanoyl moiety replacing the hydrogen of the hydroxyl group of Ser3. The *n*-octanoic acid was determined to be essential for ghrelin activity as des-acyl-ghrelin did not increase intracellular Ca^{2+} concentration through the ghrelin-R (Kojima *et al.*, 2001).

1.10.2 Ghrelin processing

Ghrelin is produced from a 117 amino acid precursor, preproghrelin. A full-length precursor of 94 amino acids, proghrelin, is produced after removal of a 23 amino acid signal peptide. The proghrelin precursor also contains the related hormone, obestatin (Zhang *et al.*, 2005) (Fig 1.9 (a)). Prohormone convertase 1/3, found within ghrelin-positive cells of the stomach, is responsible for the post-translational proteolytic cleavage of proghrelin, at arginine residues, to produce ghrelin and obestatin (Kojima *et al.*, 1999; Zhu *et al.*, 2006).

The octanoylation of Ser3 is essential for ghrelin biological activity. Although des-acyl ghrelin is found at 10-50 times the concentration of the *n*-octanoyl ghrelin in the circulation (Kojima *et al.*, 2005) des-acyl ghrelin does not have the same physiological roles as ghrelin

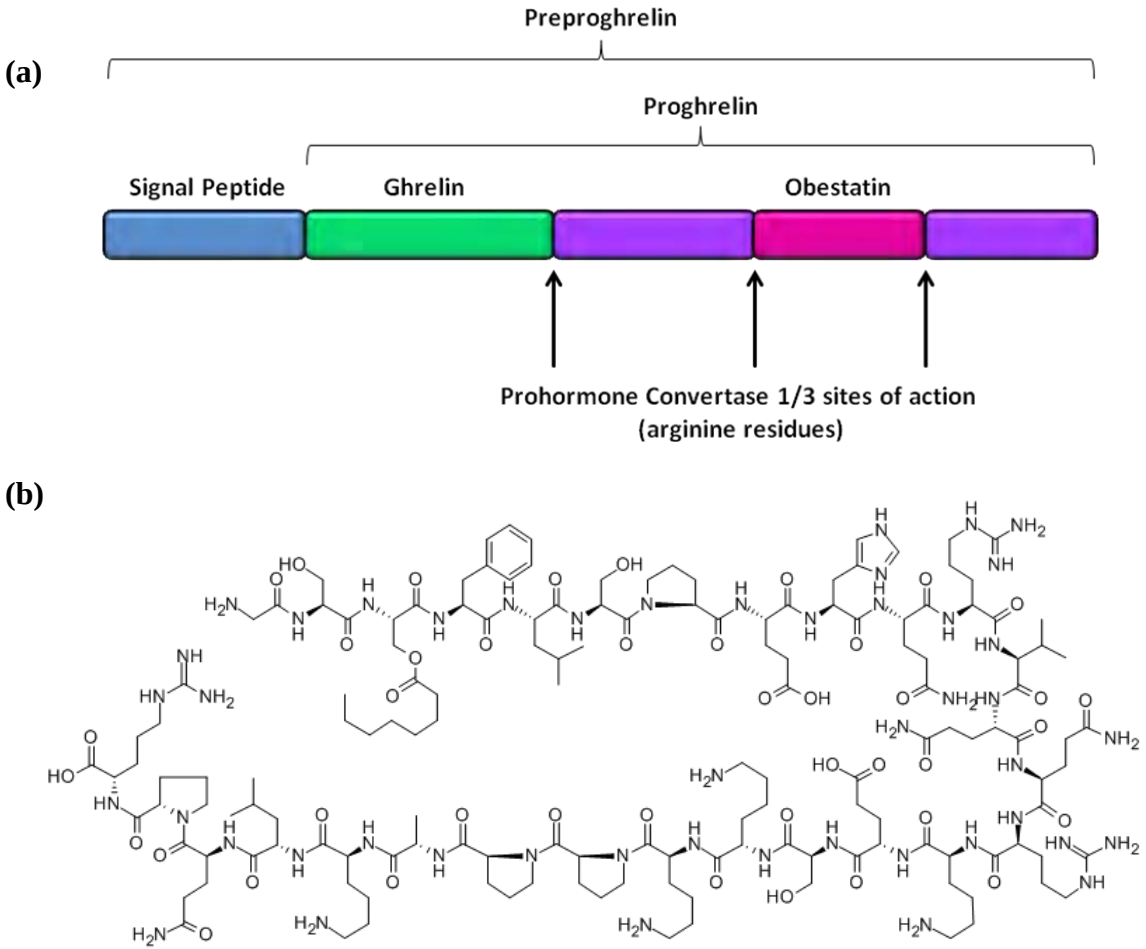


Figure 1.9 Preproghrelin and the Human ghrelin sequence: (a) the 117 amino acid preproghrelin contains ghrelin and obestatin. The signal peptide is removed to give the 94 amino acid proghrelin. Proghrelin is cleaved at arginine residues by prohormone convertase 1/3 to produce mature ghrelin and obestatin, (b) sequence of human ghrelin showing the *n*-octanoyl modification of Ser3.

(Kojima *et al.*, 1999; Gauna *et al.*, 2007). The acyl modification is made to Ser3 of ghrelin, before its cleavage from proghrelin in the Golgi, by the enzyme ghrelin *O*-acyltransferase (GOAT) (Gutierrez *et al.*, 2008; Yang *et al.*, 2008). The ghrelin-GOAT system is an important mechanism in the control of energy homeostasis as des-acyl ghrelin has been found to antagonise the effects of ghrelin *in vivo*, and may therefore negatively regulate ghrelin function, probably via alternative receptors (Gauna *et al.*, 2007; Romero *et al.*, 2010).

1.10.3 Ghrelin physiology and pathology

Ghrelin is highly conserved in vertebrates, highlighting a crucial role in vertebrate physiology (Fig 1.10). Ghrelin has many functional roles, demonstrating both endocrine and paracrine effects (Pedretti *et al.*, 2007). Initially identified for its GH secreting effects in the pituitary (Kojima *et al.*, 1999; Arvat *et al.*, 2000), it has since been implicated in the stimulation of appetite (Wren *et al.*, 2000; Nakazato *et al.*, 2001), gastric acid secretion (Masuda *et al.*, 2000; Date *et al.*, 2001; Sibilia *et al.*, 2002), adrenocorticotropin hormone and arginine vasopressin (AVP) release (Mozid *et al.*, 2003), carbohydrate metabolism (Cruz *et al.*, 2010), fat utilization (Tschop *et al.*, 2000; Choi *et al.*, 2003) and cell proliferation (Rak-Mardyla *et al.*, 2010). Ghrelin is also involved in behavioural (Asakawa *et al.*, 2001) cardiovascular (Nagaya *et al.*, 2001b; Okumura *et al.*, 2002), gastrointestinal (Masuda *et al.*, 2000) and reproductive (Fernandez-Fernandez *et al.*, 2004) functions.

Ghrelin has a significant effect on energy homeostasis due to its orexigenic (appetite-stimulating) and adipogenic (fat-storing) activity. Although ghrelin is the only currently identified orexigenic hormone released from the gut (Suzuki *et al.*, 2010) ghrelin exerts its main activity on the hypothalamic arcuate (ARC) nuclei, stimulating the release of the orexigenic peptides, neuropeptide Y (NPY) and AgRP (Lu *et al.*, 2002; Cowley *et al.*, 2003). The ablation of NPY/AgRP neurons results in a loss of ghrelin orexigenic activity (Chen *et al.*, 2004). Ghrelin also stimulates GABA which exerts an inhibitory effect on proopiomelanocortin (POMC) and cocaine and amphetamine-regulated transcript (CART)-expressing neurons resulting in a decrease in α-melanocyte-stimulating hormone (α-MSH) release and subsequent reduction in stimulation of the melanocortin pathway. α-MSH usually displays an anorexigenic effect and increases energy expenditure (Nogueiras *et al.*, 2008).

NPY and AgRP appetite-stimulating effects are inhibited by leptin, an anorexigenic hormone which also acts on the ARC, stimulating the POMC/CART neurons, and subsequently the melanocortin pathway, inhibiting the NPY/AgRP neurons, exerting the opposite

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	
G	S	S[#]	F	L	S	P	E	H	Q	R	V	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	**Human**
G	S	S[#]	F	L	S	P	E	H	Q	K	T	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	**Gerbil**
G	S	S[#]	F	L	S	P	E	H	Q	K	A	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	**Mouse**
G	S	S[#]	F	L	S	P	E	H	Q	K	A	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	**Rat**
G	S	S[#]	F	L	S	P	E	H	Q	K	L	Q	Q	R	K	E	S	K	K	P	P	A	K	L	Q	P	R	**Dog**
G	S	S[#]	F	L	S	P	E	H	Q	K	V	Q	Q	R	K	E	S	K	K	P	A	A	K	L	K	P	R	**Pig**
G	S	S[#]	F	L	S	P	E	H	Q	K	L	-	Q	R	K	E	A	K	K	P	S	G	R	L	K	P	R	**Cattle***
G	S	S[#]	F	L	S	P	E	H	Q	K	L	-	Q	R	K	E	P	K	K	P	S	G	R	L	K	P	R	**Sheep***

Figure 1.10 Comparison of the human ghrelin peptide and ghrelin from other mammalian species: Cattle* and sheep* ghrelin have only 27 amino acids. [#]Ser3 has an *n*-octanoyl modification. Green represents a conserved side chain characteristic between human ghrelin and the other species, but not an identical amino acid. Pink is a non-conservative amino acid substitution when compared to human ghrelin. Yellow is a missing amino acid compared to human ghrelin (Image adapted from van der Lely *et al.*, 2004).

effects to those of ghrelin (Morton *et al.*, 2001; Zigman *et al.*, 2003; Nogueiras *et al.*, 2008).

Ghrelin is released from the X/A-like cells in response to fasting situations. The concentration of ghrelin in the blood is determined by the nutritional state of the individual, with plasma levels of the hormone being significantly elevated shortly before meals (Cummings *et al.*, 2001) and decreasing after eating (Tschop *et al.*, 2001). The role of ghrelin in various eating disorders has been questioned; ghrelin concentration and the role that it plays in catabolism has been studied in anorexia nervosa (Otto *et al.*, 2001), obesity (le Roux *et al.*, 2005), cardiac cachexia (Nagaya *et al.*, 2004) and cancer cachexia (Garcia *et al.*, 2005). Ghrelin has been found to exist at high concentrations in patients suffering from anorexia nervosa (Otto *et al.*, 2001) and at low concentrations in obese subjects (le Roux *et al.*, 2005). These observations suggest that ghrelin plays a substantial role in long-term energy homeostasis (Janssen *et al.*, 2004; Davenport *et al.*, 2005). Despite this, ghrelin$^{-/-}$ mice have been shown display growth rate, appetite, feeding behaviour and body composition comparable to ghrelin$^{+/+}$ mice, implying that ghrelin is not an essential orexigenic factor or that ghrelin can be compensated for by other mechanisms (Sun *et al.*, 2003). A high ghrelin concentration has been observed in patients with Prader-Willi syndrome (PWS) suggesting that the hyperphagia associated with PWS may be related to the increase in ghrelin (Cummings *et al.*, 2002; DelParigi *et al.*, 2002).

The applications of ghrelin as a therapeutic agent are potentially many as ghrelin is involved in a diverse range of physiological functions (Espelund *et al.*, 2003), although its uses in some areas may be limited, especially as its diverse distribution and extensive roles could lead to lack of specificity. In obesity, blocking ghrelin's actions may provide some therapeutic advantage, although compensatory mechanisms may prevent the full antagonistic activity from being observed, as demonstrated in the ghrelin$^{-/-}$ mice (Sun *et al.*, 2003; Bays, 2004; Kojima *et al.*, 2005). Similarly, in anorexia nervosa, ghrelin levels are elevated suggesting that ghrelin sensitivity is affected and that ghrelin treatment, although having the potential to induce an orexigenic effect, is unlikely to be a successful therapeutic agent (Otto *et al.*, 2001; Muccioli *et al.*, 2002; Kojima *et al.*, 2005). As a treatment for chronic heart failure, ghrelin holds more potential (Kojima *et al.*, 2005). Ghrelin positively affects cardiac structure and function and decreases the development of cardiac cachexia (Nagaya *et al.*, 2003). Similarly, ghrelin may hold therapeutic use as a treatment for other forms of disease-associated cachexia and lipodystophy (Koutkia *et al.*, 2004; Garcia *et al.*, 2005).

1.11 The ghrelin receptor

The ghrelin-R belongs to a subfamily of GPCRs that are all activated by gastrointestinal peptides or neuropeptides. Other receptors in the subfamily are the motilin receptor, neuromedin U receptors, the neurotensin receptors (NT-Rs) and the orphan receptor, GPR39 (Kojima *et al.*, 2005).

The ghrelin-R is expressed primarily in the somatotroph cells of the pituitary and the ARC nucleus of the hypothalamus (Howard *et al.*, 1996), but detectable levels of ghrelin-R mRNA have also been found in the hippocampus and a number of peripheral organs such as the pancreas (Guan *et al.*, 1997), the heart (Nagaya *et al.*, 2001a), the adrenal cortex (Carraro *et al.*, 2004), the ovaries (Gaytan *et al.*, 2005) and the stomach (Date *et al.*, 2000). The ghrelin-R, as with ghrelin, is well conserved in vertebrates, highlighting an important physiological function (Kojima *et al.*, 2005). The relatively high conservation of ghrelin and the ghrelin-R in vertebrates supports both a common mechanism of action and an important physiological role as both are so well conserved throughout evolution.

The discovery of the ghrelin-R by Howard *et al*. (1996) occurred after a synthetic peptide, growth hormone releasing peptide-6 (a GHS), was found to release GH independently of the growth hormone releasing hormone receptor (GHRH-R). It was apparent that another receptor was involved in this GH-releasing pathway. The GHS-induced rise in intracellular Ca^{2+} and $InsP_3$ indicated that the target of the GHSs was likely to be a GPCR. Two receptors were identified in the pituitary that bound the GHSs; the GHS-R1a (ghrelin-R) and the GHS-R1b (Howard *et al*., 1996).

The human ghrelin-R consists of 366 amino acids and forms the typical 7TM helical domains of a GPCR (Feighner *et al.*, 1998). The ghrelin-R has been identified as a Family A GPCR that couples to $G\alpha_{q/11}$ G-proteins. The GHS-R1b cDNA encodes only 289 amino acids; a splice-variant truncated protein, containing 5TM domains identical to the ghrelin-R, with 24 additional amino acids at the C-terminus (Feighner *et al.*, 1998) (Fig 1.11). The GHS-R1b failed to produce a response to the GHSs (Carraro *et al.*, 2004) and the precise functional role of GHS-R1b has not yet been determined. Evidence suggests that the GHS-R1b acts as a dominant negative modulator of the ghrelin-R (Leung *et al.*, 2007). Previous studies on the α1a adrenergic receptor (α_{1a}AR) (Coge *et al.*, 1999) and the V_2R (Zhu *et al.*, 1998) amongst others, have indicated that truncated variants of GPCRs may act as dominant negative

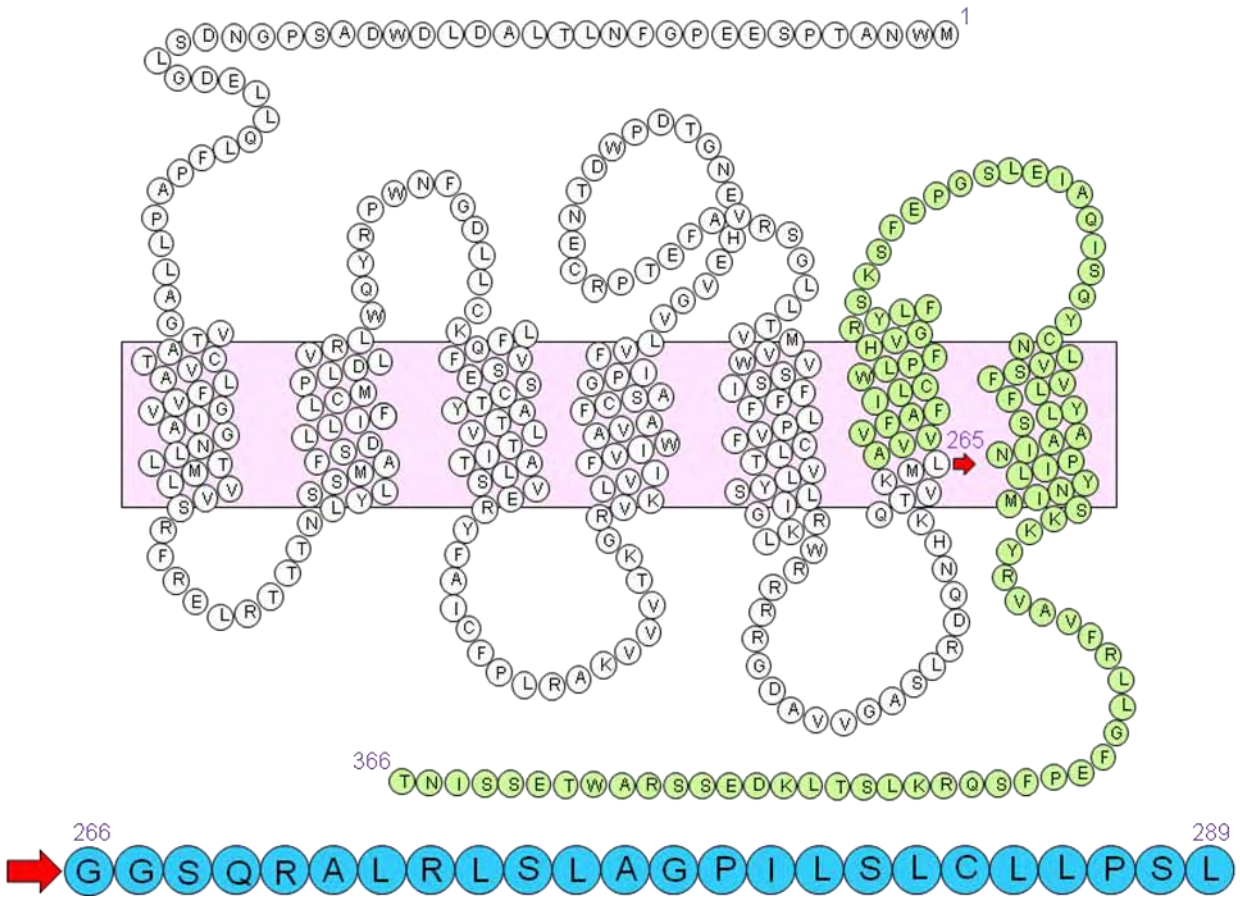


Figure 1.11 Schematic representation of the ghrelin-R and GHS-R1b: 366 amino acids constitute the full length ghrelin-R (white and **green**). The truncated GHS-R1b consists of only 289 amino acids, 265 of which are identical to the ghrelin-R (white), with 24 additional amino acids at the C-terminal end (**blue**).

modulators of the full length GPCR. The ghrelin-R has been predicted to function as a homodimer (Holst et al., 2005) and coimmunoprecipitation and BRET studies indicated that homodimers, or oligomers, can form *in vitro* (Leung *et al.*, 2007). When ghrelin-R and GHS-R1b cDNAs were expressed in the same cells, ghrelin-R/GHS-R1b heterodimers were produced (Leung *et al.*, 2007). The GHS-R1b appeared to act as a dominant negative when overexpressed, resulting in changes in the intracellular localisation of the ghrelin-R. Ghrelin-R/GHS-R1b heterodimers co-localised inside the cell, resulting in a reduction in constitutive activity and [^{125}I]ghrelin binding, without affecting ghrelin-R mRNA expression (Leung *et al.*, 2007).

1.11.1 The ghrelin-R displays high constitutive activity

The ghrelin-R is a highly constitutively active GPCR, signalling at approximately 50 % of the ghrelin-induced maximum in the absence of agonist (Holst *et al.*, 2004b). This was first identified using InsP-InsP_3 accumulation assays in COS-7 cells transiently transfected with ghrelin-R cDNA (Holst *et al.*, 2003a) and has since been confirmed in all cell lines tested to date. Also, *in vivo*, naturally occurring mutations of the ghrelin-R were identified that caused decreased constitutive activity (Holst *et al.*, 2006b; Pantel *et al.*, 2006). The Substance-P analogue [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}]-Substance P (SP-analogue) has been reported to reduce the basal activity of the ghrelin-R in COS-7 cells down to levels similar to that seen for cells transfected with empty expression vector in InsP-InsP_3 accumulation assays. The SP-analogue was therefore identified as being a full inverse agonist at the ghrelin-R (Holst *et al.*, 2003a). However, in cAMP-responsive element (CRE) reporter assays, which measure phosphorylation of the CRE binding protein (CREB) by the downstream kinases in the Gα_q pathway, SP-analogue was identified as a partial inverse agonist (Holst *et al.*, 2003a). The ghrelin-R has also been shown to signal through the MAP kinase pathway, stimulating ERK1/2 phosphorylation, although this only occurs in an agonist-dependent manner; the pathway is not activated constitutively (Holst *et al.*, 2004a).

Holst *et al*. (2004) studied the constitutively active ghrelin receptor and the related receptors, NT-R2 and GPR39, and identified an aromatic cluster within TMs VI and VII that was thought to be structurally important for the constitutive activity. This aromatic cluster allows TMVI and TMVII to approach TMIII in the absence of an agonist, shifting the receptor to its active conformation (Holst *et al.*, 2004b; Pedretti *et al.*, 2007). Mutational analysis of these residues showed that the size, aromaticity and hydrophobicity of residue 6.51 could alter the constitutive activity of, not only the ghrelin-R, but also the related GPR39. The high

constitutive activity also required a tyrosine or phenylalanine at position 7.42 (Holst *et al.*, 2004a), which is more commonly a small residue in Family A GPCRs (Mirzadegan *et al.*, 2003; Holst *et al.*, 2004a).

It has been suggested that the high level of ghrelin-R constitutive activity may be a "signalling set point," designed to counterbalance the inhibitory effects of other hormones such as leptin and insulin (Holst *et al.*, 2004b). As constitutive activity is related to the expression level of the receptor, the ghrelin-R expression is extensively regulated *in vivo* (Holst *et al.*, 2004b).

The mechanisms of action of ghrelin and the ghrelin-R are complex: a single nucleotide polymorphism that results in a point mutation (A204E in ECL2) in the ghrelin-R affects constitutive activity, but retains ghrelin sensitivity. This same mutation has been found to be responsible for both short stature (Pantel *et al.*, 2006) and obesity (Wang *et al.*, 2004) in different individuals. Ghrelin is an orexigenic hormone and therefore stimulation of the ghrelin-R results in an increase in appetite; loss of ghrelin-R constitutive activity would be expected to decrease hunger (Liu *et al.*, 2007b). The reduction in constitutive activity might be expected to cause a decrease in GH release, and subsequent short stature, however the development of obesity from the same point mutation highlights the complexity of this system. Ghrelin-$R^{-/-}$ mice have been found to have normal feeding behaviour and growth, again proving that other mechanisms compensate for the loss of the receptor (Sun *et al.*, 2003; Sun *et al.*, 2004).

1.11.2 Alternative receptors for ghrelin

There are still some uncertainties with respect to ghrelin-R subtypes. Alternative ghrelin-R have been identified *in vivo* which mediate the effects of both ghrelin and des-acyl ghrelin (Cassoni *et al.*, 2001; Baldanzi *et al.*, 2002; Cassoni *et al.*, 2004). Uncharacterised receptors for ghrelin and des-acyl ghrelin have been identified in cardiomyocytes (Baldanzi *et al.*, 2002) and breast cancer cells (Cassoni *et al.*, 2001) and it appears that a distinct population of receptors are responsible for some non-endocrine actions of ghrelin (Camina *et al.*, 2004). Separate des-acyl ghrelin receptors are also thought to exist, again indicating that the mechanisms involved in ghrelin physiology are many and complex (Camina, 2006).

1.12 Aims of this study

The overall aim of this study is to investigate the structural and functional aspects of ghrelin-R activity, specifically focusing on agonist-independent constitutive activity through the $InsP_3$ pathway. The high constitutive activity observed for the ghrelin-R is of great physiological importance and holds much therapeutic potential.

Site-directed mutagenesis and subsequent characterisation of mutant ghrelin-R constructs enables investigation into the role of specific amino acids within the receptor. The TM domains have been found to be essential for ligand binding and intracellular signalling in Family A GPCRs with TMs III and VI being extensively involved in maintaining both the inactive and active receptor conformations. Chapters 4 and 5 within this thesis will focus on residues within TMIII and TMVI with the aim of elucidating the role of individual amino acids in maintaining ghrelin-R high basal signalling. Chapter 6 will address the role of ECL2 of the ghrelin-R in both ghrelin binding and intracellular signalling, specifically aiming to identify residues which support the high basal signalling of the ghrelin-R.

The effect of alanine substitution mutagenesis of the individual ghrelin-R residues examined in this study are summarised in Appendix Fig 9.4-9.8.

CHAPTER 2.

MATERIALS AND METHODS

2.1 Materials

2.1.1 Antibodies

Monoclonal anti-haemagglutinin (HA) (mouse clone HA-7) primary antibody was from Sigma (Dorset, UK). Anti-mouse IgG whole molecule, Horse radish peroxidase (HRP) - linked, produced in horse was from New England Biolabs (NEB, Hitchin, UK).

2.1.2 Cell culture reagents

Dulbecco's modified Eagles medium (DMEM) and Dulbecco's phosphate-buffered saline (PBS) were from Lonza (Slough, UK). The inositol-free DMEM was custom synthesised by Lonza (Slough, UK) and Gibco (Paisley, UK). Foetal bovine serum (FBS) was from PAA (Pasching, Austria). Polyethyleneimine (PEI) and poly-D-lysine (PDL) were purchased from Sigma. Cell-culture plastic-ware was purchased from Triple Red (Long Crendon, UK) and Fisher Scientific (Loughborough, UK).

2.1.3 Molecular biology reagents

The DNA polymerase, *Pfu*, was from Promega (Southampton, UK). Restriction endonucleases, *Eco*RI and *Dpn*I and *Not*I were from NEB. dNTPs were from Bioline (London, UK) and were stored at a stock concentration of 40 mM. Calf intestinal alkaline phosphatase and T4 DNA ligase were from NEB. The kits used for the isolation and purification of plasmid DNA were Promega Wizard® Plus SV Miniprep Kit (Southampton, UK) and High Purity Maxiprep System, Marligen Biosciences (High Wycombe, UK). The QIAquick® Gel Extraction Kit from Qiagen (Crawley, UK) was used for the purification of DNA from agarose gel.

2.1.4 Oligonucleotides

Oligonucleotides for QuikChange™ and PCR were custom synthesised on the 200 or 500 nM scale and Sephadex G25 purified by Invitrogen (Paisley, UK). Oligonucleotides to introduce mutations were designed with minimal base changes to achieve the correct amino acid substitution. The specific sequences of oligonucleotide designed for each mutation are

described in the relevant chapters. Lyophilised oligonucleotides were dissolved in sterile distilled water, to a concentration of 100 pmol/µl and stored at -20°C. The oligonucleotide primer sequences for the expression vector pcDNA3.1(+) (S) and pcDNA3.1(+) (AS) were 5'-TAATACGACTCACTAT-3' and 5'-TAGAAGGCACAGTCGAGGCTG-3' respectively. These oligonucleotides were used to confirm mutant receptor sequences.

2.1.5 Peptide ligands

Human ghrelin, [D-Arg^{1}, D-Phe^{5}, D-Trp$^{7, 9}$, Leu^{11}]-Substance P and [Arginine^{8}]vasopressin (AVP) were purchased from Bachem (Weil am Rhine, Germany).

2.1.6 Plasmid expression vectors

The mammalian expression vector used to express the ghrelin-R and ghrelin-R mutants was pcDNA3.1(+) (Invitrogen). pcDNA3.1(+) (Fig 2.1) is a 5.4 kb vector. The vector contains ampicillin and neomycin resistance genes for selection in *E.coli* and production of stable cell lines respectively. It also contains the human cytomegalovirus (CMV) intermediate-early promoter, required for high level expression in a wide range of mammalian cell lines and the SV40 enhancer promoter region for high level expression and episomal replication in cell lines that express the large T antigen.

2.1.7 Radiochemicals

The radiochemicals [His [^{125}I]-ghrelin (human) (specific activity of 2200 Ci/mmol) and myo-[2-^{3}H]inositol (specific activity of 22 Ci/mmol) were purchased from Perkin Elmer (Stevenage, UK).

2.1.8 Substrates

Sigma-fast *O*-phenylenediamine dihydrochloride (OPD) substrate for the enzyme-linked immunosorbent assay (ELISA) protocol was from Sigma.

2.2 Methods

2.2.1 Polymerase chain reaction (PCR)

PCR was used to introduce the HA-epitope tag at the N-terminus of the ghrelin-R. Two separate reactions were carried out using the HA-tagged human vasopressin 1a receptor (HA-hV_{1a}R) and the ghrelin-R as templates (discussed further in Chapter 3). PCR reactions were set up using 2 µl of 10 pmol/µl sense and antisense primers, 1 µl of 40 mM dNTPs, 1 µl *Pfu*

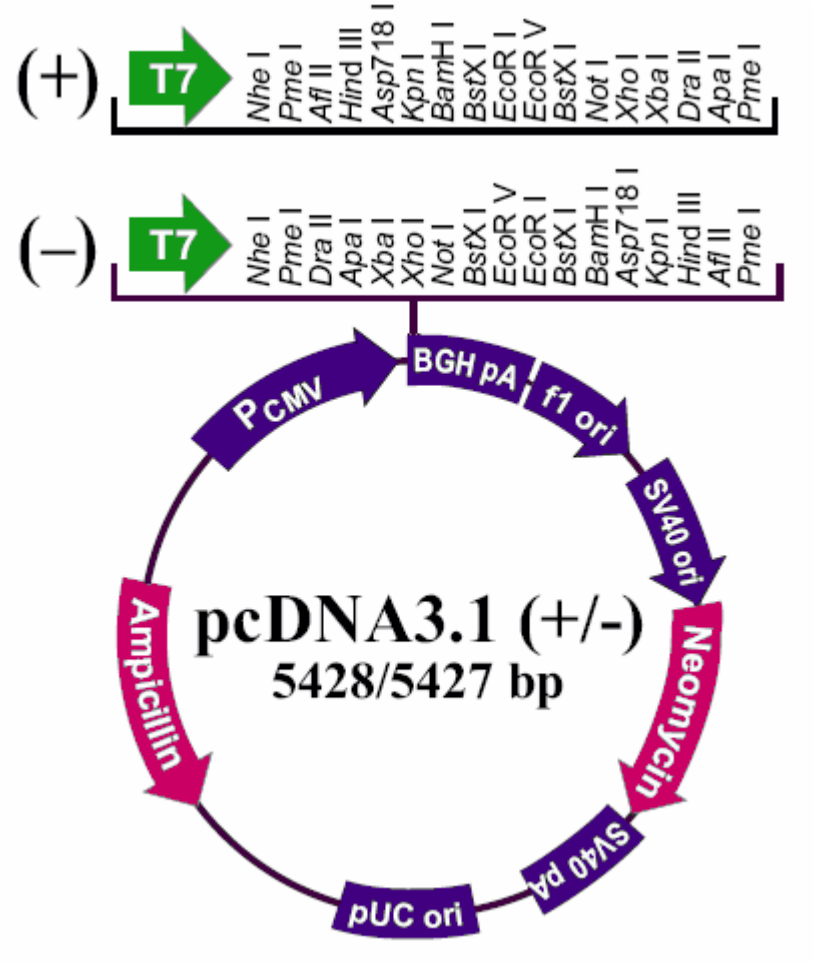


Figure 2.1 Mammalian expression vector, pcDNA3.1: The main restriction sites found within the multiple cloning site are shown for pcDNA3.1(+) and pcDNA3.1(-). The ampicillin and neomycin resistance genes and SV40 enhancer promoter regions are indicated (Image adapted from Invitrogen.com).

polymerase or 0.5 µl High Fidelity DNA polymerase (Roche, Burgess Hill, UK) and 5 µl DMSO (Sigma) in a final volume of 50 µl with sterile distilled water and 1x polymerase buffer. The program used was as follows; initial hot start 95 °C for 1 min, followed by denaturing (temperature 95 °C for 30 sec), annealing (temperature 60 °C for 1 min) and extension (temperature 68 °C for 14 min) for 12 cycles. The reactions were stored at 4 °C once the cycle was complete. The PCR products were then analysed using agarose gel electrophoresis.

2.2.2 Site-directed mutagenesis

The nine amino acid haemagglutinin (HA) epitope tag (YPYDVPDYA) was engineered at the N-terminus of the human ghrelin-R and the tagged receptor was subcloned into pcDNA3.1(+) (discussed further in Chapter 3). Mutagenesis was conducted using the QuikChange™ site-directed mutagenesis kit by Strategene (Cambridge, UK) according to the manufacturers' protocol. 100 ng plasmid cDNA in pcDNA3.1(+) was used as a template. The WT ghrelin-R or a mutant ghrelin-R construct previously constructed, was used as the template. The reaction also contained; 2 µl sense and antisense oligonucleotides, to introduce the desired mutation, at a stock concentration of 10 pmol/µl, 1 µl of 40 mM dNTPs, *Pfu* polymerase (0.5-2.0 units), *Pfu* buffer and sterile distilled water to make a final volume of 50 µl. Using a Biometra T3000 Thermocycler, the amplification cycling conditions were; denaturing, 94 °C, 1 min; annealing, 55 °C, 1 min; extension, 72 °C, 14 mins, for 12 cycles. Methylated template DNA was digested with *Dpn*I for 90 min at 37 °C.

2.2.3 Restriction enzyme digests

Restriction digests were carried out as recommended in the manufacturer's protocols. Using approximately 5 µg of cDNA and 2-10 units of restriction enzyme with 1 x (or 2 x) restriction enzyme buffer in a final volume of 10 µl the samples were incubated for 3-6 h at 37 °C. The restriction digests were analysed using agarose gel electrophoresis.

2.2.4 Agarose gel electrophoresis

QuikChange™ reactions, PCR products and restriction digests were analysed using horizontal gel electrophoresis on a 1 % agarose gel containing ethidium bromide (0.5 µg/ml). The samples were mixed (10:1) with a loading dye buffer (0.25 % (w/v) bromophenol blue, 10 mM Tris, 1 mM EDTA and 30 % (v/v) glycerol). Molecular markers were used to determine the size of DNA fragments (NEB). The system was run at 80 mV for approximately 60 min.

The gels were visualised using an ultraviolet transilluminator. They were photographed using the Ultraviolet Products gel documentation system image store 5000 programme.

2.2.5 Agarose gel cDNA purification

cDNA bands were cut from the agarose gel and purified using the QIAquick® Gel Extraction Kit from Qiagen according to the manufacturer's protocol.

2.2.6 Ligation of cDNA

Ligation reactions contained approximately a 3:1 ratio of insert:vector (concentrations of DNA estimated using gel electrophoresis). 1-3 units of T4 DNA ligase, 1 x ligase buffer and sterile distilled water were combined to make a final volume of 20-50 µl. Ligations were incubated at 16 °C for 16 h.

2.2.7 Transformation

XL-10 gold competent *E.coli* cells were made using a standard protocol (Sambrook *et al*., 1989). The cells were aliquoted to reduce freeze-thaw cycles and stored at -80 °C. 30 µl of ice-cold (thawed on ice prior to transformation) cells were incubated on ice with cDNA for 30 min. After incubation, cells were heat shocked at 42 °C for 30 sec. This was followed by a further 2 min incubation on ice, after which 0.8 ml of Luria broth (LB) (1 % (w/v) peptone, 0.5 % (w/v) NaCl, 0.5 % yeast extract) was added and the cells were incubated at 37 °C for 1 h. After incubation, the cells were sedimented by centrifugation at 13,000 rpm for 10 min. The pellet was resuspended in approximately 50 µl of LB, and spread on LB agar plates, containing 100 µg/ml ampicillin, and incubated overnight at 37 °C. Single colonies were selected from the plate and grown overnight in LB containing 100 µg/ml ampicillin at 37 °C for cDNA extraction.

2.2.8 Plasmid cDNA preparation

The plasmid cDNA was extracted from the XL-10 gold cells and purified in a "mini-prep" using the Wizard® Plus SV kit (1-10 µg yield), from Promega following the centrifugation method in the manufacturers' protocol. The cDNA was eluted into 100 µl of sterile distilled water. "Mini-prep" allowed small scale preparation of the cDNA for automated fluorescent sequencing. "Maxi-prep" cDNA preparation (0.5-3.0 mg) was carried out using the purified columns Powerprep™ HP Plasmid Purification Systems (Marligen) according to the manufacturers' protocol. The extracted cDNA was analysed by restriction digest and gel

electrophoresis. The “maxi-prep” cDNA concentration was determined by DNA absorbance spectroscopy, measuring the absorbance of a 1 in 100 dilution of the sample at 260 nm. The cDNA purity was determined by measuring the absorbance ratio at 260/280 nm.

2.2.9 Automated fluorescent DNA sequencing

The DNA sequence of all WT and mutant receptor constructs were obtained by fluorescent automated sequencing using the pcDNA3.1(+) sequencing primers at pM concentrations (Functional Genomics and Proteomics Laboratories, University of Birmingham).

2.2.10 Cell culture

Human embryonic kidney (HEK) 293T cells were cultured in DMEM supplemented with 10 % (v/v) FBS, in a humidified 5 % (v/v) CO_2 incubator at 37 °C. Cells were subcultured twice weekly to maintain approximately 50 % confluence. For inositol phosphate accumulation assays, cells were seeded onto PDL-coated 12-well plates at a density of approximately 2.5 x 10^5 cells per well. For ligand binding assays cells were seeded at a density of approximately 5 x 10^5 cells/100 mm dish. For ELISA measurement of cell-surface expression, cells were seeded onto PDL-coated 24-well plates at an approximate density of 1.5 x10^5 cells per well.

2.2.11 PEI transfections

For inositol phosphate accumulation assays and ELISA, transfections were carried out 24 h after seeding cells. HEK 293T cells were transfected using (per 1 μg cDNA), 8 μl 10 mM PEI and 60 μl 5 % glucose solution. Before transfection, this mixture was allowed to incubate at room temperature for 30 min. After incubation, DMEM was added to make an appropriate final volume, and the transfection mixture was added to cells. For ligand binding assays, cells were transfected 48 h after seeding with 5 μg cDNA per plate, 60 μl 10 mM PEI and 1.0 ml 5 % glucose solution. Cells were incubated for 48 h at 37 °C, 5 % CO_2 (v/v) before characterisation. The dominant negative K44A dynamin mutant construct was a gift from Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, USA) and was transfected in a similar manner to the receptor cDNA.

2.2.12 Inositol phosphate accumulation assays

16-24 h post-transfection, the cell culture DMEM was replaced with inositol-free DMEM containing 1 μCi/ml myo-[2-^{3}H]inositol and incubated for 24 h at 37 °C, 5 % CO_2 (v/v). Cells were then gently washed with 1 x PBS and incubated with inositol-free DMEM containing 10

mM LiCl for 30 min at 37 °C. This was followed by a further incubation with ligand at concentrations ranging from 10^{-11} to 10^{-5} M for 45 min (see Chapter 3). The reaction was terminated by aspiration of media from each well, followed by the addition of 0.5 ml 5 % (v/v) perchloric acid, containing 1 mM EDTA and 1 mg/ml phytic acid, for 15 min at room temperature. Neutralisation with 1.2 M KOH, containing 10 mM EDTA and 50 mM HEPES, was followed by incubation on ice for at least 1 h, preferably overnight. Precipitate was sedimented by centrifugation at 13,000 rpm for 10 min, then the supernatant was loaded onto Bio-Rad AG1-X8 (formate form), (Hemel Hempstead, UK) filled columns. 10 ml of 60 mM ammonium formate (NH_4COOH) with 0.1 M formic acid (HCOOH) was added to elute free inositol and glycerophosphoinositol. 10 ml 850 mM NH_4COOH, 0.1 M HCOOH was used to elute inositol mono-, bis-, and trisphosphate (InsP-$InsP_3$). The eluant was mixed with 10 ml UltimaFlo AF scintillation cocktail (Perkin Elmer) and radioactivity was quantified using liquid scintillation counting. Data was analysed by non-linear regression using GraphPad Prism 4.0 software (GraphPad, San Diego, USA). The ghrelin EC_{50} for each receptor construct was determined. pEC_{50} ± SEM are recorded in Appendix Table 9.2.

2.2.13 Harvesting and preparation of cell membranes

The harvesting of cell membrane extracts for the radioligand binding assays were carried out as described (Wheatley *et al.*, 1997). Briefly, cells were washed twice in ice cold 1 x PBS. The cells were then scraped from the plates using a standard membrane harvesting buffer containing 20 mM HEPES, 1 mM EGTA, 10 mM $Mg(CH_3COO)_2$ at pH 7.4, with the addition of 250 mM sucrose and 0.1 mg/ml of bacitracin. Cells were centrifuged at 4000 rpm at 4 °C for 10 minutes and then resuspended in a harvesting buffer that contained 0.1 mg/ml bacitracin. The cells were then incubated for 20 min on ice. After incubation the cells were centrifuged as before and resuspended in a harvesting buffer containing 250 mM sucrose. The membranes were stored at –20 °C in 500 µl aliquots.

2.2.14 Protein Assays

The approximate total protein concentration of membrane preparations was determined with the Pierce BCA (bicinchoninic acid) protein assay kit (Pierce, Northumberland, UK) following the manufacturers' protocol. A standard curve using bovine serum albumin (BSA) was produced to determine the unknown protein concentrations.

2.2.15 Radioligand binding assays

The binding assays were performed using 10 pM His-[^{125}I]ghrelin as a tracer. Membranes, harvested previously, containing 50-300 μg total protein (determined using protein assays) were diluted in 10.5 ml assay buffer (20 mM HEPES, 1 mM EGTA, 10 mM $Mg(CH_3COO)_2$, 1 mg/ml BSA; pH 7.4). Competing ligand was added at a concentration range of 10^{-6} to 10^{-11} M to make a final volume of 500 μl. Incubation was at 30 ˚C for 60 min to establish binding equilibrium. The bound and the free ligands were separated by centrifugation at 13,000 rpm for 10 min and the pelleted membranes were then washed and dried. 50 μl Soluene 350 (Perkin Elmer) was added to each tube to solubilise the membranes. After solubilisation, 1 ml of ScintiSafe™ liquid scintillation cocktail (Fisher Scientific) was added and the radioactive content was counted using liquid scintillation counting. The experimental binding data was analysed using non-linear regression. A theoretical Langmuir binding isotherm was fitted to the experimental data using GraphPad Prism Version 4.0 software. pIC_{50} values were determined from the experimental data.

2.2.16 Enzyme-linked immunosorbant assay (ELISA)

48 h post-transfection, cell culture media was aspirated from the wells and cells were fixed for 15 min with 0.5 ml 3.7 % formaldehyde. For internalisation experiments, cells were stimulated prior to fixing with 10^{-7} M ligand. After fixing, cells were washed three times with 1 x PBS and treated for 45 min with 0.5 ml blocking solution (1 % BSA in 1 x PBS) to limit non-specific antibody binding. After treatment, 250 μl anti-HA primary antibody (made in mouse) (1:3000) was added to each well and incubated for 1 h at room temperature followed by three washes with 1 x PBS. Blocking treatment was reapplied for a further 15 min after which 250 μl anti-mouse secondary antibody (made in horse) conjugated to HRP (1:2000) was added for 1 h. Cells were washed again three times and OPD-substrate was added according to manufacturers' instructions. The reaction was terminated by the addition of 100 μl, 1 M H_2SO_4 and the absorbance read at 492 nM.

CHAPTER 3.

THE GHRELIN-R: EPITOPE TAGGING, CONSTITUTIVE ACTIVITY AND FAMILY A CONSERVED RESIDUES

3.1 The ghrelin-R haemagglutinin epitope tag

3.1.1 Introduction

Epitope tags can be incorporated into recombinant proteins to allow identification, characterisation and *in vitro* localisation of target proteins (Jarvik *et al.*, 1998). By producing a protein that is immunoreactive to readily available commercial antibodies, proteins of interest can be isolated efficiently. Antibodies to the protein itself can be raised directly, but this is often costly and unreliable, particularly with GPCRs. Epitope tags can be introduced to make antibody detection easier, cheaper and more reliable. Epitope tags are generally small peptides consisting of between 6 and 30 amino acids and are engineered at a specific point within a protein to preserve biological activity, most commonly at the N- or C-terminus (Jarvik *et al.*, 1998). Common epitope tags are the HA (YPYDVPDYA), derived from the human influenza virus (Wilson *et al.*, 1984), the c-myc tag (EQKLISEEDL), and the FLAG™-tag (DYKDDDDK), a synthetic epitope designed by Strategene (Hopp *et al.*, 1988).

As yet, no useful antibody raised to the ghrelin-R has been reported, therefore it was necessary to introduce an epitope into the ghrelin-R sequence. The introduction of the tag enables ELISAs, Western blots and immunoprecipitation assays to be conducted using antibodies to the tag. To facilitate down-stream characterisation of both the WT receptor and the mutant ghrelin-R constructs, an HA-epitope tag was engineered at the N-terminus of the receptor. Studies on the ghrelin-R (Pantel *et al.*, 2006), and other GPCRs (Schoneberg *et al.*, 1995; Hawtin *et al.*, 1997; Gaylinn, 1999), have indicated that an N-terminal HA-tag is rarely obstructive and is unlikely to affect ligand binding or subsequent intracellular signalling cascades. The N-terminus is accessible in intact cells and therefore has greater utility than a C-terminal tag. Furthermore, an N-terminal tag is far removed from any receptor interactions with intracellular proteins.

3.1.2 Results

The nine amino acid HA-tag (YPYDVPDYA) was engineered, using PCR-based techniques, into the N-terminus of the ghrelin-R, immediately after the initiation methionine. The HA-tag has been shown, with other GPCRs, to have no effect on hormone binding, receptor signalling or the translocation and cell-surface expression of the receptor and was thought therefore unlikely to have any detrimental effect on the WT ghrelin-R pharmacology. The Kozak consensus sequence was identified as a sequence necessary for the optimal initiation of translation (Kozak, 1991). The Kozak consensus sequence (GCCACC) and an *Eco*RI restriction endonuclease site (GAATTC) were engineered upstream of the ATG start codon to aid transcription and enable subcloning of the receptor constructs, respectively.

The HA-tag was introduced using a PCR-based method that required the production of two fragments by two PCR reactions, in parallel. A third reaction was required to incorporate these fragments into a final product (Fig 3.1).

For the first fragment (reaction 1) the template was the ghrelin-R clone in pcDNA5.1(+). A sense primer was required, that was complementary to the N-terminus of the receptor, minus the ATG, with a 5'-end that was complementary to the C-terminal end of the HA-tag. The antisense primer annealed to the pcDNA5.1(+) vector downstream of the receptor (Table 3.1). The Bgh site at the 3'-end of the multiple cloning site was used as the antisense primer region.

The second reaction (reaction 2) required the HA-tagged $V_{1a}R$ in the pcDNA3.1(+) expression vector (tagged previously in our laboratory) as a template. The HA-tag, initiation methionine, Kozak consensus sequence, *Eco*RI restriction site and a section of pcDNA3.1 were amplified using a sense primer, complementary to the T7 site found at the 5'-end of the multiple cloning site of pcDNA3.1(+). The antisense primer was complementary to the C-terminal end of the HA-tag.

Two PCR-based reactions were carried out to amplify these regions producing two products, one encompassing the ghrelin-R, with the C-terminal end of the HA-tag located at the N-terminus, and the other, smaller fragment, encompassing expression vector, the *Eco*RI restriction site, Kozak sequence, ATG start codon and the complete HA-tag. A third PCR reaction (reaction 3) was carried out using the two purified fragments from reactions 1 and 2 as the template cDNA. Where the fragments were complementary, at the HA-tag sequence, the cDNA strands annealed, producing a template fragment that contained the HA-tag at the

HA-tag: YPYDVPDYA

ATG-TAC-CCC-TAC-GAC-GTC-CCC-GAC-TAC-GCC

Start Tyr Pro Tyr Asp Val Pro Asp Tyr Ala

	Sense Oligonucleotide	Antisense Oligonucleotide
Reaction 1	5'-**CGTCCCCGACTACGCC**TGGAA CGCGACGCCCAGCGAAG-3'	5'-TAGAAGGCACAGTCGAGGCTG-3'
Reaction 2	5'-TAATACGACTCACTAT-3'	5'-**GGCGTAGTCGGGGACG**-3'
Reaction 3	5'-TAATACGACTCACTAT-3'	5'-TAGAAGGCACAGTCGAGGCTG-3'

Table 3.1 Primers for introducing the HA-tag to the N-terminus of the ghrelin-R: Reaction 1, 2 and 3 primers for the introduction of the HA-tag to the ghrelin-R. The ghrelin-R was used as a template in reaction 1 and HA-V_{1a}R as a template in reaction 2. Codons for the introduction of the HA-tag are shown in **red**.

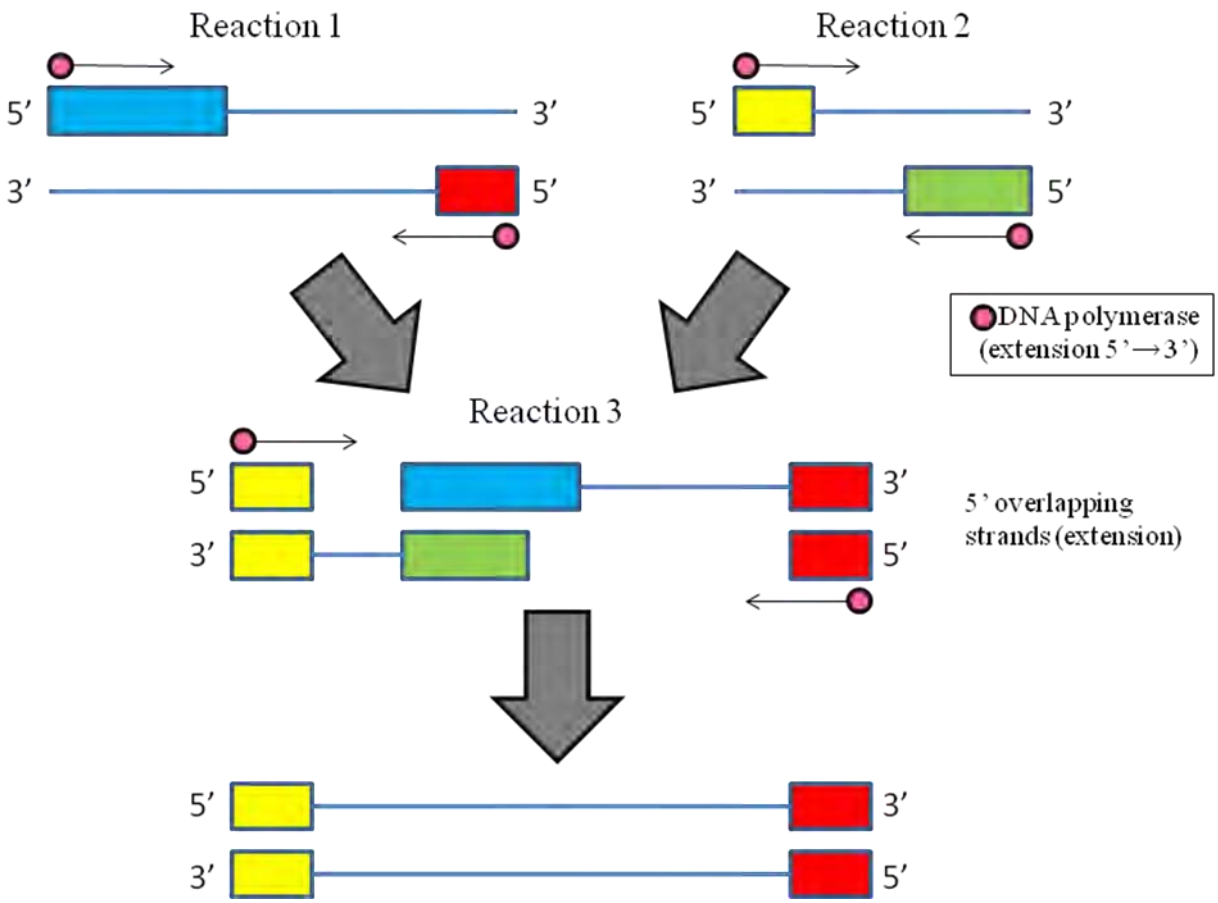


Figure 3.1 PCR-based method for HA-tagging of the ghrelin-R: Reaction 1 template was the ghrelin-R in pcDNA5.1(+) and used a sense primer that was complementary to the N-terminus of the ghrelin-R with a 5’-overhanging end that was complementary to the HA-epitope sequence (**blue**). The antisense primer was complementary to the multiple cloning site at the 3’-end of the ghrelin-R (Bgh- **red**). Reaction 2 template was the HA-V_{1a}R and used a sense primer complementary to the 5’-end of the multiple cloning site (T7- **yellow**) and an antisense primer complementary to the C-terminal end of the HA-tag (**green**). Reaction 3 required the products of reactions 1 and 2 and the T7 and Bgh primers to amplify a fragment containing the full HA-epitope tag sequence at the 5’-end of the ghrelin-R.

N-terminus of the ghrelin-R. The T7 and Bgh primers were then used to amplify the complete fragment, which contained the restriction sites, Kozak consensus sequence, initiation methionine, HA-tag and ghrelin-R. Restriction digests using *Eco*RI, at the N-terminus, and *Not*I, located in the multiple cloning site at the C-terminal end of the ghrelin-R, were carried out and the insert ligated into purified pcDNA3.1(+). Automated DNA sequencing revealed the presence of the HA-tag at the N-terminus of the ghrelin-R, and that the receptor sequence was otherwise unchanged, compared to untagged ghrelin-R (Fig 3.2).

3.1.2.1 Characterisation of the HA-ghrelin-R

The HA-ghrelin-R was characterised pharmacologically with respect to ligand binding, intracellular signalling and cell-surface expression (Fig 3.3, Table 3.2). The HA-ghrelin-R was compared to the untagged ghrelin-R to determine i) whether the tag affected the pharmacology of the receptor and ii) that the HA-tag enabled detection of the receptor using antibodies. The radioligand binding assay using [^{125}I]ghrelin vs. ghrelin demonstrated that the HA-ghrelin-R was able to bind ghrelin with an affinity comparable to the untagged receptor. The tag also had no effect on the intracellular signalling capabilities of the ghrelin-R, with the HA-ghrelin-R demonstrating basal signalling, ghrelin potency and efficacy comparable to untagged ghrelin-R and similar to the values reported by others (Matsumoto *et al.*, 2001). The ELISA indicated that the HA-ghrelin-R could be detected at the cell surface using anti-HA antibodies. The untagged ghrelin-R did not appear to be detected at the cell-surface in transiently transfected HEK 293T cells using anti-HA antibody, as the expression was only 6 ± 2 % of the HA-ghrelin-R cell-surface expression.

3.1.2.2 HA-ghrelin-R internalisation study

The ghrelin-R has previously been shown to internalise in a time-dependant manner after ghrelin stimulation (Camina *et al.*, 2004; Holliday *et al.*, 2007). Holliday *et al*. (2007) demonstrated ghrelin-R internalisation using immunofluorescence microscopy after incubation of transiently transfected cells with 100 nM ghrelin. Furthermore, kinetic studies using confocal microscopy, GFP-tagged ghrelin-R and radioligand binding have demonstrated ghrelin-R internalisation that is maximal 20 min after stimulation with ghrelin (Camina *et al.*, 2004). Holst *et al*. (2004) conducted experiments on an N-terminally FLAG-tagged ghrelin-R which demonstrated a high degree of constitutive internalisation. It was found, however, in contrast to the results of a previous kinetic study (Camina *et al.*, 2004) that stimulation of the FLAG-tagged ghrelin-R with ghrelin did not result in further receptor internalisation.

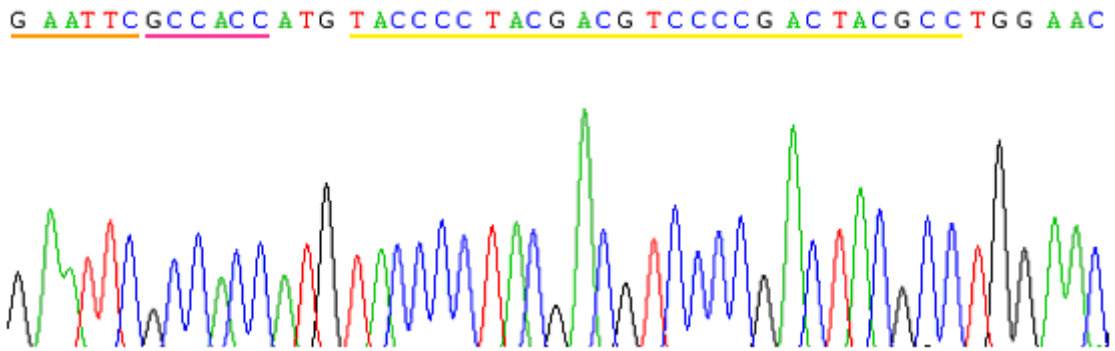


Figure 3.2 Chromatogram displaying sequence of the HA-ghrelin-R: The *Eco*RI restriction site (underlined in **orange**), the Kozak consensus sequence (underlined in **pink**) and the HA-epitope tag (underlined in **yellow**) were engineered at the N-terminus of the ghrelin-R. The ATG start codon is located upstream of the HA-tag.

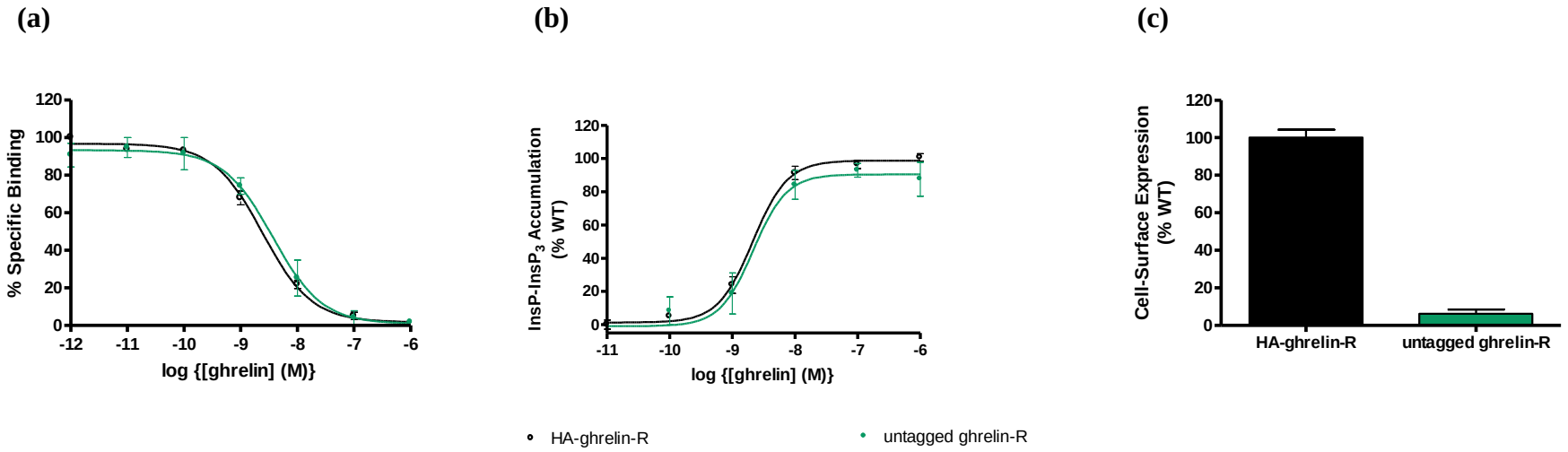


Figure 3.3 Ligand binding, intracellular signalling and cell-surface expression profiles of the HA-ghrelin-R: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with untagged ghrelin-R or HA-ghrelin-R. Values expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with untagged ghrelin-R or HA-ghrelin-R. Values are expressed as a percentage of the HA-ghrelin-R (WT), ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of HA-ghrelin-R and untagged ghrelin-R expressed as a percentage of the HA-ghrelin-R (WT). All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation			Cell-Surface Expression (% HA-ghrelin-R) ± SEM
		EC_{50} (nM)	Basal (% HA-ghrelin-R) ± SEM	E_{max} (% HA-ghrelin-R) ± SEM	
untagged ghrelin-R	8.43 ± 0.02	2.16 ± 1.33	-7 ± 4	88 ± 10	6 ± 2
HA-ghrelin-R	8.61 ± 0.02	2.12 ± 0.23	0	100	100

Table 3.2 Ligand binding, intracellular signalling and cell-surface expression for the HA-ghrelin-R and untagged ghrelin-R: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Red indicates no detectable cell-surface expression.

One suggestion for this lack of agonist-induced receptor internalisation was that there is a steric hindrance of the FLAG-tagged receptor with ghrelin (Camina, 2006).

To investigate the effects of ghrelin stimulation on the HA-ghrelin-R in our system, the HA-ghrelin-R was transiently transfected into HEK 293T cells and time-course ELISA experiments were conducted. Transfected cells were stimulated with 100 nM ghrelin and cell-surface expression compared to that of unstimulated transfected cells. As a control experiment, the HA-V_{1a}R was transfected in parallel and stimulated with the agonist, arginine vasopressin (AVP). The HA-V_{1a}R is known to internalise upon receptor stimulation with 100 nM AVP (Hawtin *et al.*, 1997). The data from the time-course ELISA experiment indicated that the HA-ghrelin-R does not internalise significantly after ghrelin stimulation in HEK 293T cells (Fig 3.4 (a)). In contrast, the HA-V_{1a}R showed a significant depletion in the number of receptors present at the cell surface after stimulation with AVP (Fig 3.4 (b)). A further time-course ELISA was carried out, over a shorter period of time, to determine whether the HA-ghrelin-R is internalised and recycled rapidly after ghrelin stimulation. In agreement with the longer time-course, rapid HA-ghrelin-R internalisation was not detectable (Fig 3.5).

A dominant negative mutant of dynamin2, K44A-dynamin (van der Bliek *et al.*, 1993), was transiently co-transfected with the HA-ghrelin-R and the HA-V_{1a}R to investigate the effect of a disruption of the clathrin-mediated internalisation mechanisms on receptor cell-surface expression and internalisation. Co-transfection of K44A-dynamin with the HA-ghrelin-R resulted in significantly increased cell-surface expression of the HA-ghrelin-R (148 ± 9 % of HA-ghrelin-R), suggesting a disruption to the ligand-independent internalisation of the receptor. The HA-V_{1a}R, which is known to display almost no constitutive activity, and is therefore unlikely to demonstrate significant ligand-independent internalisation, did not show a significant increase in the cell-surface expression of the receptor (114 ± 6 % of HA-V_{1a}R) when co-transfected with K44A-dynamin (Fig 3.6, Table 3.3).

Throughout the remainder of this study, WT ghrelin-R refers to the HA-ghrelin-R, which was used as a template for all subsequent site-directed mutagenesis, thereby enabling quantification of mutant receptor constructs at the cell-surface.

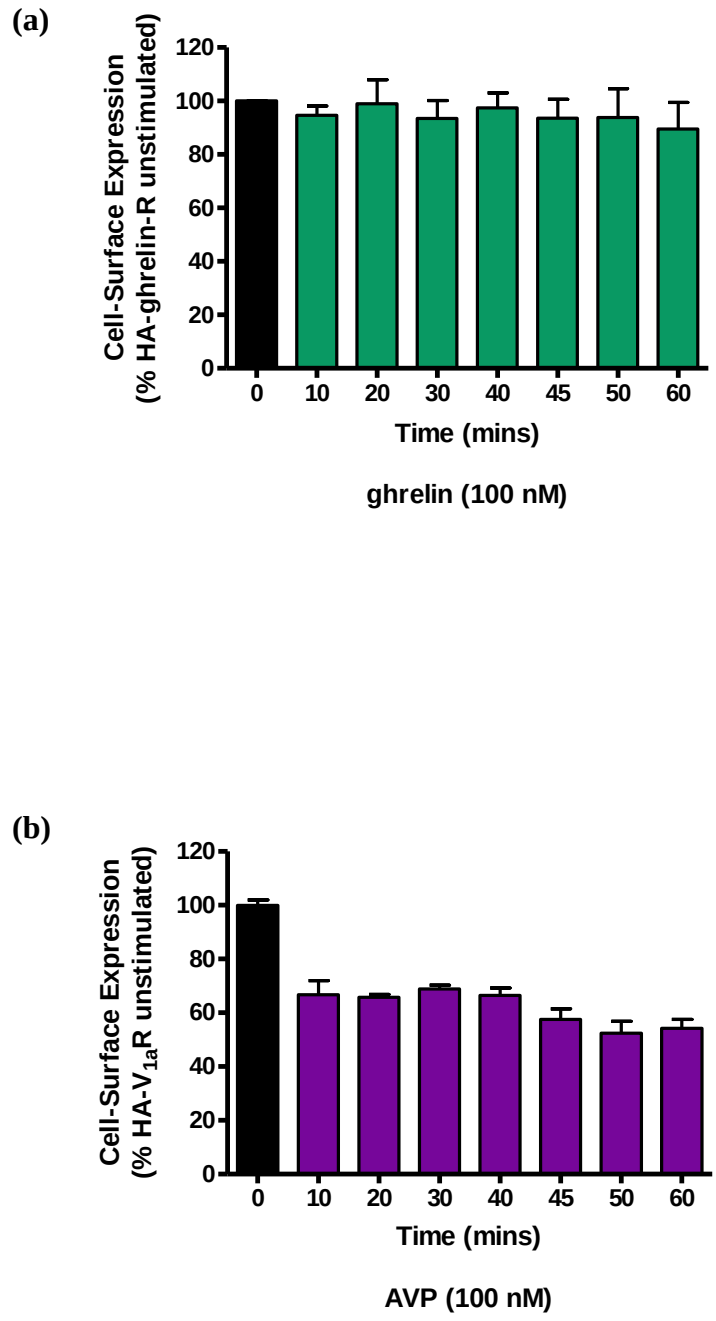


Figure 3.4 Time-course of cell-surface expression to determine agonist-induced internalisation: Time-course ELISA performed on HEK 293T cell transiently transfected with (a) HA-ghrelin-R or (b) HA-V_{1a}R. The time-course was conducted over 60 minutes to investigate the effect of (a) 100 nM ghrelin on the HA-ghrelin-R cell-surface expression or (b) 100 nM AVP on HA-V_{1a}R cell-surface expression. Data are presented as a percentage of the unstimulated receptor ± SEM of three separate experiments performed in triplicate.

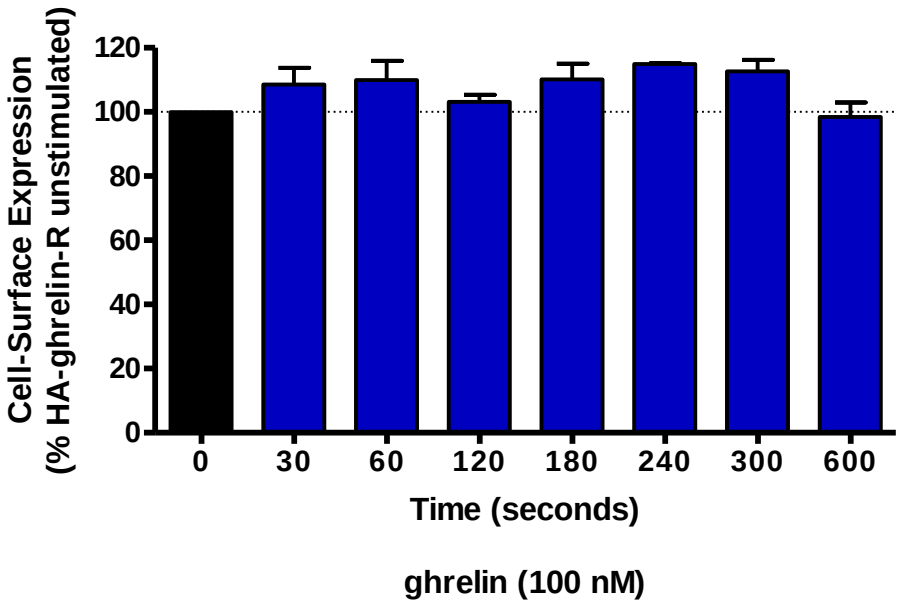
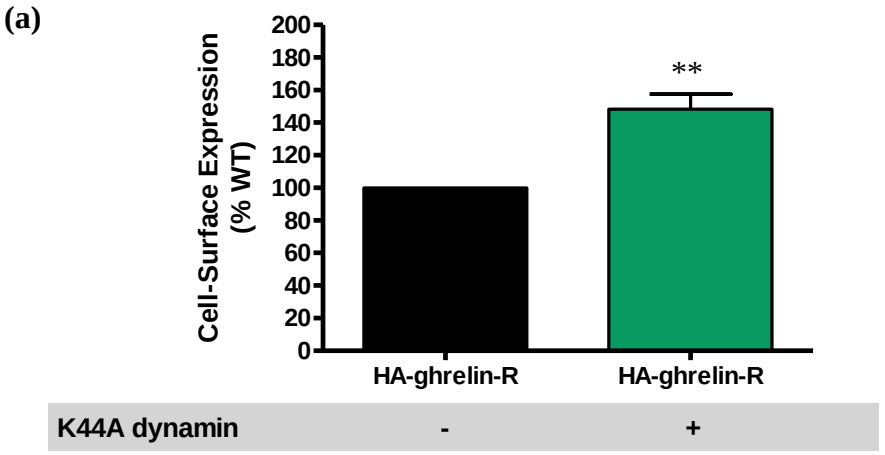


Figure 3.5 Time-course of cell-surface HA-ghrelin-R expression following ghrelin (100 nM) challenge: Time-course ELISA conducted on HEK 293T cell transiently transfected with HA-ghrelin-R and stimulated with 100 nM ghrelin. Data presented as a percentage of the unstimulated receptor ± SEM of three separate experiments performed in triplicate.



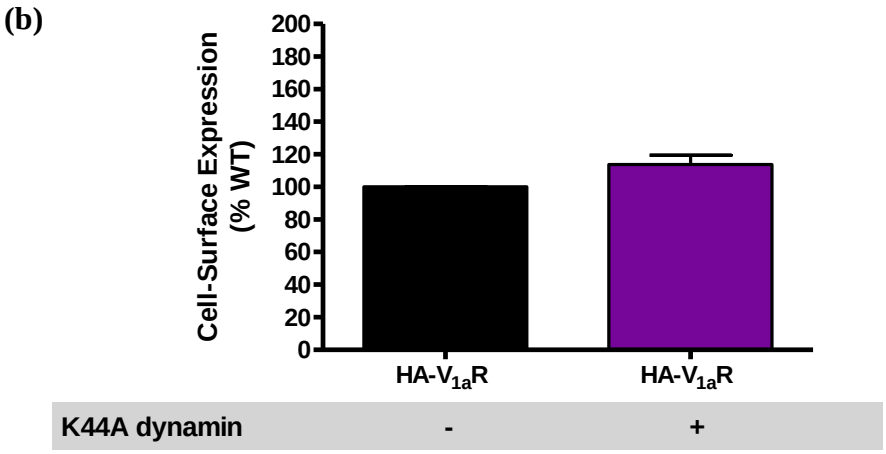


Figure 3.6 Receptor cell-surface expression when co-transfected with K44A-dynamin: (a) HA-ghrelin-R cell-surface expression and co-transfected HA-ghrelin-R with K44A-dynamin and (b) HA-V_{1a}R cell-surface expression and co-transfected HA-V_{1a}R with K44A-dynamin. Data are a percentage of HA-ghrelin-R (WT) or HA-V_{1a}R (WT) expression ± SEM of three separate experiments performed in triplicate.

Receptor	Cell-Surface Expression (% HA-tagged receptor)	Cell-Surface Expression + K44A-dynamin (% HA-tagged receptor) ± SEM
HA-ghrelin-R	100	148 ± 9 **
HA-V_{1a}R	100	114 ± 6

Table 3.3 Receptor cell-surface expression when co-transfected with K44-dynamin: HA-ghrelin-R and HA-V_{1a}R expression, alone, and when co-transfected with K44A-dynamin. Data shown are the mean ± SEM of three separate experiments performed in triplicate. Pink indicates a significant increase in cell-surface expression compared to the receptor transfected alone (** $P < 0.05$).

3.2 The ghrelin-R displays high constitutive activity

3.2.1 Introduction

It is well established that the ghrelin-R displays a high level of constitutive activity, signalling at approximately 50 % of the ghrelin-induced maximum in the absence of agonist (Holst *et al.*, 2003a). The ghrelin-R has been found to signal to this extent in the absence of ligand, *in vivo* (Petersen *et al.*, 2009), and *in vitro* (Holst *et al.*, 2003a).

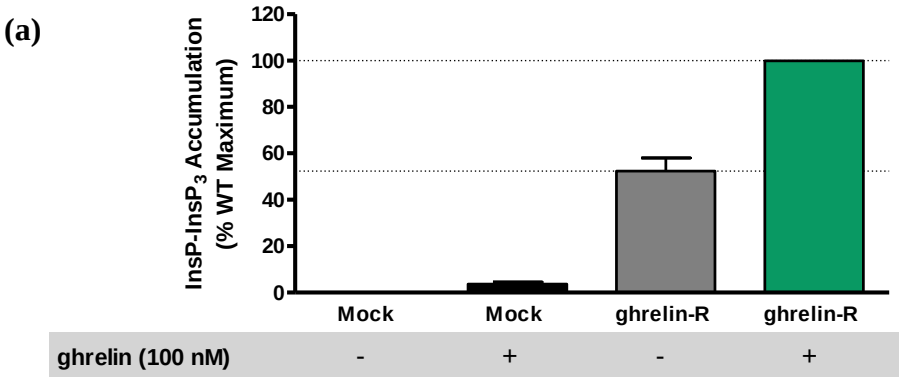
3.2.2 Results

To determine the level of constitutive activity of the ghrelin-R when expressed in HEK 293T cells, InsP-$InsP_3$ accumulation assays were conducted on the ghrelin-R and the $V_{1a}R$ in parallel. The $V_{1a}R$ has been extensively studied and is known to demonstrate little constitutive activation of the InsP pathway in HEK 293T cells. InsP-$InsP_3$ accumulation assays for mock-transfected cells and for the ghrelin-R and $V_{1a}R$, alone, and after stimulation with ghrelin or AVP respectively, were conducted and comparisons made between the ghrelin-R and $V_{1a}R$. The data supported the high level of constitutive activity previously reported for the ghrelin-R (Fig 3.7, Table 3.4).

Throughout this study, data have been presented normalised to the WT ghrelin-R basal activity and the ghrelin-induced maximum and are expressed as a percentage of the WT ghrelin-R maximum. The basal signalling for mutant receptor constructs that decrease constitutive activity are therefore expressed as negative values. Mutations that caused substantial decreases in constitutive activity were further analysed and normalised to mock-transfected cells and to WT ghrelin-R basal, to gain a more comprehensive insight into the agonist-independent activity of the ghrelin-R and are expressed as a percentage of WT basal.

3.2.2.1 Inositol phosphate accumulation assays

A time-course of InsP-$InsP_3$ accumulation was conducted to investigate the optimum period of ghrelin stimulation for InsP-$InsP_3$ production. Figure 3.8 depicts a representative graph of the time-course assay, indicating the dpm for each time period. The basal InsP-$InsP_3$ accumulation corresponds to each period of stimulation. InsP-$InsP_3$ production was near maximum after 15 min, increasing slightly up to 45 min post ghrelin stimulation, and little difference was observed between the accumulation after 45, 60 and 75 min.



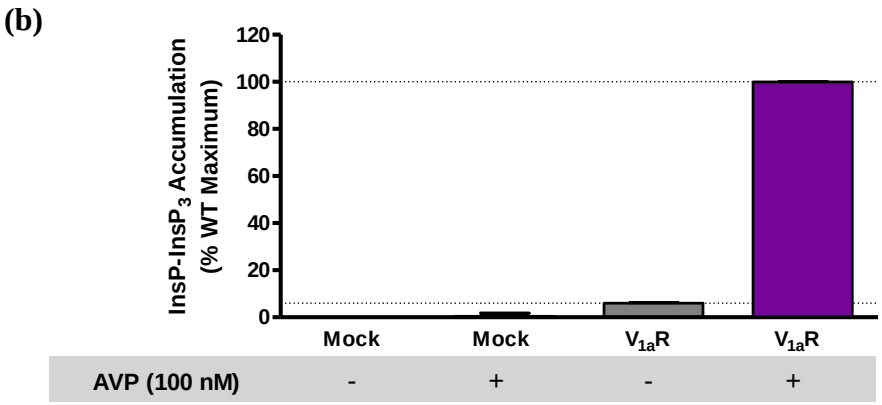


Figure 3.7 Ghrelin-R and $V_{1a}R$ constitutive activity: InsP-$InsP_3$ accumulation assays demonstrating the agonist-independent activity of (a) the ghrelin-R and (b) the $V_{1a}R$, expressed as a percentage of the maximal agonist-induced response (ghrelin and AVP respectively).

	InsP-$InsP_3$ Accumulation (% WT max) ± SEM		
	Unstimulated	**100 nM ghrelin**	**100 nM AVP**
Mock	0	3.6 ± 0.9	0.5 ± 1.4
WT ghrelin-R	52.0 ± 5.7	100	-
WT $V_{1a}R$	6.1 ± 0.1	-	100

Table 3.4 InsP-$InsP_3$ accumulation assays demonstrating the agonist independent activity of the ghrelin-R and the $V_{1a}R$: All data presented are the mean ± SEM of three or more separate experiments performed in triplicate. Data normalised to mock unstimulated cells and WT receptor agonist-induced maximum.

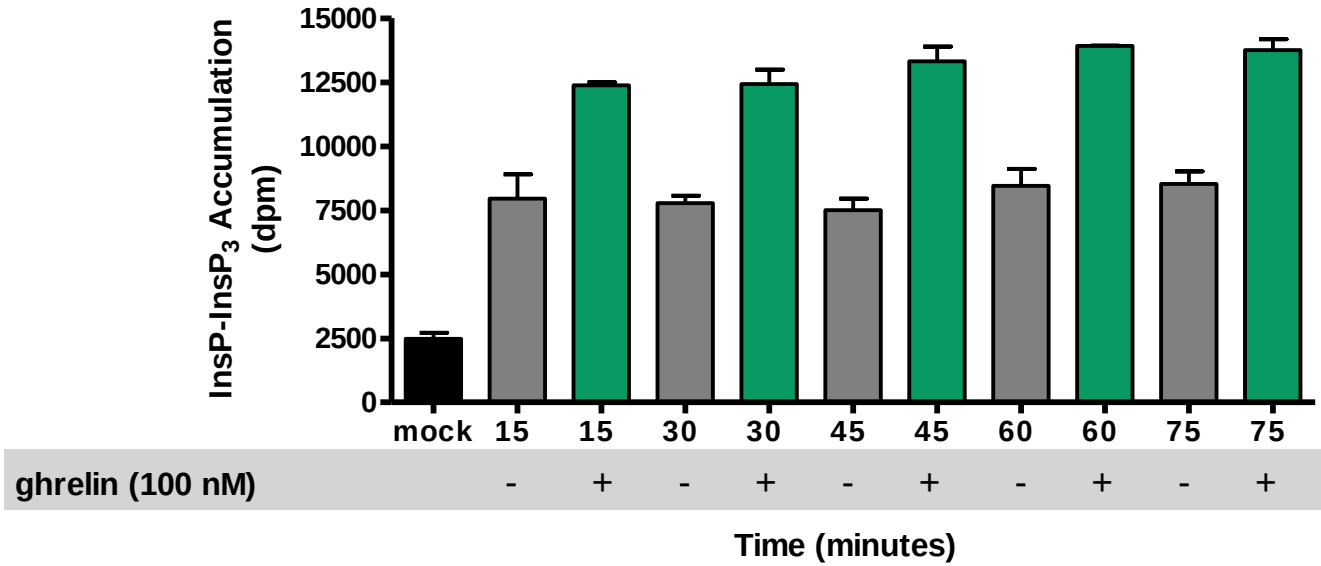


Figure 3.8 Time-course InsP-InsP$_3$ accumulation assay for the WT ghrelin-R: HEK 293T cell transiently transfected with ghrelin-R were stimulated with 100 nM ghrelin for 15, 30, 45, 60 or 75 min. Basal ghrelin-R InsP-InsP$_3$ accumulation was determined in parallel and the difference between basal and ghrelin-induced maximum determined for each time period, in dpm. Data shown are representative of the results of three individual experiments. Graph shows the results of a single experiment, performed in triplicate.

3.2.2.2 Radioligand binding

Radioligand binding curves were generated for the WT ghrelin-R and mock-transfections to determine the binding affinity of ghrelin for the WT ghrelin-R in transiently transfected HEK 293T cells. Mock transfections were included to ensure that ghrelin did not bind appreciably in cells that were not transfected with the ghrelin-R. Specific binding of ghrelin was determined in crude HEK 293T membrane preparations and determined as total binding of [^{125}I]ghrelin minus non-specific binding defined by the presence of 1 μM unlabelled ghrelin, in parallel. The mock-transfected cells displayed no detectable binding of 10 pM [^{125}I]ghrelin. The WT ghrelin-R bound ghrelin with a pIC_{50} of 8.61 which was comparable to values reported by others (Bedendi *et al.*, 2003) (Fig 3.9, Table 3.5).

3.2.2.3 [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-Substance P is an inverse agonist at the ghrelin-R

The SP-analogue has been reported to be an inverse agonist at the ghrelin-R (Holst *et al.*, 2003a). This was confirmed in our system. The affinity of SP-analogue was determined in competition radioligand binding assays using [^{125}I]ghrelin. SP-analogue pIC_{50} = 7.92 in agreement with published values (Holst *et al.*, 2003a) (Fig 3.10 (a), Table 3.6). 1 μM ghrelin was used to determine non-specific binding. InsP-InsP_3 accumulation assays were conducted on the WT ghrelin-R in transiently transfected HEK 293T and the SP-analogue potency and efficacy were determined. SP-analogue inhibited basal signalling with a pIC_{50} = 7.72 (19 nM) in agreement with published values (Holst *et al.*, 2006a). The maximum inhibition of ghrelin-R constitutive signalling was 51 % of ghrelin-R basal (Fig 3.10 (c) and (d), Table 3.6). The SP-analogue was not able to reduce the constitutive activity of the ghrelin-R down to the levels observed for the mock-transfected controls. This is in contrast to data previously reported by Holst *et al*. (2003) which stated that SP-analogue is a full inverse agonist of the ghrelin-R and reduces ligand-independent signalling down to the levels observed for the empty expression vector controls.

Some GPCR inverse agonists are known to cause an increase in cell-surface expression, potentially due to the reduction of constitutive internalisation (Shinyama *et al.*, 2003; Pula *et al.*, 2004). SP-analogue has been shown previously to increase the cell-surface expression of the WT ghrelin-R as indicated by ELISA (Liu *et al.*, 2007a) and immunofluorescence microscopy (Holliday *et al.*, 2007) in HEK 293 cells. In this study, the HEK 293T cells transiently transfected with the ghrelin-R were stimulated with 100 nM SP-analogue to

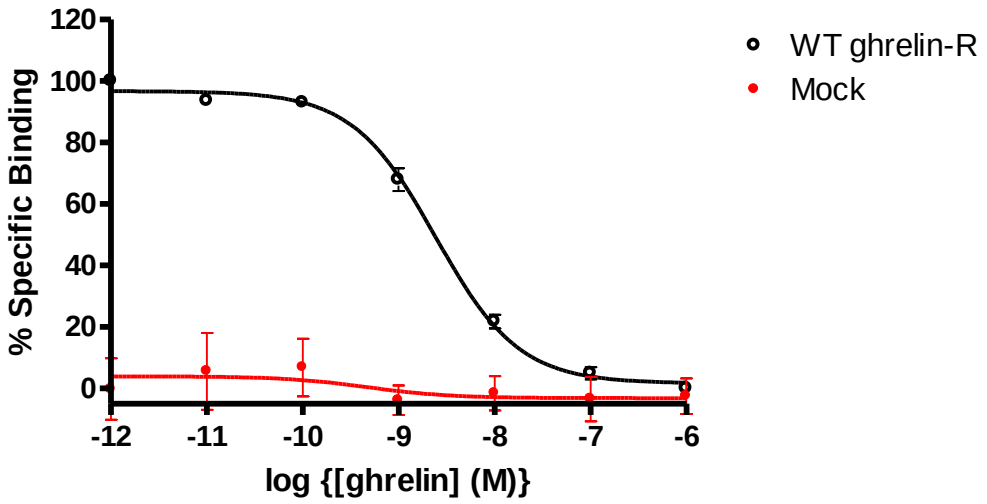


Figure 3.9 Radioligand binding for WT ghrelin-R and mock-transfected membranes: Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or on mock-transfected cells using [^{125}I]ghrelin vs. ghrelin. Data are expressed as a percentage of WT ghrelin-R specific binding. Data are the mean ± SEM of three or more separate experiments, performed in triplicate. To determine specific binding, ghrelin-R and mock-transfection binding assays were performed in parallel.

	Binding Affinity pIC_{50} ± SEM
WT ghrelin-R	8.61 ± 0.02
Mock	NDB

Table 3.5 Radioligand binding for WT ghrelin-R and mock-transfected membranes: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate and in parallel. Red indicates no detectable binding.

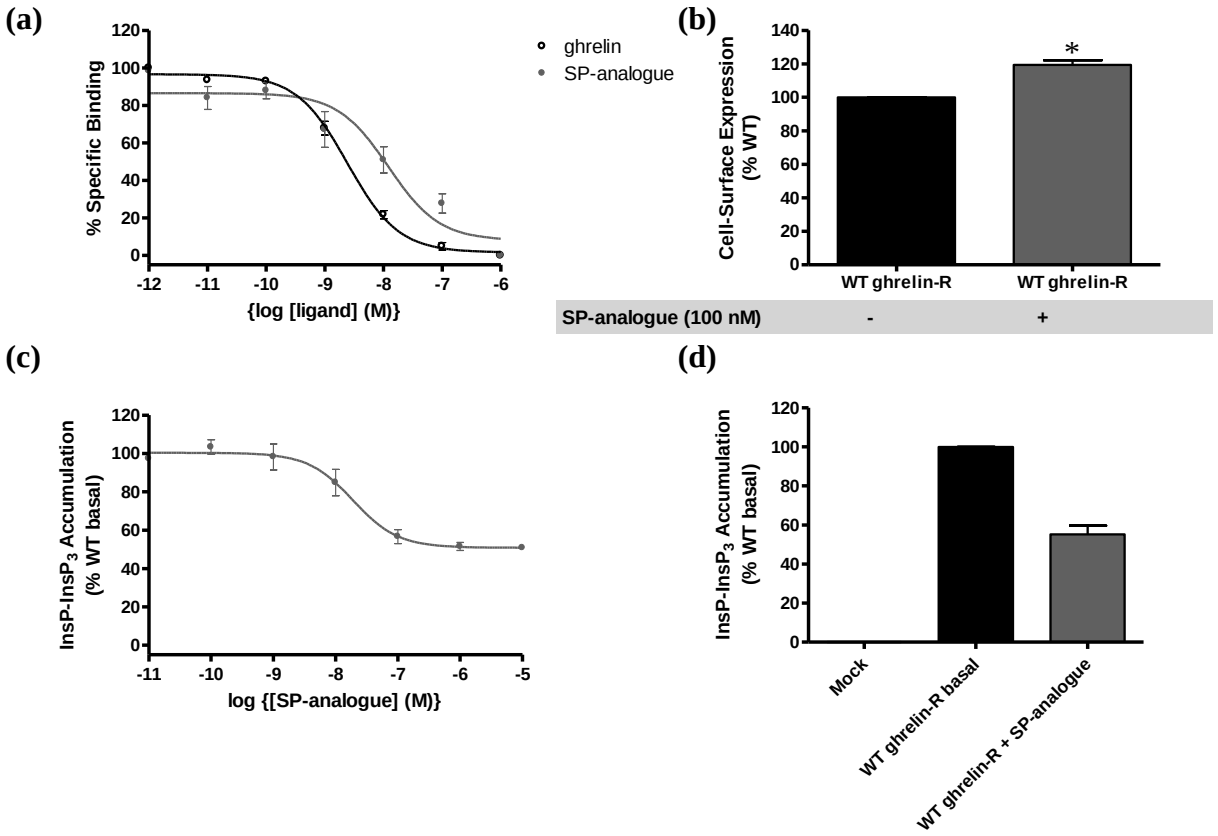


Figure 3.10 Ligand binding, cell-surface expression and intracellular signalling profiles for [D-Arg^{1}, D-Phe^{5}, D-Trp$^{7, 9}$, Leu^{11}]-Substance P: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R. [^{125}I]ghrelin vs. ghrelin or [^{125}I]ghrelin vs. SP-analogue binding is expressed as a percentage of specific binding. (b) Cell-surface expression of the ghrelin-R alone or stimulated with SP-analogue (100 nM) expressed as a percentage of the WT ghrelin-R (* $P < 0.05$). (c) SP-analogue-induced InsP-InsP$_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R. Values are expressed as a percentage of the WT ghrelin-R basal. (d) SP-analogue-induced reduction in constitutive activity as a percentage of WT ghrelin-R basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation		Cell-Surface Expression + SP-analogue (100 nM) (% WT) ± SEM
		pIC_{50} ± SEM	I_{max} (% WT basal) ± SEM	
WT-ghrelin-R	7.92 ± 0.12	7.72 ± 0.04	51 ± 0.4	119 ± 3 *

Table 3.6 Pharmacological characterisation of the SP-analogue at the WT ghrelin-R: pIC_{50} for competition binding assays for [^{125}I]ghrelin vs. SP-analogue, pIC_{50} and I_{max} values for InsP-$InsP_3$ accumulation assays and cell-surface expression of the WT ghrelin-R after SP-analogue challenge. Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Pink indicates an increase in cell-surface expression compared to unstimulated WT ghrelin-R (* $P < 0.05$).

determine the effect on cell-surface expression in this system. Treatment with SP-analogue (30 min) resulted in a significant increase in the number of receptors located at the cell surface (119 ± 3 % of WT unstimulated receptor) (Fig 3.10 (b), Table 3.6).

3.3 Functional importance of residues conserved in Family A GPCRs

3.3.1 Introduction

The TM helices of GPCRs display a relatively high degree of conservation. The most conserved residues in each helix in Family A GPCRs are $Asn^{1.50}$, $Asp^{2.50}$, $Arg^{3.50}$, $Trp^{4.50}$, $Pro^{5.50}$, $Pro^{6.50}$ and $Pro^{7.50}$ (Fig 3.11). As these residues are so highly conserved amongst Family A GPCRs, they were chosen for mutation to alanine in the ghrelin-R. Point mutations were introduced into the ghrelin-R as described in Chapter 2. The effects of alanine substitution of the residues in this chapter are summarised in the Appendix (Fig 9.4-9.8).

3.3.2 Results

Alanine was introduced in order to delete the functional side chain from the β-carbon. Seven individual point mutations were introduced; the resulting mutant receptor constructs were N1.50A, D2.50A, R3.50A, W4.50A, P5.50A, P6.50A and P7.50A. The oligonucleotide primer sequences for each mutation are presented in Table 3.7.

Each mutant receptor construct was characterised by radioligand binding using [^{125}I]ghrelin. N1.50A, D2.50A, R3.50A and P5.50A all had similar binding affinities to the WT ghrelin-R, whereas W4.50A, P6.50A and P7.50A were unable to bind [^{125}I]ghrelin at the concentrations used in the binding experiment (Fig 3.12, Table 3.8).

Each of the mutant receptor constructs was then further characterised for their ability to signal through the $InsP_3$ pathway. All of the mutant receptor constructs affected the basal signalling activity of the ghrelin-R to some degree, except for P7.50A, which retained constitutive activity similar to that seen for the WT receptor.

N1.50A and **D2.50A** had similar characteristics, both presenting decreased constitutive activity (-67 % and -50 % respectively) and decreased E_{max} (61 % and 50 % respectively), but retaining sensitivity to ghrelin, supporting a functional link between these residues. N1.50A reduced cell-surface expression (41 %), whereas D2.50A retained 'WT-like' expression characteristics (94 %) (Figs 3.13-3.14, Table 3.8).

R3.50A was unable to signal further through the $InsP_3$ pathway when stimulated with ghrelin, but maintained constitutive activity (-22 %) that was only slightly reduced when compared to that seen for the WT ghrelin-R. The cell-surface expression (113 %) was comparable to the WT ghrelin-R (Figs 3.13-3.14, Table 3.8).

W4.50A was the most detrimental mutation, resulting in loss of constitutive activity, down to levels observed for the mock-transfected cells and total loss of ghrelin-induced InsP-$InsP_3$ accumulation. The W4.50A mutation severely impaired cell-surface expression (19 % of WT) (Figs 3.13-3.14, Table 3.8).

P6.50A caused an increase in EC_{50} (3.9-fold), a decrease in constitutive activity (-53 %) and a decrease in E_{max} (10 % of WT ghrelin-induced maximum). P6.50A also decreased cell-surface expression (37 %) compared to the WT ghrelin-R (Figs 3.13-3.14, Table 3.8).

P5.50A and **P7.50A** produced increases in ghrelin-induced maximal responses (136 % and 176 % respectively) whereas none of the other mutants in this Chapter were able to reach the WT ghrelin-R ghrelin-induced E_{max}. P5.50A increased the cell-surface expression to 157 % of the WT ghrelin-R and increased the EC_{50} (4.4-fold), whereas P7.50A did not affect ghrelin potency, basal activity or cell-surface expression (Figs 3.13-3.14, Table 3.8).

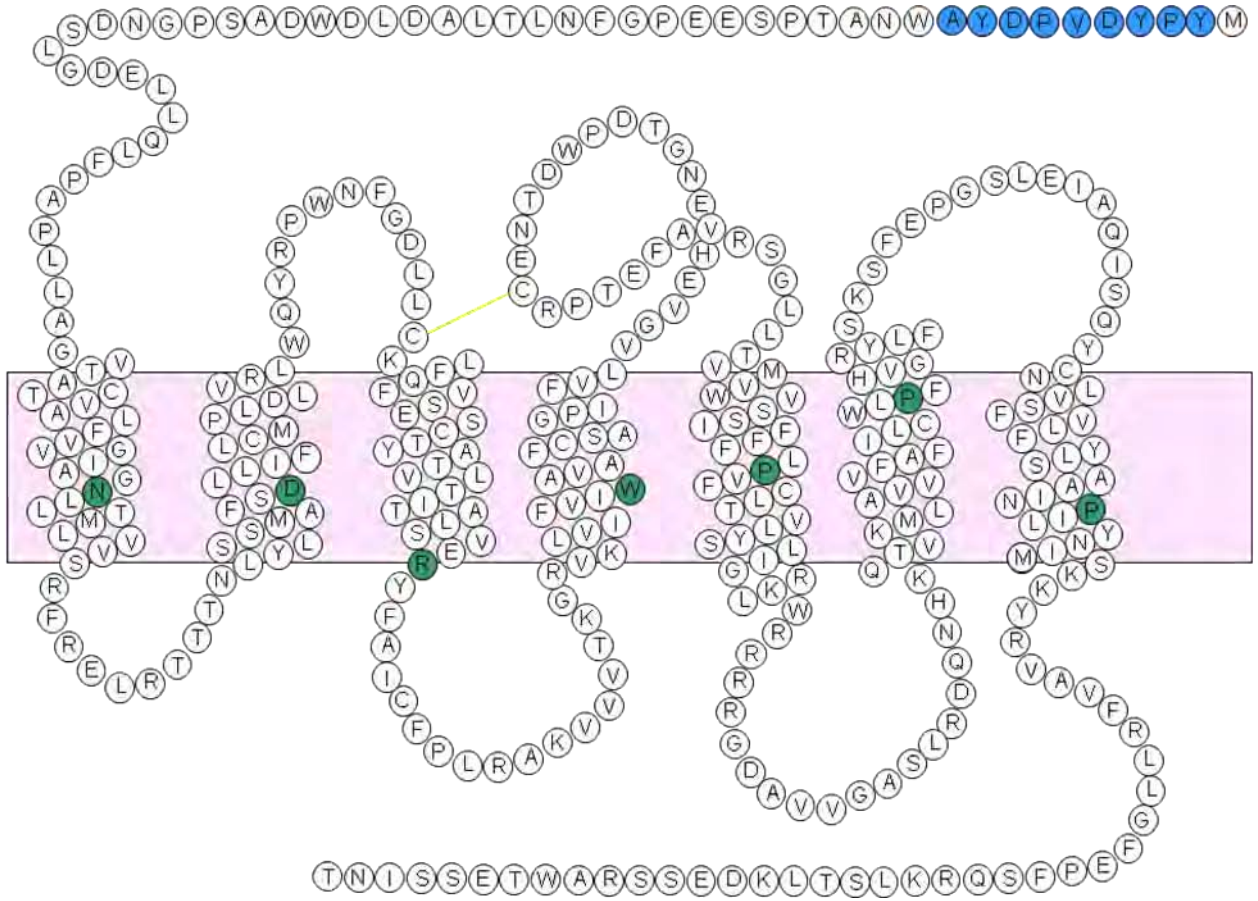


Figure 3.11 Schematic representation of the ghrelin-R: the intramolecular disulphide bond is shown in **yellow**. Residues that represent the HA-epitope tag introduced at the N-terminus (**blue**) and the individual residues mutated in this chapter (**green**) are illustrated.

Mutant Receptor Construct	Sense Oligonucleotide	Antisense Oligonucleotide
N1.50A	5’-GGC-ATC-GCT-GGC-GCC-CTG-CTC-ACC-ATG-3’	5’-CAT-GGT-GAG-CAG-GGC-GCC-AGC-GAT-GCC-3’
D2.50A	5’-CC-AGC-ATG-GCC-TTC-TCC-GCT-CTG-CTC-ATC-TTC-C-3’	5’-G-GAA-GAT-GAG-CAG-AGC-GGA-GAA-GGC-CAT-GCT-GG-3’
R3.50A	5’-CTG-AGC-GTC-GAG-GCC-TAC-TTC-GCC-ATC-3’	5’-GAT-GGC-GAA-GTA-GGC-CTC-GAC-GCT-CAG-3’
W4.50A	5’-GTC-ATC-TTC-GTC-ATC-GCG-GCC-GTG-GCC-TTC-TGC-3’	5’-GCA-GAA-GGC-CAC-GGC-CGC-GAT-GAC-GAA-GAT-GAC-3’
P5.50A	5’-C-TTC-TTC-TTC-CTT-GCT-GTC-TTC-TGT-CTC-ACG-3’	5’-CGT-GAG-ACA-GAA-GAC-AGC-AAG-GAA-GAA-GAA-G-3’
P6.50A	5’-CTC-TGC-TGG-CTC-GCC-TTC-CAC-GTA-GGG-3’	5’-CCC-TAC-GTG-GAA-GGC-GAG-CCA-GCA-GAG-3’
P7.50A	5’-GCT-GCC-ATC-AAC-GCC-ATT-CTG-TAC-AAC-3’	5’-GTT-GTA-CAG-AAT-GGC-GTT-GAT-GGC-AGC-3’

Table 3.7 Oligonucleotide primer sequences for QuikChange™ site-directed mutagenesis: Codon changes to introduce the mutations are shown in **red**.

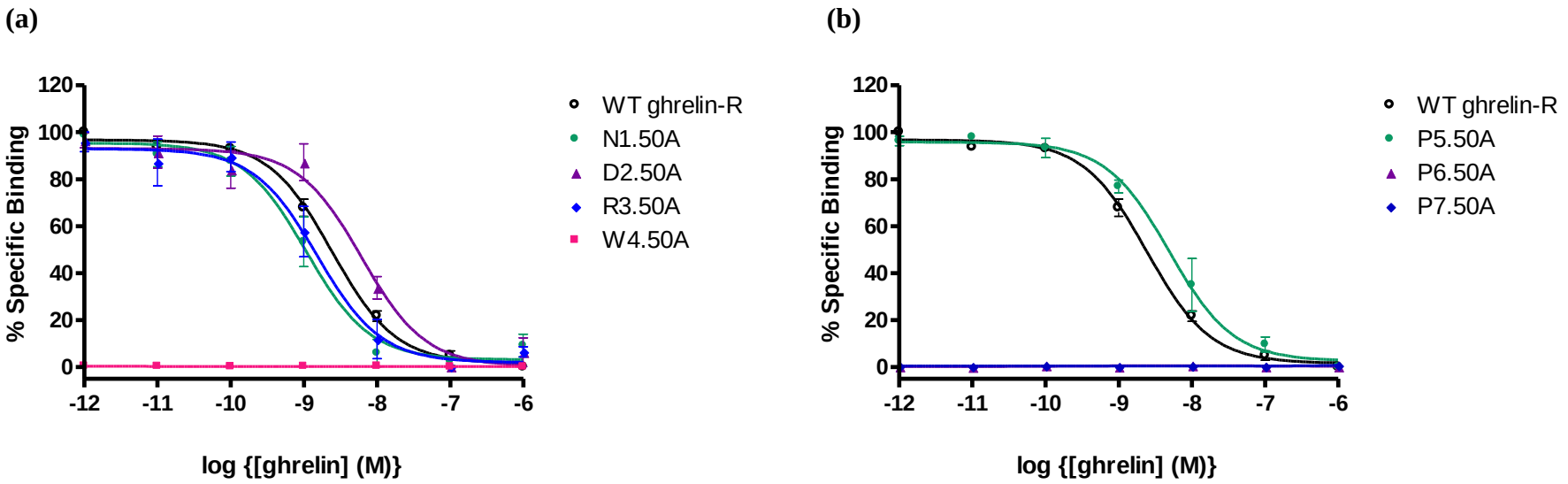


Figure 3.12 Ligand binding profiles for conserved transmembrane residue mutant receptor constructs: Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with (a) WT ghrelin-R, N1.50A, D2.50A, R3.50A or W4.50A and (b) WT ghrelin-R, P5.50A, P6.50A or P7.50A. Values are expressed as a percentage of specific binding. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

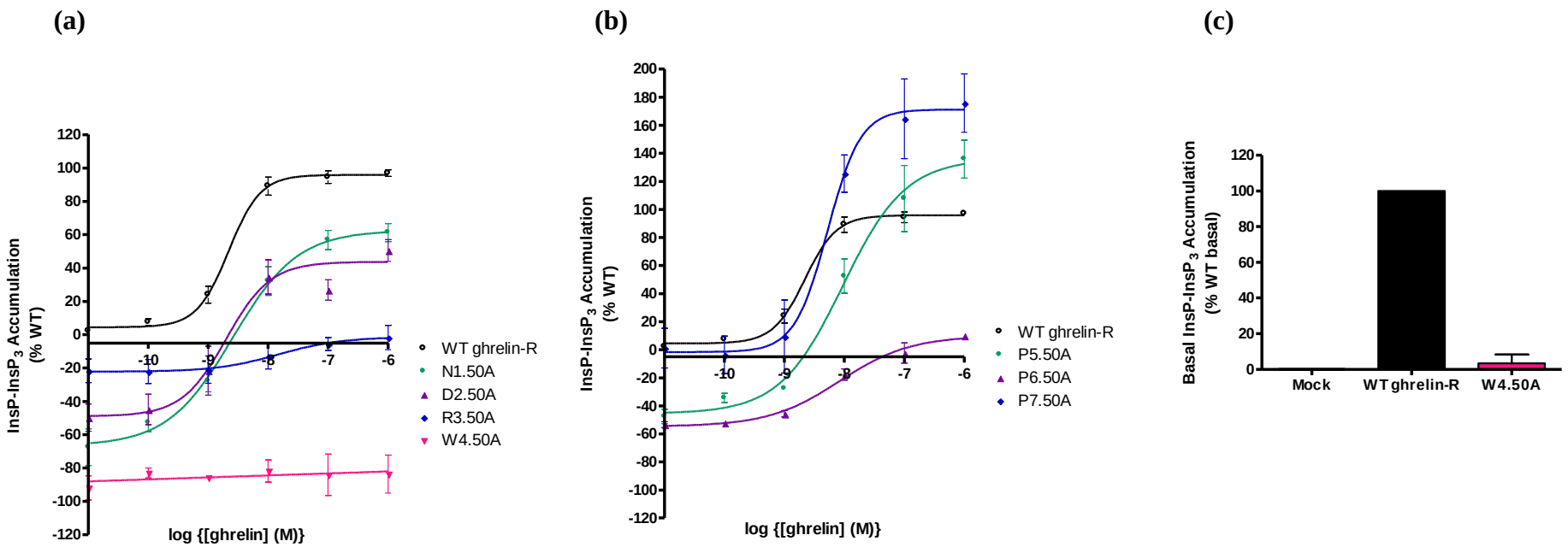


Figure 3.13 Inositol phosphate accumulation assay profiles for conserved transmembrane residue mutant receptor constructs: Assays were performed on HEK 293T cells transiently transfected with (a) WT ghrelin-R, N1.50A, D2.50A, R3.50A or W4.50A and (b) WT ghrelin-R, P5.50A, P6.50A or P7.50A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Basal InsP-$InsP_3$ accumulation for W4.50A as a percentage of WT basal signalling. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

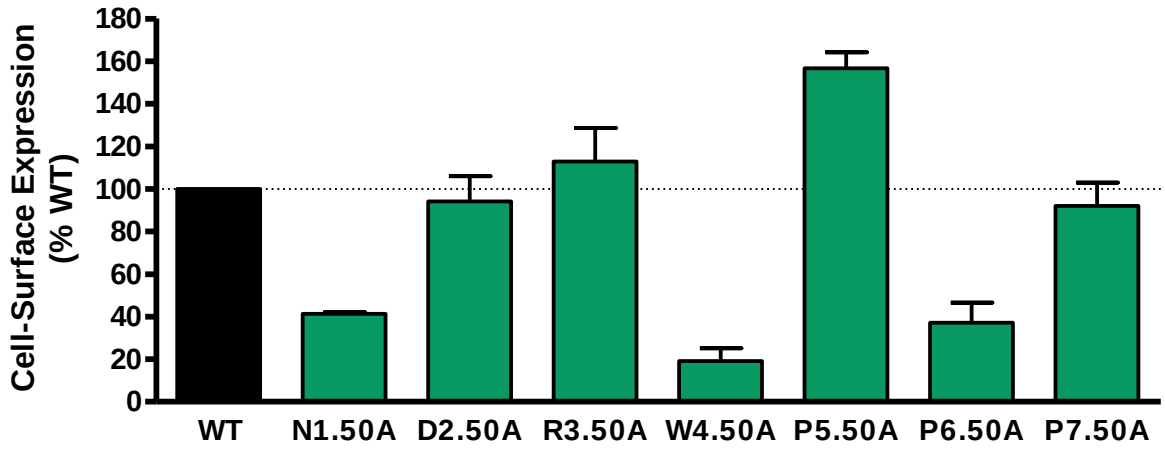


Figure 3.14 Cell-surface expression profiles for conserved transmembrane residue mutant receptor constructs: Assays were performed on HEK 293T cells transiently transfected with WT ghrelin-R, N1.50A, D2.50A, R3.50A, W4.50A, P5.50A, P6.50A and P7.50A. Values are expressed as a percentage of the WT ghrelin-R expression from experiments performed in parallel. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation				Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	Basal (% WT basal) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**	**100**
N1.50A	8.97 ± 0.05	2.55 ± 0.09	-67 ± 11	-	61 ± 5	41 ± 1
D2.50A	8.21 ± 0.07	2.00 ± 0.32	-50 ± 8	-	50 ± 6	94 ± 12
R3.50A	8.82 ± 0.05	NDS	-22 ± 7	-	NDS	113 ± 16
W4.50A	NDB	NDS	-92 ± 7	3 ± 5	NDS	19 ± 6
P5.50A	8.31 ± 0.03	9.40 ± 4.70	-48 ± 5	-	136 ± 14	157 ± 8
P6.50A	NDB	8.33 ± 5.34	-53 ± 2	-	10 ± 2	37 ± 9
P7.50A	NDB	5.27 ± 0.92	1 ± 14	-	176 ± 21	92 ± 11

Table 3.8 Ligand binding, intracellular signalling and cell-surface expression for conserved transmembrane residue mutant receptor constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Red indicates no detectable binding, > 5-fold increase in EC_{50}, > 60 % reduction in basal activity compared to WT, E_{max} < WT basal or a > 70 % reduction in cell-surface expression. Yellow indicates > 20 % reduction in basal activity or a > 30 % reduction in E_{max}. Orange indicates a > 4-fold increase in EC_{50}, a reduction in basal activity > 40 % of WT, a reduction in E_{max} > 60 % of WT, or a reduction in cell-surface expression > 50 %. Green indicates an increase in E_{max} > 130 % of WT. Blue indicates an increase in E_{max} > 160 % of WT or an increase in cell-surface expression > 150 % of WT.

3.4 Discussion

3.4.1 The ghrelin-R haemagglutinin epitope tag

The HA-epitope tag was introduced at the N-terminus of the ghrelin-R using a PCR-based method and the sequence was confirmed using fluorescence automated DNA sequencing. HA-epitope tags have been introduced previously at the N-terminus of GPCRs without any disruption to ligand binding, G-protein coupling or receptor cell-surface expression (Schoneberg *et al.*, 1995; Hawtin *et al.*, 1997; Gaylinn, 1999; Pantel *et al.*, 2006). To determine whether the introduction of the HA-tag perturbed ghrelin-R function in HEK 293T cells, radioligand binding, InsP-$InsP_3$ accumulation assays and ELISA were carried out on the HA-ghrelin-R. These results were compared to those obtained for the untagged ghrelin-R. Both the HA-tagged and the untagged ghrelin-R bound ghrelin with similar affinities and signalled through the $InsP_3$ pathway with comparable ghrelin potency and efficacy. Basal InsP-$InsP_3$ accumulation was similar for the HA-ghrelin-R and untagged ghrelin-R. Cell-surface expression was detected for the HA-ghrelin-R, unlike the untagged receptor, for which cell-surface expression was not detectable (6 ± 2 % of HA-ghrelin-R). The introduction of the HA-epitope tag was concluded to have no adverse effects on ghrelin-R function. The HA-ghrelin-R was used as the WT receptor throughout the remainder of this investigation, and as template to introduce point mutations within the receptor, enabling analysis of mutant receptor cell-surface expression characteristics.

3.4.1.1 HA-ghrelin-R internalisation study

Internalisation of GPCRs after agonist stimulation is an important process in the regulation of receptor signalling. Rapid desensitisation of GPCRs by the uncoupling of receptors from G-proteins occurs quickly after agonist stimulation and is followed by sequestration of receptors, involving their physical removal from the cell-surface and re-localisation to intracellular membranes (Krupnick *et al.*, 1998). Sequestration of GPCRs attenuates signal transduction preventing excessive stimulation, which is important for the regulation of physiology.

There is conflicting evidence about ghrelin-induced internalisation of the ghrelin-R in cell lines. In stably transfected HEK 293 and CHO cells, the ghrelin-R was found to internalise in a time-dependant manner after ghrelin stimulation, as determined by radioligand binding and confocal microscopy (Camina *et al.*, 2004; Holliday *et al.*, 2007). In contrast, an experiment using a FLAG-tagged ghrelin-R did not present any ghrelin-induced receptor internalisation, although constitutive internalisation was observed (Holst *et al.*, 2004). A suggested reason for

this lack of observable agonist-induced internalisation is that the N-terminal FLAG-tag causes a steric hindrance between ghrelin and the receptor, potentially disrupting ligand binding and altering the ability of the receptor to internalise after agonist stimulation (Camina, 2006). This was not the case in a study by Holliday *et al*. (2007), who found that an N-terminally FLAG-tagged receptor was able to internalise after stimulation with 100 nM ghrelin to a greater extent than the unstimulated receptor.

To investigate the ghrelin-R ghrelin-induced internalisation in more detail, time-course ELISA were conducted on the HA-ghrelin-R transiently transfected in HEK 293T cells. The time-course assays indicated that the HA-ghrelin-R internalisation was not increased after agonist stimulation. In contrast, the time-course conducted in parallel on the HA-V_{1a}R demonstrated ~35 % AVP-induced receptor internalisation after 10 min as demonstrated by the reduction in receptor number detected at the cell-surface. This indicates that the lack of ghrelin-R internalisation is related to the function of the ghrelin-R rather than an artefact of the assay in HEK 293T cells. The idea that the tag may be disrupting receptor-ghrelin interactions is not supported by the radioligand binding data on the HA-tagged ghrelin-R, which was found to bind ghrelin with a similar affinity to the untagged-ghrelin-R. It may be that a high level of constitutive internalisation is responsible for the lack of ghrelin-induced internalisation observed in the ELISA as agonist-dependent internalisation is masked by the constitutive internalisation.

Dynamin is a large GTPase that is recruited to the neck of clathrin-coated pits to facilitate the pinching-off of vesicles from the membrane. Dynamin polymerises at the neck of the vesicle bud where it recruits other proteins that together bend and distort the membrane, allowing the membrane leaflets to fuse and produce a new vesicle. The intrinsic GTPase activity of dynamin is aided by the GTPase effector domain (GED) which acts as a GAP. GTP hydrolysis follows the dynamin recruitment and the energy released is thought to drive dynamin contraction, pinching-off the vesicle and allowing its release into the cytosol. The mutant dynamin2, K44A-dynamin, lacks the ability to bind GTP and act as a GTPase, thus preventing GTP hydrolysis. K44A-dynamin acts as a dominant negative of dynamin, preventing the pinching-off of vesicles in cells overexpressing the mutant form (van der Bliek *et al.*, 1993; Damke *et al.*, 1994) (Fig 3.15). GPCR agonist-induced endocytosis commonly involves clathrin and dynamin, although not all GPCRs are internalised using clathrin-coated pits (Zhang *et al.*, 1996).

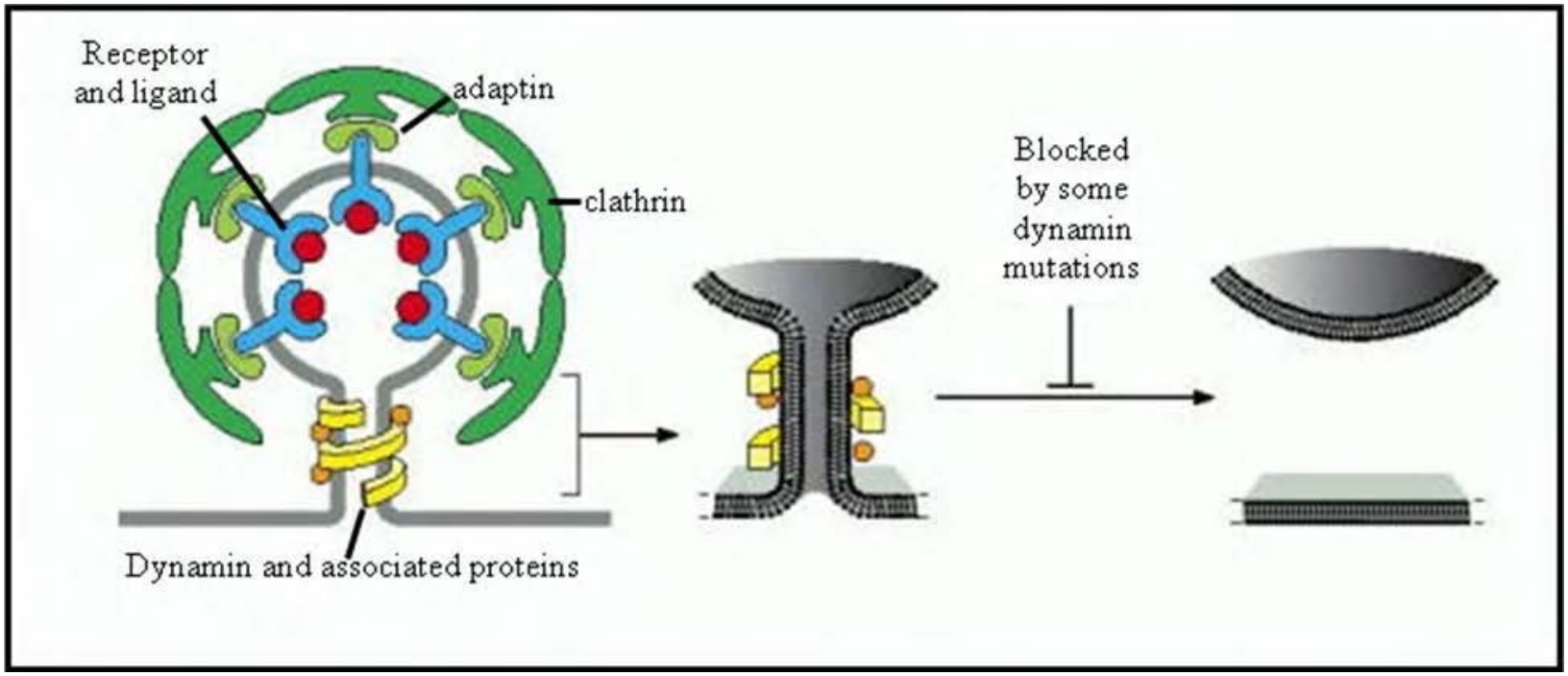


Figure 3.15 Dynamin action in the formation of membrane vesicles: Dynamin and the associated proteins bind to the forming vesicle neck and act to destabilise the membrane allowing the pinching off of the vesicle. Mutations of dynamin, such as the K44A mutation, block dynamin action, preventing the vesicle from pinching off (Image adapted from Alberts, 2002).

Co-transfection of the HA-ghrelin-R with the dominant negative K44A-dynamin resulted in a significant increase in receptor expression at the cell-surface (148 % of HA-ghrelin-R expression), suggesting that constitutive agonist-independent internalisation of the ghrelin-R occurs in a clathrin/dynamin-dependent manner. In contrast, the non-constitutively active $V_{1a}R$ did not present a significant increase in cell-surface expression when co-transfected with K44A-dynamin (114 % of HA-$V_{1a}R$ expression). This suggests that the $V_{1a}R$ does not undergo constitutive internalisation to any significant degree, or that constitutive internalisation does not involve clathrin/dynamin mechanisms. The high degree of constitutive internalisation of the ghrelin-R may go some way towards explaining the lack of ghrelin-induced receptor internalisation. It is possible that the constitutive internalisation of the ghrelin-R masks the agonist-induced internalisation. Rapid recycling of the constitutively internalised ghrelin-R may prevent detection of ghrelin-induced receptor internalisation.

3.4.2 The ghrelin-R displays high constitutive activity

3.4.2.1 Inositol phosphate accumulation assays

The ghrelin-R is known to signal at approximately 50 % of the ghrelin-induced maximum in the absence of agonist (Holst *et al.*, 2003a). To investigate ghrelin-R constitutive activity in HEK 293T cells, the ghrelin-R and the $V_{1a}R$, which has not been reported to be constitutively active, were transiently transfected in parallel in HEK 293T cells for InsP-$InsP_3$ accumulation assays. Mock-transfected cells were also included to determine basal InsP-$InsP_3$ accumulation in the absence of transfected receptor. The mock-transfected cells, stimulated with ghrelin or with AVP, did not show any appreciable signalling through the $InsP_3$ pathway compared to the mock unstimulated cells. The transfected ghrelin-R displayed constitutive InsP-$InsP_3$ accumulation of 52 ± 5.7 % of the ghrelin-induced maximum. The $V_{1a}R$ did not show any substantial increase in InsP-$InsP_3$ accumulation (6.1 ± 0.1 % of AVP-induced maximum) when compared to the mock-transfected cells. After stimulation with 100 nM AVP however, there was an increase in InsP-$InsP_3$ accumulation, confirming the $InsP_3$ signalling of the receptor in response to AVP in HEK 293T cells.

The time-course InsP-$InsP_3$ accumulation assay was designed to investigate the optimal period of ghrelin stimulation in HEK 293T cells. InsP-$InsP_3$ accumulation was greatest 45 min after ghrelin stimulation. Stimulation for longer than 45 min was not beneficial to the InsP-$InsP_3$ accumulation assay and therefore, in the remainder of this study, all InsP-$InsP_3$ accumulation assays were conducted with a period of 45 min for ghrelin stimulation.

3.4.2.2 Radioligand binding

Radioligand binding curves were generated for the WT ghrelin-R and mock-transfected crude cell membrane preparations, as described in Chapter 2. The WT ghrelin-R bound ghrelin with a pIC_{50} of 8.61, similar to the published ghrelin affinity (Bedendi *et al.*, 2003; Davenport *et al.*, 2005). The mock transfections enabled detection of ghrelin binding in HEK 293T cells in the absence of the ghrelin-R. It was established that there was no appreciable binding of ghrelin in mock-transfected HEK 293T cells.

3.4.2.3 [D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-Substance P is an inverse agonist at the ghrelin-R

The SP-analogue was found to be an inverse agonist at the ghrelin-R in the system used in this study. In contrast to previous reports, the SP-analogue was only a partial inverse agonist and so was unable to inhibit WT ghrelin-R constitutive signalling down to levels observed for the mock-transfected cells. Instead SP-analogue reduced basal signalling to an I_{max} of 51 % of WT ghrelin-R basal. The pIC_{50} of 7.72 (IC_{50} = 19 nM) was comparable to published data (Holst *et al.*, 2003a).

Stimulation of the ghrelin-R with 100 nM SP-analogue for 30 min resulted in an increase in cell-surface expression similar to the data from other studies (Holliday *et al.*, 2007; Liu *et al.*, 2007a). This supports ghrelin-R constitutive internalisation, as determined using the mutant K44A-dynamin, as constitutive internalisation could be reduced with an inverse agonist of the system.

3.4.3 Functional importance of residues conserved in Family A GPCRs

The TM domains of Family A GPCRs are the most highly conserved regions, supporting a common mechanism of receptor activation that involves conserved TM residues and motifs. The Ballesteros-Weinstein reference residue is the most highly conserved in each helix. These residues are Asn$^{1.50}$ (100 %), Asp$^{2.50}$ (94 %), Arg$^{3.50}$ (96 %), Trp$^{4.50}$ (96 %), Pro$^{5.50}$ (77 %), Pro$^{6.50}$ (100 %) and Pro$^{7.50}$ (96 %) (Mirzadegan *et al.*, 2003). Due to the high level of conservation, it is probable that each of these residues plays an important role in Family A GPCR function. To investigate the role of the well-conserved residues in ghrelin-R function, each was mutated individually to alanine. Comparisons were drawn between the WT ghrelin-R and mutant receptor constructs to determine the role of specific residues in receptor function.

Asn$^{1.50}$, **Asp$^{2.50}$** and a conserved residue within the NPxxY motif of TMVII, Asn$^{7.49}$ (Chapter 4), have been shown to participate in a network of hydrogen bonds with water molecules in Family A GPCRs. In the Rho crystal structure the distance between Asp$^{2.50}$ and Asn$^{7.49}$ is too great to allow a direct hydrogen bond, so a water molecule mediates the interhelical interaction (Palczewski *et al.*, 2000). These three integral residues along with Tyr$^{7.53}$ of the NPxxY (Chapter 4) form a polar pocket within the TM bundle (Scheer *et al.*, 1996). Alanine mutations of Asn$^{1.50}$, Asp$^{2.50}$ and Asn$^{7.49}$ in the thyrotropin-releasing hormone receptor (TRHR) revealed that the conserved side chains are important in activation of the receptor, but not in ligand binding, suggesting conformational roles, rather than direct contacts with the ligand (Perlman *et al.*, 1997b). In the $5HT_{2A}R$, Asp$^{2.50}$ has been shown to be essential for receptor activation, with point mutations resulting in inactivation of the receptor. This inactivity could be overcome, in part, by a double reciprocal mutation of D2.50N/N7.49D supporting a direct interaction (Sealfon *et al.*, 1995). Asn$^{1.50}$ and Asp$^{2.50}$ have been found to hydrogen bond in a number of Family A GPCRs, including the bRho crystal structure (Palczewski *et al.*, 2000). Molecular modelling in the α_{1b}AR suggested that mutation of Asn$^{1.50}$ to alanine would result in constitutive activity, due to removal of a polar residue and disruption of the conserved hydrogen bonding network (Scheer *et al.*, 1996). In the GnRH-R, asparagine is found at position 2.50 and aspartate at position 7.49 suggesting that similar interactions are occurring in this receptor as in most Family A GPCRs, where Asp$^{2.50}$ and Asn$^{7.49}$ are more highly conserved (Flanagan *et al.*, 1999). Mutation of Asn$^{1.50}$ and Asn$^{2.50}$ to alanine resulted in a severe disruption to cell-surface expression making it difficult to determine the role of both of these residues in terms of activation (Flanagan *et al.*, 1999).

In the ghrelin-R mutation of Asn$^{1.50}$ to alanine also caused substantially decreased cell-surface expression compared to WT although the mutant was expressed sufficiently for InsP-$InsP_3$ accumulation assays to be conducted. The N1.50A mutant retained "WT-like" binding affinity and signalling potency although there was a pronounced disruption to basal signalling and ghrelin efficacy. Agonist-independent signalling was reduced to -67 % of that seen for the WT ghrelin-R, suggesting a disruption to the mechanisms involved in ghrelin-R constitutive activity.

Studies have demonstrated that mutation of Asp$^{2.50}$ often results in a decrease in agonist affinity due to a disruption of the ability of the receptor to form the active state which has the highest affinity for agonist (Strader *et al.*, 1988; Huang *et al.*, 1995; Donnelly *et al.*, 1999). In the ghrelin-R, the D2.50A mutation maintained 'WT-like' affinity for ghrelin and WT

potency. As with the N1.50A mutation, basal activity and ghrelin efficacy were reduced, in parallel, suggesting that ghrelin maintains the same ability to activate the D2.50A mutant as the WT receptor, but that the decreased basal results in a decrease in E_{max}. $Asp^{2.50}$ appears to participate in similar interactions in the ghrelin-R as seen in other Family A GPCRs as indicated by the decreased constitutive activity, which suggests that the mutant receptor is unable to form the WT basally active conformation, but is able to adopt an active conformation after stimulation with agonist.

$Arg^{3.50}$ is an integral residue in Family A GPCR activation, being involved in stabilising interactions with TMII, TMIII, TMV, TMVI and TMVII in various activation states (Chapter 4). A number of studies have suggested that $Arg^{3.50}$ of the D/ERY motif in TMIII interacts with $Asp^{2.50}$ and participates in the hydrogen bonding network in the inactive receptor conformation (Cohen *et al.*, 1993; Scheer *et al.*, 1996). In contrast, a study on the histamine receptor 1 (H_1R) suggested that the $Asp^{2.50}$-$Arg^{3.50}$ interaction occurs in the active receptor conformation (Bakker *et al.*, 2008). A study on the GnRH-R demonstrated that $Asp^{7.49}$ (instead of the asparagine found at position 2.50) interacts with $Arg^{3.50}$, but that in other Family A GPCRs, where the more highly conserved $Asn^{7.49}$ and $Asp^{2.50}$ are found, $Asp^{2.50}$ and $Arg^{3.50}$ are likely to stabilise the active state (Ballesteros *et al.*, 1998). $Arg^{3.50}$ has a key role in activation of GPCRs, changing contacts during the R→R* transition. The ghrelin-R R3.50A mutation resulted in a mutant construct that only decreased constitutive activity to -22 % of the WT ghrelin-induced maximum, but was unable to be further stimulated by ghrelin. This suggests that introduction of alanine resulted in a receptor that is unable to adopt the active conformation after ghrelin stimulation, suggesting a disruption to interactions that stabilise the active state. This is supported by the reduction in constitutive activity and E_{max} observed with the D2.50A mutation, which suggests that the fully active receptor conformation cannot be achieved in the absence of $Asp^{2.50}$, but that D2.50A is not as detrimental to agonist-induced receptor activation as the R3.50A mutation, and conversely, $Arg^{3.50}$ is not as important for basal ghrelin-R activity as $Asp^{2.50}$. In the m3 mAChR $Asp^{2.50}$, $Arg^{3.50}$ and $Tyr^{5.58}$ have been found to be interdependent with mutations of $Arg^{3.50}$ and $Tyr^{5.58}$ being able to functionally rescue the inactive D2.50N mutant receptor (Li *et al.*, 2005). The opsin crystal structures both demonstrate that $Arg^{3.50}$ interacts with $Tyr^{5.58}$, indicating that $Arg^{3.50}$ and $Tyr^{5.58}$ stabilise the active receptor conformation. $Arg^{3.50}$ is then able to interact with $G\alpha_t$ (Park *et al.*, 2008; Scheerer *et al.*, 2008). $Arg^{3.50}$ and $Tyr^{5.58}$ are mutated further and discussed in depth as part of the mutagenesis study in Chapter 4.

Trp$^{4.50}$ is the most conserved residue in TMIV, found in 96 % of Family A GPCRs (Mirzadegan *et al.*, 2003). In the ghrelin-R, mutation of Trp$^{4.50}$ to alanine was severely detrimental to receptor function with the W4.50A mutant resulting in loss of [^{125}I]ghrelin binding and of signalling, both in an agonist-independent and agonist-dependent manner. W4.50A also resulted in a loss of cell-surface expression. Tryptophan has been shown to be important at position 4.50 in a number of Family A GPCRs (Wess, 1993b; Roth *et al.*, 1997; Rhee *et al.*, 2000a). In the cannabinoid 2 (CB_2) receptor only phenylalanine was found to be able to substitute for tryptophan at position 4.50 (Rhee *et al.*, 2000a). Similarly, in the m3 mAChR, a W4.50F mutation maintained WT signalling characteristics, although binding affinity was reduced for all ligand classes (Wess *et al.*, 1993) and in the δ-OR a W4.50A mutation revealed that Trp$^{4.50}$ was directly involved in ligand binding (Befort *et al.*, 1996). In the m1 mAChR (Lu *et al.*, 2001) and H_1R, (Wieland *et al.*, 1999), Trp$^{4.50}$ was identified as being essential for correct receptor expression at the cell surface, as determined by alanine substitution mutagenesis. Interestingly, a non-conservative W4.50L mutation introduced into Rho retained WT characteristics (Nakayama *et al.*, 1991). Trp$^{4.50}$ therefore appears to demonstrate different roles in various Family A GPCRs, sometimes being essential for receptor function, but in other receptors, it appears to be less significant. In the ghrelin-R Trp$^{4.50}$ is essential for all WT receptor characteristics.

Pro$^{6.50}$ is 100 % conserved in Family A GPCRs and is a critical residue in GPCR activation. A significant proline-induced kink was observed within TMVI in the bRho crystal structure (Palczewski *et al.*, 2000). Rearrangement of interactions around Pro$^{6.50}$ in TMVI, results in the movement of TMVI, via the straightening of the proline-induced kink. Conserved residues surrounding Pro$^{6.50}$ modulate the bend angle of TMVI; residues 6.47, 6.48 and 6.52 within TMVI are believed to adopt different rotamer conformations and interact with different residues during receptor activation (Shi *et al.*, 2002b). The crucial role of residue 6.50 is supported by its conservation in Family B GPCRs, where it has a similar function (Conner *et al.*, 2005). In the ghrelin-R the P6.50A mutation was particularly detrimental to receptor function. The mutant was unable to bind [^{125}I]ghrelin at the concentration used in the binding assay. Basal activity was reduced in the P6.50A mutant to -53 % of the WT ghrelin-R basal signalling. The mutant was able to respond to ghrelin and signal through the $InsP_3$ pathway, although both the potency and efficacy were reduced. Various effects have been observed after mutation of Pro$^{6.50}$ in Family A GPCRs. In the m3 mAChR, P6.50A retained functional activity, although a small reduction in agonist affinity was seen, as was substantially

decreased cell-surface expression (Wess, 1993a). As in the m3 mAChR, the ghrelin-R P6.50A mutation resulted in a substantial decrease in ghrelin-R expression (37 %). The P6.50A ghrelin-R mutant supports the high conservation of $Pro^{6.50}$ in Family A GPCRs. $Pro^{6.50}$ appears to be vital for correct ghrelin-R function, enabling both agonist-independent, and agonist-dependent activation and ensuring high affinity ghrelin binding. Loss of signalling capabilities, may, in part, be due to the loss of receptor expression, which is particularly pronounced with the ghrelin-R P6.50A mutation.

$Pro^{5.50}$ and **$Pro^{7.50}$** mutation to alanine in the ghrelin-R both resulted in increases in ghrelin efficacy (136 % and 176 % respectively), although both had varying effects on binding, expression, basal signalling and ghrelin potency. P5.50A reduced the basal constitutive activity (-48 %) suggesting that the alanine substitution mutation adopts a more stable, inactive conformation in the absence of agonist. This is further supported by the increase in cell-surface expression found for this mutant, which implies that there may be reduced constitutive internalisation (section 3.1.2.2), and this therefore allows greater ghrelin-induced signalling and cell-surface expression. Alternatively, the P5.50A mutant might generate a receptor conformation that has a higher affinity for G-protein when occupied by an agonist, although ghrelin potency was decreased (4.4-fold increase in EC_{50}), suggesting a disruption to ghrelin-induced activation. This disruption can be overcome at high ghrelin concentrations, resulting in increased efficacy. Different results were observed when a P5.50A mutation was carried out in the m3 mAChR. The proline was predicted to be essential for receptor folding or trafficking, as the P5.50A mutation disrupted expression and resulted in decreased agonist affinity, and efficacy. As seen with the ghrelin-R however, the alanine mutation did not critically affect receptor activation, suggesting that $Pro^{5.50}$ is not directly involved in stabilising the active receptor state, or in G-protein coupling (Wess, 1993a).

P7.50A was the only conserved residue mutation that retained 'WT-like' constitutive activity. $Pro^{7.50}$ forms part of the NPxxY motif (Chapter 4) in TMVII and has also been shown to induce a kink within the helix (Palczewski *et al.*, 2000). Mutation of $Pro^{7.50}$ to alanine did not impair ghrelin-R function, as the P7.50A mutant construct retained 'WT-like' basal signalling and ghrelin potency. Cell-surface expression of the mutant was also comparable to WT and, although ghrelin binding was not detected at the radioligand concentration used, P7.50A retained 'WT-like' ghrelin potency. Interestingly, there was also increased ghrelin efficacy with the P7.50A mutation, as seen with the P5.50A mutation, suggesting that the mutant

receptor construct is able to adopt a more active conformation than the WT ghrelin-R after ghrelin binding. In the LHR, P7.50F did not result in disruption of binding, signalling or cell-surface expression (Hong *et al.*, 1997) supporting the ghrelin-R data that suggests that $Pro^{7.50}$ is not essential for correct receptor folding and that removal of the proline, and thus possible straightening of the proline-induced kink, does not impair receptor function. In contrast, in the m3 mAChR, P7.50A reduced agonist efficacy and cell-surface expression (Wess, 1993a) implying that $Pro^{7.50}$, as $Trp^{4.50}$, might play different roles in various members of the superfamily. $Pro^{7.50}$ is discussed in more detail in Chapter 4 as part of the investigation into the role of the NPxxY motif.

CHAPTER 4.

MECHANISMS OF GHRELIN-R ACTIVATION

4.1 Introduction

GPCRs can be activated by a diverse range of natural ligands, however evidence suggests that there is a common method of activation within the superfamily. Supporting the evidence of a common mechanism, GPCRs that are activated by very different ligands can activate the same G-protein. Furthermore, sequence analysis has demonstrated that GPCRs are similar at the cytoplasmic ends of TMIII-TMVII, regions known, along with the ICLs, to interact with G-proteins (Mirzadegan *et al.*, 2003).

The mechanisms of agonist-induced activation of Family A GPCRs have been studied in great depth. After agonist binding, conformational changes occur within the TM portion of the receptor, as well as in the extracellular and intracellular loop regions. This cascade of structural changes results in the binding and activation of associated G-proteins and subsequent intracellular signalling pathways. Activation of GPCRs involves the breaking of certain intramolecular ground-state interactions and the formation of new interactions that stabilise a more active receptor conformation. It has been clearly demonstrated that activation of GPCRs involves movement of the cytoplasmic end of TMVI away from TMIII, opening a G-protein binding site. Studies on Rho using electron paramagnetic resonance revealed that receptor activation requires a rotation and tilting of TMVI in relation to TMIII (Farrens *et al.*, 1996). Since this study, further studies have been conducted that support this helical movement during activation (Gether *et al.*, 1997; Dunham *et al.*, 1999; Jensen *et al.*, 2000; Hubbell *et al.*, 2003; Elling *et al.*, 2006). The movement of TMVI relative to TMIII is thought to be centred at the highly conserved $Pro^{6.50}$ (discussed in Chapter 3). TMVI is believed to rotate around $Pro^{6.50}$ leading to a straightening of the proline-induced kink and subsequent "see-saw-like" movement of the helix referred to as the 'global toggle switch' (Elling *et al.*, 2006; Schwartz *et al.*, 2006).

$Trp^{6.48}$ in TMVI forms the rotamer toggle switch of activation. $Trp^{6.48}$ undergoes a conformational rearrangement during receptor activation, releasing interactions with TMVII and forming new interactions with TMs V and III. The altered conformations of $Trp^{6.48}$ and

the neighbouring $Cys^{6.47}$ modulate the proline-induced kink, straightening TMVI and moving the cytoplasmic end of the helix away from TMIII (Shi *et al.*, 2002a; Ruprecht *et al.*, 2004; Schwartz *et al.*, 2006).

The highly conserved D/ERY motif is a key participant in both ground state interactions and in stabilising the R* state after receptor activation (Scheer *et al.*, 1996; Ballesteros *et al.*, 1998; Ballesteros *et al.*, 2001; Rovati *et al.*, 2007; Park *et al.*, 2008). The "ionic lock" found in some Family A GPCRs constitutes an interaction between $Arg^{3.50}$ and a conserved charged residue in TMVI, $Glu^{6.30}$. This interaction maintains the cytoplasmic ends of TMs III and VI in close proximity. The interaction can be disrupted on ligand binding to the receptor, releasing the ionic lock and facilitating the movement of the cytoplasmic end of TMVI away from TMIII, allowing receptor activation (Scheer *et al.*, 1996; Ballesteros *et al.*, 2001).

The ghrelin-R is unusual amongst GPCRs in that it possesses high constitutive activity in the absence of agonist. To investigate the interactions occurring in the ghrelin-R and the role of individual residues in maintaining constitutive activity, key residues found within TMs III, VI and VII of the ghrelin-R were mutated and comparisons made to other GPCRs which do not exhibit high constitutive activity. The mutant receptor constructs were created using site-directed mutagenesis following the QuikChange™ protocol and characterised by radioligand binding, intracellular signalling and cell-surface expression as described in Chapter 2. The effects of alanine substitution of the residues in this chapter are summarised in the Appendix (Fig 9.4-9.8).

4.2 Results

The residues selected for mutation in this chapter were substituted individually for alanine in order to delete the side chain from the β-carbon. Various other amino acids were incorporated at certain positions to investigate the effect of alteration of amino acid side chains. The amino acids selected for mutation were $Thr^{2.39}$, $Cys^{3.35}$, $Thr^{3.36}$, $Glu^{3.49}$, $Arg^{3.50}$, $Tyr^{3.51}$, $Ala^{3.60}$, $Tyr^{5.58}$, $His^{6.30}$, $Gln^{6.32}$, $Thr^{6.33}$, $Val^{6.34}$, $Trp^{6.48}$, $Ser^{7.45}$, $Asn^{7.49}$, $Pro^{7.50}$, $Ile^{7.51}$, $Leu^{7.52}$, $Tyr^{7.53}$, $Lys^{7.58}$ and $Lys^{7.59}$ (Fig 4.1). The oligonucleotide sequences used to introduce each of the mutations are shown in Table 4.1. The resulting ghrelin-R mutant constructs were expressed in HEK 293T cells. Competition radioligand binding studies were conducted to demonstrate mutant receptor affinity for ghrelin compared to the WT ghrelin-R. InsP-$InsP_3$ accumulation assays demonstrated the ability of each construct to signal through the $InsP_3$ pathway after

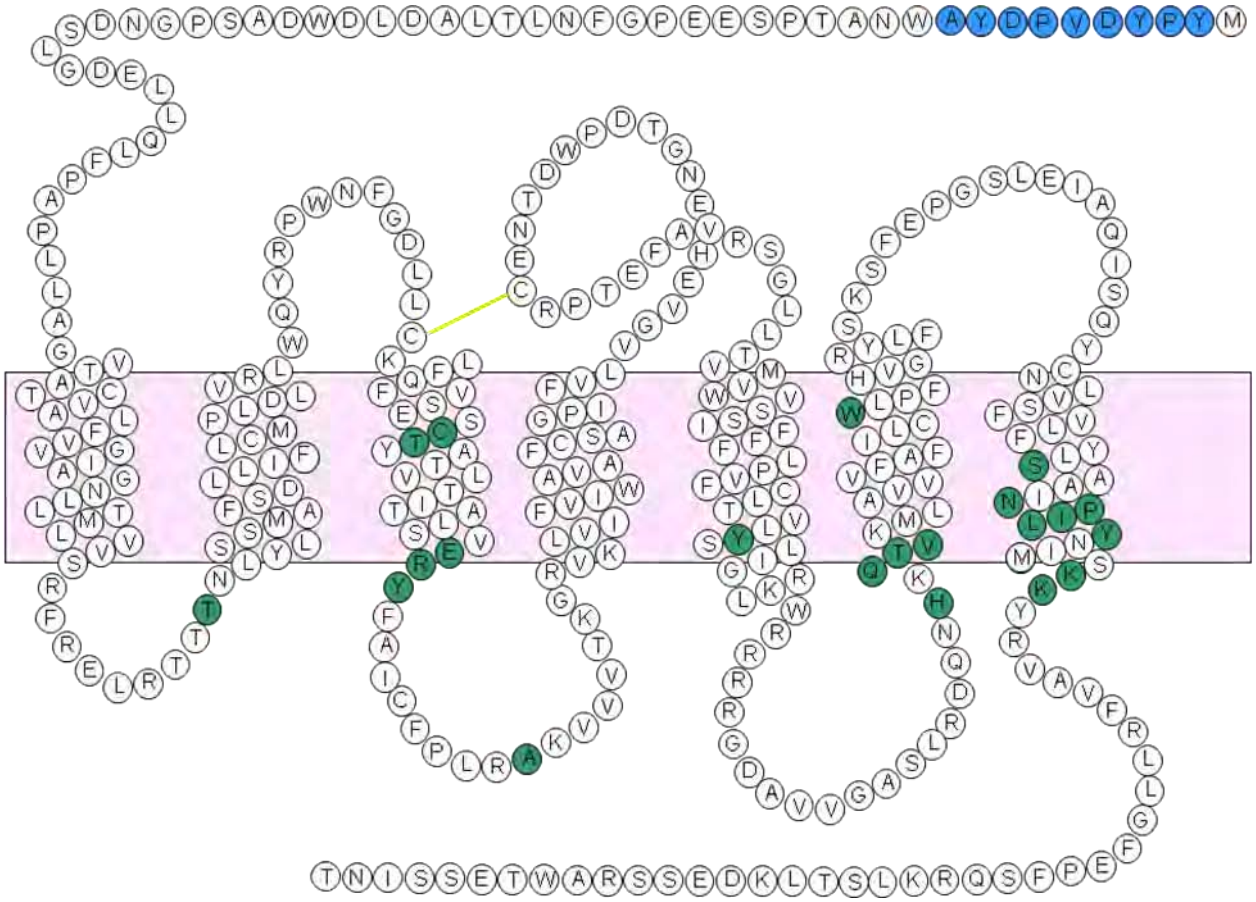


Figure 4.1 Schematic representation of the ghrelin-R: the intramolecular disulphide bond is shown in **yellow**. Residues that represent the HA-epitope tag introduced at the N-terminus (**blue**) and the individual residues mutated in this chapter (**green**) are illustrated.

Mutant Receptor Construct	Sense Oligonucleotide	Antisense Oligonucleotide
T2.39A	5’-CTG-CGC-ACC-ACC-GCC-AAC-CTC-TAC-CTG-3’	5’-CAG-GTA-GAG-GTT-GGC-GTT-GGT-GCG-CAG-3’
C3.35A	5’-GTC-AGT-GAG-AGC-GCC-ACC-TAC-GCC-ACG-3’	5’-CGT-GGC-GTA-GGT-GGC-GCT-CTC-ACT-GAC-3’
T3.36A	5’-C-AGT-GAG-AGC-TGC-GCC-TAC-GCC-ACG-GTG-3’	5’-CAC-CGT-GGC-GTA-GGC-GCA-GCT-CTC-ACT-G-3’
T3.36C	5’-GT-GAG-AGC-TGC-TGC-TAC-GCC-ACG-GTG-3’	5’-CAC-CGT-GGC-GTA-GCA-GCA-GCT-CTC-AC-3’
T3.36F	5’-GT-GAG-AGC-TGC-TTC-TAC-GCC-ACG-GTG-3’	5’-CAC-CGT-GGC-GTA-GAA-GCA-GCT-CTC-AC-3’
T3.36M	5’-GT-GAG-AGC-TGC-ATG-TAC-GCC-ACG-GTG-3’	5’-CAC-CGT-GGC-GTA-CAT-GCA-GCT-CTC-AC-3’
T3.36S	5’-GT-GAG-AGC-TGC-AGC-TAC-GCC-ACG-GTG-3’	5’-CAC-CGT-GGC-GTA-GCT-GCA-GCT-CTC-AC-3’
E3.49A	5’-GCG-CTG-AGC-GTC-GCG-CGC-TAC-TTC-GCC-3’	5’-GGG-GAA-GTA-GCG-CGC-GAC-GCT-CAG-CGC-3’
E3.49D	5’-GCG-CTG-AGC-GTC-GAC-CGC-TAC-TTC-GCC-3’	5’-GGG-GAA-GTA-GCG-GTC-GAC-GCT-CAG-CGC-3’
E3.49R	5’-GCG-CTG-AGC-GTC-CGG-CGC-TAC-TTC-GCC-3’	5’-GGC-GAA-GTA-GCG-CCG-GAC-GCT-CAG-CGC-3’
E3.49Q	5’-CA-GCG-CTG-AGC-GTC-CAG-CGC-TAC-TTC-GCC-ATC-3’	5’-GAT-GGC-GAA-GTA-GCG-CTG-GAC-GCT-CAG-CGC-TG-3’
R3.50A	5’-CTG-AGC-GTC-GAG-GCC-TAC-TTC-GCC-ATC-3’	5’-GAT-GGC-GAA-GTA-GGC-CTC-GAC-GCT-CAG-3’
R3.50E	5’-CTG-AGC-GTC-GAG-GAG-TAC-TTC-GCC-ATC-3’	5’-GAT-GGC-GAA-GTA-CTC-CTC-GAC-GCT-CAG-3’
R3.50H	5’-CTG-AGC-GTC-GAG-CAC-TAC-TTC-GCC-ATC-3’	5’-GAT-GGC-GAA-GTA-GTG-CTC-GAC-GCT-CAG-3’
R3.50K	5’-CTG-AGC-GTC-GAG-AAG-TAC-TTC-GCC-ATC-3’	5’-GAT-GGC-GAA-GTA-CTT-CTC-GAC-GCT-CAG-3’
R3.50L	5’-CTG-AGC-GTC-GAG-CTC-TAC-TTC-GCC-ATC-3’	5’-GAT-GGC-GAA-GTA-GAG-CTC-GAC-GCT-CAG-3’
R3.50Q	5’-CTG-AGC-GTC-GAG-CAG-TAC-TTC-GCC-ATC-3’	5’-GAT-GGC-GAA-GTA-CTG-CTC-GAC-GCT-CAG-3’
Y3.51A	5’-G-AGC-GTC-GAG-CGC-GCC-TTC-GCC-ATC-TGC-3’	5’-GCA-GAT-GGC-GAA-GGC-GCG-CTC-GAC-GCT-C-3’
A3.60Y	5’-C-CCA-CTC-CGG-TAC-AAG-GTG-GTG-GTC-3’	5’-GAC-CAC-CAC-CTT-GTA-CCG-GAG-TGG-G-3’
Y5.58A	5’-GT-CTC-ACG-GTC-CTC-GCC-AGT-CTC-ATC-GGC-AGG-3’	5’-CCT-GCC-GAT-GAG-ACT-GGC-GAG-GAC-CGT-GAG-AC-3’
Y5.58Q	5’-CTC-ACG-GTC-CTC-CAG-CGT-CTC-ATC-GGC-3’	5’-GCC-GAT-GAG-ACT-CTG-GAG-GAC-CGT-GAG-3’
H6.30A	5’-C-AGG-GAC-CAG-AAC-GCC-AAG-CAA-ACC-GTG-3’	5’-CAC-GGT-TTG-CTT-GGC-GTT-CTG-GTC-CCT-G-3’
H6.30D	5’-C-AGG-GAC-CAG-AAC-GAC-AAG-CAA-ACC-GTG-3’	5’-CAC-GGT-TTG-CTT-GTC-GTT-CTG-GTC-CCT-G-3’
H6.30E	5’-GG-GAC-CAG-AAC-GAA-AAG-CAA-ACC-GTG-3’	5’-CAC-GGT-TTG-CTT-TTC-GTT-CTG-GTC-CC-3’
H6.30K	5’-C-AGG-GAC-CAG-AAC-AAG-AAG-CAA-ACC-GTG-3’	5’-CAC-GGT-TTG-CTT-CTT-GTT-CTG-GTC-CCT-G-3’
H6.30Q	5’-C-AGG-GAC-CAG-AAC-CAG-AAG-CAA-ACC-GTG-3’	5’-CAC-GGT-TTG-CTT-CTG-GTT-CTG-GTC-CCT-G-3’
H6.30R	5’-GG-GAC-CAG-AAC-CGC-AAG-CAA-ACC-GTG-3’	5’-CAC-GGT-TTG-CTT-GCG-GTT-CTG-GTC-CC-3’
Q6.32A	5’-C-CAG-AAC-CAC-AAG-GCA-ACC-GTG-AAA-ATG-C-3’	5’-G-CAT-TTT-CAC-GGT-TGC-CTT-GTG-GTT-CTG-G-3’
Q6.32E	5’-C-CAG-AAC-CAC-AAG-GAA-ACC-GTG-AAA-ATG-C-3’	5’-G-CAT-TTT-CAC-GGT-TTC-CTT-GTG-GTT-CTG-G-3’

Mutant Receptor Construct	Sense Oligonucleotide	Antisense Oligonucleotide
T6.33A	5’-G-AAC-CAC-AAG-CAA-GCC-GTG-AAA-ATG-CTG-G-3’	5’-C-CAG-CAT-TTT-CAC-GGC-TTG-CTT-GTG-GTT-C-3’
T6.33V	5’-G-AAC-CAC-AAG-CAA-GTC-GTG-AAA-ATG-CTG-G-3’	5’-C-CAG-CAT-TTT-CAC-GAC-TTG-CTT-GTG-GTT-C-3’
V6.34A	5’-C-CAC-AAG-CAA-ACC-GCG-AAA-ATG-CTG-GC-3’	5’-GC-CAG-CAT-TTT-CGC-GGT-TTG-CTT-GTG-G-3’
V6.34K	5’-C-CAC-AAG-CAA-ACC-AAG-AAA-ATG-CTG-GC-3’	5’-GC-CAG-CAT-TTT-CTT-GGT-TTG-CTT-GTG-G-3’
V6.34T	5’-C-CAC-AAG-CAA-ACC-ACG-AAA-ATG-CTG-GC-3’	5’-GC-CAG-CAT-TTT-CGT-GGT-TTG-CTT-GTG-G-3’
W6.48A	5’-GCC-TTC-ATC-CTC-TGC-GCG-CTC-CCC-TTC-CAC-G-3’	5’-C-GTG-GAA-GGG-GAG-CGC-GCA-GAG-GAT-GAA-GGC-3’
W6.48F	5’-C-TTC-ATC-CTC-TGC-TTC-CTC-CCC-TTC-CAC-3’	5’-GTG-GAA-GGG-GAG-GAA-GCA-GAG-GAT-GAA-G-3’
W6.48Y	5’-C-TTC-ATC-CTC-TGC-TAC-CTC-CCC-TTC-CAC-3’	5’-GTG-GAA-GGG-GAG-GTA-GCA-GAG-GAT-GAA-G-3’
S7.45A	5’-CTC-TTC-TAC-CTC-GCT-GCT-GCC-ATC-AAC-3’	5’-GTT-GAT-GGC-AGC-AGC-GAG-GTA-GAA-GAG-3’
S7.45N	5’-CTC-TTC-TAC-CTC-AAT-GCT-GCC-ATC-AAC-3’	5’-GTT-GAT-GGC-AGC-ATT-GAG-GTA-GAA-GAG-3’
N7.49A	5’-CTC-AGT-GCT-GCC-ATC-GCC-CCC-ATT-CTG-TAC-3’	5’-GTA-CAG-AAT-GGG-GGC-GAT-GGC-AGC-ACT-GAG-3’
P7.50A	5’-GCT-GCC-ATC-AAC-GCC-ATT-CTG-TAC-AAC-3’	5’-GTT-GTA-CAG-AAT-GGC-GTT-GAT-GGC-AGC-3’
I7.51A	5’-GCC-ATC-AAC-CCC-GCT-CTG-TAC-AAC-ATC-3’	5’-GAT-GTT-GTA-CAG-AGC-GGG-GTT-GAT-GGC-3’
L7.52A	5’-C-ATC-AAC-CCC-ATT-GCG-TAC-AAC-ATC-ATG-3’	5’-CAT-GAT-GTT-GTA-CGC-AAT-GGG-GTT-GAT-G-3’
Y7.53A	5’-C-AAC-CCC-ATT-CTG-GCC-AAC-ATC-ATG-TCC-3’	5’-GGA-CAT-GAT-GTT-GGC-CAG-AAT-GGG-GTT-G-3’
K7.58A	5’-C-AAC-ATC-ATG-TCC-GCG-AAG-TAC-CGG-GTG-G-3’	5’-C-CAC-CCG-GTA-CTT-CGC-GGA-CAT-GAT-GTT-G-3’
K7.59A	5’-C-ATC-ATG-TCC-AAG-GCG-TAC-CGG-GTG-GCA-G-3’	5’-C-TGC-CAC-CCG-GTA-CGC-CTT-GGA-CAT-GAT-G-3

Table 4.1 Oligonucleotide primer sequences for QuikChange™ site-directed mutagenesis: Codons changed to introduce mutations shown in **red**.

stimulation with ghrelin. Some of the constructs were further characterised by InsP-$InsP_3$ accumulation assays after stimulation with the ghrelin-R inverse agonist, SP-analogue. Cell-surface expression of the mutant receptor constructs were determined by ELISA using the HA-tag engineered at the N-terminus of the receptor (Chapter 3). The cell-surface expression characteristics were indicative of the ability of the mutant receptors to be trafficked to the cell membrane and to be functionally folded prior to trafficking.

4.2.1 The role of the highly conserved ERY motif

$Glu^{3.49}$, $Arg^{3.50}$ and $Tyr^{3.51}$ of the ERY motif were substituted individually for alanine to produce E3.49A, R3.50A and Y3.51A mutant ghrelin-R constructs. E3.49A demonstrated 'WT-like' binding affinity for ghrelin (Fig 4.2 (a), Table 4.2), but was unable to signal through the $InsP_3$ pathway when stimulated by ghrelin. The constitutive activity of the ghrelin-R was unchanged in the E3.49A mutant receptor construct, despite the inability to further signal after agonist stimulation (Fig 4.2 (c)). Cell-surface expression of E3.49A was greatly reduced (30 %) compared to the WT receptor (Fig 4.2 (b)). R3.50A displayed WT-like binding affinity (Fig 4.3 (a)), but prevented ghrelin-stimulated signalling and slightly reduced the constitutive activity (-22 %) compared to WT (Fig 4.4 (a), Table 4.3). The cell-surface expression of this mutant was similar to that seen for the WT ghrelin-R (Fig 4.5). Y3.51A was essentially WT in all respects (Fig 4.6, Table 4.4).

4.2.2 Further analysis of the ERY motif

In order to more extensively characterise the ghrelin-R and further investigate the role of the residues within the ERY motif in ghrelin-R function, $Glu^{3.49}$ and $Arg^{3.50}$ were mutated to other amino acids. The mutant receptor constructs E3.49D, E3.49Q, E3.49R, R3.50E, R3.50H, R3.50K, R3.50L and R3.50Q were produced to probe the functional effects of altering the side chain characteristics within the ERY motif. The mutant receptor constructs produced could then be characterised and analysed based on the new side-chain properties. $Tyr^{3.51}$ is not as well conserved (67 %) as Asp/$Glu^{3.49}$ or $Arg^{3.50}$ and the initial Y3.51A mutation was essentially WT with respect to all characteristics, therefore $Tyr^{3.51}$ was not mutated further.

4.2.2.1 Extended Analysis of $Glu^{3.49}$

All of the $Glu^{3.49}$ mutants retained 'WT-like' binding affinities for ghrelin (Fig 4.2, Table 4.2). Aspartate and glutamate are very similar, however, aspartate is often found at position 3.49 (66 %) whereas glutamate, as found in the ghrelin-R, is only found in 20 % of Family A receptors (Mirzadegan *et al*., 2003). An aspartate was therefore introduced at position 3.49 of

the ghrelin-R in the E3.49D mutant to study the effect of shortening the amino acid side chain, whilst still retaining the overall negative charge at this position. This resulted in a mutant receptor construct with 'WT-like' signalling and expression, but almost total ablation of constitutive activity (-78 % of WT ghrelin-induced maximum) revealing that glutamate at this position is important for maintaining ghrelin-R constitutive activity (Fig 4.2 (c)). The charge neutralisation mutation of E3.49Q resulted in a receptor with 'WT-like' expression, EC_{50} and basal constitutive activity, but one that was unable to be maximally stimulated by ghrelin, with an E_{max} of only 35 % of WT ghrelin-R. The E3.49R mutation reversed the charge found at this locus. E3.49R resulted in a receptor that lost its ability to signal through the $InsP_3$ pathway and displayed reduced cell-surface expression at only 29 % of the WT, similar to that seen for the E3.49A mutant (Fig 4.2, Table 4.2). E3.49D and E3.49R were analysed to determine their constitutive activity compared to WT ghrelin-R and mock-transfected cells and were found to reduce basal signalling substantially to 13 % and 21 % of WT basal, respectively (Fig 4.2 (d)).

4.2.2.2 Extended Analysis of $Arg^{3.50}$

All of the $Arg^{3.50}$ mutants displayed binding affinities for ghrelin similar to that seen for the WT ghrelin-R and all presented 'WT-like' cell-surface expression, except for R3.50K and R3.50L which increased expression (134 and 150 % respectively) (Fig 4.3 & 4.5, Table 4.3). R3.50E was introduced to investigate the effects of a reversal of charge at such a highly conserved residue. Constitutive ghrelin-R activity was almost totally abolished however ghrelin sensitivity was maintained, with the mutant demonstrating an EC_{50} similar to WT. The E_{max} for this mutant was also dramatically reduced, reflecting the diminished constitutive activity. R3.50H displayed greatly reduced constitutive activity, an increase in EC_{50} (8-fold), but was still responsive to ghrelin, albeit with reduced E_{max}. R3.50K, conserving a positive charge, retained some of the ghrelin-R constitutive activity but had an increased EC_{50} (4.7-fold). The decrease in E_{max} observed for this mutant corresponded to its decrease in basal signalling. R3.50L greatly diminished constitutive activity, and was effectively unable to signal through the $InsP_3$ pathway after ghrelin stimulation. The charge neutralising mutation, R3.50Q displayed a 3-fold increase in EC_{50}, only a slight decrease in basal level of signalling and a 'WT-like' E_{max} (Fig 4.4, Table 4.3).

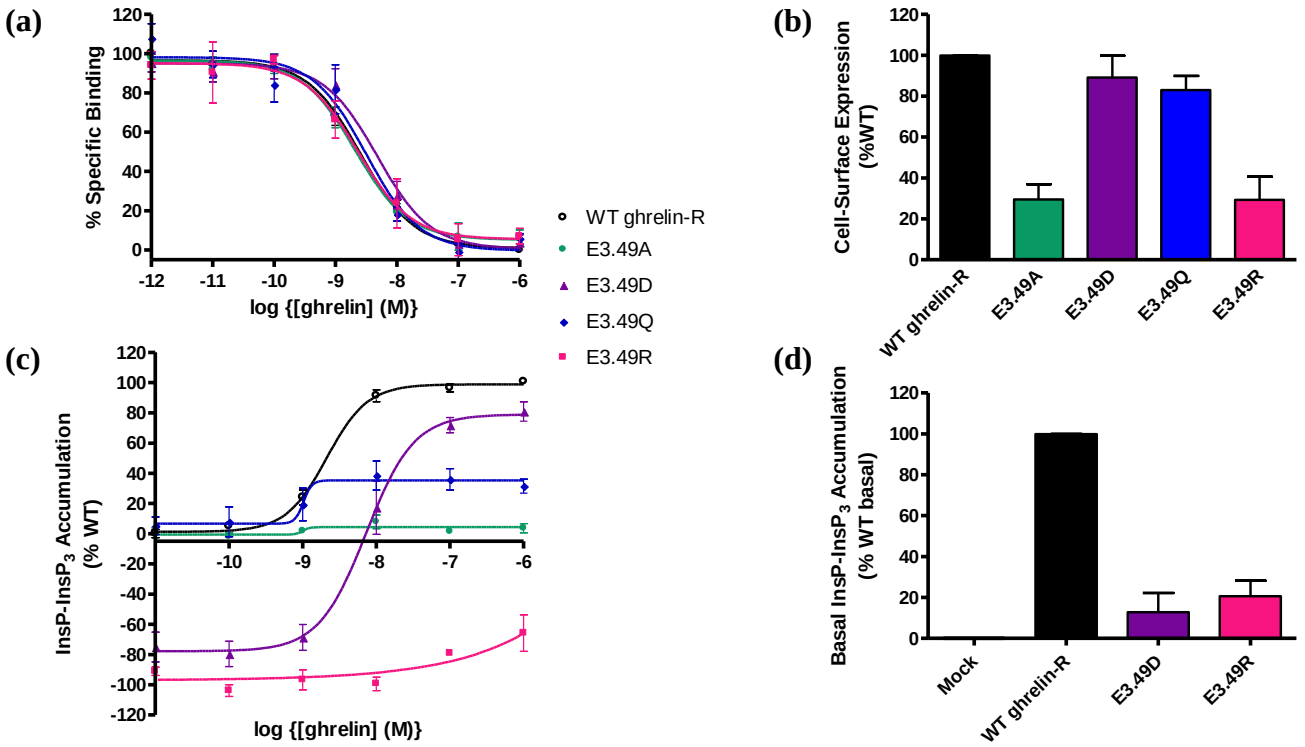


Figure 4.2 Ligand binding, intracellular signalling and cell-surface expression profiles for Glu$^{3.49}$ mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, E3.49A, E3.49D, E3.49Q or E3.49R and are expressed as a percentage of specific binding. (b) Cell-surface expression of mutant receptor constructs expressed as a percentage of the WT ghrelin-R. (c) Ghrelin-induced InsP-InsP$_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, E3.49A, E3.49D, E3.49Q or E3.49R. Values are expressed as a percentage of the WT ghrelin-R, ghrelin-induced maximum from experiments performed in parallel. (d) Basal InsP-InsP$_3$ accumulation for E3.49D and E3.49R as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation				Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	Basal (% WT basal) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**	**100**
E3.49A	8.68 ± 0.03	NDS	-1 ± 1	-	NDS	30 ± 7
E3.49D	8.32 ± 0.04	7.31 ± 0.05	-78 ± 1	13 ± 9	79 ± 1	89 ± 11
E3.49Q	8.50 ± 0.10	1.03 ± 0.66	7 ± 1	-	35 ± 1	83 ± 7
E3.49R	8.64 ± 0.04	NDS	-97 ± 3	21 ± 8	NDS	29 ± 11

Table 4.2 Ligand binding, intracellular signalling and cell-surface expression for $Glu^{3.49}$ mutant receptor constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Red indicates no detectable signalling, > 60 % reduction in basal activity compared to WT or > 70 % reduction in cell-surface expression. Orange indicates > 60 % reduction in E_{max}.

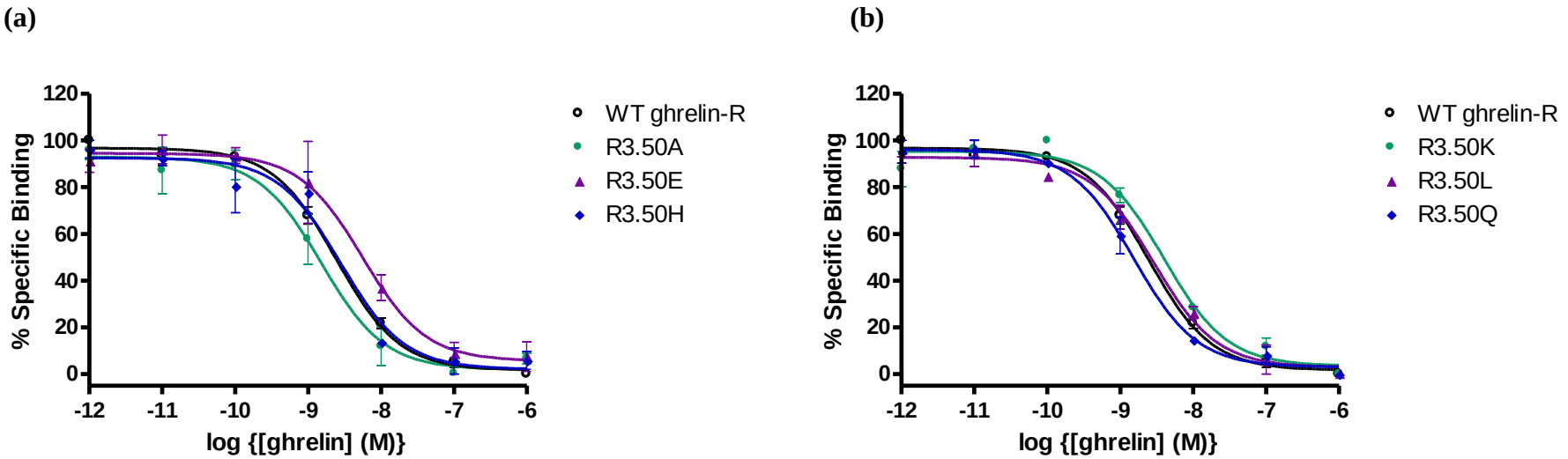


Figure 4.3 Ligand binding profiles for Arg$^{3.50}$ mutant receptor constructs: Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with (a) WT ghrelin-R, R3.50A, R3.50E or R3.50H and (b) WT ghrelin-R, R3.50K, R3.50L or R3.50Q. Values are expressed as a percentage of specific binding. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

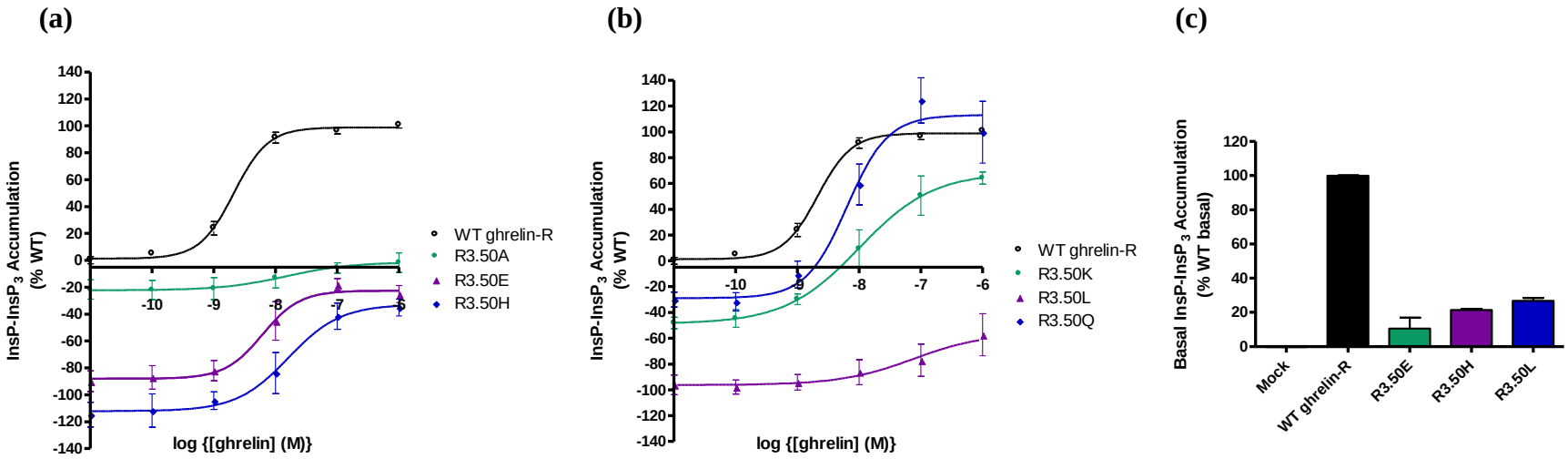


Figure 4.4 Inositol phosphate accumulation assay profiles for Arg$^{3.50}$ mutant receptor constructs: Assays were performed on HEK 293T cells transiently transfected with (a) WT ghrelin-R, R3.50A, R3.50E or R3.50H and (b) WT ghrelin-R, R3.50K, R3.50L or R3.50Q. Values are expressed as a percentage of the WT ghrelin-R, ghrelin-induced maximum from experiments performed in parallel. (c) Basal InsP-InsP$_3$ accumulation for R3.50E, R3.50H and R3.50L as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

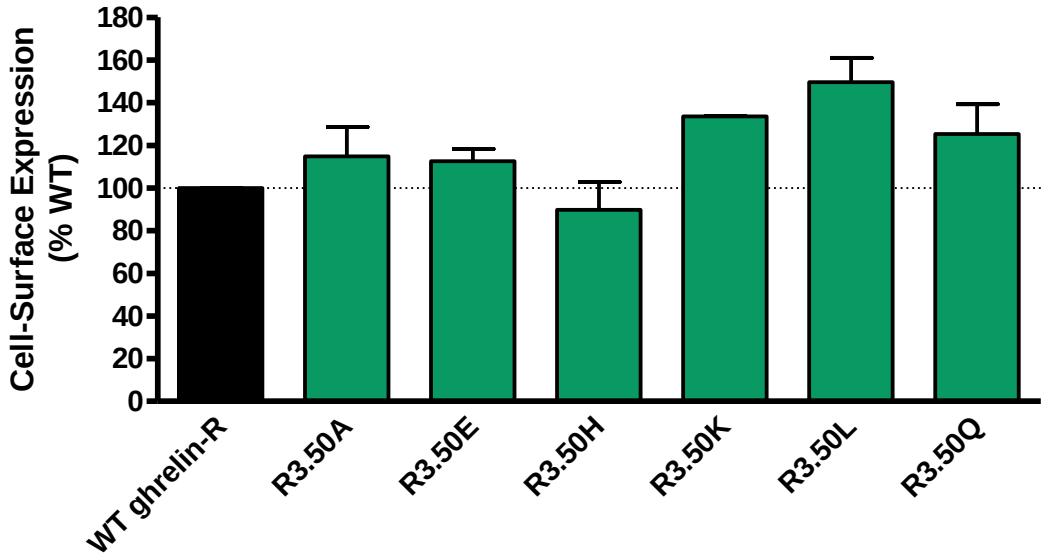


Figure 4.5 ELISA cell-surface expression profiles for Arg$^{3.50}$ mutant receptor constructs: Assays were performed on HEK 293T cells transiently transfected with WT ghrelin-R, R3.50A, R3.50E, R3.50H, R3.50K, R3.50L or R3.50Q. Values are expressed as a percentage of WT ghrelin-R expression from experiments performed in parallel. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation				Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	Basal (% WT basal) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**	**100**
R3.50A	8.82 ± 0.05	NDS	-22 ± 7	-	NDS	115 ± 14
R3.50E	8.25 ± 0.02	6.13 ± 2.36	-88 ± 1	11 ± 6	-23 ± 1	113 ± 6
R3.50H	8.55 ± 0.09	16.2 ± 6.51	-112 ± 1	21 ± 1	-33 ± 2	90 ± 13
R3.50K	8.40 ± 0.08	9.99 ± 0.72	-45 ± 1	-	68 ± 1	134 ± 1
R3.50L	8.54 ± 0.06	NDS	-76 ± 1	27 ± 2	NDS	150 ± 11
R3.50Q	8.82 ± 0.03	6.38 ± 4.14	-29 ± 4	-	113 ± 4	125 ± 14

Table 4.3 Ligand binding, intracellular signalling and cell-surface expression for $Arg^{3.50}$ mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Yellow indicates > 3-fold increase in EC_{50}, >20 % reduction in basal activity and >30 % reduction in E_{max}. Orange indicates > 4-fold increase in EC_{50} reduction of basal activity > 40 % compared to WT. Red indicates no detectable signalling, > 5-fold shift in EC_{50}, > 60 % reduction in basal activity compared to WT or an E_{max} < WT basal. Green indicates > 130 % cell-surface expression and blue indicates > 150 % cell-surface expression compared to WT.

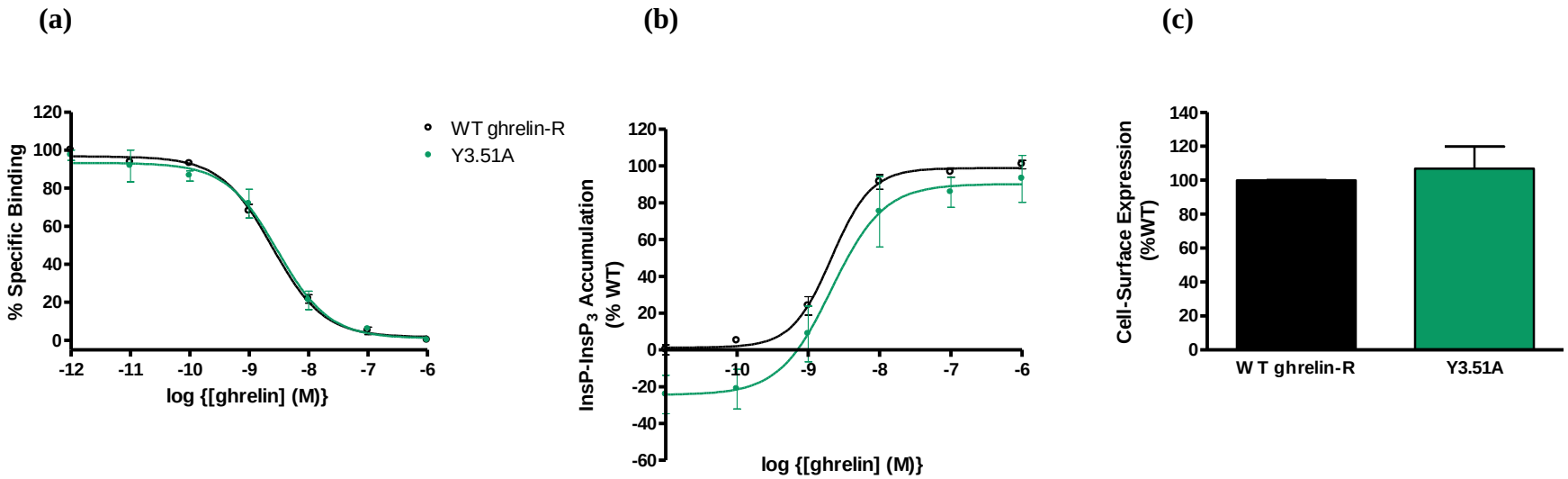


Figure 4.6 Ligand binding, intracellular signalling and cell-surface expression profiles for Y3.51A: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or Y3.51A and are expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, or Y3.51A. Values are expressed as a percentage of the WT ghrelin-R, ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation			Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**
Y3.51A	8.52 ± 0.05	2.12 ± 0.31	-24 ± 10	93 ± 13	107 ± 13

Table 4.4 Ligand binding, intracellular signalling and cell-surface expression for Y3.51A: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Yellow indicates > 20 % reduction in basal activity.

4.2.2.3 Investigation of the ERY motif using an inverse agonist

The ghrelin-R inverse agonist, SP-analogue, was used to determine the responsiveness of ERY mutant receptor constructs to inverse agonist challenge. E3.49A, E3.49Q and R3.50A were chosen for investigation due to their ability to retain 'WT-like' constitutive activity, despite a severely detrimental effect on ghrelin-induced signalling. E3.49A, E3.49Q and R3.50A were all able to respond to SP-analogue in a similar manner to the WT ghrelin-R, retaining 'WT-like' pIC_{50} and I_{max} (Fig 4.7, Table 4.5).

4.2.3 The role of residue 6.30 in ghrelin-R function

An acidic residue is found at position 6.30 in 32 %, and a robust basic residue (arginine or lysine) is found in 34 % of Family A GPCRs (Mirzadegan *et al.*, 2003). In Rho and the biogenic amine receptors, a glutamate is highly conserved at this position and forms the ionic lock interaction with $Arg^{3.50}$ (Ballesteros *et al.*, 2001). A histidine is found at position 6.30 in the constitutively active ghrelin-R, indicating that an "ionic lock-like" interaction does not occur with $Arg^{3.50}$ of the ERY motif. In order to investigate the role of the residue at position 6.30, $His^{6.30}$ was mutated to alanine (H6.30A). This mutation effectively removed the functional amino acid side-chain and perturbed potential any intramolecular interactions, in which histidine usually participates. H6.30A resulted in a receptor that was functionally similar to the WT receptor, displaying 'WT-like' binding affinity, EC_{50}, basal signalling and cell-surface expression (Fig 4.8-4.10, Table 4.6). The E_{max} for this mutant receptor construct was slightly increased (130 %) compared to WT, suggesting that this mutant receptor has a greater efficacy than WT (Fig 4.9 (a), Table 4.6).

In order to investigate the role of residue 6.30 in more depth, further mutant receptor constructs were created, substituting $His^{6.30}$ for amino acids that altered the side chain properties. The constructs H6.30D, H6.30E, H6.30K, H6.30Q and H6.30R were produced. All of the $His^{6.30}$ mutants displayed high affinity for ghrelin as demonstrated by radioligand binding studies (Fig 4.8, Table 4.6). H6.30D and H6.30E were introduced to investigate the effects of the introduction of a negative charge, as found in Rho and the biogenic amine GPCRs. Both constructs had 'WT-like' EC_{50} values. The basal constitutive activity was reduced to similar levels (approximately -40 % compared to WT ghrelin-induced maximum) in each case, suggesting that a negative charge at this position created a more stable ground state receptor conformation. Interestingly, H6.30E displayed increased cell-surface expression (161 %) compared to WT, suggesting a more stable receptor conformation at the cell surface and H6.30D demonstrated an increase in E_{max} of 169 % compared to the WT ghrelin-R (Fig

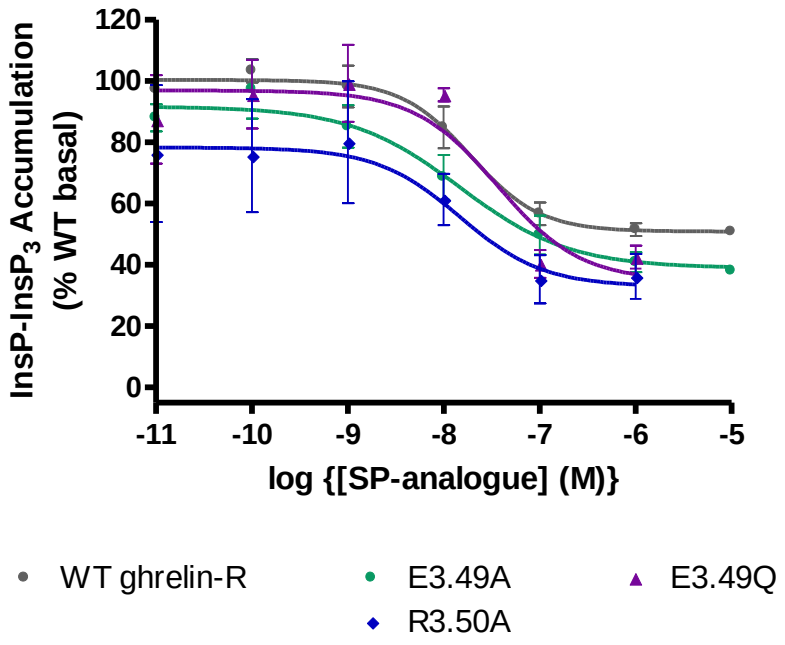


Figure 4.7 Intracellular signalling profile for ghrelin-R ERY motif mutant constructs using SP-analogue: InsP-InsP$_3$ accumulation assays were performed on cells transiently transfected with (a) WT ghrelin-R, E3.49A, E3.49Q or R3.50A. Values are expressed as a percentage of the WT ghrelin-R basal from experiments performed in parallel. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	SP-analogue-induced InsP-InsP$_3$ Accumulation		
	pIC$_{50}$ ± SEM	**Basal (% WT basal) ± SEM**	**I$_{max}$ (% WT basal) ± SEM**
WT ghrelin-R	**7.72 ± 0.04**	**97 ± 2**	**51 ± 1**
E3.49A	7.83 ± 0.07	89 ± 6	40 ± 4
E3.49Q	7.64 ± 0.20	88 ± 14	43 ± 4
R3.50A	7.83 ± 0.10	76 ± 22	36 ± 7

Table 4.5 Intracellular signalling of ERY mutant receptor constructs using SP-analogue: Values were obtained using the methods described in Chapter 2. Data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Yellow indicates a > 20 % reduction in basal.

4.9 & 4.10, Table 4.6). H6.30K and H6.30R introduced a robust positive charge at position 6.30 as found in many Family A peptide hormone GPCRs. Both constructs, H6.30K and H6.30R, reduced the basal constitutive activity (-87 and -48 % of WT ghrelin-induced maximum, respectively), but retained the ability to signal through the $InsP_3$ pathway after ghrelin stimulation, with potencies similar to that seen for the WT receptor. H6.30K demonstrated a slight increase in E_{max} compared to WT, whereas the H6.30R E_{max} was decreased, in parallel with its decreased basal. Both constructs were expressed at levels similar to the WT ghrelin-R at the cell surface (Fig 4.9 & 4.10, Table 4.6). The H6.30Q mutant, neutralising the charge on the side-chain, resulted in a receptor that demonstrated 'WT-like' basal activity and EC_{50}, but had a significantly increased E_{max} (165 %) compared to WT, suggesting an increase in efficacy. The cell-surface expression for this construct was comparable to the WT ghrelin-R (Fig 4.9 & 4.10, Table 4.6). H6.30E and H6.30K were further analysed with respect to basal signalling. H6.30E and H6.30K reduced basal signalling to 60 % and 41 % of WT basal, respectively (Fig 4.9 (c), Table 4.6).

4.2.3.1 Investigation of $His^{6.30}$ using an inverse agonist

H6.30E and H6.30K were chosen for further analysis using the SP-analogue to investigate the effect of the inverse agonist on mutant receptor constructs with reduced constitutive activity. The constitutive activity of the H6.30E and H6.30K mutants was not reduced further by the addition of the SP-analogue suggesting that neither mutant responded to inverse agonist and that the basal signalling could not be reduced further after inverse agonist challenge. The H6.30E basal signalling was comparable to the I_{max} value for the WT ghrelin-R after stimulation with SP-analogue. The data suggest that the basal signalling cannot be reduced further after SP-analogue addition in a mutant receptor construct that demonstrates substantially decreased basal signalling (Fig 4.11, Table 4.7).

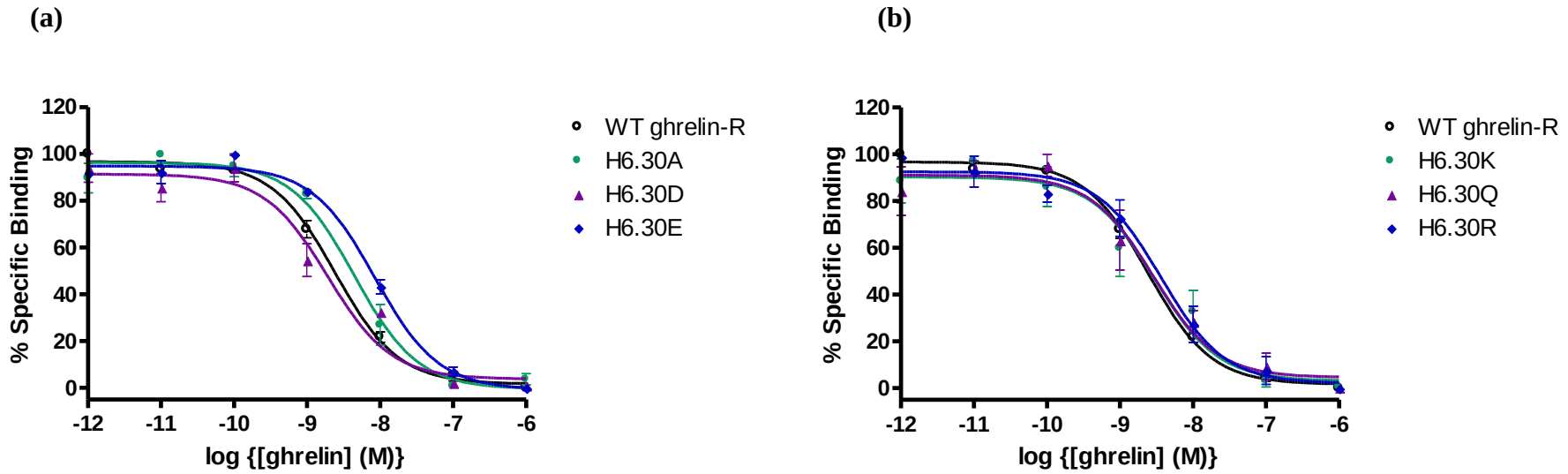


Figure 4.8 Ligand binding profiles for His$^{6.30}$ mutant receptor constructs: Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with (a) WT ghrelin-R, H6.30A, H6.30D or H6.30E and (b) WT ghrelin-R, H6.30K, H6.30Q or H6.30R. Values are expressed as a percentage of specific binding. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

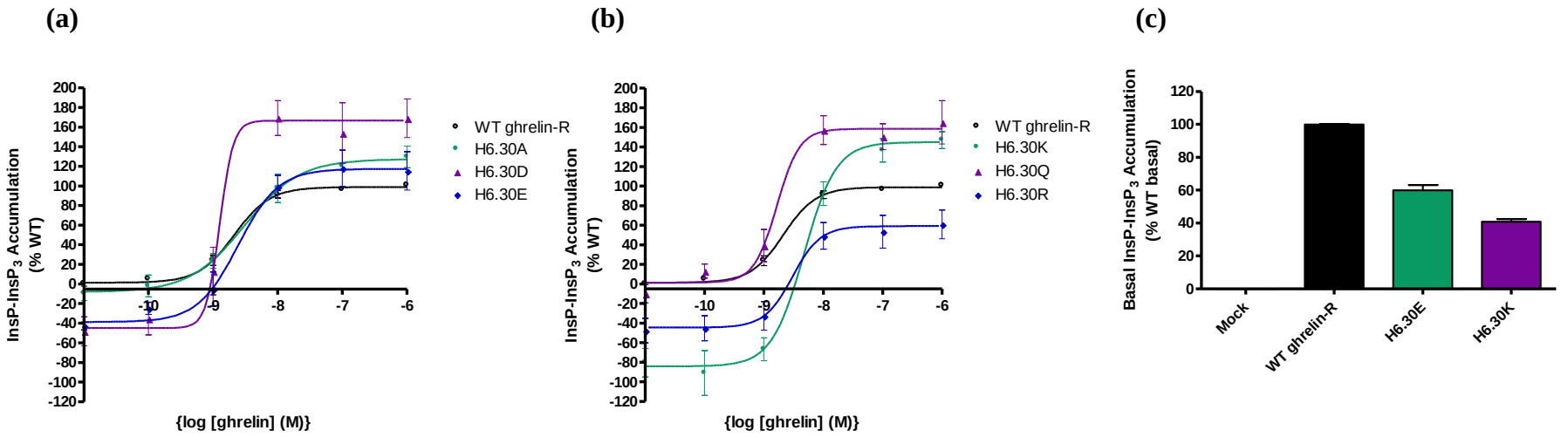


Figure 4.9 Inositol phosphate accumulation assay profiles for His$^{6.30}$ mutant receptor constructs: Assays were performed on HEK 293T cells transiently transfected with (a) WT ghrelin-R, H6.30A, H6.30D or H6.30E and (b) WT ghrelin-R, H6.30K, H6.30Q or H6.30R. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Basal InsP-InsP$_3$ accumulation for H6.30E and H6.30K as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments performed in triplicate.

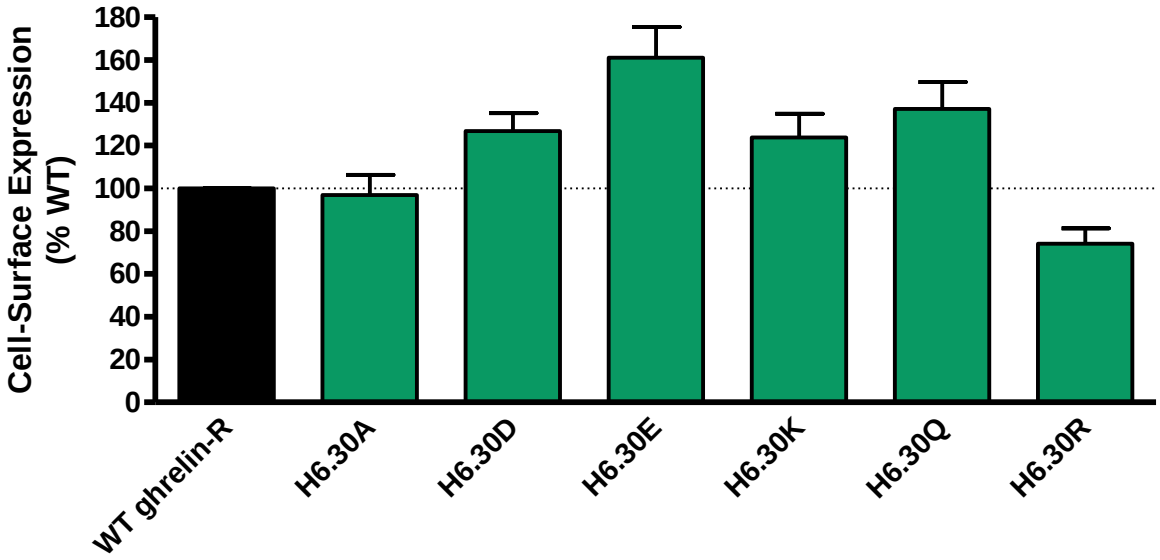


Figure 4.10 ELISA cell-surface expression profiles for His$^{6.30}$ mutant receptor constructs: Assays were performed on HEK 293T cells transiently transfected with WT ghrelin-R, H6.30A, H6.30D, H6.30E, H6.30K, H6.30Q or H6.30R. Values are expressed as a percentage of the WT ghrelin-R from experiments performed in parallel. All data are the mean ± SEM of three or more separate experiments performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation				Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	Basal (% WT basal) ± SEM	E_{max} (% WT max) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**	**100**
H6.30A	8.35 ± 0.05	3.06 ± 0.58	-9 ± 7	-	130 ± 11	97 ± 9
H6.30D	8.71 ± 0.10	1.26 ± 30.3	-48 ± 15	-	169 ± 20	127 ± 8
H6.30E	8.07 ± 0.04	2.43 ± 0.86	-44 ± 4	60 ± 3	115 ± 20	161 ± 14
H6.30K	8.52 ± 0.09	4.41 ± 0.57	-87 ± 17	41 ± 1	147 ± 9	124 ± 11
H6.30Q	8.54 ± 0.08	1.66 ± 1.46	-10 ± 9	-	165 ± 22	137 ± 13
H6.30R	8.44 ± 0.06	3.05 ± 0.83	-48 ± 12	-	61 ±15	74 ± 7

Table 4.6 Ligand binding, intracellular signalling and cell-surface expression for $His^{6.30}$ mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Yellow indicates > 0.5 log unit decrease in agonist affinity or > 30 % reduction in E_{max}. Green indicates and an increase in E_{max} > 130 %. Orange indicates a reduction in basal activity > 40 % of WT. Blue indicates an increase in E_{max} > 160 % or > 160 % cell-surface expression compared to WT. Red indicates > 60 % reduction in basal activity compared to WT.

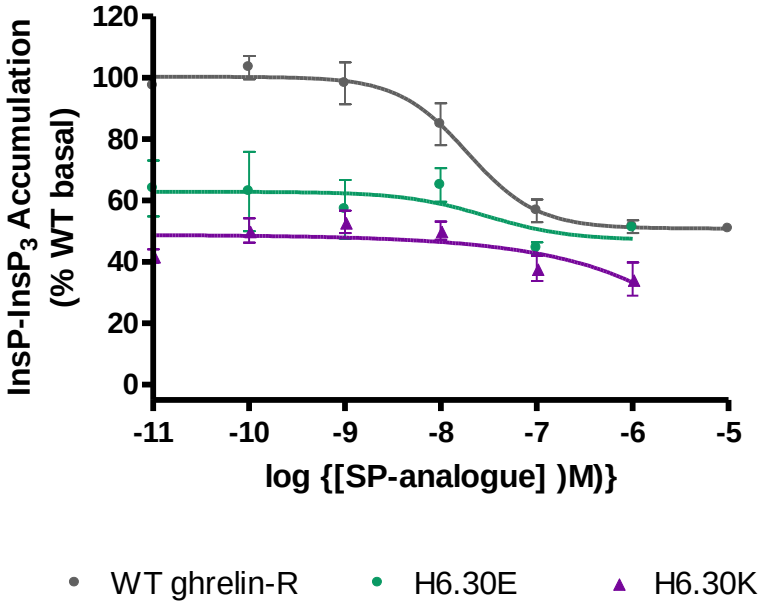


Figure 4.11 Intracellular signalling profile for His$^{6.30}$ mutant constructs using SP-analogue: InsP-InsP$_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, H6.30E or H6.30K. Values are expressed as a percentage of the WT ghrelin-R basal from experiments performed in parallel. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	SP-analogue-induced InsP-InsP$_3$ Accumulation		
	pIC$_{50}$ ± SEM	**Basal (% WT basal) ± SEM**	**I$_{max}$ (% WT basal) ± SEM**
WT ghrelin-R	**7.72 ± 0.04**	**97 ± 2**	**51 ± 0.4**
H6.30E	NDS	64 ± 9	NDS
H6.30K	NDS	42 ± 1	NDS

Table 4.7 Intracellular signalling of His$^{6.30}$ mutant receptor constructs using SP-analogue: Values were obtained using the methods described in Chapter 2. Data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Red indicates no detectable signalling. Orange indicates > 40 % reduction in basal activity.

4.2.4 Investigation of further stabilising interactions

4.2.4.1 Interactions with the D/ERY motif

In the β_1AR (Warne *et al.*, 2008), β_2AR (Rasmussen *et al.*, 2007), and A_{2A}R (Jaakola *et al.*, 2008) crystal structures, the D/ERY motif is involved in constraining interactions with residues found within ICL2 and with residue 2.39 in TMII. At present, the only crystal structure that demonstrates the presence of the ionic lock in the inactive conformation is that of bRho (Palczewski *et al.*, 2000) and therefore other constraining interactions are likely to be occurring in Family A GPCRs that stabilise the ground state. One residue, identified in the A_{2A}R crystal structure as forming important stabilising interactions with the D/ERY motif is $Tyr^{3.60}$ (Jaakola *et al.*, 2008). An alanine is found at position 3.60 in the ghrelin-R, whereas the A_{2A}R and the βARs have a tyrosine at position 3.60 in ICL2. To investigate the potential of an interaction between the ERY motif and ICL2 in the ghrelin-R, $Ala^{3.60}$ was mutated to tyrosine. A threonine is present at position 2.39 in the ghrelin-R, A_{2A}R and βARs. $Thr^{2.39}$ was mutated to alanine in the ghrelin-R in this study. The agonist affinity and the EC_{50} for the T2.39A and the A3.60Y mutants were similar to the WT ghrelin-R (Fig 4.12 & 4.13, Table 4.8). T2.39A had reduced E_{max} (63 %) and slightly decreased cell-surface expression (69 %), but maintained 'WT-like' basal signalling (Fig 4.12, Table 4.8). A3.60Y caused a substantial reduction in the constitutive activity. The E_{max} for A3.60Y was also decreased in parallel with the basal (44 % of WT). A3.60Y cell-surface expression was 'WT-like' (Fig 4.13, Table 4.8).

To further probe the potential of interactions within this region of the ghrelin-R, a double mutant of T2.39A/A3.60Y was produced. By removing the threonine in the T2.39A mutant, the introduction of tyrosine at position 3.60 can be investigated in isolation, rather than in combination with a threonine at position 2.39, with which it may form an interaction. T2.39A/A3.60Y maintained 'WT-like' affinity and cell-surface expression. The constitutive activity was diminished to a similar level to that seen for the A3.60Y single mutant; however, ghrelin-induced InsP-$InsP_3$ accumulation was no longer detectable revealing that the double mutant was more detrimental than either of the single point mutations (Fig 4.14, Table 4.8).

4.2.4.2 The role of residues at the cytoplasmic ends of TMVI and TMVII

In inactive Rho, $Glu^{6.32}$ at the cytoplasmic end of TMVI appears to interact with the backbone of $Lys^{7.58}$ at the C-terminal end of TMVII (Vogel *et al.*, 2008). This interaction is predicted to be broken upon receptor activation as the TMVI-TMVII interface changes conformation (Abdulaev *et al.*, 1998; Vogel *et al.*, 2008). Neutralisation of $Glu^{6.32}$ by mutation

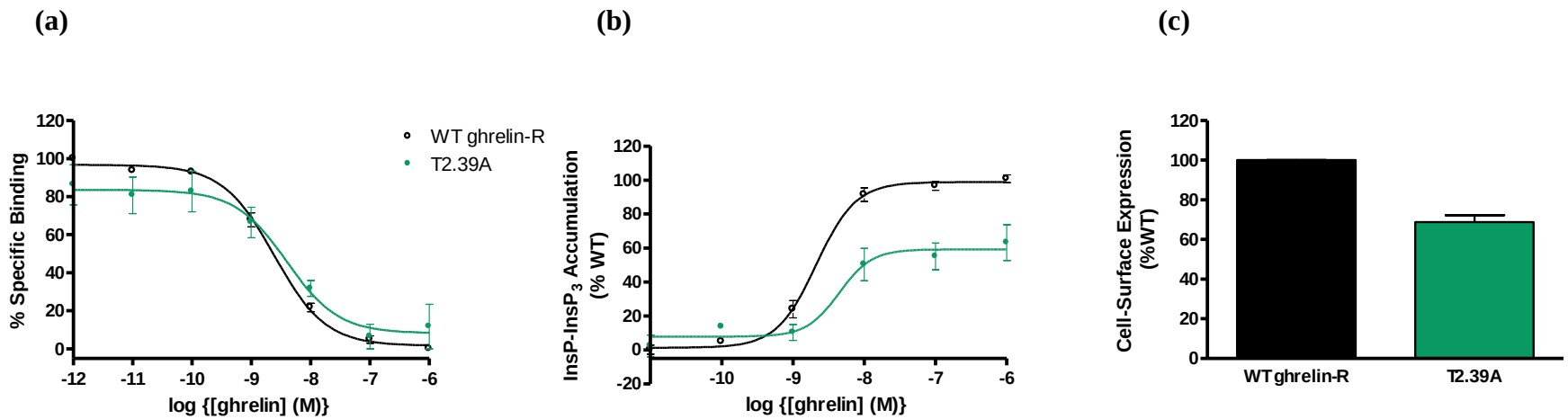


Figure 4.12 Ligand binding, intracellular signalling and cell-surface expression profiles for T2.39A: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or T2.39A and are expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or T2.39A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

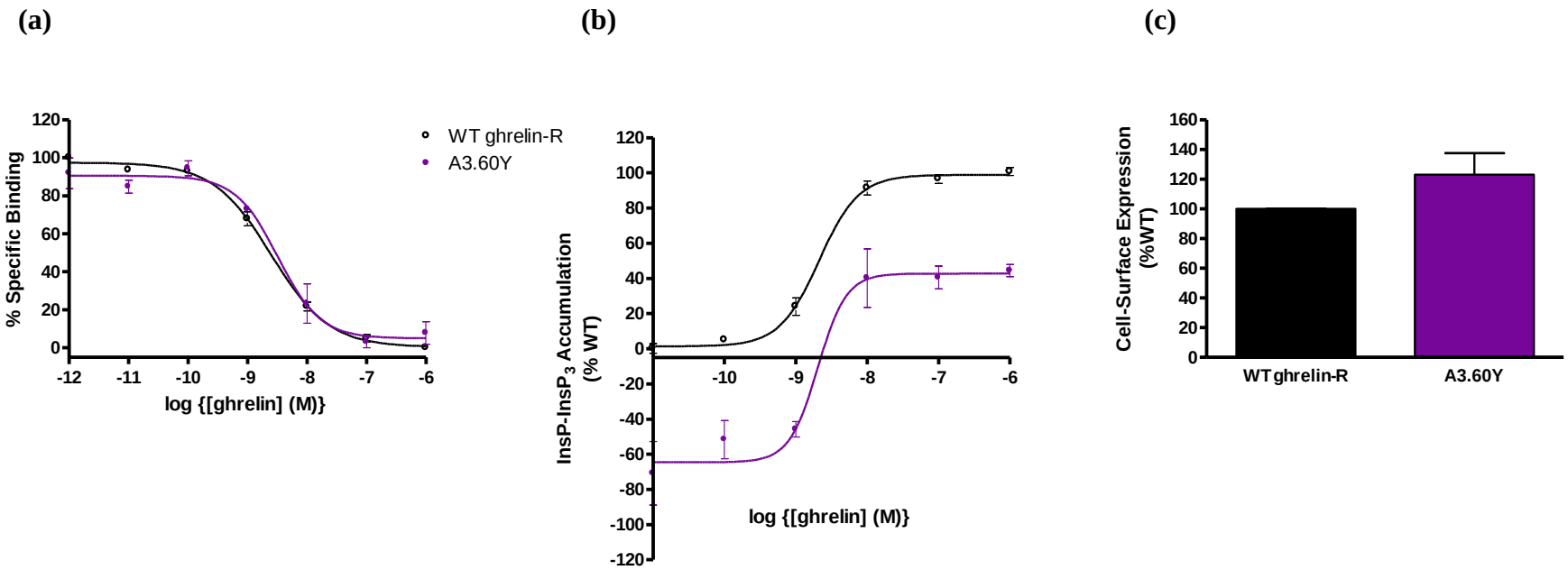


Figure 4.13 Ligand binding, intracellular signalling and cell-surface expression profiles for A3.60Y: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or A3.60Y and are expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or A3.60Y. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

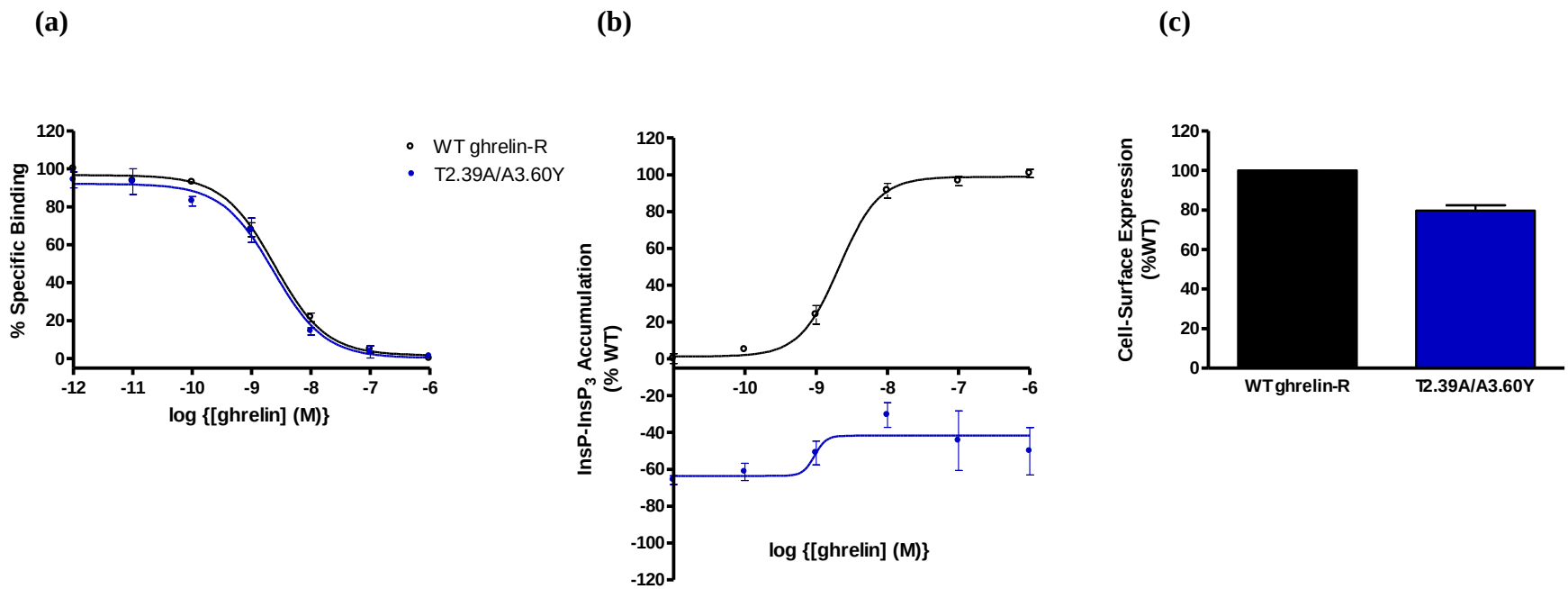


Figure 4.14 Ligand binding, intracellular signalling and cell-surface expression profiles for T2.39A/A3.60Y: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or T2.39A/A3.60Y and are expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or T2.39A/A3.60Y. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation			Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**
T2.39A	8.40 ± 0.05	4.45 ± 8.04	2 ± 6	63 ± 11	69 ± 3
A3.60Y	8.49 ± 0.05	2.03 ± 0.25	-71 ± 18	44 ± 3	123 ± 14
T2.39A/A3.60Y	8.63 ± 0.04	NDS	-66 ± 2	NDS	80 ± 3

Table 4.8 Ligand binding, intracellular signalling and cell-surface expression for T2.39A, A3.60Y and T2.39A/A3.60Y mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Yellow indicates > 30 % reduction in E_{max} or > 30 % reduction in cell-surface expression. Red indicates no detectable signalling or a > 60 % reduction in basal activity compared to WT.

to glutamine in Rho resulted in a shift towards Meta II, indicative of a more active receptor conformation (Vogel *et al.*, 2008). Although a lysine is found at position 7.58 in the WT ghrelin-R, there is a glutamine at 6.32 in the WT receptor. This lack of charge may contribute to the ghrelin-R constitutive activity. $Gln^{6.32}$ was mutated to alanine and glutamate in order to investigate whether the constitutive activity of the ghrelin-R is related to the neutral charge found at position 6.32 and the subsequent lack of a stabilising interaction with TMVII. $Lys^{7.58}$ and the neighbouring $Lys^{7.59}$ were both mutated to alanine as part of the extended investigation. All of the mutant receptor constructs retained high affinity binding to ghrelin and 'WT-like' cell-surface expression (Fig 4.15 & 4.16, Table 4.9). Q6.32A was 'WT-like' with respect to the basal activity, potency and efficacy. Introducing a negative charge in the Q6.32E mutant resulted in a receptor with basal activity and EC_{50} similar to the WT ghrelin-R, suggesting that an inactive stabilising interaction is not formed. The Q6.32E construct did however have greatly increased ghrelin efficacy (E_{max} 199 %) compared to WT (Fig 4.15, Table 4.9), supporting the formation of a more active receptor conformation after agonist binding. K7.58A displayed characteristics similar to WT, but had a small decrease in constitutive activity (-24 %). K7.59A had the greatest affect on ghrelin-R function, having decreased constitutive activity (-53 %), and E_{max} (46 %) and, interestingly, decreased EC_{50} (3.5-fold) compared to WT (Fig 4.16, Table 4.9).

$Thr^{6.33}$ is found at the cytoplasmic end of TMVI in the ghrelin-R and is ideally positioned to interact with TMIII in the inactive receptor conformation. Mutation of $Thr^{6.33}$ to alanine in the T6.33A mutant resulted in a receptor that displayed increased agonist-independent signalling (18 % of WT ghrelin-induced maximum or 132 % of WT basal) compared to the WT ghrelin-R although the ability of T6.33A to signal through the InsP-$InsP_3$ pathway when stimulated with ghrelin was affected (Fig 4.17 (c) & (d)). Despite the increased basal activity, the E_{max} was reduced to only 60 % of WT. Cell-surface expression of this mutant was also decreased (54 %). The agonist affinity remained comparable to the WT receptor (Fig 4.17, Table 4.10). To further investigate $Thr^{6.33}$, valine was introduced at this position. T6.33V retained 'WT-like' binding affinity, constitutive activity and EC_{50} and only presented a very slight decrease in E_{max} (75 % of WT). The cell-surface expression for this mutant was decreased (57 %), similar to the expression of the T6.33A mutant (Fig 4.17, Table 4.10).

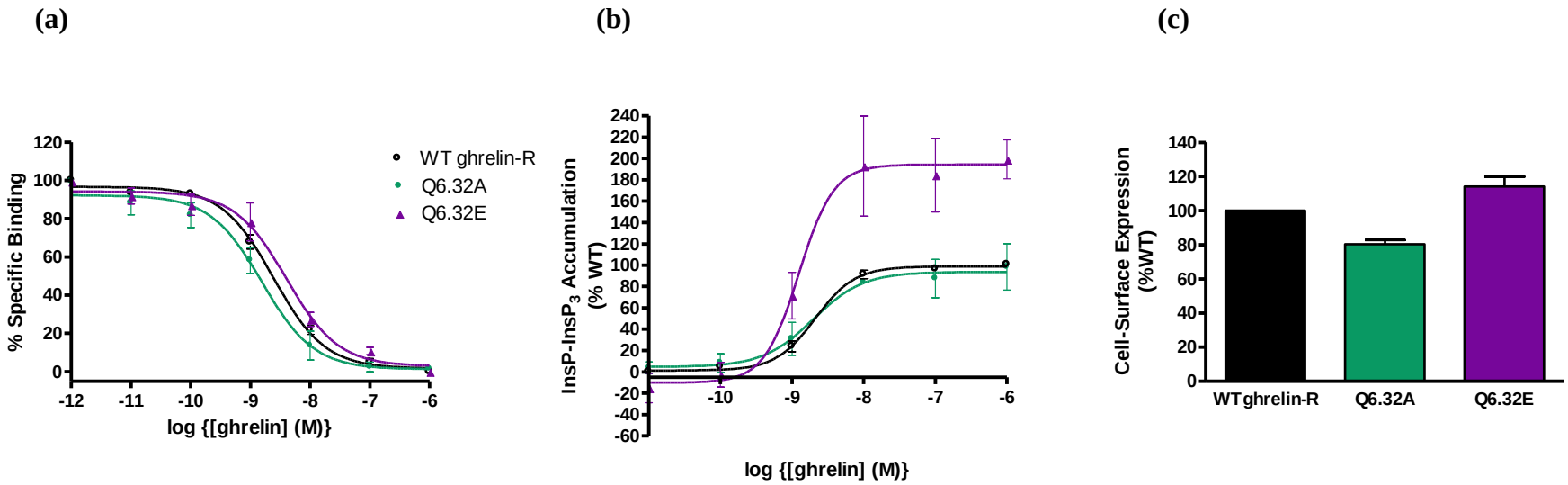


Figure 4.15 Ligand binding, intracellular signalling and cell-surface expression profiles for Gln$^{6.32}$ mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, Q6.32A or Q6.32E and are expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-InsP$_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, Q6.32A or Q6.32E. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

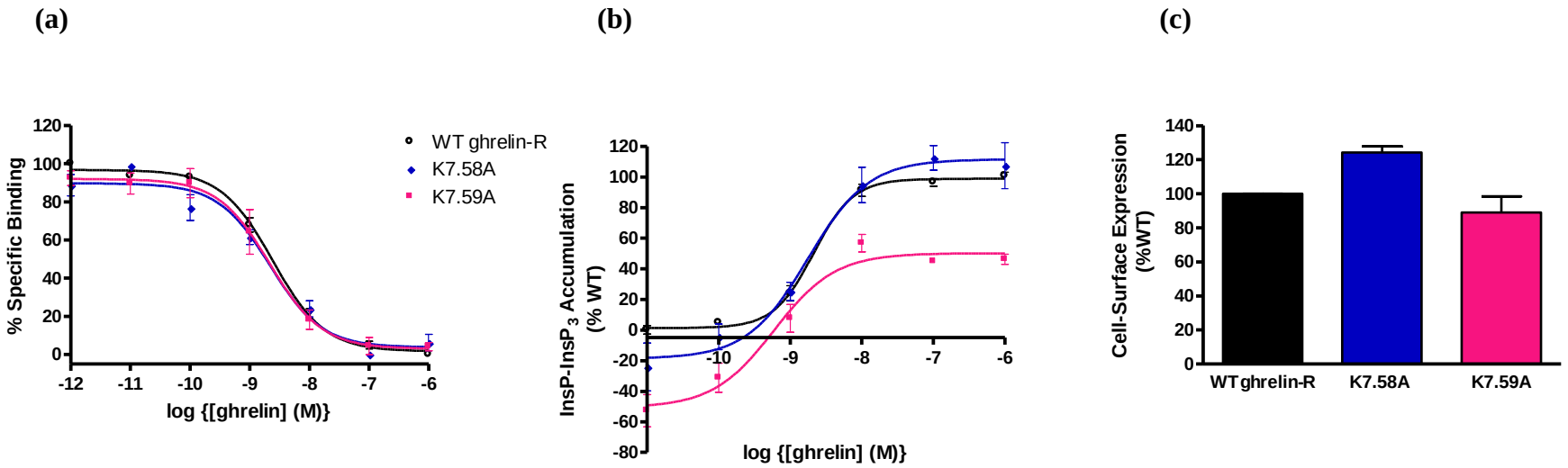


Figure 4.16 Ligand binding, intracellular signalling and cell-surface expression profiles for K7.58A and K7.59A mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, K7.58A or K7.59A and are expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, K7.58A or K7.59A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation			Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**
Q6.32A	8.80 ± 0.05	1.96 ± 0.88	4 ± 6	98 ± 22	80 ± 2
Q6.32E	8.39 ± 0.05	1.25 ± 0.29	-15 ± 14	199 ± 18	114 ± 6
K7.58A	8.66 ± 0.09	1.76 ± 0.89	-24 ± 16	107 ± 15	124 ± 4
K7.59A	8.66 ± 0.02	0.60 ± 0.91	-53 ± 11	46 ± 3	89 ± 10

Table 4.9 Ligand binding, intracellular signalling and cell-surface expression for Q6.32A, Q6.32E, K7.58A and K7.59A mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Green indicates a > 3-fold decrease in EC_{50}. Yellow indicates a reduction in basal activity > 20 % or > 30 % reduction in E_{max}. Orange indicates reduction of basal activity > 40 % of WT. Pink indicates an increase in E_{max} > 190 % of WT.

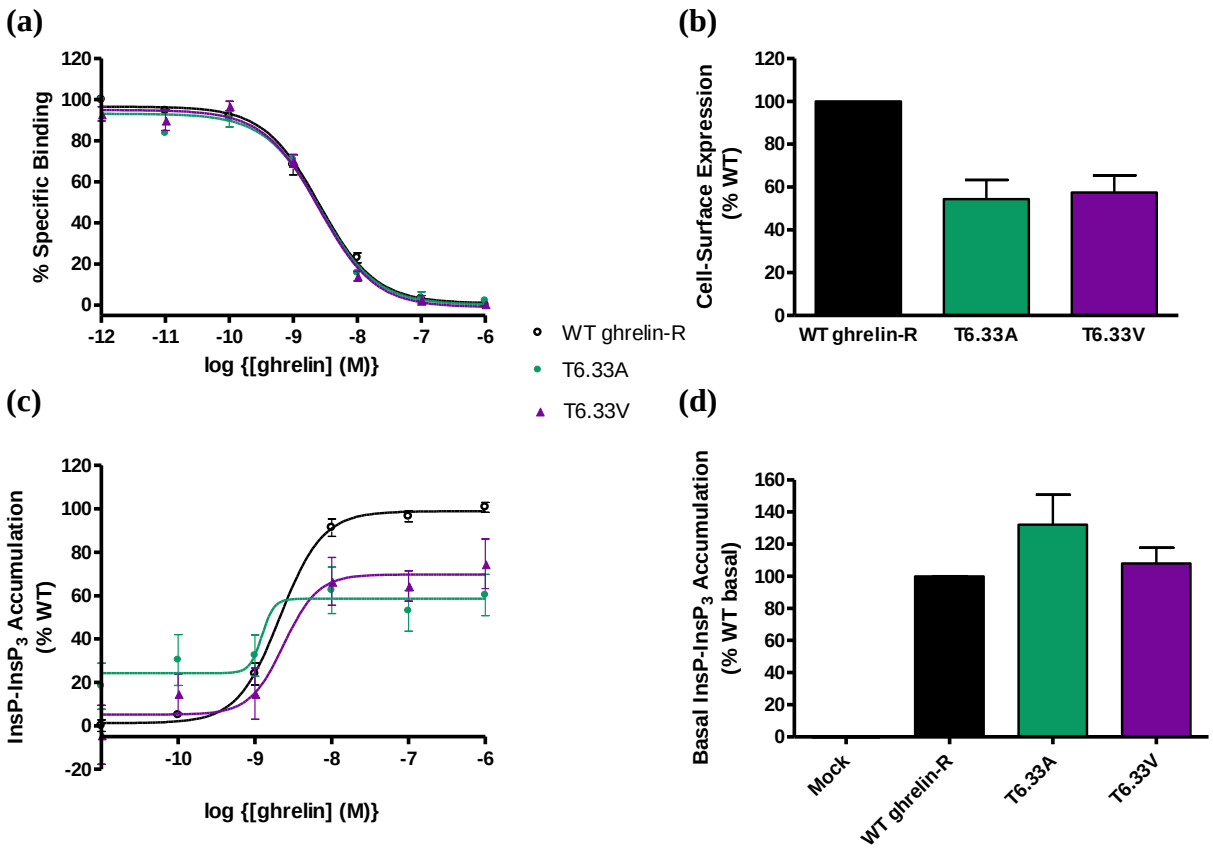


Figure 4.17 Ligand binding, intracellular signalling and cell-surface expression profiles for Thr$^{6.33}$ mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, T6.33A or T6.33V and are expressed as a percentage of specific binding. (b) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. (c) Ghrelin-induced InsP-InsP$_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, T6.33A or T6.33V. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum and ghrelin-R basal from experiments performed in parallel. (d) Basal InsP-InsP$_3$ accumulation as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation				Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	Basal (% WT basal) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**0**	**100**	**100**
T6.33A	8.58 ± 0.07	1.24 ± 1.75	18 ± 11	132 ± 19	60 ± 9	54 ± 9
T6.33V	8.59 ± 0.05	2.38 ± 2.79	4 ± 14	108 ± 10	75 ± 11	57 ± 8

Table 4.10 Ligand binding, intracellular signalling and cell-surface expression for Thr$^{6.33}$ mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Green indicates increased basal signalling > 10 % of WT. Yellow indicates a decrease in E_{max} > 30 % of WT or decreased cell surface expression > 30 % of WT.

The residue at position 6.34 has been shown to be highly important in a number of Family A GPCRs. Mutation of $Ala^{6.34}$ to any other amino acid in the α_{1b}AR resulted in a constitutively active receptor (Kjelsberg *et al.*, 1992). Other receptors have also demonstrated changes in basal signalling activity after mutation of 6.34 (Ren *et al.*, 1993; Samama *et al.*, 1993; Lattion *et al.*, 1999; Huang *et al.*, 2001) and the bRho crystal structure demonstrated an interaction between $Arg^{3.50}$ and $Thr^{6.34}$ (Palczewski *et al.*, 2000). $Val^{6.34}$ in the ghrelin receptor was mutated to alanine, lysine and threonine to investigate the effects of amino acid size and polarity at this position. V6.34A and V6.34T were comparable to the WT ghrelin-R in all characteristics (Fig 4.18, Table 4.11). V6.34K produced a receptor that was unable to bind [^{125}I]ghrelin at the concentrations used in assay. The EC_{50} and cell-surface expression were similar to the WT receptor, but the introduction of lysine resulted in a decrease in basal constitutive activity (- 56 %) and E_{max} (48 %) (Fig 4.18, Table 4.11).

4.2.4.3 Analysis of T6.33A using an inverse agonist

The ghrelin-R inverse agonist, SP-analogue, was used to characterise the InsP-$InsP_3$ accumulation profile of the T6.33A CAM. Despite the increased basal signalling of the T6.33A mutant, the pIC_{50} and I_{max} for SP-analogue were similar to that observed for the WT ghrelin-R (Fig 4.19, Table 4.12), indicating that the high basal signalling could be reduced after inverse agonist stimulation.

4.2.5 The role of residue 5.58 in ghrelin-R activity

In the active G-protein-interacting opsin crystal structure, $Tyr^{5.58}$ interacts with $Arg^{3.50}$ of the D/ERY motif and stabilises the $Arg^{3.50}$ interaction with the G-protein (Scheerer *et al.*, 2008). Residue 5.58 has also been implicated in G-protein specificity. A neutrally charged amino acid, glutamine, is found at position 5.58 in the V_2R, which couples to $G\alpha_s$ G-proteins.

To investigate the role of $Tyr^{5.58}$ in the ghrelin-R, Y5.58A and Y5.58Q mutants were produced. Y5.58A had 'WT-like' ghrelin affinity (Fig 4.20 (a)), but a profound effect on signalling through the $InsP_3$ pathway. Basal constitutive activity was substantially reduced and the mutant displayed a 4-fold increase in EC_{50}. Interestingly, the efficacy of the Y5.58A mutant was significantly increased with an E_{max} of 224 % of WT (Fig 4.20 (c), Table 4.13). Y5.58Q displayed 'WT-like' potency, with decreased basal and related decreased E_{max} (Fig 4.20 (Fig 4.20 (c) & (d), Table 4.13). Ghrelin affinity was reduced; the mutant was unable to bind at the radioligand concentration used (Fig 4.20 (a), Table 4.13). Both of the $Tyr^{5.58}$ mutants expressed at levels similar to WT at the cell-surface (Fig 4.20 (b), Table 4.13).

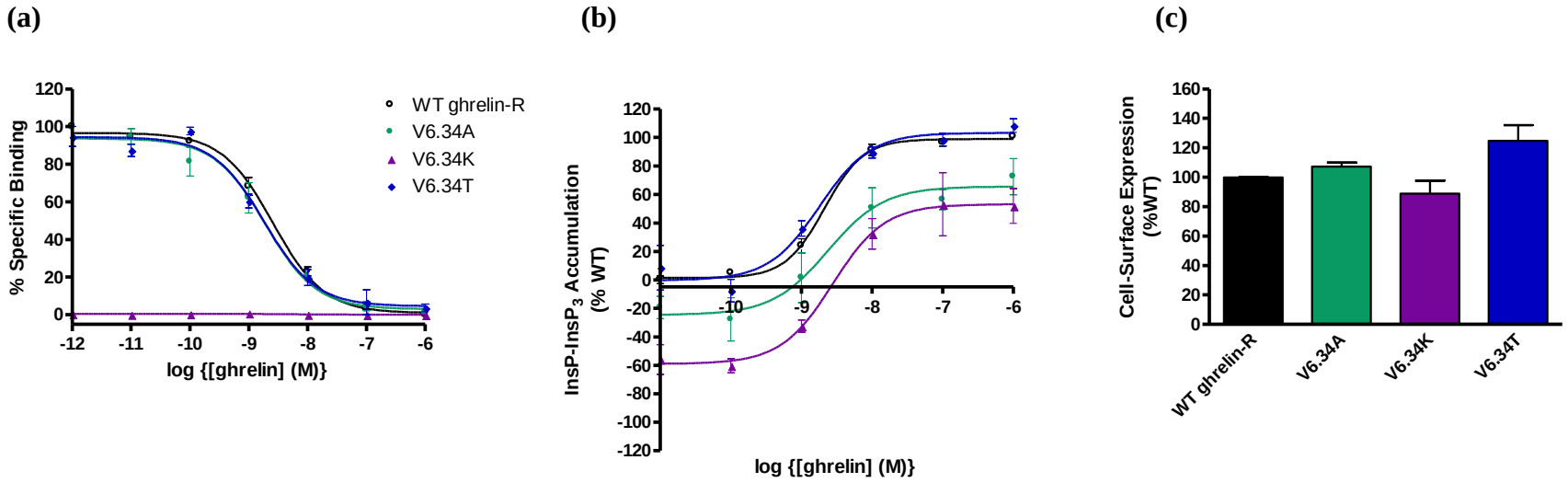


Figure 4.18 Ligand binding, intracellular signalling and cell-surface expression profiles for Val$^{6.34}$ mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, V6.34A, V6.34K or V6.34T and are expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-InsP$_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, V6.34A, V6.34K or V6.34T. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation			Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	0	100	100
V6.34A	8.72 ± 0.06	2.39 ± 1.59	-19 ± 8	72 ± 13	107 ± 3
V6.34K	NDB	2.77 ± 0.79	-56 ± 10	52 ± 12	89 ± 9
V6.34T	8.74 ± 0.06	1.80 ± 1.39	9 ± 16	108 ± 5	125 ± 11

Table 4.11 Ligand binding, intracellular signalling and cell-surface expression for Val$^{6.34}$ mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Red indicates no detectable binding. Orange indicates reduction of basal activity > 40 % of WT. Yellow indicates > 30 % reduction in E_{max}.

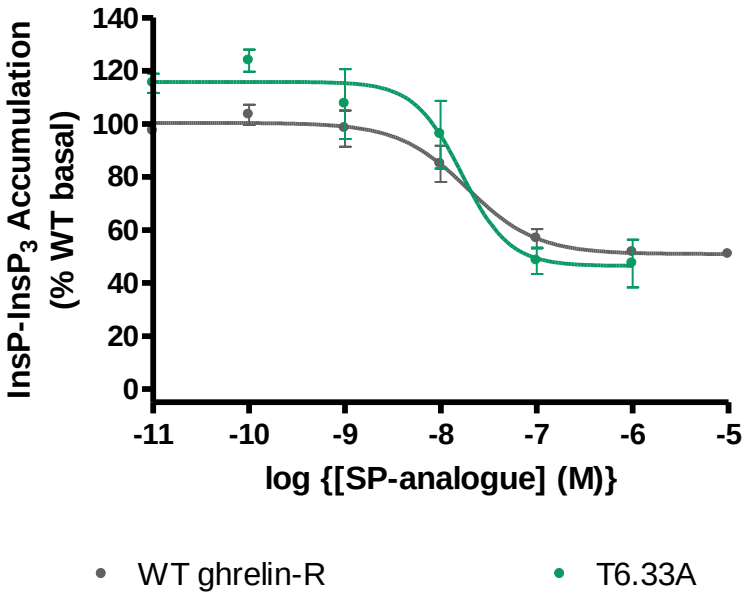


Figure 4.19 Intracellular signalling profile for T6.33A ghrelin-R mutant construct using SP-analogue: SP-analogue-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or T6.33A. Values are expressed as a percentage of the WT ghrelin-R basal from experiments performed in parallel. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	SP-analogue-induced InsP-$InsP_3$ Accumulation		
	pIC_{50} ± SEM	**Basal (% WT basal) ± SEM**	**I_{max} (% WT basal) ± SEM**
WT ghrelin-R	**7.72 ± 0.04**	**97 ± 2**	**51 ± 0.4**
T6.33A	7.78 ± 0.09	115 ± 4	47 ± 9

Table 4.12 Intracellular signalling of the T6.33A mutant receptor construct using SP-analogue: Values were obtained using the methods described in Chapter 2. Data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Green indicates increased constitutive activity > 10 % of WT.

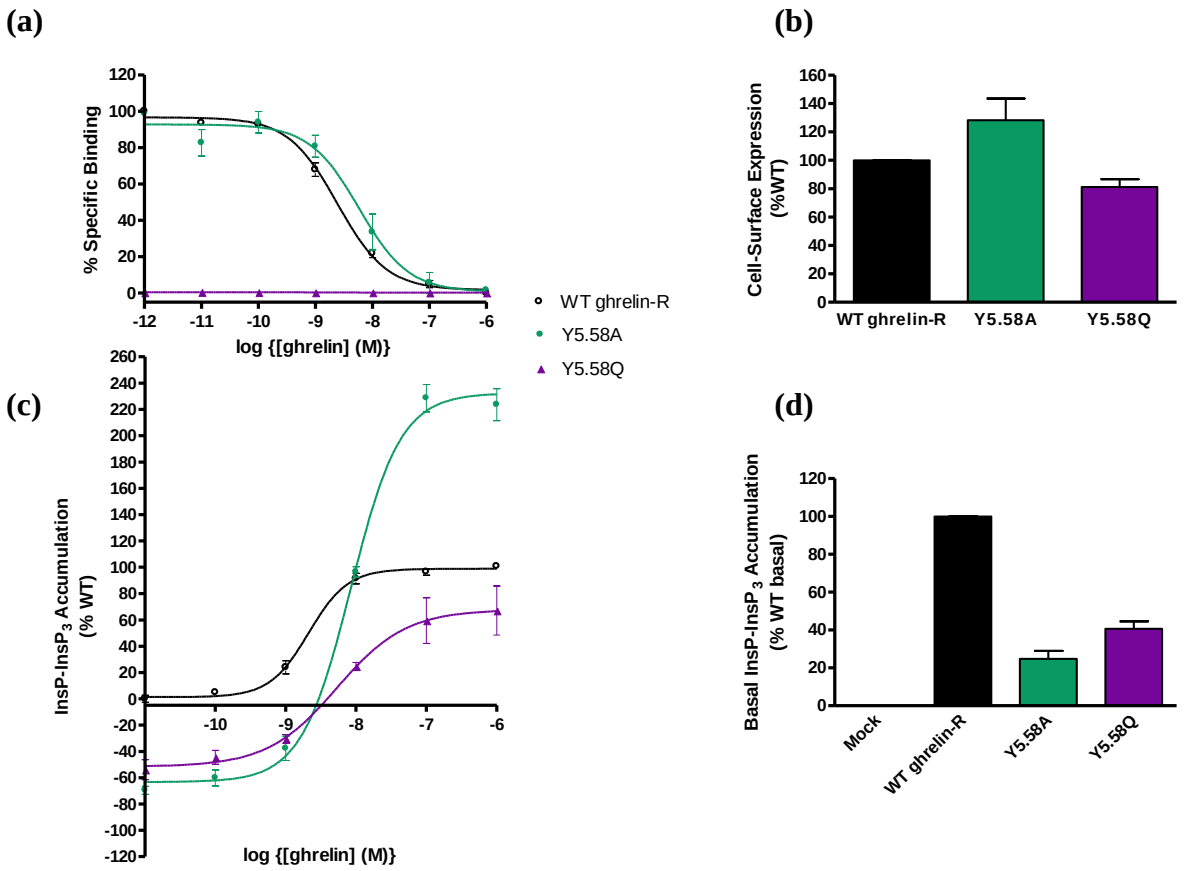


Figure 4.20 Ligand binding, intracellular signalling and cell-surface expression profiles for Tyr$^{5.58}$ mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, Y5.58A or Y5.58Q and are expressed as a percentage of specific binding. (b) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. (c) Ghrelin-induced InsP-InsP$_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, Y5.58A or Y5.58Q. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (d) Basal InsP-InsP$_3$ accumulation as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation				Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	Basal (% WT basal) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**	**100**
Y5.58A	8.24 ± 0.08	8.47 ± 1.98	-70 ± 3	25 ± 4	224 ± 12	128 ± 15
Y5.58Q	NDB	5.32 ± 1.04	-54 ± 8	41 ± 4	67 ± 19	81 ± 5

Table 4.13 Ligand binding, intracellular signalling and cell-surface expression for $Tyr^{5.58}$ mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Red indicates no detectable binding or a decrease in basal signalling > 60 %. Orange indicates an increase in EC_{50} > 4-fold or a reduction in basal activity > 40 %. Yellow indicates a decrease in E_{max} > 30 % of WT. Pink indicates an increase in E_{max} > 190 % of WT.

4.2.6 Investigation of the rotamer toggle switch in the ghrelin-R

The activation of Family A GPCRs involves the toggling of $Trp^{6.48}$ and the formation of stabilising interactions with a number of amino acids within TMs III, VI and VII. $Trp^{6.48}$ of the ghrelin-R was mutated to alanine, phenylalanine and tyrosine to investigate the effect of both side chain removal and the introduction of other aromatic residues at this position. The W6.48A mutant resulted in a receptor that had a 'WT-like' affinity for ghrelin (Fig 4.21 (a)), but was detrimental to signalling via the $InsP_3$ pathway. W6.48A resulted in a 13-fold increase in EC_{50}, completely diminished basal signalling and had an E_{max} below the WT basal signalling (-22 %), however, the receptor was still responsive to ghrelin to some degree (Fig 4.21, Table 4.14). Retaining an aromatic residue at position 6.48 in W6.48F and W6.48Y resulted in mutant receptors with similar phenotypes; both had 7-fold increases in EC_{50}, reduced basal (-58 and -56 % respectively) and reduced ghrelin-induced maximum, to approximately the level observed for WT basal. Both mutants had a binding affinity similar the WT (Fig 4.21, Table 4.14). W6.48A and W6.48Y displayed increased cell-surface expression (170 and 137 % of WT, respectively) whereas W6.48F expression was comparable to WT (Fig 4.21 (b), Table 4.14).

$Thr^{3.36}$ was mutated to alanine to produce the T3.36A mutant. Interestingly, this mutant displayed a very pronounced increase in basal constitutive activity, signalling at 70 % of the WT ghrelin-R, ghrelin-induced maximum (172 % of WT basal) (Fig 4.22 (c) & (d)). There was however, a 6-fold increase in EC_{50} indicating a reduced ghrelin potency. The WT E_{max} was reached, but not exceeded, despite the dramatic increase in the basal signalling. The binding affinity and cell-surface expression were comparable to WT (Fig 4.22, Table 4.15). To further investigate the role of $Thr^{3.36}$ in the ghrelin-R, T3.36C, T3.36F, T3.36M and T3.36S mutants were produced to examine various side chain lengths and characteristics. None of these additional $Thr^{3.36}$ mutants displayed constitutive activity (Fig 4.22 (c), Table 4.15). All of these mutants had 'WT-like' affinity for ghrelin. T3.36C and T3.36M EC_{50}, basal signalling and expression were comparable to WT however both had increased E_{max} (146 and 137 % respectively). T3.36F displayed increased potency (3-fold). Cell surface expression of this mutant was also increased (143 %). Basal signalling was slightly reduced in the T3.36F and T3.36S mutants (-25 and -21 %, respectively), but T3.36S signalling was very impaired with an E_{max} of only 36 % of the WT ghrelin-R (Fig 4.22, Table 4.15).

An asparagine is highly conserved at position 7.45 in Family A GPCRs (67 %) (Mirzadegan *et al.*, 2003) and forms part of the NSxx motif in TMVII of many Family A GPCRs. In the

ghrelin-R, a serine is found at this locus. Serine is the second most common residue at this position, found in 12 % of Family A receptors including Rho (Mirzadegan *et al.*, 2003). $Asn^{7.45}$ has been shown to be important for both ligand binding and intracellular signalling in some Family A GPCRs (Van Sande *et al.*, 1995; Jongejan *et al.*, 2005).

To investigate the role of $Ser^{7.45}$ in the ghrelin-R, mutant receptor constructs were produced. Alanine was introduced in the S7.45A mutant to investigate the effect of removing the side chain. S7.45N was produced to investigate introduction of the more conserved asparagine residue at this position. Both mutants retained 'WT-like' affinity for ghrelin (Fig 4.23 (a), Table 4.16). S7.45A had increased basal constitutive activity (134 % of WT basal) and retained the ability to signal further after stimulation with ghrelin with a resultant increase in E_{max} (178 %) and 'WT-like' potency. The S7.45N mutant reduced constitutive activity, but increased ghrelin potency (3.5-fold). The E_{max} was similar to the WT ghrelin-R. The cell-surface expression for S7.45A and S7.45N were 115 and 145 % respectively (Fig 4.23, Table 4.16).

Residue 3.35 has been proposed to participate in the network of stabilising hydrogen bonds within Family A GPCRs. In the ghrelin-R $Cys^{3.35}$ was mutated to alanine to examine the specific role of the cysteine side chain at this position. The C3.35A mutant retained 'WT-like' ghrelin affinity, basal signalling and cell-surface expression, but affected the ghrelin-induced signalling with a reduced E_{max} of 48 % of the WT (Fig 4.24, Table 4.17).

4.2.6.1 Investigation of T3.36A using an inverse agonist

InsP-$InsP_3$ accumulation assays were conducted on T3.36A using the inverse agonist, SP-analogue. T3.36A retained a pIC_{50} comparable to the WT ghrelin-R. The constitutive activity of the T3.36A mutant was decreased on addition of the SP-analogue with an I_{max} similar to that seen for the WT ghrelin-R, suggesting that despite the increased basal activity, SP-analogue was able to inhibit the T3.36A mutant receptor signalling to the same extent as seen for the WT ghrelin-R (Fig 4.25, Table 4.18).

4.2.6.2 Investigation of S7.45A using an inverse agonist

The constitutive activity of the S7.45A mutant was decreased on addition of the SP-analogue and was able to approach a value near to the WT I_{max} at μM concentrations (Fig 4.26, Table 4.19). From the data gather for S7.45A, when stimulated with inverse agonist, it is unclear whether the mutant receptor construct retains a 'WT-like' pIC_{50} and I_{max} as a greater

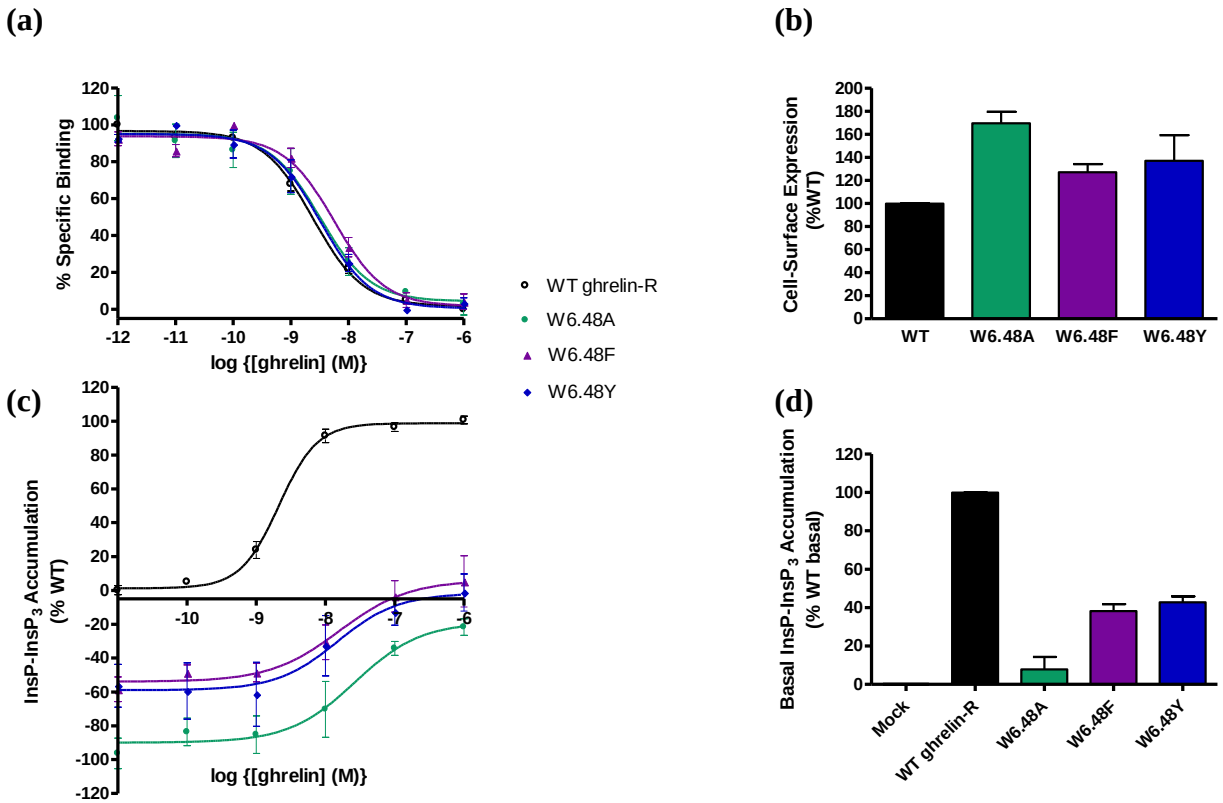


Figure 4.21 Ligand binding, cell-surface expression and intracellular signalling profiles for Trp$^{6.48}$ mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, W6.48A, W6.48F or W6.48Y and are expressed as a percentage of specific binding. (b) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. (c) Ghrelin-induced InsP-InsP$_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, W6.48A, W6.48F or W6.48Y. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (d) Basal InsP-InsP$_3$ accumulation as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation				Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	Basal (% WT basal) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**	**100**
W6.48A	8.49 ± 0.06	26.62 ± 25.3	-96 ± 9	8 ± 6	-22 ± 5	170 ± 10
W6.48F	8.24 ± 0.06	15.88 ± 8.29	-58 ± 7	38 ± 4	5 ± 15	127 ± 7
W6.48Y	8.49 ± 0.05	14.86 ± 8.82	-56 ± 15	43 ± 3	-1 ± 11	137 ± 22

Table 4.14 Ligand binding, intracellular signalling and cell-surface expression for $Trp^{6.48}$ mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Red indicates > 5-fold increase in EC_{50}, > 60 % decrease in constitutive activity or E_{max} < WT basal signalling. Orange indicates reduction of basal activity > 40 % of WT or decreased E_{max} > 60 % of WT. Pink indicates cell-surface expression > 170 % of WT. Green indicates increased cell-surface expression > 130 % of WT.

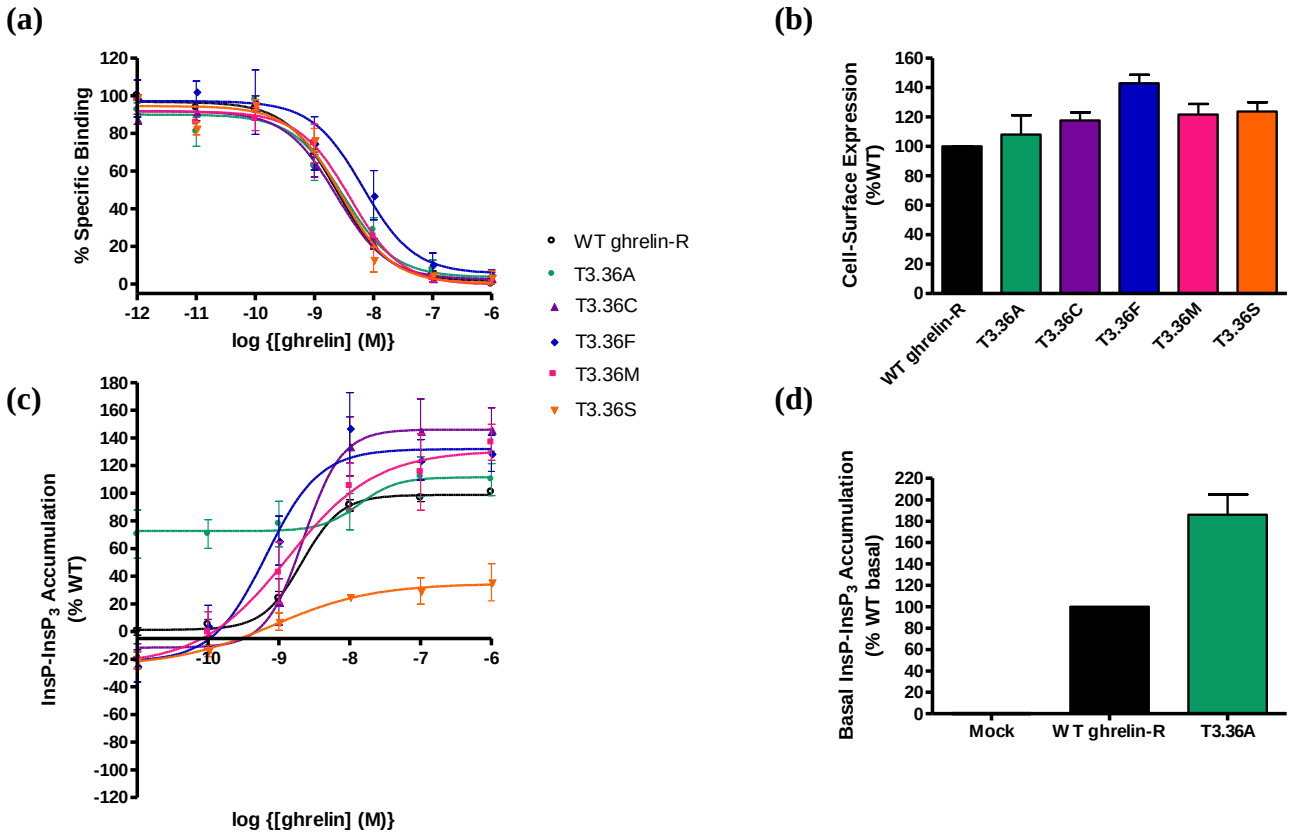


Figure 4.22 Ligand binding, intracellular signalling and cell-surface expression profiles for Thr$^{3.36}$ mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, T3.36A, T3.36C, T3.36F, T3.36M or T3.36S and are expressed as a percentage of specific binding. (b) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. (c) Ghrelin-induced InsP-InsP$_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, T3.36A, T3.36C, T3.36F, T3.36M or T3.36S. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (d) Basal InsP-InsP$_3$ accumulation as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation				Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	Basal (% WT basal) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**	**100**
T3.36A	8.51 ± 0.10	13.63 ± 8.73	70 ± 17	172 ± 14	110 ± 11	108 ± 13
T3.36C	8.63 ± 0.04	2.24 ± 0.75	-18 ± 9	-	146 ± 16	118 ± 5
T3.36F	8.18 ± 0.06	0.66 ± 0.50	-25 ± 12	-	129 ± 13	143 ± 6
T3.36M	8.39 ± 0.05	1.34 ± 0.93	-20 ± 6	-	137 ± 13	122 ± 7
T3.36S	8.55 ± 0.09	0.85 ± 0.60	-21 ± 6	-	36 ± 13	124 ± 6

Table 4.15 Ligand binding, intracellular signalling and cell-surface expression for $Thr^{3.36}$ mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Red indicates > 5-fold increase in EC_{50}. Pink indicates an increase in basal signalling of > 60 % of WT. Yellow indicates a reduction in basal activity > 20 % of WT. Orange indicates reduction in E_{max} > 60 % of WT. Green indicates a decrease in EC_{50} > 3-fold, an increase in E_{max} > 130 % or an increase in cell-surface expression > 130 % of WT.

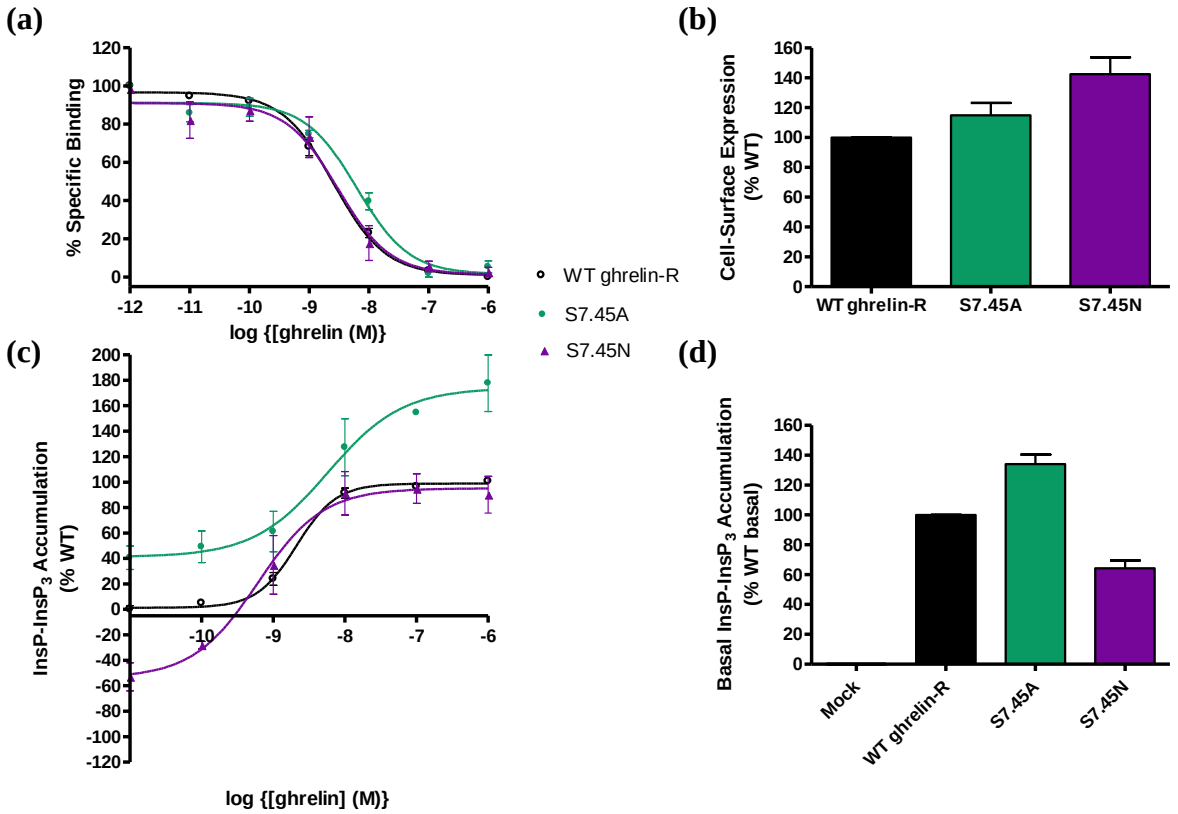


Figure 4.23 Ligand binding, intracellular signalling and cell-surface expression profiles for Ser$^{7.45}$ mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, S7.45A or S7.45N and are expressed as a percentage of specific binding. (b) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. (c) Ghrelin-induced InsP-InsP$_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, S7.45A or S7.45N. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (d) Basal InsP-InsP$_3$ accumulation as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation				Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	Basal (% WT basal) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**	**100**
S7.45A	8.20 ± 0.07	5.82 ± 3.76	41 ± 9	134 ± 6	178 ± 22	115 ± 8
S7.45N	8.52 ± 0.07	0.61 ± 0.24	-53 ± 11	64 ± 5	90 ± 14	142 ± 11

Table 4.16 Ligand binding, intracellular signalling and cell-surface expression for $Ser^{7.45}$ mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Green indicates an increase in EC_{50} > 3-fold, an increase in basal > 10 % of WT basal or an increase in cell-surface expression > 130 % of WT. Blue indicates an increase in basal signalling > 40 % or an increase in E_{max} > 160 %. Orange indicates a reduction in basal activity > 40 %. Yellow indicates a reduction in basal signalling > 20 % of WT.

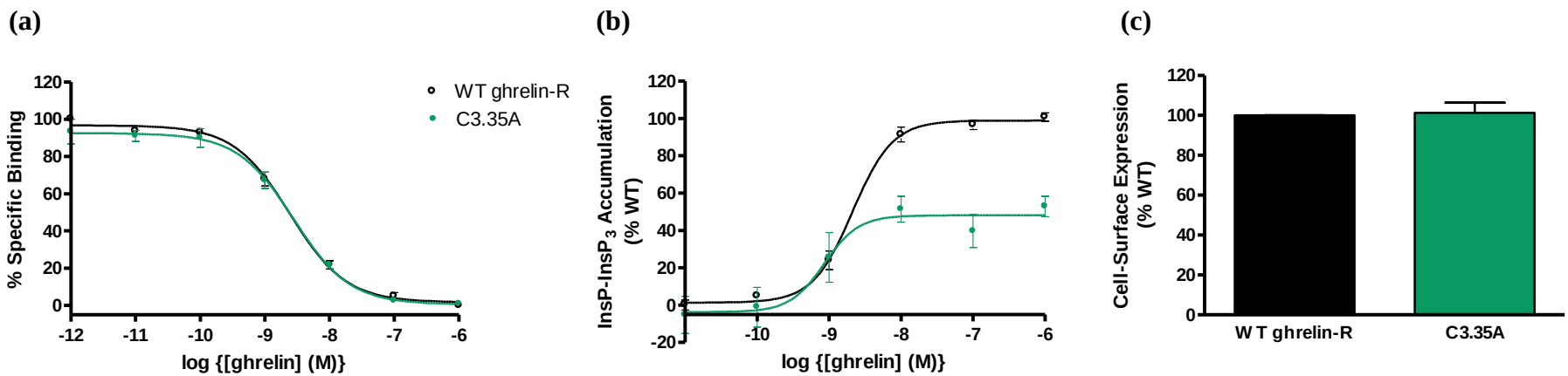


Figure 4.24 Ligand binding, intracellular signalling and cell-surface expression profiles for the C3.35A mutant receptor construct: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or C3.35A and are expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or C3.35A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation			Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**
C3.35A	8.56 ± 0.01	0.86 ± 0.58	-3 ± 2	48 ± 2	101 ± 5

Table 4.17 Ligand binding, intracellular signalling and cell-surface expression for C3.35A mutant construct: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Yellow indicates > 30 % reduction in E_{max}.

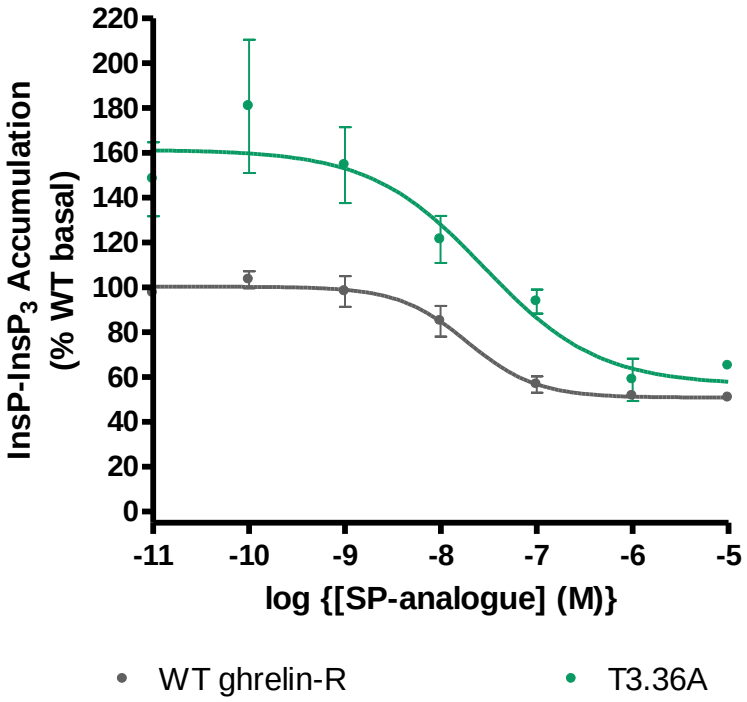


Figure 4.25 Intracellular signalling profile for T3.36A using SP-analogue: InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or T3.36A. Values are expressed as a percentage of the WT ghrelin-R basal from experiments performed in parallel. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	SP-analogue-induced InsP-$InsP_3$ Accumulation		
	pIC_{50} ± SEM	**Basal (% WT basal) ± SEM**	**I_{max} (% WT basal) ± SEM**
WT ghrelin-R	**7.72 ± 0.04**	**97 ± 2**	**51 ± 1**
T3.36A	7.55 ± 0.25	161 ± 11	57 ± 6

Table 4.18 Intracellular signalling of the T3.36A mutant receptor construct using SP-analogue: Values were obtained using the methods described in Chapter 2. Data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Pink indicates increase in constitutive activity > 60 % of WT basal.

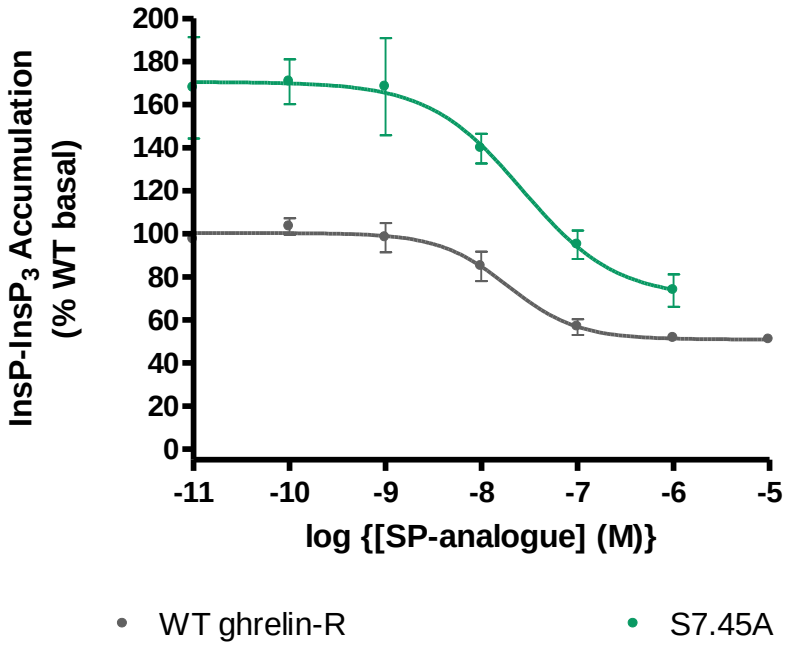


Figure 4.26 Intracellular signalling profile for S7.45A using SP-analogue: InsP-$InsP_3$ accumulation assays were performed on HEK 293T cells transiently transfected with WT ghrelin-R or S7.45A. Values are expressed as a percentage of the WT ghrelin-R basal from experiments performed in parallel. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	SP-analogue-induced InsP-$InsP_3$ Accumulation		
	pIC_{50} ± SEM	**Basal (% WT basal) ± SEM**	**I_{max} (% WT basal) ± SEM**
WT ghrelin-R	**7.72 ± 0.04**	**97 ± 2**	**51 ± 1**
S7.45A	-	168 ± 24	-

Table 4.19 Intracellular signalling of S7.45A mutant receptor construct using SP-analogue: Values were obtained using the methods described in Chapter 2. Data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Pink indicates an increase in constitutive activity > 60 % of WT basal.

concentration of SP-analogue is required, although it appears to retain 'WT-like' SP-analogue potency and efficacy, despite the substantial increase in basal activity.

4.2.7 The role of the highly conserved NPxxY motif in ghrelin-R activity

The NPxxY motif located at the C-terminal end of TMVII is a highly conserved motif involved in activation in Family A GPCRs. To investigate the role of these amino acids in the ghrelin-R, each residue was individually mutated to alanine to produce N7.49A, P7.50A, I7.51A, L7.52A and Y7.53A mutant receptor constructs. Although $Asp^{7.49}$, $Pro^{7.50}$, and $Tyr^{7.53}$ are the most highly conserved, an isoleucine is found at position 7.51 and a leucine at position 7.52 in 32 % and 36 % of Family A GPCRs respectively (Mirzadegan *et al.*, 2003). All mutants displayed 'WT-like' ghrelin potency (Fig 4.27 (c), Table 4.20). N7.49A and Y7.53A retained 'WT-like' affinity for ghrelin as determined by radioligand binding. P7.50A was unable to bind [^{125}I]ghrelin at the concentrations used. Interestingly, both I7.51A and L7.52A demonstrated increased affinity for ghrelin, ~3-fold, relative to the WT ghrelin-R (Fig 4.27 (a), Table 4.20). The mutants displayed moderately decreased constitutive activity except for P7.50A, which retained basal activity similar to WT, but produced a dramatic increase in ghrelin efficacy (E_{max} 176 % of WT ghrelin-R). I7.52A and Y7.53A had reduced E_{max}, possibly in relation to their decreased basal signalling activity (Fig 4.27 (b), Table 7.20). None of the mutations had a large effect on cell-surface expression; only L7.52A had any effect, reducing expression to 67 % of WT (Fig 4.27 (c), Table 4.20).

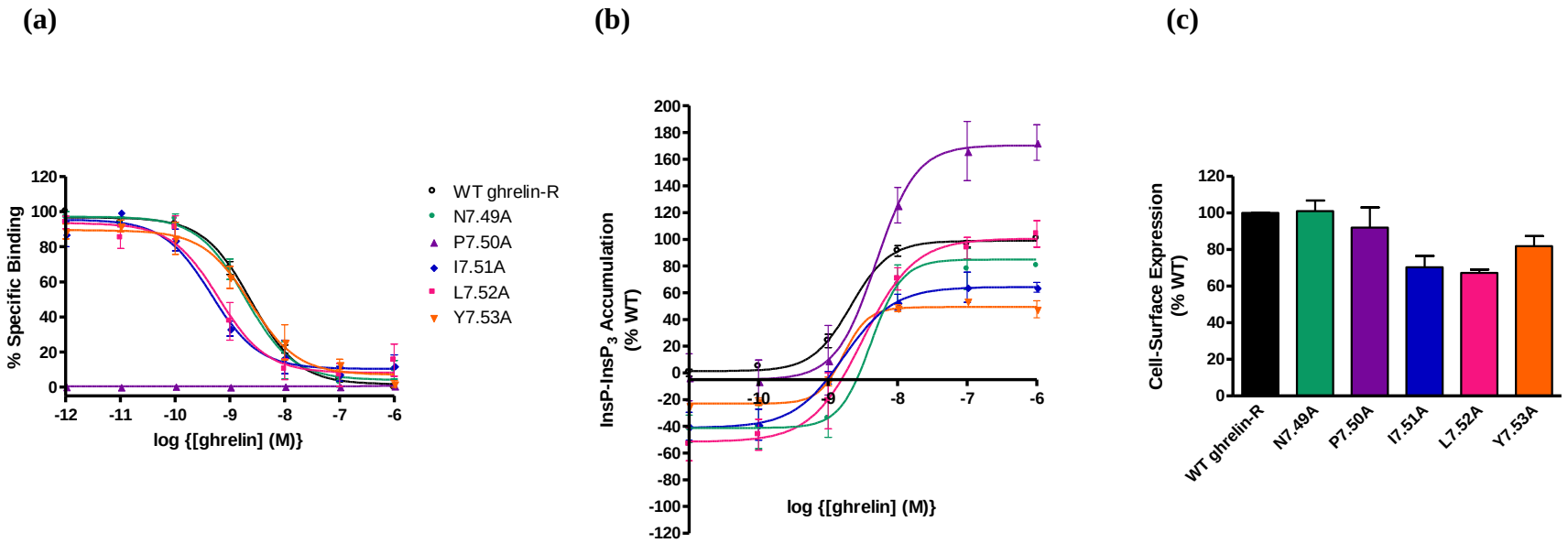


Figure 4.27 Ligand binding, intracellular signalling and cell-surface expression profiles for N7.49A, P7.50A, I7.51A, L7.52A and Y7.53A mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, N7.49A, P7.50A, I7.51A, L7.52A or Y7.53A and are expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, N7.49A, P7.50A, I7.51A, L7.52A or Y7.53A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation			Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**
N7.49A	8.72 ± 0.05	4.12 ± 2.52	-42 ± 10	93 ± 23	101 ± 6
P7.50A	NDB	5.27 ± 0.92	1 ± 14	176 ± 21	92 ± 11
I7.51A	9.34 ± 0.07	1.66 ± 0.11	-40 ± 11	64 ± 4	70 ± 6
L7.52A	9.20 ± 0.07	3.14 ± 0.77	-53 ± 12	104 ± 10	67 ± 2
Y7.53A	8.63 ± 0.04	1.65 ± 1.11	-24 ± 2	48 ± 6	82 ± 5

Table 4.20 Ligand binding, intracellular signalling and cell-surface expression for N7.49A, P7.50A, I7.51A, L7.52A and Y7.53A mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Red indicates no detectable binding. Orange indicates reduction basal activity > 40 % of WT. Blue indicates a > 160 % increase in E_{max}. Green indicates an increase in pIC_{50} > 0.5 log units. Yellow indicates a decrease in basal activity > 20 %, a decrease in E_{max} > 30 % of WT or a decrease in cell-surface expression > 30 % of WT.

4.3 Discussion

A number of conformational changes take place within the receptors after agonist binding that are similar in all Family A members, despite pronounced variations in ligand and in receptor sequence. Studies of receptor activation have revealed a universal molecular mechanism, termed the 'global toggle switch', which incorporates the structural rearrangements of TMIII and TMVI, involving the "see-saw-like" movements of TMVI around $Pro^{6.50}$ (Schwartz *et al.*, 2006). Arguably the most important feature of GPCR activation is the movement of TMVI away from TMIII at the cytoplasmic face of the receptor, resulting in the exposure of key amino acids to the G-protein, enabling coupling and activation. Despite a proposed general overall mechanism of GPCR activation, the precise interactions that occur within the receptors vary throughout the superfamily.

Most GPCRs display very little agonist-independent activity although some, like the ghrelin-R, are able to signal to a large extent in the absence of an agonist. It would therefore be expected that the mechanisms that are involved in ghrelin-R activation may differ from those of a GPCR that does not display substantial constitutive activity, as the ghrelin-R might be expected to exist in partial transition between the R and R* states. GPCR constitutive activity may be a result of inherent receptor flexibility and an ability to adopt multiple conformational states in the absence of ligands, or it might be a reflection of high affinity for a G-protein, despite a constrained state (Kobilka *et al.*, 2007). Rho is restrained in the inactive conformation by the endogenous, covalently bound, inverse agonist, 11-*cis* retinal. In the absence of retinal, the opsin protein adopts a more active conformation. The crystal structures of opsin, both alone and in the G-protein-interacting conformation, are the only crystal structures that denote active receptors (Park *et al.*, 2008; Scheerer *et al.*, 2008). The β_2AR has been shown to display a certain amount of constitutive activity although the crystal structure of the β_2AR is of an inactive receptor bound to the inverse agonist, carazolol (Rasmussen *et al.*, 2007).

To gain an insight into the underlying mechanisms of ghrelin-R constitutive and agonist-induced activation, key motifs and individual residues, implicated in Family A GPCR activation, were extensively investigated.

4.3.1 The role of the ERY motif in ghrelin-R activity

Activation of Family A GPCRs involves the rearrangement of interactions with and within the highly conserved D/ERY motif in TMIII. In Rho, protonation of $Glu^{3.49}$ is predicted to allow movement of this residue to a non-polar environment resulting in the loss of the salt bridge interaction with the neighbouring $Arg^{3.50}$ (Palczewski *et al.*, 2000; Li *et al.*, 2004). Mutation of $Asp/Glu^{3.49}$ in a number of GPCRs has allowed the characterisation of Family A receptors into two distinct phenotypes: phenotype 1 is characterised by increased constitutive receptor activity as a result of the aspartate/glutamate mutation, whereas phenotype 2 receptors do not demonstrate increased agonist-independent activity (Chung *et al.*, 2002). It has therefore been concluded that despite an acknowledged essential role in GPCR function, $Asp/Glu^{3.49}$ may participate in different interactions in different GPCRs of the same family. Examples of phenotype 1 receptors, where mutation of $Asp/Glu^{3.49}$ to alanine results in constitutive activity include the V_2R (Morin *et al.*, 1998), the β_2AR (Rasmussen *et al.*, 1999), the $\alpha_{1b}AR$ (Scheer *et al.*, 1996), the MC4R (Yamano *et al.*, 2004) and the H_2R (Alewijnse *et al.*, 2000). However, mutation of $Asp^{3.49}$ to alanine in the GnRHR (Ballesteros *et al.*, 1998), and the CB_2 receptor (Feng *et al.*, 2003), eliminated detectable activation and agonist binding. The mutation of $Asp/Glu^{3.49}$ of the D/ERY motif is thought to result in constitutive activity due to the induction of conformational changes which result in a shift of $Arg^{3.50}$ out of its constraining polar pocket. The disruption of the $Asp/Glu^{3.49}$ interaction with $Arg^{3.50}$ is believed to be a significant factor influencing the ability of a number of Family A GPCRs to adopt the R* active conformation (Scheer *et al.*, 1996). In this study, substitution of $Glu^{3.49}$ with alanine did not result in constitutive activity in the ghrelin-R; the E3.49A mutant was able to retain WT ghrelin-R constitutive activity. Ghrelin-induced signalling however was abolished, implicating $Glu^{3.49}$ in the formation of the active conformation and not in the stabilisation of the inactive state implicated in phenotype 1, above.

Mutation of $Asp/Glu^{3.49}$ to residues other than alanine has also resulted in increased agonist-independent activity in Family A GPCRs (Cohen *et al.*, 1993; Scheer *et al.*, 1996; Scheer *et al.*, 1997; Morin *et al.*, 1998; Alewijnse *et al.*, 2000; Ballesteros *et al.*, 2001; Favre *et al.*, 2005). In the $\alpha_{1b}AR$ mutation of $Asp^{3.49}$ to glutamate, arginine or asparagine resulted in significant increases in basal activity (Scheer *et al.*, 1996). Mutation of $Glu^{3.49}$ to glutamine in opsin resulted in increased constitutive activity, whereas mutation to aspartate resulted in inhibition of opsin activation highlighting the importance of a negative charge at this locus in maintaining an inactive state (Cohen *et al.*, 1993; Arnis *et al.*, 1994).

The E3.49D mutation converted the ghrelin-R into a 'typical GPCR' with very little basal signalling, but a robust response to ghrelin. E3.49R ablates signalling whether constitutive, or ghrelin-induced, revealing that the charge reversal is not tolerated, possibly due to a repulsion of the neighbouring $Arg^{3.50}$. Interestingly, mutation of $Glu^{3.49}$ to aspartate was equally as detrimental to constitutive activity as reversing the charge by the introduction of arginine, and reduced basal activity to levels similar to that seen for the mock-transfected cells. The introduction of a glutamine at position 3.49, neutralising the charge found at this locus, did not affect the ghrelin-R constitutive activity although the receptor was unable to respond to ghrelin stimulation with anything like the same efficacy as observed for the WT ghrelin-R. This result highlights the requirement of a negative charge at this position for ghrelin-induced activation. The H_2R has previously been studied to determine the effects of mutations within the DRY motif on $G\alpha_s$-coupled receptors which display constitutive activity. Mutation of $Asp^{3.49}$ to both alanine and asparagine produced increases in basal cAMP production indicating that the constitutive activity is able to be increased, even in receptors that already exhibit endogenous constitutive activity (Alewijnse *et al.*, 2000). This is not found to be the case for the ghrelin-R.

The introduction of alanine or a reversal of charge at position 3.49 in the E3.49A and E3.49R mutants respectively, were severely detrimental to cell-surface expression and were unable to signal after stimulation with ghrelin. E3.49A, however, retained 'WT-like' constitutive activity, indicating that the 70 % reduction in cell-surface expression was not the reason underlying the loss of signalling capabilities. Ligand binding was not affected by either mutation suggesting that the decrease in cell-surface expression might be a result of a disruption of receptor trafficking, rather than gross miss-folding. Both the E3.49D and E3.49Q mutant receptor constructs expressed at the cell-surface at levels similar to that seen for the WT ghrelin-R. Consequently, the reduction in constitutive activity observed for the E3.49D mutant was not a result of decrease receptor expression and was a genuine characteristic of the introduction of an aspartate. Cell-surface expression has been shown to be reduced in a number of $Asp/Glu^{3.49}$ mutations, possibly due to disruption of stabilising interactions, resulting in increased internalisation (Lu *et al.*, 1997; Morin *et al.*, 1998; Alewijnse *et al.*, 2000).

One of the most highly conserved residues in Family A GPCRs is the arginine located at the cytoplasmic side of TMIII. Forming part of the highly conserved D/ERY motif, $Arg^{3.50}$ is involved in stabilising the ground state of GPCRs and in stabilising interactions that occur in

the active state (Ballesteros *et al.*, 1998; Alewijnse *et al.*, 2000; Scheer *et al.*, 2000; Scheerer *et al.*, 2008). $Arg^{3.50}$ has also been reported to be directly involved in binding to G-proteins in a number of Family A GPCRs (Zhu *et al.*, 1994; Scheer *et al.*, 1996; Feng *et al.*, 2003; Scheerer *et al.*, 2008;). Studies have also shown that mutation of $Arg^{3.50}$ is often particularly detrimental to GPCR function, suggesting a crucial role of this residue in receptor activation (Ballesteros *et al.*, 1998; Alewijnse *et al.*, 2000; Scheer *et al.*, 2000; Lagane *et al.*, 2005). Mutation of $Arg^{3.50}$ has resulted in impaired signalling and complete loss of G-protein coupling in different receptors and the effect produced is often dependant on the amino acid that is introduced. A study by Scheer *et al*. (2000) mutated $Arg^{3.50}$ of the α_{1b}AR to several amino acids. Most substitutions resulted in receptors with impaired signalling capabilities, however, the R3.50K, R3.50D and R3.50H mutants all produced increases in agonist-independent signalling through the $InsP_3$ pathway. Mutation of $Arg^{3.50}$ to alanine or asparagine did not produce constitutive activity, but resulted in receptors that were unable to signal through the $InsP_3$ pathway (Scheer *et al.*, 1996). This is in agreement with studies on the m1 mAChR and m2 mAChR (Zhu *et al.*, 1994) and the V_2R (Rosenthal *et al.*, 1993). In contrast, in the oxytocin receptor, mutation of $Arg^{3.50}$ to alanine resulted in constitutive activity (Fanelli *et al.*, 1999).

The mutations carried out at position 3.50 of the ghrelin-R had varying effects on receptor activation, however, none of the mutations induced further constitutive activation as seen for some GPCRs. The R3.50A receptor construct was unable to signal after stimulation with ghrelin, but retained a substantial level of constitutive activity suggesting that the introduction of a small, hydrophobic amino acid did not substantially affect G-protein coupling itself, but instead altered the ability of the receptor to adopt a fully active conformation. R3.50L was unable to signal after ghrelin stimulation but also diminished the WT ghrelin-R constitutive activity to levels near that seen for the mock-transfected cells. R3.50L was far more detrimental to receptor function than the alanine mutation which implies that the larger side chain of leucine obstructs interactions that allow the ghrelin-R to be constitutively active, possibly direct interactions with the G-protein. Leucine might be stabilising ground state interactions and these cannot be overcome by stimulation with ghrelin, or might interfere with receptor-G-protein coupling. The polar mutation, R3.50Q, was the least detrimental, maintaining characteristics similar to WT except for a slight reduction in constitutive activity and 3-fold increase in EC_{50}. This suggests that the positive charge at position 3.50 is not essential for receptor activation. Supporting this finding, mutation of $Arg^{3.50}$ to glutamine in the GnRHR was more tolerated than mutation to a conserved positive residue, lysine

(Ballesteros *et al.*, 1998). For the ghrelin-R, constitutive activity was affected in the conservative R3.50K mutation and completely abolished in the R3.50H mutation, indicating that substitution of arginine with another positive residue is not capable of restoring ghrelin-R agonist-independent activity. R3.50K and R3.50H were more detrimental to constitutive activity than the R3.50A mutant and this suggests that introduction of lysine or histidine disrupts an interaction that is essential for constitutive activity. Although both the R3.50K and R3.50H mutants were capable of signalling after stimulation with ghrelin, neither mutant was able to signal to the same extent as the WT ghrelin-R and both displayed increases in EC_{50} indicating that the ability of the receptor to adopt a fully active state was compromised. Reversing the charge found at position 3.50 by the introduction of glutamate eliminated any ghrelin-R constitutive activity but maintained ghrelin sensitivity. Interestingly, this mutant had a 'WT-like' EC_{50} despite a severe reduction in E_{max}. These results suggest that, as found in many Family A GPCRs, an arginine at position 3.50 is necessary for WT ghrelin-R activity and that other amino acids cannot successfully substitute.

Studies have suggested that, despite detrimental effects on G-protein coupling, agonist affinity can be increased with $Arg^{3.50}$ mutation (Seibold *et al.*, 1998; Scheer *et al.*, 2000). This implies that substitution of $Arg^{3.50}$ removes a stabilising interaction that keeps the receptor in the ground state, thereby allowing an R* conformation to be adopted and resulting in increased agonist affinity, but that G-protein coupling and activation is affected due to removal of a G-protein interaction site. There was no observed increase in agonist affinity for any of the $Arg^{3.50}$ mutant receptor constructs. All of the $Arg^{3.50}$ substitutions in the ghrelin-R retained 'WT-like' affinity for ghrelin and 'WT-like' expression at the cell-surface, indicative of correct folding and trafficking of the receptor constructs to the cell-surface. Any detrimental effects of the mutations on intracellular signalling were not, therefore, a result of reduced receptor expression or of a disruption to ghrelin binding.

Further analysis of E3.49A, E3.49Q and R3.50A using SP-analogue demonstrated that the detrimental effect of these mutations on receptor responsiveness is related only to the agonist-induced InsP-$InsP_3$ accumulation. The effect of the SP-analogue on the mutant receptor constructs was comparable to the data for the WT ghrelin-R, despite the lack of ghrelin responsiveness. This suggests that a more inactive conformation is able to be formed after inverse agonist stimulation, and supports and highlights the retention of some level of constitutive activity in each of these mutants.

The tyrosine residue found at position 3.51 in TMIII is the least well conserved residue (67 %) of the D/ERY motif in Family A GPCRs (Mirzadegan *et al.*, 2003) and is generally accepted to have little significance in receptor function (Lu *et al.*, 1997; Wess, 1998; Rhee *et al.*, 2000b). Mutation of $Tyr^{3.51}$ to alanine resulted in a mutant ghrelin-R which displayed 'WT-like' binding affinity, cell-surface expression and intracellular signalling capabilities and presented only a small decrease in basal activity. It is clear that a tyrosine at position 3.51 is not essential for correct receptor functioning in the ghrelin-R and therefore this residue was not mutated further in this investigation.

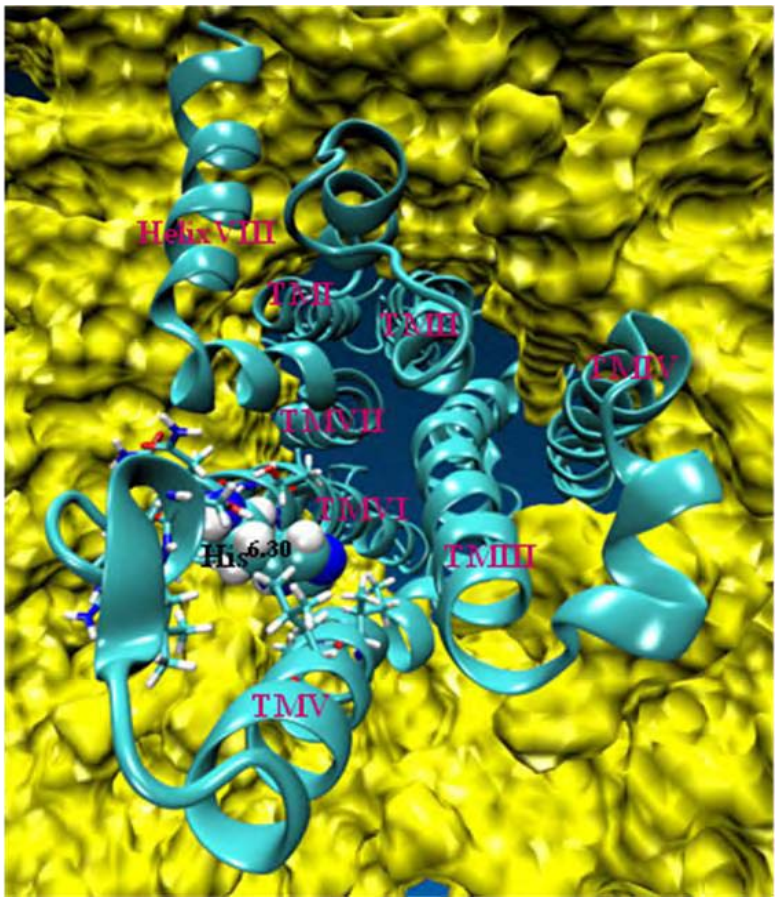
4.3.2 Investigation of 'ionic lock' interactions within the ghrelin-R

A number of residues within TMVI have been identified as being important for maintaining the inactive state of Family A receptors. A conserved glutamate residue at position 6.30 has been shown to interact with the D/ERY motif, through a hydrogen bonding network, creating the ionic lock (Ballesteros *et al.*, 2001). The crystal structure of bRho demonstrated that, in the inactive conformation, TMIII and TMVI are constrained at their cytoplasmic ends by a salt bridge interaction (Palczewski *et al.*, 2000). $Glu^{6.30}$ at the cytoplasmic face of TMVI can form an ionic bond with the highly conserved $Arg^{3.50}$ in the D/ERY motif of TMIII. $Arg^{3.50}$ is, in turn, interacting with the neighbouring $Glu^{3.49}$ creating a stabilising network of ionic bonds which maintain the inactive state. A negative charge is found at position 6.30 in 32 % of Family A GPCRs (Mirzadegan *et al.*, 2003) including all of the receptors that currently have determined crystal structures. Despite this, only the dark-state Rho crystal structures demonstrate the presence of the ionic lock interaction. The crystal structure of the β_2AR, where ICL3 was replaced by the T4L and the receptor is bound to a partial inverse agonist, carazolol, demonstrated that $Arg^{3.50}$ and $Glu^{6.30}$ are 10 Å apart and are therefore not capable of interacting to produce the proposed ionic lock (Cherezov *et al.*, 2007). However, the presence of the T4L has been suggested to influence the positioning of the helices in the crystal structure due to the interaction of $Glu^{6.30}$ and Arg8 of the T4L and similar disruptions to the cytoplasmic conformation of the receptor have been observed in the β_2AR-Fab crystal structure (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Rosenbaum *et al.*, 2007). Carazolol is only a partial inverse agonist of the β_2AR, and so there may be some constitutive activity retained in the β_2AR-T4L and this may also account for the absence of the ionic lock interaction (Rasmussen *et al.*, 2007). Furthermore, it is thought that the T4L may, in itself, be inducing a partial constitutively active phenotype due to its presence in ICL3 and subsequent disruption of the close contacts between TMIII and TMVI (Cherezov *et al.*, 2007; Rosenbaum *et al.*, 2007).

In peptide GPCRs, a negative charge is found at position 6.30 in only 14 % of receptors indicating that the classical 'ionic lock' does not occur in most members of this family. In a constitutively active GPCR such as the ghrelin-R, it could be predicted that a number of the constraining intramolecular interactions seen in Family A GPCRs do not occur and that this results in the more active phenotype. In the ghrelin-R, a histidine is found at position 6.30, indicative of the absence of an amine-GPCR-like "ionic lock" interaction (Fig 4.28). Despite this, molecular modelling of the ghrelin-R has indicated that activation of the receptor involves the classical movement of TMVI and TMVII toward each other and the movement of the cytoplasmic end of TMVI away from TMIII (Floquet *et al.*, 2010). In order to investigate the activation mechanisms of the ghrelin-R in detail, $His^{6.30}$ was mutated to various other amino acids. Each mutation was designed to probe potential interactions occurring at the cytoplasmic face of the receptor and to determine interactions that are integral to the ghrelin-R constitutive activity.

The introduction of alanine in the H6.30A mutant resulted in a receptor that displayed 'WT-like' characteristics suggesting that removal of the histidine side chain at the β-carbon did not perturb the receptor. In the WT ghrelin-R, $His^{6.30}$ does not appear to be structurally or functionally significant as substitution of $His^{6.30}$ for alanine results in very little overall effect. Introduction of a negative charge in both the H6.30E and H6.30D mutants resulted in decreased constitutive activity of the ghrelin-R, suggesting that a more inactive receptor conformation was adopted (Fig 4.29). Both mutants were able to signal when stimulated with ghrelin, indicating that a fully active conformation could still be achieved. The introduction of glutamate at this position appeared to result in a more stable receptor conformation. Cell-surface expression of this mutant construct was increased to 161 % of WT, suggesting a stabilised receptor at the cell surface, potentially with less constitutive internalisation than seen for the WT ghrelin-R (Chapter 3). Mutation of $Arg^{6.30}$ to glutamate or aspartate in the CCR5 resulted in ablation of constitutive activity, suggesting that introduction of a potential ionic interaction with $Arg^{3.50}$ stabilises the inactive receptor (Springael *et al.*, 2007). In the constitutively active H_4R, alanine is found at position 6.30, unusually for a biogenic amine receptor. $Ala^{6.30}$ was mutated to glutamate to investigate the introduction of an "ionic lock-like" residue on the constitutive receptor activity. No significant reduction in receptor constitutive activity was observed in the H_4R A6.30E mutant suggesting that the introduction of an "ionic lock-type" interaction is not sufficient to significantly reduce constitutive activity (Schneider *et al.*, 2010). Interestingly, histidine and alanine are found in only 4 % of Family A GPCRs (Mirzadegan *et al.*, 2003) possibly being a factor in receptor constitutive activity.

(a)



(b)

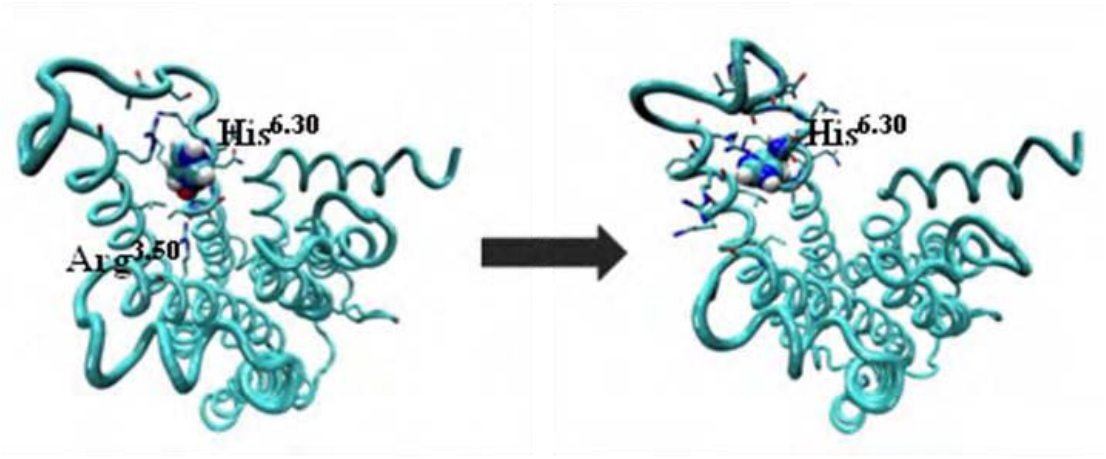


Figure 4.28 ghrelin-R model: (a) Model of the ghrelin-R viewed from the cytoplasmic surface showing the position of $His^{6.30}$ on TMVI and neighbouring residues that might engage in interactions in the receptor ground state. (b) Forced activation of the ghrelin-R model based on the adenosine $A_{2A}R$ crystal structure (Jaakola *et al.*, 2008) and the GαCT-bound opsin (Scheerer *et al*., 2008). Model produced by N. Floquet, Montpellier, France.

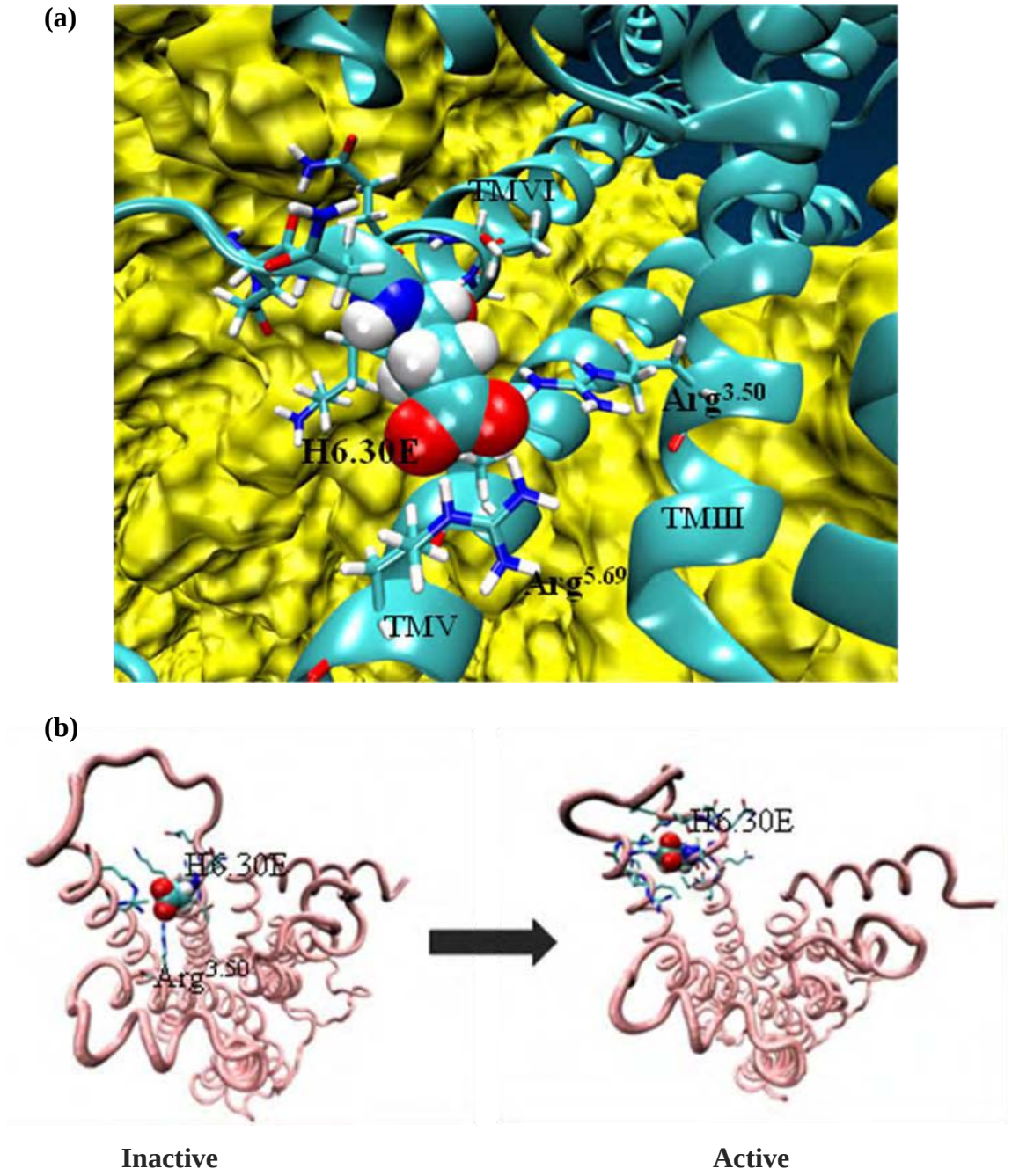


Figure 4.29 ghrelin-R model: (a) Mutation of $His^{6.30}$ to glutamate in the H6.30E mutant resulted in the formation of ionic lock-type interaction with $Arg^{3.50}$ in TMIII (b) Molecular dynamic simulations were conducted based on the crystal structures of the $A_{2A}R$ (Jaakola *et al.*, 2008) and the GαCT-bound opsin (Scheerer *et al.*, 2008). The data show the disruption of the ionic lock as the model is forced into the active conformation. Model produced by N. Floquet, Montpellier, France.

In other Family A GPCRs, mutation of residue 6.30 has varied effects on constitutive activity. The basal activity of the constitutively active mutant, T6.34K, in the μ-OR, could be reduced when a $Leu^{6.30}$ to glutamate mutation was introduced (Huang *et al.*, 2002) and the naturally occurring $Asp^{6.30}$ has been shown to form stabilising interactions in the glycoprotein hormone receptors. Mutation of $Asp^{6.30}$ increased constitutive activity in the thyrotropin-stimulating hormone receptor (TSHR) (Parma *et al.*, 1993), FSHR (Montanelli *et al.*, 2004a) and the LHR (Laue *et al.*, 1995; Kosugi *et al.*, 1996). In contrast, in the constitutively active CB_1 and CB_2 receptors, which also have an aspartate located at position 6.30, the charge-neutralising mutation of $Asp^{6.30}$ to asparagine did not increase the constitutive activity, indicating that $Asp^{6.30}$ does not form an ionic bond interaction with $Arg^{3.50}$ in these receptors, or if it does, that the ionic bond is not essential to the maintenance of ground-state interactions (Nebane *et al.*, 2006).

Charge-neutralising mutations of $Asp^{3.49}$ and $Glu^{6.30}$ in the β_2AR demonstrated that removal of both the $Asp^{3.49}$-$Arg^{3.50}$ interaction and the $Arg^{3.50}$-$Glu^{6.30}$ interaction resulted in a receptor that was more readily able to adopt an active conformation (Ballesteros *et al.*, 2001). Mutagenesis and modelling carried out on the α_{1b}AR suggested that similar interactions are occurring between $Glu^{6.30}$ and $Arg^{3.50}$ as those found in Rho and the β_2AR. The mutation of $Glu^{6.30}$ to glutamine did not change the receptor constitutive activity however it did result in a mutant α_{1b}AR that displayed a 50 % increase in maximal response after agonist stimulation. Computer simulations were inconclusive when attempting to provide explanation for this observation (Greasley *et al.*, 2002). A similar result was observed when $His^{6.30}$ was mutated to glutamine in the H6.30Q ghrelin-R mutant. No change was observed in the constitutive activity; however, the maximal ghrelin-induced signal was increased to 165 % of WT.

The introduction of a positive charge in the H6.30K and H6.30R mutations both resulted in receptors with decreased constitutive activity relative to the WT ghrelin-R. H6.30K had a particularly profound effect, reducing the constitutive activity to -87 % of the WT (41 % of WT basal). It might have been predicted that the introduction of a robust positive charge would repel $Arg^{3.50}$, resulting in a more active receptor conformation, as found with the $5HT_{2A}$R (Shapiro *et al.*, 2002), and the α_{1b}AR (Greasley *et al.*, 2002). However, 54 % of peptide receptors have a positive charge at this locus, and most present no appreciable constitutive activity, suggesting that other interactions must be occurring that stabilise the ground state. This is supported by the H6.30K and H6.30R ghrelin-R mutants, where agonist-independent activity was reduced. Modelling can provide mechanistic insight into the loss of

constitutive activity observed with the H6.30K and H6.30R mutants. H6.30K loss of constitutive activity is likely caused by repulsion between H6.30K and neighbouring positively charged residues found in TMV ($Lys^{5.69}$) and TMVI ($Lys^{6.31}$) (Fig 4.30). A large cluster of positive residues are located at the cytoplasmic face of TMV. These positive residues inhibit the TM movement associated with activation in the H6.30K and H6.30R mutants, thereby decreasing constitutive activity. Interactions also occurred between $Arg^{3.50}$ and $Thr^{6.33}$ in the H6.30K mutant ghrelin-R, stabilising the inactive receptor conformation by maintaining the cytoplasmic ends of TMIII and TMVI in close proximity.

It is apparent from the molecular modelling and mutagenesis of the ghrelin-R that when $His^{6.30}$ is substituted by either a negative or a positive residue, different interactions are formed which act to stabilise the inactive R state of the receptor, but that the mechanisms involved are distinct in the different mutant receptor constructs. H6.30D and H6.30E may act to produce an "ionic lock-type" interaction with $Arg^{3.50}$ (Fig 4.29 (a) & (b)), whereas H6.30K and H6.30R appear to repel TMV and allow $Thr^{6.33}$ to form a stabilising hydrogen bond interaction with $Arg^{3.50}$ (Fig 4.30).

4.3.3 Investigation of further stabilising interactions

4.3.3.1 Interactions with the ERY motif

In the β_1AR (Warne *et al.*, 2008), β_2AR (Rasmussen *et al.*, 2007) and A_{2A}R (Jaakola *et al.*, 2008) crystal structures, the D/ERY motif is involved in constraining interactions with ICL2. In the A_{2A}R and the β_1AR, $Asp^{3.49}$ forms hydrogen bond interactions with $Tyr^{3.60}$ found in ICL2 and in the A_{2A}R stabilising interactions also include $Thr^{2.39}$ located at the cytoplasmic face of TMII (Fig 4.31 (insert)). These interactions are thought to be possible due to a short helical section within ICL2 that is present in the β_1AR (Warne *et al.*, 2008), and the A_{2A}R (Jaakola *et al.*, 2008), but not in the other crystal structures, and positions $Tyr^{3.60}$ close to $Asp^{3.49}$. In the β_1AR, mutation of $Tyr^{3.60}$ results in a thermally unstable receptor supporting the proposed stabilising interaction that occurs between $Asp^{3.49}$ and $Tyr^{3.60}$ (Warne *et al.*, 2008). Mutation of $Tyr^{3.60}$ in the m5 mAChR resulted in increased constitutive activity and this receptor also displays an α-helical conformation of ICL2 (Burstein *et al.*, 1998). The interaction between $Tyr^{3.60}$ and $Asp^{3.49}$ is lacking in the constitutively active β_2AR. In this receptor other interactions have been proposed to form between the D/ERY motif and ICL2 at residue 3.62 (Jaakola *et al.*, 2008).

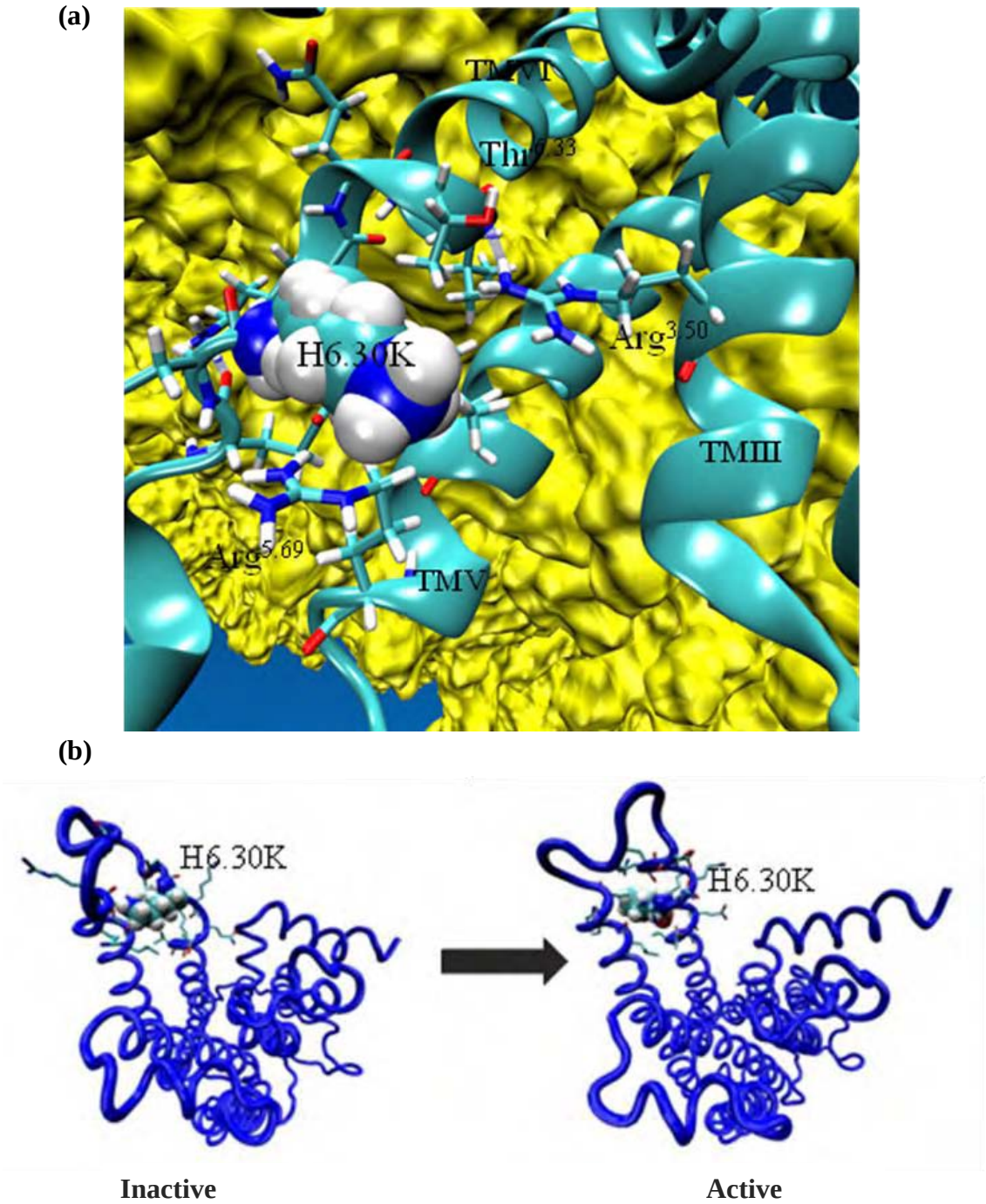


Figure 4.30 ghrelin-R model: (a) Mutation of $His^{6.30}$ to lysine in the H6.30K mutant resulted in repulsion of positively charged residues within TMV and allowed $Thr^{6.33}$ to interact with $Arg^{3.50}$ accounting for the reduction in constitutive activity observed, (b) Molecular dynamic simulations were conducted based on the crystal structures of the $A_{2A}R$ (Jaakola *et al.*, 2008) and the GαCT-bound opsin (Scheerer *et al*., 2008). Lysine was energetically unfavourable at position 6.30 due to charge-charge repulsions with TMV residues. Model produced by N. Floquet, Montpellier, France.

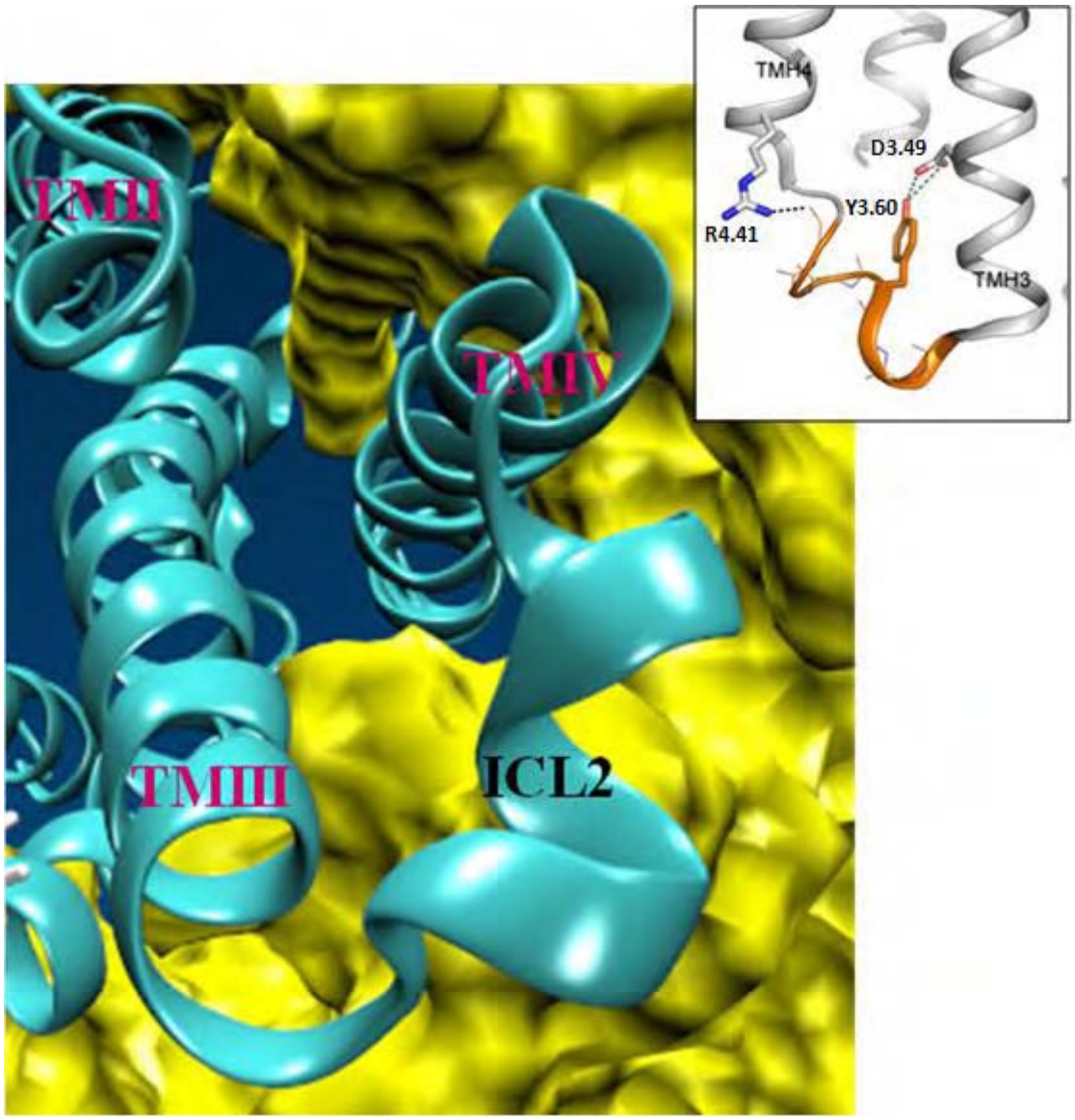


Figure 4.31 ghrelin-R model: the ghrelin-R viewed from the cytoplasmic face showing the short helical region of ICL2. Model produced by N. Floquet (Montpellier, France). Insert: Crystal structure of ICL2 of the $A_{2A}R$ showing the hydrogen bond between $Asp^{3.49}$ and $Tyr^{3.60}$ (image adapted from Worth *et al*., 2009).

The ghrelin-R model produced by N. Floquet (Montpellier, France), demonstrated the presence of a short helical region in ICL2, as found in the β_1AR, m5 mAChR and A_{2A}R (Fig 4.31). To investigate the potential of interactions between the ERY motif, $Thr^{2.39}$ and $Ala^{3.60}$ were mutated in the ghrelin-R. T2.39A, A3.60Y and a double mutant, T2.39A/A3.60Y, were introduced into the ghrelin-R.

Removing the threonine side chain at position 2.39 in the T2.39A mutant had very little effect on ghrelin-R function. The introduction of alanine did not affect the constitutive activity of the ghrelin-R indicating that threonine is not essential at this locus for maintaining the WT basal signalling. In contrast, A3.60Y caused a substantial reduction in the constitutive activity of the receptor, suggesting that new ground-state stabilising interactions may have been formed, perhaps with $Glu^{3.49}$, similar to those interactions observed in the A_{2A}R crystal structure (Jaakola *et al.*, 2008) and β_1AR (Warne *et al.*, 2008). The A3.60Y mutant was still able to respond to ghrelin stimulation, but with reduced efficacy, in parallel with the decreased constitutive activity, which implies that the receptor is unable to adopt the fully active conformation in the presence of ghrelin.

To further probe the potential of interactions within this region of the ghrelin-R, a double mutant of T2.39A/A3.60Y was produced. By removing the threonine at position 2.39, but maintaining a tyrosine at 3.60, as in the A3.60Y mutant, the effect of the introduction of $Tyr^{3.60}$, in the absence of the potentially stabilising interaction with $Thr^{2.39}$, can be investigated. T2.39A/A3.60Y maintained the ability to bind ghrelin with 'WT-like' affinity and to express at the cell-surface. The constitutive activity was diminished to a similar level to that seen for the A3.60Y single mutant; however, ghrelin-induced InsP-$InsP_3$ accumulation was no longer detectable. This suggests that there may be an interaction occurring between $Thr^{2.39}$, $Glu^{3.49}$ and $Tyr^{3.60}$ in the A3.60Y ghrelin-R mutant. Although both the single T2.39A and A3.60Y mutants reduced ghrelin efficacy, a response was still able to be elicited. Removal of the threonine after introduction of the tyrosine resulted in a receptor that was unable to adopt an active conformation after ghrelin stimulation.

4.3.3.2 The role of residues at the cytoplasmic ends of TMVI and TMVII

Crystal structures and molecular modelling have demonstrated that the cytoplasmic ends of TMVI and TMVII participate in significant conformational changes that are critical to receptor activation (Rosenbaum *et al.*, 2009). In Rho, antibodies specific to the cytoplasmic end of TMVII were only able to bind the Meta II state, suggesting that a section of TMVII is

exposed in the Meta II state and is inaccessible in the dark state (Abdulaev *et al.*, 1998) and this supports data for conformational rearrangements of TMVII as well as TMVI.

In the dark state of Rho, $Glu^{6.32}$ at the cytoplasmic end of TMVI is proposed to interact with the backbone of a lysine residue, located at the TMVII-HelixVIII interface, $Lys^{7.58}$. It was suggested, through mutagenesis studies, that this interaction is broken upon receptor activation and subsequent transition to Meta II (Vogel *et al.*, 2008). Mutation of $Glu^{6.32}$ to glutamine in Rho resulted in a small shift in the equilibrium between Meta I and Meta II towards Meta II, suggesting a weakening of the stabilising interhelical interactions, facilitating a transition towards a more active state (Vogel *et al.*, 2008). In the ghrelin-R, a glutamine is present at position 6.32 and a lysine at position 7.58. $Gln^{6.32}$ was mutated to glutamate to investigate the effect of the introduction of a potentially stabilising interaction between TMVI and TMVII. This residue was also substituted by alanine to investigate interactions occurring with and around position 6.32 in more detail.

The Q6.32A mutation has no effect on radioligand binding, intracellular signalling or cell-surface expression in the ghrelin-R, suggesting that glutamine is not important for normal receptor function. Mutation to glutamate did not affect ligand binding, constitutive activity or cell-surface expression. This suggests that stabilising interactions with TMVII are not formed. However, the Q6.32E mutation did have a profound effect on the ghrelin-induced InsP-$InsP_3$ accumulation profile. The efficacy of ghrelin was greatly increased, with the mutant receptor construct displaying an E_{max} of 199 % of WT. Consequently, in contrast to Rho, new interactions must be occurring when a glutamate is introduced at position 6.32, that stabilise the active receptor conformation after agonist stimulation, or that support a different form of active state. The introduction of glutamate may influence G-protein coupling itself, increasing the affinity of the receptor for G-protein, in the ghrelin-induced active conformation.

$Lys^{7.58}$ and $Lys^{7.59}$, located at the TMVII-HelixVIII interface, were mutated to alanine to investigate the potential of an interaction with residues in TMVI similar to those found in Rho. K7.58A was 'WT-like' with respect to binding affinity, cell-surface expression and intracellular signalling characteristics. Only a slight decrease in basal constitutive activity was observed. The K7.59A mutant had a more detrimental effect on receptor function. Ghrelin binding affinity was 'WT-like', whilst the signalling was affected by the alanine substitution. Constitutive activity was diminished to -53 % of that seen for the WT ghrelin-R and the

K7.59A mutant was unable to signal to the same extent as the WT ghrelin-R (E_{max} = 46 %). A small decrease in EC_{50} (3.5-fold) was observed, indicative of increased potency of ghrelin. The data for the $Gln^{6.32}$, $Lys^{7.58}$ and $Lys^{7.59}$ mutations suggest that an interaction does not occur between these residues in the WT ghrelin-R, nor in the Q6.32E mutant receptor construct. The Q6.32E mutation must form new stabilising interactions that allow a more active conformation to be adopted after ghrelin stimulation and must support interactions that maintain ghrelin-R constitutive activity, although, currently, it is unclear as to what these interactions may be.

Another residue at the cytoplasmic end of TMVI, $Thr^{6.33}$, might be involved in interactions that stabilise either the ground state, or the active receptor conformation. The positioning of $Thr^{6.33}$ at the cytoplasmic end of TMVI in the ghrelin-R means that this residue may interact with TMIII in the inactive receptor conformation. $Thr^{6.33}$ was mutated to alanine in the ghrelin-R to investigate its role in the WT receptor. Mutation of $Thr^{6.33}$ to alanine resulted in increased constitutive activity of the ghrelin-R suggesting that threonine in the WT receptor engages in stabilising interactions that maintain the ghrelin-R basal activity at appropriate levels. The T6.33A mutant appears to release a constraint allowing the receptor to adopt a more active conformation in the absence of the agonist, ghrelin. After stimulation with ghrelin, the T6.33A mutant receptor construct was unable to reach the WT E_{max} suggesting that $Thr^{6.33}$ contributes to interactions that stabilise the R and the R* state. Molecular modelling of the H6.30K mutant ghrelin-R implicated $Thr^{6.33}$ in the stabilising of the inactive state through the formation of a hydrogen bond with $Arg^{3.50}$. It is possible that $Thr^{6.33}$ plays a similar role in stabilising the WT ghrelin-R, as the T6.33A mutant increased constitutive activity.

Valine was introduced as it has a similar side chain size as threonine, but lacks the polar character, to further probe the role of $Thr^{6.33}$. The T6.33V mutant did not affect ghrelin-R constitutive activity suggesting that the side chain size, and not the polarity of the residue, is responsible for maintaining the basal activity of the WT receptor. Neither mutation at position 6.33 affected agonist affinity however both reduced the receptor cell-surface expression. Mutation of $Thr^{6.33}$ in the $V_{1a}R$ to valine resulted in increased basal activity suggesting that in the $V_{1a}R$, the ability of the residue at position 6.33 to hydrogen bond is essential for suppressing basal signalling. This was further supported by the conservative T6.33S mutation that retained 'WT-like' characteristics (Wootten & Wheatley, manuscript in preparation).

The intracellular signalling capabilities of the T6.33A mutant were investigated further using the ghrelin-R inverse agonist SP-analogue. The T6.33A mutant affected the efficacy of ghrelin; however, the potency and efficacy of SP-analogue were not affected by the mutation. The basal signalling of the T6.33A mutant was decreased using the SP-analogue with 'WT-like' I_{max} and pIC_{50}. This suggests that T6.33A only affects the agonist response and the ability of the receptor to adopt the fully active conformation, and not the ability of the receptor to form a more stable inactive conformation after stimulation with an inverse agonist.

Residue 6.34 has been studied extensively in Family A GPCRs due to its relationship with receptor activation and the maintenance of ground-state interactions. In the crystal structure of bRho, an interaction has been demonstrated between $Arg^{3.50}$ and $Thr^{6.34}$ in the ground state (Palczewski *et al.*, 2000).

Both V6.34A and V6.34T in the ghrelin-R were 'WT-like' with respect to all receptor characteristics. The V6.34K mutant displayed a reduction in agonist affinity, demonstrated by the lack of radioligand binding at the concentrations used. V6.34K also had decreased constitutive activity and decreased efficacy, but was 'WT-like' in relation to EC_{50}. In the biogenic amine receptors, mutation of residue 6.34 often results in increased constitutive activity (Kjelsberg *et al.*, 1992; Ren *et al.*, 1993; Samama *et al.*, 1993; Lattion *et al.*, 1999; Parnot *et al.*, 2002). In the α_{1b}AR mutation of $Ala^{6.34}$ to all other amino acids resulted in constitutive activity, highlighting the requirement of the WT alanine in maintaining the inactive receptor conformation. There appeared to be no correlation between the properties of the amino acid side chain and the increase in constitutive activity, nor between the constitutive activity and the effect on agonist affinity (Kjelsberg *et al.*, 1992; Parnot *et al.*, 2002). Mutation of $Leu^{6.34}$ to alanine in the β_2AR resulted in increased constitutive activity suggesting that the intracellular end of TMVI is crucial for maintaining the inactive β_2AR conformation (Samama *et al.*, 1993). In the β_2AR $Leu^{6.34}$ has been shown to interact via van der Waals forces with $Tyr^{5.58}$. $Leu^{6.34}$ was identified as producing constitutive activity when mutated to alanine and that the stabilising van der Waals interactions minimise basal activity (Rasmussen *et al.*, 2007). Similarly, mutation of $Thr^{6.34}$ to phenylalanine, alanine, glutamate, cysteine or lysine resulted in increased constitutive activity in the α_2AR (Ren *et al.*, 1993). In the β_1AR mutation of $Leu^{6.34}$ also increased the constitutive activity, with the L6.34K mutant displaying the greatest increase in basal cAMP accumulation (Lattion *et al.*, 1999). The T6.34K mutant in the μ-OR resulted in constitutive receptor activation presumably, by disrupting a stabilising interaction between 6.34 and $Arg^{3.50}$, after the introduction of the

repulsive positive charge. In contrast, the T6.34D mutant stabilised the inactive state of the receptor by the introduction of a negative charge (Huang *et al.*, 2001). In the $G\alpha_i$-coupled CB_2 receptor, mutation of $Ala^{6.34}$ to glutamate reduced the WT constitutive inhibition of AC suggesting that this mutation alters the receptor conformation to a more inactive state, possibly due to the introduction of a negative charge. This may have resulted in stabilising interactions with $Arg^{3.50}$ of the D/ERY motif and would therefore explain the lack of agonist-independent activation observed with this mutation (Feng *et al.*, 2003). In the TSHR molecular modelling predicted that a hydrogen bond is formed between $Tyr^{5.58}$ and $Ala^{6.34}$ that is essential for WT receptor signalling capabilities (Biebermann *et al.*, 1998).

Interactions may be occurring between TMVI, TMIII and TMV in the ghrelin-R, supporting the high basal signalling in the WT receptor that can be decreased after introduction of charged residues, although the introduction of a positive lysine did not increase constitutive activity as observed in other Family A GPCRs. This suggests that the V6.34K mutant does not disrupt an interaction with $Arg^{3.50}$, and does not repel the cytoplasmic end of TMIII. It is unclear as to the role of $Val^{6.34}$ in the WT ghrelin-R and further investigation is required at this locus to interpret the current findings with more accuracy.

4.3.4 The role of residue $Tyr^{5.58}$ in ghrelin-R activity

$Tyr^{5.58}$ is conserved in 77 % of Family A GPCRs (Mirzadegan *et al.*, 2003) and has been implicated in receptor activation in a number of receptors. To investigate the functional role of residue 5.58 in the ghrelin-R, $Tyr^{5.58}$ was substituted with alanine and with glutamine. Alanine substitution removed the side chain and therefore was likely to disrupt potential intramolecular interactions. Y5.58A was able to bind ghrelin with a similar affinity to the WT ghrelin-R but had a dramatic effect on intracellular signalling. There was a substantial decrease in constitutive activity, indicative of the stabilisation of a relatively inactive receptor conformation or decreased $G\alpha_{q/11}$ activation. Interestingly, Y5.58A had a very large increase in ghrelin efficacy, resulting in a receptor that had an E_{max} of 224 % of the WT ghrelin-R. This suggests that the Y5.58A mutant produces a receptor that can adopt a more active state, when stimulated by ghrelin, than the WT ghrelin-R. Removal of the tyrosine side chain at position 5.58 therefore has an interesting effect on signalling and coupling to G-proteins. Agonist-independent signalling was severely perturbed, but the mutant receptor was still particularly responsive to ghrelin, and was capable of signalling to a much greater extent than the WT receptor.

Due to the high conservation of tyrosine at position 5.58 in Family A GPCRs, $Tyr^{5.58}$ has been extensively studied. (Hunyady *et al.*, 1995a; Biebermann *et al.*, 1998; Erlenbach *et al.*, 1998; Palczewski *et al.*, 2000; Li *et al.*, 2005). One role that has been proposed for $Tyr^{5.58}$ is in the selectivity of receptor-G-protein interactions. The location of $Tyr^{5.58}$ at the cytoplasmic interface of TMV and ICL3 suggests that this residue may be involved in G-protein selectivity and this has been confirmed in the V_2R, where the side chain of $Gln^{5.58}$ is predicted to play a role in G-protein recognition and specificity. Glutamine is required at position 5.58, in the V_2R, for efficient activation of the $G\alpha_s$ G-proteins (Erlenbach *et al.*, 1998). Other members of the neurohypophysial hormone receptor family have a tyrosine at position 5.58 and all couple to $G\alpha_{q/11}$, further supporting the role of $Tyr/Gln^{5.58}$ in G-protein coupling, selectivity and activation. In the TSHR $Tyr^{5.58}$ was mutated to various amino acids to investigate the effects of side chain substitution. The TSHR has dual signalling capabilities, signalling through $G\alpha_{q/11}$ and $G\alpha_s$ G-proteins. Some of the mutations resulted in a loss of activation through one pathway, again suggesting that $Tyr^{5.58}$ is important for G-protein specificity, as found with the V_2R. Constitutive activity, cell-surface expression and agonist-induced signalling were all affected in the TSHR mutants, highlighting an essential role of tyrosine at this position (Biebermann *et al.*, 1998). Mutational studies on the $AT_{1a}R$ found that a $Tyr^{5.58}$ mutation to phenylalanine resulted in a loss of agonist-induced InsP-$InsP_3$ accumulation, again suggesting that tyrosine is required for efficient G-protein coupling (Hunyady *et al.*, 1995a).

The Y5.58Q mutant was produced in the ghrelin-R to investigate whether this mutation affected coupling or altered G-protein specificity. The mutant receptor was still able to signal through the $InsP_3$ pathway, demonstrating $G\alpha_{q/11}$ selectivity, with 'WT-like' ghrelin potency, but had a decreased efficacy and decreased basal activity. The Y5.58Q mutant was unable to bind ghrelin at the radioligand concentrations used. Combined, this data suggests that glutamine substitution of $Tyr^{5.58}$ was more detrimental to ghrelin-R function than the alanine substitution.

The crystal structure of Rho demonstrated that the phenolic ring of $Tyr^{5.58}$ extends into the interhelical region between TMV and TMVI (Palczewski *et al.*, 2000). Baldwin *et al.* (1997) conducted molecular modelling of typical Family A GPCRs and predicted that the side chains of residues 5.58 and 3.50 project into the cavity formed between TMIII and TMVI. The crystal structures of opsin revealed that the hydroxyl group of $Tyr^{5.58}$ interacts with $Arg^{3.50}$ of the ERY motif, replacing the carboxyl group of $Glu^{3.49}$ in the active state. Release of $Arg^{3.50}$ from $Glu^{3.49}$ and $Glu^{6.30}$ also allows $Arg^{3.50}$ to interact with $G\alpha_t$ (Park *et al.*, 2008; Scheerer *et*

al., 2008). This interaction forms the basis of the hydrogen bonding network between Rho and $G\alpha_t$ and potentially forms the basis for activation of all Family A GPCRs. The results of the $Tyr^{5.58}$ mutations in the ghrelin-R, clearly implicate the tyrosine residue in receptor activation and imply that $Tyr^{5.58}$ may have a number of functions including maintaining ghrelin-R constitutive activity and stabilising an active receptor conformation, perhaps preventing hyperstimulation, as indicated by the increased efficacy with the Y5.58A mutation.

4.3.5 Investigation of the rotamer toggle switch in the ghrelin-R

The highly conserved tryptophan at position 6.48 is an important residue in GPCR activation, making up part of the CWxP motif in TMVI. Changes in the rotameric conformation of $Trp^{6.48}$ and other residues within an aromatic cluster in TMVI are thought to accompany Family A receptor activation and constitute the rotamer toggle switch (Schwartz *et al.*, 1996; Shi *et al.*, 2002b; Schwartz *et al.*, 2006). The aromatic cluster is believed to aid receptor activation by stabilising the various conformations of the side chains of each residue within the cluster. The toggling of $Trp^{6.48}$ modulates the proline kink in TMVI at $Pro^{6.50}$, straightening the bend angle of the helix and resulting in the movement of the cytoplasmic end of TMVI away from TMIII, facilitating receptor activation (Shi *et al.*, 2002b; Bhattacharya *et al.*, 2008). The $Trp^{6.48}$ side chain is proposed to adopt different conformations, being an important microswitch in GPCR activation (Nygaard *et al.*, 2009).

Studies on Rho measuring the UV-absorbance of specific tryptophan residues identified the change in position of TMVI relative to TMIII based on the absorbance of $Trp265^{(6.48)}$ (Lin *et al.*, 1996). This movement was further supported by computer simulations of the β_2AR (Shi *et al.*, 2002b), and the crystal structure of the Meta I state of Rho (Ruprecht *et al.*, 2004). In all of the crystal structures to date $Trp^{6.48}$ is found in a similar position and rotameric conformation, although none of the structures are of agonist-bound receptors, and this may account for the stability of the $Trp^{6.48}$ side chain in the crystal structures (Nygaard *et al.*, 2009). In Rho, $Trp^{6.48}$ forms a direct contact with retinal in the binding pocket (Palczewski *et al.*, 2000), whereas in the β_2AR crystal structure, there is no direct ligand-$Trp^{6.48}$ contact observed (Cherezov *et al.*, 2007). In fact, there is great variation in the contact area between ligand and $Trp^{6.48}$ in all of the resolved GPCR crystal structures, perhaps reflecting the observed level of constitutive activity for each receptor (Jaakola *et al.*, 2008). In the inactive Rho, $Trp^{6.48}$ points towards TMVII and is constrained by a network of hydrogen bonds. $Trp^{6.48}$ forms a hydrogen bond with $Asn^{7.49}$ which is disrupted upon isomerisation of retinal and subsequent receptor activation. Upon activation and isomerisation of 11-*cis* retinal to all-

trans retinal, $Trp^{6.48}$ is released from this constraint and moves to point towards TMV (Crocker *et al.*, 2006). $Trp^{6.48}$ flips around to face TMV and forms a new hydrogen bond with $His^{5.46}$ (Bhattacharya *et al.*, 2008). Molecular modelling of the inactive ghrelin-R has demonstrated that $Trp^{6.48}$ establishes hydrophobic contacts with surrounding phenylalanine residues (Floquet *et al.*, 2010), which have previously been implicated in ghrelin-R activation (Holst *et al.*, 2004a). In the active state $Trp^{6.48}$ assumes a different rotamer conformation, making contact with $His^{6.52}$ (Floquet *et al.*, 2010).

In this study, $Trp^{6.48}$ of the ghrelin-R was substituted by alanine, phenylalanine and tyrosine to look at the effects of a dramatic reduction in side chain size and of conservative substitution with other aromatic residues. All three $Trp^{6.48}$ mutations were detrimental to ghrelin-R function with the W6.48A being the most pronounced. There was an increase in EC_{50} of 12.5-fold indicating that the mutant receptor is still able to signal in response to ghrelin, despite ablation of agonist-independent signalling (basal signalling 8 % of WT basal). The efficacy was affected by the alanine substitution with the mutant receptor construct only able to signal at -22 % of the ghrelin-R ghrelin-induced maximum. The W6.48A mutant retained a ghrelin binding affinity that was comparable to WT and increased cell-surface expression to 170 % of the WT ghrelin-R implying that the mutant adopted a more stable inactive conformation at the cell-surface, perhaps reducing the constitutive internalisation observed for the ghrelin-R (Chapter 3). Any adverse effect on ghrelin-induced signalling was not therefore a result of decreased receptor expression or disruption to the ghrelin binding pocket. Holst *et al*. (2010) recently also mutated $Trp^{6.48}$ in the ghrelin-R. Similar results were seen; the W6.48A mutant was severely detrimental to constitutive and ghrelin-induced signalling, without any adverse effect on ghrelin binding affinity or cell-surface expression. In fact, as observed in our study, the cell-surface expression was increased beyond the levels observed for the WT ghrelin-R (Holst *et al.*, 2010). Alanine substitution of $Trp^{6.48}$ results in disruption of the rotamer toggle switch that has been shown to be integral to receptor activation in a number of Family A GPCRs. Although the mutant receptor construct is still able to adopt an active receptor conformation after ghrelin stimulation, the ghrelin efficacy and potency are affected and the effect is not related to a disruption to ghrelin binding or to cell-surface expression. The location of $Trp^{6.48}$ within TMVI suggests that disruption of a G-protein interaction, directly, is not responsible for the detrimental effect on receptor activation. Instead, an inability of the receptor to adopt a fully active conformation is likely to be involved. To further investigate the role of $Trp^{6.48}$, this residue was replaced by phenylalanine and tyrosine, retaining the aromatic character. Both the W6.48F and W6.48Y mutants retained 'WT-like' binding

affinity and cell-surface expression. Both affected basal and agonist-induced $InsP_3$ signalling to the same extent, with an increase in EC_{50} of 7.5-fold and 7-fold respectively and both displayed similar reductions in basal signalling (38 and 43 % of WT basal, respectively). Neither construct was able to signal to the same extent as the WT receptor after stimulation with ghrelin, only presenting E_{max} similar to the WT constitutive activity. Maintaining an aromatic at position 6.48 was unable to retain WT signalling properties, revealing that only tryptophan can reside at position 6.48 and supporting the important functional role of $Trp^{6.48}$ in GPCR activation.

$Gly^{3.36}$ forms packing interactions with $Trp^{6.48}$ in the Rho inactive state and this interaction is removed upon receptor activation due to the relative movement of TMVI away from TMIII (Crocker *et al.*, 2006). In the ghrelin-R, $Thr^{3.36}$ mutation to alanine resulted in a dramatic increase in constitutive activity revealing that removal of the threonine side chain releases constraining intramolecular interactions. Introduction of conservative cysteine or serine residues did not induce further ghrelin-R agonist-independent activity, but instead retained basal signalling at levels similar to that seen for the WT ghrelin-R. This result, along with the 'WT-like' binding affinity and EC_{50} suggested that cysteine or serine can successfully substitute for threonine at this position. Interestingly however, the ghrelin efficacy was affected in both cases, with the T3.36C mutant having an increased E_{max} (146 % of WT) and the T3.36S mutant displaying a decrease (36% of WT). This indicates that, despite having binding affinity, constitutive activity and ghrelin potency similar to the WT ghrelin-R, both the T3.36C and T3.36S mutants affected ghrelin-R function in a different manner. None of the $Thr^{3.36}$ mutations affected ghrelin binding or cell-surface expression suggesting that all of the mutant constructs are correctly folded and expressed in HEK 293T cells.

In the H_1R (Jongejan *et al.*, 2005), the $5HT_4R$ (Pellissier *et al.*, 2009) and the CB_1 receptor (McAllister *et al.*, 2004), as in Rho (Crocker *et al.*, 2006), residue 3.36 has been shown to participate in the rotamer toggle switch, although in these cases, residues 3.36 appears to have different roles, stabilising the inactive and active receptor conformations. In the H_1R, mutation of $Ser^{3.36}$ to alanine resulted in a reduction in the WT basal signalling and agonist affinity, suggesting that serine in the WT receptor acts to stabilise the active conformation. Mutation of $Ser^{3.36}$ to cysteine or threonine increased the constitutive activity and resulted in mutant receptor constructs that could not be activated further by histamine, but that could be inhibited by an inverse agonist. $Ser^{3.36}$ was shown to interact with $Asn^{7.45}$ as part of a toggle

switch of activation, representing an early stage in receptor activation before important and pronounced structural changes occur within the TM helices (Jongejan *et al.*, 2005). Similarly, in the constitutively active $5HT_4R$, the T3.36A mutation resulted in ablation of constitutive activity, suggesting an important role of the WT threonine in stabilising active receptor conformations (Pellissier *et al.*, 2009). In contrast, substitution of $Phe^{3.36}$ with alanine in the CB_1 receptor resulted in increased constitutive activity. This may be due to the removal of a steric block of the conformational change of $Trp^{6.48}$, allowing greater ligand-independent activation (McAllister *et al.*, 2004) and suggests that similar interactions may be occurring in the CB_1 receptor as the ghrelin-R.

The conserved TMVI aromatic cluster is not found in the CB_1 receptor, although a phenylalanine is found at position 3.36. $Phe^{3.36}$ may act to stabilise the ground state of the receptor via aromatic-aromatic interactions with $Trp^{6.48}$ and other aromatics found within TMs III-V (McAllister *et al.*, 2004). Phenylalanine was introduced at position 3.36 in the ghrelin-R to investigate introduction of an aromatic at this locus. T3.36F had no effect on ghrelin binding, but had an interesting effect on intracellular signalling. The T3.36F mutant resulted in a 3.2-fold increase in ghrelin potency and a small reduction in basal signalling without affecting the efficacy. There was also a small increase in cell-surface expression (143 %). This suggested that the T3.36F mutant is more readily able to be activated by ghrelin and adopt the active conformation, without a direct effect on ghrelin binding.

Serine is found at position 7.45 in Rho and has been suggested to form an interaction with $Trp^{6.48}$ via a water molecule as $Trp^{6.48}$ toggles during the activation of Rho (Bhattacharya *et al.*, 2008). The more highly conserved $Asn^{7.45}$ has been proposed to restrain the side chain of $Trp^{6.48}$ in the inactive conformation in the H_1R. Mutation of $Asn^{7.45}$ in the H_1R had various effects on constitutive activity depending on the amino acid introduced (Jongejan *et al.*, 2005). In the ghrelin-R, mutation of $Ser^{7.45}$ to alanine resulted in increased constitutive activity indicating that removal of the polar side chain released a stabilising interaction, possibly with $Trp^{6.48}$, and allowed the ghrelin-R to adopt a more active conformation in the absence of ghrelin. Introduction of the more polar asparagine at position 7.45 restrained an inactive receptor conformation, with the S7.45N ghrelin-R construct displaying decreased basal activity compared to WT, and suggesting that similar interactions can be introduced into the ghrelin-R as those observed in many WT Family A GPCRs. The WT ghrelin-R may display similar interactions as those observed in Rho, involving hydrogen bonding networks

with water molecules. The presence of serine and alanine instead of the more highly conserved asparagine and serine at positions 7.45 and 7.46 in the ghrelin-R and Rho further supports the idea that interactions may be conserved in the two receptors. These interactions may dictate the constitutive activity of the receptor, as supported by opsin constitutive activity when 11-*cis* retinal is absent (Cohen *et al.*, 1993).

$Trp^{6.48}$, $Thr^{3.36}$ and $Ser^{7.45}$ appear to be involved in a stabilising network of interactions which can be disrupted by alanine mutations. The T3.36A mutation removes a stabilising packing interaction allowing $Trp^{6.48}$ to adopt the active rotamer conformation in the absence of agonist. In turn, S7.45A may allow the release of $Trp^{6.48}$ from the inactive rotamer conformation, pointing toward TMVII, again allowing the receptor to adopt a more active conformation without ghrelin binding.

The extended network of hydrogen bonds stabilising GPCRs also includes residue 3.35. Asparagine is located at position 3.35 in 29 % of Family A GPCRs (Mirzadegan *et al.*, 2003) and has been found to make important hydrogen bond interactions. Cysteine is found at this locus in the ghrelin-R. $Cys^{3.35}$ was mutated to alanine and the resultant C3.35A mutant had no effect on ghrelin binding, basal activity or cell-surface expression. The introduction of an alanine at this position was however detrimental to ghrelin-induced signalling efficacy (E_{max} 48 % of WT).

Mutation of $Asn^{3.35}$ in the CXC chemokine receptor type 4 (CXCR4) to alanine or serine resulted in constitutive activity, whereas introduction of a positive charge (N3.35K) stabilised the inactive receptor conformation. Positive charges are never found at position 3.35 in Family A GPCRs (Mirzadegan *et al.*, 2003), supporting the inactivity found in the N3.35K CXCR4 mutant (Zhang *et al.*, 2002). Mutation of $Asn^{3.35}$ in the AT_1R (Groblewski *et al.*, 1997) and $Cys^{3.35}$ in the $\alpha_{1b}AR$ (Perez *et al.*, 1996) also resulted in constitutive receptor activity. In Rho, interactions were proposed between $Gly^{3.35}$ and $Met^{2.53}$ in the inactive state and in Meta II, suggesting that TMIII is stationary during receptor activation and that toggling and movement of TMVI, not TMIII, is responsible for receptor activation (Crocker *et al.*, 2006). Any detrimental effect on receptor activation caused by mutation of $Cys^{3.35}$ in the ghrelin-R is therefore unlikely to be related to a disruption of the formation of the active receptor conformation itself, and is more likely to be due to a disruption of the formation of the fully active conformation via alteration of TMIII-TMVI-TMVII interactions.

4.3.6 The role of the highly conserved NPxxY motif in ghrelin-R function

The NPxxY motif is a highly conserved sequence in Family A GPCRs that is found at the cytoplasmic end of TMVII. The NPxxY motif has been extensively studied in Family A GPCRs, however, studies have indicated that the residues have various roles despite their conserved nature. In Rho, the D/ERY motif and the NPxxY motif have been proposed to provide a dual control in the structural changes leading to receptor activation (Fritze *et al.*, 2003). The NPxxY participates in a network of interactions with water molecules, stabilising the inactive receptor conformation, but allowing ease of breaking for rapid formation of the active state (Rosenbaum *et al.*, 2009).

Alanine substitution of the residues within the NPxxY of the ghrelin-R had no effect on ghrelin potency, but each mutant affected ghrelin binding affinity or constitutive activity. N7.49A retained 'WT-like' binding affinity and efficacy although constitutive activity was reduced, with basal signalling being -42 % of the WT ghrelin-induced maximum. In the prostaglandin EP3 receptor (Audoly *et al.*, 1997) and the cholecystokinin type B receptor (Gales *et al.*, 2000), mutation of residue 7.49 disrupted signalling but did not affect ligand binding. In the ghrelin-R, only constitutive receptor activation was affected by the $Asn^{7.49}$ mutation to alanine indicating that, despite its high conservation, $Asn^{7.49}$ is not essential for ghrelin-induced receptor activation. The alanine substitution may have affected interactions with other residues that normally act to stabilise the more active ghrelin-R conformation required for WT constitutive activity. In contrast, in the TSHR, $Asn^{7.49}$ was found to be required for agonist-induced receptor activation (Claeysen *et al.*, 2002).

$Asn^{7.49}$ has been proposed to form an interaction, via water molecules, with the highly conserved aspartic acid at position 2.50 ($Asp^{2.50}$). The crystal structure of Rho revealed that the distance between $Asp^{2.50}$ and $Asn^{7.49}$ is too great for a direct interaction, but that water can mediate the contact, and that this interaction stabilises the active receptor conformation (Palczewski *et al.*, 2000; Okada *et al.*, 2002). Molecular modelling of the TSHR based on the 2.2 Å Rho crystal structure (Okada *et al.*, 2004) implicated a water molecule as a hydrogen bond acceptor in the $Asp^{2.50}$-$Asn^{7.49}$ interaction and suggested that this water-mediated interaction restrains the side chain of $Asn^{7.49}$, maintaining an active receptor conformation (Urizar *et al.*, 2005). Mutation of $Asp^{2.50}$ to alanine in the ghrelin-R affected ghrelin-R basal signalling and efficacy suggesting that removal of the putative $Asp^{2.50}$-$Asn^{7.49}$ interaction by alanine substitution, acted to produce a more inactive receptor conformation (Chapter 3). The interaction between $Asp^{2.50}$ and $Asn^{7.49}$ has been shown in a number of Family A GPCRs

including the $5HT_{2A}R$ (Sealfon *et al.*, 1995), the m1 mAChR (Bee *et al.*, 2007), the CB_1 receptor (Shim, 2009), the GnRHR (Flanagan *et al.*, 1999), the TRHR (Perlman *et al.*, 1997b) and the tachykinin NK2 receptor (Donnelly *et al.*, 1999). Alanine substitution of the residues at either position 2.50 or 7.49 resulted in a similar reduction in ghrelin-R constitutive activity supporting the potential interaction between the residues. Neither mutation resulted in a change in binding affinity or ghrelin potency, indicating that these residues do not form part of the ghrelin binding pocket. Similarly, high affinity agonist and antagonist binding were retained after mutation of $Asn^{7.49}$ in the $5HT_{2A}R$ (Sealfon *et al.*, 1995).

The network of interactions that involve $Asn^{7.49}$ are both extensive and complex. CAMs of the H_1R, induced by mutation at position 6.40, highlighted a network of interactions known as the asparagine cage that appear to restrain $Asn^{7.49}$ in the inactive conformation and pointing towards TMVI. Mutation of $Ile^{6.40}$, which is a highly conserved hydrophobic residue, to a charged residue, removed hydrophobic constraints and allowed for constitutive receptor activation (Bakker *et al.*, 2008) (discussed further in Chapter 5).

Prolines within TM domains have been shown to induce kinks within the helical structure due to steric conflicts with the previous residue and the lack of ability of the proline backbone to hydrogen bond (von Heijne, 1991). Helical distortions in membrane proteins are well tolerated due to the stability provided by the membrane environment and can provide weak points that add flexibility to allow movement of the helix (Yohannan *et al.*, 2004). Studies on Rho and bacteriorhodopsin have shown that alanine substitution of prolines found within TM helices does not necessarily result in straightening of proline-induced kinks. Stabilising interactions are often present which support the structure of the helix even after removal of the proline residue (Faham *et al.*, 2004; Yohannan *et al.*, 2004). In the ghrelin-R, mutation of $Pro^{7.50}$ of the NPxxY to alanine, caused a decrease in ghrelin binding affinity so that binding was not detectable at the [^{125}I]ghrelin concentrations used in assay. Despite this, both agonist-independent signalling and ghrelin potency were unaffected by the P7.50A mutation. Interestingly, ghrelin efficacy was increased, with the P7.50A mutant having an E_{max} of 176 % of the WT receptor. This suggests that the P7.50A mutant receptor construct is able to adopt a more active conformation than the WT ghrelin-R without having an effect on constitutive activity. In contrast, in the m3 mAChR mutation of $Pro^{7.50}$ to alanine affected cell-surface expression and intracellular signalling but not agonist binding affinity suggesting that $Pro^{7.50}$ might be involved directly in G-protein coupling and agonist-induced

conformational changes (Wess *et al.*, 1993). Although the P7.50A mutation in the ghrelin-R was not detrimental to ghrelin-R signalling, there was a detrimental effect on ghrelin binding suggesting that $Pro^{7.50}$ may form part of the ghrelin binding pocket or might act to stabilise the binding pocket through its interaction with other residues. The P7.50A mutant affected ghrelin affinity, as indicated by the radioligand binding, although ghrelin binding cannot have been eliminated as ghrelin-induced signalling was not impaired.

$Ile^{7.51}$ and $Leu^{7.52}$ are found at the 'xx' positions of the NPxxY motif. These residues are the least conserved within the NPxxY motif although hydrophobic amino acids in general are highly conserved (Mirzadegan *et al.*, 2003). Compared to the asparagine, proline and tyrosine residues of the NPxxY motif, the 'xx' are also the least well studied. Interestingly, alanine mutation of both $Ile^{7.51}$ and $Leu^{7.52}$ resulted in an increase in ghrelin binding affinity. Mutations within the NPxxY motif in other GPCRs have been found to increase receptor function; mutation of $Tyr^{7.53}$ has been found to increase agonist binding affinity in the $5HT_{2C}R$ (Rosendorff *et al.*, 2000). The NPxxY may be involved in interactions with ligands as supported by the increased affinity found with the I7.51A and L7.52A ghrelin-R mutants and the disruption to binding caused by the P7.50A mutation. Mutation of $Phe^{7.52}$ in the $AT_{1a}R$ to alanine resulted in severe impairment of agonist binding without affecting G-protein coupling (Hunyady *et al.*, 1995b).

The constitutive activity of the two alanine mutants, I7.51A and L7.52A, was decreased compared to the WT ghrelin-R suggesting that the increase in affinity is not a result of a constitutively active conformation and that the residues may instead be involved directly in ghrelin binding or in moulding the binding pocket. The decreased constitutive activity observed for the I7.51A and L7.52A mutants was not unexpected, despite the increased agonist affinity. Mutations within such a highly conserved motif would be unlikely to have no effect on signalling, especially when the mutations are at the cytoplasmic end of a TM helix known to be closely involved in the coupling and activation of G-proteins. Both the I7.51A and L7.52A mutants were able to signal in response to ghrelin with 'WT-like' EC_{50}. I7.51A altered the ghrelin efficacy with an E_{max} of 64 % of WT suggesting that this mutation was moderately detrimental to agonist-induced signalling.

$Tyr^{7.53}$ of the NPxxY motif is 92 % conserved in Family A GPCRs (Mirzadegan *et al.*, 2003). $Tyr^{7.53}$ has been implicated in the network of hydrogen bonds that stabilise the inactive GPCR conformation, forming a hydrogen bond with $Asn^{1.50}$ in TMI, which interacts with $Asp^{2.50}$

(Chapter 3). This hydrogen bond network is proposed to occur in the inactive receptor, maintaining the ground state (Konvicka *et al.*, 1998; Okada *et al.*, 2002). Mutation of $Tyr^{7.53}$ to alanine in the ghrelin-R affected both constitutive and ghrelin-induced receptor activation. The basal signalling was reduced to -24 % of the ghrelin-induced maximum and the efficacy was reduced, with the E_{max} being only 48 % of WT. Mutation of $Tyr^{7.53}$ has been found to have various effects in different Family A GPCRs. Mutation of $Tyr^{7.53}$ to all other naturally occurring amino acids gave rise to mutant $5HT_{2C}R$ with varying levels of constitutive activity, implicating $Tyr^{7.53}$ in the transition of receptors between inactive and active states (Prioleau *et al.*, 2002). In the formyl peptide receptor (FPR) a Y7.53F mutation resulted in constitutive activity suggesting that a tyrosine is essential at this position (He *et al.*, 2001). In the V_2R, phenylalanine could substitute successfully for tyrosine and retain high affinity AVP binding and signal transduction (Bouley *et al.*, 2003). As seen in the ghrelin-R, the Y7.53A mutant in the B2 bradykinin receptor had no effect on ligand binding affinity or EC_{50}. This mutant was however poorly expressed at the cell membrane and underwent substantial constitutive internalisation (Kalatskaya *et al.*, 2004). In contrast, the ghrelin-R Y7.53A mutant was expressed at near-WT levels at the cell surface, suggesting no enhanced constitutive internalisation. A $Tyr^{7.53}$ to phenylalanine mutation was found in the MCR4 in a patient suffering from severe childhood obesity. The mutation resulted in decreased cell-surface expression and signal transduction (Roth *et al.*, 2009). This data provides evidence in a clinical study, that a $Tyr^{7.53}$ mutation is detrimental to GPCR function. In the ghrelin-R Y7.53A was the most detrimental mutation within the NPxxY in terms of ghrelin-induced activation, reducing the E_{max} to only 48 % of that seen for the WT receptor.

Mutation of $Tyr^{7.53}$ to alanine, cysteine or phenylalanine in the $5HT_{2C}$ receptor increased the binding affinity of 5-hydroxytryptamine despite differing effects of each mutant on constitutive activity. This suggests that activating and inactivating mutations at position 7.53 stabilise a high affinity state of the $5HT_{2C}R$ (Rosendorff *et al.*, 2000). Although no increase in agonist affinity was observed with the ghrelin-R Y7.53A mutation, 'WT-like' ghrelin affinity was maintained, despite a detrimental effect on intracellular signalling, suggesting that inactivating mutations at this locus also retain high agonist affinity.

$Tyr^{7.53}$ and a highly conserved phenylalanine located seven residues away, within HelixVIII have been shown to be essential for activation of Rho. Mutation of the residues within the $NPxxY_{5,6}F$ motif to alanine identified $Tyr306^{(7.53)}$ and $Phe313^{(7.60)}$ as being essential for $G\alpha_t$ activation. Mutation of these conserved aromatics released stabilising constraints, facilitating

Meta II formation, but did not aid $G\alpha_t$-coupling (Fritze *et al.*, 2003). An extensive investigation of $Tyr^{7.53}$ in the serotonin $5HT_{2C}R$ highlighted this residue along with $Tyr^{7.60}$ as a functional microdomain that influences receptor activation (Prioleau *et al.*, 2002). Similar aromatic stacking interactions may be occurring in the ghrelin-R between $Tyr^{7.53}$ and $Tyr^{7.60}$, located at the TMVII-HelixVIII interface, supporting the role of $Tyr^{7.53}$ in efficient activation of the WT receptor.

Figure 4.32 presents schematic representations of the ghrelin-R showing each of the alanine substitution mutations in this Chapter and their effects on ghrelin-R signalling. The location of each residue within the TM domain is indicated and the effect of the mutation on ghrelin potency, basal signalling and ghrelin efficacy are indicated by colour.

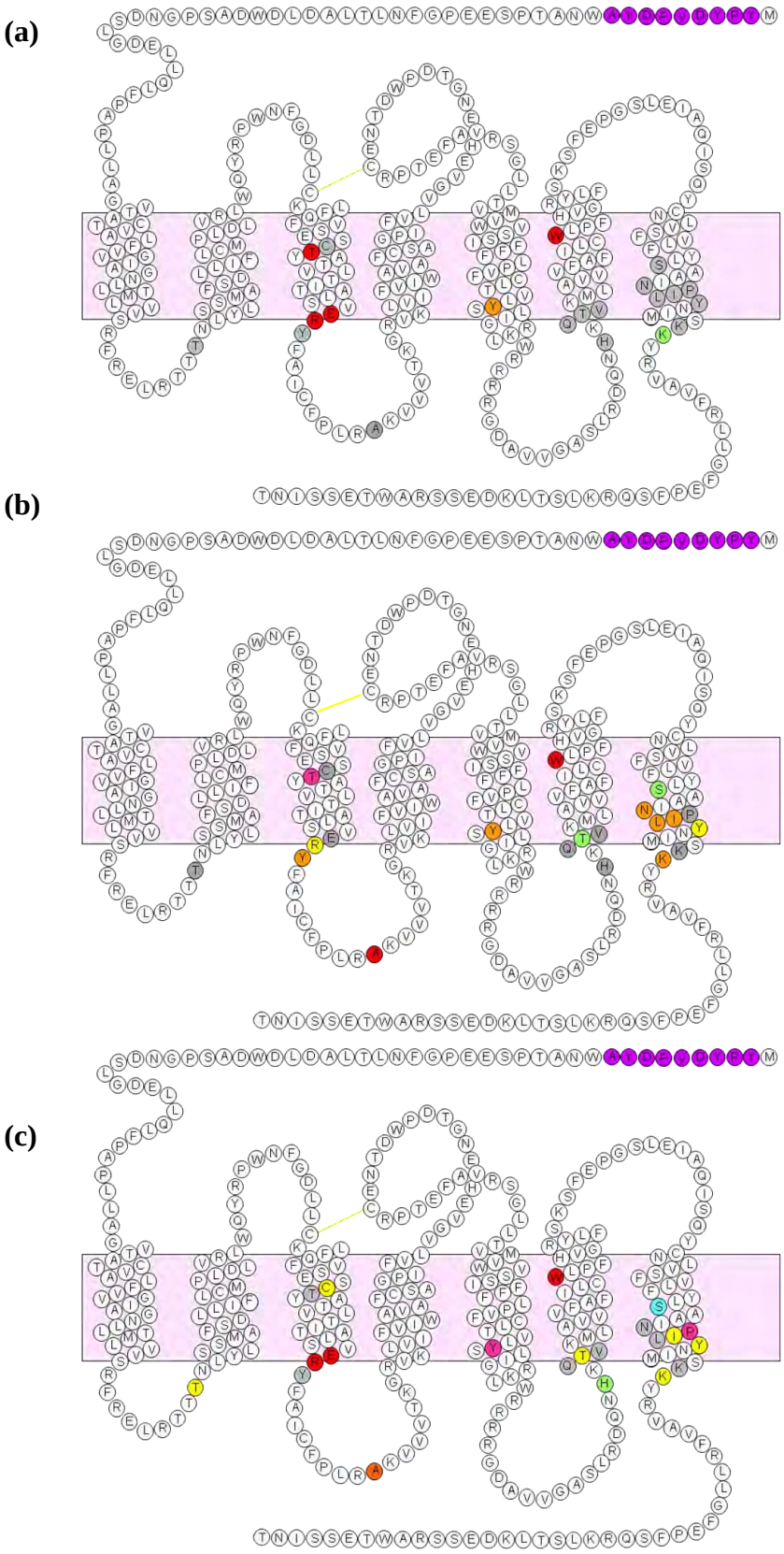


Figure 4.32 Schematic representations of the ghrelin-R demonstrating signalling characteristics for ghrelin-R alanine substitutions in Chapter 4: each residue mutated to alanine ($Ala^{3.60}$ to tyrosine) was characterised using InsP-$InsP_3$ accumulation assays to determine (a) ghrelin potency (b) basal constitutive activity and (c) ghrelin efficacy (see Appendix Table 9.1 for colour code). HA-epitope tag at the N-terminus is shown in purple.

CHAPTER 5.

GHRELIN RECEPTOR CONSTITUTIVE ACTIVITY:

THE ROLE OF HYDROPHOBIC AND POLAR RESIDUES WITHIN THE TRANSMEMBRANE DOMAINS

5.1 Introduction

The TM domains of GPCRs are the most conserved regions within the receptor superfamily. Hydrophobic interactions occur frequently within α-helices and contribute in a significant manner to the stabilisation of protein tertiary structure. Mutation of conserved hydrophobic residues in Family A GPCRs can result in a disruption to receptor activation, either in an agonist-independent (Latronico *et al.*, 1998; Tao *et al.*, 2000; Shapiro *et al.*, 2002; Urizar *et al.*, 2005; Proneth *et al.*, 2006; Ringkananont *et al.*, 2006), or an agonist-dependent manner (Blin *et al.*, 1995; Latronico *et al.*, 1998).

Polar residues are not commonly found within α-helices of membrane proteins, but can be shielded from the non-polar membrane environment by being clustered within the helical bundle (Dube *et al.*, 1998). Although small and polar residues are not highly conserved individually in GPCR TM domains, when considered as a group, they are often relatively well conserved (Eilers *et al.*, 2005). Small and polar amino acids are important in protein tertiary structure as they mediate tight helical packing via van der Waals interactions between, and within, helices. Within tightly packed helices, the ability of residues to form stabilising hydrogen bonds is increased and therefore both strong and weakly polar amino acids can facilitate further stabilising and structural interactions (Liu *et al.*, 2004).

A number of conserved hydrophobic and polar residues in TMIII and TMVI have been implicated in preventing or supporting constitutive activity in Family A GPCRs. To investigate the role of some of these residues in the ghrelin-R, mutations were introduced within the TM helices. The mutant receptor constructs were characterised to determine the role of these amino acids in both constitutive activity and agonist-induced signalling. The effects of alanine substitution of the residues in this chapter are summarised in the Appendix (Fig 9.4-9.8).

5.2 Results

Mutations were introduced within the ghrelin-R to investigate the effect of substitution of conserved hydrophobic and polar amino acids within the TM domains. The constructs were expressed in HEK 293T cells and pharmacologically characterised by competition radioligand binding studies and InsP-$InsP_3$ accumulation assays. Specific residues were selected for mutagenesis based on their reported role in constitutive activation in other Family A GPCRs or their relative positions within the TM domains. In TMIII, $Ser^{3.32}$, $Thr^{3.42}$, $Ile^{3.43}$, $Thr^{3.44}$, $Leu^{3.46}$ and $Ser^{3.47}$ were mutated and in TMVI, $Met^{6.36}$, $Leu^{6.37}$ and $Val^{6.40}$. $Met^{2.46}$ was included due to its role in stabilising interactions in other Family A GPCRs (Fig 5.1) (Bakker *et al.*, 2008; Okada *et al.*, 2001b; Urizar *et al.*, 2005). The oligonucleotide sequences used to introduce each of the mutations are shown in Table 5.1.

5.2.1 The role of the conserved hydrophobic residues in ghrelin-R function

A leucine is highly conserved at position 2.46 in Family A GPCRs (91 %) (Mirzadegan *et al.*, 2003). Methionine (4 % abundance) replaces the more conserved leucine in the ghrelin-R. To investigate the role of $Met^{2.46}$ in the ghrelin-R an M2.46A mutant construct was produced. The M2.46A mutant had a detrimental effect on ghrelin-R function. M2.46A retained 'WT-like' agonist affinity and potency, however the basal signalling was slightly reduced to -26 % of the WT ghrelin-induced maximum and the E_{max} was only 55 % of WT. Cell-surface expression was also found to be reduced (55 % of WT) (Fig 5.2, Table 5.2).

A hydrophobic residue is almost absolutely conserved at position 3.43 in Family A GPCRs and has been implicated in maintaining the inactive receptor conformation (Lu *et al.*, 2000). Mutation of residue 3.43 to alanine and arginine has been found to increase constitutive activity in some Family A GPCRs (Latronico *et al.*, 1998; Lu *et al.*, 2000; Tao *et al.*, 2000). To investigate the role of $Ile^{3.43}$ in the ghrelin-R, two mutant receptor constructs were produced; I3.43A and I3.43R. The I3.43A mutant displayed a dramatic increase in agonist-independent activity (140 % of WT basal), comparable to stimulation of the WT ghrelin-R with ~3 nM ghrelin. The mutant was still slightly responsive to further stimulation with ghrelin, reaching an E_{max} similar to that seen for the WT receptor. Agonist affinity and cell-surface expression were both comparable to WT (Fig 5.3, Table 5.3). In contrast, the I3.43R was detrimental to ghrelin-R function. The mutant did not display constitutive activity, with basal $InsP_3$ signalling reduced to levels similar to those seen for the mock-transfection (12 % of WT basal). The I3.43R mutant retained some agonist responsiveness, increasing the EC_{50}

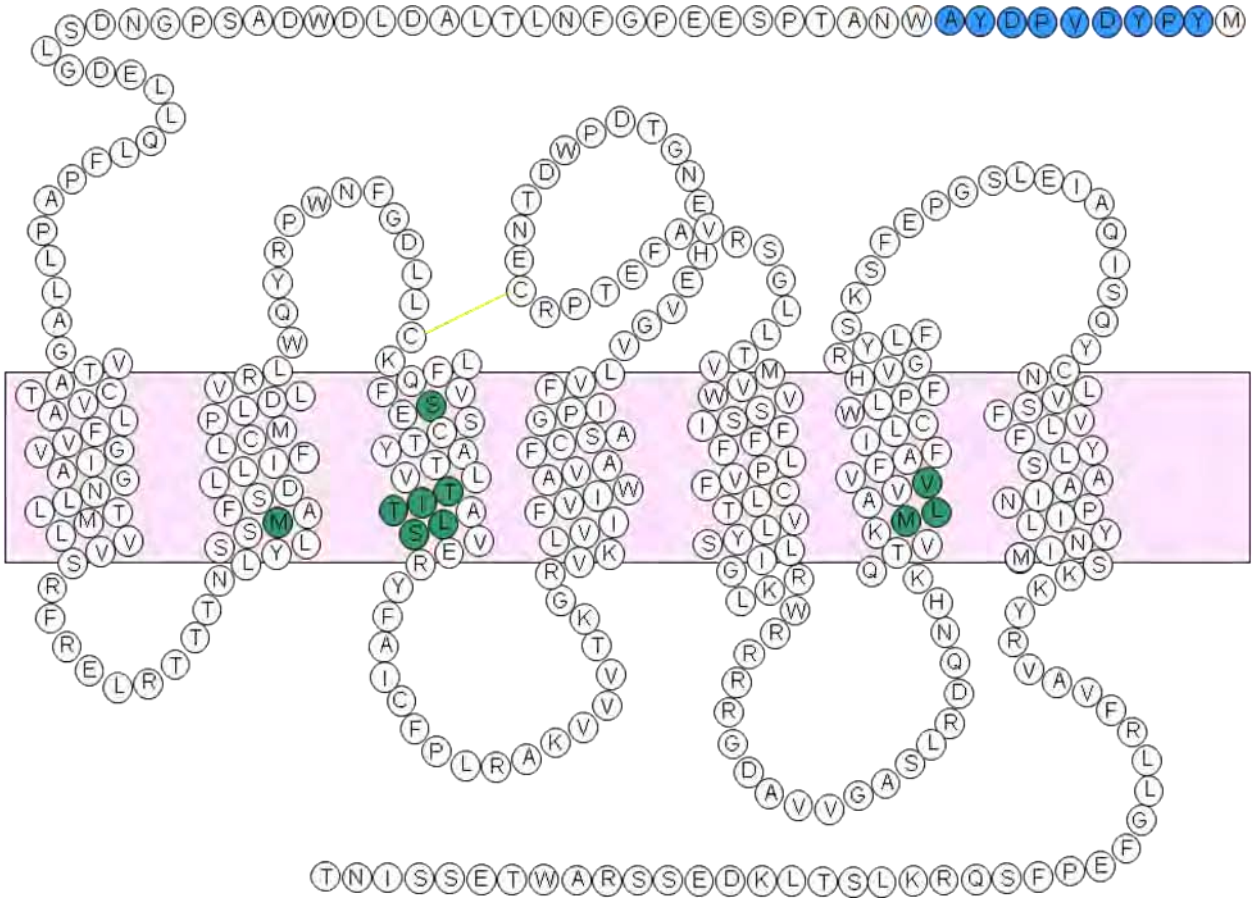


Figure 5.1 Schematic representation of the ghrelin-R: the intramolecular disulphide bond is shown in **yellow**. Residues that represent the HA-epitope tag introduced at the N-terminus (**blue**) and the individual residues mutated in this chapter (**green**) are illustrated.

Mutant Receptor Construct	Sense Oligonucleotide	Antisense Oligonucleotide
M2.46A	5’-C-TAC-CTG-TCC-AGC-GCG-GCC-TTC-TCC-GAT-C-3’	5’-G-ATC-GGA-GAA-GGC-CGC-GCT-GGA-CAG-GTA-G-3’
S3.32A	5’-C-TTC-CAA-TTC-GTC-GCT-GAG-AGC-TGC-ACC-3’	5’-GGT-GCA-GCT-CTC-AGC-GAC-GAA-TTG-GAA-G-3’
T3.42A	5’-C-GCC-ACG-GTG-CTC-GCC-ATC-ACA-GCG-CTG-3’	5’-CAG-CGC-TGT-GAT-GGC-GAG-CAC-CGT-GGC-G-3’
I3.43A	5’-GCC-ACG-GTG-CTC-ACC-GCC-ACA-GCG-CTG-AGC-GTC-3’	5’-GAC-GCT-CAG-CGC-TGT-GGC-GGT-GAG-CAC-CGT-GGC-3’
I3.43R	5’-GCC-ACG-GTG-CTC-ACC-CGC-ACA-GCG-CTG-AGC-GTC-3’	5’-GAC-GCT-CAG-CGC-TGT-GCG-GGT-GAG-CAC-CGT-GGC-3’
T3.44A	5’-CG-GTG-CTC-ACC-ATC-GCA-GCG-CTG-AGC-GTC-G-3’	5’-C-GAC-GCT-CAG-CGC-TGC-GAT-GGT-GAG-CAC-CG-3’
L3.46A	5’-C-ACC-ATC-ACA-GCG-GCG-AGC-GTC-GAG-CGC-3’	5’-GCG-CTC-GAC-GCT-CGC-CGC-TGT-GAT-GGT-G-3’
L3.46I	5’-C-ACC-ATC-ACA-GCG-ATC-AGC-GTG-CAG-CGC-3’	5’-GCG-CTC-GAC-GCT-GAT-CGC-TGT-GAT-GGT-G-3’
L3.46M	5’-C-ACC-ATC-ACA-GCG-ATG-AGC-GTC-GAG-CGC-3’	5’-GCG-CTC-GAC-GCT-CAT-CGC-TGT-GAT-GGT-G-3’
S3.47A	5’-C-CAT-CAC-AGC-GCT-GCC-GTC-GAG-CGC-TAC-3’	5’-GTA-GCG-CTC-GAC-GGC-CAG-CGC-TGT-GAT-GG-3’
M6.36A	5’-G-CAA-ACC-GTG-AAA-GCG-CTG-GCT-GTA-GTG-G-3’	5’-C-CAC-TAC-AGC-CAG-CGC-TTT-CAC-GGT-TTG-C-3’
L6.37A	5’-C-CGT-GAA-ATG-GCG-GCT-GTA-GTG-GTG-3’	5’-CAC-CAC-TAC-AGC-CGC-CAT-TTT-CAC-GG-3’
L6.37M	5’-CC-GTG-AAA-ATG-ATG-GCT-GTA-GTG-GTG-3’	5’-CAC-CAC-TAC-AGC-CAT-CAT-TTT-CAC-GG-3’
L6.37I	5’-CC-GTG-AAA-ATG-ATC-GCT-GTA-GTG-GTG-3’	5’-CAC-CAC-TAC-AGC-GAT-CAT-TTT-CAC-GG-3’
L6.37T	5’-CC-GTG-AAA-ATG-ACG-GCT-GTA-GTG-GTG-3’	5’-CAC-CAC-TAC-AGC-CGT-CAT-TTT-CAC-GG-3’
V6.40A	5’-CTG-GCT-GTA-GCG-GTG-TTT-GCC-TTC-3’	5’-GAA-GGC-AAA-CAC-CGC-TAC-AGC-CAG-3’
V6.40F	5’-CTG-GCT-GTA-TTC-GTG-TTT-GCC-TTC-3’	5’-GAA-GGC-AAA-CAC-GAA-TAC-AGC-CAG-3’
V6.40M	5’-CTG-GCT-GTA-ATG-GTG-TTT-GCC-TTC-3’	5’-GAA-GGC-AAA-CAC-CAT-TAC-AGC-CAG-3’
V6.40Y	5’-G-AAA-ATG-CTG-GCT-GTA-TAC-GTG-TTT-GCC-TTC-ATC-3’	5’-GAT-GAA-GGC-AAA-CAC-GTA-TAC-AGC-CAG-CAT-TTT-C-3’

Table 5.1 Oligonucleotide primer sequences for QuikChange™ site-directed mutagenesis: Codons to introduce mutations are shown in red.

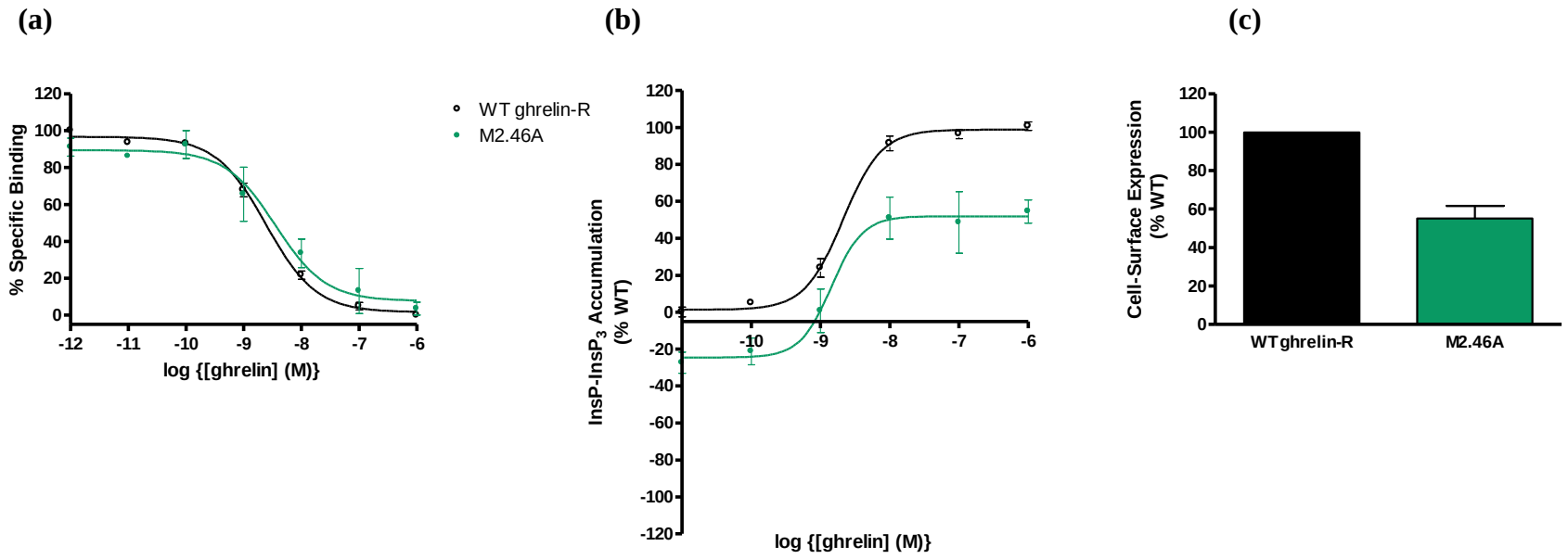


Figure 5.2 Ligand binding, intracellular signalling and cell-surface expression profiles for M2.46A mutant receptor construct: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or M2.46A and are expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or M2.46A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation			Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**
M2.46A	8.45 ± 0.07	1.44 ± 0.62	-27 ± 6	55 ± 6	55 ± 7

Table 5.2 Ligand binding, intracellular signalling and cell-surface expression for M2.46A mutant construct: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Yellow indicates a decrease basal signalling > 20 % of WT, a decrease in E_{max} > 30 % of WT and a decrease in cell-surface expression > 30 % of WT.

4.7-fold and reaching an E_{max} comparable to WT basal, reflecting the greatly reduced basal activity. [^{125}I]ghrelin binding was not detectable at the radioligand concentrations used in the binding assay and cell-surface expression was drastically reduced to only 43 % of WT (Fig 5.3, Table 5.3).

In the TSHR a constitutively activating mutation was found with a methionine to isoleucine substitution in TMVI (M6.37I). The constitutive activity was believed to be a result of steric hindrance with the side chain of Ile$^{3.46}$ as a reciprocal mutation, I3.46M in the M6.37I background, resulted in constitutive activity comparable to the WT TSHR. Furthermore, M6.37A, M6.37L and M6.37V mutants all displayed 'WT-like' constitutive activity (Ringkananont *et al.*, 2006). In the ghrelin-R Leu$^{6.37}$ and Leu$^{3.46}$ were mutated, initially to alanine, and then to other amino acids to investigate the role of these residues in receptor activity. All of the Leu$^{3.46}$ and Leu$^{6.37}$ mutants retained 'WT-like' affinity for ghrelin (Fig 5.4 & 5.5, Table 5.4 & 5.5). L6.37A and L6.37I displayed 'WT-like' characteristics. L3.46A and L3.46M caused almost complete ablation of ghrelin-R constitutive activity and L6.37T reduced basal signalling to -37 % of WT ghrelin-induced maximum, whereas L3.46I, L6.37A, L6.37I and L6.37M retained 'WT-like' basal activity. L3.46I and L6.37M were detrimental to agonist-induced receptor activation, with E_{max} values of 38 % and 67 % respectively, although potency was increased 3.3-fold compared to WT for the L3.46I mutant. Despite the reduction in constitutive activity in the L3.46A and L3.46M mutants, both were able to signal through the $InsP_3$ pathway maintaining ghrelin efficacy. L3.46A increased the EC_{50} 7.6-fold compared to WT. All of the mutants expressed at the cell-surface at levels comparable to WT, except L6.37M, which was reduced to 66 %, and L3.46A, which expressed at a greater extent than the WT ghrelin-R (142 %) (Fig 5.4 & 5.5, Table 5.4 & 5.5).

Met$^{6.36}$ was mutated to alanine as part of the investigation into the conserved hydrophobic residues in TMVI. Residue 6.36 is of hydrophobic character in 61 % of Family A GPCRs (Mirzadegan *et al.*, 2003). The M6.36A mutation in the ghrelin-R displayed 'WT-like' characteristics in relation to agonist binding, cell-surface expression and agonist-induced signalling, but displayed a reduction in agonist-independent basal signalling (-40 % of WT ghrelin-induced maximum) (Fig 5.6, Table 5.6).

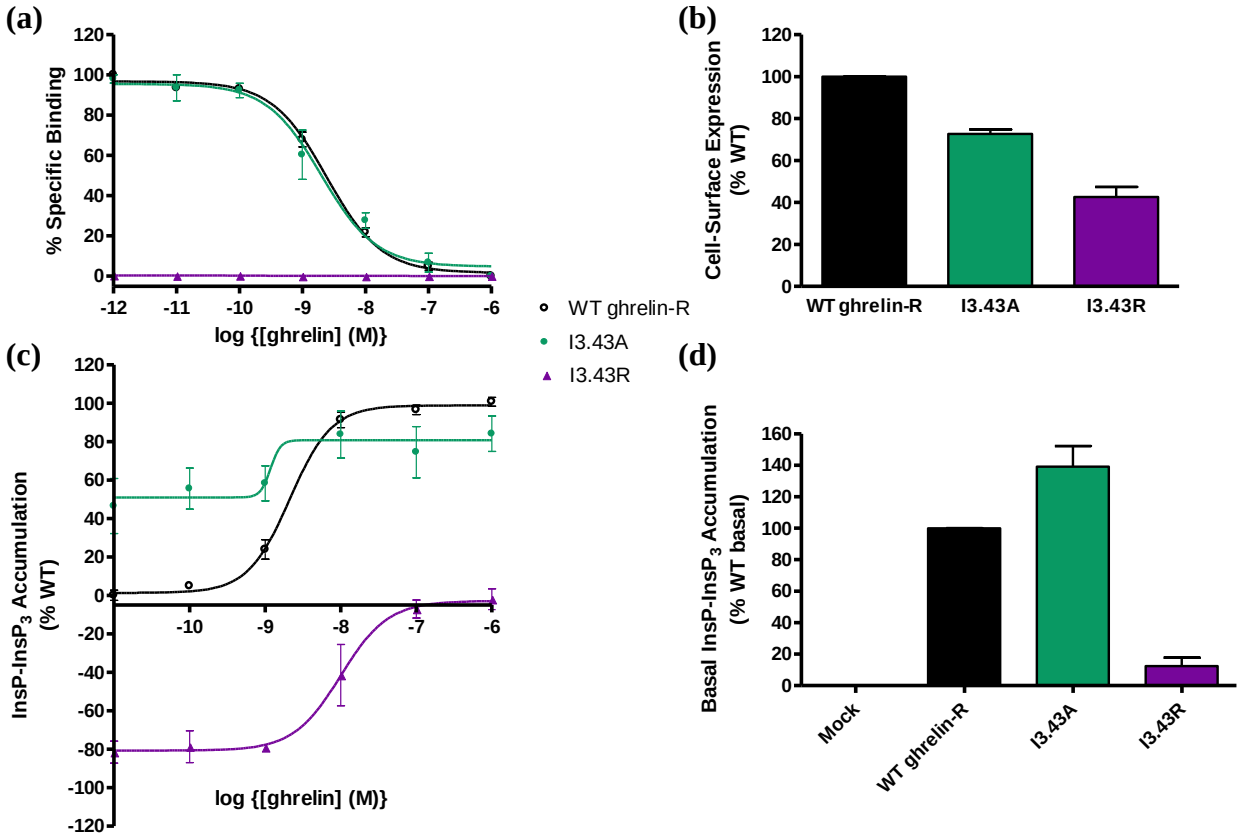


Figure 5.3 Ligand binding, intracellular signalling and cell-surface expression profiles for Ile$^{3.43}$ mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, I3.43A or I3.43R and are expressed as a percentage of specific binding. (b) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. (c) Ghrelin-induced InsP-InsP$_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, I3.43A or I3.43R. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (d) Basal InsP-InsP$_3$ accumulation as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation				Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	Basal (% WT basal) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**	**100**
I3.43A	8.70 ± 0.07	1.18 ± 0.56	46 ± 14	140 ± 13	84 ± 9	73 ± 2
I3.43R	NDB	10.00 ± 16.37	-82 ± 6	12 ± 5	-2 ± 5	43 ± 5

Table 5.3 Ligand binding, intracellular signalling and cell-surface expression for $Ile^{3.43}$ mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Blue indicates an increase in constitutive activity > 40 % of WT. Red indicates no detectable binding, a decrease in constitutive activity > 60 % or a decrease in E_{max} to less than the value observed for WT basal. Orange indicates an increase in EC_{50} > 4-fold or a reduction in cell-surface expression > 50 % of WT.

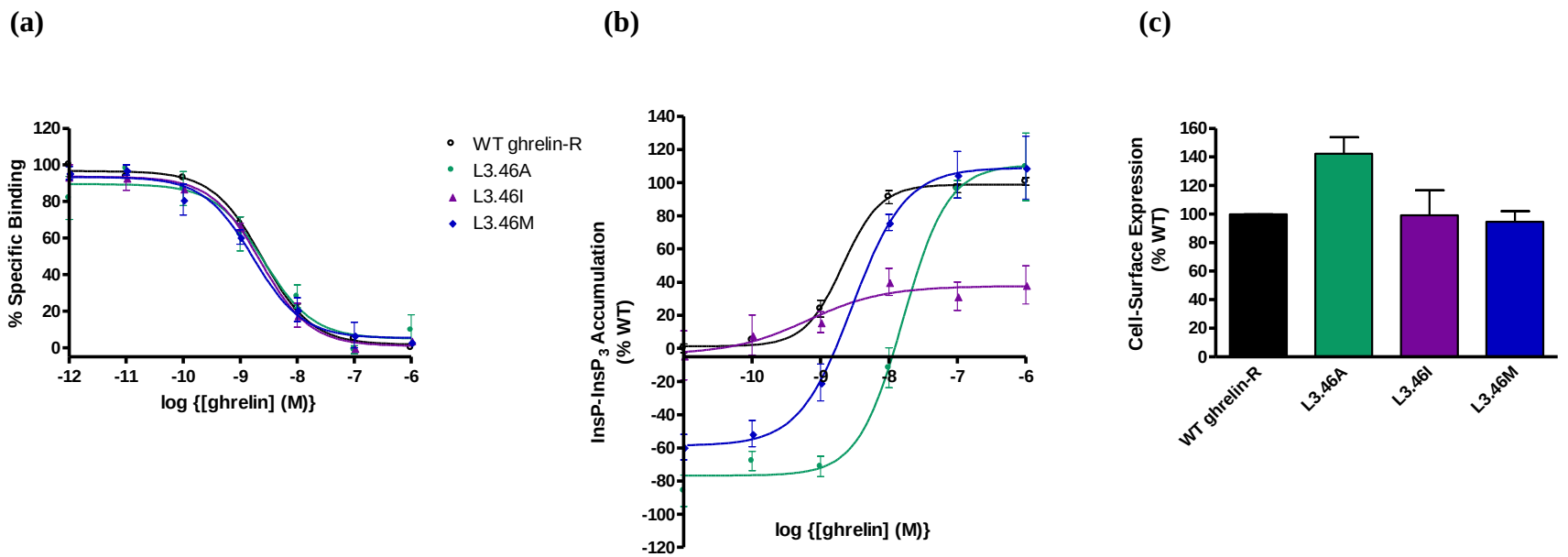


Figure 5.4 Ligand binding, intracellular signalling and cell-surface expression profiles for Leu$^{3.46}$ mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, L3.46A, L3.46I or L3.46M and are expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-InsP$_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, L3.46A, L3.46I or L3.46M. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation			Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**
L3.46A	8.58 ± 0.10	16.1 ± 5.82	-86 ± 9	110 ± 20	142 ± 12
L3.46I	8.64 ± 0.04	0.65 ± 1.26	4 ± 15	38 ± 11	99 ± 18
L3.46M	8.77 ± 0.05	2.91 ± 0.24	-59 ± 8	109 ± 19	95 ± 7

Table 5.4 Ligand binding, intracellular signalling and cell-surface expression for Leu$^{3.46}$ mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Red indicates an > 5-fold increase in EC_{50} or a decrease in basal activity > 60 %. Green indicates a decrease in EC_{50} > 3-fold or an increase in cell-surface expression > 130 %. Orange indicates reduction of basal activity > 40 % of WT or a reduction in E_{max} > 60 %.

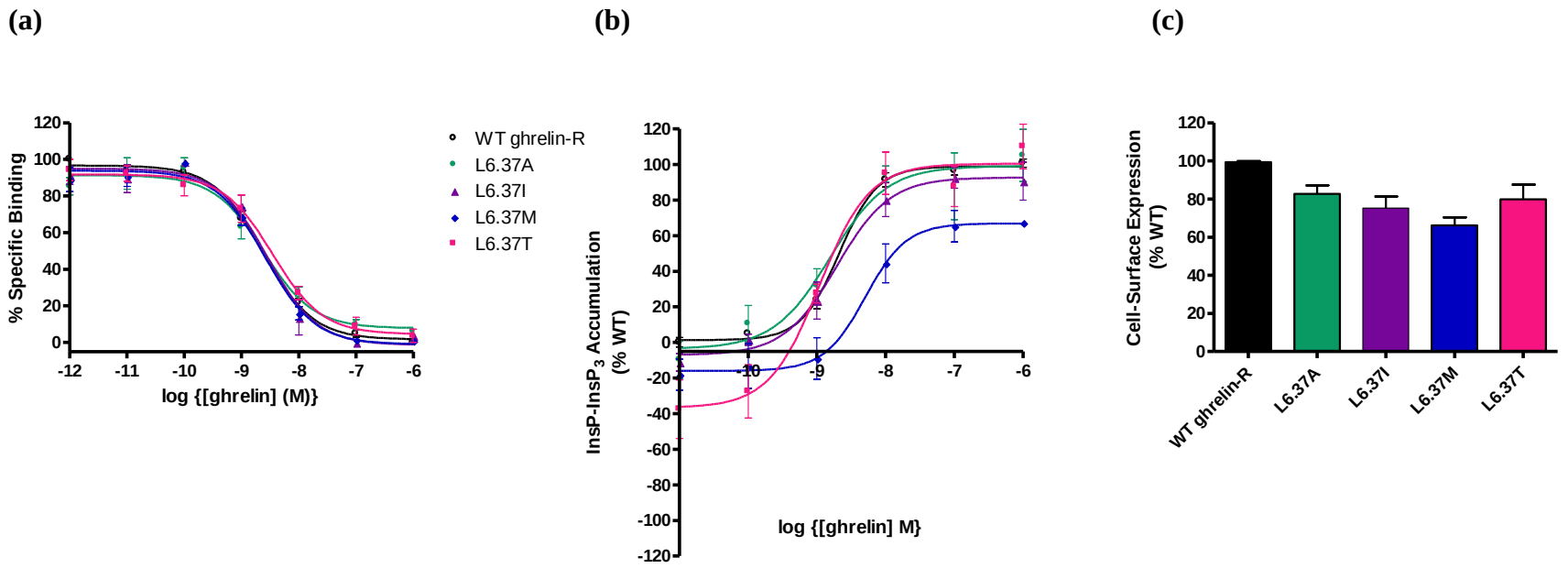


Figure 5.5 Ligand binding, intracellular signalling and cell-surface expression profiles for Leu$^{6.37}$ mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, L6.37A, L6.37I, L6.37M or L6.37T and are expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, L6.37A, L6.37I, L6.37M or L6.37T. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation			Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**
L6.37A	8.61 ± 0.07	1.56 ± 1.64	-10 ± 10	105 ± 15	83 ± 4
L6.37I	8.55 ± 0.07	1.95 ± 0.68	-11 ± 5	91 ± 11	75 ± 6
L6.37M	8.58 ± 0.05	5.08 ± 1.02	-18 ± 9	67 ± 1	66 ± 4
L6.37T	8.45 ± 0.03	1.09 ± 0.49	-37 ± 16	110 ± 13	80 ± 8

Table 5.5 Ligand binding, intracellular signalling and cell-surface expression for Leu$^{6.37}$ mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Yellow indicates a reduction in basal activity > 20 %, a reduction in E_{max} > 30 % or a reduction in cell surface expression > 30 %.

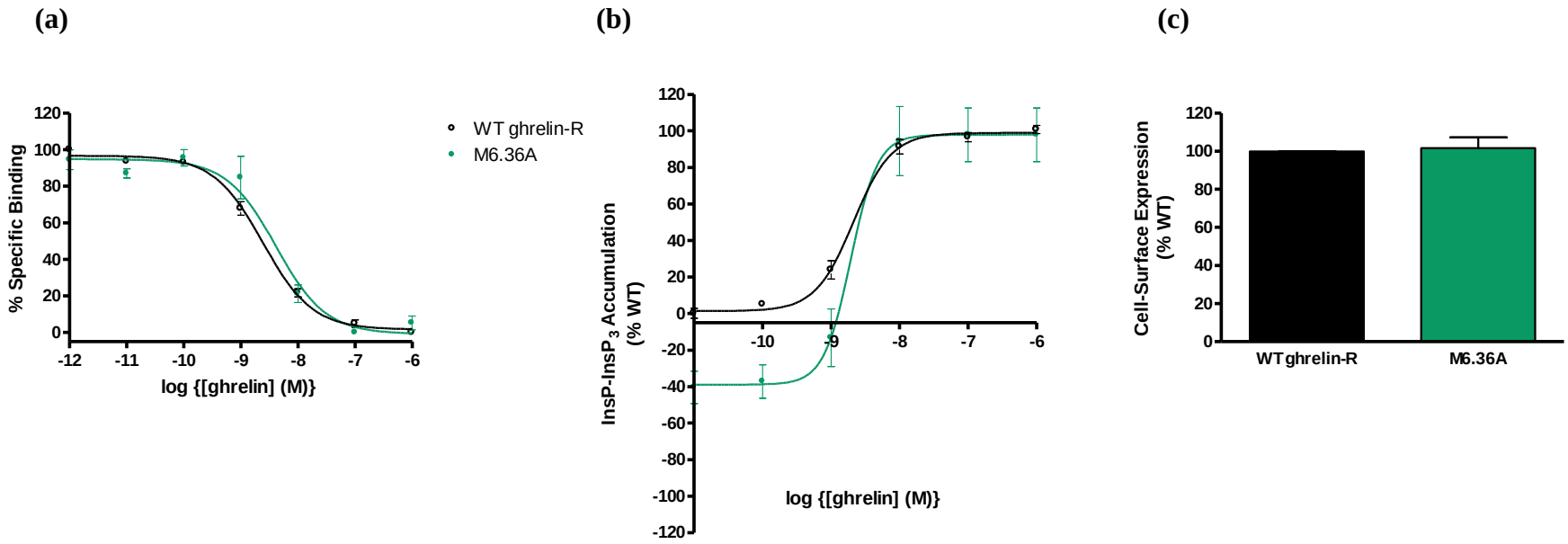


Figure 5.6 Ligand binding, intracellular signalling and cell-surface expression profiles for M6.36A mutant receptor construct: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or M6.36A and are expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or M6.36A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation			Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**
M6.36A	8.38 ± 0.10	1.94 ± 0.25	-40 ± 9	98 ± 15	102 ± 6

Table 5.6 Ligand binding, intracellular signalling and cell-surface expression for M6.36A mutant construct: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Orange indicates reduction of basal activity > 40 % of WT.

Residue 6.40 has been extensively studied in Family A GPCRs. $Met^{6.40}$ in opsin was substituted by the remaining 19 amino acids. All of the mutant receptors except for M6.40L and M6.40R resulted in the constitutive activation of opsin to varying degrees. M6.40L displayed basal activity comparable to WT and M6.40R failed to express at the cell surface meaning characterisation was not possible (Han *et al.*, 1998). Valine is found at position 6.40 in the ghrelin-R. Valine was found to produce significant constitutive activity (~110 % of WT) when substituting for methionine in opsin (Han *et al.*, 1998). $Val^{6.40}$ was mutated to various amino acids in the ghrelin-R to investigate whether this residue has a similar role in regulating constitutive activity in the ghrelin-R as found in Rho. V6.40A, V6.40M, V6.40F and V6.40Y receptor constructs were produced. All $Val^{6.40}$ mutants demonstrated 'WT-like' ghrelin binding affinity (Fig 5.7 (a), Table 5.7). V6.40A resulted in an increase in basal activity (124 % of WT basal) but was unable to be further stimulated by ghrelin. V6.40F and V6.40Y both increased basal constitutive activity in the ghrelin-R, to 111 % and 142 % of WT basal, respectively. V6.40F was still responsive to further stimulation with ghrelin although there was a 3.9-fold increase in EC_{50}. V6.40Y basal was approaching WT maximum signalling and this mutant did not respond further to ghrelin stimulation. V6.40M was detrimental to ghrelin-R constitutive activity (56 % of WT basal), however this mutant had a 'WT-like' EC_{50} and E_{max} (Fig 5.7 (c) & (d), Table 5.7). V6.40F, V6.40M and V6.40Y all displayed 'WT-like' cell-surface expression characteristics whereas V6.40A was detrimental to expression (65 % of WT) (Fig 5.7 (b), Table 5.7).

5.2.2 Investigation of the conserved hydrophobic residues using an inverse agonist

Based on the results of the ghrelin-induced InsP-$InsP_3$ accumulation assays, I3.43A, V6.40A, V6.40M and V6.40Y were chosen for characterisation with the ghrelin-R inverse agonist, SP-analogue. I3.43A was investigated using the inverse agonist as the mutant displays an increase in basal activity. The pIC_{50} for the I3.43A mutant was 'WT-like' although the mutant receptor construct was unable to reach the same maximal inhibition of basal activity as seen for the WT (I_{max} 83 % compared to the WT I_{max} 51 %). This may reflect the increased basal signalling observed with this mutant receptor construct (Fig 5.8, Table 5.8). V6.40A and V6.40Y were also more constitutively active than the WT ghrelin-R and were therefore characterised with SP-analogue. V6.40A had a WT pIC_{50} and I_{max} despite the increased basal, suggesting that the introduction of alanine at position 6.40 does not affect the inverse agonist response despite having a lower E_{max} for ghrelin-induced signalling (Fig 5.9, Table 5.9). The constitutive activity of the V6.40Y mutant was not reduced by stimulation with the

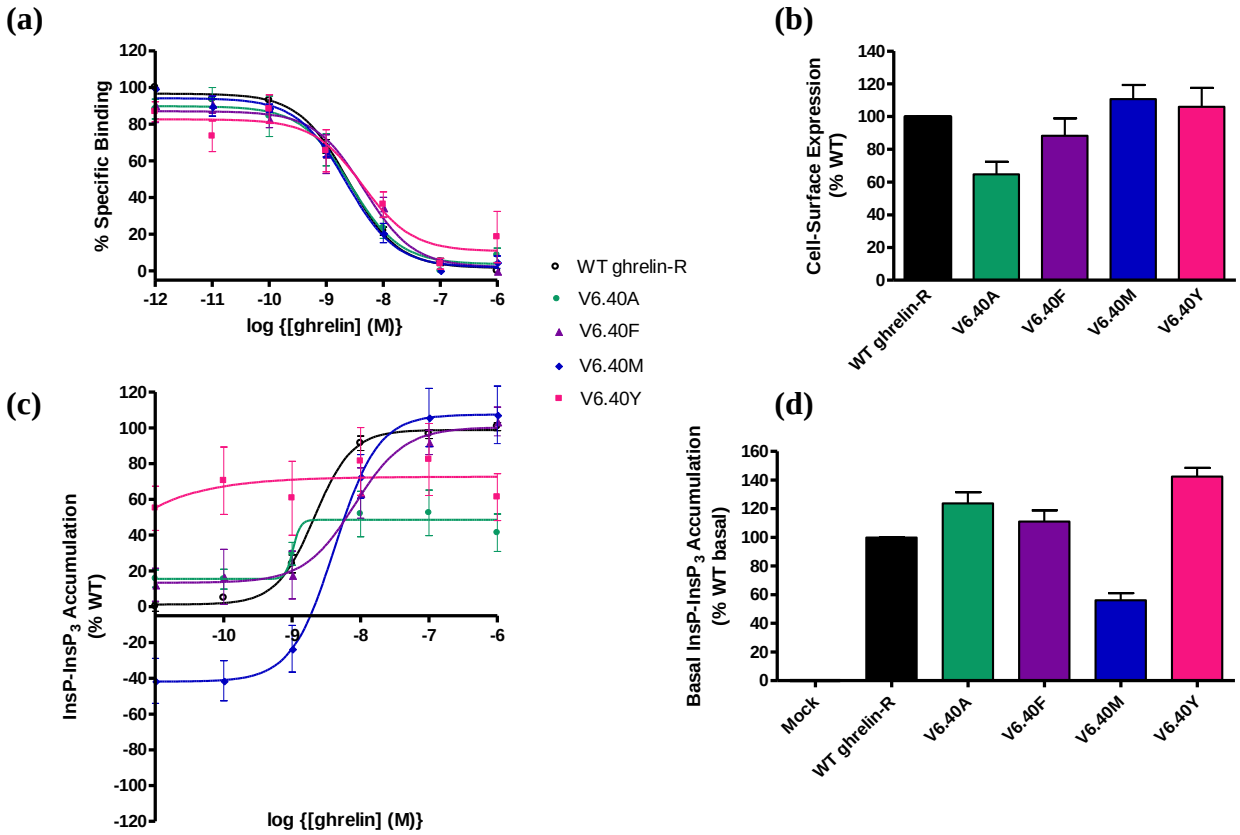


Figure 5.7 Ligand binding, intracellular signalling and cell-surface expression profiles for Val$^{6.40}$ mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, V6.40A, V6.40F, V6.40M or V6.40Y and are expressed as a percentage of specific binding. (b) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. (c) Ghrelin-induced InsP-InsP_3 accumulation assays were performed on cells transiently transfected with WT ghrelin-R, V6.40A, V6.40F, V6.40M or V6.40Y. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (d) Basal InsP-InsP_3 accumulation as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation				Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	Basal (% WT basal) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**	**100**
V6.40A	8.57 ± 0.05	NDS	16 ± 5	124 ± 8	NDS	65 ± 8
V6.40F	8.34 ± 0.07	8.20 ± 2.77	12 ± 9	111 ± 8	104 ± 8	83 ± 11
V6.40M	8.65 ± 0.04	4.62 ± 2.49	-41 ± 13	56 ± 5	107 ± 16	111 ± 9
V6.40Y	8.36 ± 0.09	NDS	55 ± 12	142 ± 6	NDS	106 ± 12

Table 5.7 Ligand binding, intracellular signalling and cell-surface expression for $Val^{6.40}$ mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Red indicates no detectable signalling. Orange indicates an increase in EC_{50} > 4-fold or a reduction in basal activity > 40 % of WT. Green indicates an increase in basal activity > 10 % of WT. Blue indicates an increase in basal activity > 40 % of WT. Yellow indicates a reduction in cell surface expression > 30 % of WT.

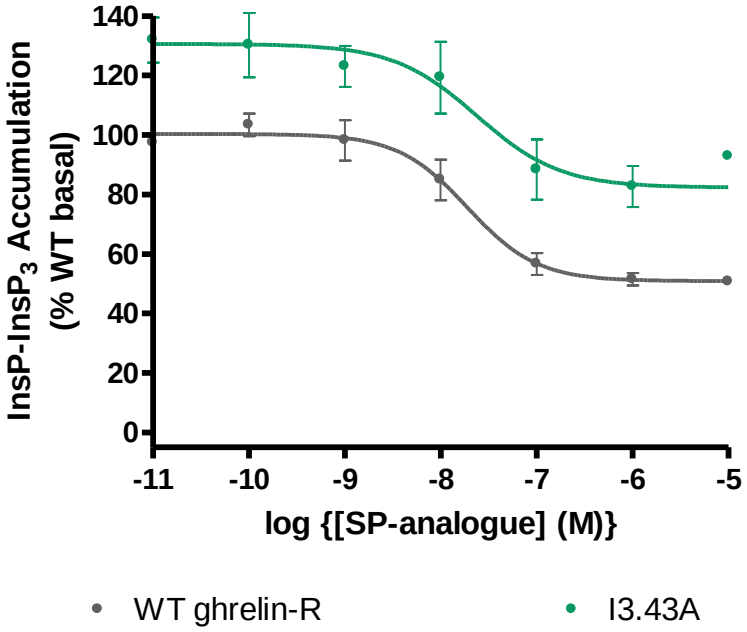


Figure 5.8 Intracellular signalling profile for I3.43A using SP-analogue: InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or I3.43A. Values are expressed as a percentage of the WT ghrelin-R basal from experiments performed in parallel. All data are mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	SP-analogue-induced InsP-$InsP_3$ Accumulation		
	pIC_{50} ± SEM	**Basal (% WT basal) ± SEM**	**I_{max} (% WT basal) ± SEM**
WT ghrelin-R	**7.72 ± 0.04**	**97 ± 2**	**51 ± 1**
I3.43A	7.62 ± 0.09	132 ± 8	83 ± 7

Table 5.8 Intracellular signalling of I3.43A mutant receptor construct using SP-analogue: Values were obtained using the methods described in Chapter 2. Data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Green indicates increase constitutive activity > 10 % of WT. Yellow indicates a reduction in I_{max} > 30 %.

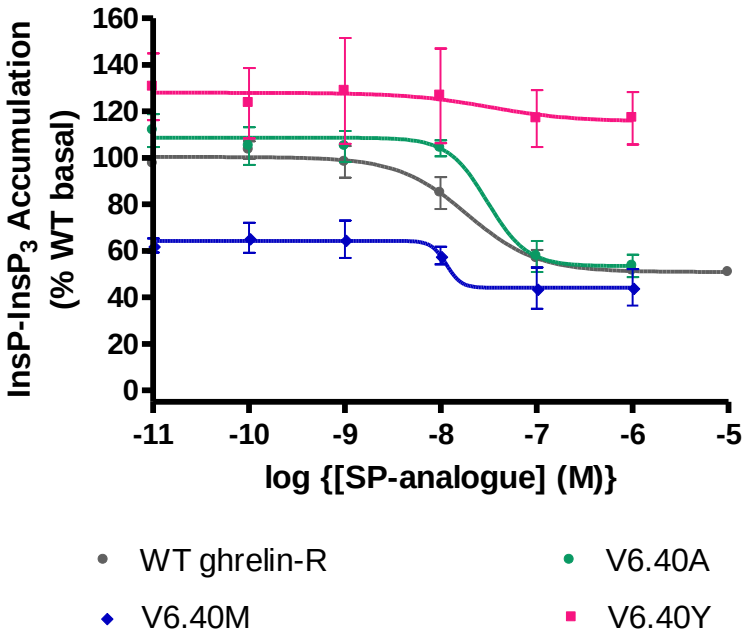


Figure 5.9 Intracellular signalling profile for Val$^{6.40}$ mutant constructs using SP-analogue: InsP-InsP$_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, V6.40A, V6.40M or V6.40Y. Values are expressed as a percentage of the WT ghrelin-R basal from experiments performed in parallel. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	SP-analogue-induced InsP-InsP$_3$ Accumulation		
	pIC$_{50}$ ± SEM	**Basal (% WT basal) ± SEM**	**I$_{max}$ (% WT basal) ± SEM**
WT ghrelin-R	**7.72 ± 0.04**	**97 ± 2**	**51 ± 1**
V6.40A	7.51 ± 0.07	112 ± 7	54 ± 5
V6.40F	Not characterised		
V6.40M	NDS	62 ± 3	NDS
V6.40Y	NDS	131 ± 14	NDS

Table 5.9 Intracellular signalling of Val$^{6.40}$ mutant constructs using SP-analogue: Values were obtained using the methods described in Chapter 2. Data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Green indicates an increase in basal > 10 % of WT. Red indicates no detectable signalling. Yellow indicates reduced basal > 20 % of WT.

inverse agonist, implying that substitution of valine with tyrosine affected agonist and inverse agonist sensitivity. V6.40M decreased ghrelin-R constitutive activity to a level comparable to the I_{max} observed with SP-analogue on the WT ghrelin-R and this was not decreased further by SP-analogue (Fig 5.9, Table 5.9).

5.2.3 The role of polar residues in TMIII of the ghrelin-R

Three polar residues in TMIII were mutated to alanine in the ghrelin-R. Although not absolutely conserved, serine and threonine residues are often found at these positions in Family A GPCRs. Residue 3.32 is polar in 15 %, 3.42 is polar in 38 %, residue 3.44 is polar in 37 % and residue 3.47 is polar in 54 % of Family A GPCRs (Mirzadegan *et al.*, 2003).

$Ser^{3.32}$ is found at the extracellular side of TMIII in the ghrelin-R. In the $V_{1a}R$, molecular modelling revealed that $Gln^{3.32}$ interacts, via a hydrogen bond, with Asp204 in ECL2 (Hawtin *et al.*, 2006) (Chapter 6). $Thr^{3.32}$ has been shown to be involved in restraining the ground state of the FSHR. Mutation of this residue in the FSHR to a number of different amino acids, including alanine, resulted in increased constitutive activity and resulted in abnormal responses to agonist (Montanelli *et al.*, 2004b). This residue is also known to play a role in binding of biogenic amine ligands (Strader *et al.*, 1988; Porter *et al.*, 1996; Kristiansen *et al.*, 2000). To investigate whether $Ser^{3.32}$ in important in maintaining the natural constitutive activity in the ghrelin-R the mutant, S3.32A, was constructed. S3.32A demonstrated 'WT-like' agonist affinity, cell-surface expression, and intracellular signalling characteristics, although the basal constitutive activity was found to be slightly reduced (-26 % of WT) (Fig 5.10, Table 5.10).

T3.42A was 'WT-like' with respect to ghrelin binding, potency, efficacy and cell-surface expression. There was an increase in basal constitutive activity to 124 % (27 % of WT ghrelin-induced maximum) suggesting that the introduction of alanine results in a more active receptor conformation in the absence of agonist (Fig 5.11, Table 5.10).

Mutation of $Thr^{3.44}$ to alanine in the T3.44A mutant resulted in a receptor that displayed decreased agonist-independent signalling (-29 %) compared to the WT ghrelin-R. The ability of T3.44A to signal when stimulated with ghrelin was also affected; the E_{max} was greatly reduced (55 %) and potency decreased 3.8-fold compared to WT. Cell-surface expression of this mutant was also slightly decreased (69 %). The agonist affinity remained similar to that seen for the WT receptor (Fig 5.12, Table 5.10).

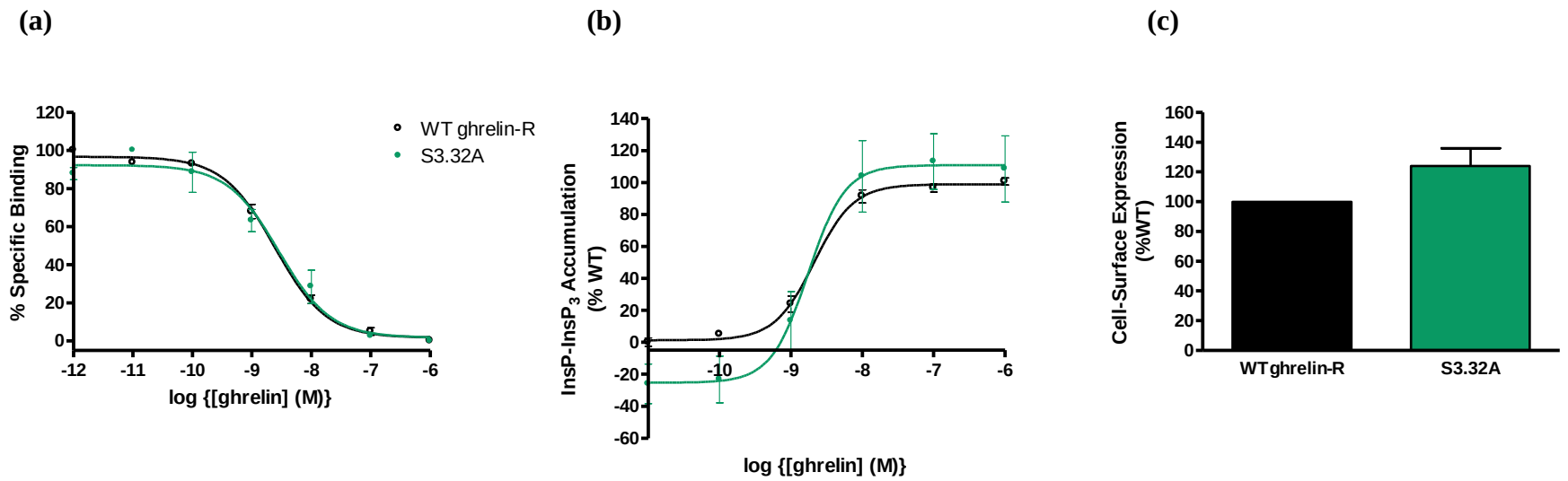


Figure 5.10 Ligand binding, intracellular signalling and cell-surface expression profiles for S3.32A: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or S3.32A and are expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or S3.32A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

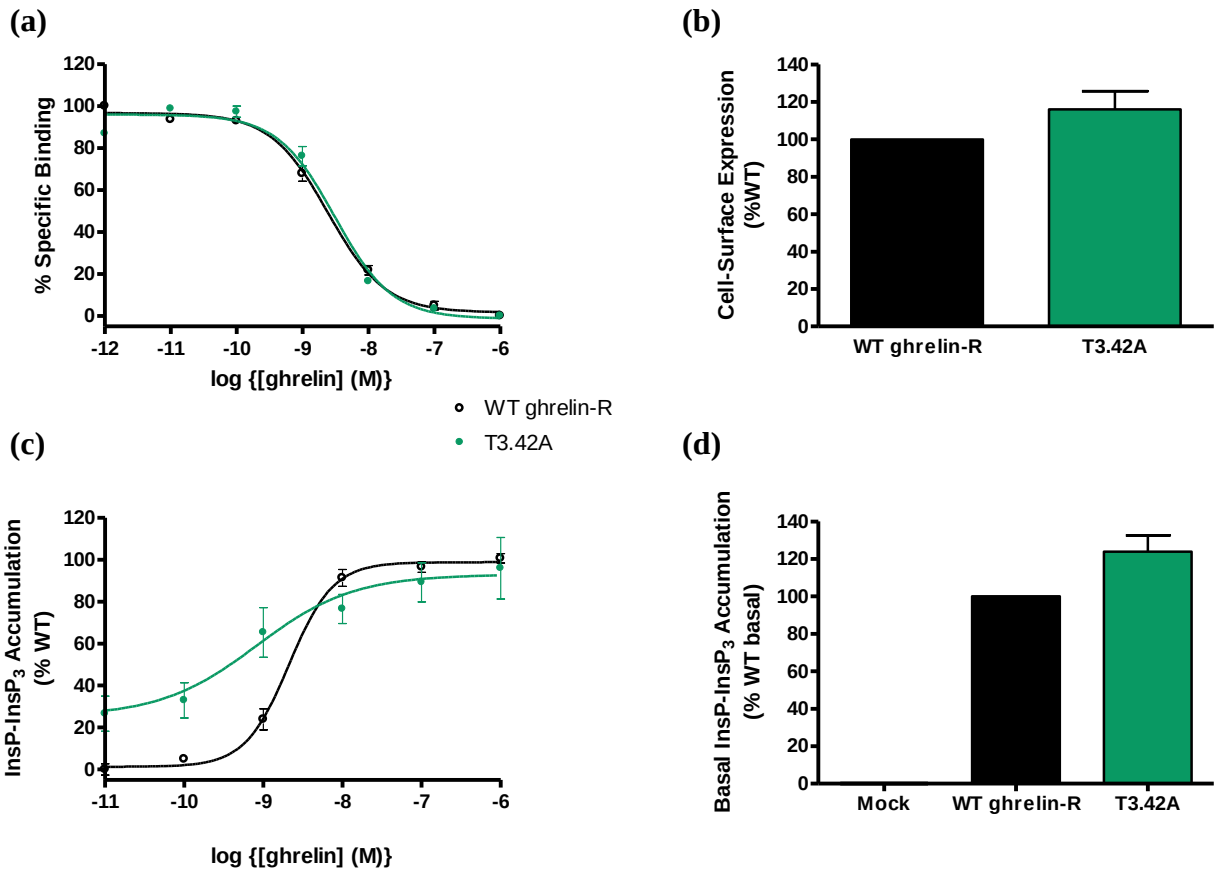


Figure 5.11 Ligand binding, intracellular signalling and cell-surface expression profiles for T3.42A mutant receptor construct: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or T3.42A and are expressed as a percentage of specific binding. (b) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. (c) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or T3.42A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (d) Basal InsP-$InsP_3$ accumulation as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

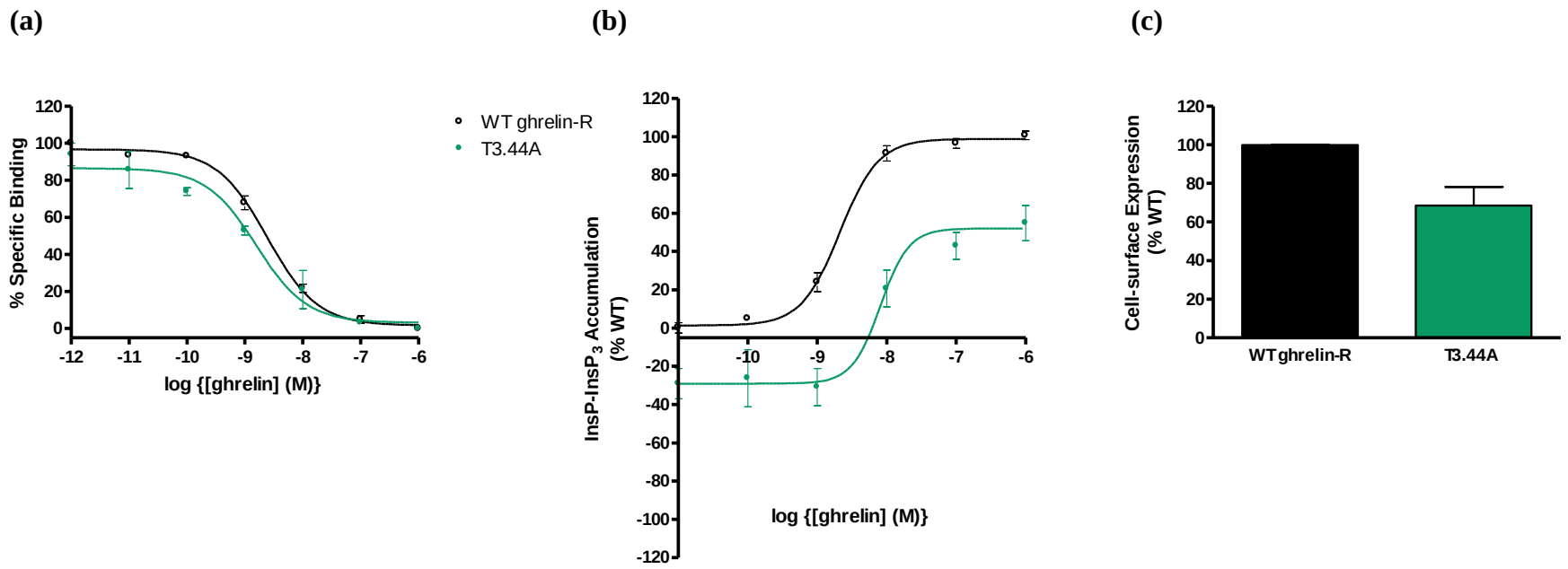


Figure 5.12 Ligand binding, intracellular signalling and cell-surface expression profiles for T3.44A mutant receptor construct: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or T3.44A and are expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or T3.44A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum and ghrelin-R basal from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

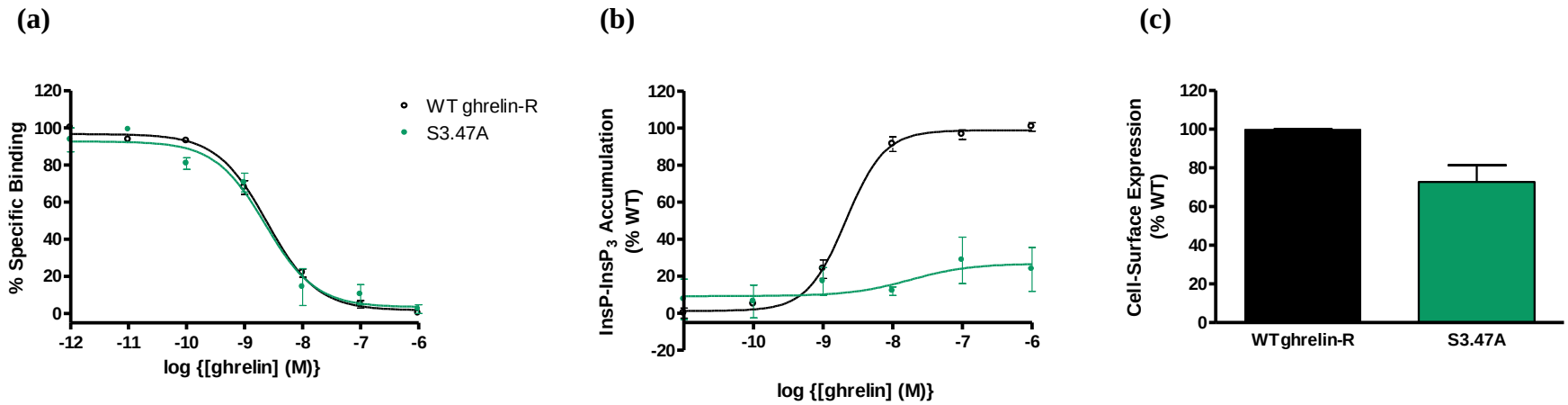


Figure 5.13 Ligand binding, intracellular signalling and cell-surface expression profiles for S3.47A mutant receptor construct: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or S3.47A and are expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or S3.47A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum and ghrelin-R basal from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R maximum. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation			Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**
S3.32A	8.54 ± 0.09	1.74 ± 0.19	-26 ± 12	108 ± 21	124 ± 12
T3.42A	8.51 ± 0.08	0.83 ± 0.43	27 ± 8	124 ± 9	96 ± 15
T3.44A	8.80 ± 0.07	8.12 ± 3.36	-29 ± 8	55 ± 9	69 ± 10
S3.47A	8.64 ± 0.08	NDS	7 ± 11	NDS	73 ± 9

Table 5.10 Ligand binding, intracellular signalling and cell-surface expression for S3.32A, T3.42A, T3.44A and S3.47A mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Yellow indicates > 20% reduction in basal activity, a decrease in E_{max} > 30 % of WT or a decrease in cell-surface expression > 30 %. Green indicates an increase basal signalling > 10 % of WT. Orange indicates an increase in EC_{50} > 4-fold. Red indicates no detectable signalling.

The S3.47A mutant was unable to signal in response to ghrelin despite retaining 'WT-like' constitutive activity, ghrelin binding affinity and cell-surface expression, suggesting that the S3.47A mutation disrupts the ability of the ghrelin-R to adopt the fully active conformation in response to agonist (Fig 5.13, Table 5.10).

5.2.4 Investigation of polar residues using an inverse agonist

T3.42A and S3.47A were analysed further using the inverse agonist, SP-analogue. InsP-$InsP_3$ accumulation assays indicated that T3.42A was 'WT-like' in its response to SP-analogue, despite the increased basal signalling compared to the WT ghrelin-R. The constitutive activity was decreased on addition of the SP-analogue and the resultant I_{max} (55 %) was comparable to the I_{max} observed for the WT ghrelin-R (51 %) (Fig 5.14, Table 5.11). The S3.47A mutation, which was unresponsive to ghrelin despite retaining WT constitutive activity, displayed 'WT-like' signalling characteristics in response to SP-analogue (Fig 5.15, Table 5.12). This suggests that the effect of the S3.47A mutation is specific to agonists and that there appears to be a disruption to the ability of the ghrelin-R to adopt a more active conformation in response to ghrelin, but that the mutation does not cause a disruption to the conformational re-arrangements induced by binding to the inverse agonist.

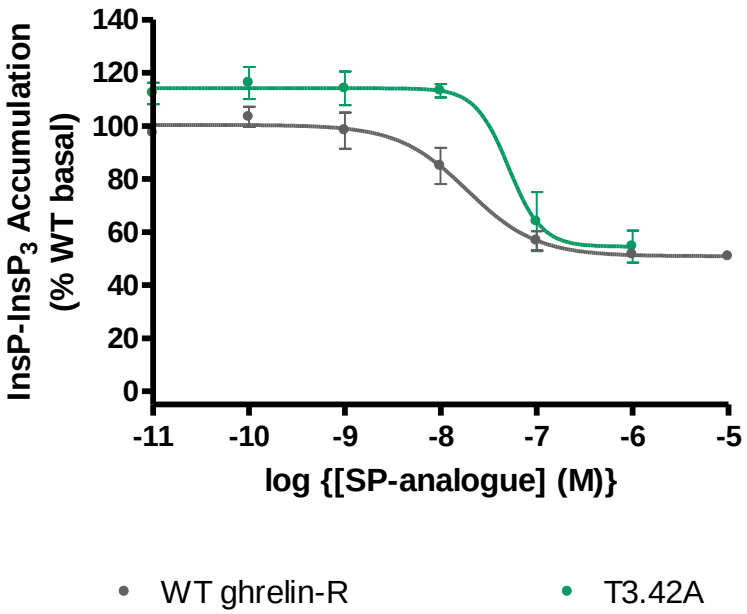


Figure 5.14 Intracellular signalling profile for T3.42A ghrelin-R mutant construct using SP-analogue: InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or T3.42A. Values are expressed as a percentage of the WT ghrelin-R basal from experiments performed in parallel. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	SP-analogue-induced Ins-$InsP_3$ Accumulation		
	pIC_{50} ± SEM	**Basal (% WT basal) ± SEM**	**I_{max} (% WT basal) ± SEM**
WT ghrelin-R	**7.72 ± 0.04**	**97 ± 2**	**51 ± 1**
T3.42A	7.29 ± 0.05	112 ± 4	55 ± 6

Table 5.11 Intracellular signalling of the T3.42A mutant using SP-analogue: Values were obtained using the methods described in Chapter 2. Data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Green indicates an increase in basal > 10 % of WT.

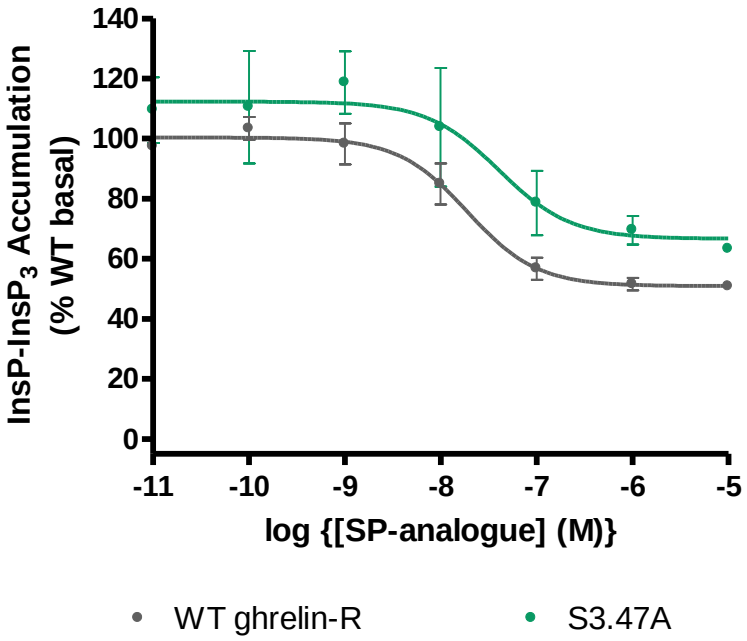


Figure 5.15 Intracellular signalling profile for S3.47A using SP-analogue: InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or S3.47A. Values are expressed as a percentage of the WT ghrelin-R basal from experiments performed in parallel. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	SP-analogue-induced InsP-$InsP_3$ Accumulation		
	pIC_{50} ± SEM	**Basal (% WT basal) ± SEM**	**I_{max} (% WT basal) ± SEM**
WT ghrelin-R	**7.72 ± 0.04**	**97 ± 2**	**51 ± 1**
S3.47A	7.39 ± 0.12	109 ± 11	69 ± 5

Table 5.12 Intracellular signalling of the S3.47A mutant receptor using SP-analogue: Values were obtained using the methods described in Chapter 2. Data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R.

5.3 Discussion

Hydrophobic and polar residues within TMs III and VI of Family A GPCRs have been found to be involved in a number of stabilising and constraining interactions, indicated by the production of constitutive activity after mutation of some of these key residues. A 'hydrophobic latch' has been proposed to occur in the TM domain of Family A GPCRs with mutation of residues within this cluster resulting in a favouring of the active receptor conformation (Meng *et al.*, 2001; Hulme *et al.*, 2003). Hydrophobic interactions occur between residues in TMs II, III, VI and VII in Rho and these interactions include $Leu^{3.43}$ and $Met^{6.40}$ investigated in this study (Okada *et al.*, 2001a).

Individual hydrophobic and polar residues within TMIII and TMVI were mutated to alanine and characterised in the ghrelin-R, probing the molecular basis of the high constitutive activity of the ghrelin-R. To gain further insight into the role of individual residues in the TM domains, highly conserved residues and some less well conserved residues, were targeted for mutagenesis. Each mutant was characterised in terms of their ability to bind ghrelin, express at the cell-surface and signal through the $InsP_3$ pathway, in both an agonist-independent and agonist-dependent manner.

5.3.1 The role of the conserved hydrophobic residues in ghrelin-R function

A leucine is highly conserved at position 2.46 in Family A GPCRs (91 %) (Mirzadegan *et al.*, 2003). $Leu^{2.46}$ has been proposed to form part of the asparagine cage, along with residue 6.40, restraining $Asn^{7.49}$ of the NPxxY motif (Chapter 4) in an inactive conformation and pointing towards TMVI (Okada *et al.*, 2001a; Bakker *et al.*, 2008). $Leu^{2.46}$ mutation to alanine in Rho resulted in constitutive activity (Okada *et al.*, 2001a), as did mutation of $Met^{6.40}$ (Han *et al.*, 1998) (discussed later in this section) highlighting the role of these residues in stabilising an inactive receptor (Madabushi *et al.*, 2004). Similarly, in the TSHR, an L2.46A mutation increased receptor constitutive activity (Urizar *et al.*, 2005). $Met^{2.46}$ in the ghrelin-R was mutated to alanine to investigate whether methionine plays a similar role in stabilising the basal state of the ghrelin-R. The M2.46A mutation moderately decreased ghrelin-R basal signalling to -27 % of the WT ghrelin-R ghrelin-induced maximal response. Ghrelin affinity and potency were maintained, but efficacy was reduced with the mutant receptor presenting an E_{max} of only 55 % of the WT. Cell-surface expression was also substantially reduced (55 % of WT), possibly providing an explanation of the decreased constitutive activity and E_{max}, if

the receptor was not present at the cell-surface in sufficient quantities to signal in a 'WT-like' manner. However, other ghrelin-R mutants that demonstrate low expression have been found to retain 'WT-like' constitutive activity (see Chapter 4, section 4.4.4.2, T6.33V), suggesting that the reduced expression is unlikely to be the cause of the decreased basal or E_{max}. It could be suggested that the presence of methionine at position 2.46, instead of the much more highly conserved leucine, may in part be responsible for the high level of constitutive activity observed for the ghrelin-R. Further mutations are required at this locus to investigate the effect of introducing a leucine, thus stabilising a potential asparagine cage, on ghrelin-R constitutive activity, as it is unclear from the single alanine mutation, whether $Met^{2.46}$ plays a crucial role in the structure and function of the constitutively active ghrelin-R.

A leucine is highly conserved (74 %) in Family A GPCRs at position 3.43 in TMIII. An isoleucine is found in the ghrelin-R, which occurs in 10 % of the superfamily (Mirzadegan *et al.*, 2003). Residue 3.43 has been proposed to form part of a cluster of hydrophobic residues connecting TMs III, VI and VII, referred to as the hydrophobic latch (Lu *et al.*, 2000; Palczewski *et al.*, 2000; Okada *et al.*, 2001a). Residue 3.43 has been extensively investigated in the glycoprotein hormone receptors. In the LHR mutation of $Leu^{3.43}$ to arginine was found to result in constitutive activity (Latronico *et al.*, 1998) and in the FSHR an L3.43R mutant also resulted in constitutive activity, whereas mutation of $Leu^{3.43}$ to alanine, aspartate or lysine did not affect basal signalling (Tao *et al.*, 2000). Similarly in the C5a anaphylatoxin chemotactic receptor (C5aR), an L3.43Q mutation resulted in weak constitutive activity (Baranski *et al.*, 1999) and in the β_2AR, three $Leu^{3.43}$ substitutions resulted in increased constitutive activity: L3.43A, L3.43K and L3.43R (Tao *et al.*, 2000).

In the ghrelin-R, mutation of $Ile^{3.43}$ to alanine resulted in increased constitutive activity (140 % of WT basal) without affecting ghrelin binding affinity, potency, efficacy or receptor cell-surface expression. Disruption of potential stabilising interactions by the I3.43A mutation in the ghrelin-R caused increased constitutive activity, suggesting that $Ile^{3.43}$ must form stabilising interactions that maintain the receptor in the basal conformation in the absence of ghrelin.

To further investigate the role of $Ile^{3.43}$ in the ghrelin-R, an I3.43R mutant was produced. Arginine introduction at this locus in the LHR, FSHR and β_2AR resulted in constitutive activity (Latronico *et al.*, 1998; Tao *et al.*, 2000). The introduction of arginine in the ghrelin-R

was particularly detrimental to receptor function, affecting ghrelin binding affinity (no detectable binding at the [^{125}I]ghrelin concentration used), potency (decreased 4.7-fold), efficacy (-2 % of WT ghrelin-induced maximum), constitutive activity (12 % of WT basal) and cell-surface expression (43 % of WT). This is in contrast to the data from the β_2AR, in which the introduction of a positive charge (L3.43K and L3.43R), resulted in increased constitutive activity as found with the L3.43A mutation (Tao *et al.*, 2000) and the LHR, where positive charges introduced at position 3.43 caused constitutive activity, possibly via the formation of a salt bridge with Asp$^{6.44}$ (Zhang *et al*., 2005).

Liu *et al*. (2007), found that an I3.43T mutant in the ghrelin-R, which has been identified as a mutation resulting from a single nucleotide polymorphism in human subjects, did not affect ghrelin-R constitutive activity, but resulted in a receptor that was unresponsive to ghrelin and only partially responsive to the inverse agonist, SP-analogue (Liu *et al.*, 2007b). I3.43A was investigated using SP-analogue in this study. The I3.43A mutant was 'WT-like' in terms of inverse agonist potency, although the mutant receptor construct was not able to reach the same maximal inhibition of basal activity as seen for the WT. Similar to that seen for the I3.43T mutation in the study by Liu *et al*. the I3.43A mutant was only partially responsive to inverse agonist. The data from Liu *et al*. (2007), combined with that of our current study, suggests that Ile$^{3.43}$ has a significant role in all aspects of ghrelin-R activity, as mutations at this locus can cause both increases and decreases in constitutive activity and can affect the ability of the receptor to be further activated by ghrelin, or inactivated by the SP-analogue, to varying degrees.

Residues 3.46 and 6.37 are highly conserved as hydrophobic amino acids in Family A GPCRs (98 % and 90 % respectively). In the TSHR, Ile$^{3.46}$ and Met$^{6.37}$ have been identified as interaction partners through site-directed mutagenesis after identification of a CAM TSHR (M6.37I) in humans suffering from hyperthyroidism. A reciprocal mutation producing I3.46M/M6.37I was able to restore WT constitutive activity, suggesting the recovery of stabilising interactions that were released through the M6.37I mutation alone, possibly due to a repulsive force between TMs III and VI (Ringkananont *et al.*, 2006).

Leu$^{3.46}$ and Leu$^{6.37}$ were both mutated in the ghrelin-R to other hydrophobic amino acids to investigate the potential of a stabilising interaction between these residues that maintains the ghrelin-R basal activity. All of the Leu$^{3.46}$ and Leu$^{6.37}$ mutants retained 'WT-like' ghrelin

binding affinity. Both the L3.46A and L3.46M mutations reduced the constitutive signalling (-86 % and -59 % respectively), with the L3.46A mutant constitutive activity being comparable to that seen for mock transfected cells. Despite this reduction in basal signalling, both mutants were able to signal to the same extent as the WT ghrelin-R in response to ghrelin. The L3.46A mutant did however show decreased ghrelin potency (7.6-fold). The L3.46I mutation had a particular effect on ghrelin-R function, affecting potency (3.3-fold increase in EC_{50}), and efficacy (E_{max} 38 % of WT maximum) although it maintained basal signalling capabilities. Similarly, in the GnRHR, mutation of $Ile^{3.46}$ to alanine or valine, were not well tolerated in terms of retaining 'WT-like' binding and signalling properties although a conservative I3.46L mutation retained 'WT-like' GnRH binding and signal transduction (Ballesteros *et al.*, 1998).

Mutation of $Leu^{6.37}$ to alanine, isoleucine and methionine, thus conserving the hydrophobicity at this locus, had no effect on ghrelin-R basal activity. L6.37A and L6.37I were 'WT-like' with regards to all characteristics. Only L6.37M demonstrated any difference in characteristics, having decreased efficacy (E_{max} 67 % of WT) and decreased cell-surface expression (66 % of WT). The constitutive activity was slightly decreased (-18 % of WT ghrelin-induced maximum) and the EC_{50} was increased compared to WT, (2.4-fold), although neither to any notable degree. The decreased efficacy was unlikely to be a result of the decreased number of receptors present at the cell surface as other mutations within the ghrelin-R have expression of approximately 66 % and still display 'WT-like' efficacy (see Chapter 4, section 4.2.7, L7.52A). Hydrophobic residues have been shown to be important at position 6.37 in the m3 mAChR for coupling to G-proteins, suggesting an essential role of the conserved hydrophobic character in activation (Blin *et al*., 1995). To investigate whether the maintenance of a hydrophobic residue is responsible for the 'WT-like' characteristics in the ghrelin-R mutants, a L6.37T mutant was produced. This introduced a polar threonine residue, which is only found at this position in 4 % of Family A GPCRs (serine found in 2 %) (Mirzadegan *et al.*, 2003). The L6.37T mutation had reduced basal signalling (-37 % of the WT ghrelin-induced maximum), without affecting other aspects of receptor function.

From the data in this study it is clear that many different residues are tolerated at position 6.37 and that, unlike the TSHR, an interaction between 6.37 and 3.46 was not contributing to the ghrelin-R constitutive activity. Alone, $Leu^{3.46}$ appears to be particularly important to ghrelin-R activity; the data demonstrate that maintaining a hydrophobic residue does not necessarily

retain WT activity. Introduction of isoleucine in the L3.46I mutant retained WT constitutive activity, suggesting that maintaining a similar side chain size and character to leucine is essential for ghrelin-R constitutive activity. This was further supported by the loss of ghrelin-R constitutive activity in the L3.46A mutant, and the substantial reduction with the L3.46M mutant.

Residue 6.36 in not as highly conserved as residue 6.37 or some of the other residues within TMs III and VI, although a hydrophobic amino acid is found in 64 % of Family A GPCRs (Mirzadegan *et al.*, 2003). An M6.36A mutant was included in the ghrelin-R mutagenesis study due to its location at the cytoplasmic end of TMVI to gain a greater insight into the role of individual residues in ghrelin-R function. The M6.36A mutant retained 'WT-like' ghrelin affinity, potency, efficacy and cell-surface expression, but reduced ghrelin-R constitutive activity to -40 % of the WT ghrelin-R ghrelin-induced maximum. Mutation of $Arg^{6.36}$ in the TSHR to alanine caused a similar effect to that seen in the ghrelin-R, with the mutant causing a small reduction in constitutive activity, but not affecting agonist responsiveness, or affinity (Urizar *et al.*, 2005). Many of the mutations introduced in to the ghrelin-R caused small decreases in constitutive activity, potentially due to small disruptions to the microenvironment surrounding each residue. This combined data suggests that residue 6.36 is not as significant in Family A receptor function as some of the other hydrophobic residues found within TMVI, and does not appear to form major stabilising interactions, although it is required for maintaining full WT constitutive activity.

Residue 6.40 is a highly conserved hydrophobic residue in Family A GPCRs (93 %) (Mirzadegan *et al.*, 2003). $Met^{6.40}$ was substituted by all 19 amino acids in opsin and all mutants, except the conservative M6.40L and M6.40R, which failed to express at the cell surface, resulted in increased constitutive activity compared to WT (Han *et al.*, 1998). Similarly, in the H_1R, mutation of $Ile^{6.40}$ to glutamate, glycine, alanine, arginine, lysine and serine resulted in increased constitutive activity (Bakker *et al.*, 2008).

The four ghrelin-R $Val^{6.40}$ mutants demonstrated different pharmacological characteristics. Mutation of $Val^{6.40}$ in the ghrelin-R resulted in varying levels of constitutive activity depending on the amino acid introduced. V6.40A, V6.40F and V6.40Y all increased constitutive activity, whereas V6.40M reduced agonist-independent signalling. The V6.40A mutant displayed CAM activity (124 % of WT ghrelin-R basal), but as seen with all the $Val^{6.40}$ substitutions, retained 'WT-like' agonist affinity, unlike many classical CAMs which

increase agonist affinity. The V6.40A mutant was unable to signal further when stimulated with ghrelin suggesting that, despite the increase in agonist-independent activity, ghrelin potency and efficacy were greatly affected. Other alanine mutations at position 6.40 also resulted in increased constitutive activity in Family A GPCRs including opsin (Han *et al.*, 1998), the H_1R (Bakker *et al.*, 2008), and the $5HT_{2A}R$ (Shapiro *et al.*, 2002).

The M6.40Y mutation in opsin resulted in the biggest increase in constitutive activity (Han *et al.*, 1998). To investigate the effects of a tyrosine at 6.40 in the ghrelin-R, a V6.40Y mutation was produced. V6.40Y was highly constitutively active (143 % of WT basal), suggesting that the introduction of tyrosine potentially stabilises an active receptor conformation. As seen with the V6.40A mutation, V6.40Y was unable to be further stimulated by ghrelin. This could be for a number of reasons; the V6.40Y conformation may resemble the active state of the receptor when stimulated with ghrelin, with TMIII and TMVI being separated by a repulsive action of tyrosine at position 6.40, or the V6.40Y mutation may represent a different receptor conformation that is partially active, but cannot be further activated by ghrelin.

V6.40F only increased constitutive activity to 111 % of WT basal, the lowest effect of all the CAMs at position 6.40 in the ghrelin-R. Similarly, in opsin, the introduction of phenylalanine did not produce a dramatic increase in constitutive activity, although some measurable increase was observed (Han *et al.*, 1998). This suggests that phenylalanine forms different interactions, in both Rho and the ghrelin-R, to the alanine and tyrosine substitutions, which both presented substantially increased constitutive activity when introduced into the receptors. This also suggests that it is unlikely that activation in the V6.40Y mutation is caused by the increased side chain bulk of tyrosine forcing TMIII and TMVI apart and thus mimicking activation, as the substitution with phenylalanine would be expected to result in a similar phenotype.

The V6.40M mutation decreased the agonist-independent activity of the ghrelin-R (56 % of WT basal) suggesting that this mutant receptor adopts a more inactive conformation. Ghrelin-induced activation was unaffected by the mutation, with V6.40M displaying 'WT-like' EC_{50} and E_{max}, therefore only the basal state of the receptor was altered and not the ability of the receptor to adopt an active conformation after stimulation with ghrelin. Methionine is found at position 6.40 in Rho, and it is assumed that $Met^{6.40}$ must retain the lowest level of constitutive activity in opsin, as mutation to any other amino acid (except leucine) resulted in constitutive activity (Han *et al.*, 1998). This result is consistent with our findings in the ghrelin-R.

Molecular modelling and mutagenesis in the $V_{1a}R$ suggested that mutations of $Ile^{6.40}$ either disrupted or strengthened TMIII and/or TMVII interactions with TMVI, depending on the effect of the introduced amino acid on receptor constitutive activity (Wootten &Wheatley, manuscript in preparation). In the current study, the data for the ghrelin-R appears to represent that found in Rho (Han *et al.*, 1998) and the $V_{1a}R$, in that there was no apparent correlation between the side chain properties of the amino acids introduced and the level of constitutive activity. In Rho and the H_1R, $Met/Ile^{6.40}$ is predicted to have a role in stabilising the side chain conformation of $Asn^{7.49}$ of the NPxxY motif in the inactive receptor, as part of the asparagine cage, and therefore mutation of 6.40 releases this constraint and allows the receptor to adopt a more active conformation causing constitutive activity (Okada *et al.*, 2001a; Bakker *et al.*, 2008). A mutagenesis study of the MC4R found that $Leu^{6.40}$ substitution with arginine, lysine, glutamate, glutamine and asparagine resulted in increased receptor constitutive activity that appeared to relate to the side chain properties of the amino acid introduced. It was suggested in the MC4R that the constitutively active mutations at 6.40 acted to stabilise the active state of the receptor rather than destabilise the inactive conformation, possibly through interactions with $Arg^{3.50}$ and $Tyr^{5.58}$, and this is supported by the high level of constitutive activity observed for L6.40E, L6.40N and L6.40Q mutants (Proneth *et al.*, 2006). Interestingly, the alanine and phenylalanine mutants did not cause increased basal signalling, and this was suggested to be due to the destabilisation of the inactive and the active state with the alanine mutant, and due to reinforcement of interactions with TMVII in the L6.40F mutant, thus affecting movement of the helices in activation (Proneth *et al.*, 2006). It can be assumed that different interactions are occurring in the ghrelin-R, as both the alanine and phenylalanine introductions at position 6.40 increased the constitutive activity of the receptor.

Characterisation of the $Val^{6.40}$ mutants using SP-analogue showed that V6.40A displayed 'WT-like' characteristics in response to the inverse agonist, despite an increase in constitutive activity. In contrast, the constitutive activity of the V6.40Y mutant was not able to be reduced by SP-analogue. This correlates with the data for ghrelin-induced activation of V6.40Y, and indicates that V6.40Y results in a reduction in both agonist and inverse agonist sensitivity, and supports the important disruptive action of tyrosine at this locus. The data suggests that tyrosine may be stabilising an active receptor conformation that is unable to be activated further by ghrelin, or disrupted by inverse agonist challenge. To investigate $Val^{6.40}$ in more detail, V6.40M was also characterised for SP-analogue sensitivity. V6.40M was unresponsive to SP-analogue stimulation, highlighting the decreased constitutive activity found for this mutation and suggesting that, despite retaining ghrelin sensitivity, the inverse agonist is

unable to reduce constitutive activity further. It can be assumed that the V6.40M mutation reduces constitutive activity down to a level similar to the observed I_{max} for the SP-analogue, meaning that basal signalling cannot be reduced further using the inverse agonist (see Chapter 4, section 4.2.3.1, H6.30E & H6.30K).

5.3.2 The role of polar residues in TMIII of the ghrelin-R

Serine, threonine and cysteine are readily able to form interhelical hydrogen bonds, and thus form stabilising interactions within TM domains (Liu *et al.*, 2004). Four of these polar residues, within TMIII of the ghrelin-R, were chosen for alanine substitution mutagenesis to investigate their role in ghrelin-R function.

Residue 3.32 has been found to be involved in ligand binding and activation in a number of Family A GPCRs, in particular, the biogenic amine receptors (Strader *et al.*, 1988; Porter *et al.*, 1996). In the $5HT_{2A}R$, $Asn^{3.32}$ was identified as being an important anchor site for agonists (Kristiansen *et al.*, 2000) and the same residue is essential for agonist and antagonist binding in the β_2AR (Strader *et al.*, 1988), and the $\alpha_{2a}AR$ (Wang *et al.*, 1991). Mutation of $Asp^{3.32}$ in the δ-OR resulted in constitutive activity (Befort *et al.*, 2001) as did mutation at this position in the $\alpha_{1b}AR$ (Porter *et al.*, 1996). In the $5HT_4R$, mutation of $Asp^{3.32}$ only affected receptor signalling, and was not particularly detrimental to ligand binding (Claeysen *et al.*, 2003). This combined data supports a multifunctional role of residue 3.32 both in individual receptors, and within Family A as a whole. $Ser^{3.32}$ mutation to alanine in the ghrelin-R did not have a profound effect on any receptor characteristic. There was a small decrease in constitutive activity (-26 % of WT ghrelin-induced maximum), but no effect on ghrelin binding or agonist-induced receptor signalling, suggesting that a serine at position 3.32 is not vital for ghrelin-R activity.

Two threonine residues within TMIII were mutated to alanine to investigate the role of these polar residues in ghrelin-R function. Residue 3.42 is not highly conserved in Family A GPCRs, but is often a hydrophobic or polar residue. The $Thr^{3.42}$ mutation to alanine in the ghrelin-R caused increased constitutive activity (124 % of WT basal), suggesting that alanine substitution either disrupts interactions that stabilise the basal state of the receptor, or that alanine allows the formation of new interactions that stabilise the R* conformation. In contrast, the T3.44A mutation was detrimental to ghrelin-R function and activity, reducing cell-surface expression, constitutive activity, ghrelin potency and efficacy. The combined data suggests that the two threonine residues within TMIII play different roles in the ghrelin-R;

either stabilising the basally active conformation, but preventing excessive constitutive activation ($Thr^{3.42}$), or affecting all aspects of receptor signalling ($Thr^{3.44}$). T3.42A was further analysed using the inverse agonist, SP-analogue, to determine whether the increased constitutive activity had any effect on the inverse agonist response. The T3.42A was 'WT-like' in its response to the SP-analogue, despite the increased basal signalling compared to the WT ghrelin-R, with the SP-analogue reducing the constitutive activity so that the I_{max} (55 %) was comparable to the I_{max} observed for the WT receptor (51 %). This revealed that the T3.42A mutation only affects basal signalling and has no effect on ligand-induced receptor conformational changes.

In the m1 mAChR three residues, including $Ser^{3.47}$, were identified as potentials for forming intramolecular interactions that stabilise the R state of the receptor. Alanine substitution of $Ser^{3.47}$ resulted in increased agonist affinity, efficacy and constitutive activity (Lu *et al*., 1999; Lu *et al.*, 2000). In contrast, mutation of $Ala^{3.47}$ in the β_2AR to serine, valine or leucine, did not result in increased agonist-independent activity (Chelikani *et al.*, 2007).

Mutation of $Ser^{3.47}$ to alanine in the ghrelin-R was particularly detrimental to agonist-induced receptor activation, although constitutive activity was retained at levels similar to WT. Ghrelin binding and cell-surface expression were not affected by the S3.47A mutation, suggesting that the detrimental effect on agonist-induced receptor activation is a direct effect on receptor activation and is not related to a reduction in expression, or loss of high affinity agonist binding. The S3.47A mutation also displayed 'WT-like' characteristics in response to the SP-analogue. This suggests that the effect of the S3.47A mutation is specific to the action of agonists. It is apparent that there is a disruption to the ability of the mutant ghrelin-R to adopt a more active R* state in response to ghrelin stimulation, but that the S3.47A mutation does not disrupt inverse agonist mechanisms of action. As $Ser^{3.47}$ is located in close proximity to the highly conserved ERY motif in TMIII (Chapter 4), it is possible that the disruption to agonist-induced activation is due to changes to the highly conserved region of the ERY motif and perturbation of $Glu^{3.49}$/$Arg^{3.50}$ interactions.

Figure 5.16 presents schematic representations of the ghrelin-R showing each of the alanine substitution mutations in this chapter and their effects on ghrelin-R signalling. The location of each residue within the TM domain is indicated and the effect of the mutation on basal signalling and ghrelin potency and efficacy are indicated by colour.

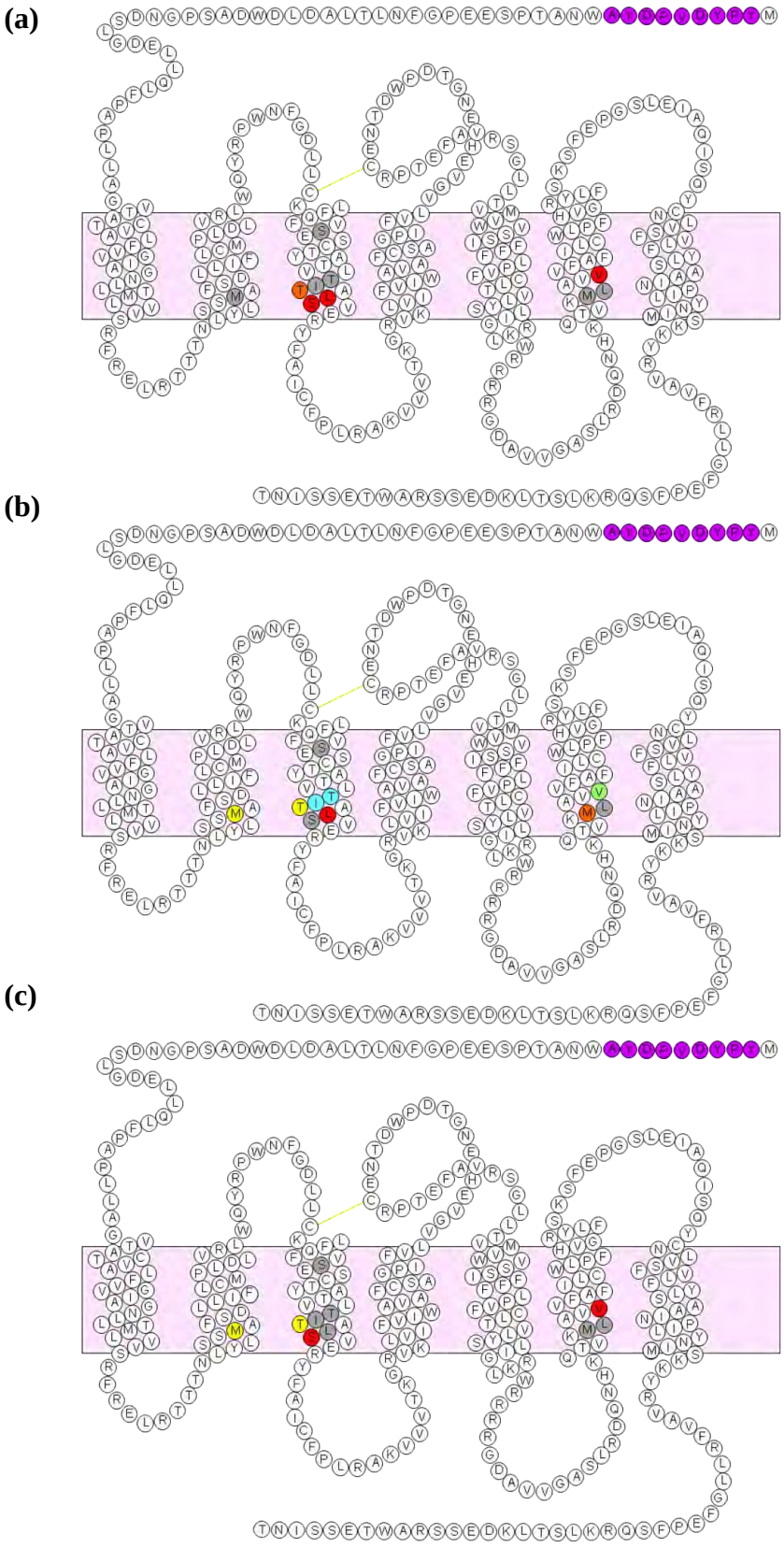


Figure 5.16 Schematic representations of the ghrelin-R demonstrating signalling characteristics for ghrelin-R alanine substitutions in Chapter 5: each residue mutated to alanine was characterised using InsP-InsP_3 accumulation assays to determine (a) ghrelin potency (b) basal constitutive activity and (c) ghrelin efficacy (see Appendix Table 9.1 for colour code). HA-epitope tag engineered at the N-terminus (purple).

CHAPTER 6.

THE ROLE OF ECL2 AND EXTRACELLULAR RESIDUES IN GHRELIN-R FUNCTION

6.1 Introduction

The extracellular domains of GPCRs are the most diverse regions and this is linked to the wide variety of ligands that are able to activate the members of the receptor superfamily. The roles of the extracellular surfaces in GPCR activation are not as well defined as the TM domains, although ECL2 has been shown to be important for ligand binding and activation in a number of GPCRs (abu Alla *et al.*, 1996; Ott *et al.*, 2002; Karnik *et al.*, 2003; Klco *et al.*, 2005; Massotte *et al.*, 2005).

The crystal structure of Rho revealed that ECL2 forms a β-hairpin which extends into the ligand binding pocket, making extensive contacts with retinal and with the extracellular domains (Palczewski *et al.*, 2000). In contrast, the crystal structure of the β_2AR demonstrated that ECL2 comprises an α-helix and a β-strand, stabilised by two disulphide bonds. This conformation allows the diffusion of ligands into, and out of, the binding pocket (Cherezov *et al.*, 2007) (Fig 6.1). It is unclear which of these structures best represents ECL2 of peptide GPCRs. It might be presumed that, in a peptide GPCR, the ECL2 structure is similar to the β_2AR ECL2 structure, as both are activated by diffusible ligands rather than by a covalently-bound ligand as in Rho. However, many peptide hormone receptors, including the ghrelin-R, lack the second disulphide bond within ECL2, which constrains the helical structure and adds rigidity to the exposed loop.

ECL regions vary in length between different members of the GPCR superfamily. As such, it is often difficult to determine the boundaries of the ECLs. Four secondary structure prediction programs (Phobius, JPred3, TMHMM and MEMSAT) were used to determine the residues comprising ECL2 in the ghrelin-R. The SwissProt entry Q92847-1 (2010) for the ghrelin-R states that ECL2 is residues 184-211. To investigate the role of ECL2 in ghrelin-R function, each residue within ECL2 was substituted by alanine. The effects of alanine substitution of residues in this chapter are summarised in the Appendix (Fig 9.4-9.8).

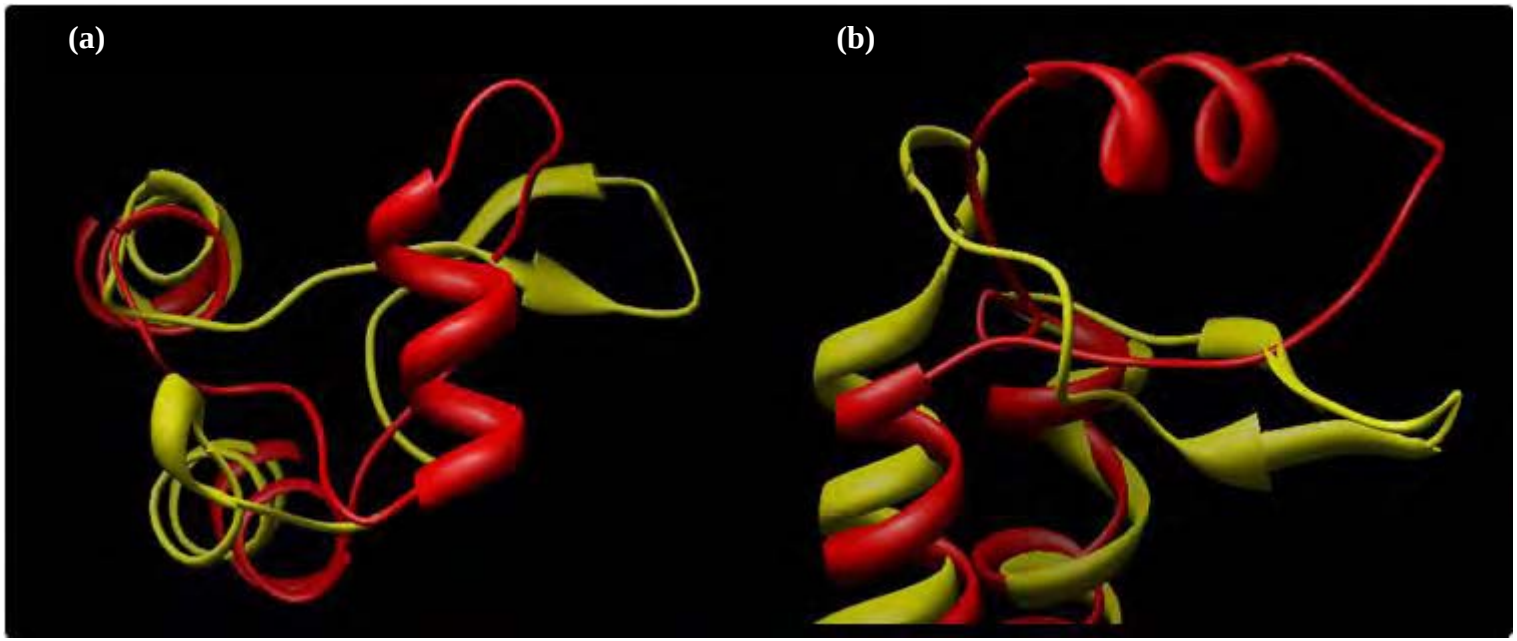


Figure 6.1 Extracellular domains of the β_2AR and rhodopsin: Crystal structure of bRho (**yellow**) and the β_2AR (**red**) extracellular domains showing the β-hairpin and α-helical region of ECL2 respectively (a) as viewed from the extracellular side, (b) as viewed through the membrane.

To ensure that all ECL2 residues were mutated in the alanine scan, residues 180-212 were mutated as part of this study (Fig 6.2). Most of these mutants were only characterised with respect to ligand binding and cell-surface expression. Further mutagenesis and analysis of intracellular signalling capabilities were conducted on those residues that have been found to have a particular functional relevance in other Family A GPCRs and therefore might play a significant role in ghrelin-R function.

Due to the large degree of variation in ECL2 length and sequence, a direct comparison between ECL2 regions in different Family A GPCRs is difficult. Conner *et al*. (2007) proposed a numbering system for residues within ECL2 based on the highly conserved cysteine that forms the intramolecular disulphide bond with $Cys^{3.25}$ at the top of TMIII in most Family A GPCRs. Residues to the C-terminal side of the conserved ECL2 cysteine (Cys198 in the ghrelin-R) are numbered C+ and residues to the N-terminal side are numbered C-, e.g. Ala204 in ECL2 of the ghrelin-R is C+6. For ease of comparison with other Family A GPCRs, this numbering system has been used throughout this investigation.

6.2 Results

6.2.1 ECL2 alanine scanning mutagenesis study

All of the residues within ECL2 were mutated to alanine ($Ala204^{(C+6)}$ to glycine) to investigate the effect of removal of the side chains. The mutant ghrelin-R constructs produced were V180A, L181A, V182A, G183A, V184A, E185A, H186A, E187A, N188A, G189A, T190A, D191A, P192A, W193A, D194A, T195A, N196A, E197A, C198A, R199A, P200A, T201A, E202A, F203A, A204G, V205A, R206A, S207A, G208A, L209A, L210A, T211A and V212A (Fig 6.2). The sequences of the oligonucleotide primers for site-directed mutagenesis are detailed in Table 6.1.

The mutant receptor constructs were expressed in HEK 293T cells and pharmacologically characterised for ghrelin binding and cell-surface expression. V180A, L181A, G183A, V184A, E185A, H186A, E187A, P192A, W193A, D194A, T195A, V205A, R206A, S207A, G208A, L209A, T211A and V212A constructs all demonstrated ‘WT-like’ binding affinity for ghrelin (Figs 6.3-6.5, Table 6.2).

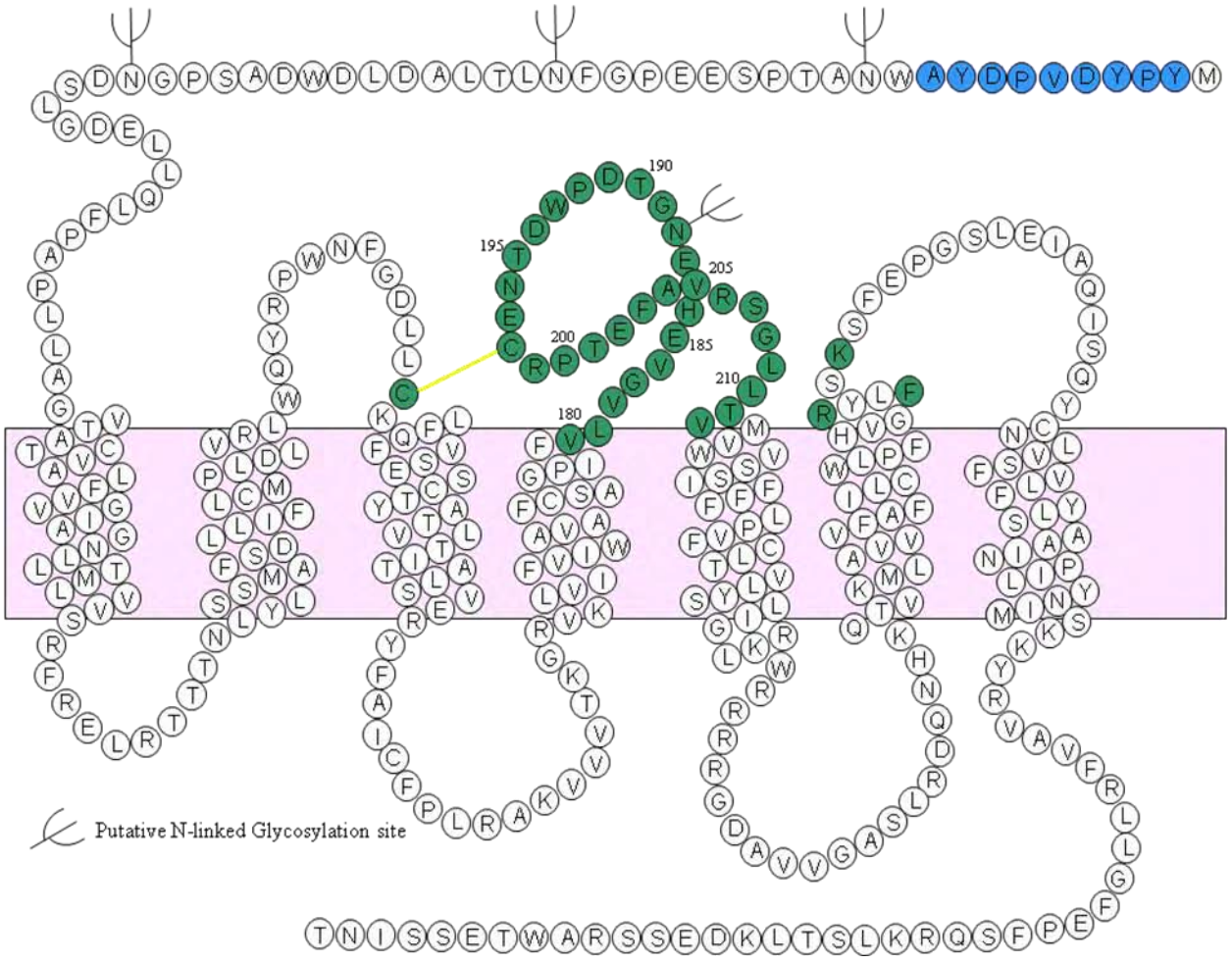


Figure 6.2 Schematic representation of the ghrelin-R: the intramolecular disulphide bond is shown in **yellow**. Residues that represent the HA-epitope tag introduced at the N-terminus (**blue**) and the individual residues mutated in this chapter (**green**) are illustrated.

Mutant Receptor Construct	Sense Oligonucleotide	Antisense Oligonucleotide
V180A	5’-GGG-CCC-ATC-TTC-GCG-CTA-GTC-GGG-GTG-3’	5’-CAC-CCC-GAC-TAG-CGC-GAA-GAT-GGG-CCC-3’
L181A	5’-CCC-ATC-TTC-GTG-GCA-GTC-GGG-GTG-GAG-3’	5’-CTC-CAC-CCC-GAC-TGC-CAC-GAA-GAT-GGG-3’
V182A	5’-ATC-TTC-GTG-CTA-GCC-GGG-GTG-GAGCAC-3’	5’-GTG-CTC-CAC-CCC-GGC-TAG-CAC-GAA-GAT-3’
G183A	5’-TTC-GTG-CTA-GTC-GCG-GTG-GAG-CAC-GAG-3’	5’-CTC-GTG-CTC-CAC-CGC-GAC-TAG-CAC-GAA-3’
V184A	5’-GTG-CTA-GTC-GGG-GCG-GAG-CAC-GAG-AAC-3’	5’-GTT-CTC-GTG-CTC-CGC-CCC-GAC-TAG-CAC-3’
E185A	5’-CTA-GTC-GGG-GTG-GCG-CAC-GAG-AAC-GGC-3’	5’-GCC-GTT-CTC-GTG-CGC-CAC-CCC-GAC-TAG-3’
H186A	5’-GTC-GGG-GTG-GAG-GCC-GAG-AAC-GGC-ACC-3’	5’-GGT-GCC-GTT-CTC-GGC-CTC-CAC-CCC-GAC-3’
E187A	5’-GGG-GTG-GAG-CAC-GCG-AAC-GGC-ACC-GAC-3’	5’-GTC-GGT-GCC-GTT-CGC-GTG-CTC-CAC-CCC-3’
N188A	5’-GTG-GAG-CAC-GAG-GCC-GGC-ACC-GAC-CC-3’	5’-GG-GTC-GGT-GCC-GGC-CTC-GTG-CTC-CAC-3’
G189A	5’-GAG-CAC-GAG-AAC-GCC-ACC-GAC-CCT-TGG-3’	5’-CCA-AGG-GTC-GGT-GGC-GTT-CTC-GTG-CTC-3’
T190A	5’-CAC-GAG-AAC-GGC-GCC-GAC-CCT-TGG-GAC-3’	5’-GTC-CCA-AGG-GTC-GGC-GCC-GTT-CTC-GTG-3’
D191A	5’-GAG-AAC-GGC-ACC-GCC-CCT-TGG-GAC-ACC-3’	5’-GGT-GTC-CCA-AGG-GGC-GGT-GCC-GTT-CTC-3’
P192A	5’-G-AAC-GGC-ACC-GAC-GCT-TGG-GAC-ACC-AAC-3’	5’-GTT-GGT-GTC-CCA-AGC-GTC-GGT-GCC-GTT-3’
W193A	5’-GGC-ACC-GAC-CCT-GCG-GAC-ACC-AAC-GAG-3’	5’-CTC-GTT-GGT-GTC-CGC-AGG-GTC-GGT-GCC-3’
D194A	5’-C-ACC-GAC-CCT-TGG-GCC-ACC-AAC-GAG-TGC-3’	5’-GCA-CTC-GTT-GGT-GGC-CCA-AGG-GTC-GGT-G-3’
T195A	5’-GAC-CCT-TGG-GAC-GCC-AAC-GAG-TGC-CGC-3’	5’-GCG-GCA-CTC-GTT-GGC-GTC-CCA-AGG-GTC-3’
N196A	5’-CT-TGG-GAC-ACC-GCC-GAG-TGC-CGC-CCC-3’	5’-GGG-GCG-GCA-CTC-GGC-GGT-GTC-CCA-AG-3’
E197A	5’-GG-GAC-ACC-AAC-GCG-TGC-CGC-CCC-ACC-3’	5’-GGT-GGG-GCG-GCA-CGC-GTT-GGT-GTC-CC-3’
C198A	5’-GAC-ACC-AAC-GAG-GCC-CGC-CCC-ACC-GAG-3’	5’-CTC-GGT-GGG-GCG-GGC-CTC-GTT-GGT-GTC-3’
R199A	5’-C-ACC-AAC-GAG-TGC-GCC-CCC-ACC-GAG-TTT-G-3’	5’-C-AAA-CTC-GGT-GGG-GGC-GCA-CTC-GTT-GGT-G-3’
P200A	5’-C-AAC-GAG-TGC-CGC-GCC-ACC-GAG-TTT-GCG-3’	5’-CGC-AAA-CTC-GGT-GGC-GCG-GCA-CTC-GTT-G-3’
T201A	5’-G-TGC-CGC-CCC-GCC-GAG-TTT-GCG-GTG-3’	5’-CAC-CGC-AAA-CTC-GGC-GGG-GCG-GCA-C-3’
E202A	5’-C-CGC-CCC-ACC-GCG-TTT-GCG-GTG-CGC-3’	5’-GCG-CAC-CGC-AAA-CGC-GGT-GGG-GCG-G-3’
F203A	5’-CGC-CCC-ACC-GAG-GCT-GCG-GTG-CGC-TCT-G-3’	5’-C-AGA-GCG-CAC-CGC-AGC-CTC-GGT-GGG-GCG-3’
A204G	5’-CCC-ACC-GAG-TTT-GGG-GTG-CGC-TCT-GG-3’	5’-CC-AGA-GCG-CAC-CCC-AAA-CTC-GGT-GGG-3’
V205A	5’-CC-GAG-TTT-GCG-GCG-CGC-TCT-GGA-CTG-3’	5’-CAG-TCC-AGA-GCG-CGC-CGC-AAA-CTC-GG-3’
R206A	5’-CC-GAG-TTT-GCG-GTG-GCC-TCT-GGA-CTG-CTC-3’	5’-GAG-CAG-TCC-AGA-GGC-CAC-CGC-AAA-CTC-GG-3’
S207A	5’-G-TTT-GCG-GTG-CGC-GCT-GGA-CTG-CTC-ACG-3’	5’-CGT-GAG-CAG-TCC-AGC-GCG-CAC-CGC-AAA-C-3’

Mutant Receptor Construct	Sense Oligonucleotide	Antisense Oligonucleotide
G208A	5’-CG-GTG-CGC-TCT-GCA-CTG-CTC-ACG-GTC-3’	5’-GAC-CGT-GAG-CAG-TGC-AGA-GCG-CAC-CG-3’
L209A	5’-G-GTG-CGC-TCT-GGA-GCG-CTC-ACG-GTC-ATG-3’	5’-CAT-GAC-CGT-GAG-CGC-TCC-AGA-GCG-CAC-C-3’
L210A	5’-G-CGC-TCT-GGA-CTG-GCC-ACG-GTC-ATG-GTG-3’	5’-CAC-CAT-GAC-CGT-GGC-CAG-TCC-AGA-GCG-C-3’
T211A	5’-CT-GGA-CTG-CTC-GCG-GTC-ATG-GTG-TGG-3’	5’-CCA-CAC-CAT-GAC-CGC-GAG-CAG-TCC-AG-3’
V212A	5’-GGA-CTG-CTC-ACG-GCC-ATG-GTG-TGG-GTG-3’	5’-CAC-CCA-CAC-CAT-GGC-CGT-GAG-CAG-TCC-3’

Table 6.1 Oligonucleotide primer sequences for QuikChange™ site-directed mutagenesis: Codons changed to introduce mutations are shown in **red**.

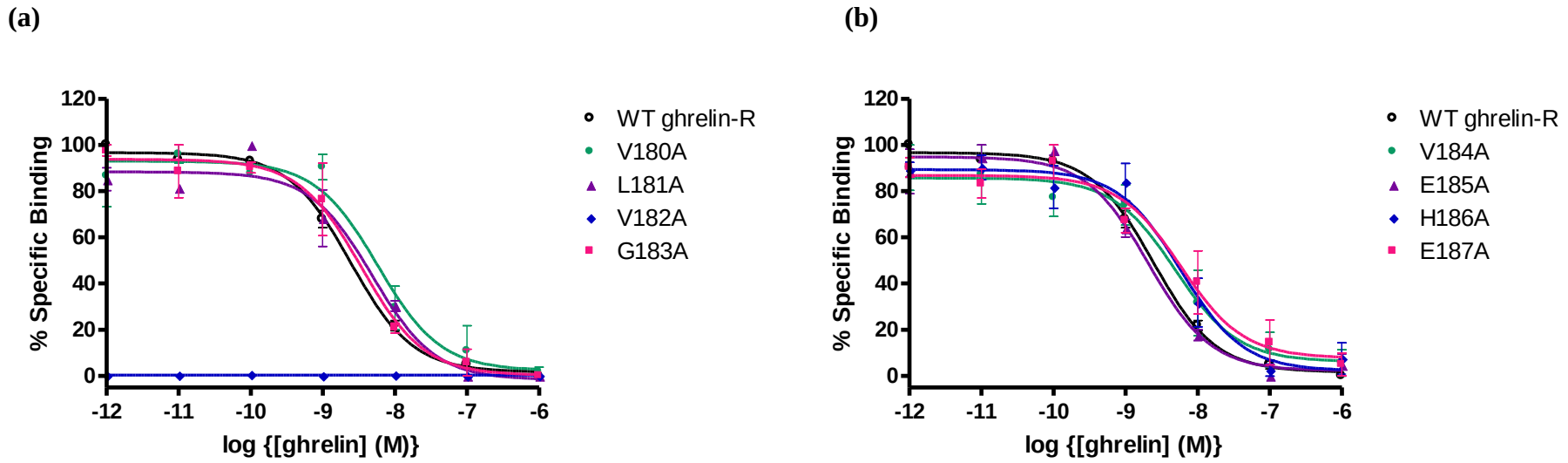


Figure 6.3 Ligand binding profiles for ECL2 mutant receptor constructs: Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with (a) WT ghrelin-R, V180A, L181A, V182A or G183A and (b) WT ghrelin-R, V184A, E185A, H186A or E187A. Values are expressed as a percentage of specific binding. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

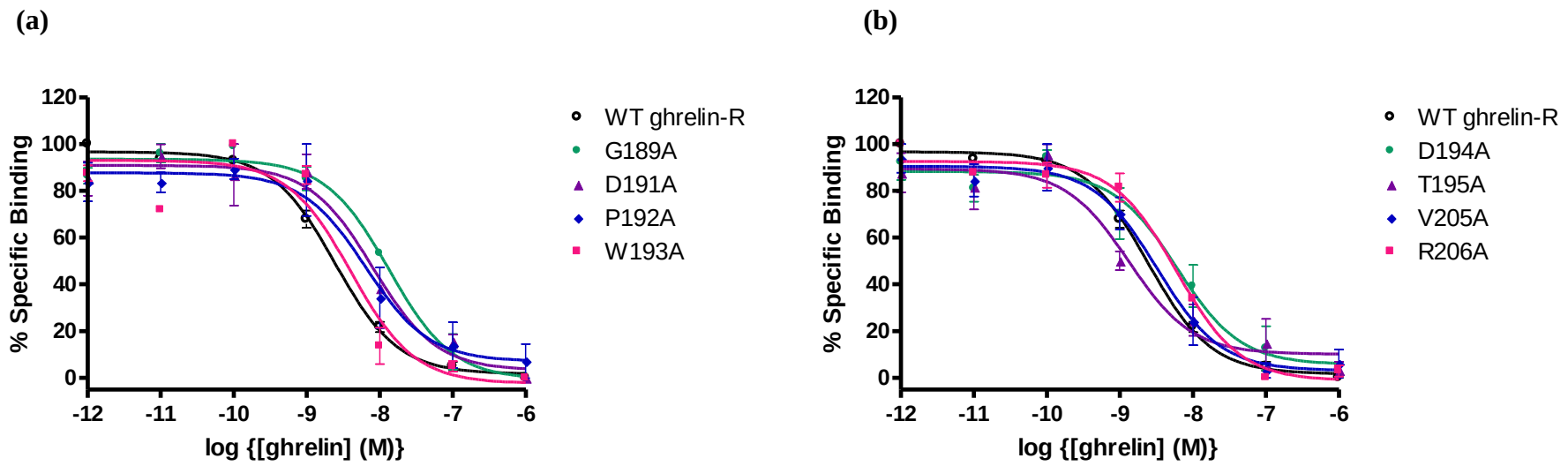


Figure 6.4 Ligand binding profiles for ECL2 mutant receptor constructs: Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with (a) WT ghrelin-R, G189A, D191A, P192A or W193A and (b) WT ghrelin-R, D194A, T195A, V205A or R206A. Values are expressed as a percentage of specific binding. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

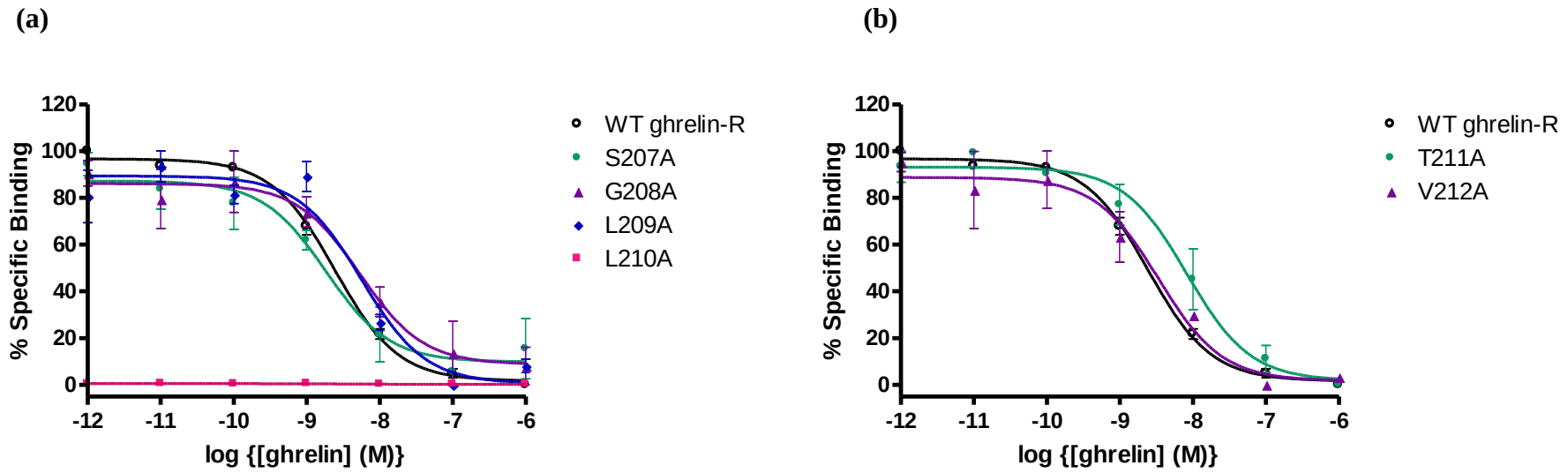


Figure 6.5 Ligand binding profiles for ECL2 mutant receptor constructs: Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with (a) WT ghrelin-R, S207A, G208A, L209A or L210A and (b) WT ghrelin-R, T211A or V212A. Values are expressed as a percentage of specific binding. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

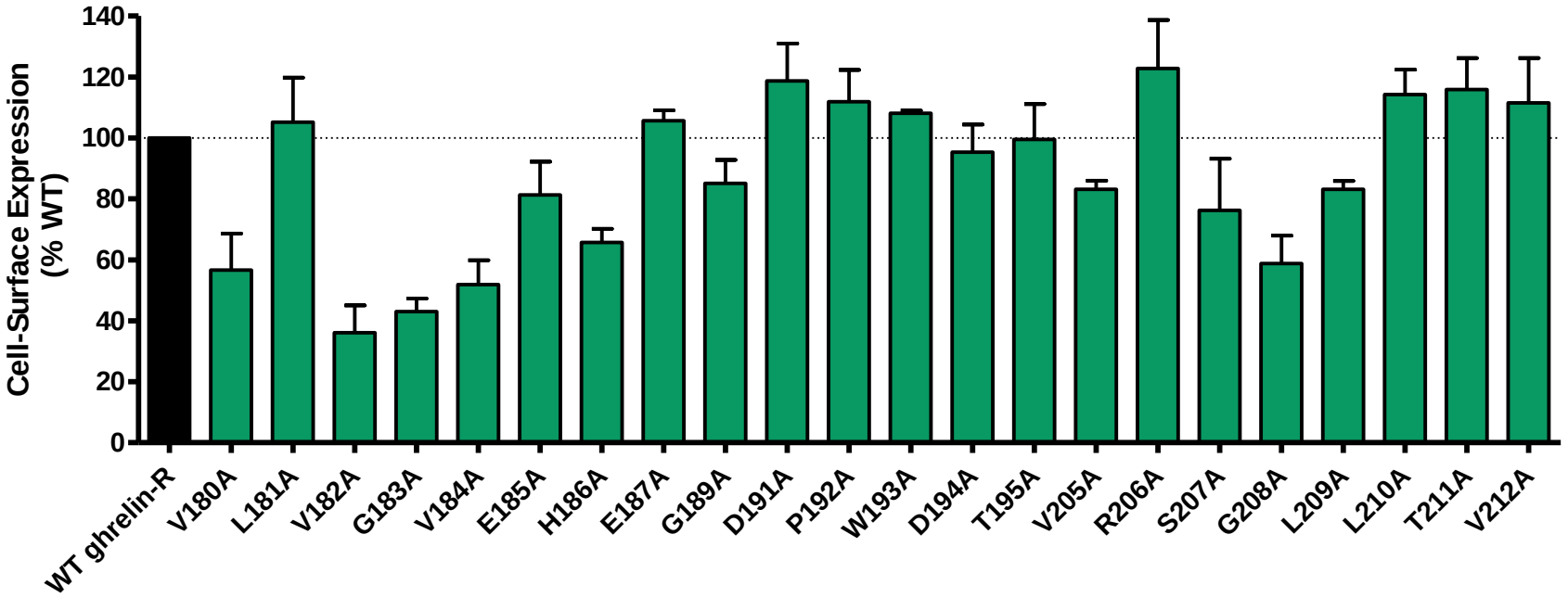


Figure 6.6 ELISA cell-surface expression profiles for ECL2 mutant receptor constructs: Assays were performed on HEK 293T cells transiently transfected with WT ghrelin-R, V180A, L181A, V182A, G183A, V184A, E185A, H186A, E187A, G189A, D191A, P192A, W193A, D194A, T195A, V205A, R206A, S207A, G208A, L209A, L210A, T211A or V212A. Values are expressed as a percentage of the WT ghrelin-R expression from experiments performed in parallel. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	Cell-Surface Expression (% WT) ± SEM
WT ghrelin-R	**8.61 ± 0.02**	**100**
V180A	8.23 ± 0.10	57 ± 12
L181A	8.31 ± 0.12	105 ± 15
V182A	NDB	36 ± 9
G183A	8.46 ± 0.06	43 ± 4
V184A	8.31 ± 0.06	52 ± 8
E185A	8.68 ± 0.07	81 ± 11
H186A	8.23 ± 0.07	66 ± 4
E187A	8.23 ± 0.07	106 ± 3
G189A	7.86 ± 0.07	85 ± 8
D191A	8.09 ± 0.09	119 ± 12
P192A	8.19 ± 0.07	112 ± 10
W193A	8.39 ± 0.18	108 ± 1
D194A	8.22 ± 0.08	95 ± 9
T195A	9.05 ± 0.10	100 ± 12
V205A	8.49 ± 0.06	83 ± 3
R206A	8.22 ± 0.08	123 ± 16
S207A	8.73 ± 0.08	76 ± 19
G208A	8.27 ± 0.06	59 ± 9
L209A	8.25 ± 0.12	83 ± 3
L210A	NDB	114 ± 8
T211A	8.08 ± 0.07	116 ± 10
V212A	8.47 ± 0.09	112 ± 15

Table 6.2 Ligand binding and cell-surface expression for ECL2 mutant receptor constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Yellow indicates an increase in pIC_{50} > 0.5 log units or a reduction in cell-surface expression >30 % of the WT ghrelin-R expression. Orange indicates an increase in pIC_{50} > 0.75 log units or a reduction in cell-surface expression > 50 % of the WT ghrelin-R expression. Red indicates no detectable binding.

The G189A, D191A and T211A mutants decreased the binding affinity of ghrelin; G189A by ~5-fold, and D191A and T211A by ~3-fold (Fig 6.4-6.5, Table 6.2). V182A and L210A exhibited no detectable binding of [^{125}I]ghrelin at the concentration used in the assay (Fig 6.3 & 6.5, Table 6.2). V182A was also particularly detrimental to cell-surface expression (36 % of WT). In contrast, L210A did not decrease cell-surface expression. V180A, G183A, V184A, H186A and G208A also exhibited decreased cell-surface expression (40-70 % of WT), although none of these mutants affected binding. All of the other alanine substitution mutants of ECL2 residues expressed at the cell surface at levels similar (70-100 %) to that seen for the WT ghrelin-R (Fig 6.6, Table 6.2).

6.2.2 Extended ECL2 mutagenesis study

The residues flanking the conserved ECL2 cysteine (Cys198$^{(C)}$) and the residues of the putative *N*-linked glycosylation site in ECL2 were chosen for extended analysis. The mutants included in the extended study were N188A, T190A, N196A, E197A, C198A, R199A, P200A, T201A, E202A, F203A, F203G, A204G, A204E and A204V. Additional residues within the TM helices, which have been implicated in potential interactions with ECL2, were also included in this extended study. These residues were Cys116$^{(3.25)}$, the highly conserved cysteine that forms a disulphide bond with Cys198$^{(C)}$, Arg$^{6.55}$, Phe$^{6.58}$ and Lys$^{6.60}$. The sequences of the oligonucleotide primers for site-directed mutagenesis are detailed in Table 6.1 and Table 6.3.

6.2.2.1 The role of Cys116$^{(3.25)}$ and Cys198$^{(C)}$

Two cysteine residues are highly conserved in GPCRs; one in ECL2 (Cys$^{(C)}$) and the other at the extracellular boundary of TMIII (Cys$^{3.25}$). These cysteines form a disulphide bond in most Family A GPCRs; Cys$^{3.25}$ is found in 90 % of Family A receptors (Mirzadegan *et al.*, 2003). Cys116$^{(3.25)}$ and Cys198$^{(C)}$ in the ghrelin-R were mutated individually to alanine and mutated in tandem as a double mutant, C116A/C198A. The C116A, C198A and C116A/C198A mutants were all unable to bind ghrelin at the radioligand concentration available, or to signal in response to ghrelin (Fig 6.7-6.8, Table 6.4). Each mutation also resulted in a loss of constitutive activity to levels similar to that seen for the mock-transfected cells and all resulted in substantially decreased cell-surface expression; C116A (23 %), C198A (33 %) and C116A/C198A (27 %) (Fig 6.7-6.8, Table 6.4).

Mutant Receptor Construct	Sense Oligonucleotide	Antisense Oligonucleotide
C116A	5’-C-GGC-GAC-CTC-CTC-GCC-AAA-CTC-TTC-CAA-TTC-G-3’	5’-C-GAA-TTG-GAA-GAG-TTT-GGC-GAG-GAG-GTC-GCC-G-3’
F203G	5’-CGC-CCC-ACC-GAG-GGT-GCG-GTG-CGC-TCT-G-3’	5’-C-AGA-GCG-CAC-CGC-ACC-CTC-GGT-GGG-GCG-3’
A204E	5’-CGC-CCC-ACC-GAG-TTT-GAG-GTG-CGC-TCT-GGA-CTG-C-3’	5’-G-CAG-TCC-AGA-GCG-CAC-CTC-AAA-CTC-GGT-GGG-GCG-3’
A204V	5’-CCC-ACC-GAG-TTT-GTG-GTG-CGC-TCT-GG-3’	5’-CC-AGA-GCG-CAC-CAC-AAA-CTC-GGT-GGG-3’
R6.55A	5’-CCC-TTC-CAC-GTA-GGG-GCA-TAT-TTA-TTT-TCC-3’	5’-GGA-AAA-TAA-ATA-TGC-CCC-TAC-GTG-GAA-GGG-3’
F6.58A	5’-GTA-GGG-CGA-TAT-TTA-GCT-TCC-AAA-TCC-TTT-GAG-C-3’	5’-G-CTC-AAA-GGA-TTT-GGA-AGC-TAA-ATA-TCG-CCC-TAC-3’
K6.60A	5’-GA-TAT-TTA-TTT-TCC-GCA-TCC-TTT-GAG-CC-3’	5’-GG-CTC-AAA-GGA-TGC-GGA-AAA-TAA-ATA-TC-3’

Table 6.3 Oligonucleotide primer sequences for QuikChange™ site-directed mutagenesis: Codons changed to introduce mutations are shown in **red**.

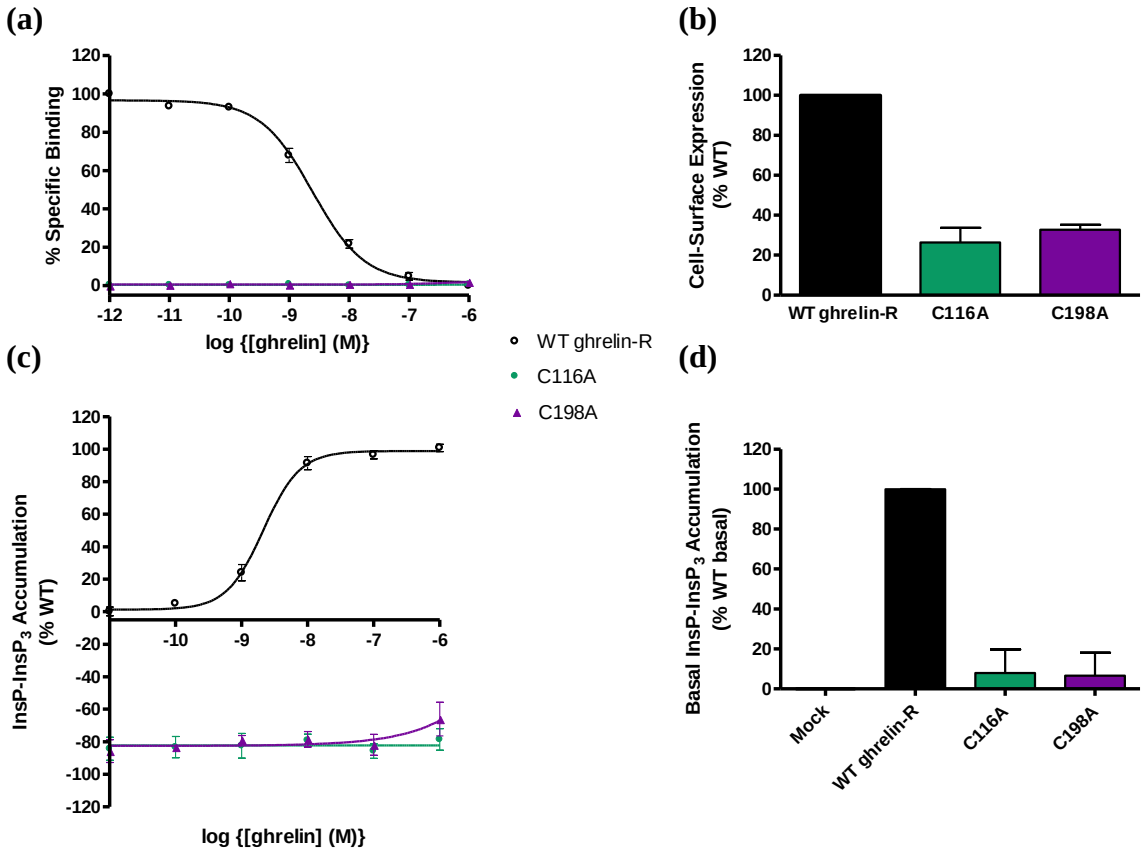


Figure 6.7 Ligand binding, cell-surface expression and intracellular signalling profiles for C116A and C198A mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, C116A or C198A expressed as a percentage of specific binding. (b) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. (c) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, C116A or C198A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (d) Basal InsP-$InsP_3$ accumulation as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

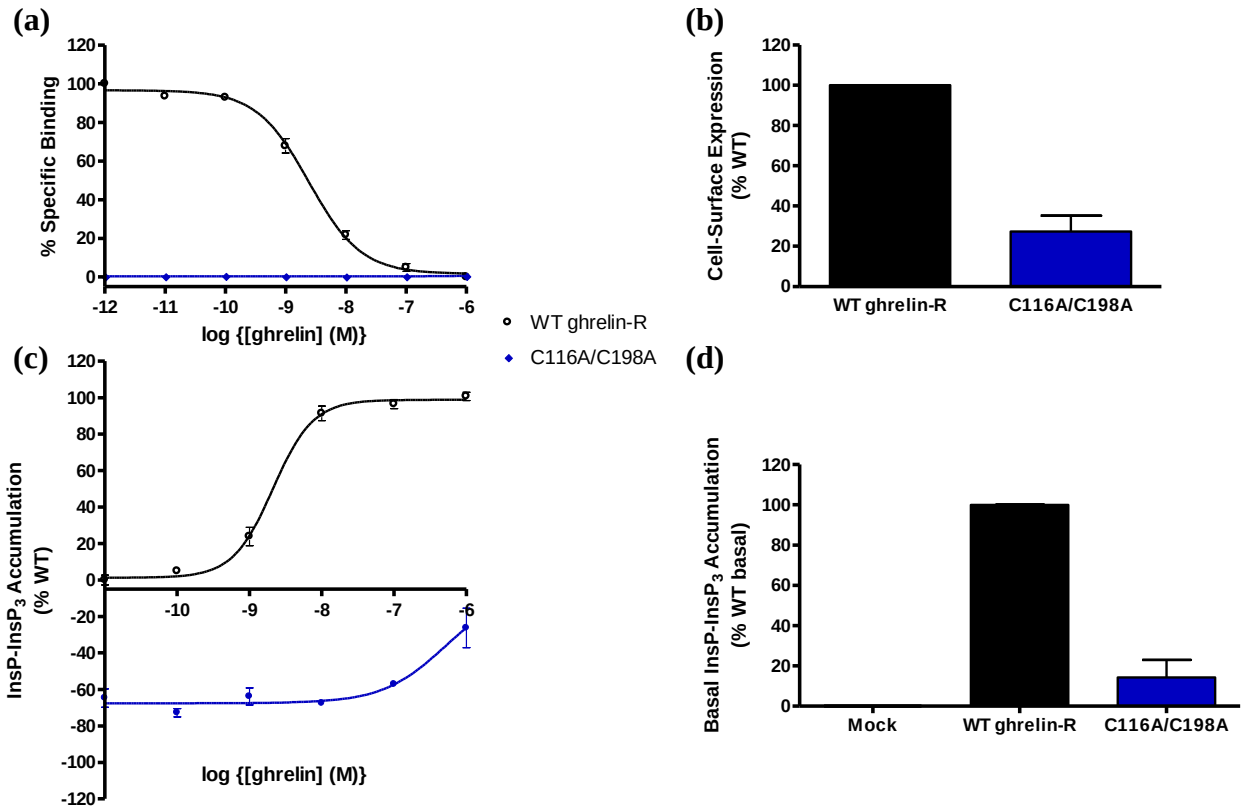


Figure 6.8 Ligand binding, cell-surface expression and intracellular signalling profiles for the C116A/C198A double mutant receptor construct: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or C116A/C198A expressed as a percentage of specific binding. (b) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. (c) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or C116A/C198A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (d) Basal InsP-$InsP_3$ accumulation as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation				Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	Basal (% WT basal) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**	**100**
C116A	NDB	NDS	-84 ± 7	8 ± 12	NDS	23 ± 7
C198A	NDB	NDS	-86 ± 7	7 ± 11	NDS	33 ±3
C116A/C198A	NDB	NDS	-65 ± 5	14 ± 9	NDS	27 ± 8

Table 6.4 Ligand binding, intracellular signalling and cell-surface expression for C116A, C198A and C116A/C198A mutant constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Red indicates no detectable binding, no detectable signalling, > 60 % reduction in basal activity and > 70 % reduction in cell-surface expression compared to WT. Orange indicates > 50 % reduction in cell-surface expression compared to WT.

6.2.2.2 Asn188 and Thr190: A potential glycosylation motif

Residues Asn188$^{(C-10)}$ and Thr190$^{(C-8)}$ form part of an *N*-linked glycosylation consensus motif (NxS/T) within ECL2, where 'x' is any amino acid except for proline. Not all GPCR glycosylation consensus sequences are utilised, however, regions within the N-terminus and ECL2 are often glycosylated suggesting the potential for glycosylation of the ghrelin-R at this locus. For many GPCRs, glycosylation is essential for normal receptor function (Wheatley *et al.*, 1999). In order to investigate the potential role of glycosylation of the ghrelin-R within ECL2, Asn188$^{(C-10)}$ and Thr190$^{(C-8)}$ were mutated individually to alanine as part of the alanine scan and were subsequently characterised. N188A displayed 'WT-like' binding affinity for ghrelin and 'WT-like' signalling except for a small reduction in constitutive activity (-28 %) (Fig 6.9, Table 6.5). T190A was unable to bind [^{125}I]ghrelin at the concentrations used and signalling through the $InsP_3$ pathway was affected to a small degree with a 2-fold increase in EC_{50} and a reduction in basal constitutive activity (-35 %) (Fig 6.9, Table 6.5). The T190A mutation also resulted in a small decrease in cell-surface expression (68 % of WT) whereas N188A was expressed at levels similar to the WT receptor (Fig 6.9, Table 6.5).

6.2.2.3 The role of key ECL2 residues in ghrelin-R function

N196A, E197A, R199A, P200A, T201A and E202A were characterised for radioligand binding, cell-surface expression and intracellular signalling capabilities. R199A, P200A and E202A displayed 'WT-like' binding affinity for ghrelin (Fig 6.12, 6.13 & 6.15, Table 6.6). N196A, E197A and T201A displayed no detectable binding at the [^{125}I]ghrelin concentration used (Fig 6.10, 6.11 & 6.14, Table 6.6). All of the mutants retained the ability to signal through the InsP pathway, although N196A, E197A and T201A displayed increases in EC_{50} (9.6-fold, 4.5-fold and 4.2-fold respectively). (Fig 6.10, 6.11 & 6.14, Table 6.6). Basal constitutive activity was affected in all mutants to varying degrees, except R199A, which retained 'WT-like' constitutive activity (Fig 6.10-6.15, Table 6.6). N196A was the mutant that was most detrimental to basal signalling, reducing the constitutive signalling to 26 % of the WT basal (Fig 6.10 (d), Table 6.6) (-120 % when normalised to WT ghrelin-induced maximum, Fig 6.10 (c)). E197A, P200A, T201A and E202A displayed constitutive activity of -35 %, -42 %, -45 % and -32 % of WT ghrelin-induced maximum, respectively. E_{max} was reduced in N196A and E197A mutants (57 % and 67 % of WT, respectively) and increased to 161 % for the T201A construct. Cell-surface expression was 'WT-like' for all mutants except N196A which reduced expression to 51 % of the WT (Fig 6.10-6.15, Table 6.6).

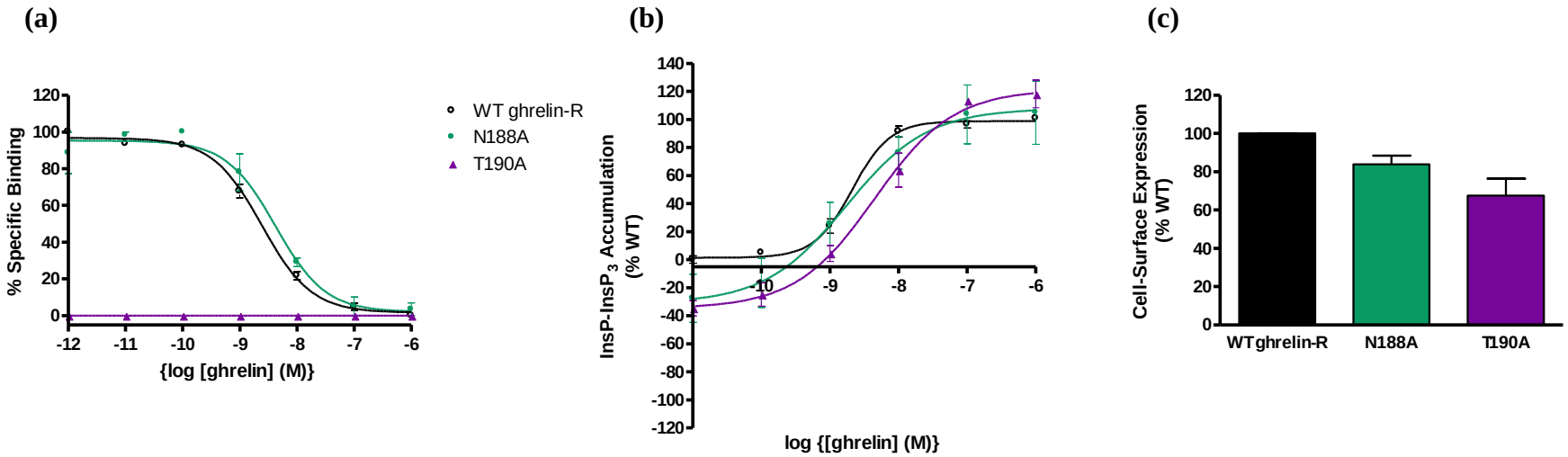


Figure 6.9 Ligand binding, intracellular signalling and cell-surface expression profiles for N188A and T190A mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, N188A or T190A expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, N188A or T190A. Values are expressed as a percentage of the WT ghrelin-R, ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation			Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**
N188A	8.37 ± 0.06	1.72 ± 0.30	-28 ± 17	105 ± 23	84 ± 4
T190A	NDB	4.46 ± 0.90	-35 ± 5	118 ± 10	68 ± 9

Table 6.5 Ligand binding, intracellular signalling and cell-surface expression for N188A and T190A mutant receptor constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Red indicates no detectable binding. Yellow indicates a > 20 % decrease in basal activity compared to WT or a decrease in cell-surface expression > 30 % of WT.

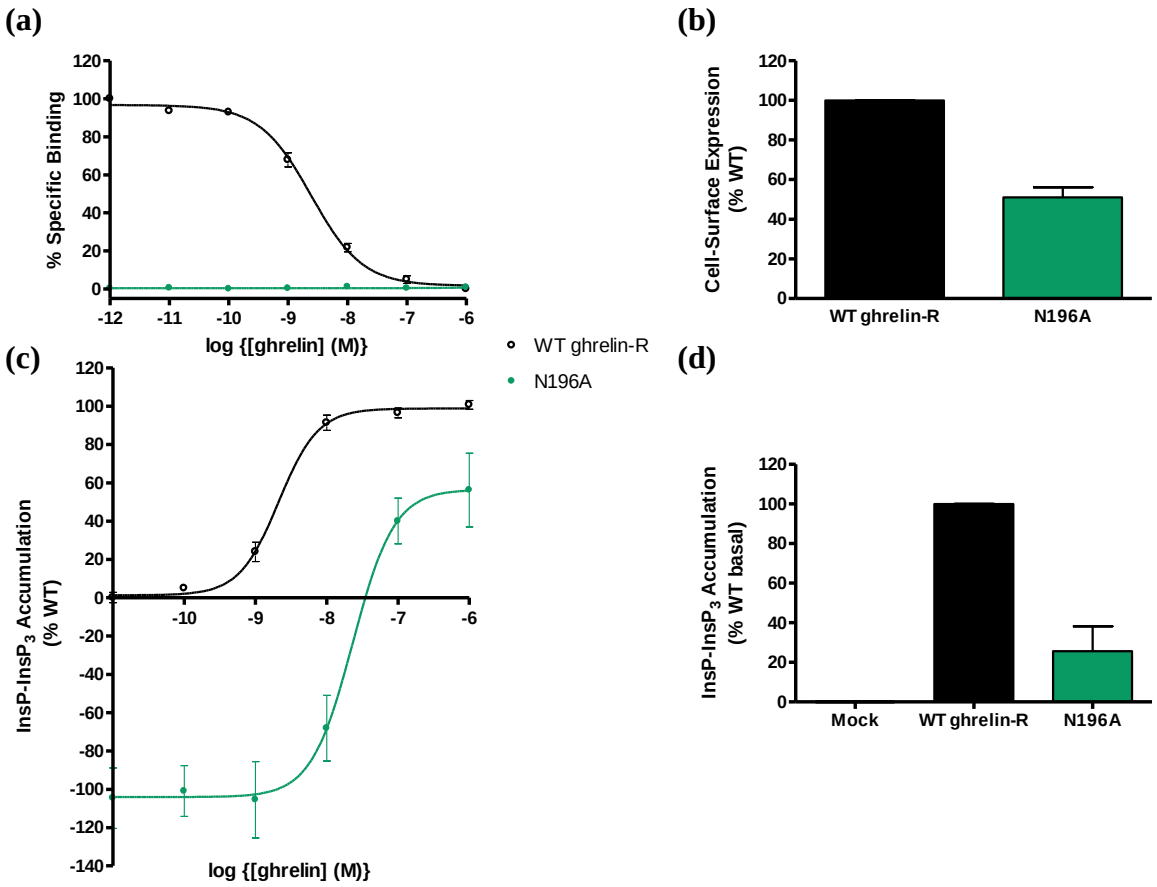


Figure 6.10 Ligand binding, intracellular signalling and cell-surface expression profiles for N196A mutant receptor construct: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or N196A expressed as a percentage of specific binding. (b) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. (c) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or N196A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (d) Basal InsP-$InsP_3$ accumulation as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

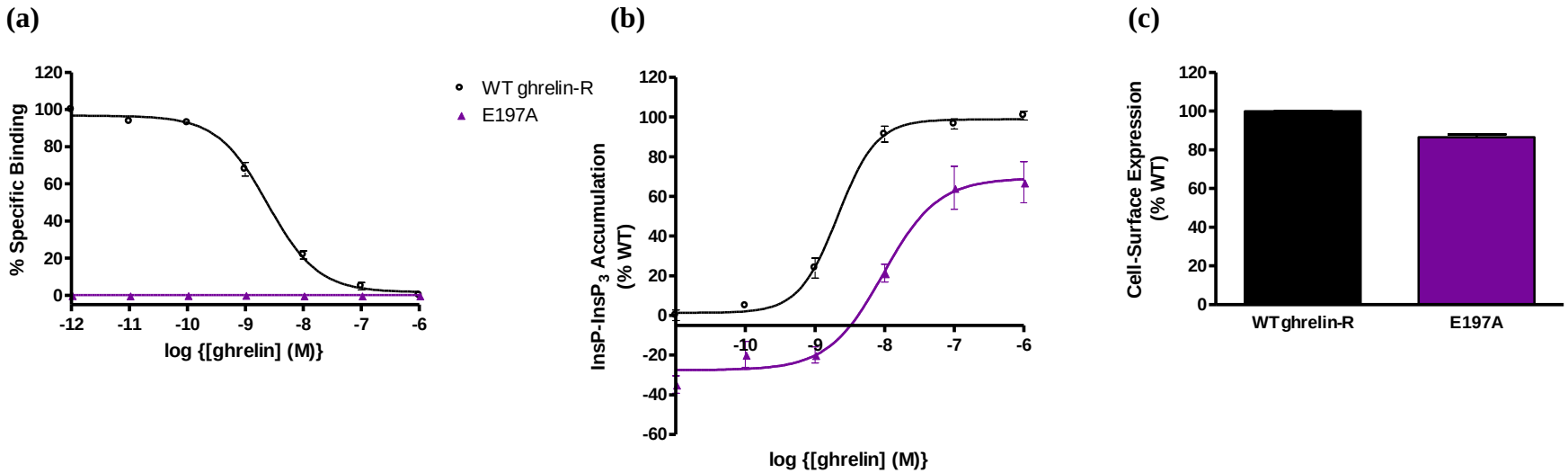


Figure 6.11 Ligand binding, intracellular signalling and cell-surface expression profiles for E197A mutant receptor construct: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or E197A expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or E197A.Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

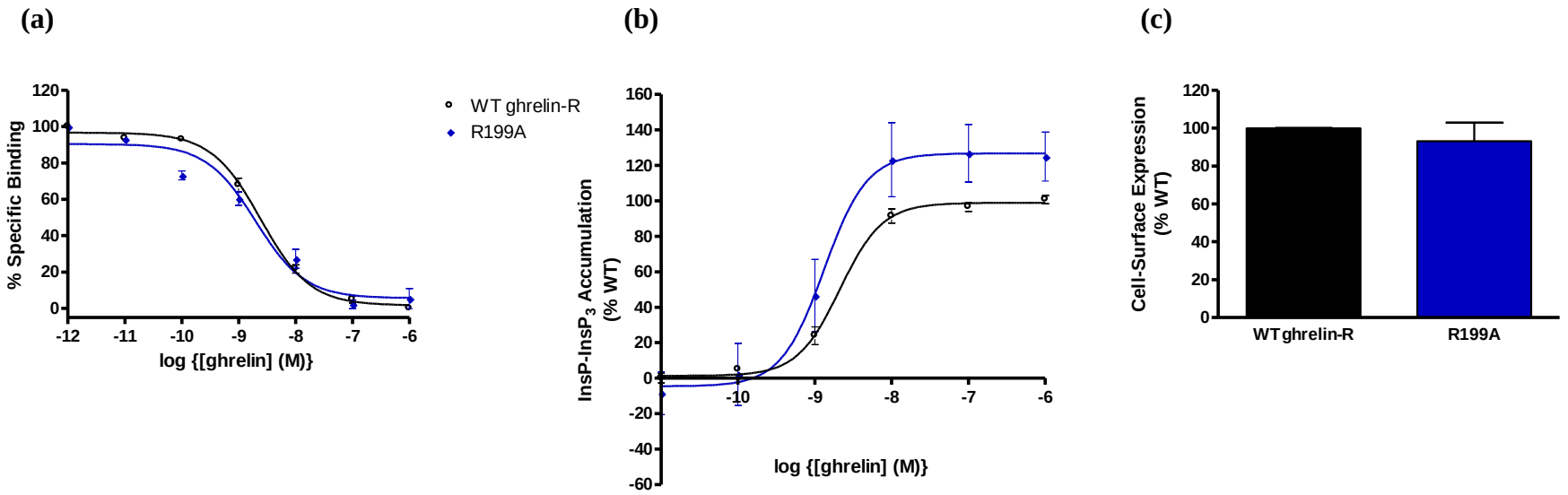


Figure 6.12 Ligand binding, intracellular signalling and cell-surface expression profiles for R199A mutant receptor construct: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or R199A expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or R199A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

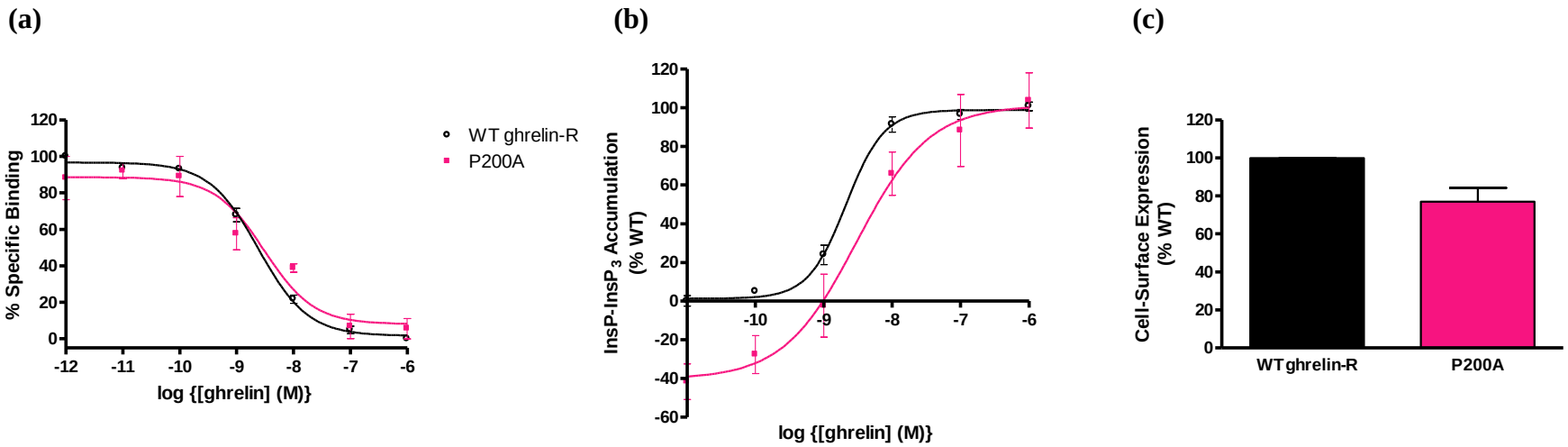


Figure 6.13 Ligand binding, intracellular signalling and cell-surface expression profiles for P200A mutant receptor construct: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or P200A expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or P200A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

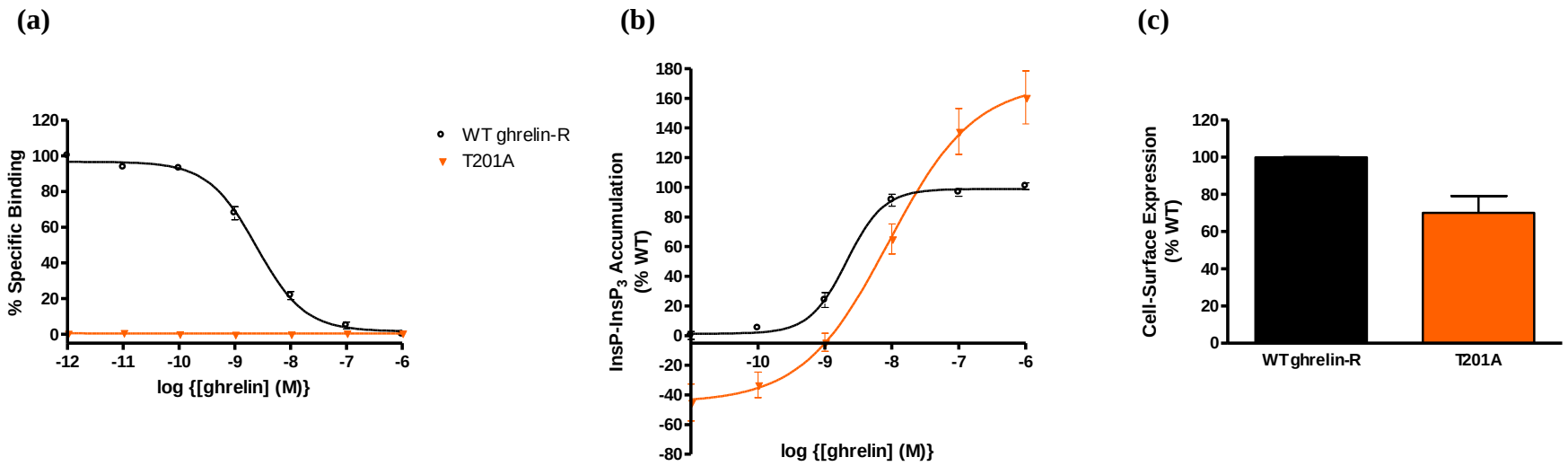


Figure 6.14 Ligand binding, intracellular signalling and cell-surface expression profiles for T201A mutant receptor construct: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or T201A expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or T201A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

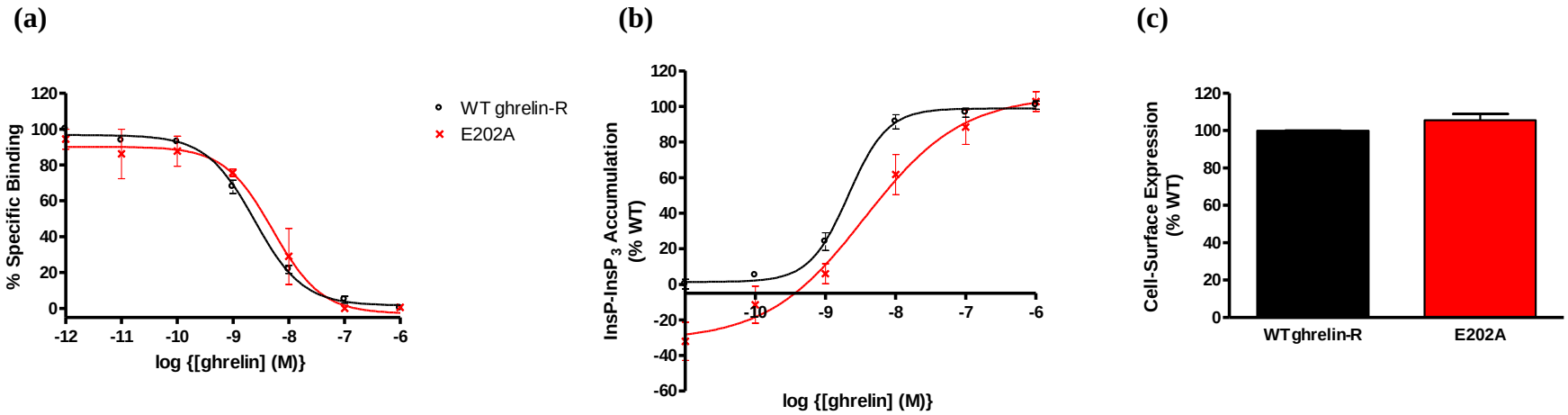


Figure 6.15 Ligand binding, intracellular signalling and cell-surface expression profiles for E202A mutant receptor construct: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or E202A expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or E202A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Mutant	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation				Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	Basal (% WT basal) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**	**100**
N196A	NDB	20.37 ± 12.38	-120 ± 17	26 ± 13	57 ± 18	51 ± 5
E197A	NDB	9.54 ± 5.25	-35 ± 4	-	67 ± 10	87 ± 1
R199A	8.70 ± 0.14	1.32 ± 0.22	-9 ± 12	-	125 ± 14	93 ± 10
P200A	8.51 ± 0.14	3.00 ± 2.97	-42 ± 9	-	101 ± 19	77 ± 7
T201A	NDB	8.89 ± 1.62	-45 ± 12	-	161 ± 18	70 ± 9
E202A	8.28 ± 0.05	3.60 ± 2.23	-32 ± 11	-	103 ± 6	106 ± 3

Table 6.6 Ligand binding, intracellular signalling and cell-surface expression for N196A, E197A, R199A, P200A, T201A and E202A mutant receptor constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Red indicates no detectable binding, > 5-fold increase in EC_{50} compared to WT or a reduction in basal activity > 60 % of WT. Orange indicates an increase in EC_{50} > 4-fold or a reduction in basal activity > 40 % of WT. Yellow indicates a > 20 % decrease in basal activity compared to WT, a decrease in E_{max} > 30 % or a decrease in cell-surface expression > 30 % of WT. Blue indicates and increase in E_{max} > 160 % of WT.

6.2.2.4 The role of Phe203$^{(C+5)}$ and Ala204$^{(C+6)}$ in ghrelin-R function

Phe203$^{(C+5)}$ was mutated to alanine and Ala204$^{(C+6)}$ to glycine as part of the "alanine scan" of ECL2. Both F203A and A204G displayed 'WT-like' affinity for ghrelin, 'WT-like' EC_{50} and 'WT-like' expression, although F203A resulted in a small decrease in basal activity (Fig 6.16 & 6.17, Table 6.7 & 6.8). Interestingly, A204G resulted in a dramatic increase in efficacy with an E_{max} of 217 % of the WT (Fig 6.17, Table 6.8). To investigate the role of these two residues in more detail, Phe203$^{(C+5)}$ was mutated to glycine examining the effects of replacing phenylalanine with a residue that would allow greater flexibility within the loop. F203G had a similar phenotype to F203A, displaying 'WT-like' characteristics except a small decrease in agonist-independent activity (Fig 6.16, Table 6.7).

Ala204$^{(C+6)}$ has previously been identified as a key residue in the ghrelin-R; the naturally occurring mutation A204E in humans, results in short stature presumably due to a loss of ghrelin-R constitutive activity (Pantel *et al.*, 2006). The A204E mutant was introduced to complete the investigation of Ala204 and to facilitate the probing of potential interactions occurring in the A204E receptor that result in the observed decrease in constitutive activity. It was confirmed that A204E results in a marked loss of ghrelin-R basal activity. Unlike previous studies (Pantel *et al.*, 2006; Liu *et al.*, 2007a) the A204E mutant was found to express well at the cell-surface (80 % of WT), but was unable to bind [^{125}I]ghrelin at the concentrations used (Fig 6.17, Table 6.8). Valine was also introduced at position 204 to examine the effects of maintaining a small hydrophobic residue at this locus. A204V displayed characteristics similar to the WT ghrelin-R (Fig 6.17, Table 6.8), suggesting a hydrophobic residue is required at this position but that there is not a specific requirement for alanine. The data suggested that the effects that occur in the A204E mutation are not due to the loss of a functionally important alanine, but rather to the introduction of a glutamate.

6.2.3 Investigation of the role of residues at the extracellular face of TMVI

Three residues located at the TMVI-ECL3 interface were mutated to investigate their roles in ligand binding and ghrelin-R activation. Studies on the free fatty acid receptor 1 (FFAR1) have demonstrated that ionic interactions occur between glutamate residues in ECL2 and arginine residues in TMs V and VII, and that these interactions act to stabilise the inactive receptor conformation, as mutation of the glutamate residues to alanine resulted in constitutive activity (Sum *et al.*, 2009). Similarly, in the β_2AR, an ionic interaction occurs between Asp192$^{(C+1)}$ in ECL2 and Lys$^{7.32}$ and this is broken upon receptor activation (Bokoch *et al.*, 2010). It was hypothesised that interactions in the ghrelin-R between A204E and

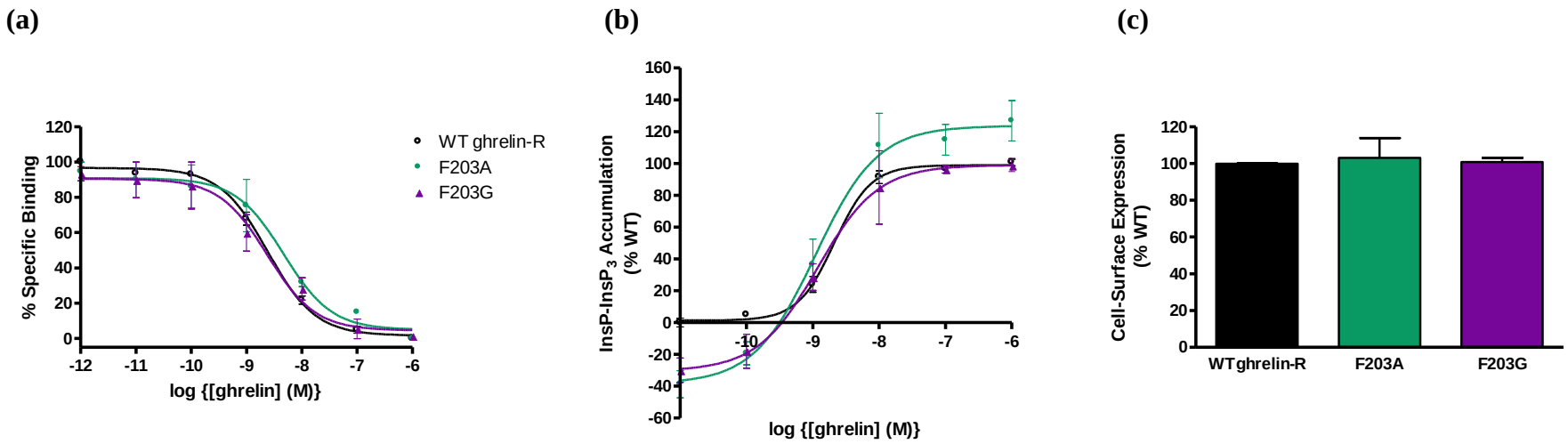


Figure 6.16 Ligand binding, intracellular signalling and cell-surface expression profiles for Phe203 mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, F203A or F203G expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, F203A or F203G. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation			Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**
F203A	8.33 ± 0.07	1.11 ± 0.43	-39 ± 8	127 ± 13	103 ± 11
F203G	8.63 ± 0.07	1.18 ± 0.12	-30 ± 8	99 ± 4	101 ± 2

Table 6.7 Ligand binding, intracellular signalling and cell-surface expression for Phe203 mutant receptor constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Yellow indicates a > 20 % decrease in basal activity compared to WT.

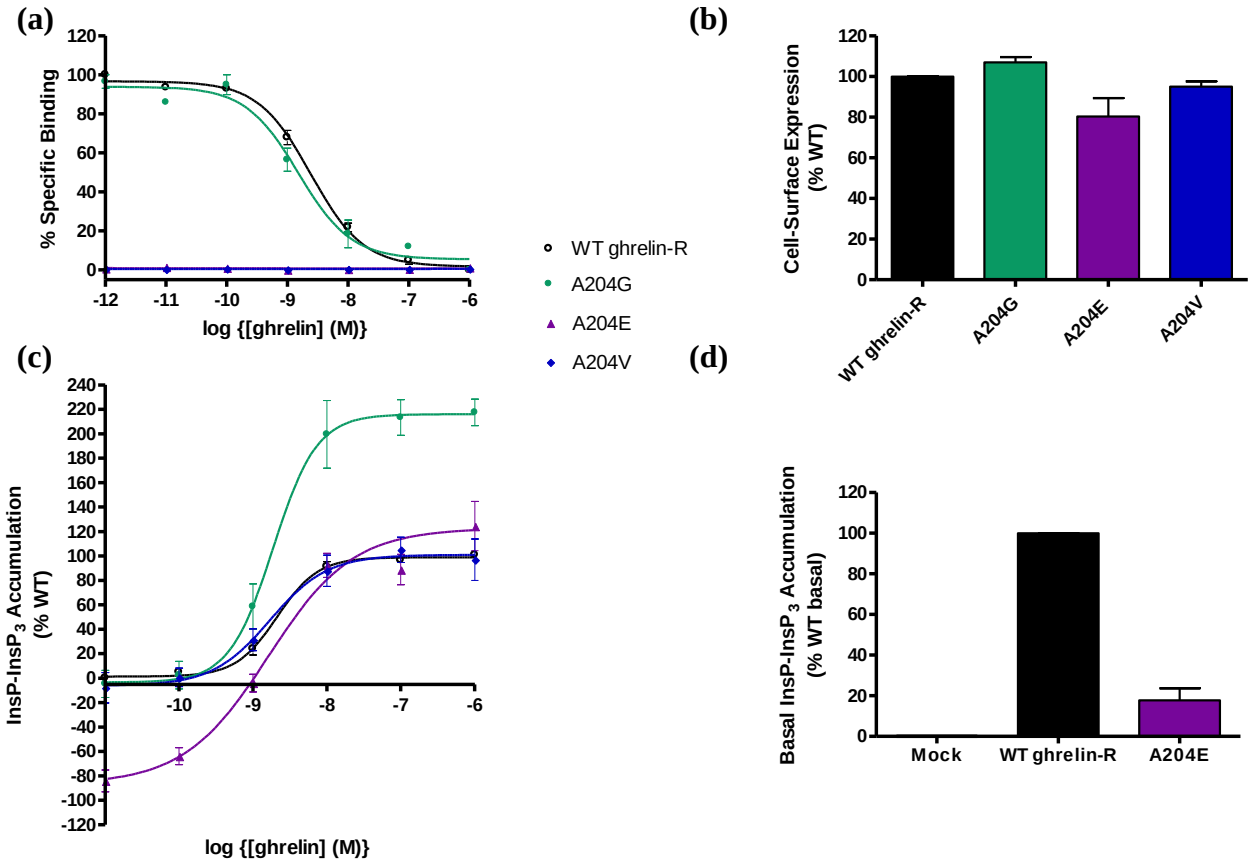


Figure 6.17 Ligand binding, intracellular signalling and cell-surface expression profiles for Ala204 mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, A204G, A204E or A204V expressed as a percentage of specific binding. (b) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. (c) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, A204G, A204E or A204. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (d) Basal InsP-$InsP_3$ accumulation as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation				Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	Basal (% WT basal) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**	**100**
A204G	8.82 ± 0.09	1.87 ± 0.14	-5 ± 11	-	217 ± 11	107 ± 3
A204E	NDB	1.62 ± 1.14	-84 ± 9	18 ± 6	124 ± 20	80 ± 9
A204V	NDB	1.70 ± 0.59	-8 ± 12	-	97 ± 17	95 ± 3

Table 6.8 Ligand binding, intracellular signalling and cell-surface expression for Ala204 mutant receptor constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Red indicates no detectable binding or a > 60 % reduction in basal activity compared to WT. Pink indicates an increase in E_{max} > 190 %.

positively charged residues at the extracellular TM boundaries might be responsible for the decreased constitutive activity found with the A204E mutant. Removal of the positive charge by mutation to alanine might therefore remove an interaction partner and allow the restoration of WT constitutive activity. $Arg^{6.55}$ and $Lys^{6.60}$ at the top of TMVI were mutated to alanine to investigate potential interactions. $Arg^{6.55}$ has previously been identified as important in both constitutive and agonist-induced InsP-$InsP_3$ accumulation in the ghrelin-R (Holst *et al.*, 2004a). The same results were found for R6.55A in this study. There was no detectable binding or signalling and cell-surface expression was diminished to 50 % of WT (Fig 6.18, Table 6.9). K6.60A retained a 'WT-like' EC_{50}, but reduced basal activity to -32 % of the WT ghrelin-induced maximal response. The E_{max} was also slightly increased (142 % of WT). Interestingly, the pIC_{50} was decreased by ~3-fold, suggesting the formation of a receptor conformation that had a higher affinity for ghrelin. Cell-surface expression for the K6.60A mutant was similar to that seen for the WT ghrelin-R (117 %) (Fig 6.18, Table 6.9).

In order to investigate the potential interactions between ECL2 and the positive charges at the top of TMVI, double mutants were produced. A204E/R6.55A was unable to bind [^{125}I]ghrelin or to signal through the InsP pathway either in response to ghrelin, or in an agonist-independent manner. Cell-surface expression of this double mutant was also dramatically decreased to only 29 % of the WT ghrelin-R (Fig 6.19, Table 6.9). These results suggest that removal of the positive charge at position 6.55 does not prevent the loss of constitutive activity observed for the A204E mutant, but this may be explained by the $Arg^{6.55}$ mutation alone being particularly detrimental to constitutive activity and ghrelin-induced signalling. The A204E/K6.60A mutant also failed to recover the loss of constitutive activity observed for the A204E mutant. This double mutant retained the ability to signal in response to ghrelin, but displayed a 6.8-fold increase in EC_{50} and was unable to bind ghrelin at the radioligand concentration used (Fig 6.19, Table 6.9).

To further investigate the extracellular domain of the ghrelin-R, $Phe^{6.58}$ was included in the study. $Phe^{6.58}$ was mutated to alanine to investigate the role of this aromatic residue in ghrelin binding and activation. Residue 6.58 has been shown to be important for ligand binding in a number of Family A GPCRs (Bhogal *et al.*, 1994; Leong *et al.*, 1994; Yamano *et al.*, 1995; Coetsee *et al.*, 2008). F6.58A expressed well at the cell surface but resulted in a loss of ghrelin binding and affected the ability of the receptor to signal. The ghrelin potency was decreased (EC_{50} = 18.32 nM), basal activity reduced to -26 % of the WT ghrelin-induced maximum and the E_{max} was decreased to only 49 % of WT (Fig 6.20, Table 6.10).

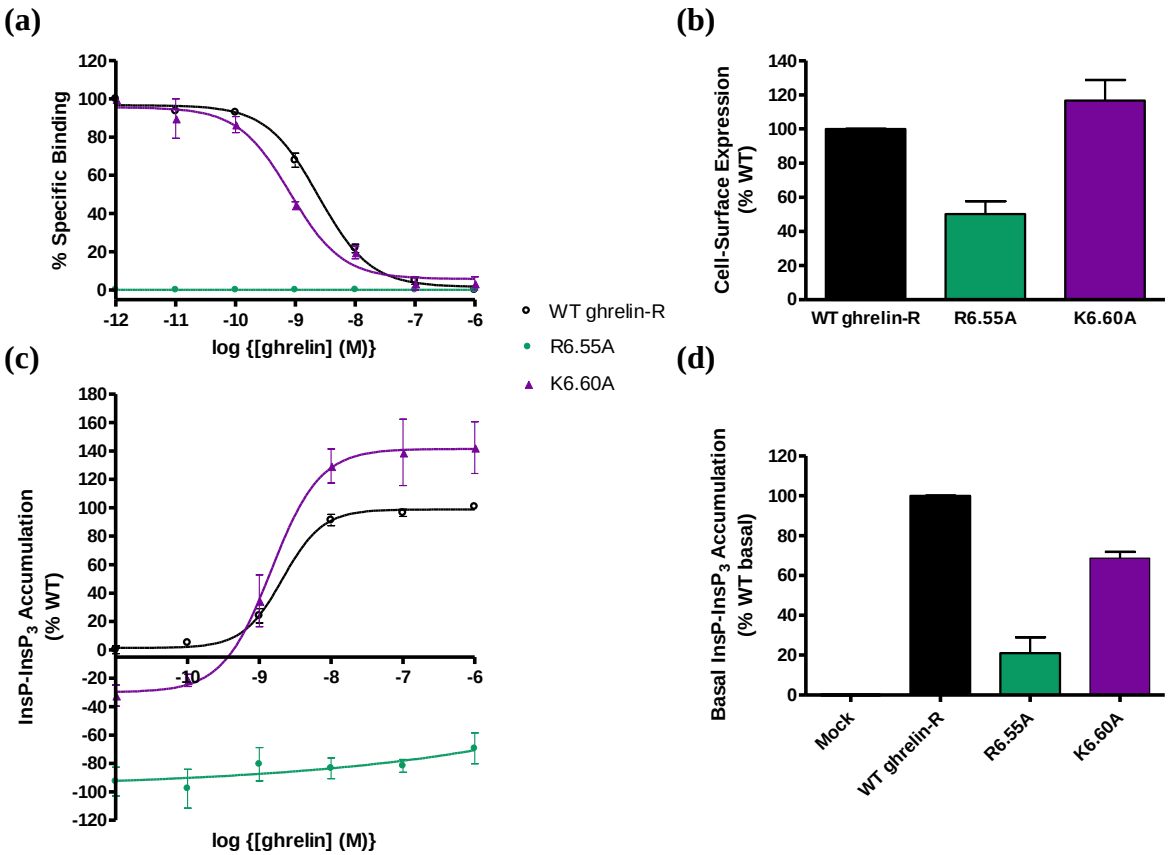


Figure 6.18 Ligand binding, intracellular signalling and cell-surface expression profiles for Arg$^{6.55}$ and Lys$^{6.60}$ mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, R6.55A or K6.60A expressed as a percentage of specific binding. (b) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. (c) Ghrelin-induced InsP-InsP$_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, R6.55A or K6.60A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (d) Basal InsP-InsP$_3$ accumulation as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

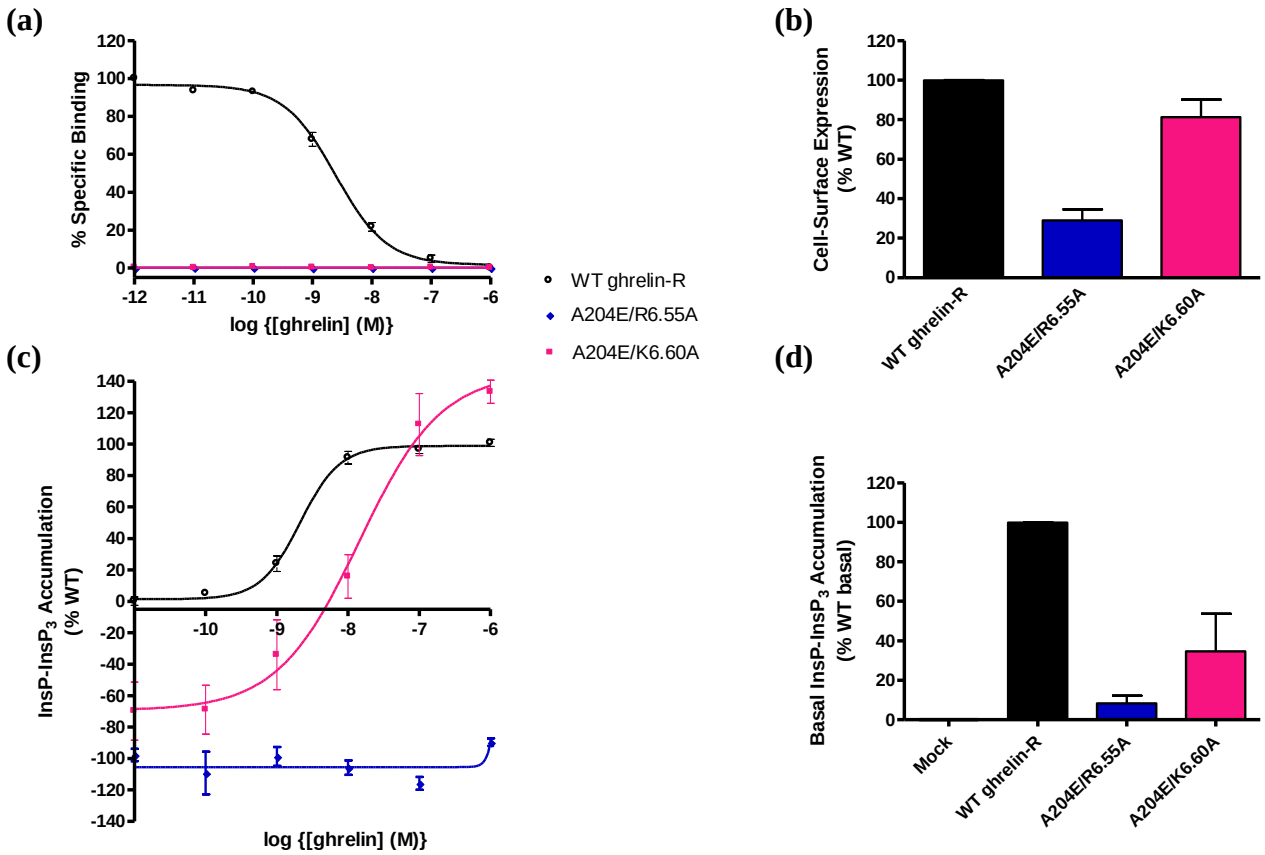


Figure 6.19 Ligand binding, intracellular signalling and cell-surface expression profiles for A204E/R6.55A and A204E/K6.60A double mutant receptor constructs: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R, A204E/R6.55A or A204E/K6.60A expressed as a percentage of specific binding. (b) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. (c) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R, A204E/R6.55A or A204E/K6.60A. Values are expressed as a percentage of the WT ghrelin-R ghrelin-induced maximum from experiments performed in parallel. (d) Basal InsP-$InsP_3$ accumulation as a percentage of WT basal. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation				Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT max) ± SEM	Basal (% WT basal) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**	**100**
R6.55A	NDB	NDS	-93 ± 10	21 ± 8	NDS	50 ± 8
K6.60A	9.09 ± 0.07	1.47 ± 0.16	-32 ± 7	69 ± 3	142 ± 18	117 ± 12
A204E/R6.55A	NDB	NDS	-98 ± 4	8 ± 4	NDS	29 ± 6
A204E/K6.60A	NDB	14.35 ± 8.79	-70 ± 18	35 ± 19	133 ± 7	81 ± 9

Table 6.9 Ligand binding, intracellular signalling and cell-surface expression for R6.55A, K6.60A and Ala204 double mutant receptor constructs: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Red indicates no detectable binding, no detectable signalling, > 5-fold increase in EC_{50}, > 60 % reduction in basal activity compared to WT or a decrease in cell-surface expression > 70 %. Orange indicates a decrease in cell-surface expression > 50 % of WT. Green indicates an increase in ghrelin affinity > 0.5 log units or an increase in E_{max} > 130 %. Yellow indicates a > 20 % decrease in basal activity compared to WT.

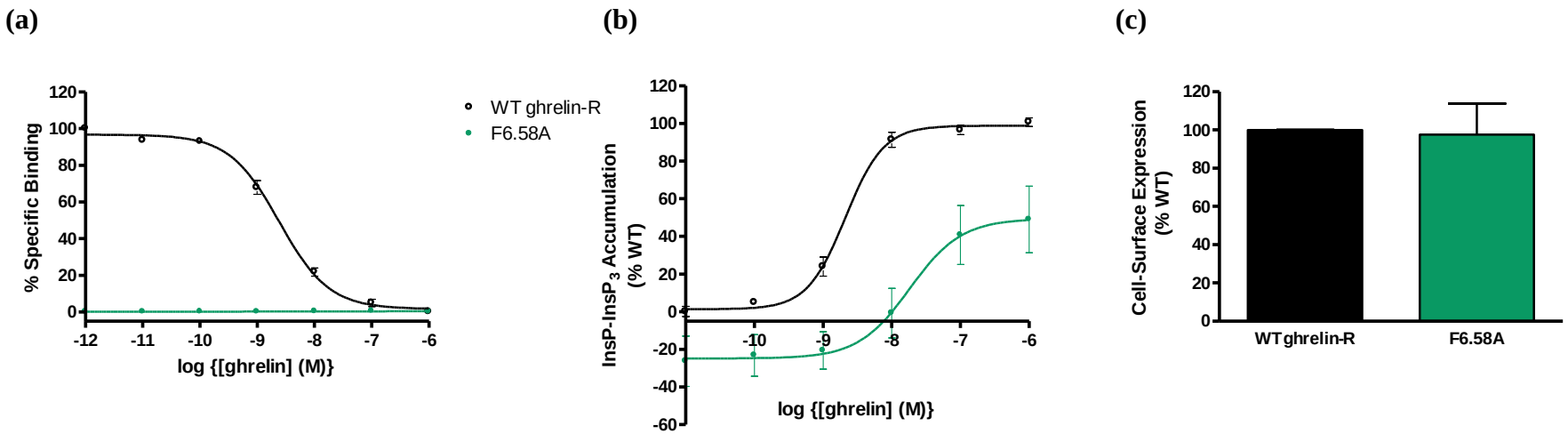


Figure 6.20 Ligand binding, intracellular signalling and cell-surface expression profiles for F6.58A mutant receptor construct: (a) Competition radioligand binding assays were performed on HEK 293T membranes from cells transiently transfected with WT ghrelin-R or F6.58A expressed as a percentage of specific binding. (b) Ghrelin-induced InsP-$InsP_3$ accumulation assays were performed on cells transiently transfected with WT ghrelin-R or F6.58A. Values are expressed as a percentage of the WT ghrelin-R, ghrelin-induced maximum from experiments performed in parallel. (c) Cell-surface expression of mutant receptor construct expressed as a percentage of the WT ghrelin-R. All data are the mean ± SEM of three or more separate experiments, performed in triplicate.

Construct	Binding Affinity pIC_{50} ± SEM	InsP-$InsP_3$ Accumulation			Cell-Surface Expression (% WT) ± SEM
		EC_{50} (nM)	Basal (% WT) ± SEM	E_{max} (% WT) ± SEM	
WT ghrelin-R	**8.61 ± 0.02**	**2.12 ± 0.23**	**0**	**100**	**100**
F6.58A	NDB	18.32 ± 2.80	-26 ± 13	49 ± 18	98 ± 16

Table 6.10 Ligand binding, intracellular signalling and cell-surface expression for F6.58A mutant receptor construct: Values were obtained using the methods described in Chapter 2. All data are the mean ± SEM of three or more separate experiments, performed in triplicate. Grey indicates WT ghrelin-R. Red indicates no detectable binding or a > 5-fold increase in EC_{50}. Yellow indicates a > 20 % decrease in basal activity compared to WT or a decrease in E_{max} > 30 % of WT.

6.3 Discussion

The differences in the ECL2 regions of Family A GPCRs are pronounced and this is highlighted by the structural differences in the Rho, β_2AR and A_{2A}R crystal structures. The β_1AR (Warne *et al.*, 2008), and β_2AR (Cherezov *et al.*, 2007) crystal structures display similar ECL2 conformations reflecting their relatedness and high sequence homology. The β_2AR and β_1AR crystal structures demonstrate a rigid conformation of ECL2, with an intra-loop disulphide bond which appears to act to stabilise ECL2 (Cherezov *et al.*, 2007; Warne *et al.*, 2008). Rho ECL2 consists of a β-hairpin that dips into the retinal binding pocket and creates extensive contacts with the extracellular domains and with retinal (Palczewski *et al.*, 2000). In contrast to these structures, in the A_{2A}R crystal structure appears to demonstrate a random coil within ECL2, with several disulphide bonds constraining a more unstructured loop (Jaakola *et al.*, 2008).

The structure of ECL2 in peptide receptors is currently unknown due to the lack of a crystal structure for this GPCR subfamily. It might be predicted that the peptide GPCR ECL2 structure would most likely resemble that of the β_1AR/β_2AR, as peptide ligands rapidly bind and dissociate from their appropriate receptor. This rapid binding requires unhindered access to the binding cavity which is prevented in Rho, not only by ECL2, but also by the N-terminal domain (Okada *et al.*, 2004).

The extracellular regions of Family A GPCRs have been shown to be involved in ligand binding in a number of receptors. Despite this, aside from the highly conserved cysteine in ECL2, the role of individual residues has not been studied in great depth. A number of different functions of ECL2 have been highlighted in studies of Family A GPCRs, including binding of peptide (Ding *et al.*, 2002) and amine (Shi *et al.*, 2002a) ligands, modulating internalisation (Li *et al.*, 2001) and participating in conformational changes that accompany receptor activation (Baneres *et al.*, 2005). The specific functioning of ECL2 is presumed to vary greatly between members of the GPCR superfamily and this is supported by the great variation in sequence and length found within the loop region.

6.3.1 Alanine scanning mutagenesis of ECL2 of the ghrelin-R

Each residue within ECL2 of the ghrelin-R was mutated individually to alanine to determine the role of single amino acids in receptor function. In order to ensure that every residue within

the loop was mutated, residues 180-212 were incorporated in the study. Mutant ECL2 receptor constructs were pharmacologically characterised after being expressed in HEK 293T cells. Radioligand binding assays and ELISA were conducted on each mutant receptor construct to investigate the effects of alanine substitution on ghrelin-R function.

All of the ghrelin-R alanine substitution mutants bound ghrelin with an affinity similar to the WT receptor except for V182A, G189A, T190A, D191A, C198A, N196A, E197A, T201A, L210A and T211A. The mutants T190A, C198A, N196A, E197A and T201A are included in the extended ECL2 study and are therefore discussed in detail in sections 6.3.2-6.3.4. V182A and L210A displayed no detectable binding at the [^{125}I]ghrelin concentrations used. D191A and T211A displayed ~3-fold increases in pIC_{50} and G189A displayed ~5-fold increase in pIC_{50}. This suggests that these mutations are detrimental to binding, possibly through a direct disruption of interactions with ghrelin, or due to changes in receptor conformation that result in modifications to the ghrelin binding pocket. In the m3 mAChR (Scarselli *et al.*, 2007) and the $V_{1a}R$ (Conner *et al.*, 2007) a number of residues have been identified within ECL2 that affect the receptor function. Interestingly, in the m3 mAChR, mutation of ECL2 residues had little effect on ligand binding characteristics, but instead affected the intracellular signalling capabilities of the receptors. The residues identified were proposed to be involved in stabilising active receptor conformations, rather than participating in agonist binding or forming part of the binding pocket (Scarselli *et al.*, 2007). Recently, Glu187 has been found to affect ghrelin binding affinity and intracellular signalling of the ghrelin-R, with E187A and E187D mutants increasing ghrelin K_i and EC_{50} (Ueda *et al.*, 2010). This is in contrast to the results of the current study, in which E187A has no effect on ghrelin binding.

The residue corresponding to the ghrelin-R Thr211$^{(C+13)}$, in the $V_{1a}R$ [Tyr218$^{(C+13)}$], has been shown to be important for high affinity agonist binding (Conner *et al.*, 2007). Both T211A and the adjacent L210A mutant had a detrimental effect on ghrelin binding, suggesting that this residue may have a similar function in the ghrelin-R as observed in the $V_{1a}R$. The $V_{1a}R$[Tyr218$^{(C+13)}$] has been proposed to form stabilising interactions with $V_{1a}R$[Phe189$^{(C-14)}$], influencing the stability and orientation of the loop region (Conner *et al.*, 2007). In the $V_{1a}R$ mutation of Phe189$^{(C-14)}$ was found to severely disrupt ligand binding and InsP-$InsP_3$ accumulation, supporting its high conservation in the neurohypophysial hormone receptor sub-family (Conner *et al.*, 2007). Val182$^{(C-16)}$, located at the TMIV-ECL2 interface in the ghrelin-R, disrupted ghrelin binding substantially when mutated to alanine in the V182A construct, being unable to bind 10 pM [^{125}I]ghrelin. Because of their localisation within

ECL2, the residues Val182$^{(C-16)}$, Leu210$^{(C+12)}$ and Thr211$^{(C+13)}$ may play a similar role in ghrelin-R function as proposed for Phe189$^{(C-14)}$ and Tyr218$^{(C+13)}$ in the V_{1a}R. Stabilising interactions of the same nature might be occurring in the ghrelin-R accounting for the disruption to ghrelin binding observed for these mutations. Interestingly, V182A was particularly detrimental to cell-surface expression, being only 36 % of that seen for the WT ghrelin-R. The substantial reduction in cell-surface expression supports the theory that Val182$^{(C-16)}$ may be participating in interactions that stabilise the ghrelin-R conformation, allowing delivery and maintenance at the cell surface. Despite conserving a small hydrophobic residue at position 182, alanine substitution is particularly disruptive to ghrelin-R function. This suggests that a valine can form interactions, either with the ligand, or with other residues, that alanine is unable to form.

The residues Gly189$^{(C-9)}$, Thr190$^{(C-8)}$ and Asp191$^{(C-7)}$ affected ghrelin binding affinity when mutated to alanine as part of the ECL2 alanine scan. These residues are gathered in a cluster within ECL2 and Gly189 and Thr190 form part of the putative glycosylation site along with Asn188$^{(C-10)}$. The effect of these mutations on ghrelin binding does not appear to be related to potential removal of a glycosylation motif (section 6.3.3). These three residues may make specific contacts with ghrelin, thus affecting ligand binding directly, or may influence the conformation of ECL2 and hence the ghrelin binding pocket.

A number of the alanine substitution mutants also affected cell-surface expression of the receptors. V180A, V182A, G183A, V184A, H186A, T190A, N196A, C198A, and G208A all affected ghrelin-R cell-surface expression, reducing the number of receptors detectable at the cell surface. C198A, T190A and N196A are discussed in detail in the extended analysis of ECL2 (sections 6.3.2-6.3.4). Disruption of cell-surface expression has been observed in other GPCRs with mutations within ECL2. As seen in the ghrelin-R, these disruptions were of little consequence to ligand binding (Conner *et al.*, 2007).

6.3.2 The role of Cys116$^{(3.25)}$ and Cys198$^{(C)}$

The most common feature of ECL2 is the conserved disulphide bond that occurs between two highly conserved cysteines at the extracellular end of TMIII and in ECL2. These cysteines have been subjected to a number of mutagenesis studies in Family A GPCRs. In the ghrelin-R, Cys116$^{(3.25)}$ and Cys198$^{(C)}$ were mutated to alanine individually and together to produce C116A, C198A and C116A/C198A mutant constructs. All three mutants were particularly detrimental to ghrelin-R function resulting in loss of ghrelin binding, loss of intracellular

signalling (both in an agonist-dependent and agonist-independent manner), and a substantial reduction in cell-surface expression. The loss of ghrelin-R constitutive activity indicates that the cysteines are essential for agonist-independent receptor activation as well as agonist-induced activation. The reduction in cell surface expression may be, in part, responsible for the loss of ghrelin-induced signalling observed for the cysteine mutants. Mutation of the disulphide cysteines has been found to have various effects in different members of the family. In Rho, mutation of $Cys110^{(3.25)}$ and $Cys187^{(C)}$ to alanine resulted in a receptor that displayed a ground-state structure that was comparable to WT suggesting that the mutant opsin still binds retinal and that the presence of the disulphide bond is not essential for receptor folding. This receptor was however compromised in activation, with the Meta II conformation being destabilised (Davidson *et al.*, 1994). In the μ-OR (Zhang *et al.*, 1999), the GnRHR (Cook *et al.*, 1997), the NK_1 receptor (Elling *et al.*, 2000), and the C5aR (Klco *et al.*, 2005), mutation of the disulphide cysteines did not affect receptor expression, but instead altered the ability of the receptor to bind ligands and to activate G-proteins. This was not seen in the m3 mAChR where mutation of the conserved cysteines only resulted in inefficient receptor localisation at the plasma membrane (Zeng *et al.*, 1999a). Interestingly, the disulphide bond in the C5aR appears not to be essential for achieving an active receptor conformation, only for agonist-induced receptor activation. Activating mutations within TMIII retained their constitutive activity when the disulphide bond was removed by mutation of $Cys188^{(C)}$ in ECL2 to alanine (Klco *et al.*, 2005). This suggests that removal of the disulphide bond affects ligand binding, rather than receptor activation directly. Mutation of the cysteines that form the disulphide bond in GPR39 eliminated agonist-induced InsP-$InsP_3$ accumulation and resulted in decreased receptor cell-surface expression, but interestingly the disruption of the disulphide bond caused an increase in agonist-independent signalling. The presence of the conserved disulphide bond and another disulphide between ECL2 and the N-terminus appears to have a dampening effect, reducing the constitutive activity of GPR39 (Storjohann *et al.*, 2008).

The inability of the cysteine mutant constructs to bind ghrelin suggests that the receptors may be incorrectly folded. Similar results were seen in the $V_{1a}R$, where mutation of $Cys205^{(C)}$ to alanine had a particularly detrimental effect on receptor structure and function resulting in ablation of ligand binding and intracellular signalling and decreasing the receptor cell-surface expression (Conner *et al.*, 2007). Receptor-mediated G-protein activation was unaffected by the conserved cysteine mutations in the m3 mAChR. The mutation of either residue only affected localization of the receptor at the cell-surface, not the receptor structure, as G-protein

coupling was still detectable (Zeng *et al.*, 1999a). This was not the case in the ghrelin-R, where the mutation of the disulphide cysteines appeared to be far more detrimental; both Cys116$^{(3.25)}$ and Cys198$^{(C)}$ are essential for ghrelin-R function. In a recent study, Cys116 and Cys198 were deleted from the ghrelin-R and the resultant Δ116C and Δ198C mutants severely affected ghrelin binding affinity and intracellular signalling (Ueda *et al.*, 2010) supporting the data from our study and the importance of these cysteine residues in ghrelin-R function. When the cysteines are mutated to alanine individually, or in parallel, the effect on ghrelin-R function is profound. Both cysteines are required for ghrelin binding and correct receptor delivery to the cell-surface, as indicated by ELISA, suggesting that the disulphide bond may be essential for ghrelin-R folding as well as cell-surface localisation.

6.3.3 Asn188 and Thr190: A potential glycosylation motif

There are four potential glycosylation sites within the extracellular domains of the ghrelin-R. Three are located within the N-terminal domain at residues Asn3, Asn13 and Asn27. The other is located within ECL2 at Asn188$^{(C-10)}$. To date, it is unknown which of the putative glycosylation sites are utilised in the ghrelin-R. In order to investigate ECL2 of the ghrelin-R, Asn188 and Thr190 were included in the alanine scan. By mutating these residues to alanine, a potential carbohydrate modification was removed. The inclusion of the Thr190 mutation was important as any effects of Asn188 mutation might be due to a disruption of glycosylation, or conversely, may be due to the loss of the asparagine, independent of glycosylation. Substitution of the threonine provided a second approach for examining the potential glycosylation site, whilst preserving the asparagine side chain. Removal of the putative glycosylation site by mutation of Asn188$^{(C-10)}$ to alanine in the ghrelin-R had little effect on receptor function. The N188A mutant displayed 'WT-like' characteristics except for a small decrease in constitutive activity (-28 %). This suggests that, if Asn188 is glycosylated, the glycosylation is not essential for receptor function. There are however three further putative sites for carbohydrate modification within the N-terminus of the ghrelin-R, therefore glycosylation of ECL2 may not be necessary for correct ghrelin-R function. In some Family A GPCRs carbohydrate modifications appear to have no direct consequences. Glycosylation of the m2 mAChR is not essential for receptor function or cell-surface localisation as determined by mutagenesis studies (Habecker *et al.*, 1993).

However, glycosylation has been shown to be essential for correct cell-surface delivery and expression of a number of Family A GPCRs. The PAF receptor has a single glycosylation site located in ECL2 which was removed by mutation of the asparagine to alanine. This mutant

PAF receptor retained 'WT-like' signalling characteristics, but cell-surface expression of the mutant receptor construct was diminished. Introduction of glycosylation sites into the N-terminus of the mutant receptor regained WT expression characteristics suggesting that although glycosylation is essential for receptor expression, the position of the carbohydrate moiety is not relevant (Garcia Rodriguez *et al.*, 1995). Similar results were found in the β-ARs, where glycosylation of the receptor was needed for cell-surface expression, but not for G-protein coupling (Rands *et al.*, 1990). Glycosylation of ECL2 of the PAR1 receptor is important for endocytosis and activation of the receptor whereas glycosylation of the N-terminus mediates trafficking to the cell-surface indicating that glycosylation at different positions within the extracellular domains of the PAR1 receptor may regulate different aspects of receptor function (Soto *et al.*, 2010). In the $V_{1a}R$ glycosylation was found to be essential for receptor expression, but of the three glycosylation sites utilised, none was found to be important alone. Only removal of all three sites resulted in a detrimental effect on expression (Hawtin *et al.*, 2001a). Glycosylation of GPCRs has also been shown to be important for receptor activation and ligand binding. Glycosylation at one of the consensus sites in the N-terminus of Rho (Asn15) was shown to be essential for correct signal transduction (Kaushal *et al.*, 1994) and in the SST receptor, removal of glycosylation resulted in reduced binding affinity of agonists (Rens-Domiano *et al.*, 1991).

Threonine and serine residues, located two residues after the asparagine of the glycosylation consensus motif, are required for their ability to hydrogen bond during precursor oligosaccharide transfer to the asparagine residue (Hortin *et al.*, 1980). Alanine mutation of $Thr190^{(C-9)}$ would result in a loss of the glycosylation consensus sequence in ECL2, and therefore mutation of Thr190 to alanine might be expected to result in similar receptor characteristics to the N188A mutant. The T190A mutant however was much more detrimental to ghrelin-R function, affecting ghrelin binding, constitutive activity and cell-surface expression. Ghrelin binding was not detectable at the radioligand concentrations used in binding assays suggesting a disruption to the binding pocket or removal of an interaction with the ligand directly. Molecular modelling of the ghrelin-R docked to a tetrapeptide based on the ghrelin active core, suggested that hydrogen bonds are formed between the ligand and the backbone atoms of both Asn188 and Thr190 (Pedretti *et al.*, 2007). Constitutive activity, but not ghrelin-induced InsP-$InsP_3$ accumulation, was impaired to a similar extent (-35 %) as that observed for the N188A mutant. Cell-surface expression of the T190A mutant was also slightly decreased (68 % of WT). Thr190 appears to have a functional role, independent of glycosylation, as indicated by the decreased binding affinity and cell-surface expression.

6.3.4 The role of key ECL2 residues in ghrelin-R function

Previous studies on Family A GPCRs have demonstrated that individual residues or regions of ECL2 are critical for ligand binding, activation and receptor trafficking. An alanine scanning study conducted on ECL2 of the CB_1 receptor identified two regions that are required for correct receptor function. This data supported a dual role of ECL2 of the CB_1 receptor in ligand binding and in stabilising GPCRs, allowing localisation at the cell surface (Ahn *et al.*, 2009). In the α_{1b}AR three residues were identified as being critical for antagonist specificity, implicating them in ligand binding. Mutation of these residues; Gly196$^{(C+1)}$, Val197$^{(C+2)}$ and Thr198$^{(C+3)}$ (producing mutants G196Q, V197I, T198N) altered antagonist specificity from α_{1b}AR-specific to α_{1a}AR-specific (Zhao *et al.*, 1996). Similarly, in the Rho crystal structure Glu181$^{(C-6)}$, Ser186$^{(C-1)}$, Cys187$^{(C)}$, Gly188$^{(C+1)}$, Ile189$^{(C+2)}$ and Tyr191$^{(C+4)}$ have been shown to contact retinal (Palczewski *et al.*, 2000). In the V_{1a}R residues within ECL2 were found to be important for high affinity agonist binding and intracellular signalling. These residues were Phe189$^{(C-14)}$, Asp204$^{(C-1)}$, Cys205$^{(C)}$, Trp206$^{(C+1)}$, Phe209$^{(C+4)}$ and Tyr218$^{(C+13)}$ (Conner *et al.*, 2007). In order to investigate the role of residues in ECL2 of the ghrelin-R, the residues corresponding to those identified as crucial in other Family A GPCRs, were extensively investigated.

Asn196$^{(C-2)}$, Glu197$^{(C-1)}$, Arg199$^{(C+1)}$, Pro200$^{(C+2)}$, Thr201$^{(C+3)}$ and Glu202$^{(C+4)}$ were all mutated to alanine as part of the initial alanine scan of ECL2. This incorporated N196A, E197A, R199A, P200A, T201A and E202A mutations independently into the ghrelin-R. N196A, E197A and T201A were all detrimental mutations within the ghrelin-R in terms of ghrelin binding. These mutations resulted in receptors that were unable to bind ghrelin at the radioligand concentration used in the binding assay. This suggests that substitution of any of these amino acids with alanine resulted in receptor constructs that demonstrated a substantial decrease in affinity for ghrelin. The negatively charged C-1 residue was found to affect V_{1a}R binding to AVP when mutated to alanine (Hawtin *et al.*, 2006). This supports the importance of Glu197$^{(C-1)}$ in ghrelin binding in the ghrelin-R. Asp204$^{(C-1)}$ in the V_{1a}R was mutated to glutamate, conserving the negative charge at this position and the resultant D204E mutant retained 'WT-like' characteristics, suggesting that glutamate can successfully substitute for aspartate at this position, whereas mutation to alanine or arginine resulted in disruption of vasopressin binding (Hawtin *et al.*, 2006). As found in the D204A mutation in the V_{1a}R, E197A in the ghrelin-R resulted in an increase in EC_{50} (4.2-fold) compared to WT. A previous study on the ghrelin-R revealed that mutation of Glu197 to glutamine resulted in decreased ghrelin potency (Holst *et al.*, 2009). Molecular modelling in the V_{1a}R revealed that

Asp204$^{(C-1)}$ is in close contact with residues located at the extracellular side of TMIII and forms a salt bridge with Lys$^{3.29}$ and a hydrogen bond with Gln$^{3.32}$. This is demonstrated in Fig 6.21. Removing the negative charge in the D204A mutant disrupted these interactions and resulted in partial unfolding of the β-hairpin that forms ECL2 (Hawtin *et al.*, 2006). From the mutagenesis data, similar interactions can be proposed to occur in the ghrelin-R. Gln$^{3.29}$ or Ser$^{3.32}$ may form a hydrogen bond with Glu197$^{(C-1)}$ and this may restrain one conformation of ECL2. Mutational analysis of Gln$^{3.29}$ in the ghrelin-R has revealed that a Q3.29A mutation resulted in a 6.5-fold increase in EC_{50} and implicated Gln$^{3.29}$ in ghrelin binding (Holst *et al.*, 2006a). A Q3.29L mutant resulted in total loss of signalling through the $InsP_3$ pathway, both constitutively, and in response to ghrelin (Holst *et al.*, 2004a). Mutation of Ser$^{3.32}$ to alanine (Chapter 5) resulted in a receptor construct that was essentially WT in its characteristics except for a slight decrease in constitutive activity and the same result was observed by Holst *et al*. (2004) in a study which included the S3.32A mutant. In the m3 mAChR a negative charge is also found at position C-1 of ECL2; however, the residue Glu219$^{(C-1)}$ was not found to be required for efficient function of the m3 mAChR as mutation to alanine produced a mutant receptor with similar binding and signalling characteristics to the WT receptor (Scarselli *et al.*, 2007).

Mutation of Asn196$^{(C-2)}$ is particularly detrimental to ghrelin-R function, with this residue being required for high affinity ghrelin binding, efficacy, potency, basal signalling and cell-surface expression. N196A substantially decreased ghrelin-R constitutive activity, but was still able to respond to ghrelin stimulation. It appeared that Asn196 contributes to interactions that stabilise the ghrelin-R constitutive activity, but does not prevent activation in the presence of agonist. Asn196 may be providing stabilising contacts with other residues in the ghrelin-R. Interestingly, a recent study found that the deletion of 11 residues in ECL2 (Δ188-197) which included Asn196, had no effect on ghrelin binding affinity (Ueda *et al.*, 2010). This is in contrast to the data presented in this study, perhaps suggesting that it is the interaction of Asn196 with surround residues that is disrupted in the N196A mutant, and that removal of neighbouring residues as well as Asn196 is less detrimental that an N196A mutant alone.

One residue that may form an interaction with Asn196 is Glu$^{3.33}$ at the extracellular end of TMIII. Glu$^{3.33}$ has been identified as a potential counterion in the binding of ghrelin and the GHSs to the ghrelin-R. An E3.33D mutation retained ‘WT-like’ characteristics, whereas an E3.33Q mutation severely disrupted binding, suggesting that the charge on this residue is

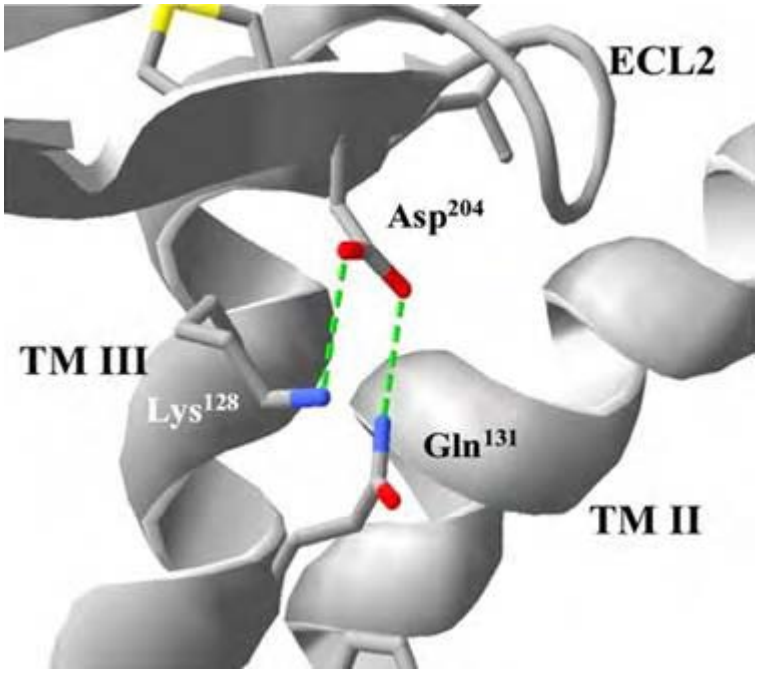


Figure 6.21 Model of the $V_{1a}R$: Model showing the hydrogen bonding and salt bridge interactions between Asp204$^{(C-1)}$ in ECL2 and Lys128$^{(3.29)}$ and Gln131$^{(3.32)}$ in TMIII of the $V_{1a}R$. Similar interactions may be occurring in the ghrelin-R between Glu197$^{(C-1)}$ and Gln$^{3.29}$ (Image taken from Hawtin *et al.*, 2006).

essential for agonist binding (Feighner *et al.*, 1998; Holst *et al.*, 2009). Removal of this putative interaction in the N196A mutation appears to cause a disruption to the ability of the ghrelin-R to signal in the absence of agonist. The loss of constitutive activity observed with the N196A mutation, but not the E3.33Q mutation, might be explained by the loss of an ionic interaction between Asn196 and $Glu^{3.33}$, in the E3.33Q receptor, whilst still retaining a hydrogen bond. Ionic interactions have previously been identified between residues within ECL2 and the TM domains in Family A GPCRs (Jiang *et al.*, 1997; Hoffmann *et al.*, 1999; Costanzi *et al.*, 2005; Hawtin *et al.*, 2006; Sum *et al.*, 2009; Wang *et al.*, 2009; Bokoch *et al.*, 2010).

In this study, the R199A mutant was 'WT-like' in all characteristics. An R199L mutant was produced by Holst *et al*. (2004) in the ghrelin-R as part of an investigation into the constitutive activity of the receptor. The R199L mutant displayed a reduction in agonist-independent activity (Holst *et al.*, 2004a). These data, combined with the results of the current study, suggest that $Arg199^{(C+1)}$ itself is not important in maintaining WT ghrelin-R functioning, as alanine substitution does not appear to affect receptor function, rather that the introduction of leucine is detrimental to receptor pharmacology.

Mutation of $Thr201^{(C+3)}$ in the T201A mutant construct had a severely detrimental effect on ghrelin binding, and also resulted in a decrease in ghrelin potency (EC_{50} 4-fold increase), and constitutive activity (-45 % of WT ghrelin-induced maximum), however, this mutant displayed an increase in efficacy (E_{max} 161 % of WT) despite 'WT-like' cell-surface expression. This suggests that, although T201A is detrimental to ghrelin binding and to agonist-induced and basal InsP-$InsP_3$ accumulation, once the agonist is bound the response is greater than that seen for the WT receptor. Mutation-induced increases in efficacy have been identified in a number of GPCRs, including the ghrelin-R (Holst *et al.*, 2004a), and reflect the complexity of GPCR activation, and the multiple signalling conformations adopted during ligand-induced activation. Investigations into mutation-induced changes in efficacy in the gastrin receptor identified mutations within the transmembrane domains that enhanced ligand efficacy without affecting binding or potency (Blaker *et al.*, 2000).

An aromatic residue at position C+4 is conserved in 59 % of peptide and amine Family A GPCRs. Aromatics at this position have been found to be important in a number of GPCRs for ligand binding (Palczewski *et al.*, 2000; Zhou *et al.*, 2001; Conner *et al.*, 2007). In the ghrelin-R a glutamate is found at position C+4 and this residue was not identified as being

important for ghrelin-R function as mutation to alanine had no effect on ligand binding, agonist-induced InsP-$InsP_3$ accumulation or receptor cell-surface expression, and only minimally affected basal signalling, reducing the constitutive activity to -32 % of WT maximal ghrelin-induced response. None of the members of the ghrelin-R subfamily have an aromatic at C+4 despite the reasonable conservation of an aromatic at this locus in peptide GPCRs therefore the ghrelin-R subfamily is somewhat unusual with respect to residue C+4.

In the C5aR, a random saturation mutagenesis study of ECL2 revealed 23 mutant receptors that demonstrated constitutive activity. This suggests that ECL2 may be acting as a negative regulator of GPCR function. However, for GPCRs in general, activating mutations are rarely reported in ECL2, perhaps highlighting the need for multiple mutations within the domain as observed for the C5aR (Klco *et al.*, 2005). However, the C5aR data demonstrate that ECL2 may play a significant role in stabilising inactive receptor conformations, and in allowing agonist binding and subsequent receptor activation. Several studies have revealed that autoantibodies to ECL2 can activate GPCRs, perhaps by mimicking ligand binding (Lebesgue *et al.*, 1998; Mobini *et al.*, 1999; Wang *et al.*, 2000). This further supports the role of ECL2 in maintaining receptor conformations and in restraining inactive states. A study on Rho demonstrated that ECL2 acts as a plug that requires rearrangement for activation of the receptor. The rearrangement of a hydrogen bonding network with ECL2 and TMV is supported by ECL2 movement after receptor activation (Ahuja *et al.*, 2009).

The highly constitutively active melanocortin receptors exhibit an almost total lack of ECL2. This unique feature is believed to be responsible, in part, for the agonist-independent activity observed for this sub-family of GPCRs, potentially due to the removal of restraining interactions between the helical bundle and ECL2 (Holst *et al.*, 2003b).

6.3.5 The role of Phe203$^{(C+5)}$ and Ala204$^{(C+6)}$ in ghrelin-R function

A single nucleotide substitution within the ghrelin-R coding exons has been reported to result in obesity (Wang *et al.*, 2004) and short stature (Pantel *et al.*, 2006) in humans. This cytosine to adenine substitution results in a missense mutation of Ala204 in ECL2 to glutamate. Ala204 is conserved in ECL2 of the ghrelin-R in all species, conveying its importance in ghrelin-R function (Pantel *et al.*, 2006). The A204E mutation has been previously shown to result in decreased surface expression of the mutant ghrelin-R, without ultimately affecting ghrelin binding affinity or agonist-responsiveness (Pantel *et al.*, 2006; Liu *et al.*, 2007a). This mutation did, however, result in a substantial decrease in ghrelin-R constitutive activity, both

as a result of decreased cell-surface expression and a loss of the intrinsic ability to signal in the absence of ghrelin (Pantel *et al.*, 2006; Liu *et al.*, 2007a). In order to investigate the role of Ala204 in ghrelin-R function in more depth, Ala204 was mutated to glycine, glutamate and valine in this study. The A204G mutant resulted in a receptor construct that presented similar binding, signalling and cell-surface expression characteristics to the WT ghrelin-R, except for a dramatic increase in the E_{max} after ghrelin stimulation. The A204G E_{max} was 217 % of that seen for the WT ghrelin-R, suggesting that the introduction of glycine resulted in a receptor that bound ghrelin in a 'WT-like' manner and had 'WT-like' potency, but displayed increased efficacy, being able to signal to a much greater extent than the WT when stimulated with ghrelin. The increased efficacy seen with the A204G mutant indicated that the introduction of glycine at this position within ECL2 resulted in the release of a stabilising constraint probably due to the inherent conformational flexibility of glycine, without drastic changes to amino acid side chain size. To investigate the increase in efficacy further, the neighbouring residue, Phe203, was also mutated to glycine. Mutation of Phe203 to alanine, as part of the alanine scan of ECL2, produced a receptor that displayed 'WT-like' characteristics except for a small decrease in basal $InsP_3$ signalling (-39 % of WT). This implies that a phenylalanine at this position is not essential to receptor function. Knowing that a small residue is well tolerated at position 203, the F203G mutant was characterised. The F203G mutant displayed 'WT-like' characteristics, similar to that seen with the F203A mutant, with the observed small decrease in constitutive activity (-30 % of WT). This suggests that the glycine introduced at position 204 in the A204G mutant produces its effects via a direct disruption at position 204 rather than by introducing flexibility within the loop, as mutation of the adjacent Phe203 to glycine did not yield the same result. To aid further understanding, Ala204 was mutated to valine, retaining a small hydrophobic residue at this position. A204V was essentially WT with regards to InsP-$InsP_3$ accumulation and cell-surface expression. This mutant was, however, unable to bind [^{125}I]ghrelin at the concentrations used, suggesting a decrease in ghrelin affinity. As the signalling characteristics were unchanged, it is clear that ghrelin does bind to the A204V mutant receptor, but that this was not detectable in the assay.

In the A204E mutation, similar results were seen to those previously reported (Pantel *et al.*, 2006; Liu *et al.*, 2007a). Ghrelin binding affinity was affected, with the A204E mutant being unable to bind [^{125}I]ghrelin at the concentrations used. Previous studies have concluded that loss of binding is related to a substantial decrease in cell-surface expression, and that expressing the A204E mutant at levels similar to WT resulted in 'WT-like' binding characteristics (Pantel *et al.*, 2006). However, in the A204E mutant in this study, expression

at the cell-surface was similar to the WT ghrelin-R (80 %) and therefore this appears not to be responsible for the reduction in ghrelin affinity observed. The ghrelin-R has however been shown to undergo substantial constitutive internalisation (Chapter 3), related to its constitutive activity, and therefore the high cell-surface expression of the A204E mutant observed in the ELISA assay may be a reflection of the reduction in constitutive internalisation as a result of the loss of basal activity. Feighner *et al*. (1998) predicted that Ala204 is distant from the ghrelin binding pocket, and therefore not directly involved in ghrelin binding. Consequently, any effect of mutation of Ala204 on binding could be assumed to be due to disruption of intramolecular interactions, resulting in conformational modifications. A ghrelin-R model produced by Pedretti *et al*. (2007) suggested that ECL2 forms the upper part of a polar binding site and that several residues, including Ala204, are directly involved in interacting with the octanoyl chain of a ghrelin active core-based tetrapeptide. The results of the current study suggest that Ala204 may be involved in high affinity ghrelin binding as binding was only observed when Ala204 was mutated to glycine and was not detectable with the A204E or A204V mutants despite both mutant constructs retaining the ability to signal in response to ghrelin stimulation.

It is still unclear as to the mechanisms involved the substantial reduction in constitutive activity observed for the A204E mutant. The results from this study allow for the proposal of a potential interaction between residues within ECL2. Asn196 mutation to alanine results in a dramatic decrease in ghrelin-R constitutive activity as seen with the A204E mutation. Asn196 may be forming an ionic interaction with $Glu^{3.33}$ in TMIII, stabilising basal signalling, and this interaction might be broken after alanine substitution of Asn196 (section 6.3.4). In the A204E mutant receptor construct, it is possible that the glutamate introduced at position 204 interacts with Asn196, thus disrupting the $Glu^{3.33}$-Asn196 interaction and resulting in the decreased constitutive activity, whilst still retaining agonist sensitivity.

Figure 6.22 is a summary schematic of the alanine scan of residues within ECL2 of the ghrelin-R. The schematics depict the effect of each mutation, colour coding the effect on; (a) radioligand binding, (b) cell-surface expression, (c) ghrelin potency and (d) constitutive activity, compared to the WT ghrelin-R.

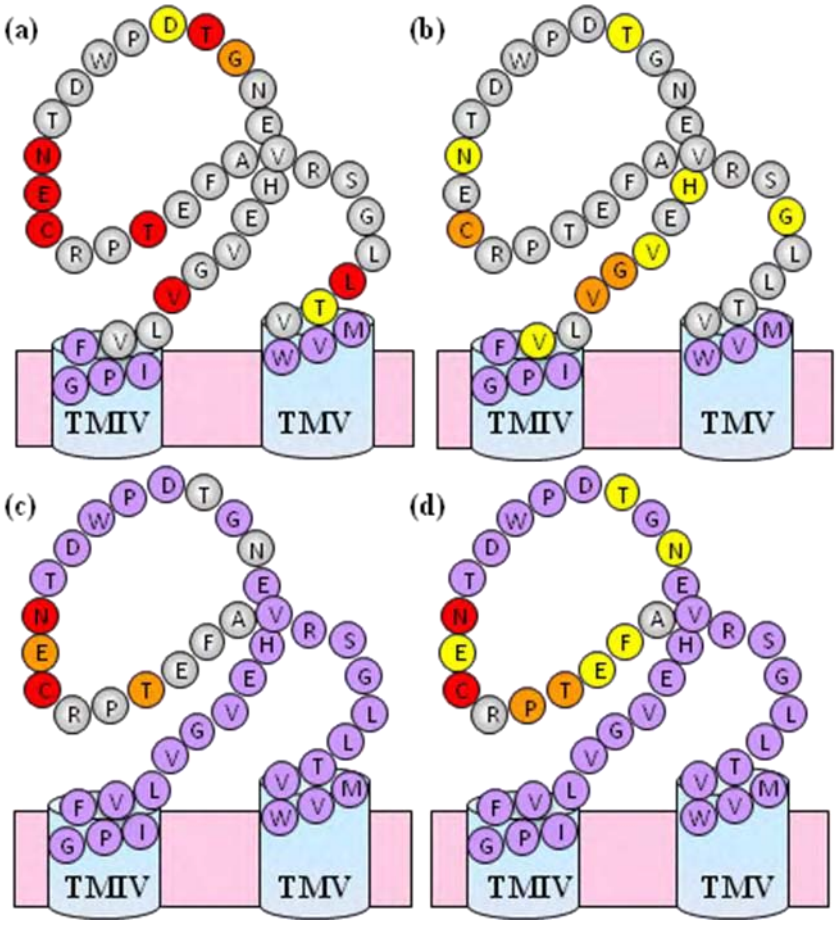


Figure 6.22 Summary schematics of the residues within ECL2 of the ghrelin-R: Individual residues coloured according to their effects, after alanine substitution, on (a) ghrelin binding, (b) cell-surface expression, (c) EC_{50} and (d) constitutive activity. See Appendix Table 9.1 for colour code. Purple = not characterised. Grey = 'WT-like' characteristics.

6.3.6 Investigation of the role of residues at the extracellular face of TMVI

In the FFAR1 two glutamate residues in ECL2 have been proposed to interact with positively charged arginine residues at the extracellular ends of TMV and TMVII (Sum *et al.*, 2009). The FFAR1 lacks the ionic lock (discussed in Chapter 4) that stabilises the inactive state of some Family A GPCRs and it has therefore been suggested that other interactions must be occurring that maintain the receptor in the inactive conformation in the absence of agonist. Activation of the FFAR1 is believed to involve the breaking of the ionic interactions that occur at the extracellular surface of the receptor. Mutation of the glutamate residues within ECL2 to alanine resulted in constitutive receptor activation suggesting that stabilising interactions are broken (Sum *et al.*, 2009). Similarly, in the purinoceptor P2Y6 (P2Y6 receptor), molecular modelling has shown that Asp179$^{(C+2)}$ in ECL2 forms a stabilising interaction with Arg$^{3.29}$ located at the extracellular side of TMIII. This interaction appears to be disrupted upon agonist binding, allowing extensive ECL2 conformational changes promoting activation (Costanzi *et al.*, 2005). In the related purinoceptor P2Y1 (P2Y1 receptor), similar interactions between the top of TMIII and ECL2 were observed (Jiang *et al.*, 1997; Hoffmann *et al.*, 1999) and in the inactive β_2AR, a salt bridge has been proposed to form between Asp192$^{(C+1)}$ in ECL2 and Lys$^{7.32}$ at the top of TMVII (Wang *et al.*, 2009). This interaction appears to be weaker in the β_2AR active state, as demonstrated by nuclear magnetic resonance (NMR) data (Bokoch *et al.*, 2010). Molecular modelling of the V_{1a}R suggested that Lys$^{3.29}$ forms a salt bridge with Asp204$^{(C-1)}$ in ECL2 which is disrupted by mutation of either residue to alanine and results in receptors that have impaired agonist binding and signalling capabilities (section 6.3.4) (Hawtin *et al.*, 2006). As the ghrelin-R is highly constitutively active, it could be assumed that no such stabilising interactions occur within ECL2, restraining the ground state. Mutation of the residues within ECL2 did not result in increased constitutive activity suggesting that none of the residues are involved in stabilising the ligand-free receptor conformation. Interestingly, introduction of a glutamate at position 204 in ECL2 results in a loss of ghrelin-R constitutive activity, whereas mutation to valine or glycine did not result in a disruption to the basal signalling. This suggests that an interaction may be occurring in the A204E mutant ghrelin-R that does not occur in the WT receptor and requires the presence of a negatively charged residue. In order to investigate potential stabilising interactions, positive charges, located in the extracellular domains of the ghrelin-R, were mutated to alanine. Arg$^{6.55}$ had previously been found to be essential for ghrelin-R function and mutation to alanine (Holst *et al.*, 2004a) or glutamine (Feighner *et al*., 1998) resulted in reduction of ghrelin-R constitutive activity. It was proposed that Arg$^{6.55}$ may interact with a glutamate residue in TMIII (Glu$^{3.33}$) as mutation of this glutamate also resulted

in perturbed receptor function (Feighner *et al.*, 1998; Holst *et al.*, 2003a). The data from the FFAR1 and the β_2AR suggested that the ghrelin-R ECL2 may potentially form stabilising interactions with the arginine when the A204E mutation is introduced into the loop. As found previously, mutation of $Arg^{6.55}$ to alanine was particularly detrimental to receptor function, resulting in a loss of [^{125}I]ghrelin binding, agonist-induced intracellular signalling, agonist-independent signalling and a reduction in cell-surface expression to 50 % of the WT receptor. To investigate the potential of an ionic interaction, the R6.55A mutant was introduced into the A204E ghrelin-R background. The A204E/R6.55A double mutant was unable to recover ghrelin-R function. The constitutive activity was diminished, as seen in the two single mutations, and the double mutant construct was also unable to signal in response to ghrelin. Although the data implies that an interaction does not occur between the glutamate at position 204 and $Arg^{6.55}$, as removal of the positive residue was unable to restore ghrelin-R function, the R6.55A mutation was severely detrimental to the receptor. Therefore, it may be that $Arg^{6.55}$ plays such a significant role in receptor function that the true effect of the double mutant is unable to be seen. The corresponding residue, $Asn^{6.55}$, was found to directly contact 5-HT in the $5HT_{2b}R$, and therefore directly influence binding (Manivet *et al.*, 2002) highlighting the crucial role of residues at this position.

Another positively charged residue is located at position 6.60 in TMVI/ECL3. $Lys^{6.60}$ was mutated to alanine. The K6.60A mutant construct was not detrimental to agonist binding; the mutation produced an increase in ghrelin affinity of ~3-fold. There was also little effect on the intracellular signalling capabilities of the ghrelin-R as a result of the K6.60A mutation. Constitutive activity was slightly reduced to -32 % of the WT maximum activity and there was a small increase in the ghrelin-induced E_{max}. Overall, this mutation retained characteristics similar to that seen for the WT ghrelin-R, suggesting that $Lys^{6.60}$ is not as vital for ghrelin-R function as $Arg^{6.55}$. The double mutation A204E/K6.60A was introduced to further investigate the potential of an ionic interaction between A204E and positive residues in the extracellular domain. A204E/K6.60A retained some of the agonist responsiveness seen for the A204E and K6.60A single point mutations, but was unable to restore WT constitutive activity. The binding affinity for ghrelin was reduced as seen for the A204E mutant. This suggests that an ionic interaction does not occur between the glutamate in the A204E mutant and $Lys^{6.60}$, as removal of the positive charge in the A204E/K6.60A double mutant construct, did not counteract the loss of constitutive activity observed for the A204E single mutation.

The residue at position 6.58 has been shown to be important for ligand binding in a number of Family A GPCRs to date. In the GnRHR, $Tyr^{6.58}$ has been shown to be essential for ligand binding and only substitution with phenylalanine retained GnRH potency (Coetsee *et al.*, 2008). A $Gly^{6.58}$ to threonine mutation in the neurokinin A receptor (NK_2R) also resulted in a loss of agonist binding, although non-peptide antagonist binding was maintained (Bhogal *et al.*, 1994). An $Asp^{6.58}$ to alanine mutant greatly reduced the binding affinity of angiotensin II for the AT_1R (Yamano *et al.*, 1995) and a D6.58A mutant in the CXCR1 resulted in reduced binding affinity and loss of agonist-induced signal transduction (Leong *et al.*, 1994).

There is relatively little conservation of this residue within the GPCR superfamily, possibly supporting a role in binding various GPCR ligands. $Phe^{6.58}$ was mutated to alanine in the ghrelin-R to investigate the role of this aromatic residue in ghrelin binding and activation. The F6.58A mutation resulted in a loss of ghrelin binding and affected the ability of the receptor to signal through the $InsP_3$ pathway, both in response to ghrelin and in an agonist-independent manner. The F6.58A mutant had no effect on cell-surface expression. This is similar to the results observed for the GnRHR where mutation of $Tyr^{6.58}$ to alanine retained 'WT-like' cell-surface expression despite other mutations at this locus altering receptor localisation (Coetsee *et al.*, 2008). The 'WT-like' cell-surface expression observed for the ghrelin-R F6.58A mutant indicates that the detrimental effect observed on ligand binding and intracellular signalling is a direct consequence of disruption of the receptor-ghrelin interaction, rather than an artefact of decreased receptor number.

Aromatic residues found within TMVI and TMVII have previously been identified as being vital for ghrelin-R constitutive activity. Mutation of $Phe^{6.51}$ to various other amino acids enabled the constitutive activity to be systematically turned up and down depending on the size and aromaticity of the residue (Holst *et al.*, 2004a). $Phe^{6.58}$ is located two turns above this aromatic cluster and is not predicted to be involved in the same interactions. Whereas the F6.51A mutation resulted in total ablation of ghrelin-R constitutive activity (Holst *et al.*, 2004a) the F6.58A mutant in this study only resulted in a partial loss of basal agonist-independent activity (-26 %) suggesting that the role of $Phe^{6.58}$ is not related to the conserved aromatic cluster in the ghrelin-R family.

CHAPTER 7.

SUMMARY, CONCLUSIONS AND FUTURE WORK

7.1 Summary and Conclusions

The structural and functional requirements of ghrelin-R constitutive activity are of potential therapeutic importance and are likely to be relevant to other Family A GPCRs. The work presented in this thesis addresses the importance of individual residues in both constitutive and ghrelin-induced receptor activation. Amino acid substitutions were performed in the ghrelin-R to investigate the role of individual residues in receptor function. Each mutation was assayed for its effects on ghrelin binding affinity and intracellular signalling, both in an agonist-dependent and agonist-independent manner, and for receptor cell-surface expression. Specifically, the residues were assessed for their ability to influence the constitutive activity of the ghrelin-R, and to extend this study some of the point mutations were further characterised using the ghrelin-R inverse agonist, SP-analogue.

Chapter 3 detailed the introduction of the HA-epitope tag at the N-terminus of the ghrelin-R. The HA-tag enabled analysis of WT and mutant receptor expression at the cell-surface when transiently transfected into HEK 293T cells. ELISA analysis of ghrelin-stimulated receptors revealed that the ghrelin-R does not internalise in response to ghrelin stimulation in HEK 293T cells. It is known that the ghrelin-R undergoes a high level of constitutive internalisation and it is predicted that this tonic internalisation is responsible for the apparent lack of ghrelin-induced internalisation in this system. In order to further assess the constitutive internalisation, the ghrelin-R was cotransfected with a dominant negative mutant of dynamin, K44A dynamin. It was subsequently found that receptor cell-surface expression could be significantly increased, supporting the high level of constitutive internalisation observed for the ghrelin-R, which occurs in a dynamin-dependent manner.

The WT ghrelin-R was extensively characterised and each assay optimised to the WT so that comparisons could be drawn between the WT and mutant ghrelin-R constructs. The third part of Chapter 3 examined the effect of alanine substitution mutations of the most conserved residue within each helix. Each residue investigated in this section appears to be important, in some respect, to ghrelin-R function, highlighting the highly conserved nature of these residues

within the family and indicating that their conservation is crucial in terms of maintaining WT receptor characteristics. The similarities in characteristics for the N1.50A and D2.50A mutants appeared to confirm their role as part of an interhelical hydrogen bonding network, identified in a number of Family A GPCRs.

Chapter 4 examined the role of specific residues within the TM domains in receptor activation. TMIII and VI have been extensively investigated in Family A GPCRs and have been proved to be critical to the formation of both the inactive and active receptor conformations. The highly conserved ERY motif was revealed to be of functional importance in activation of the ghrelin-R in both an agonist-independent and agonist-dependent manner. The residues within the motif were found to influence constitutive activity of the ghrelin-R, although no substitutions within the ERY increased the constitutive activity, in contrast to a number of other Family A GPCRs.

The "ionic lock" is found between TMs III and VI some Family A GPCRs, but is not present in the ghrelin-R. Mutations of $His^{6.30}$ provided an insight into potential intramolecular interactions between TMs III and VI. Substitution of $His^{6.30}$ with acidic residues decreased constitutive activity in a manner consistent with the introduction of an "ionic lock"-type interaction between $Arg^{3.50}$ and residue 6.30. Substitution of $His^{6.30}$ with basic amino acids also decreased the basal signalling of the ghrelin-R, but by different intramolecular mechanisms. It is thought that the introduction of a positive charge repels the cytoplasmic end of TMV and allows other residues at the bottom of TMVI to form stabilising contacts. This indicates that the cytoplasmic end of TMVI is extensively involved in maintaining ground state and active state interactions.

Despite the high level of constitutive activity observed for the WT ghrelin-R, it was apparent from this study that the ghrelin-R basal signalling could be increased with a number of individual point mutations. In Chapters 4 and 5, residues within TMs III, VI and VII were identified for their role in maintaining ghrelin-R constitutive activity, with substitutions either impairing ($Thr^{3.44}$, $Leu^{3.46}$, $Glu^{3.49}$, $Arg^{3.50}$, $Ala^{3.60}$, $His^{6.30}$, $Val^{6.34}$, $Met^{6.36}$, $Val^{6.40}$, $Trp^{6.48}$, $Pro^{6.50}$, $Ser^{7.45}$, $Asn^{7.49}$, $Ile^{7.51}$, $Leu^{7.52}$, $Tyr^{7.53}$, $Lys^{7.59}$) or enhancing ($Thr^{3.36}$, $Thr^{3.42}$, $Ile^{3.43}$, $Thr^{6.33}$, $Val^{6.40}$, $Ser^{7.45}$) basal $InsP_3$ signalling. Interactions between TMs III, V, VI and VII have been found to be responsible for stabilising both the inactive and active receptor conformations. As discussed in Chapter 4, the $Trp^{6.48}$ 'rotamer toggle switch' involves residues 3.36 and 7.45. Mutation of these later residues to alanine resulted in increased

constitutive activity revealing their importance in maintaining the inactive Trp$^{6.48}$ conformation. Mutating Ser$^{7.45}$ to alanine may release Trp$^{6.48}$ from its inactive conformation, pointing towards TMVII, whereas mutating Thr$^{3.36}$ to alanine appears to allow Trp$^{6.48}$ to adopt the active rotamer conformation, pointing towards TMV, due to the removal of the restraining threonine side chain.

In Chapter 5, Val$^{6.40}$ was found to be important for ghrelin-R basal activation, with mutations at this position being able to turn constitutive activity up and down, and therefore implicating position 6.40 in the transition from the R to the R* state. The mechanisms by which residue 6.40 alters ghrelin-R basal signalling activity are unknown, but it might be assumed that removal of stabilising interactions with TMVII are responsible for the increased constitutive activity in the V6.40Y mutant. Similar interactions would however be expected in the V6.40F mutant, but this mutation did not increase basal signalling to the same extent as V6.40Y, implicating the tyrosine alone in the formation of specific interactions that stabilise a more active receptor conformation and a possible role for the hydroxyl which is absent in phenylalanine.

Apart from Thr$^{3.36}$, two other residues within TMIII were found to increase constitutive activity when mutated to alanine. These were Thr$^{3.42}$ and Ile$^{3.43}$, located adjacent to each other at the cytoplasmic end of the helix. Both the T3.42A and I3.43A mutants retained the ability to signal further when stimulated with ghrelin and the constitutive signalling of both mutants could be inhibited by the inverse agonist, SP-analogue. This suggests that the introduction of alanine at these loci disrupted interactions which usually stabilise the basal receptor conformation, or introduced interactions which stabilise new, increased basal levels of signalling, without affecting the ability of the receptors to be activated further by agonist, or inactivated by inverse agonist.

Unlike many CAMs of Family A GPCRs, the CAMs of the ghrelin-R did not affect ghrelin binding affinity. ‘Classical’ CAMs usually increase agonist affinity as they stabilise the R* state for which agonists have a greater affinity. The lack of increased ghrelin affinity in any of the CAM ghrelin-R might reflect a different receptor conformation that does not resemble the classical R* state. Theoretically, any number of receptor conformations are possible; the ghrelin-R CAMs may reflect receptor states that are not typically active and therefore do not display increased agonist affinity, and can usually be activated further after ghrelin stimulation. Conversely, the mutations that decrease constitutive activity do not appear to

decrease ghrelin affinity suggesting that agonist affinity is not a sensitive gauge of constitutive activity in this system.

Chapter 6 was an investigation into the role of ECL2 in the ghrelin-R. A systematic alanine scan was conducted, mutating all residues within ECL2 to alanine to investigate the effect of removing the amino acid side chains on ghrelin binding and cell-surface expression. Most amino acids substitutions within ECL2 had little effect on ghrelin-R function. A few residues were more extensively characterised using InsP-$InsP_3$ accumulation assays. This identified Asn196 as a residue that is essential for ghrelin-R receptor function, with the N196A mutation affecting all aspects pharmacology studied. It was proposed that Asn196 may be forming stabilising interactions with $Glu^{3.33}$ in TMIII, a residue identified previously as essential for agonist binding (Feighner *et al.*, 1998; Holst *et al.*, 2009). Stabilising ionic interactions have been found between ECL2 and the extracellular ends of the TM domains in other Family A GPCRs. These interactions were further investigated in relation to the effects of the naturally occurring A204E mutation, previously identified as causing a substantial decrease in ghrelin-R constitutive activity (Pantel *et al.*, 2006). The glutamate in the A204E mutant did not appear to interact with basic residues at the top of TMVI, although it is unclear whether interactions are occurring with $Arg^{6.55}$, as the R6.55A mutation is particularly detrimental to receptor function. We propose a potential interaction may be occurring between the glutamate and Asn196, pulling Asn196 away from $Glu^{3.33}$ and resulting in the basally inactive phenotype observed for the A204E mutant construct.

7.2 Future Work

The study on the ghrelin-R can be extended to enhance the knowledge surrounding ghrelin-R constitutive activity. In Chapter 3 the internalisation study revealed that the HA-ghrelin-R does not display agonist-dependent internalisation in HEK 293T cells. It was however concluded that the ghrelin-R undergoes a high degree of constitutive internalisation in a dynamin-dependent manner, as indicated by the increase in cell-surface expression after co-transfection with a dominant negative mutant of dynamin. It would be interesting to examine the consequences of a reduction or increase in constitutive signalling through the $InsP_3$ pathway on receptor agonist-independent internalisation. Although some of the mutant receptor constructs that displayed reduced constitutive $InsP_3$ signalling also had increased cell-surface expression (e.g. L3.46A, H6.30E, W6.48A, S7.45N), this was not the case for all mutants studied as the remainder displayed ‘WT-like’ (or decreased) cell-surface expression.

Conversely, the T6.33A and V6.40A mutations displayed increased basal signalling and deceased cell-surface expression, possibly suggesting increased constitutive internalisation, although this was not found with any of the other CAM ghrelin-R constructs. Further analysis of both the WT and the mutant ghrelin-R constructs, in terms of cell-surface expression using K44A dynamin, may indicate whether constitutive signalling through the $InsP_3$ pathway is necessary for constitutive internalisation, or whether the two are functionally distinct. From the data in this study, no correlation can be made between cell-surface expression and the level of constitutive activity observed for each mutant receptor.

In relation to cell-surface expression, it would be of interest to characterise the constitutive activity of the E3.49A mutant after increasing its level of expression at the cell-surface. The E3.49A construct retains 'WT-like' constitutive activity, despite its cell-surface expression being reduced to only 30 % of the WT expression. Greater insight into the mechanism of action of $Glu^{3.49}$ could be gained by expressing E3.49A at levels comparable to the WT expression in HEK 293T cells. It may be that E3.49A does adopt a more constitutively active conformation than the WT ghrelin-R, but that the CAM effect is masked by the substantial decrease in cell-surface expression. It would also be interesting to investigate whether the decreased cell-surface expression of the E3.49A mutant is due to increased constitutive internalisation caused by a disruption of stabilising interactions as found in some Family A GPCRs, or is a result of decreased trafficking of the receptor to the cell surface initially.

The T2.39A, A3.60Y and T2.39/A3.60Y mutations indicated that there was a potential interaction between TMII, TMIII and ICL2 in the ghrelin-R, as found in the $A_{2A}R$ (Jaakola *et al*., 2008). To investigate these potential interactions further, reciprocal mutations within the ERY motif and at $Thr^{2.39}$ and $Ala^{3.60}$, could be introduced into the ghrelin-R. Difficulties may arise in the reciprocal mutations, as introduction of mutations within the ERY motif have been found to be particularly detrimental to receptor function. However, they may provide a means for investigating the stabilising interactions in the ghrelin-R further and may give more detailed insight into the mechanisms involved in ghrelin-R constitutive activity. Residue 3.62 has been found to be involved in stabilising interactions in the constitutively active β_2AR (Rasmussen *et al.*, 2007) and it may therefore be advantageous to extend this investigation to incorporate $Val^{3.62}$ of the ghrelin-R.

In Chapter 4, $Tyr^{5.58}$ was found to be important in maintaining ghrelin-R constitutive activity and in restraining ghrelin-induced activation, preventing hyperstimulation of the receptor. The

alanine substitution resulted in a dramatic increase in ghrelin efficacy, increasing signalling to 224 % of the WT receptor after stimulation with 1 μM ghrelin. In contrast, the Y5.58Q mutation reduced signalling E_{max}. Residue 5.58 has been implicated in G-protein coupling and in G-protein selectivity in other Family A GPCRs and it would therefore be of interest to determine whether mutation of $Tyr^{5.58}$ in the ghrelin-R alters G-protein selectivity and to further investigate the mechanisms by which alanine substitution causes such a dramatic increase in ghrelin efficacy.

The results from the mutagenesis study and the molecular modelling carried out by N. Floquet (Montpellier, France) implicated residues 6.33 and 6.34 in ghrelin-R constitutive and agonist-induced activation. Further mutagenesis studies could be conducted at these two positions to determine the roles that $Thr^{6.33}$ and $Val^{6.34}$ play in the WT ghrelin-R. Mutating $Thr^{6.33}$ to a negatively charged residue may stabilise an inactive receptor conformation through an interaction with $Arg^{3.50}$, especially in the context of an H6.30K background. Similarly, mutation of $Val^{6.34}$ to a negative charge as conducted in the CB_2 receptor (Feng *et al.*, 2003) and μ-OR (Huang *et al.*, 2001), might reveal more about the molecular mechanisms involved in ghrelin-R activity. The positive charge introduction in the ghrelin-R V6.34K mutant did not increase the constitutive activity, and so had the opposite effect to that observed in many other Family A GPCRs. It would therefore be of interest to see the effect of a negative charge at this position on basal signalling.

In ECL2, interactions have been proposed between residues within the loop and charged residues at the extracellular ends of the TM domains in other members of the GPCR superfamily. The results of this study have lead to the proposal of an interaction between Asn196 and $Glu^{3.33}$ at the top of TMIII in the ghrelin-R. Further site-directed mutagenesis including reciprocal mutations would aid the investigation into potential interactions within the extracellular domain of the ghrelin-R. Furthermore, the effect of the A204E mutation can be more extensively investigated with extended mutagenesis of ECL2. The proposed interaction between Asn196 and the glutamate in the A204E construct needs to be investigated further to probe the exact molecular mechanisms involved in the loss of constitutive activity observed in the A204E and N196A mutations.

The future work suggested here may help to build on our findings and extend the molecular insight into ghrelin-R structure and the underlying mechanisms of ghrelin-R function, aiding Family A GPCR research.

CHAPTER 8.

REFERENCES

Abdulaev, NG, Ridge, KD (1998) Light-induced exposure of the cytoplasmic end of transmembrane helix seven in rhodopsin. *Proc Natl Acad Sci U S A* **95**(22)**:** 12854-12859.

abu Alla, S, Quitterer, U, Grigoriev, S, Maidhof, A, Haasemann, M, Jarnagin, K, Muller-Esterl, W (1996) Extracellular domains of the bradykinin B2 receptor involved in ligand binding and agonist sensing defined by anti-peptide antibodies. *J Biol Chem* **271**(3)**:** 1748-1755.

Adan, RA (2006) Constitutive receptor activity series: endogenous inverse agonists and constitutive receptor activity in the melanocortin system. *Trends Pharmacol Sci* **27**(4)**:** 183-186.

Adan, RA, Kas, MJ (2003) Inverse agonism gains weight. *Trends Pharmacol Sci* **24**(6)**:** 315-321.

Ahn, KH, Bertalovitz, AC, Mierke, DF, Kendall, DA (2009) Dual role of the second extracellular loop of the cannabinoid receptor 1: ligand binding and receptor localization. *Mol Pharmacol* **76**(4)**:** 833-842.

Ahuja, S, Hornak, V, Yan, EC, Syrett, N, Goncalves, JA, Hirshfeld, A, Ziliox, M, Sakmar, TP, Sheves, M, Reeves, PJ, Smith, SO, Eilers, M (2009) Helix movement is coupled to displacement of the second extracellular loop in rhodopsin activation. *Nat Struct Mol Biol* **16**(2)**:** 168-175.

Alberts, B (2002) *Molecular biology of the cell*. 4th edn. Garland Science: New York.

Alewijnse, AE, Timmerman, H, Jacobs, EH, Smit, MJ, Roovers, E, Cotecchia, S, Leurs, R (2000) The effect of mutations in the DRY motif on the constitutive activity and structural instability of the histamine H(2) receptor. *Mol Pharmacol* **57**(5)**:** 890-898.

Angers, S, Salahpour, A, Bouvier, M (2002) Dimerization: an emerging concept for G protein-coupled receptor ontogeny and function. *Annu Rev Pharmacol Toxicol* **42:** 409-435.

Archer-Lahlou, E, Escrieut, C, Clerc, P, Martinez, J, Moroder, L, Logsdon, C, Kopin, A, Seva, C, Dufresne, M, Pradayrol, L, Maigret, B, Fourmy, D (2005) Molecular mechanism underlying partial and full agonism mediated by the human cholecystokinin-1 receptor. *J Biol Chem* **280**(11)**:** 10664-10674.

Arnis, S, Fahmy, K, Hoffman, KP, Sakmar, TP (1994) A conserved carboxylic acid group mediates light-dependent proton uptake and signaling by rhodopsin. J Biol Chem **269**(39): 23879-81

Arvat, E, Di Vito, L, Broglio, F, Papotti, M, Muccioli, G, Dieguez, C, Casanueva, FF, Deghenghi, R, Camanni, F, Ghigo, E (2000) Preliminary evidence that Ghrelin, the natural GH secretagogue (GHS)-receptor ligand, strongly stimulates GH secretion in humans. *J Endocrinol Invest* **23**(8)**:** 493-495.

Asakawa, A, Inui, A, Kaga, T, Yuzuriha, H, Nagata, T, Fujimiya, M, Katsuura, G, Makino, S, Fujino, MA, Kasuga, M (2001) A role of ghrelin in neuroendocrine and behavioral responses to stress in mice. *Neuroendocrinology* **74**(3)**:** 143-147.

Audet, M, Bouvier, M (2008) Insights into signaling from the beta2-adrenergic receptor structure. *Nat Chem Biol* **4**(7)**:** 397-403.

Audoly, L, Breyer, RM (1997) Substitution of charged amino acid residues in transmembrane regions 6 and 7 affect ligand binding and signal transduction of the prostaglandin EP3 receptor. *Mol Pharmacol* **51**(1)**:** 61-68.

Azzi, M, Charest, PG, Angers, S, Rousseau, G, Kohout, T, Bouvier, M, Pineyro, G (2003) Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. *Proc Natl Acad Sci U S A* **100**(20)**:** 11406-11411.

Baker, JG (2008) A study of antagonist affinities for the human histamine H2 receptor. *Br J Pharmacol* **153**(5)**:** 1011-1021.

Bakker, RA, Jongejan, A, Sansuk, K, Hacksell, U, Timmerman, H, Brann, MR, Weiner, DM, Pardo, L, Leurs, R (2008) Constitutively active mutants of the histamine H1 receptor suggest a conserved hydrophobic asparagine-cage that constrains the activation of class A G protein-coupled receptors. *Mol Pharmacol* **73**(1)**:** 94-103.

Baldanzi, G, Filigheddu, N, Cutrupi, S, Catapano, F, Bonissoni, S, Fubini, A, Malan, D, Baj, G, Granata, R, Broglio, F, Papotti, M, Surico, N, Bussolino, F, Isgaard, J, Deghenghi, R, Sinigaglia, F, Prat, M, Muccioli, G, Ghigo, E, Graziani, A (2002) Ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. *J Cell Biol* **159**(6)**:** 1029-1037.

Baldwin, JM, Schertler, GF, Unger, VM (1997) An alpha-carbon template for the transmembrane helices in the rhodopsin family of G-protein-coupled receptors. *J Mol Biol* **12**;272(1); 144-64

Ballesteros, J, Kitanovic, S, Guarnieri, F, Davies, P, Fromme, BJ, Konvicka, K, Chi, L, Millar, RP, Davidson, JS, Weinstein, H, Sealfon, SC (1998) Functional microdomains in G-protein-coupled receptors. The conserved arginine-cage motif in the gonadotropin-releasing hormone receptor. *J Biol Chem* **273**(17)**:** 10445-10453.

Ballesteros, JA, Jensen, AD, Liapakis, G, Rasmussen, SG, Shi, L, Gether, U, Javitch, JA (2001) Activation of the beta 2-adrenergic receptor involves disruption of an ionic lock between the cytoplasmic ends of transmembrane segments 3 and 6. *J Biol Chem* **276**(31)**:** 29171-29177.

Ballesteros, JA, Weinstein, H (1992) Analysis and refinement of criteria for predicting the structure and relative orientations of transmembranal helical domains. *Biophys J* **62**(1)**:** 107-109.

Baneres, JL, Mesnier, D, Martin, A, Joubert, L, Dumuis, A, Bockaert, J (2005) Molecular characterization of a purified 5-HT4 receptor: a structural basis for drug efficacy. *J Biol Chem* **280**(21)**:** 20253-20260.

Bannert, N, Craig, S, Farzan, M, Sogah, D, Santo, NV, Choe, H, Sodroski, J (2001) Sialylated O-glycans and sulfated tyrosines in the NH2-terminal domain of CC chemokine receptor 5 contribute to high affinity binding of chemokines. *J Exp Med* **194**(11)**:** 1661-1673.

Baranski, TJ, Herzmark, P, Lichtarge, O, Gerber, BO, Trueheart, J, Meng, EC, Iiri, T, Sheikh, SP, Bourne, HR (1999) C5a receptor activation. Genetic identification of critical residues in four transmembrane helices. *J Biol Chem* **274**(22)**:** 15757-15765.

Bayburt, TH, Leitz, AJ, Xie, G, Oprian, DD, Sligar, SG (2007) Transducin activation by nanoscale lipid bilayers containing one and two rhodopsins. *J Biol Chem* **282**(20)**:** 14875-14881.

Bays, HE (2004) Current and investigational antiobesity agents and obesity therapeutic treatment targets. *Obes Res* **12**(8)**:** 1197-1211.

Bedendi, I, Alloatti, G, Marcantoni, A, Malan, D, Catapano, F, Ghe, C, Deghenghi, R, Ghigo, E, Muccioli, G (2003) Cardiac effects of ghrelin and its endogenous derivatives des-octanoyl ghrelin and des-Gln14-ghrelin. *Eur J Pharmacol* **476**(1-2)**:** 87-95.

Bee, MS, Hulme, EC (2007) Functional analysis of transmembrane domain 2 of the M1 muscarinic acetylcholine receptor. *J Biol Chem* **282**(44)**:** 32471-32479.

Befort, K, Filliol, D, Decaillot, FM, Gaveriaux-Ruff, C, Hoehe, MR, Kieffer, BL (2001) A single nucleotide polymorphic mutation in the human mu-opioid receptor severely impairs receptor signaling. *J Biol Chem* **276**(5)**:** 3130-3137.

Befort, K, Tabbara, L, Kling, D, Maigret, B, Kieffer, BL (1996) Role of aromatic transmembrane residues of the delta-opioid receptor in ligand recognition. *J Biol Chem* **271**(17)**:** 10161-10168.

Benovic, JL, Pike, LJ, Cerione, RA, Staniszewski, C, Yoshimasa, T, Codina, J, Caron, MG, Lefkowitz, RJ (1985) Phosphorylation of the mammalian beta-adrenergic receptor by cyclic AMP-dependent protein kinase. Regulation of the rate of receptor phosphorylation and dephosphorylation by agonist occupancy and effects on coupling of the receptor to the stimulatory guanine nucleotide regulatory protein. *J Biol Chem* **260**(11)**:** 7094-7101.

Bhattacharya, M, Babwah, AV, Ferguson, SS (2004) Small GTP-binding protein-coupled receptors. *Biochem Soc Trans* **32**(Pt 6)**:** 1040-1044.

Bhattacharya, S, Hall, SE, Vaidehi, N (2008) Agonist-induced conformational changes in bovine rhodopsin: insight into activation of G-protein-coupled receptors. *J Mol Biol* **382**(2)**:** 539-555.

Bhogal, N, Donnelly, D, Findlay, JB (1994) The ligand binding site of the neurokinin 2 receptor. Site-directed mutagenesis and identification of neurokinin A binding residues in the human neurokinin 2 receptor. *J Biol Chem* **269**(44)**:** 27269-27274.

Biebermann, H, Schoneberg, T, Schulz, A, Krause, G, Gruters, A, Schultz, G, Gudermann, T (1998) A conserved tyrosine residue (Y601) in transmembrane domain 5 of the human thyrotropin receptor serves as a molecular switch to determine G-protein coupling. *Faseb J* **12**(14)**:** 1461-1471.

Birdsall, NJ, Lazareno, S, Matsui, H (1996) Allosteric regulation of muscarinic receptors. *Prog Brain Res* **109:** 147-151.

Blin, N, Yun, J, Wess, J (1995) Mapping of single amino acid residues required for selective activation of Gq/11 by the m3 muscarinic acetylcholine receptor. *J Biol Chem* **270**(30)**:** 17741-17748.

Bokoch, MP, Zou, Y, Rasmussen, SG, Liu, CW, Nygaard, R, Rosenbaum, DM, Fung, JJ, Choi, HJ, Thian, FS, Kobilka, TS, Puglisi, JD, Weis, WI, Pardo, L, Prosser, RS, Mueller, L, Kobilka, BK (2010) Ligand-specific regulation of the extracellular surface of a G-protein-coupled receptor. *Nature* **463**(7277)**:** 108-112.

Bond, RA, Leff, P, Johnson, TD, Milano, CA, Rockman, HA, McMinn, TR, Apparsundaram, S, Hyek, MF, Kenakin, TP, Allen, LF, et al. (1995) Physiological effects of inverse agonists in transgenic mice with myocardial overexpression of the beta 2-adrenoceptor. *Nature* **374**(6519)**:** 272-276.

Bouley, R, Sun, TX, Chenard, M, McLaughlin, M, McKee, M, Lin, HY, Brown, D, Ausiello, DA (2003) Functional role of the NPxxY motif in internalization of the type 2 vasopressin receptor in LLC-PK1 cells. *Am J Physiol Cell Physiol* **285**(4)**:** C750-762.

Bowers, CY, Momany, FA, Reynolds, GA, Hong, A (1984) On the in vitro and in vivo activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. *Endocrinology* **114**(5)**:** 1537-1545.

Burstein, ES, Spalding, TA, Brann, MR (1998) The second intracellular loop of the m5 muscarinic receptor is the switch which enables G-protein coupling. *J Biol Chem* **273**(38)**:** 24322-24327.

Camina, JP (2006) Cell biology of the ghrelin receptor. *J Neuroendocrinol* **18**(1)**:** 65-76.

Camina, JP, Carreira, MC, El Messari, S, Llorens-Cortes, C, Smith, RG, Casanueva, FF (2004) Desensitization and endocytosis mechanisms of ghrelin-activated growth hormone secretagogue receptor 1a. *Endocrinology* **145**(2)**:** 930-940.

Carraro, G, Albertin, G, Abudukerimu, A, Aragona, F, Nussdorfer, GG (2004) Growth hormone secretagogue receptor subtypes 1a and 1b are expressed in the human adrenal cortex. *Int J Mol Med* **13**(2)**:** 295-298.

Cassoni, P, Ghe, C, Marrocco, T, Tarabra, E, Allia, E, Catapano, F, Deghenghi, R, Ghigo, E, Papotti, M, Muccioli, G (2004) Expression of ghrelin and biological activity of specific receptors for ghrelin and des-acyl ghrelin in human prostate neoplasms and related cell lines. *Eur J Endocrinol* **150**(2)**:** 173-184.

Cassoni, P, Papotti, M, Ghe, C, Catapano, F, Sapino, A, Graziani, A, Deghenghi, R, Reissmann, T, Ghigo, E, Muccioli, G (2001) Identification, characterization, and biological activity of specific receptors for natural (ghrelin) and synthetic growth hormone secretagogues and analogs in human breast carcinomas and cell lines. *J Clin Endocrinol Metab* **86**(4)**:** 1738-1745.

Ceresa, BP, Limbird, LE (1994) Mutation of an aspartate residue highly conserved among G-protein-coupled receptors results in nonreciprocal disruption of alpha 2-adrenergic receptor-G-protein interactions. A negative charge at amino acid residue 79 forecasts alpha 2A-adrenergic receptor sensitivity to allosteric modulation by monovalent cations and fully effective receptor/G-protein coupling. *J Biol Chem* **269**(47)**:** 29557-29564.

Charest, PG, Oligny-Longpre, G, Bonin, H, Azzi, M, Bouvier, M (2007) The V2 vasopressin receptor stimulates ERK1/2 activity independently of heterotrimeric G protein signalling. *Cell Signal* **19**(1)**:** 32-41.

Chelikani, P, Hornak, V, Eilers, M, Reeves, PJ, Smith, SO, RajBhandary, UL, Khorana, HG (2007) Role of group-conserved residues in the helical core of beta2-adrenergic receptor. *Proc Natl Acad Sci U S A* **104**(17)**:** 7027-7032.

Chen, HY, Trumbauer, ME, Chen, AS, Weingarth, DT, Adams, JR, Frazier, EG, Shen, Z, Marsh, DJ, Feighner, SD, Guan, XM, Ye, Z, Nargund, RP, Smith, RG, Van der Ploeg, LH, Howard, AD, MacNeil, DJ, Qian, S (2004) Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. *Endocrinology* **145**(6)**:** 2607-2612.

Cherezov, V, Rosenbaum, DM, Hanson, MA, Rasmussen, SG, Thian, FS, Kobilka, TS, Choi, HJ, Kuhn, P, Weis, WI, Kobilka, BK, Stevens, RC (2007) High-resolution crystal structure of an engineered human beta2-adrenergic G protein-coupled receptor. *Science* **318**(5854)**:** 1258-1265.

Choi, K, Roh, SG, Hong, YH, Shrestha, YB, Hishikawa, D, Chen, C, Kojima, M, Kangawa, K, Sasaki, S (2003) The role of ghrelin and growth hormone secretagogues receptor on rat adipogenesis. *Endocrinology* **144**(3)**:** 754-759.

Christopoulos, A, Christopoulos, G, Morfis, M, Udawela, M, Laburthe, M, Couvineau, A, Kuwasako, K, Tilakaratne, N, Sexton, PM (2003) Novel receptor partners and function of receptor activity-modifying proteins. *J Biol Chem* **278**(5)**:** 3293-3297.

Christopoulos, A, Kenakin, T (2002) G protein-coupled receptor allosterism and complexing. *Pharmacol Rev* **54**(2)**:** 323-374.

Chung, DA, Wade, SM, Fowler, CB, Woods, DD, Abada, PB, Mosberg, HI, Neubig, RR (2002) Mutagenesis and peptide analysis of the DRY motif in the alpha2A adrenergic receptor: evidence for alternate mechanisms in G protein-coupled receptors. *Biochem Biophys Res Commun* **293**(4)**:** 1233-1241.

Claeysen, S, Govaerts, C, Lefort, A, Van Sande, J, Costagliola, S, Pardo, L, Vassart, G (2002) A conserved Asn in TM7 of the thyrotropin receptor is a common requirement for activation by both mutations and its natural agonist. *FEBS Lett* **517**(1-3)**:** 195-200.

Claeysen, S, Joubert, L, Sebben, M, Bockaert, J, Dumuis, A (2003) A single mutation in the 5-HT4 receptor (5-HT4-R D100(3.32)A) generates a Gs-coupled receptor activated exclusively by synthetic ligands (RASSL). *J Biol Chem* **278**(2)**:** 699-702.

Clark, AJ (1937) Individual Variation in Response to Drugs. *Br Med J* **2**(3997)**:** 307-310.

Coetsee, M, Millar, RP, Flanagan, CA, Lu, ZL (2008) Identification of Tyr(290(6.58)) of the human gonadotropin-releasing hormone (GnRH) receptor as a contact residue for both GnRH I and GnRH II: importance for high-affinity binding and receptor activation. *Biochemistry* **47**(39)**:** 10305-10313.

Coge, F, Guenin, SP, Renouard-Try, A, Rique, H, Ouvry, C, Fabry, N, Beauverger, P, Nicolas, JP, Galizzi, JP, Boutin, JA, Canet, E (1999) Truncated isoforms inhibit [3H]prazosin binding and cellular trafficking of native human alpha1A-adrenoceptors. *Biochem J* **343 Pt 1:** 231-239.

Cohen, GB, Oprian, DD, Robinson, PR (1992) Mechanism of activation and inactivation of opsin: role of Glu113 and Lys296. *Biochemistry* **31**(50)**:** 12592-12601.

Cohen, GB, Yang, T, Robinson, PR, Oprian, DD (1993) Constitutive activation of opsin: influence of charge at position 134 and size at position 296. *Biochemistry* **32**(23)**:** 6111-6115.

Conn, PJ, Christopoulos, A, Lindsley, CW (2009) Allosteric modulators of GPCRs: a novel approach for the treatment of CNS disorders. *Nat Rev Drug Discov* **8**(1)**:** 41-54.

Conner, AC, Hay, DL, Simms, J, Howitt, SG, Schindler, M, Smith, DM, Wheatley, M, Poyner, DR (2005) A key role for transmembrane prolines in calcitonin receptor-like receptor agonist binding and signalling: implications for family B G-protein-coupled receptors. *Mol Pharmacol* **67**(1)**:** 20-31.

Conner, AC, Simms, J, Barwell, J, Wheatley, M, Poyner, DR (2007) Ligand binding and activation of the CGRP receptor. *Biochem Soc Trans* **35**(Pt 4)**:** 729-732.

Cook, JV, Eidne, KA (1997) An intramolecular disulfide bond between conserved extracellular cysteines in the gonadotropin-releasing hormone receptor is essential for binding and activation. *Endocrinology* **138**(7)**:** 2800-2806.

Cornea, A, Janovick, JA, Maya-Nunez, G, Conn, PM (2001) Gonadotropin-releasing hormone receptor microaggregation. Rate monitored by fluorescence resonance energy transfer. *J Biol Chem* **276**(3)**:** 2153-2158.

Costa, T, Cotecchia, S (2005) Historical review: Negative efficacy and the constitutive activity of G-protein-coupled receptors. *Trends Pharmacol Sci* **26**(12)**:** 618-624.

Costa, T, Herz, A (1989) Antagonists with negative intrinsic activity at delta opioid receptors coupled to GTP-binding proteins. *Proc Natl Acad Sci U S A* **86**(19)**:** 7321-7325.

Costanzi, S, Joshi, BV, Maddileti, S, Mamedova, L, Gonzalez-Moa, MJ, Marquez, VE, Harden, TK, Jacobson, KA (2005) Human P2Y(6) receptor: molecular modeling leads to the rational design of a novel agonist based on a unique conformational preference. *J Med Chem* **48**(26)**:** 8108-8111.

Cowley, MA, Smith, RG, Diano, S, Tschop, M, Pronchuk, N, Grove, KL, Strasburger, CJ, Bidlingmaier, M, Esterman, M, Heiman, ML, Garcia-Segura, LM, Nillni, EA, Mendez, P, Low, MJ, Sotonyi, P, Friedman, JM, Liu, H, Pinto, S, Colmers, WF, Cone, RD, Horvath, TL (2003) The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron* **37**(4)**:** 649-661.

Crocker, E, Eilers, M, Ahuja, S, Hornak, V, Hirshfeld, A, Sheves, M, Smith, SO (2006) Location of Trp265 in metarhodopsin II: implications for the activation mechanism of the visual receptor rhodopsin. *J Mol Biol* **357**(1)**:** 163-172.

Cruz, SA, Tseng, YC, Kaiya, H, Hwang, PP (2010) Ghrelin affects carbohydrate-glycogen metabolism via insulin inhibition and glucagon stimulation in the zebrafish (Danio rerio) brain. *Comp Biochem Physiol A Mol Integr Physiol* **156**(2)**:** 190-200.

Cummings, DE, Clement, K, Purnell, JQ, Vaisse, C, Foster, KE, Frayo, RS, Schwartz, MW, Basdevant, A, Weigle, DS (2002) Elevated plasma ghrelin levels in Prader Willi syndrome. *Nat Med* **8**(7)**:** 643-644.

Cummings, DE, Purnell, JQ, Frayo, RS, Schmidova, K, Wisse, BE, Weigle, DS (2001) A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* **50**(8)**:** 1714-1719.

Cvejic, S, Devi, LA (1997) Dimerization of the delta opioid receptor: implication for a role in receptor internalization. *J Biol Chem* **272**(43)**:** 26959-26964.

Daaka, Y, Luttrell, LM, Ahn, S, Della Rocca, GJ, Ferguson, SS, Caron, MG, Lefkowitz, RJ (1998) Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J Biol Chem* **273**(2)**:** 685-688.

Damke, H, Baba, T, Warnock, DE, Schmid, SL (1994) Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J Cell Biol* **127**(4)**:** 915-934.

Date, Y, Kojima, M, Hosoda, H, Sawaguchi, A, Mondal, MS, Suganuma, T, Matsukura, S, Kangawa, K, Nakazato, M (2000) Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology* **141**(11)**:** 4255-4261.

Date, Y, Nakazato, M, Murakami, N, Kojima, M, Kangawa, K, Matsukura, S (2001) Ghrelin acts in the central nervous system to stimulate gastric acid secretion. *Biochem Biophys Res Commun* **280**(3)**:** 904-907.

Davenport, AP, Bonner, TI, Foord, SM, Harmar, AJ, Neubig, RR, Pin, JP, Spedding, M, Kojima, M, Kangawa, K (2005) International Union of Pharmacology. LVI. Ghrelin receptor nomenclature, distribution, and function. *Pharmacol Rev* **57**(4)**:** 541-546.

Davidson, FF, Loewen, PC, Khorana, HG (1994) Structure and function in rhodopsin: replacement by alanine of cysteine residues 110 and 187, components of a conserved disulfide bond in rhodopsin, affects the light-activated metarhodopsin II state. *Proc Natl Acad Sci U S A* **91**(9)**:** 4029-4033.

Davis, D, Liu, X, Segaloff, DL (1995) Identification of the sites of N-linked glycosylation on the follicle-stimulating hormone (FSH) receptor and assessment of their role in FSH receptor function. *Mol Endocrinol* **9**(2)**:** 159-170.

De Lean, A, Stadel, JM, Lefkowitz, RJ (1980) A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor. *J Biol Chem* **255**(15)**:** 7108-7117.

de Ligt, RA, Kourounakis, AP, AP, IJ (2000) Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery. *Br J Pharmacol* **130**(1)**:** 1-12.

DelParigi, A, Tschop, M, Heiman, ML, Salbe, AD, Vozarova, B, Sell, SM, Bunt, JC, Tataranni, PA (2002) High circulating ghrelin: a potential cause for hyperphagia and obesity in prader-willi syndrome. *J Clin Endocrinol Metab* **87**(12)**:** 5461-5464.

Deslauriers, B, Ponce, C, Lombard, C, Larguier, R, Bonnafous, JC, Marie, J (1999) N-glycosylation requirements for the AT1a angiotensin II receptor delivery to the plasma membrane. *Biochem J* **339 (Pt 2):** 397-405.

Devi, LA (2001) Heterodimerization of G-protein-coupled receptors: pharmacology, signaling and trafficking. *Trends Pharmacol Sci* **22**(10)**:** 532-537.

Ding, FX, Lee, BK, Hauser, M, Patri, R, Arshava, B, Becker, JM, Naider, F (2002) Study of the binding environment of alpha-factor in its G protein-coupled receptor using fluorescence spectroscopy. *J Pept Res* **60**(1)**:** 65-74.

Donnelly, D, Maudsley, S, Gent, JP, Moser, RN, Hurrell, CR, Findlay, JB (1999) Conserved polar residues in the transmembrane domain of the human tachykinin NK2 receptor: functional roles and structural implications. *Biochem J* **339 (Pt 1):** 55-61.

Dube, P, Konopka, JB (1998) Identification of a polar region in transmembrane domain 6 that regulates the function of the G protein-coupled alpha-factor receptor. *Mol Cell Biol* **18**(12)**:** 7205-7215.

Dunham, TD, Farrens, DL (1999) Conformational changes in rhodopsin. Movement of helix f detected by site-specific chemical labeling and fluorescence spectroscopy. *J Biol Chem* **274**(3)**:** 1683-1690.

Eilers, M, Hornak, V, Smith, SO, Konopka, JB (2005) Comparison of class A and D G protein-coupled receptors: common features in structure and activation. *Biochemistry* **44**(25)**:** 8959-8975.

Elling, CE, Frimurer, TM, Gerlach, LO, Jorgensen, R, Holst, B, Schwartz, TW (2006) Metal ion site engineering indicates a global toggle switch model for seven-transmembrane receptor activation. *J Biol Chem* **281**(25)**:** 17337-17346.

Elling, CE, Raffetseder, U, Nielsen, SM, Schwartz, TW (2000) Disulfide bridge engineering in the tachykinin NK1 receptor. *Biochemistry* **39**(4)**:** 667-675.

Elling, CE, Thirstrup, K, Nielsen, SM, Hjorth, SA, Schwartz, TW (1997) Metal-ion sites as structural and functional probes of helix-helix interactions in 7TM receptors. *Ann N Y Acad Sci* **814:** 142-151.

Erlenbach, I, Wess, J (1998) Molecular basis of V2 vasopressin receptor/Gs coupling selectivity. *J Biol Chem* **273**(41)**:** 26549-26558.

Espelund, U, Hansen, TK, Orskov, H, Frystyk, J (2003) Assessment of ghrelin. *APMIS Suppl*(109)**:** 140-145.

Faham, S, Yang, D, Bare, E, Yohannan, S, Whitelegge, JP, Bowie, JU (2004) Side-chain contributions to membrane protein structure and stability. *J Mol Biol* **335**(1)**:** 297-305.

Fan, T, Varghese, G, Nguyen, T, Tse, R, O'Dowd, BF, George, SR (2005) A role for the distal carboxyl tails in generating the novel pharmacology and G protein activation profile of mu and delta opioid receptor hetero-oligomers. *J Biol Chem* **280**(46)**:** 38478-38488.

Fanelli, F, Barbier, P, Zanchetta, D, de Benedetti, PG, Chini, B (1999) Activation mechanism of human oxytocin receptor: a combined study of experimental and computer-simulated mutagenesis. *Mol Pharmacol* **56**(1)**:** 214-225.

Farrens, DL, Altenbach, C, Yang, K, Hubbell, WL, Khorana, HG (1996) Requirement of rigid-body motion of transmembrane helices for light activation of rhodopsin. *Science* **274**(5288)**:** 768-770.

Favre, N, Fanelli, F, Missotten, M, Nichols, A, Wilson, J, di Tiani, M, Rommel, C, Scheer, A (2005) The DRY motif as a molecular switch of the human oxytocin receptor. *Biochemistry* **44**(30)**:** 9990-10008.

Feighner, SD, Howard, AD, Prendergast, K, Palyha, OC, Hreniuk, DL, Nargund, R, Underwood, D, Tata, JR, Dean, DC, Tan, CP, McKee, KK, Woods, JW, Patchett, AA, Smith, RG, Van der Ploeg, LH (1998) Structural requirements for the activation of the human growth hormone secretagogue receptor by peptide and nonpeptide secretagogues. *Mol Endocrinol* **12**(1)**:** 137-145.

Feng, W, Song, ZH (2003) Effects of D3.49A, R3.50A, and A6.34E mutations on ligand binding and activation of the cannabinoid-2 (CB2) receptor. *Biochem Pharmacol* **65**(7)**:** 1077-1085.

Fernandez-Fernandez, R, Tena-Sempere, M, Aguilar, E, Pinilla, L (2004) Ghrelin effects on gonadotropin secretion in male and female rats. *Neurosci Lett* **362**(2)**:** 103-107.

Ferre, S, Karcz-Kubicha, M, Hope, BT, Popoli, P, Burgueno, J, Gutierrez, MA, Casado, V, Fuxe, K, Goldberg, SR, Lluis, C, Franco, R, Ciruela, F (2002) Synergistic interaction between adenosine A2A and glutamate mGlu5 receptors: implications for striatal neuronal function. *Proc Natl Acad Sci U S A* **99**(18)**:** 11940-11945.

Filipek, S, Teller, DC, Palczewski, K, Stenkamp, R (2003) The crystallographic model of rhodopsin and its use in studies of other G protein-coupled receptors. *Annu Rev Biophys Biomol Struct* **32:** 375-397.

Flanagan, CA, Zhou, W, Chi, L, Yuen, T, Rodic, V, Robertson, D, Johnson, M, Holland, P, Millar, RP, Weinstein, H, Mitchell, R, Sealfon, SC (1999) The functional microdomain in transmembrane helices 2 and 7 regulates expression, activation, and coupling pathways of the gonadotropin-releasing hormone receptor. *J Biol Chem* **274**(41)**:** 28880-28886.

Floquet, N, M'Kadmi, C, Perahia, D, Gagne, D, Berge, G, Marie, J, Baneres, JL, Galleyrand, JC, Fehrentz, JA, Martinez, J (2010) Activation of the ghrelin receptor is described by a privileged collective motion: a model for constitutive and agonist-induced activation of a sub-class A G-protein coupled receptor (GPCR). *J Mol Biol* **395**(4)**:** 769-784.

Fong, TM, Huang, RC, Yu, H, Swain, CJ, Underwood, D, Cascieri, MA, Strader, CD (1995) Mutational analysis of neurokinin receptor function. *Can J Physiol Pharmacol* **73**(7)**:** 860-865.

Fraser, CM, Wang, CD, Robinson, DA, Gocayne, JD, Venter, JC (1989) Site-directed mutagenesis of m1 muscarinic acetylcholine receptors: conserved aspartic acids play important roles in receptor function. *Mol Pharmacol* **36**(6)**:** 840-847.

Fredriksson, R, Lagerstrom, MC, Lundin, LG, Schioth, HB (2003) The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol* **63**(6)**:** 1256-1272.

Freedman, NJ, Lefkowitz, RJ (1996) Desensitization of G protein-coupled receptors. *Recent Prog Horm Res* **51:** 319-351; discussion 352-313.

Fritze, O, Filipek, S, Kuksa, V, Palczewski, K, Hofmann, KP, Ernst, OP (2003) Role of the conserved NPxxY(x)5,6F motif in the rhodopsin ground state and during activation. *Proc Natl Acad Sci U S A* **100**(5)**:** 2290-2295.

Fuchs, AR, Fuchs, F, Husslein, P, Soloff, MS (1984) Oxytocin receptors in the human uterus during pregnancy and parturition. *Am J Obstet Gynecol* **150**(6)**:** 734-741.

Gales, C, Kowalski-Chauvel, A, Dufour, MN, Seva, C, Moroder, L, Pradayrol, L, Vaysse, N, Fourmy, D, Silvente-Poirot, S (2000) Mutation of Asn-391 within the conserved NPXXY motif of the cholecystokinin B receptor abolishes Gq protein activation without affecting its association with the receptor. *J Biol Chem* **275**(23)**:** 17321-17327.

Galvez, T, Duthey, B, Kniazeff, J, Blahos, J, Rovelli, G, Bettler, B, Prezeau, L, Pin, JP (2001) Allosteric interactions between GB1 and GB2 subunits are required for optimal GABA(B) receptor function. *Embo J* **20**(9)**:** 2152-2159.

Garcia, JM, Garcia-Touza, M, Hijazi, RA, Taffet, G, Epner, D, Mann, D, Smith, RG, Cunningham, GR, Marcelli, M (2005) Active ghrelin levels and active to total ghrelin ratio in cancer-induced cachexia. *J Clin Endocrinol Metab* **90**(5)**:** 2920-2926.

Garcia Rodriguez, C, Cundell, DR, Tuomanen, EI, Kolakowski, LF, Jr., Gerard, C, Gerard, NP (1995) The role of N-glycosylation for functional expression of the human platelet-activating factor receptor. Glycosylation is required for efficient membrane trafficking. *J Biol Chem* **270**(42)**:** 25178-25184.

Gauna, C, van de Zande, B, van Kerkwijk, A, Themmen, AP, van der Lely, AJ, Delhanty, PJ (2007) Unacylated ghrelin is not a functional antagonist but a full agonist of the type 1a growth hormone secretagogue receptor (GHS-R). *Mol Cell Endocrinol* **274**(1-2)**:** 30-34.

Gaylinn, BD (1999) Molecular and cell biology of the growth hormone-releasing hormone receptor. *Growth Horm IGF Res* **9 Suppl A:** 37-44.

Gaytan, F, Morales, C, Barreiro, ML, Jeffery, P, Chopin, LK, Herington, AC, Casanueva, FF, Aguilar, E, Dieguez, C, Tena-Sempere, M (2005) Expression of growth hormone secretagogue receptor type 1a, the functional ghrelin receptor, in human ovarian surface epithelium, mullerian duct derivatives, and ovarian tumors. *J Clin Endocrinol Metab* **90**(3)**:** 1798-1804.

George, SR, Fan, T, Xie, Z, Tse, R, Tam, V, Varghese, G, O'Dowd, BF (2000) Oligomerization of mu- and delta-opioid receptors. Generation of novel functional properties. *J Biol Chem* **275**(34)**:** 26128-26135.

George, SR, Lee, SP, Varghese, G, Zeman, PR, Seeman, P, Ng, GY, O'Dowd, BF (1998) A transmembrane domain-derived peptide inhibits D1 dopamine receptor function without affecting receptor oligomerization. *J Biol Chem* **273**(46)**:** 30244-30248.

Gether, U (2000) Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocr Rev* **21**(1)**:** 90-113.

Gether, U, Lin, S, Ghanouni, P, Ballesteros, JA, Weinstein, H, Kobilka, BK (1997) Agonists induce conformational changes in transmembrane domains III and VI of the beta2 adrenoceptor. *Embo J* **16**(22)**:** 6737-6747.

Gnanapavan, S, Kola, B, Bustin, SA, Morris, DG, McGee, P, Fairclough, P, Bhattacharya, S, Carpenter, R, Grossman, AB, Korbonits, M (2002) The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *J Clin Endocrinol Metab* **87**(6)**:** 2988.

Gomes, I, Jordan, BA, Gupta, A, Trapaidze, N, Nagy, V, Devi, LA (2000) Heterodimerization of mu and delta opioid receptors: A role in opiate synergy. *J Neurosci* **20**(22)**:** RC110.

Gotze, K, Jakobs, KH (1994) Unoccupied beta-adrenoceptor-induced adenylyl cyclase stimulation in turkey erythrocyte membranes. *Eur J Pharmacol* **268**(2)**:** 151-158.

Gouldson, PR, Higgs, C, Smith, RE, Dean, MK, Gkoutos, GV, Reynolds, CA (2000) Dimerization and domain swapping in G-protein-coupled receptors: a computational study. *Neuropsychopharmacology* **23**(4 Suppl)**:** S60-77.

Govaerts, C, Lefort, A, Costagliola, S, Wodak, SJ, Ballesteros, JA, Van Sande, J, Pardo, L, Vassart, G (2001) A conserved Asn in transmembrane helix 7 is an on/off switch in the activation of the thyrotropin receptor. *J Biol Chem* **276**(25)**:** 22991-22999.

Grant, M, Kumar, U (2010) The role of G-proteins in the dimerisation of human somatostatin receptor types 2 and 5. *Regul Pept* **159**(1-3)**:** 3-8.

Greasley, PJ, Fanelli, F, Rossier, O, Abuin, L, Cotecchia, S (2002) Mutagenesis and modelling of the alpha(1b)-adrenergic receptor highlight the role of the helix 3/helix 6 interface in receptor activation. *Mol Pharmacol* **61**(5)**:** 1025-1032.

Groblewski, T, Maigret, B, Larguier, R, Lombard, C, Bonnafous, JC, Marie, J (1997) Mutation of Asn111 in the third transmembrane domain of the AT1A angiotensin II receptor induces its constitutive activation. *J Biol Chem* **272**(3)**:** 1822-1826.

Guan, XM, Yu, H, Palyha, OC, McKee, KK, Feighner, SD, Sirinathsinghji, DJ, Smith, RG, Van der Ploeg, LH, Howard, AD (1997) Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. *Brain Res Mol Brain Res* **48**(1)**:** 23-29.

Gurevich, VV, Gurevich, EV (2006) The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. *Pharmacol Ther* **110**(3)**:** 465-502.

Gutierrez, JA, Solenberg, PJ, Perkins, DR, Willency, JA, Knierman, MD, Jin, Z, Witcher, DR, Luo, S, Onyia, JE, Hale, JE (2008) Ghrelin octanoylation mediated by an orphan lipid transferase. *Proc Natl Acad Sci U S A* **105**(17)**:** 6320-6325.

Habecker, BA, Tietje, KM, van Koppen, CJ, Creason, SA, Goldman, PS, Migeon, JC, Parenteau, LA, Nathanson, NM (1993) Regulation of expression and function of muscarinic receptors. *Life Sci* **52**(5-6)**:** 429-432.

Hakalahti, AE, Vierimaa, MM, Lilja, MK, Kumpula, EP, Tuusa, JT, Petaja-Repo, UE (2010) Human {beta}1 adrenergic receptor is subject to constitutive and regulated N-terminal cleavage. *J Biol Chem* **285**(37)**:** 28850-61

Han, M, Smith, SO, Sakmar, TP (1998) Constitutive activation of opsin by mutation of methionine 257 on transmembrane helix 6. *Biochemistry* **37**(22)**:** 8253-8261.

Han, SJ, Hamdan, FF, Kim, SK, Jacobson, KA, Brichta, L, Bloodworth, LM, Li, JH, Wess, J (2005) Pronounced conformational changes following agonist activation of the M(3) muscarinic acetylcholine receptor. *J Biol Chem* **280**(26)**:** 24870-24879.

Haskell-Luevano, C, Monck, EK (2001) Agouti-related protein functions as an inverse agonist at a constitutively active brain melanocortin-4 receptor. *Regul Pept* **99**(1)**:** 1-7.

Hawtin, SR, Davies, AR, Matthews, G, Wheatley, M (2001a) Identification of the glycosylation sites utilized on the V1a vasopressin receptor and assessment of their role in receptor signalling and expression. *Biochem J* **357**(Pt 1)**:** 73-81.

Hawtin, SR, Simms, J, Conner, M, Lawson, Z, Parslow, RA, Trim, J, Sheppard, A, Wheatley, M (2006) Charged extracellular residues, conserved throughout a G-protein-coupled receptor family, are required for ligand binding, receptor activation, and cell-surface expression. *J Biol Chem* **281**(50)**:** 38478-38488.

Hawtin, SR, Tobin, AB, Patel, S, Wheatley, M (2001b) Palmitoylation of the vasopressin V1a receptor reveals different conformational requirements for signaling, agonist-induced receptor phosphorylation, and sequestration. *J Biol Chem* **276**(41)**:** 38139-38146.

Hawtin, SR, Wheatley, M (1997) Characterization of an epitope-tagged vasopressin V1a receptor: a probe for receptor structure and function. *Biochem Soc Trans* **25**(3)**:** 437S.

Hay, DL, Conner, AC, Howitt, SG, Smith, DM, Poyner, DR (2004a) The pharmacology of adrenomedullin receptors and their relationship to CGRP receptors. *J Mol Neurosci* **22**(1-2)**:** 105-113.

Hay, DL, Conner, AC, Howitt, SG, Takhshid, MA, Simms, J, Mahmoud, K, Poyner, DR (2004b) The pharmacology of CGRP-responsive receptors in cultured and transfected cells. *Peptides* **25**(11)**:** 2019-2026.

He, J, Xu, J, Castleberry, AM, Lau, AG, Hall, RA (2002) Glycosylation of beta(1)-adrenergic receptors regulates receptor surface expression and dimerization. *Biochem Biophys Res Commun* **297**(3)**:** 565-572.

He, R, Browning, DD, Ye, RD (2001) Differential roles of the NPXXY motif in formyl peptide receptor signaling. *J Immunol* **166**(6)**:** 4099-4105.

Hebert, TE, Moffett, S, Morello, JP, Loisel, TP, Bichet, DG, Barret, C, Bouvier, M (1996) A peptide derived from a beta2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. *J Biol Chem* **271**(27)**:** 16384-16392.

Hepler, JR (1999) Emerging roles for RGS proteins in cell signalling. *Trends Pharmacol Sci* **20**(9)**:** 376-382.

Hicke, L, Dunn, R (2003) Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins. *Annu Rev Cell Dev Biol* **19:** 141-172.

Hicke, L, Schubert, HL, Hill, CP (2005) Ubiquitin-binding domains. *Nat Rev Mol Cell Biol* **6**(8)**:** 610-621.

Hill-Eubanks, D, Burstein, ES, Spalding, TA, Brauner-Osborne, H, Brann, MR (1996) Structure of a G-protein-coupling domain of a muscarinic receptor predicted by random saturation mutagenesis. *J Biol Chem* **271**(6)**:** 3058-3065.

Hoffmann, C, Moro, S, Nicholas, RA, Harden, TK, Jacobson, KA (1999) The role of amino acids in extracellular loops of the human P2Y1 receptor in surface expression and activation processes. *J Biol Chem* **274**(21)**:** 14639-14647.

Holliday, ND, Holst, B, Rodionova, EA, Schwartz, TW, Cox, HM (2007) Importance of constitutive activity and arrestin-independent mechanisms for intracellular trafficking of the ghrelin receptor. *Mol Endocrinol* **21**(12)**:** 3100-3112.

Holst, B, Cygankiewicz, A, Jensen, TH, Ankersen, M, Schwartz, TW (2003a) High constitutive signaling of the ghrelin receptor--identification of a potent inverse agonist. *Mol Endocrinol* **17**(11)**:** 2201-2210.

Holst, B, Frimurer, TM, Mokrosinski, J, Halkjaer, T, Cullberg, KB, Underwood, CR, Schwartz, TW (2009) Overlapping binding site for the endogenous agonist, small-molecule agonists, and ago-allosteric modulators on the ghrelin receptor. *Mol Pharmacol* **75**(1)**:** 44-59.

Holst, B, Holliday, ND, Bach, A, Elling, CE, Cox, HM, Schwartz, TW (2004a) Common structural basis for constitutive activity of the ghrelin receptor family. *J Biol Chem* **279**(51)**:** 53806-53817.

Holst, B, Lang, M, Brandt, E, Bach, A, Howard, A, Frimurer, TM, Beck-Sickinger, A, Schwartz, TW (2006a) Ghrelin receptor inverse agonists: identification of an active peptide core and its interaction epitopes on the receptor. *Mol Pharmacol* **70**(3)**:** 936-946.

Holst, B, Nygaard, R, Valentin-Hansen, L, Bach, A, Engelstoft, MS, Petersen, PS, Frimurer, TM, Schwartz, TW (2010) A conserved aromatic lock for the tryptophan rotameric switch in TM-VI of seven-transmembrane receptors. *J Biol Chem* **285**(6)**:** 3973-3985.

Holst, B, Schwartz, TW (2004b) Constitutive ghrelin receptor activity as a signaling set-point in appetite regulation. *Trends Pharmacol Sci* **25**(3)**:** 113-117.

Holst, B, Schwartz, TW (2006b) Ghrelin receptor mutations--too little height and too much hunger. *J Clin Invest* **116**(3)**:** 637-641.

Holst, B, Schwartz, TW (2003b) Molecular mechanism of agonism and inverse agonism in the melanocortin receptors: Zn(2+) as a structural and functional probe. *Ann N Y Acad Sci* **994:** 1-11.

Hong, S, Ryu, KS, Oh, MS, Ji, I, Ji, TH (1997) Roles of transmembrane prolines and proline-induced kinks of the lutropin/choriogonadotropin receptor. *J Biol Chem* **272**(7)**:** 4166-4171.

Hopp, TP, Prickett, KS, Price, VL, Libby, RT, March, CJ, Cerretti, DP, Urdal, DL, Conlon, PJ (1988) A Short Polypeptide Marker Sequence Useful for Recombinant Protein Identification and Purification. *Bio-Technology* **6**(10)**:** 1204-1210.

Horstman, DA, Brandon, S, Wilson, AL, Guyer, CA, Cragoe, EJ, Jr., Limbird, LE (1990) An aspartate conserved among G-protein receptors confers allosteric regulation of alpha 2-adrenergic receptors by sodium. *J Biol Chem* **265**(35)**:** 21590-21595.

Horstmeyer, A, Cramer, H, Sauer, T, Muller-Esterl, W, Schroeder, C (1996) Palmitoylation of endothelin receptor A. Differential modulation of signal transduction activity by post-translational modification. *J Biol Chem* **271**(34)**:** 20811-20819.

Hortin, G, Boime, I (1980) Inhibition of asparagine-linked glycosylation by incorporation of a threonine analog into nascent peptide chains. *J Biol Chem* **255**(17)**:** 8007-8010.

Howard, AD, Feighner, SD, Cully, DF, Arena, JP, Liberator, PA, Rosenblum, CI, Hamelin, M, Hreniuk, DL, Palyha, OC, Anderson, J, Paress, PS, Diaz, C, Chou, M, Liu, KK, McKee, KK, Pong, SS, Chaung, LY, Elbrecht, A, Dashkevicz, M, Heavens, R, Rigby, M, Sirinathsinghji, DJ, Dean, DC, Melillo, DG, Patchett, AA, Nargund, R, Griffin, PR, DeMartino, JA, Gupta, SK, Schaeffer, JM, Smith, RG, Van der Ploeg, LH (1996) A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science* **273**(5277)**:** 974-977.

Huang, P, Li, J, Chen, C, Visiers, I, Weinstein, H, Liu-Chen, LY (2001) Functional role of a conserved motif in TM6 of the rat mu opioid receptor: constitutively active and inactive receptors result from substitutions of Thr6.34(279) with Lys and Asp. *Biochemistry* **40**(45)**:** 13501-13509.

Huang, P, Visiers, I, Weinstein, H, Liu-Chen, LY (2002) The local environment at the cytoplasmic end of TM6 of the mu opioid receptor differs from those of rhodopsin and monoamine receptors: introduction of an ionic lock between the cytoplasmic ends of helices 3 and 6 by a L6.30(275)E mutation inactivates the mu opioid receptor and reduces the constitutive activity of its T6.34(279)K mutant. *Biochemistry* **41**(40)**:** 11972-11980.

Huang, RR, Vicario, PP, Strader, CD, Fong, TM (1995) Identification of residues involved in ligand binding to the neurokinin-2 receptor. *Biochemistry* **34**(31)**:** 10048-10055.

Hubbell, WL, Altenbach, C, Hubbell, CM, Khorana, HG (2003) Rhodopsin structure, dynamics, and activation: a perspective from crystallography, site-directed spin labeling, sulfhydryl reactivity, and disulfide cross-linking. *Adv Protein Chem* **63:** 243-290.

Hulme, EC, Lu, ZL, Saldanha, JW, Bee, MS (2003) Structure and activation of muscarinic acetylcholine receptors. *Biochem Soc Trans* **31**(Pt 1)**:** 29-34.

Hunyady, L, Bor, M, Balla, T, Catt, KJ (1995a) Critical role of a conserved intramembrane tyrosine residue in angiotensin II receptor activation. *J Biol Chem* **270**(17)**:** 9702-9705.

Hunyady, L, Bor, M, Baukal, AJ, Balla, T, Catt, KJ (1995b) A conserved NPLFY sequence contributes to agonist binding and signal transduction but is not an internalization signal for the type 1 angiotensin II receptor. *J Biol Chem* **270**(28)**:** 16602-16609.

Jaakola, VP, Griffith, MT, Hanson, MA, Cherezov, V, Chien, EY, Lane, JR, Ijzerman, AP, Stevens, RC (2008) The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. *Science* **322**(5905)**:** 1211-1217.

Janssen, JA, van der Lely, AJ, Lamberts, SW (2004) Is there a role of ghrelin in preventing catabolism? *J Endocrinol Invest* **27**(4)**:** 400-403.

Jarvik, JW, Telmer, CA (1998) Epitope tagging. *Annu Rev Genet* **32:** 601-618.

Jean-Alphonse, F, Hanyaloglu, AC (2010) Regulation of GPCR signal networks via membrane trafficking. *Mol Cell Endocrinol*.

Jensen, AD, Gether, U (2000) Assessing adrenergic receptor conformation using chemically reactive fluorescent probes. *Methods Mol Biol* **126:** 345-361.

Jiang, Q, Guo, D, Lee, BX, Van Rhee, AM, Kim, YC, Nicholas, RA, Schachter, JB, Harden, TK, Jacobson, KA (1997) A mutational analysis of residues essential for ligand recognition at the human P2Y1 receptor. *Mol Pharmacol* **52**(3)**:** 499-507.

Jones, KA, Borowsky, B, Tamm, JA, Craig, DA, Durkin, MM, Dai, M, Yao, WJ, Johnson, M, Gunwaldsen, C, Huang, LY, Tang, C, Shen, Q, Salon, JA, Morse, K, Laz, T, Smith, KE, Nagarathnam, D, Noble, SA, Branchek, TA, Gerald, C (1998) GABA(B) receptors function as a heteromeric assembly of the subunits GABA(B)R1 and GABA(B)R2. *Nature* **396**(6712)**:** 674-679.

Jongejan, A, Bruysters, M, Ballesteros, JA, Haaksma, E, Bakker, RA, Pardo, L, Leurs, R (2005) Linking agonist binding to histamine H1 receptor activation. *Nat Chem Biol* **1**(2)**:** 98-103.

Juaneda, C, Dumont, Y, Quirion, R (2000) The molecular pharmacology of CGRP and related peptide receptor subtypes. *Trends Pharmacol Sci* **21**(11)**:** 432-438.

Kalatskaya, I, Schussler, S, Blaukat, A, Muller-Esterl, W, Jochum, M, Proud, D, Faussner, A (2004) Mutation of tyrosine in the conserved NPXXY sequence leads to constitutive phosphorylation and internalization, but not signaling, of the human B2 bradykinin receptor. *J Biol Chem* **279**(30)**:** 31268-31276.

Karnik, SS, Gogonea, C, Patil, S, Saad, Y, Takezako, T (2003) Activation of G-protein-coupled receptors: a common molecular mechanism. *Trends Endocrinol Metab* **14**(9)**:** 431-437.

Kaushal, S, Ridge, KD, Khorana, HG (1994) Structure and function in rhodopsin: the role of asparagine-linked glycosylation. *Proc Natl Acad Sci U S A* **91**(9)**:** 4024-4028.

Kenakin, T (1995) Agonist-receptor efficacy. II. Agonist trafficking of receptor signals. *Trends Pharmacol Sci* **16**(7)**:** 232-238.

Kenakin, T (2004) Efficacy as a vector: the relative prevalence and paucity of inverse agonism. *Mol Pharmacol* **65**(1)**:** 2-11.

Kenakin, T (2001) Inverse, protean, and ligand-selective agonism: matters of receptor conformation. *Faseb J* **15**(3)**:** 598-611.

Kimura, T, Takemura, M, Nomura, S, Nobunaga, T, Kubota, Y, Inoue, T, Hashimoto, K, Kumazawa, I, Ito, Y, Ohashi, K, Koyama, M, Azuma, C, Kitamura, Y, Saji, F (1996) Expression of oxytocin receptor in human pregnant myometrium. *Endocrinology* **137**(2)**:** 780-785.

Kjelsberg, MA, Cotecchia, S, Ostrowski, J, Caron, MG, Lefkowitz, RJ (1992) Constitutive activation of the alpha 1B-adrenergic receptor by all amino acid substitutions at a single site. Evidence for a region which constrains receptor activation. *J Biol Chem* **267**(3)**:** 1430-1433.

Kjoller, L, Hall, A (1999) Signaling to Rho GTPases. *Exp Cell Res* **253**(1)**:** 166-179.

Klco, JM, Wiegand, CB, Narzinski, K, Baranski, TJ (2005) Essential role for the second extracellular loop in C5a receptor activation. *Nat Struct Mol Biol* **12**(4)**:** 320-326.

Kobilka, BK (2007) G protein coupled receptor structure and activation. *Biochim Biophys Acta* **1768**(4)**:** 794-807.

Kobilka, BK, Deupi, X (2007) Conformational complexity of G-protein-coupled receptors. *Trends Pharmacol Sci* **28**(8)**:** 397-406.

Kojima, M, Hosoda, H, Date, Y, Nakazato, M, Matsuo, H, Kangawa, K (1999) Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* **402**(6762)**:** 656-660.

Kojima, M, Hosoda, H, Matsuo, H, Kangawa, K (2001) Ghrelin: discovery of the natural endogenous ligand for the growth hormone secretagogue receptor. *Trends Endocrinol Metab* **12**(3)**:** 118-122.

Kojima, M, Kangawa, K (2005) Ghrelin: structure and function. *Physiol Rev* **85**(2)**:** 495-522.

Kolakowski, LF, Jr. (1994) GCRDb: a G-protein-coupled receptor database. *Receptors Channels* **2**(1)**:** 1-7.

Konvicka, K, Guarnieri, F, Ballesteros, JA, Weinstein, H (1998) A proposed structure for transmembrane segment 7 of G protein-coupled receptors incorporating an asn-Pro/Asp-Pro motif. *Biophys J* **75**(2)**:** 601-611.

Korbonits, M, Kojima, M, Kangawa, K, Grossman, AB (2001) Presence of ghrelin in normal and adenomatous human pituitary. *Endocrine* **14**(1)**:** 101-104.

Kornfeld, R, Kornfeld, S (1985) Assembly of asparagine-linked oligosaccharides. *Annu Rev Biochem* **54:** 631-664.

Kosugi, S, Mori, T, Shenker, A (1996) The role of Asp578 in maintaining the inactive conformation of the human lutropin/choriogonadotropin receptor. *J Biol Chem* **271**(50)**:** 31813-31817.

Koutkia, P, Meininger, G, Canavan, B, Breu, J, Grinspoon, S (2004) Metabolic regulation of growth hormone by free fatty acids, somatostatin, and ghrelin in HIV-lipodystrophy. *Am J Physiol Endocrinol Metab* **286**(2)**:** E296-303.

Kozak, M (1991) Structural features in eukaryotic mRNAs that modulate the initiation of translation. *J Biol Chem* **266**(30)**:** 19867-19870.

Krebs, A, Edwards, PC, Villa, C, Li, J, Schertler, GF (2003) The three-dimensional structure of bovine rhodopsin determined by electron cryomicroscopy. *J Biol Chem* **278**(50)**:** 50217-50225.

Kristiansen, K, Kroeze, WK, Willins, DL, Gelber, EI, Savage, JE, Glennon, RA, Roth, BL (2000) A highly conserved aspartic acid (Asp-155) anchors the terminal amine moiety of tryptamines and is involved in membrane targeting of the 5-HT(2A) serotonin receptor but does not participate in activation via a "salt-bridge disruption" mechanism. *J Pharmacol Exp Ther* **293**(3)**:** 735-746.

Krupnick, JG, Benovic, JL (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. *Annu Rev Pharmacol Toxicol* **38:** 289-319.

Krupnick, JG, Santini, F, Gagnon, AW, Keen, JH, Benovic, JL (1997) Modulation of the arrestin-clathrin interaction in cells. Characterization of beta-arrestin dominant-negative mutants. *J Biol Chem* **272**(51)**:** 32507-32512.

Kuhne, W (1977) Chemical processes in the retina. *Vision Res* **17**(11-12)**:** 1269-1316.

Lagane, B, Ballet, S, Planchenault, T, Balabanian, K, Le Poul, E, Blanpain, C, Percherancier, Y, Staropoli, I, Vassart, G, Oppermann, M, Parmentier, M, Bachelerie, F (2005) Mutation of the DRY motif reveals different structural requirements for the CC chemokine receptor 5-mediated signaling and receptor endocytosis. *Mol Pharmacol* **67**(6)**:** 1966-1976.

Laporte, SA, Miller, WE, Kim, KM, Caron, MG (2002) beta-Arrestin/AP-2 interaction in G protein-coupled receptor internalization: identification of a beta-arrestin binging site in beta 2-adaptin. *J Biol Chem* **277**(11)**:** 9247-9254.

Latronico, AC, Abell, AN, Arnhold, IJ, Liu, X, Lins, TS, Brito, VN, Billerbeck, AE, Segaloff, DL, Mendonca, BB (1998) A unique constitutively activating mutation in third transmembrane helix of luteinizing hormone receptor causes sporadic male gonadotropin-independent precocious puberty. *J Clin Endocrinol Metab* **83**(7)**:** 2435-2440.

Lattion, A, Abuin, L, Nenniger-Tosato, M, Cotecchia, S (1999) Constitutively active mutants of the beta1-adrenergic receptor. *FEBS Lett* **457**(3)**:** 302-306.

Laue, L, Chan, WY, Hsueh, AJ, Kudo, M, Hsu, SY, Wu, SM, Blomberg, L, Cutler, GB, Jr. (1995) Genetic heterogeneity of constitutively activating mutations of the human luteinizing hormone receptor in familial male-limited precocious puberty. *Proc Natl Acad Sci U S A* **92**(6)**:** 1906-1910.

Law, PY, Erickson-Herbrandson, LJ, Zha, QQ, Solberg, J, Chu, J, Sarre, A, Loh, HH (2005) Heterodimerization of mu- and delta-opioid receptors occurs at the cell surface only and requires receptor-G protein interactions. *J Biol Chem* **280**(12)**:** 11152-11164.

Lawson, Z, Wheatley, M (2004) The third extracellular loop of G-protein-coupled receptors: more than just a linker between two important transmembrane helices. *Biochem Soc Trans* **32**(Pt 6)**:** 1048-1050.

le Roux, CW, Patterson, M, Vincent, RP, Hunt, C, Ghatei, MA, Bloom, SR (2005) Postprandial plasma ghrelin is suppressed proportional to meal calorie content in normal-weight but not obese subjects. *J Clin Endocrinol Metab* **90**(2)**:** 1068-1071.

Lebesgue, D, Wallukat, G, Mijares, A, Granier, C, Argibay, J, Hoebeke, J (1998) An agonist-like monoclonal antibody against the human beta2-adrenoceptor. *Eur J Pharmacol* **348**(1)**:** 123-133.

Leff, P (1995) The two-state model of receptor activation. *Trends Pharmacol Sci* **16**(3)**:** 89-97.

Leong, SR, Kabakoff, RC, Hebert, CA (1994) Complete mutagenesis of the extracellular domain of interleukin-8 (IL-8) type A receptor identifies charged residues mediating IL-8 binding and signal transduction. *J Biol Chem* **269**(30)**:** 19343-19348.

Leung, PK, Chow, KB, Lau, PN, Chu, KM, Chan, CB, Cheng, CH, Wise, H (2007) The truncated ghrelin receptor polypeptide (GHS-R1b) acts as a dominant-negative mutant of the ghrelin receptor. *Cell Signal* **19**(5)**:** 1011-1022.

Li, B, Nowak, NM, Kim, SK, Jacobson, KA, Bagheri, A, Schmidt, C, Wess, J (2005) Random mutagenesis of the M3 muscarinic acetylcholine receptor expressed in yeast: identification of second-site mutations that restore function to a coupling-deficient mutant M3 receptor. *J Biol Chem* **280**(7)**:** 5664-5675.

Li, J, Edwards, PC, Burghammer, M, Villa, C, Schertler, GF (2004) Structure of bovine rhodopsin in a trigonal crystal form. *J Mol Biol* **343**(5)**:** 1409-1438.

Li, JG, Chen, C, Liu-Chen, LY (2007) N-Glycosylation of the human kappa opioid receptor enhances its stability but slows its trafficking along the biosynthesis pathway. *Biochemistry* **46**(38)**:** 10960-10970.

Li, S, Liu, X, Min, L, Ascoli, M (2001) Mutations of the second extracellular loop of the human lutropin receptor emphasize the importance of receptor activation and de-emphasize the importance of receptor phosphorylation in agonist-induced internalization. *J Biol Chem* **276**(11)**:** 7968-7973.

Limbird, LE (1984) GTP and Na+ modulate receptor-adenyl cyclase coupling and receptor-mediated function. *Am J Physiol* **247**(1 Pt 1)**:** E59-68.

Lin, SW, Sakmar, TP (1996) Specific tryptophan UV-absorbance changes are probes of the transition of rhodopsin to its active state. *Biochemistry* **35**(34)**:** 11149-11159.

Liu, G, Fortin, JP, Beinborn, M, Kopin, AS (2007a) Four missense mutations in the ghrelin receptor result in distinct pharmacological abnormalities. *J Pharmacol Exp Ther* **322**(3)**:** 1036-1043.

Liu, W, Eilers, M, Patel, AB, Smith, SO (2004) Helix packing moments reveal diversity and conservation in membrane protein structure. *J Mol Biol* **337**(3)**:** 713-729.

Liu, X, Yang, N, Wu, S, Mai, S (2007b) [Relationship of ghrelin and obesity induced by high-fat diet in rats]. *Wei Sheng Yan Jiu* **36**(3)**:** 317-319.

Lohse, MJ, Benovic, JL, Codina, J, Caron, MG, Lefkowitz, RJ (1990) beta-Arrestin: a protein that regulates beta-adrenergic receptor function. *Science* **248**(4962)**:** 1547-1550.

Lu, S, Guan, JL, Wang, QP, Uehara, K, Yamada, S, Goto, N, Date, Y, Nakazato, M, Kojima, M, Kangawa, K, Shioda, S (2002) Immunocytochemical observation of ghrelin-containing neurons in the rat arcuate nucleus. *Neurosci Lett* **321**(3)**:** 157-160.

Lu, ZL, Curtis, CA, Jones, PG, Pavia, J, Hulme, EC (1997) The role of the aspartate-arginine-tyrosine triad in the m1 muscarinic receptor: mutations of aspartate 122 and tyrosine 124 decrease receptor expression but do not abolish signaling. *Mol Pharmacol* **51**(2)**:** 234-241.

Lu, ZL, Hulme, EC (1999) The functional topography of transmembrane domain 3 of the M1 muscarinic acetylcholine receptor, revealed by scanning mutagenesis. *J Biol Chem* **274**(11)**:** 7309-15

Lu, ZL, Hulme, EC (2000) A network of conserved intramolecular contacts defines the off-state of the transmembrane switch mechanism in a seven-transmembrane receptor. *J Biol Chem* **275**(8)**:** 5682-5686.

Lu, ZL, Saldanha, JW, Hulme, EC (2001) Transmembrane domains 4 and 7 of the M(1) muscarinic acetylcholine receptor are critical for ligand binding and the receptor activation switch. *J Biol Chem* **276**(36)**:** 34098-34104.

Luttrell, LM, Daaka, Y, Lefkowitz, RJ (1999) Regulation of tyrosine kinase cascades by G-protein-coupled receptors. *Curr Opin Cell Biol* **11**(2)**:** 177-183.

Madabushi, S, Gross, AK, Philippi, A, Meng, EC, Wensel, TG, Lichtarge, O (2004) Evolutionary trace of G protein-coupled receptors reveals clusters of residues that determine global and class-specific functions. *J Biol Chem* **279**(9)**:** 8126-8132.

Maggio, R, Vogel, Z, Wess, J (1993) Coexpression studies with mutant muscarinic/adrenergic receptors provide evidence for intermolecular "cross-talk" between G-protein-linked receptors. *Proc Natl Acad Sci U S A* **90**(7)**:** 3103-3107.

Manivet, P, Schneider, B, Smith, JC, Choi, DS, Maroteaux, L, Kellermann, O, Launay, JM (2002) The serotonin binding site of human and murine 5-HT2B receptors: molecular modeling and site-directed mutagenesis. *J Biol Chem* **277**(19)**:** 17170-17178.

Marchese, A, Benovic, JL (2001) Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. *J Biol Chem* **276**(49)**:** 45509-45512.

Marinissen, MJ, Gutkind, JS (2001) G-protein-coupled receptors and signaling networks: emerging paradigms. *Trends Pharmacol Sci* **22**(7)**:** 368-376.

Massotte, D, Kieffer, BL (2005) The second extracellular loop: a damper for G protein-coupled receptors? *Nat Struct Mol Biol* **12**(4)**:** 287-288.

Masuda, Y, Tanaka, T, Inomata, N, Ohnuma, N, Tanaka, S, Itoh, Z, Hosoda, H, Kojima, M, Kangawa, K (2000) Ghrelin stimulates gastric acid secretion and motility in rats. *Biochem Biophys Res Commun* **276**(3)**:** 905-908.

Matsumoto, M, Hosoda, H, Kitajima, Y, Morozumi, N, Minamitake, Y, Tanaka, S, Matsuo, H, Kojima, M, Hayashi, Y, Kangawa, K (2001) Structure-activity relationship of ghrelin: pharmacological study of ghrelin peptides. *Biochem Biophys Res Commun* **287**(1)**:** 142-146.

Matsuyama, T, Yamashita, T, Imai, H, Shichida, Y (2010) Covalent bond between ligand and receptor required for efficient activation in rhodopsin. *J Biol Chem* **285**(11)**:** 8114-8121.

McAllister, SD, Hurst, DP, Barnett-Norris, J, Lynch, D, Reggio, PH, Abood, ME (2004) Structural mimicry in class A G protein-coupled receptor rotamer toggle switches: the importance of the F3.36(201)/W6.48(357) interaction in cannabinoid CB1 receptor activation. *J Biol Chem* **279**(46)**:** 48024-48037.

Means, AR, Dedman, JR (1980) Calmodulin--an intracellular calcium receptor. *Nature* **285**(5760)**:** 73-77.

Meng, EC, Bourne, HR (2001) Receptor activation: what does the rhodopsin structure tell us? *Trends Pharmacol Sci* **22**(11)**:** 587-593.

Mercier, JF, Salahpour, A, Angers, S, Breit, A, Bouvier, M (2002) Quantitative assessment of beta 1- and beta 2-adrenergic receptor homo- and heterodimerization by bioluminescence resonance energy transfer. *J Biol Chem* **277**(47)**:** 44925-44931.

Meyer, D, Liu, A, Margolis, B (1999) Interaction of c-Jun amino-terminal kinase interacting protein-1 with p190 rhoGEF and its localization in differentiated neurons. *J Biol Chem* **274**(49)**:** 35113-35118.

Michineau, S, Alhenc-Gelas, F, Rajerison, RM (2006) Human bradykinin B2 receptor sialylation and N-glycosylation participate with disulfide bonding in surface receptor dimerization. *Biochemistry* **45**(8)**:** 2699-2707.

Milligan, G (2003) Constitutive activity and inverse agonists of G protein-coupled receptors: a current perspective. *Mol Pharmacol* **64**(6)**:** 1271-1276.

Milligan, G, Bond, RA, Lee, M (1995) Inverse agonism: pharmacological curiosity or potential therapeutic strategy? *Trends Pharmacol Sci* **16**(1)**:** 10-13.

Milligan, G, Kostenis, E (2006) Heterotrimeric G-proteins: a short history. *Br J Pharmacol* **147 Suppl 1:** S46-55.

Mirzadegan, T, Benko, G, Filipek, S, Palczewski, K (2003) Sequence analyses of G-protein-coupled receptors: similarities to rhodopsin. *Biochemistry* **42**(10)**:** 2759-2767.

Mitsuhashi, M, Payan, DG (1987) The mitogenic effects of vasoactive neuropeptides on cultured smooth muscle cell lines. *Life Sci* **40**(9)**:** 853-861.

Mobini, R, Magnusson, Y, Wallukat, G, Viguier, M, Hjalmarson, A, Hoebeke, J (1999) Probing the immunological properties of the extracellular domains of the human beta(1)-adrenoceptor. *J Autoimmun* **13**(2)**:** 179-186.

Monczor, F, Fernandez, N, Legnazzi, BL, Riveiro, ME, Baldi, A, Shayo, C, Davio, C (2003) Tiotidine, a histamine H2 receptor inverse agonist that binds with high affinity to an inactive G-protein-coupled form of the receptor. Experimental support for the cubic ternary complex model. *Mol Pharmacol* **64**(2)**:** 512-520.

Monod, J, Changeux, JP, Jacob, F (1963) Allosteric proteins and cellular control systems. *J Mol Biol* **6:** 306-329.

Montanelli, L, Delbaere, A, Di Carlo, C, Nappi, C, Smits, G, Vassart, G, Costagliola, S (2004a) A mutation in the follicle-stimulating hormone receptor as a cause of familial spontaneous ovarian hyperstimulation syndrome. *J Clin Endocrinol Metab* **89**(3)**:** 1255-1258.

Montanelli, L, Van Durme, JJ, Smits, G, Bonomi, M, Rodien, P, Devor, EJ, Moffat-Wilson, K, Pardo, L, Vassart, G, Costagliola, S (2004b) Modulation of ligand selectivity associated with activation of the transmembrane region of the human follitropin receptor. *Mol Endocrinol* **18**(8)**:** 2061-2073.

Morin, D, Cotte, N, Balestre, MN, Mouillac, B, Manning, M, Breton, C, Barberis, C (1998) The D136A mutation of the V2 vasopressin receptor induces a constitutive activity which permits discrimination between antagonists with partial agonist and inverse agonist activities. *FEBS Lett* **441**(3)**:** 470-475.

Morton, GJ, Schwartz, MW (2001) The NPY/AgRP neuron and energy homeostasis. *Int J Obes Relat Metab Disord* **25 Suppl 5:** S56-62.

Mozid, AM, Tringali, G, Forsling, ML, Hendricks, MS, Ajodha, S, Edwards, R, Navarra, P, Grossman, AB, Korbonits, M (2003) Ghrelin is released from rat hypothalamic explants and stimulates corticotrophin-releasing hormone and arginine-vasopressin. *Horm Metab Res* **35**(8)**:** 455-459.

Muccioli, G, Tschop, M, Papotti, M, Deghenghi, R, Heiman, M, Ghigo, E (2002) Neuroendocrine and peripheral activities of ghrelin: implications in metabolism and obesity. *Eur J Pharmacol* **440**(2-3)**:** 235-254.

Muller, G (2000) Towards 3D structures of G protein-coupled receptors: a multidisciplinary approach. *Curr Med Chem* **7**(9)**:** 861-888.

Murakami, M, Kouyama, T (2008) Crystal structure of squid rhodopsin. *Nature* **453**(7193)**:** 363-367.

Nagatomo, T, Koike, K (2000) Recent advances in structure, binding sites with ligands and pharmacological function of beta-adrenoceptors obtained by molecular biology and molecular modeling. *Life Sci* **66**(25)**:** 2419-2426.

Nagaya, N, Kangawa, K (2003) Ghrelin improves left ventricular dysfunction and cardiac cachexia in heart failure. *Curr Opin Pharmacol* **3**(2)**:** 146-151.

Nagaya, N, Moriya, J, Yasumura, Y, Uematsu, M, Ono, F, Shimizu, W, Ueno, K, Kitakaze, M, Miyatake, K, Kangawa, K (2004) Effects of ghrelin administration on left ventricular function, exercise capacity, and muscle wasting in patients with chronic heart failure. *Circulation* **110**(24)**:** 3674-3679.

Nagaya, N, Uematsu, M, Kojima, M, Date, Y, Nakazato, M, Okumura, H, Hosoda, H, Shimizu, W, Yamagishi, M, Oya, H, Koh, H, Yutani, C, Kangawa, K (2001a) Elevated circulating level of ghrelin in cachexia associated with chronic heart failure: relationships between ghrelin and anabolic/catabolic factors. *Circulation* **104**(17)**:** 2034-2038.

Nagaya, N, Uematsu, M, Kojima, M, Ikeda, Y, Yoshihara, F, Shimizu, W, Hosoda, H, Hirota, Y, Ishida, H, Mori, H, Kangawa, K (2001b) Chronic administration of ghrelin improves left ventricular dysfunction and attenuates development of cardiac cachexia in rats with heart failure. *Circulation* **104**(12)**:** 1430-1435.

Nakayama, TA, Khorana, HG (1991) Mapping of the amino acids in membrane-embedded helices that interact with the retinal chromophore in bovine rhodopsin. *J Biol Chem* **266**(7)**:** 4269-4275.

Nakazato, M, Murakami, N, Date, Y, Kojima, M, Matsuo, H, Kangawa, K, Matsukura, S (2001) A role for ghrelin in the central regulation of feeding. *Nature* **409**(6817)**:** 194-198.

Nebane, NM, Kellie, B, Song, ZH (2006) The effects of charge-neutralizing mutation D6.30N on the functions of CB1 and CB2 cannabinoid receptors. *FEBS Lett* **580**(22)**:** 5392-5398.

Neve, KA, Cox, BA, Henningsen, RA, Spanoyannis, A, Neve, RL (1991) Pivotal role for aspartate-80 in the regulation of dopamine D2 receptor affinity for drugs and inhibition of adenylyl cyclase. *Mol Pharmacol* **39**(6)**:** 733-739.

Ng, GY, O'Dowd, BF, Lee, SP, Chung, HT, Brann, MR, Seeman, P, George, SR (1996) Dopamine D2 receptor dimers and receptor-blocking peptides. *Biochem Biophys Res Commun* **227**(1)**:** 200-204.

Nogueiras, R, Tschop, MH, Zigman, JM (2008) Central nervous system regulation of energy metabolism: ghrelin versus leptin. *Ann N Y Acad Sci* **1126:** 14-19.

Nygaard, R, Frimurer, TM, Holst, B, Rosenkilde, MM, Schwartz, TW (2009) Ligand binding and micro-switches in 7TM receptor structures. *Trends Pharmacol Sci* **30**(5)**:** 249-259.

O'Brien, PJ, Zatz, M (1984) Acylation of bovine rhodopsin by [3H]palmitic acid. *J Biol Chem* **259**(8)**:** 5054-5057.

O'Dowd, BF, Hnatowich, M, Caron, MG, Lefkowitz, RJ, Bouvier, M (1989) Palmitoylation of the human beta 2-adrenergic receptor. Mutation of Cys341 in the carboxyl tail leads to an uncoupled nonpalmitoylated form of the receptor. *J Biol Chem* **264**(13)**:** 7564-7569.

Okada, T, Ernst, OP, Palczewski, K, Hofmann, KP (2001a) Activation of rhodopsin: new insights from structural and biochemical studies. *Trends Biochem Sci* **26**(5)**:** 318-324.

Okada, T, Fujiyoshi, Y, Silow, M, Navarro, J, Landau, EM, Shichida, Y (2002) Functional role of internal water molecules in rhodopsin revealed by X-ray crystallography. *Proc Natl Acad Sci U S A* **99**(9)**:** 5982-5987.

Okada, T, Palczewski, K (2001b) Crystal structure of rhodopsin: implications for vision and beyond. *Curr Opin Struct Biol* **11**(4)**:** 420-426.

Okada, T, Sugihara, M, Bondar, AN, Elstner, M, Entel, P, Buss, V (2004) The retinal conformation and its environment in rhodopsin in light of a new 2.2 A crystal structure. *J Mol Biol* **342**(2)**:** 571-583.

Okamoto, Y, Ninomiya, H, Tanioka, M, Sakamoto, A, Miwa, S, Masaki, T (1997) Palmitoylation of human endothelinB. Its critical role in G protein coupling and a differential requirement for the cytoplasmic tail by G protein subtypes. *J Biol Chem* **272**(34)**:** 21589-21596.

Okumura, H, Nagaya, N, Enomoto, M, Nakagawa, E, Oya, H, Kangawa, K (2002) Vasodilatory effect of ghrelin, an endogenous peptide from the stomach. *J Cardiovasc Pharmacol* **39**(6)**:** 779-783.

Ott, TR, Troskie, BE, Roeske, RW, Illing, N, Flanagan, CA, Millar, RP (2002) Two mutations in extracellular loop 2 of the human GnRH receptor convert an antagonist to an agonist. *Mol Endocrinol* **16**(5)**:** 1079-1088.

Otto, B, Cuntz, U, Fruehauf, E, Wawarta, R, Folwaczny, C, Riepl, RL, Heiman, ML, Lehnert, P, Fichter, M, Tschop, M (2001) Weight gain decreases elevated plasma ghrelin concentrations of patients with anorexia nervosa. *Eur J Endocrinol* **145**(5)**:** 669-673.

Overton, MC, Blumer, KJ (2000) G-protein-coupled receptors function as oligomers in vivo. *Curr Biol* **10**(6)**:** 341-344.

Palczewski, K, Kumasaka, T, Hori, T, Behnke, CA, Motoshima, H, Fox, BA, Le Trong, I, Teller, DC, Okada, T, Stenkamp, RE, Yamamoto, M, Miyano, M (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* **289**(5480)**:** 739-745.

Pantel, J, Legendre, M, Cabrol, S, Hilal, L, Hajaji, Y, Morisset, S, Nivot, S, Vie-Luton, MP, Grouselle, D, de Kerdanet, M, Kadiri, A, Epelbaum, J, Le Bouc, Y, Amselem, S (2006) Loss of constitutive activity of the growth hormone secretagogue receptor in familial short stature. *J Clin Invest* **116**(3)**:** 760-768.

Park, JH, Scheerer, P, Hofmann, KP, Choe, HW, Ernst, OP (2008) Crystal structure of the ligand-free G-protein-coupled receptor opsin. *Nature*.

Parker, SL, Parker, MS, Sah, R, Balasubramaniam, A, Sallee, FR (2008) Pertussis toxin induces parallel loss of neuropeptide Y Y1 receptor dimers and Gi alpha subunit function in CHO cells. *Eur J Pharmacol* **579**(1-3)**:** 13-25.

Parker, SL, Parker, MS, Sallee, FR, Balasubramaniam, A (2007) Oligomerization of neuropeptide Y (NPY) Y2 receptors in CHO cells depends on functional pertussis toxin-sensitive G-proteins. *Regul Pept* **144**(1-3)**:** 72-81.

Parma, J, Duprez, L, Van Sande, J, Cochaux, P, Gervy, C, Mockel, J, Dumont, J, Vassart, G (1993) Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. *Nature* **365**(6447)**:** 649-651.

Parnot, C, Miserey-Lenkei, S, Bardin, S, Corvol, P, Clauser, E (2002) Lessons from constitutively active mutants of G protein-coupled receptors. *Trends Endocrinol Metab* **13**(8)**:** 336-343.

Pedretti, A, Vistoli, G (2007) Modeling of human ghrelin receptor (hGHS-R1a) in its close state and validation by molecular docking. *Bioorg Med Chem* **15**(8)**:** 3054-3064.

Pellissier, LP, Sallander, J, Campillo, M, Gaven, F, Queffeulou, E, Pillot, M, Dumuis, A, Claeysen, S, Bockaert, J, Pardo, L (2009) Conformational toggle switches implicated in basal constitutive and agonist-induced activated states of 5-hydroxytryptamine-4 receptors. *Mol Pharmacol* **75**(4)**:** 982-990.

Perez, DM, Hwa, J, Gaivin, R, Mathur, M, Brown, F, Graham, RM (1996) Constitutive activation of a single effector pathway: evidence for multiple activation states of a G protein-coupled receptor. *Mol Pharmacol* **49**(1)**:** 112-122.

Perez, DM, Karnik, SS (2005) Multiple signaling states of G-protein-coupled receptors. *Pharmacol Rev* **57**(2)**:** 147-161.

Perlman, JH, Colson, AO, Jain, R, Czyzewski, B, Cohen, LA, Osman, R, Gershengorn, MC (1997a) Role of the extracellular loops of the thyrotropin-releasing hormone receptor: evidence for an initial interaction with thyrotropin-releasing hormone. *Biochemistry* **36**(50)**:** 15670-15676.

Perlman, JH, Colson, AO, Wang, W, Bence, K, Osman, R, Gershengorn, MC (1997b) Interactions between conserved residues in transmembrane helices 1, 2, and 7 of the thyrotropin-releasing hormone receptor. *J Biol Chem* **272**(18)**:** 11937-11942.

Petaja-Repo, UE, Hogue, M, Leskela, TT, Markkanen, PM, Tuusa, JT, Bouvier, M (2006) Distinct subcellular localization for constitutive and agonist-modulated palmitoylation of the human delta opioid receptor. *J Biol Chem* **281**(23)**:** 15780-15789.

Petersen, PS, Woldbye, DP, Madsen, AN, Egerod, KL, Jin, C, Lang, M, Rasmussen, M, Beck-Sickinger, AG, Holst, B (2009) In vivo characterization of high Basal signaling from the ghrelin receptor. *Endocrinology* **150**(11)**:** 4920-4930.

Pierce, KL, Lefkowitz, RJ (2001) Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors. *Nat Rev Neurosci* **2**(10)**:** 727-733.

Porter, JE, Hwa, J, Perez, DM (1996) Activation of the alpha1b-adrenergic receptor is initiated by disruption of an interhelical salt bridge constraint. *J Biol Chem* **271**(45)**:** 28318-28323.

Prioleau, C, Visiers, I, Ebersole, BJ, Weinstein, H, Sealfon, SC (2002) Conserved helix 7 tyrosine acts as a multistate conformational switch in the 5HT2C receptor. Identification of a novel "locked-on" phenotype and double revertant mutations. *J Biol Chem* **277**(39)**:** 36577-36584.

Proneth, B, Xiang, Z, Pogozheva, ID, Litherland, SA, Gorbatyuk, OS, Shaw, AM, Millard, WJ, Mosberg, HI, Haskell-Luevano, C (2006) Molecular mechanism of the constitutive activation of the L250Q human melanocortin-4 receptor polymorphism. *Chem Biol Drug Des* **67**(3)**:** 215-229.

Pula, G, Mundell, SJ, Roberts, PJ, Kelly, E (2004) Agonist-independent internalization of metabotropic glutamate receptor 1a is arrestin- and clathrin-dependent and is suppressed by receptor inverse agonists. *J Neurochem* **89**(4)**:** 1009-1020.

Qanbar, R, Bouvier, M (2003) Role of palmitoylation/depalmitoylation reactions in G-protein-coupled receptor function. *Pharmacol Ther* **97**(1)**:** 1-33.

Rak-Mardyla, A, Gregoraszczuk, E (2010) Effect of ghrelin on proliferation, apoptosis and secretion of progesterone and hCG in the placental JEG-3 cell line. *Reprod Biol* **10**(2)**:** 159-165.

Ramsay, D, Kellett, E, McVey, M, Rees, S, Milligan, G (2002) Homo- and hetero-oligomeric interactions between G-protein-coupled receptors in living cells monitored by two variants of bioluminescence resonance energy transfer (BRET): hetero-oligomers between receptor subtypes form more efficiently than between less closely related sequences. *Biochem J* **365**(Pt 2)**:** 429-440.

Rands, E, Candelore, MR, Cheung, AH, Hill, WS, Strader, CD, Dixon, RA (1990) Mutational analysis of beta-adrenergic receptor glycosylation. *J Biol Chem* **265**(18)**:** 10759-10764.

Rang, HP (2003) *Pharmacology*. 5th ed. edn. Churchill Livingstone: Edinburgh.

Rao, VR, Cohen, GB, Oprian, DD (1994) Rhodopsin mutation G90D and a molecular mechanism for congenital night blindness. *Nature* **367**(6464)**:** 639-642.

Rasmussen, SG, Choi, HJ, Rosenbaum, DM, Kobilka, TS, Thian, FS, Edwards, PC, Burghammer, M, Ratnala, VR, Sanishvili, R, Fischetti, RF, Schertler, GF, Weis, WI, Kobilka, BK (2007) Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature* **450**(7168)**:** 383-387.

Rasmussen, SG, Jensen, AD, Liapakis, G, Ghanouni, P, Javitch, JA, Gether, U (1999) Mutation of a highly conserved aspartic acid in the beta2 adrenergic receptor: constitutive activation, structural instability, and conformational rearrangement of transmembrane segment 6. *Mol Pharmacol* **56**(1)**:** 175-184.

Reiter, E, Lefkowitz, RJ (2006) GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling. *Trends Endocrinol Metab* **17**(4)**:** 159-165.

Ren, Q, Kurose, H, Lefkowitz, RJ, Cotecchia, S (1993) Constitutively active mutants of the alpha 2-adrenergic receptor. *J Biol Chem* **268**(22)**:** 16483-16487.

Rens-Domiano, S, Hamm, HE (1995) Structural and functional relationships of heterotrimeric G-proteins. *Faseb J* **9**(11)**:** 1059-1066.

Rens-Domiano, S, Reisine, T (1991) Structural analysis and functional role of the carbohydrate component of somatostatin receptors. *J Biol Chem* **266**(30)**:** 20094-20102.

Rhee, MH, Nevo, I, Bayewitch, ML, Zagoory, O, Vogel, Z (2000a) Functional role of tryptophan residues in the fourth transmembrane domain of the CB(2) cannabinoid receptor. *J Neurochem* **75**(6)**:** 2485-2491.

Rhee, MH, Nevo, I, Levy, R, Vogel, Z (2000b) Role of the highly conserved Asp-Arg-Tyr motif in signal transduction of the CB2 cannabinoid receptor. *FEBS Lett* **466**(2-3)**:** 300-304.

Riek, RP, Rigoutsos, I, Novotny, J, Graham, RM (2001) Non-alpha-helical elements modulate polytopic membrane protein architecture. *J Mol Biol* **306**(2)**:** 349-362.

Ringkananont, U, Van Durme, J, Montanelli, L, Ugrasbul, F, Yu, YM, Weiss, RE, Refetoff, S, Grasberger, H (2006) Repulsive separation of the cytoplasmic ends of transmembrane helices 3 and 6 is linked to receptor activation in a novel thyrotropin receptor mutant (M626I). *Mol Endocrinol* **20**(4)**:** 893-903.

Rios, CD, Jordan, BA, Gomes, I, Devi, LA (2001) G-protein-coupled receptor dimerization: modulation of receptor function. *Pharmacol Ther* **92**(2-3)**:** 71-87.

Rocheville, M, Lange, DC, Kumar, U, Patel, SC, Patel, RC, Patel, YC (2000a) Receptors for dopamine and somatostatin: formation of hetero-oligomers with enhanced functional activity. *Science* **288**(5463)**:** 154-157.

Rocheville, M, Lange, DC, Kumar, U, Sasi, R, Patel, RC, Patel, YC (2000b) Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers. *J Biol Chem* **275**(11)**:** 7862-7869.

Romero, A, Kirchner, H, Heppner, K, Pfluger, PT, Tschop, MH, Nogueiras, R (2010) GOAT: the master switch for the ghrelin system? *Eur J Endocrinol* **163**(1)**:** 1-8.

Rosenbaum, DM, Cherezov, V, Hanson, MA, Rasmussen, SG, Thian, FS, Kobilka, TS, Choi, HJ, Yao, XJ, Weis, WI, Stevens, RC, Kobilka, BK (2007) GPCR engineering yields high-resolution structural insights into beta2-adrenergic receptor function. *Science* **318**(5854)**:** 1266-1273.

Rosenbaum, DM, Rasmussen, SG, Kobilka, BK (2009) The structure and function of G-protein-coupled receptors. *Nature* **459**(7245)**:** 356-363.

Rosendorff, A, Ebersole, BJ, Sealfon, SC (2000) Conserved helix 7 tyrosine functions as an activation relay in the serotonin 5HT(2C) receptor. *Brain Res Mol Brain Res* **84**(1-2)**:** 90-96.

Rosenkilde, MM, Schwartz, TW (2000) Potency of ligands correlates with affinity measured against agonist and inverse agonists but not against neutral ligand in constitutively active chemokine receptor. *Mol Pharmacol* **57**(3)**:** 602-609.

Rosenthal, W, Antaramian, A, Gilbert, S, Birnbaumer, M (1993) Nephrogenic diabetes insipidus. A V2 vasopressin receptor unable to stimulate adenylyl cyclase. *J Biol Chem* **268**(18)**:** 13030-13033.

Ross, EM, Wilkie, TM (2000) GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu Rev Biochem* **69:** 795-827.

Roth, BL, Shoham, M, Choudhary, MS, Khan, N (1997) Identification of conserved aromatic residues essential for agonist binding and second messenger production at 5-hydroxytryptamine2A receptors. *Mol Pharmacol* **52**(2)**:** 259-266.

Roth, CL, Ludwig, M, Woelfle, J, Fan, ZC, Brumm, H, Biebermann, H, Tao, YX (2009) A novel melanocortin-4 receptor gene mutation in a female patient with severe childhood obesity. *Endocrine* **36**(1)**:** 52-59.

Rovati, GE, Capra, V, Neubig, RR (2007) The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state. *Mol Pharmacol* **71**(4)**:** 959-964.

Ruprecht, JJ, Mielke, T, Vogel, R, Villa, C, Schertler, GF (2004) Electron crystallography reveals the structure of metarhodopsin I. *Embo J* **23**(18)**:** 3609-3620.

Sadeghi, H, Birnbaumer, M (1999) O-Glycosylation of the V2 vasopressin receptor. *Glycobiology* **9**(7)**:** 731-737.

Sadeghi, HM, Innamorati, G, Dagarag, M, Birnbaumer, M (1997) Palmitoylation of the V2 vasopressin receptor. *Mol Pharmacol* **52**(1)**:** 21-29.

Sah, VP, Seasholtz, TM, Sagi, SA, Brown, JH (2000) The role of Rho in G protein-coupled receptor signal transduction. *Annu Rev Pharmacol Toxicol* **40:** 459-489.

Sakmar, TP, Franke, RR, Khorana, HG (1989) Glutamic acid-113 serves as the retinylidene Schiff base counterion in bovine rhodopsin. *Proc Natl Acad Sci U S A* **86**(21)**:** 8309-8313.

Samama, P, Cotecchia, S, Costa, T, Lefkowitz, RJ (1993) A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model. *J Biol Chem* **268**(7)**:** 4625-4636.

Sambrook, J, Fritsch, E.F, Maniatis, T (1989) Molecular Cloning, 2nd ed. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, N.Y.

Scarselli, M, Li, B, Kim, SK, Wess, J (2007) Multiple residues in the second extracellular loop are critical for M3 muscarinic acetylcholine receptor activation. *J Biol Chem* **282**(10)**:** 7385-7396.

Scheer, A, Costa, T, Fanelli, F, De Benedetti, PG, Mhaouty-Kodja, S, Abuin, L, Nenniger-Tosato, M, Cotecchia, S (2000) Mutational analysis of the highly conserved arginine within the Glu/Asp-Arg-Tyr motif of the alpha(1b)-adrenergic receptor: effects on receptor isomerization and activation. *Mol Pharmacol* **57**(2)**:** 219-231.

Scheer, A, Cotecchia, S (1997) Constitutively active G protein-coupled receptors: potential mechanisms of receptor activation. *J Recept Signal Transduct Res* **17**(1-3)**:** 57-73.

Scheer, A, Fanelli, F, Costa, T, De Benedetti, PG, Cotecchia, S (1996) Constitutively active mutants of the alpha 1B-adrenergic receptor: role of highly conserved polar amino acids in receptor activation. *Embo J* **15**(14)**:** 3566-3578.

Scheerer, P, Park, JH, Hildebrand, PW, Kim, YJ, Krauss, N, Choe, HW, Hofmann, KP, Ernst, OP (2008) Crystal structure of opsin in its G-protein-interacting conformation. *Nature* **455**(7212)**:** 497-502.

Schetz, JA, Chu, A, Sibley, DR (1999) Zinc modulates antagonist interactions with D2-like dopamine receptors through distinct molecular mechanisms. *J Pharmacol Exp Ther* **289**(2)**:** 956-964.

Schetz, JA, Sibley, DR (1997) Zinc allosterically modulates antagonist binding to cloned D1 and D2 dopamine receptors. *J Neurochem* **68**(5)**:** 1990-1997.

Schneider, EH, Schnell, D, Strasser, A, Dove, S, Seifert, R (2010) Impact of the DRY motif and the missing "ionic lock" on constitutive activity and G-protein coupling of the human histamine H4 receptor. *J Pharmacol Exp Ther* **333**(2)**:** 382-392.

Schoneberg, T, Liu, J, Wess, J (1995) Plasma membrane localization and functional rescue of truncated forms of a G protein-coupled receptor. *J Biol Chem* **270**(30)**:** 18000-18006.

Schulte, G, Levy, FO (2007) Novel aspects of G-protein-coupled receptor signalling--different ways to achieve specificity. *Acta Physiol (Oxf)* **190**(1)**:** 33-38.

Schwartz, TW, Frimurer, TM, Holst, B, Rosenkilde, MM, Elling, CE (2006) Molecular mechanism of 7TM receptor activation--a global toggle switch model. *Annu Rev Pharmacol Toxicol* **46:** 481-519.

Schwartz, TW, Rosenkilde, MM (1996) Is there a 'lock' for all agonist 'keys' in 7TM receptors? *Trends Pharmacol Sci* **17**(6)**:** 213-216.

Sealfon, SC, Chi, L, Ebersole, BJ, Rodic, V, Zhang, D, Ballesteros, JA, Weinstein, H (1995) Related contribution of specific helix 2 and 7 residues to conformational activation of the serotonin 5-HT2A receptor. *J Biol Chem* **270**(28)**:** 16683-16688.

Seibold, A, Dagarag, M, Birnbaumer, M (1998) Mutations of the DRY motif that preserve beta 2-adrenoceptor coupling. *Receptors Channels* **5**(6)**:** 375-385.

Seifert, R, Wenzel-Seifert, K (2002) Constitutive activity of G-protein-coupled receptors: cause of disease and common property of wild-type receptors. *Naunyn Schmiedebergs Arch Pharmacol* **366**(5)**:** 381-416.

Seta, K, Nanamori, M, Modrall, JG, Neubig, RR, Sadoshima, J (2002) AT1 receptor mutant lacking heterotrimeric G protein coupling activates the Src-Ras-ERK pathway without nuclear translocation of ERKs. *J Biol Chem* **277**(11)**:** 9268-9277.

Shapiro, DA, Kristiansen, K, Weiner, DM, Kroeze, WK, Roth, BL (2002) Evidence for a model of agonist-induced activation of 5-hydroxytryptamine 2A serotonin receptors that involves the disruption of a strong ionic interaction between helices 3 and 6. *J Biol Chem* **277**(13)**:** 11441-11449.

Sheikh, SP, Zvyaga, TA, Lichtarge, O, Sakmar, TP, Bourne, HR (1996) Rhodopsin activation blocked by metal-ion-binding sites linking transmembrane helices C and F. *Nature* **383**(6598)**:** 347-350.

Shenoy, SK, Lefkowitz, RJ (2003) Multifaceted roles of beta-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling. *Biochem J* **375**(Pt 3)**:** 503-515.

Shenoy, SK, McDonald, PH, Kohout, TA, Lefkowitz, RJ (2001) Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin. *Science* **294**(5545)**:** 1307-1313.

Shi, L, Javitch, JA (2002a) The binding site of aminergic G protein-coupled receptors: the transmembrane segments and second extracellular loop. *Annu Rev Pharmacol Toxicol* **42:** 437-467.

Shi, L, Liapakis, G, Xu, R, Guarnieri, F, Ballesteros, JA, Javitch, JA (2002b) Beta2 adrenergic receptor activation. Modulation of the proline kink in transmembrane 6 by a rotamer toggle switch. *J Biol Chem* **277**(43)**:** 40989-40996.

Shim, JY (2009) Transmembrane helical domain of the cannabinoid CB1 receptor. *Biophys J* **96**(8)**:** 3251-3262.

Shinyama, H, Masuzaki, H, Fang, H, Flier, JS (2003) Regulation of melanocortin-4 receptor signaling: agonist-mediated desensitization and internalization. *Endocrinology* **144**(4)**:** 1301-1314.

Sibilia, V, Pagani, F, Guidobono, F, Locatelli, V, Torsello, A, Deghenghi, R, Netti, C (2002) Evidence for a central inhibitory role of growth hormone secretagogues and ghrelin on gastric acid secretion in conscious rats. *Neuroendocrinology* **75**(2)**:** 92-97.

Smit, MJ, Vischer, HF, Bakker, RA, Jongejan, A, Timmerman, H, Pardo, L, Leurs, R (2007) Pharmacogenomic and structural analysis of constitutive g protein-coupled receptor activity. *Annu Rev Pharmacol Toxicol* **47:** 53-87.

Soto, AG, Trejo, J (2010) N-linked glycosylation of protease-activated receptor-1 second extracellular loop: a critical determinant for ligand-induced receptor activation and internalization. *J Biol Chem* **285**(24)**:** 18781-18793.

Springael, JY, de Poorter, C, Deupi, X, Van Durme, J, Pardo, L, Parmentier, M (2007) The activation mechanism of chemokine receptor CCR5 involves common structural changes but a different network of interhelical interactions relative to rhodopsin. *Cell Signal* **19**(7)**:** 1446-1456.

Storjohann, L, Holst, B, Schwartz, TW (2008) A second disulfide bridge from the N-terminal domain to extracellular loop 2 dampens receptor activity in GPR39. *Biochemistry* **47**(35)**:** 9198-9207.

Strader, CD, Sigal, IS, Candelore, MR, Rands, E, Hill, WS, Dixon, RA (1988) Conserved aspartic acid residues 79 and 113 of the beta-adrenergic receptor have different roles in receptor function. *J Biol Chem* **263**(21)**:** 10267-10271.

Strange, PG (2002) Mechanisms of inverse agonism at G-protein-coupled receptors. *Trends Pharmacol Sci* **23**(2)**:** 89-95.

Sullivan, R, Chateauneuf, A, Coulombe, N, Kolakowski, LF, Jr., Johnson, MP, Hebert, TE, Ethier, N, Belley, M, Metters, K, Abramovitz, M, O'Neill, GP, Ng, GY (2000) Coexpression of full-length gamma-aminobutyric acid(B) (GABA(B)) receptors with truncated receptors and metabotropic glutamate receptor 4 supports the GABA(B) heterodimer as the functional receptor. *J Pharmacol Exp Ther* **293**(2)**:** 460-467.

Sum, CS, Tikhonova, IG, Costanzi, S, Gershengorn, MC (2009) Two arginine-glutamate ionic locks near the extracellular surface of FFAR1 gate receptor activation. *J Biol Chem* **284**(6)**:** 3529-3536.

Sun, Y, Ahmed, S, Smith, RG (2003) Deletion of ghrelin impairs neither growth nor appetite. *Mol Cell Biol* **23**(22)**:** 7973-7981.

Sun, Y, Wang, P, Zheng, H, Smith, RG (2004) Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. *Proc Natl Acad Sci U S A* **101**(13)**:** 4679-4684.

Suzuki, K, Simpson, KA, Minnion, JS, Shillito, JC, Bloom, SR (2010) The role of gut hormones and the hypothalamus in appetite regulation. *Endocr J* **57**(5)**:** 359-372.

Tao, YX, Abell, AN, Liu, X, Nakamura, K, Segaloff, DL (2000) Constitutive activation of G protein-coupled receptors as a result of selective substitution of a conserved leucine residue in transmembrane helix III. *Mol Endocrinol* **14**(8)**:** 1272-1282.

Teller, DC, Okada, T, Behnke, CA, Palczewski, K, Stenkamp, RE (2001) Advances in determination of a high-resolution three-dimensional structure of rhodopsin, a model of G-protein-coupled receptors (GPCRs). *Biochemistry* **40**(26)**:** 7761-7772.

Tschop, M, Smiley, DL, Heiman, ML (2000) Ghrelin induces adiposity in rodents. *Nature* **407**(6806)**:** 908-913.

Tschop, M, Wawarta, R, Riepl, RL, Friedrich, S, Bidlingmaier, M, Landgraf, R, Folwaczny, C (2001) Post-prandial decrease of circulating human ghrelin levels. *J Endocrinol Invest* **24**(6)**:** RC19-21.

Ueda, T, Matsuura, B, Miyake, T, Furukawa, S, Abe, M, Hiasa, Y, Onji, M (2010) Mutational analysis of predicted extracellular domains of human growth hormone secretagogue receptor 1a. *Regul Pept*.

Urizar, E, Claeysen, S, Deupi, X, Govaerts, C, Costagliola, S, Vassart, G, Pardo, L (2005) An activation switch in the rhodopsin family of G protein-coupled receptors: the thyrotropin receptor. *J Biol Chem* **280**(17)**:** 17135-17141.

van der Bliek, AM, Redelmeier, TE, Damke, H, Tisdale, EJ, Meyerowitz, EM, Schmid, SL (1993) Mutations in human dynamin block an intermediate stage in coated vesicle formation. *J Cell Biol* **122**(3)**:** 553-563.

van der Lely, AJ, Tschop, M, Heiman, ML, Ghigo, E (2004) Biological, physiological, pathophysiological, and pharmacological aspects of ghrelin. *Endocr Rev* **25**(3)**:** 426-457.

van Koppen, CJ, Nathanson, NM (1991) The cysteine residue in the carboxyl-terminal domain of the m2 muscarinic acetylcholine receptor is not required for receptor-mediated inhibition of adenylate cyclase. *J Neurochem* **57**(6)**:** 1873-1877.

Van Sande, J, Parma, J, Tonacchera, M, Swillens, S, Dumont, J, Vassart, G (1995) Somatic and germline mutations of the TSH receptor gene in thyroid diseases. *J Clin Endocrinol Metab* **80**(9)**:** 2577-2585.

Vogel, R, Mahalingam, M, Ludeke, S, Huber, T, Siebert, F, Sakmar, TP (2008) Functional role of the "ionic lock"--an interhelical hydrogen-bond network in family A heptahelical receptors. *J Mol Biol* **380**(4)**:** 648-655.

von Heijne, G (1991) Proline kinks in transmembrane alpha-helices. *J Mol Biol* **218**(3)**:** 499-503.

von Zastrow, Mp (2001) Endocytosis and downregulation of G protein-coupled receptors. **7**(3)**:** 265-271.

Wald, G (1968) The molecular basis of visual excitation. *Nature* **219**(5156)**:** 800-807.

Wang, CD, Buck, MA, Fraser, CM (1991) Site-directed mutagenesis of alpha 2A-adrenergic receptors: identification of amino acids involved in ligand binding and receptor activation by agonists. *Mol Pharmacol* **40**(2)**:** 168-179.

Wang, HJ, Geller, F, Dempfle, A, Schauble, N, Friedel, S, Lichtner, P, Fontenla-Horro, F, Wudy, S, Hagemann, S, Gortner, L, Huse, K, Remschmidt, H, Bettecken, T, Meitinger, T, Schafer, H, Hebebrand, J, Hinney, A (2004) Ghrelin receptor gene: identification of several sequence variants in extremely obese children and adolescents, healthy normal-weight and underweight students, and children with short normal stature. *J Clin Endocrinol Metab* **89**(1)**:** 157-162.

Wang, T, Duan, Y (2009) Ligand entry and exit pathways in the beta2-adrenergic receptor. *J Mol Biol* **392**(4)**:** 1102-1115.

Wang, W, Guo, G, Tang, J, Li, J, Zhao, R, Hjalmarson, A, Fu, LX (2000) Stimulatory activity of anti-peptide antibodies against the second extracellular loop of human M2 muscarinic receptors. *Chin Med J (Engl)* **113**(10)**:** 867-871.

Ward, DG, Brewer, SM, Cornes, MP, Trayer, IP (2003) A cross-linking study of the N-terminal extension of human cardiac troponin I. *Biochemistry* **42**(34)**:** 10324-10332.

Warne, T, Serrano-Vega, MJ, Baker, JG, Moukhametzianov, R, Edwards, PC, Henderson, R, Leslie, AG, Tate, CG, Schertler, GF (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature* **454**(7203)**:** 486-491.

Weiss, JM, Morgan, PH, Lutz, MW, Kenakin, TP (1996) The cubic ternary complex receptor-occupancy model. III. resurrecting efficacy. *J Theor Biol* **181**(4)**:** 381-397.

Wess, J (2005) Allosteric binding sites on muscarinic acetylcholine receptors. *Mol Pharmacol* **68**(6)**:** 1506-1509.

Wess, J (1997) G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. *Faseb J* **11**(5)**:** 346-354.

Wess, J (1993a) Molecular basis of muscarinic acetylcholine receptor function. *Trends Pharmacol Sci* **14**(8)**:** 308-313.

Wess, J (1998) Molecular basis of receptor/G-protein-coupling selectivity. *Pharmacol Ther* **80**(3)**:** 231-264.

Wess, J (1993b) Mutational analysis of muscarinic acetylcholine receptors: structural basis of ligand/receptor/G protein interactions. *Life Sci* **53**(19)**:** 1447-1463.

Wess, J, Nanavati, S, Vogel, Z, Maggio, R (1993) Functional role of proline and tryptophan residues highly conserved among G protein-coupled receptors studied by mutational analysis of the m3 muscarinic receptor. *Embo J* **12**(1)**:** 331-338.

Westphal, RS, Sanders-Bush, E (1996) Differences in agonist-independent and -dependent 5-hydroxytryptamine2C receptor-mediated cell division. *Mol Pharmacol* **49**(3)**:** 474-480.

Wheatley, M, Hawtin, SR (1999) Glycosylation of G-protein-coupled receptors for hormones central to normal reproductive functioning: its occurrence and role. *Hum Reprod Update* **5**(4)**:** 356-364.

Wheatley, M, Howl, J, Yarwood, NJ, Hawtin, SR, Davies, AR, Matthews, G, Parslow, RA (1997) Structure and function of neurohypophysial hormone receptors. *Biochem Soc Trans* **25**(3)**:** 1046-1051.

Whorton, MR, Bokoch, MP, Rasmussen, SG, Huang, B, Zare, RN, Kobilka, B, Sunahara, RK (2007) A monomeric G protein-coupled receptor isolated in a high-density lipoprotein particle efficiently activates its G protein. *Proc Natl Acad Sci U S A* **104**(18)**:** 7682-7687.

Wieland, K, Laak, AM, Smit, MJ, Kuhne, R, Timmerman, H, Leurs, R (1999) Mutational analysis of the antagonist-binding site of the histamine H(1) receptor. *J Biol Chem* **274**(42)**:** 29994-30000.

Wilson, IA, Niman, HL, Houghten, RA, Cherenson, AR, Connolly, ML, Lerner, RA (1984) The structure of an antigenic determinant in a protein. *Cell* **37**(3)**:** 767-778.

Worth, CL, Kleinau, G, Krause, G (2009) Copmarative sequence and structural analyses of G-protein-coupled receptor crystal structures and implications for molecular models.*PLoS One* **16**;4(9); e7011

Wren, AM, Small, CJ, Ward, HL, Murphy, KG, Dakin, CL, Taheri, S, Kennedy, AR, Roberts, GH, Morgan, DG, Ghatei, MA, Bloom, SR (2000) The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* **141**(11)**:** 4325-4328.

Yamano, Y, Kamon, R, Yoshimizu, T, Toda, Y, Oshida, Y, Chaki, S, Yoshioka, M, Morishima, I (2004) The role of the DRY motif of human MC4R for receptor activation. *Biosci Biotechnol Biochem* **68**(6)**:** 1369-1371.

Yamano, Y, Ohyama, K, Kikyo, M, Sano, T, Nakagomi, Y, Inoue, Y, Nakamura, N, Morishima, I, Guo, DF, Hamakubo, T, et al. (1995) Mutagenesis and the molecular modeling of the rat angiotensin II receptor (AT1). *J Biol Chem* **270**(23)**:** 14024-14030.

Yang, J, Brown, MS, Liang, G, Grishin, NV, Goldstein, JL (2008) Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell* **132**(3)**:** 387-396.

Yohannan, S, Faham, S, Yang, D, Whitelegge, JP, Bowie, JU (2004) The evolution of transmembrane helix kinks and the structural diversity of G protein-coupled receptors. *Proc Natl Acad Sci U S A* **101**(4)**:** 959-963.

Zeng, FY, Soldner, A, Schoneberg, T, Wess, J (1999a) Conserved extracellular cysteine pair in the M3 muscarinic acetylcholine receptor is essential for proper receptor cell surface localization but not for G protein coupling. *J Neurochem* **72**(6)**:** 2404-2414.

Zeng, FY, Wess, J (1999b) Identification and molecular characterization of m3 muscarinic receptor dimers. *J Biol Chem* **274**(27)**:** 19487-19497.

Zezula, J, Freissmuth, M (2008) The A(2A)-adenosine receptor: a GPCR with unique features? *Br J Pharmacol* **153 Suppl 1:** S184-190.

Zhang, J, Ferguson, SS, Barak, LS, Menard, L, Caron, MG (1996) Dynamin and beta-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. *J Biol Chem* **271**(31)**:** 18302-18305.

Zhang, JV, Ren, PG, Avsian-Kretchmer, O, Luo, CW, Rauch, R, Klein, C, Hsueh, AJ (2005) Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science* **310**(5750)**:** 996-999.

Zhang, M, Mizrachi, D, Fanelli, F, Segaloff, DL (2005) The formation of a salt bridge between helices 3 and 6 is responsible for the constitutive activity and lack of hormone responsiveness of the naturally occurring L457R mutation of the human lutropin receptor. *J Biol Chem* **280**(28)**:** 26169-76.

Zhang, P, Johnson, PS, Zollner, C, Wang, W, Wang, Z, Montes, AE, Seidleck, BK, Blaschak, CJ, Surratt, CK (1999) Mutation of human mu opioid receptor extracellular "disulfide cysteine" residues alters ligand binding but does not prevent receptor targeting to the cell plasma membrane. *Brain Res Mol Brain Res* **72**(2)**:** 195-204.

Zhang, R, Tsai-Morris, CH, Kitamura, M, Buczko, E, Dufau, ML (1991) Changes in binding activity of luteinizing hormone receptors by site directed mutagenesis of potential glycosylation sites. *Biochem Biophys Res Commun* **181**(2)**:** 804-808.

Zhang, WB, Navenot, JM, Haribabu, B, Tamamura, H, Hiramatu, K, Omagari, A, Pei, G, Manfredi, JP, Fujii, N, Broach, JR, Peiper, SC (2002) A point mutation that confers constitutive activity to CXCR4 reveals that T140 is an inverse agonist and that AMD3100 and ALX40-4C are weak partial agonists. *J Biol Chem* **277**(27)**:** 24515-24521.

Zhao, MM, Hwa, J, Perez, DM (1996) Identification of critical extracellular loop residues involved in alpha 1-adrenergic receptor subtype-selective antagonist binding. *Mol Pharmacol* **50**(5)**:** 1118-1126.

Zhou, N, Luo, Z, Luo, J, Liu, D, Hall, JW, Pomerantz, RJ, Huang, Z (2001) Structural and functional characterization of human CXCR4 as a chemokine receptor and HIV-1 co-receptor by mutagenesis and molecular modeling studies. *J Biol Chem* **276**(46)**:** 42826-42833.

Zhu, SZ, Wang, SZ, Hu, J, el-Fakahany, EE (1994) An arginine residue conserved in most G protein-coupled receptors is essential for the function of the m1 muscarinic receptor. *Mol Pharmacol* **45**(3)**:** 517-523.

Zhu, X, Cao, Y, Voogd, K, Steiner, DF (2006) On the processing of proghrelin to ghrelin. *J Biol Chem* **281**(50)**:** 38867-38870.

Zhu, X, Wess, J (1998) Truncated V2 vasopressin receptors as negative regulators of wild-type V2 receptor function. *Biochemistry* **37**(45)**:** 15773-15784.

Zigman, JM, Elmquist, JK (2003) Minireview: From anorexia to obesity--the yin and yang of body weight control. *Endocrinology* **144**(9)**:** 3749-3756.

CHAPTER 9.

APPENDIX

Table 9.1: Colour code for changes in mutant receptor characteristics compared to the WT ghrelin-R.

	Increase in pIC_{50} > 1 log unit Increase in EC_{50} > 5-fold Decrease in basal signalling > 60 % Decrease in E_{max} ≥ WT basal signalling (0 % or less) Decrease in expression > 70 %
	Increase in pIC_{50} > 0.75 log unit Increase in EC_{50} > 4-fold Decrease in basal signalling > 40 % Decrease in E_{max} > 60 % Decrease in expression > 50 %
	Increase in pIC_{50} > 0.50 log unit Increase in EC_{50} > 3-fold Decrease in basal signalling > 20 % Decrease in E_{max} > 30 % Decrease in expression > 70 %
	Decrease in pIC_{50} > 0.50 log unit Decrease in EC_{50} > 3-fold Increase in basal signalling > 10 % Increase in E_{max} > 130 % Increase in expression > 130 %
	Decrease in pIC_{50} > 0.75 log unit Decrease in EC_{50} > 4-fold Increase in basal signalling > 40 % Increase in E_{max} > 160 % Increase in expression > 150 %
	Decrease in pIC_{50} > 1 log unit Decrease in EC_{50} > 5-fold Increase in basal signalling > 60 % Increase in E_{max} > 190 % Increase in expression > 170 %

Table 9.2: Data in main text for InsP-InsP$_3$ accumulation is presented as EC_{50} (nM) ± spread of the 95 % confidence interval. For clarity and ease of comparison, EC_{50} are quoted in nM. To complete the data and enable correct analysis of the SEM of the EC_{50}, this table is included of pEC_{50} ± SEM for the WT ghrelin-R and all mutant ghrelin-R constructs studied.

Construct	pEC_{50} ± SEM
WT ghrelin-R	8.67 ± 0.01
N1.50A	8.59 ± 0.03
T2.39A	8.35 ± 0.15
M2.46A	8.84 ± 0.04
D2.50A	8.70 ± 0.10
C3.25A	No detectable signal
S3.32A	8.76 ± 0.01
C3.35A	9.07 ± 0.07
T3.36A	7.87 ± 0.07
T3.36C	8.65 ± 0.03
T3.36F	9.18 ± 0.08
T3.36M	8.87 ± 0.07
T3.36S	9.07 ± 0.07
T3.42A	9.08 ± 0.05
I3.43A	8.93 ± 8.13
I3.43R	8.00 ± 0.02
T3.44A	8.09 ± 0.04
L3.46A	7.79 ± 0.04
L3.46I	9.18 ± 0.18
L3.46M	8.54 ± 0.01
S3.47A	No detectable signal
E3.49A	No detectable signal
E3.49D	8.14 ± 0.01
E3.49Q	8.97 ± 29.3
E3.49R	No detectable signal
R3.50A	No detectable signal
R3.50E	8.21 ± 0.04
R3.50H	7.79 ± 0.04
R3.50L	7.12 ± 0.07
R3.50K	8.00 ± 0.07
R3.50Q	8.20 ± 0.01
Y3.51A	8.67 ± 0.02
A3.60Y	8.69 ± 0.10
T2.39A/A3.60Y	No detectable signal
W4.50A	No detectable signal
N188A	8.76 ± 0.02
T190A	8.35 ± 0.02
N196A	7.65 ± 0.02
E197A	8.02 ± 0.06
C198A	No detectable signal
C116A/C198A	No detectable signal
R199A	8.88 ± 0.02
P200A	8.52 ± 0.03
T201A	8.05 ± 0.02
E202A	8.44 ± 0.06
F203A	8.96 ± 0.04

Construct	pEC_{50} ± SEM
F203G	8.93 ± 0.01
A204G	8.73 ± 0.01
A204E	8.79 ± 0.07
A204V	8.77 ± 0.04
P5.50A	8.03 ± 0.05
Y5.58A	8.07 ± 0.02
Y5.58Q	8.27 ± 0.02
H6.30A	8.52 ± 0.02
H6.30D	8.90 ± 0.68
H6.30E	8.62 ± 0.04
H6.30K	8.36 ± 0.01
H6.30Q	8.78 ± 0.09
H6.30R	8.52 ± 0.02
Q6.32A	8.70 ± 0.05
Q6.32E	8.90 ± 0.02
T6.33A	8.91 ± 60.8
T6.33V	8.62 ± 0.12
V6.34A	8.62 ± 0.07
V6.34K	8.56 ± 0.02
V6.34T	8.75 ± 0.08
M6.36A	8.71 ± 0.01
L6.37A	8.81 ± 0.10
L6.37I	8.71 ± 0.04
L6.37M	8.29 ± 0.02
L6.37T	8.96 ± 0.05
V6.40A	No detectable signal
V6.40F	8.09 ± 0.03
V6.40M	8.34 ± 0.05
V6.40Y	No detectable signal
W6.48A	7.58 ± 0.09
W6.48F	7.80 ± 0.05
W6.48Y	7.83 ± 0.06
P6.50A	8.08 ± 0.06
R6.55A	No detectable signal
A204E/R6.55A	No detectable signal
K6.60A	8.83 ± 0.01
A204E/K6.60A	7.84 ± 0.06
S7.45A	8.24 ± 0.06
S7.45N	9.22 ± 0.04
N7.49A	8.39 ± 0.03
P7.50A	8.31 ± 0.02
I7.51A	8.78 ± 0.01
L7.52A	8.50 ± 0.02
Y7.53A	8.78 ± 0.06
K7.58A	8.75 ± 0.05
K7.59A	9.22 ± 0.10

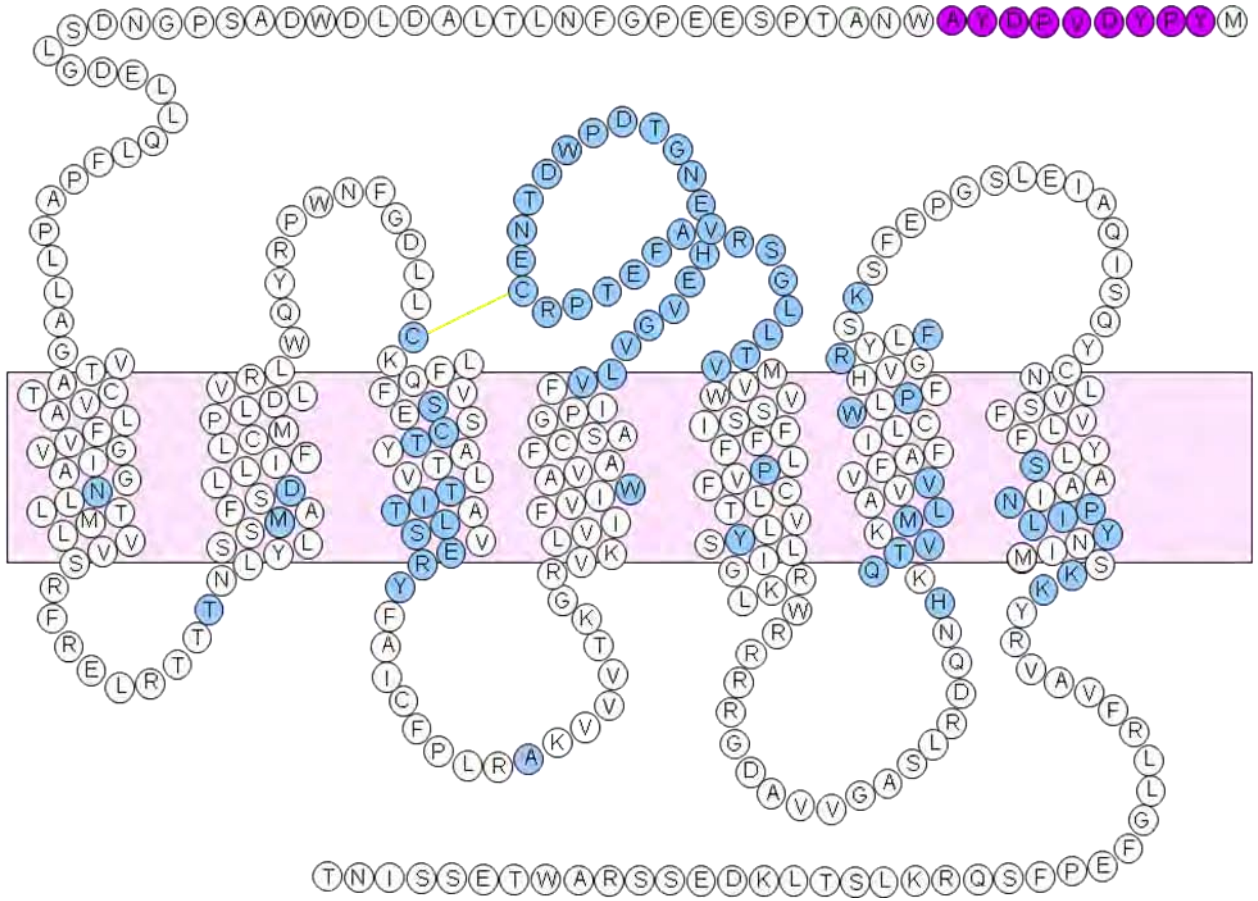


Figure 9.3 Residues mutated in the ghrelin-R as part of this study: schematic representation of the ghrelin-R showing the residues mutated in this study (**blue**). HA-epitope tag introduced at the N-terminus is shown in **purple**.

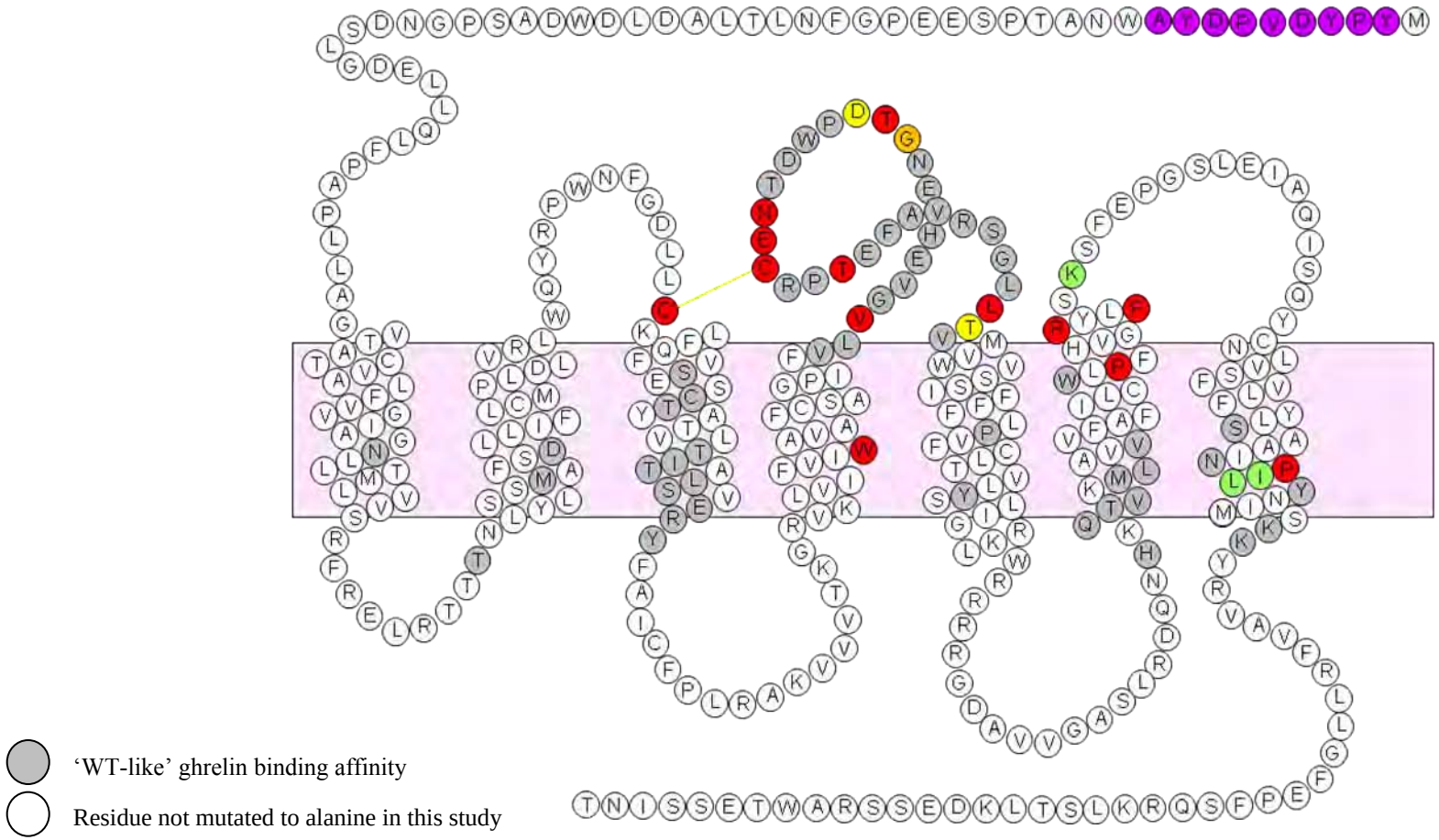


Figure 9.4 Ghrelin binding affinity: schematic representation of the ghrelin-R summarising the effects of individual alanine substitutions on ghrelin binding affinity. See Table 9.1 for colour code. HA-epitope tag introduced at the N-terminus is shown in **purple**.

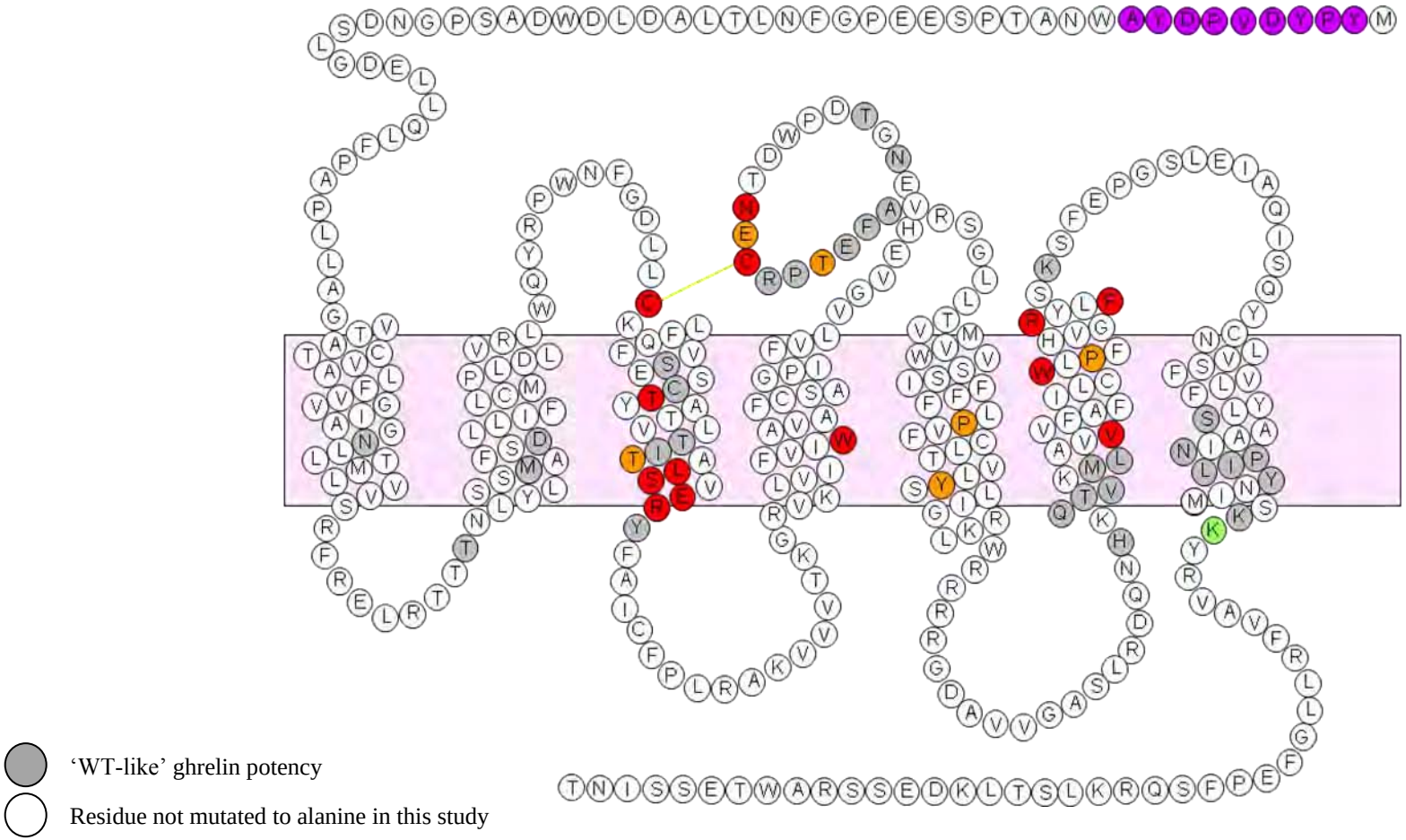


Figure 9.5 Ghrelin potency: schematic representation of the ghrelin-R summarising the effects of individual alanine substitutions on ghrelin potency. See Table 9.1 for colour code. HA-epitope tag introduced at the N-terminus is shown in **purple**.

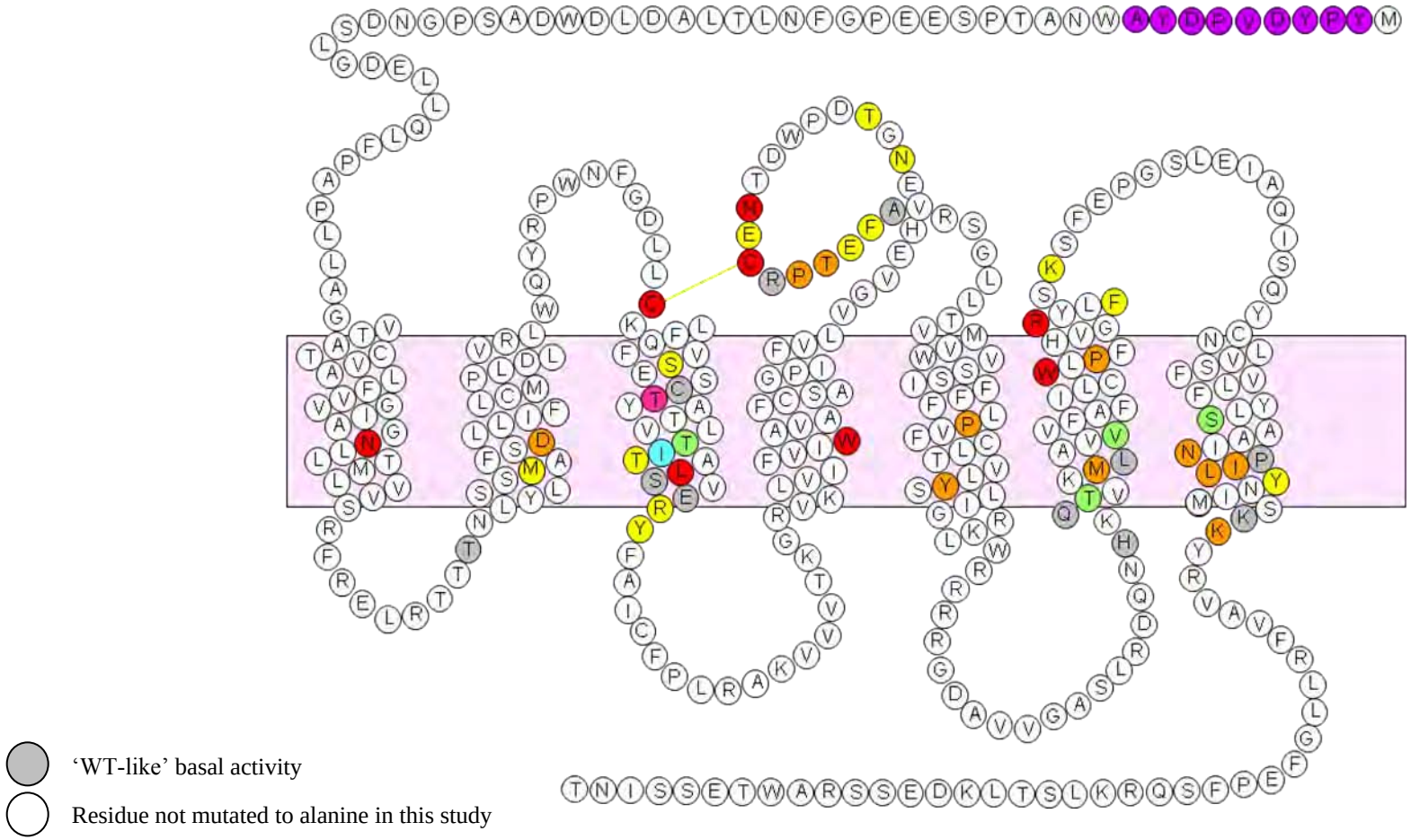


Figure 9.6 Ghrelin-R basal activity: schematic representation of the ghrelin-R summarising the effects of individual alanine substitutions on basal signalling. See Table 9.1 for colour code. HA-epitope tag introduced at the N-terminus is shown in **purple**.

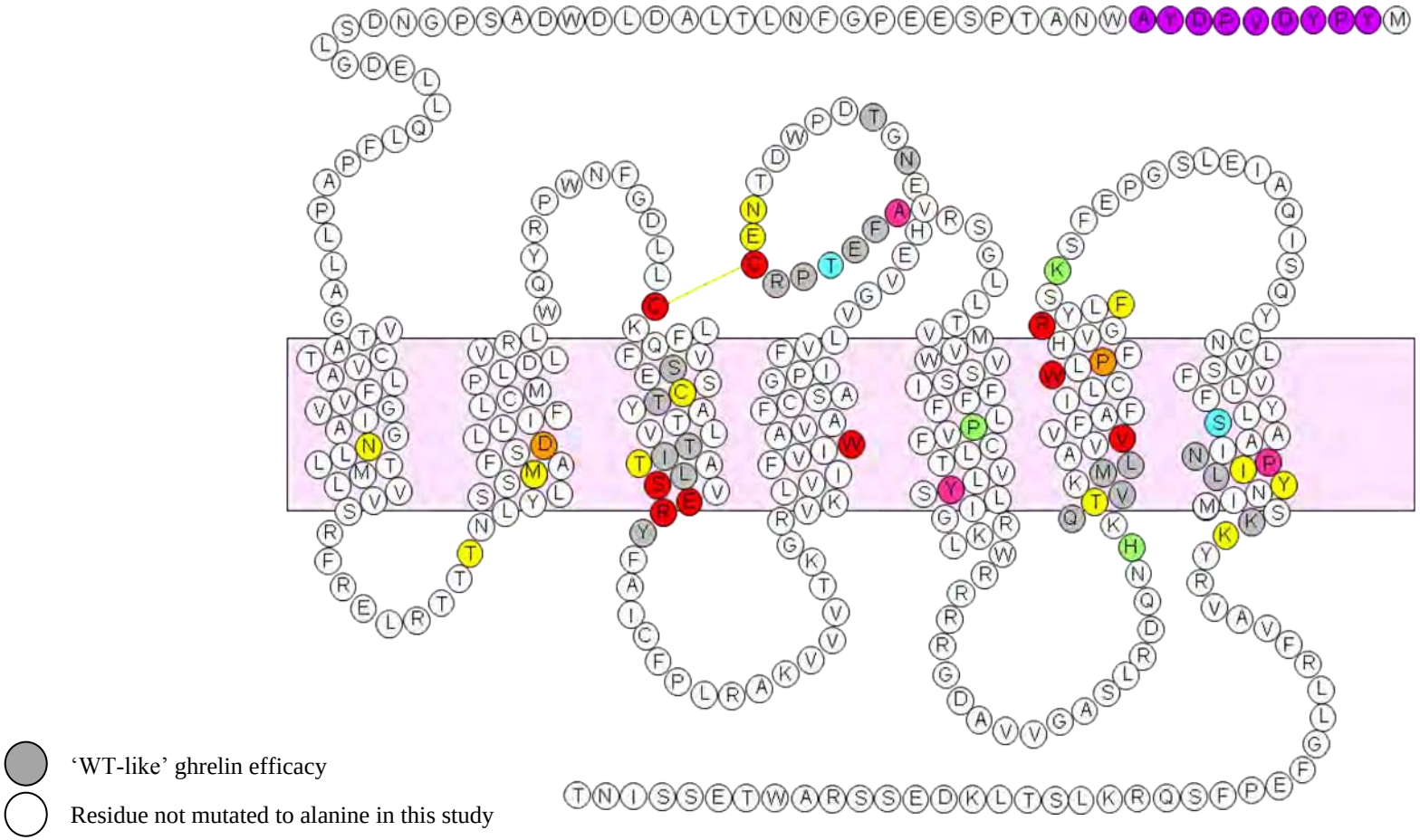


Figure 9.7 Ghrelin efficacy: schematic representation of the ghrelin-R summarising the effects of individual alanine substitutions on ghrelin efficacy. See Table 9.1 for colour code. HA-epitope tag introduced at the N-terminus is shown in **purple**.

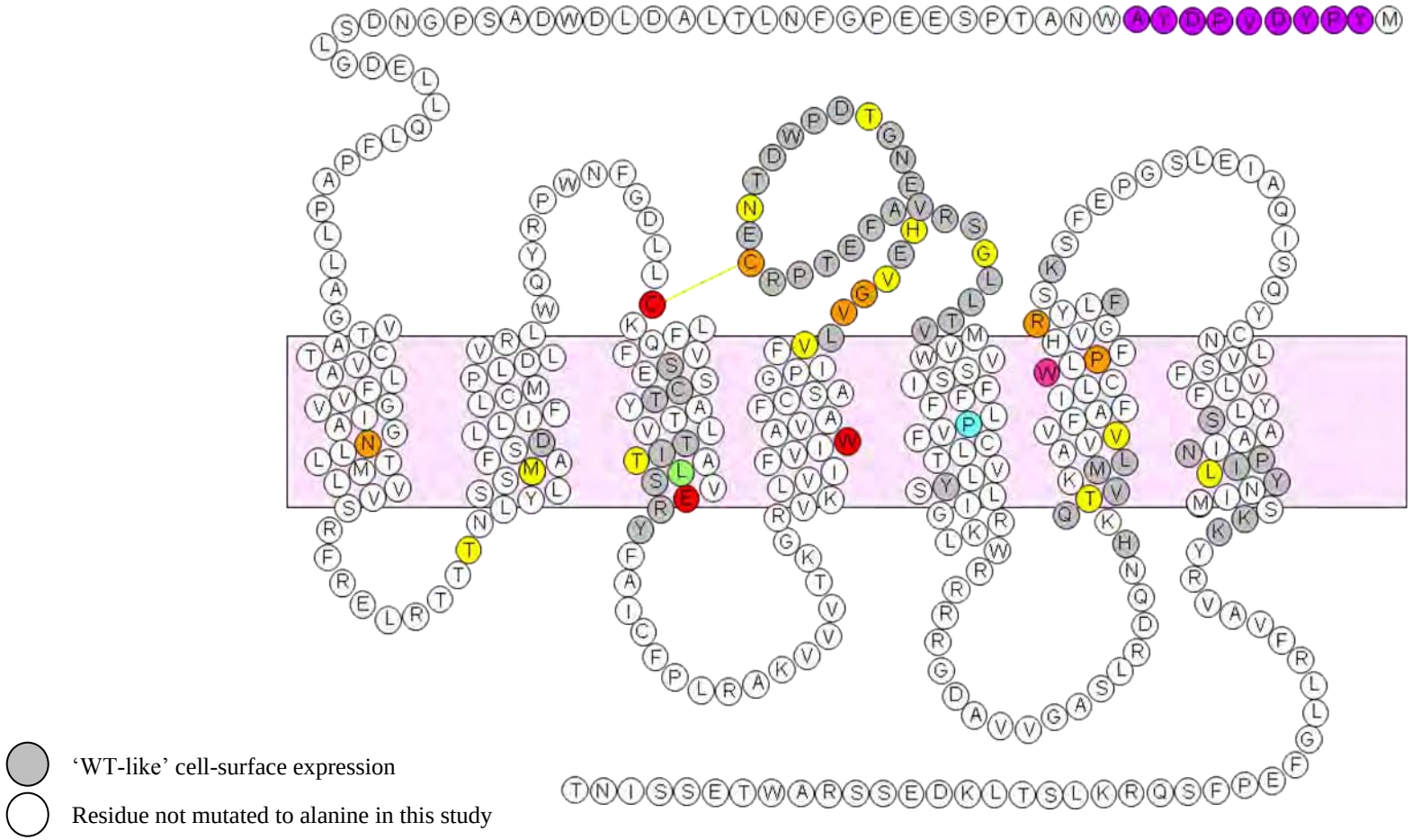


Figure 9.8 Receptor cell-surface expression: schematic representation of the ghrelin-R summarising the effects of individual alanine substitutions on receptor cell-surface expression. See Table 9.1 for colour code. HA-epitope tag introduced at the N-terminus is shown in **purple**.