An Investigation into the Pharmacology of the Ghrelin Receptor

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Abstract

The ghrelin receptor (GRLN-R) was cloned in 1996 after the discovery that a series of synthetic growth hormone-releasing compounds (the growth hormone secretagogues) acted through a receptor distinct from the growth hormone-releasing hormone receptor. In 1999 the endogenous ligand of the receptor, ghrelin, was discovered. As well as stimulating growth hormone release, ghrelin has been shown to be involved in many other processes such as appetite stimulation and the regulation of energy homeostasis, making the ghrelin/GRLN-R system an attractive pharmaceutical target for the treatment of disorders such as growth hormone deficiency, cachexia and obesity. The GRLN-R displays a high level of ligand-independent (constitutive) activity and has been suggested to couple to $G_{\alpha q/11}$, $G_{\alpha i/o}$, $G_{\alpha s}$ and $G_{\alpha 12/13}$ G protein pathways, although little is known about the signalling of ghrelin and the growth hormone secretagogues in all but the $G_{\alpha q/11}$ pathway. Two of the growth hormone secretagogues, GHRP-6 and L-692,429, have been described as 'ago-allosteric modulators' of the GRLN-R as, when co-administered with ghrelin, GHRP-6 and L-692,429 were reported to act both as co-agonists (increasing the efficacy of the ghrelin response) and as negative or positive (respectively) regulators of the potency of ghrelin. This study sought to investigate the pharmacology of the GRLN-R through the $G_{\alpha i/o}$ pathway.

[^{35}S]GTPγS binding assays were used to measure activation of the $G_{\alpha i/o}$ pathway, demonstrating that GHRP-6, L-692,585 (a commercially available analogue of L-692,429) and a third growth hormone secretagogues, MK-677, acted with higher efficacy than ghrelin. At least in the system tested, upon co-administration with ghrelin each of the growth hormone secretagogues acted in a simple competitive fashion with ghrelin. Radioligand binding experiments showed that the dissociation kinetics of [His[^{125}I]]-ghrelin from the GRLN-R were not altered by co-administration of the growth hormone secretagogues. Fitting data to a modified operational model of allosterism demonstrated that GHRP-6, L-692,585 and MK-677 were not ago-allosteric modulators of the GRLN-R but simple orthosteric agonists.

In order to further examine the receptor-specific effects of ghrelin and the growth hormone secretagogues, a Flp-In™ T-REx™ HEK293 cell line expressing the GRLN-R was constructed. [^{35}S]GTPγS binding assays confirmed that the GRLN-R was constitutively active through both the $G_{\alpha q/11}$ and $G_{\alpha i/o}$ pathways, demonstrated by an increase in [^{35}S]GTPγS loading upon receptor expression which could be reduced by the

administration of the GRLN-R inverse agonist SPA. Upon expression of the GRLN-R a considerable level of cell detachment was observed with the remaining cells appearing rounded compared to parental HEK293 cells, an affect that appeared to be mediated by the constitutive activity of the receptor.

In contrast to the [^{35}S]GTPγS assays used to measure activation of $G_{\alpha i/o,}$ [^{35}S]GTPγS assays with a $G_{\alpha q}$-immunoprecipitation step demonstrated that the growth hormone secretagogues acted with equal efficacies to that of ghrelin, demonstrating functional selectivity at the GRLN-R. Intact cell assays were also used to measure $G_{\alpha q/11}$ and $G_{\alpha i/o}$ responses, however, a $G_{\alpha i/o}$-mediated response could not be measured in cAMP accumulation assays, suggesting that the GRLN-R could signal via activation of $G_{\alpha o}$ but not $G_{\alpha i1\text{-}3}$. Although the activation of the $G_{\alpha s}$ pathway by the GRLN-R remains controversial, in this study ghrelin and the growth secretagogues could evoke a $G_{\alpha s}$-mediated cAMP response (although L-692,585 acted with a lower efficacy than ghrelin).

Finally, two naturally occurring missense mutations of the GRLN-R (A204E in the second extracellular loop and I134T in the third transmembrane helix) were analysed to investigate whether these mutations led to retention of the receptor within the endoplasmic reticulum and to investigate whether the mutations affected the ability of the GRLN-R to signal to the growth hormone secretagogues. The A204E mutation caused partial retention of the GRLN-R within the endoplasmic reticulum, whilst receptor that was transported to the plasma membrane did not display any measurable constitutive activity. In contrast, the I134T mutation did not alter receptor localisation, nor did it have any effect on the constitutive or ligand-induced activation of the GRLN-R, however, it appeared to lower the efficacy of the inverse agonist SPA.

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Author’s declaration

The work presented in this thesis was conducted by the author (except where otherwise acknowledged) and has not previously been submitted for a degree or diploma at this University or any other institution.

Abbreviations

Abbreviation	Full name
[^{35}S]GTPγS	guanosine 5'-O-(3-[^{35}S]thio)triphosphate
AC	adenylate cyclase
AGRP	agouti-related protein
ANOVA	analysis of variance
AP2	adaptor protein 2
AT_{1A}	angiotensin II type 1A receptor
ATP	adenosine triphosphate
β_2AR	β_2 adrenergic receptor
BCA	bicinchoninic acid
BSA	bovine serum albumin
Ca^{2+}	calcium
$[Ca^{2+}]_I$	intracellular calcium
CAMP	cyclic adenosine 3',5'-monophosphate
CART	cocaine-and amphetamine-related transcript
CD36	multi-functional class B scavenger receptor
CHO	Chinese hamster ovary
CDNA	complementary deoxyribonucleic acid
CGMP	cyclic guanosine 3',5'-monophosphate
CMV	cytomegalovirus
C-terminus	carboxy terminus
DAG	1,2 diacylglycerol
DNA	deoxyribonucleic acid
DMEM	Dulbecco's modified Eagle's medium

Abbreviation	Full name
DNTP	deoxynucleoside triphosphate
ECFP	enhanced cyan fluorescent protein
ECL	extracellular loop
EEA1	early endosome autoantigen 1
Endo H	endo-β-acetylglucosaminidase H
ER	endoplasmic reticulum
ERK1/2	extracellular signal regulated kinases 1 and 2
FBS	foetal bovine serum
FFA1	Free fatty acid receptor 1
FRET	fluorescence resonance energy transfer
FRT	Flp recombinase target
G protein	guanine nucleotide binding protein
GABA	γ-aminobutyric acid
GAP	guanosine 5'-triphosphate activating protein
GDP	guanosine 5'-diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GHRP-6	growth hormone-releasing peptide 6
GHs	growth hormone secretagogues
GHs-R1a	growth hormone secretagogues receptor type 1a
GHs-R1b	growth hormone secretagogues receptor type 1b
GHRH	growth hormone releasing hormone
GOAT	ghrelin O-Acetyltransferase
GPCR	G protein-coupled receptor
GPR38	motilin receptor

Abbreviation	Full name
GPR39	putative obestatin receptor
GRK	G protein receptor kinase
GRLN-R	ghrelin receptor
GTP	guanosine 5'-triphosphate
GTPγS	guanosine 5'-O-(3-thio)triphosphate
HA	haemagglutinin protein
HBSS	Hank's buffered saline solution
HEK	human embryonic kidney
HRP	horse radish peroxidase
ICL	intracellular loop
IP_3	inositol [1,4,5]-triphosphate
JNK	c-Jun N-terminal kinase
LB medium	Luria-Bertani medium
MAPK	mitogen-activated protein kinase
MEM	minimal essential medium
mRNA	messenger ribonucleic acid
N-terminus	amino terminus
NBCS	newborn calf serum
NPY	neuropeptide Y
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethylenimine
PIP_2	phosphatidyl inositol [4,5] bisphosphate
PKA	protein kinase A
PKC	protein kinase C

Abbreviation	Full name
PLA_2	phospholipase A_2
PLCβ	phospholipase Cβ
PNGaseF	peptide-N^4-(acetyl-β-glucosaminyl)-asparagine amidase
POMC	pro-opiomelanocortin
PTx	*Bordella pertussis* toxin
REM	rapid eye movement
Rho GEF	rho guanine nucleotide exchange factor
RIPA	radioimmunoprecipitation assay buffer
RGS	regulator of G protein signalling
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
S.E.M	standard error of the mean
siRNA	small interfering ribonucleic acid
SPA	substance P analogue ([D-Arg^1,D-Phe^5, D-$Trp^{7,9}$,Leu^{11}]-substance P)
TAE	Tris acetate-EDTA
TE	Tris-EDTA
$TeTO_2$	tetracycline operon system
TM	transmembrane
Tr-FRET	time resolved fluorescence energy transfer
VSV-G	vesicular stomatitis virus glycoprotein
WGA	wheat germ agglutinin

1 Introduction

1.1 Introduction to ghrelin and the ghrelin receptor

In 1981 the ability of two enkephalin analogues, met-enkephalin and leu-enkephalin, to stimulate growth hormone (GH) release in primary cultures of rat pituitary cells was described (Momany et al., 1981). Seven years later Bowers and colleagues described the first synthetic GH–releasing hormone peptide (GHRP-6) displaying *in vivo* GH-releasing activity; both in human and animal models (Bowers et al., 1984). In an attempt to increase bioavailability the structure of GHRP-6 was used as a template in the development of a series of synthetic ligands, termed the 'growth hormone secretagogues' (GHs; in which GHRP-6 is usually included), including the benzolactams L-692,429 (Smith et al., 1993) and L-692,585 (Jacks et al., 1994) and the spiroperidine MK-677 (Patchett et al., 1995) (Table 1-1 lists some of the growth hormone secretagogues).

Around the same time as the studies by Bowers and colleagues, a naturally occurring hypothalamic hormone, growth hormone-releasing hormone (GHRH), was discovered (Rivier et al., 1982; Guillemin et al., 1982). However, it was clear that the growth hormone secretagogues were mediating their GH-releasing effects through a distinct pathway from the GHRH (Smith et al., 1996). For example, [^{35}S]-MK-677 binding was competitively inhibited by L-692,429 but not by GHRH (Pong et al., 1996).

In 1996 the receptor for the growth hormone secretagogues was cloned and named the 'growth hormone secretagogues receptor' (GHs-R) (Howard et al., 1996). The GHs-R was shown to belong to the G protein coupled receptor (GPCR) superfamily, a family of receptors characterised by the presence of 7 transmembrane (TM) spanning alpha helical domains separated by 3 intracellular loops and 3 extracellular loops. The GHs-R gene is located at the chromosomal location of 3q26.2 and consists of a single intron of around 2 kb that divides the open reading frame into two exons, one encoding TM I-V of the GHs-R and the other encoding TM VI-VII (McKee et al., 1997). Two types of cDNA arise from the gene by alternative mRNA processing; the cDNA for the full length GHs-R (GHs-R1a; 366 amino acids in length) and cDNA for GHs-R1b, a truncated receptor (289 amino acids in length), which, as the intron is not removed (probably due to the use of an alternative splice site and an alternative polyadenylation site), encodes a receptor containing TMI-V fused to a short conserved reading frame of 24 amino acids followed by a translational stop codon (Howard et al., 1996; McKee et al., 1997). GHs-R1b has been shown to be unable to bind MK-677 and GHRP-6 (Howard et al., 1996).

Table 1-1 – Ligands acting at the growth hormone secretagogues receptor

Agonists ('the growth hormone secretagogues')	**Company (where appropriate)**	**Ligand type**	**Reference**
Met-enkephalin		Peptide	Momany et al., 1981
Leu-enkephalin		Peptide	Momany et al., 1981
GHRP-6		Peptide	Bowers et al., 1984
GHRP-1		Peptide	Bower, 1993
GHRP-2		Peptide	Bowers, 1993
Examorelin/hexarelin	Mediolanum Farmaceutici	Peptide	Deghenghi et al., 1994
L-692,429	Merck	Benzolactam	Cheng et al., 1993
L-692,585	Merck	Benzolactam	Jacks et al., 1994
MK-677	Merck	Spiropiperidine	Patchett et al., 1995
SM-130686 (partial agonist)	Sumitomo Pharmaceuticals	Oxindole derivative	Nagamine et al., 2006
Antagonists			
BIM-28163 (also binds to somatostatin receptors)		Only detail given is that BIM-28163 is a ghrelin analogue	Halem et al., 2004
YIL-781	Bayer Healthcare	Quinazolinone derivative	Esler et al., 2007
Inverse agonists			
[D-lys^{3}]-GHRP-6, originally classed as an antagonist by Cheng et al. (1993)	Merck	Peptide	Chan et al., 2004
[D-Arg^{1}, D-Phe^{5},D-Trp^{7,9}, Leu^{11}]-substance P, originally classed as an antagonist by Cheng et al. (1997)	Merck	Peptide	Holst et al., 2003

Interestingly cloning of GHs-R1a from other species has demonstrated that the sequence of the GHs-R1a is highly conserved, with mouse, ferret, pig, rabbit and rat cDNA showing over 90 % sequence homology to the human receptor.

The GHs-R1a remained an orphan receptor (i.e. the endogenous ligand remained unknown) for a further three years until Kojima and colleagues isolated a peptide from rat stomach extract that could activate the GHs-R1a, a peptide which they called ghrelin ('ghre' from the Proto-Indo-European root of the word 'grow') (Kojima et al., 1999). This led to the suggestion that the GHs-R1a should be renamed the ghrelin receptor (GRLN-R) (Davenport et al., 2005).

The human ghrelin gene is located at a chromosomal location of 3q25-56 and comprises five exons and three introns (Soares and Leite-Moreira, 2008). This encodes mRNA for pre-pro-ghrelin, a 117-amino acid ghrelin precursor (Kojima et al., 1999). Pre-pro-ghrelin contains a 23 amino acid signal peptide and a 94 amino acid peptide pro-ghrelin (Soares and Leite-Moreira, 2008). Pro-ghrelin consists of the 28 amino acid long mature ghrelin (see Figure 1-1) plus a 66 amino acid 'tail' (Kojima et al., 1999; Jeffery et al., 2005) and is cleaved by pro-hormone convertase 1/3 to generate mature ghrelin (Zhu et al., 2006). Like the GRLN-R, ghrelin appears to be highly conserved across multiple species, with human and rat pre-pro-ghrelin showing 82.9 % sequence homology, with only two amino acids replaced in the 28-amino acid ghrelin segment (Kojima et al., 1999).

Interestingly ghrelin undergoes a unique modification at serine 3 where the residue is octanylated (the hydrogen atom is replaced by a $C_7H_{15}CO$ moiety; see Figure 1-1) (Kojima et al., 1999), a modification that appears to be essential for the activity of ghrelin as removal of the octanoyl group decreases the potency of ghrelin by more than 2300-fold (Matsumoto et al., 2001). In 2008 the acyltransferase responsible for the octanylation of ghrelin was identified and named ghrelin O-Acyltransferase (GOAT) (Yang et al., 2008), a member of the membrane bound O-Acyltransferase family that attach fatty acids to lipids and proteins (Hofmann, 2000). GOAT appears to be located in the endoplasmic reticulum (in stomach and pancreas of humans; Gutierrez et al., 2008) where it acts to octanylate pro-ghrelin before pro-ghrelin is transported to the Golgi and cleaved to form mature ghrelin (Yang et al., 2008). Interestingly, although ghrelin is a peptide ligand, there is some evidence to suggest that ghrelin can cross the blood-brain barrier via a saturatable transporter system (Banks et al., 2002).

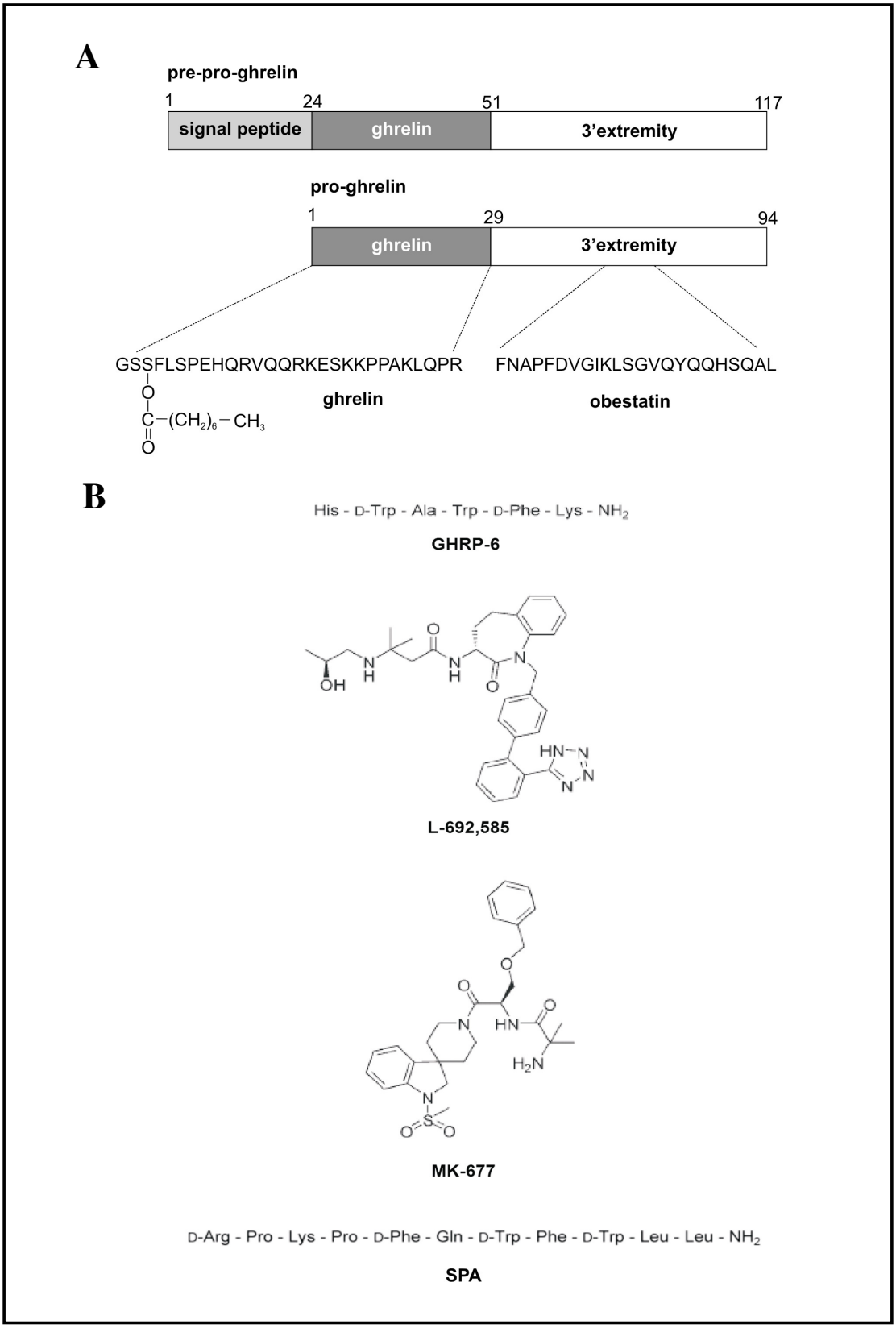

Figure 1-1 - The structure of ghrelin, GHRP-6, L-692,585, MK-677 and SPA. **A.** Pre-pro-ghrelin comprises of a 23 amino acid signal peptide and pro-ghrelin. Pro-ghrelin is cleaved by pro-hormone convertase 1/3 to form mature ghrelin (which undergoes octanylation at the third residue, S), whilst processing of the 3' extremity results in the formation of obestatin (adapted from De Vriese and Delporte, 2008). **B.** The structure of the growth hormone secretagogues GHRP-6, L-692,585, MK-677 and the inverse agonist SPA (adapted from Moulin et al., 2007). A basic amine group is conserved across GRLN-R ligands and is required for their bioactivity.

Alternative splicing of pre-pro-ghrelin results in the formation of the biologically active peptide des-Gln^{14}-ghrelin, which is also octanylated at serine 3 but lacks a glutamic acid residue in position 14 (Kojima et al., 1999). Des-Gln^{14}-ghrelin appears to possess the same hormonal activities as ghrelin (Hosoda et al., 2000). Processing of the 66 amino acid tail of pro-ghrelin results in the formation of obestatin (Soares & Leite-Moreira, 2008) – a ligand proposed to be the endogenous ligand for a receptor related to the GRLN-R, GPR39 (Zhang et al., 2005; Zhang et al., 2008) although this needs further confirmation as there are now reports that obestatin does not activate GPR39 (Holst et al., 2007; Chartrel et al., 2007).

Interestingly, the GRLN-R can signal in the absence of ghrelin and the growth horomone secretagogues i.e. the receptor exhibits a high level of constitutive (ligand-independent) activity, which has been shown to be a key requirement for constitutive (ligand-independent) receptor internalisation (Holliday et al., 2007). Depending on the system used, the constitutive activity of the GRLN-R has been recorded to be up to 50 % of the maximum activity measured in the presence of ghrelin (Holst et al., 2003). This ligand-independent activity was shown to be reduced by administration of a substance P analogue (SPA) (Holst et al., 2003); thus demonstrating that SPA acts as an inverse agonist, favouring the formation of an inactive receptor state. Intriguingly, the constitutive activity of the GRLN-R appears to be important *in vivo* as naturally occurring point mutations of the GRLN-R which ablate the constitutive activity of the receptor, whilst preserving the reponse to ghrelin, are associated with the development of obesity and short stature (Pantel et al., 2006; Wang et al., 2004).

The GRLN-R has become an attractive therapeutic target not only due to its effects on growth hormone release (per mol, ghrelin is more potent at stimulating GH release than GHRH; Gil-Campos et al., 2006) but also because ghrelin and the GRLN-R have been demonstrated to possess a wide-range of other central and peripheral actions, ranging from modulating appetite and energy homeostasis to opposing inflammation of the cardiovascular system (van der Lely et al., 2004).

The aim of this chapter is to provide background on the distribution and biological effects of ghrelin and the GRLN-R, before focusing on the structure and signalling mechanisms of the GRLN-R.

1.2 Ghrelin tissue distribution

The main source of endogenous ghrelin appears to be from X/A-like cells in the oxyntic mucosa of the stomach (Date et al., 2000). However it is clear that there are other sources of ghrelin as plasma ghrelin levels increase after a total gastrectomy (Hosoda et al., 2003) suggesting that other tissues can compensate for the loss of ghrelin production (Moller et al., 2003). Indeed ghrelin mRNA has also been detected in the pancreas (Date et al., 2002b), placenta (Gualillo et al., 2001), the adrenal glands, oesophagus, adipocytes, gall bladder, muscle, myocardium, ovary, prostate, skin, spleen, liver, thyroid gland, blood vessels (Gnanapavan et al., 2002), T-lymphocytes, B-lymphocytes and neutrophils (Hattori et al., 2001). However the levels of ghrelin expression in these tissues is relatively low compared to that of the stomach (van der Lely et al., 2004).

Ghrelin has been detected in the brain (e.g. the pituitary; Korbonits et al., 2001) using mRNA amplification and immunofluorescence histochemistry (Hou et al., 2006). However GOAT, which is responsible for the octanylation of ghrelin, is not expressed in mouse brain, therefore casting doubts on whether brain cells produce biologically active ghrelin (Yang et al., 2008).

1.3 GRLN-R tissue distribution

In contrast to ghrelin, levels of GRLN-R are highest in the central nervous system. GRLN-R mRNA expression has been recorded in the hypothalamus (especially the arcuate nucleus) and pituitary gland with GRLN-R expression also reported in other brain areas such as the hippocampus, *pars compacta* of the *substantia nigra*, the ventral tegmental area, and in the dorsal and medial raphe nuclei (van der Lely et al., 2004). In addition much lower levels of mRNA expression (than seen in the pituitary) are detected in peripheral organs and tissues, such as: the thyroid gland, pancreas, spleen, myocardium and adrenal glands (Gnanapavan et al., 2002). Specific binding of [^{125}I]-ghrelin to the GRLN-R has also been detected in the aorta, pulmonary arteries, saphenous veins, and the arcuate arteries of the kidney (Katugampola et al., 2001).

Interestingly although GHs-R1b does not signal to ghrelin (Leung et al., 2007) or the growth hormone secretagogues (Howard et al., 1996) the receptor is much more widely expressed than the GRLN-R; being detected in the skin, myocardium, pituitary, thyroid, pancreas, ileum, colon, liver, breast, spleen, duodenum, placenta, lung, adrenal glands, buccal mucosa, the stomach (fundus and antrum), lymph node, gall bladder, atrium,

lymphocytes, kidney, bladder, prostate, fallopian tubes, vein, muscle, ovaries, testes, fat, oesophagus and jejunum (Gnanapavan et al., 2002).

A study by Gnanapavan et al. (2002) revealed that GHs-R1b mRNA copy number varied depending on the tissue measured, from an mRNA copy number (per total μg tissue) of 1 in jejunum to 10^7 in skin. GRLN-R mRNA copy number (per total μg tissue) varied from 10^3 in adrenal glands to 10^5 in the pituitary. In some tissues, such as the myocardium, GHs-R1b mRNA copy number was greater than GRLN-R mRNA copy number (GRLN-R mRNA copy number = 10^3; whilst GHs-R1b copy number = 10^7) (Gnanapavan et al., 2002).

1.4 Biological actions of the GHs-R1b

The exact function of the GHs-R1b remains unknown. In HEK293 cells transfected to express the GHs-R1b, the receptor was detected predominantly expressed in the nucleus of the cells but some receptor was also detected at the cell surface (Leung et al., 2007). There is some evidence that GHs-R1b inhibits growth hormone secretagogues-mediated calcium mobilisation in HEK293 cells transfected to express the seabream GRLN-R (Chan and Cheng, 2004). Meanwhile, another report has suggested that the receptor might act as a dominant negative mutant of the GRLN-R (Leung et al., 2007; this finding is further discussed in section 1.9.2).

1.5 Biological actions of ghrelin, the growth hormone secretagogues and the GRLN-R

The two most studied actions of ghrelin and the GRLN-R are in modulating GH release and in the regulation of appetite and energy homeostasis.

1.5.1 GH release

GH is released by somatotroph cells in the anterior pituitary gland in response to hormones produced from the hypothalamus, such as GHRH and somatostatin. Multiple studies have demonstrated that ghrelin and the growth hormone secretagogues can stimulate GH release from somatotrophs *in vitro* (Kojima et al., 1999; Sartor et al., 1985; Bowers et al., 1991). *In vivo*, administration of the growth hormone secretagogues has been demonstrated to have a synergistic effect on GH-release mediated by the GHRH (Bowers et al., 1991; Wu et al., 1994). In contrast to GHRH, which increases GH release *in vivo*, somatostatin acts to inhibit GH secretion (Brazeau et al., 1973). There is evidence to suggest that, in addition to

increasing GH release, the growth hormone secretagogues may also act to antagonise the inhibitory effect of somatostatin on GH release, by counteracting its hyperpolarising effect on somatotroph cell membranes (Goth et al., 1992).

As well as acting on the anterior pituitary there is evidence to suggest that ghrelin and the growth hormone secretagogues act directly at the hypothalamus. For example, GH-releasing activity of the growth hormone secretagogues is greater in hypothalamic-pituitary preparations than in pituitary preparations (Mazza et al., 1989) whilst GH release is reduced, but not completely abolished, in animals with lesions of the pituitary stalk (Fletcher et al., 1994; Hickey et al., 1996). Indeed it is evident that an intact GHRH system is required for the effects of ghrelin and the growth hormone secretagogues *in vivo*. For example, GH release pulsatility and responsiveness are decreased by the administration of antibodies against GHRH in rats (Pandya et al., 1998). In humans GH response to the growth hormone secretagogues is inhibited in subjects with deficiencies in the GHRH receptor (Maheshwari et al., 1999).

The main target of ghrelin action in the hypothalamus is the arcuate nucleus of the hypothalamus where it may bind and activate the GRLN-R, here ghrelin and the growth hormone secretagogues act to increase the electrical activity and *c-fos* expression (a marker of neuronal activity) in a subpopulation of cells, of which some are GHRH neurones (Dickson et al., 1995a; Dickson et al., 1995b). Activation of GHRH producing neurones in the arcuate nucleus amplifies the effect of GHRH in somatotrophs (Lengyel, 2006). Interestingly, a recent study has shown that GHRH can act as an agonist of the GRLN-R, binding to an area on the receptor distinct from the ghrelin binding pocket where is acts to increase the binding capacity or the potency of ghrelin in the pathways tested (i.e. it acts as an allosteric modulator; see section 1.10.6) (Casanueva et al., 2008). Thus GHRH could potentially act on the GRLN-R to further increase GH release *in vivo*.

1.5.2 Appetite and energy homeostasis

Evidence for the role of ghrelin in appetite regulation was first obtained by Arvat and colleagues whose studies on the effect of ghrelin in human volunteers showed that, when injected with ghrelin, along with a prompt, marked increase in GH release, three out of the four healthy participants also reported the feeling of hunger (Arvat et al., 2000). In 2000-01 further reports were published demonstrating that ghrelin was involved in the hypothalamic regulation of energy homeostasis (Wren et al., 2000; Wren et al., 2001; Nakazato et al., 2001). For example, exogenous ghrelin was found to increase adiposity in

rodents by stimulating an acute increase in food intake as well as reducing fat utilisation (Nazazato et al., 2001; Wren et al., 2000; Tschop et al., 2000; Shitani et al., 2001; Asakawa et al., 2001). This effect appeared to be mediated by the GRLN-R as intracerebroventricular injections of ghrelin strongly stimulated feeding in rats in a dose-dependent manner, causing an increase in body weight gain, an effect that was suppressed by an antagonist/inverse agonist of the GRLN-R ([D-lys^3]-GHRP-6; Cheng et al., 1989) (Nakazato et al., 2001).

Further studies have demonstrated that plasma ghrelin levels are dependent on food-intake, with ghrelin levels rising before a meal and declining sharply after eating, suggesting that ghrelin plays a major role in meal initiation (Cummings et al., 2001). After a meal the depth and duration of ghrelin suppression is related to the energy intake (Callahan et al., 2004). Ghrelin has also been shown to stimulate gastric acid secretion and motility in rats (Masuda et al., 2000) and circulating ghrelin levels are correlated with gastric emptying time in humans (Tschop et al., 2001). Factors that promote ghrelin secretion include fasting, hypoglycaemia and the hormone leptin whilst the main inhibiting factors are food intake, hyperglycaemia and obesity (Cummings et al., 2001; Tschop et al., 2001; Toshinai et al., 2001; Shiiya et al., 2002). Interestingly during prolonged fasting an eight-fold increase in expression of the GRLN-R in the hypothalamus has been measured (Kim et al., 2003). Together these studies demonstrate that ghrelin is involved in appetite stimulation.

The cellular mechanism underlying the orexigenic effect of ghrelin has been clarified. Ghrelin travels in the bloodstream to the arcuate nuclues of the hypothalamus (Hewson and Dickson, 2000). Here the major target for ghrelin appears to be the appetite-stimulating neuropeptide Y- (NPY) and agouti-related protein- (AGRP) containing neurones which increase their firing rate in response to ghrelin (Cowley et al., 2003). The appetite-supressing pro-opiomelanocortin- (POMC) and cocaine- and amphetamine-regulated transcript- (CART) containing neurones do not possess GRLN-Rs however, projections from the NPY/AGRP-containing neurones terminate on POMC/CART-containing neurones which are indirectly inhibited by ghrelin by the release of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Cowley et al., 2003). Other peripheral hormones that are involved in appetite and energy regulation include leptin, insulin and PYY_{3-36} which all act to decrease food intake (Schwartz et al., 2000). Thus on NPY/AGRP containing neurones ghrelin and the GRLN-R provide the only hormonal appetite-stimulatory input to counterbalance the inhibitory signals (Schwartz et al., 2000; Holst and Schwartz, 2004).

Following food ingestion signals from the gastrointestinal tract are conveyed to the nucleus solitary tract in the brain stem through afferent vagal nerves and other sympathetic nervous system afferents (Gil-Campos et al., 2006). In addition to ghrelin travelling in the bloodstream, it appears that an intact vagus nerve is required for exogenous ghrelin to stimulate appetite in both man and rodents (Date et al., 2002a; le Roux et al., 2005) suggesting that a neuronal pathway (involving the vagal nerves) is an alternative and important mechanism for ghrelin signalling. As GRLN-R are also found near the nucleus solitary tract (such as in the dorsal vagus complex; Date et al., 2002a) several sites other than the hypothalamus may be important in regulating ghrelin-induced appetite (Holst and Schwartz, 2004). Indeed recently the area postrema was shown to be excited by stimulation with ghrelin in a concentration-dependent manner, thus ghrelin may partially regulate appetite regulation via the area postrema (Fry and Ferguson, 2008).

Interestingly, ghrelin levels are low in obese and high in lean individuals, suggesting that ghrelin is not only important for the acute regulation of food intake but also plays an important role in the regulation of long term energy homeostasis (Cummings et al., 2005). Ghrelin levels increase in response to weight-loss resulting from, for example, low-energy diets, cancer cachexia and anorexia (Cummings et al., 2005; Horvath et al., 2001; Tolle et al., 2003; Soriano-Guillen et al., 2004); suggesting that it signals the need to conserve energy. Indeed plasma ghrelin concentration rises rapidly after fasting in normal weight animals but is delayed in fatty Zucker rats suggesting that short-term energy storage is modified by an excess energy deposit (Ariyasu et al., 2002). Intraperitoneal injection of ghrelin in mice was shown to decrease energy expenditure (Asakawa et al., 2001); in rats and mice ghrelin was shown to reduce fat catabolism and lipolysis (Tschop et al., 2000; Muccioli et al., 2004) (although this may be via a non-GRLN-R mediated mechanism as GRLN-R has not been detected in adipose tissue) and chronic central administration of ghrelin in rats has been suggested to decrease adipocyte apoptosis (Kim et al., 2004a).

1.5.3 Other ghrelin/GRLN-R effects

As well as the effects of ghrelin and the GRLN-R on GH release and appetite/energy homeostasis there has been a wealth of studies linking ghrelin and the GRLN-R to a number of other functions such as regulation of sleep, bone formation and gonadotroph secretion; these studies are summarised in Table 1-2. The distribution of the GRLN-R supports the idea that ghrelin has functions in addition to regulation of GH release and appetite regulation/energy homeostasis.

1.5.4 Existence of receptors for ghrelin/the growth hormone secretagogues other than the GRLN-R

There is increasing evidence to suggest that the GRLN-R is not the sole receptor for ghrelin and/or the growth hormone secretagogues. For example, 1 μM ghrelin (a concentration of ghrelin which has been shown to be maximally effective at stimulating human GRLN-R-mediated inositol phosphate accumulation in HEK293 cells; Holst et al., 2005) exerts a cardioprotective effect in H9c2 cardiomyocytes, cells that do not express the GRLN-R (Baldanzi et al., 2002). Meanwhile, in CALU-1 lung carcinoma cells, DNA synthesis and proliferation was inhibited by peptidyl growth hormone secretagogues (at a concentration of 1 nM to 1 μM) but not by ghrelin or the non-peptidyl growth hormone secretagogues (Ghe et al., 2002). Interestingly in the heart a binding site for peptidyl growth hormone secretagogues has been identified as CD36 (Kim et al., 2005), a multi-functional class B scavenger receptor (also known as glycoprotein IV) which has been implicated in multiple physiological functions (e.g. cellular adhesion and fatty acid transportation) and pathological functions related to macrophage foam cell formation and pathogenesis of atherosclerosis (Bodart et al., 2002). Thus the reported effects of ghrelin and the growth hormone secretagogues in some cases may be mediated through a distinct GRLN-R subtype or by a completely different receptor.

Table 1-2 - Reported effects of ghrelin, the growth hormone secretagogues and the GRLN-R both *in vivo* and *in vitro*.

Effects	Details	Reference(s)
Ghrelin defends against depressive-like symptoms of chronic stress	Ghrelin administered in a rodent model of depression produced anxiolytic and anti-depressive-like responses in elevated maze and forced swim tests. GRLN-R-null mice showed deleterious effects of chronic defeat.	Lutter et al., 2008
Ghrelin controls hippocampal spine synapse density and memory performance	Circulating ghrelin enters hippocampus, binding to neurones of hippocampal formation where it promotes dendritic spine synapse formation and generation of long term potentiation, paralleled by enhanced spatial learning and memory. Disrupting ghrelin gene in mice impairs performance in behavioural memory testing, an effect reversible by ghrelin administration.	Diano et al., 2006
Ghrelin influences gonadotroph secretion	In rats intracerebroventricular injection of ghrelin decreases frequency of pulsatile luteinising hormone secretion, probably reflecting a modulation of the activity of the gonadotrophin releasing hormone pulse generator.	Furuta et al., 2001; Fernandez-Fernandez et al., 2004
Constitutive activity of the GRLN-R attenuates apoptosis	Cadmium-induced activation of caspase-3 (a protease involved in apoptosis) is attenuated in HEK293 cells expressing the seabream GRLN-R but not in wild-type HEK293 cells. GRLN-R ligands have no effect on caspase-3 activation.	Lau et al., 2009
MK-677 increases rapid eye movement (REM) sleep	In humans MK-677 was shown to increase REM sleep both in healthy young adults as well as in elderly subjects.	Copinschi et al., 1997
Ghrelin has diverse cardiovascular effects	*In vitro* ghrelin inhibits apoptosis of cardiomyocytes. Ghrelin may also oppose inflammation of the cardiovascular system as it inhibits NF-κB activation in human endothelial cells. Ghrelin exerts an endothelium-independent vasodilatory effect. Ghrelin administration decreases mean arterial pressure without changing heart rate and improves cardiac contractility and left ventricular function in chronic heart failure.	Isgaard and Johansson, 2005

Ghrelin stimulates bone formation	Ghrelin stimulates proliferation and differentiation of oestoblasts *in vitro*. Proliferation of osteoblasts was inhibited by the GRLN-R antagonist [D-lys^3]-GHRP-6. *In vivo* ghrelin increases bone mineral density in both normal and GH-deficient rats	Fukushima et al., 2005
Ghrelin may inhibit insulin secretion	Depending on the system used to measure the response, ghrelin has been shown to both increase and decrease insulin secretion. For example, in rat pancreatic islets and *in vivo* ghrelin was able to increase insulin secretion. In humans acute administration of ghrelin inhibits both spontaneous and arginine-stimulated insulin secretion.	Broglio et al., 2001; Date et al., 2002b; Broglio et al., 2003; Salehi et al., 2004
Ghrelin increases glucose release	Along with a transient decrease in insulin secretion, acute ghrelin administration in humans increases plasma glucose levels.	Broglio et al., 2003
Ghrelin can increase cell proliferation in some cell lines, but the GHs may have an anti-proliferative effect	Ghrelin and the GRLN-R have been detected in a number of neoplastic cells and *in vitro* experiments suggest that ghrelin and GHs can modulate proliferation of several human tumour cell lines. However, GHs have been shown to have an anti-proliferative effect in breast cancer cell lines not expressing the GRLN-R. DNA synthesis and proliferation of CALU-1 lung carcinoma cells that do not express the GRLN-R were shown to be inhibited by peptidyl GHs but not by non-peptidyl GHs and ghrelin.	Ghe et al., 2002; Cassoni et al., 2004; Ghigo et al., 2005

1.6 Ghrelin-null or GRLN-R-null models

There have been multiple studies investigating the effects of knocking-out the GRLN-R *in vivo*. Initially the physiological importance of the GRLN-R in the hypothalamus of rats was tested by using antisense GRLN-R mRNA expression under the control of a tyrosine hydroxylase promoter, to impair GRLN-R function in the arcuate nucleus (Shuto et al., 2002). This confirmed that ghrelin and the GRLN-R were involved in energy homeostasis and appetite stimulation as rats with attenuated GRLN-R expression displayed lower body weights, less adipose tissue, reduced daily food intake and smaller birth weights than their wild-type counterparts. In contrast to the wild-type rats, administration of exogenous ghrelin or growth hormone secretagogues failed to increase feeding. In female rats GH secretion and insulin-like growth factor-1 levels were reduced, however there was no effect

in males, suggesting that the ghrelin/GRLN-R system is more physiologically important in the regulation of GH secretion in females.

The development of complete GRLN-R knock-out animals showed that the GRLN-R is essential for the stimulatory effects of ghrelin on GH secretion and orexigenic properties (Sun et al., 2004). However, GRLN-R-null mice showed no difference in body composition or growth compared to wild-type mice littermates (Sun et al., 2004); suggesting that the GRLN-R does not have a significant role in the determination of body composition or growth.

In studies of ghrelin-null mice, one group showed that: size, growth rate, food intake, body composition, reproduction, gross behaviour and tissue pathology were indistinguishable from wild-type littermates (Sun et al., 2003). Another group suggested the only difference between ghrelin-null mice and their wild-type littermates was the preferential use of lipids rather than carbohydrates in maintaining energy balance particularly under high fat conditions; which led to a trend towards ghrelin-null mice having leaner body composition after 6 weeks on a high-fat diet (Wortley et al., 2004). However, it is possible that other hormonal systems may compensate for the lack of ghrelin or for the inability of the GRLN-R to respond to ghrelin.

In contrast to the previous studies, Zigman and colleagues demonstrated that GRLN-R-null mice did not show any differences in body weight and composition compared to wild-type mice until 19 weeks of age. After 19 weeks on a calorie-dense diet, GRLN-R-null mice showed significantly less accumulation of body weight and body fat content; in female mice body fat mass was almost 50 % less than wild-type mice (Zigman et al., 2005). Several suggestions were given as to why the results of this study were different to previously published studies. These included the duration of exposure to a high-fat diet, animal age when the high-fat diet was administered and fat content of the food (Zigman et al., 2005). Indeed, in a follow-up study in ghrelin-null mice Wortley and colleagues demonstrated that exposure of ghrelin-null mice to a high-fat diet at a young age (6 weeks of age) reduced the weight gain (associated with decreased adiposity, increased energy expenditure and increased locomotor activity) compared to wild-type mice (Wortley et al.,2005). These studies suggest that ghrelin/GRLN-R plays a role in energy regulation causing an increase in adiposity

In a different approach, transgenic animals were engineered to over-express the GRLN-R in GHRH neurones; these animals showed a basal up-regulation of activity in the GHRH-

GH axis and a reduction of adiposity (particularly in female rats) without effect on other GRLN-R-mediated signals (Lall et al., 2004). In mice engineered to display increased ghrelin expression and production in the stomach and brain, increased circulatory ghrelin was associated with hyperphagia, increased energy expenditure, glucose intolerance, decreased glucose-stimulated insulin secretion and reduced leptin sensitivity (Bewick et al., 2009). By knocking-out the gene for the G protein α subunit $G_{\alpha 11}$ in neuronal and glial precursor cells of mice, Wettschureck and colleagues demonstrated that mice died 3-6 weeks after birth as anorectic dwarfs. Both plasma GH and hypothalamic GHRH were reduced and postnatal proliferation of pituitary cells was strongly impaired. The effects of ghrelin were reduced at the hypothalamic level due to impairment of GRLN-R signalling which may have contributed to the GHRH deficiency and abnormal feeding behaviour (Wettschureck et al., 2005).

Together these studies support the role of ghrelin/the GRLN-R in appetite regulation and energy homeostasis but also demonstrate that other mechanisms may take over to compensate for a lack of ghrelin or its receptor. Interestingly, two of these studies have also shown that is a difference in ghrelin/GRLN-R regulation between sexes as the GRLN-R seems to be more important in maintaining body weight and regulating GH secretion in female rats and mice than in males (Shuto et al., 2002; Zigman et al., 2005). This, potentially, could lead to differences in the effectiveness of ghrelin/GRLN-R treatments in males and females, although further studies are needed to investigate this.

1.7 Clinical trials and therapeutic potential of GRLN-R agonists/antagonists

Due to the affects of ghrelin and the growth hormone secretagogues in amplifying GH release and appetite stimulation, it has been suggested that agonists of the GRLN-R would be clinically useful in the treatment of GH deficiency and the treatment of cachexia whilst inverse agonists could potentially be useful in the treatment of obesity; here the reasoning behind these suggestions are briefly discussed.

1.7.1 Cachexia

Cachexia is a syndrome of muscle wasting, increased metabolic rate and a decrease in appetite that accompanies a variety of chronic diseases such as cancer and heart failure (Tisdale et al., 1997). Thus agonists of the GRLN-R could be useful in the treatment of cachexia by increasing appetite and muscle mass. Indeed a recent phase II study into the

effects of the growth hormone secretagogues, RC-1291, showed that after 12 weeks of administration in subjects predominantly presenting with lung cancer, RC-1291 led to a significant improvement in total body mass as well as a trend towards increased lean mass (Garcia et al., 2007). An increase in appetite was also reported in another study where subjects with melanoma or breast or colon cancers were treated with a single intravenous infusion of ghrelin, this led to a 31 % increase in food intake in a single meal compared to subjects receiving placebo (Neary et al., 2004).

1.7.2 GH deficiency

GH deficiency in children is usually treated by GH injections administered daily; although this is effective it has a number of drawbacks including the need for parenteral administration and high cost (Chapman et al., 1997). This makes the non-peptidyl growth hormone secretagogues attractive targets for the treatment of GH deficiency. In one small trial (9 men) oral administration of MK-677 for four days significantly increased GH and insulin-like growth factor-1 levels in men with childhood onset GH deficiency (Chapman et al., 1997). In a study of 18 children with idiopathic GH deficiency treatment with MK-677 increased GH and insulin-like growth factor-1 levels. However, in the most severely GH-deficient children there was an apparent lack of response to MK-677 (Codner et al., 2001). The authors of this study suggested that the lack of an MK-677 response in these children was due to a disconnection between the hypothalamus and pituitary (Codner et al., 2001), although this was not demonstrated directly.

GH secretion decreases with age, leading to decreases in fat-free (muscle) mass (Zadik et al., 1985; Janssen et al., 2000), which is a major risk factor for frailty, loss of independence and physical disability in elderly persons (Fantin et al., 2007). This makes the GH releasing effects of ghrelin and the growth hormone secretagogues attractive therapeutic targets for the treatment of age-related GH decrease. A short-term (2 year) pilot study (65 people) by Nass and colleagues on the effects of MK-677 in healthy older adults demonstrated that subjects who received MK-677 showed sustained increases in the amplitude of pulsatile growth hormone secretion and insulin-like growth factor 1 levels to those seen in young adults. MK-677 also increased fat-free mass compared to placebo, likely due to the concomitant increase in intracellular water. Possibly as a result of appetite stimulation, body mass increased more in MK-677 recipients, indeed a reported side-effect of MK-677 was a feeling of increased appetite which subsided after a few months of treatment (Nass et al., 2008).

1.7.3 Obesity

Due to the high constitutive activity of the GRLN-R a compound that acts as an efficient inverse agonist could be used as an anti-obesity agent, as a neutral antagonist may not be effective *in vivo* (Holst et al., 2003). However, a naturally occurring mutation of the GRLN-R, which results in the change of an alanine residue for a glutamic acid residue in ECL2 of the GRLN-R, has been associated with the development of an obese phenotype *in vivo* that develops during puberty (Pantel et al., 2006). This suggests that the constitutive activity of the GRLN-R is important *in vivo* and that other mechanisms may compensate for the loss of constitutive receptor activity leading to an obese phenotype. However, the obese phenotype could be due to the persistent lack of constitutive activity of the GRLN-R in a particular period in the development of the carrier (Holst and Schwartz, 2006). Interestingly, a vaccine against ghrelin has been developed and in adult male rats this has the effect of slowing weight gain by decreasing feed efficiency (Zorrilla et al., 2006). However the benefits were not passed on to humans, with phase I and II clinical trials showing ghrelin vaccination had no effect on weight loss (http://www.cytos.com/doc/Cytos_Press_E_061107.pdf); perhaps not that surprising when food intake in humans is not always driven by hunger but by comfort and 'boredom-eating'.

Future anti-obesity therapies may now focus on inhibiting the actions of GOAT, as inhibiting GOAT is predicted to decrease the amount of octanylated ghrelin in the body, leading to a reduction in the orixegenic effects of ghrelin *in vivo*.

1.8 GPCR structure

1.8.1 Introduction to the G protein-coupled receptor superfamily

The GRLN-R belongs to the G protein-coupled receptor (GPCR) family, the largest superfamily of cell surface receptors, encoded for by approximately 3-4 % of the human genome (Foord, 2002). The GPCR superfamily includes receptors for diverse types of endogenous ligands, such as amines, peptides, amino acids, glycoproteins, prostanoids, phospholipids, fatty acids, nucleosides, nucleotides and a variety of sensory stimuli such as odorants, pheromones and photons of light (Kristiansen, 2004). As their name suggests, GPCRs transduce an extracellular signal into a cellular response via activation of guanine nucleotide binding proteins (G proteins) (although there is also evidence that GPCRs may signal through G protein-independent pathways, see section 1.10.3).

GPCRs are an important pharmacological target as mutations within GPCR genes have been linked to the development of many diseases such as: retinitis pigmentosa, hypo- and hyper-thyroidism, nephrogenic *diabetes insipidus* and even carcinomas (Schoneberg et al 2004). Indeed of the clinically marketed drugs around 30 % are targeted against GPCRs, however, out of the 800 or so receptors that make up the GPCR superfamily these drugs only act on approximately 30 receptors (Wise et al., 2004) leaving enormous potential for this family in future drug discovery programmes.

1.8.2 GPCR classification

Members of the GPCR family can be split into three major classes (classes A-C) and three smaller classes (D-F), with receptors within a class showing greater than 20 % sequence homology within the TM domains (Kristiansen, 2004). Apart from the 7TM architecture there are no obvious similarities between classes.

1.8.2.1 Class A

The GRLN-R belongs to the class A or rhodopsin-like receptors. This is the largest and most studied class of GPCRs, consisting of around 670 human receptors (Gloriam et al., 2007). Within the class A GPCRs the GRLN-R belongs to small subset of receptors for peptide hormones and neuropeptides, which include the motilin receptor (GPR38), the putative obestatin receptor (GPR39), neurotensin receptors 1 and 2 and the neuromedin receptors 1 and 2 (Camina, 2006). The GRLN-R shows the highest sequence homology

with GPR38, showing 52 % overall homology or 87 % sequence homology within the TM domains (Peeters, 2005). Unlike the GRLN-R, GPR38 does not exhibit any constitutive activity.

Class A GPCRs contain highly conserved residues, mainly within their TM domains. Thus to facilitate comparison of residues across class A GPCRs, Ballesteros and Weinstein devised a numbering system that assigns two numbers to each residue within the TM domains. The first number refers to the TM domain the residue is found in i.e. 1 for TMI. The second number is relative to the most conserved residue in the helix, which is assigned the number 50; with the numbers decreasing towards the N-terminus and increasing towards the C-terminus (Ballesteros & Weinstein, 1995). The most highly conserved residues in each TM domains are: an asparagine ($N^{1.50}$) residue in TMI; an aspartic acid ($D^{2.50}$) in TMII; an arginine ($R^{3.50}$) residue in TMIII; a tryptophan ($W^{4.50}$) in TMIV and proline residues in TMV, TMVI and TMVII ($P^{5.50}$, $P^{6.50}$ and $P^{7.50}$). Where appropriate, Ballesteros-Weinstein numbering will be used throughout this thesis to allow comparison of residues in the GRLN-R with other class A GPCRs.

1.8.2.2 Class B

Class B GPCRs consist of the secretin and adhesion family of receptors – including receptors such as the GHRH receptor. The receptors are characterised by a long N-terminus which (in almost all cases) contain conserved cysteine residues that form a network of three disulphide bonds – creating a globular domain involved in ligand binding (Bazarsuren et al., 2002).

1.8.2.3 Class C

Class C GPCRs (also known as the glutamate family) consist of 22 receptors including those for the neurotransmitters glutamate (the eight metabotropic glutamate receptors) and γ-aminobutyric acid (GABA – the $GABA_B$ receptors). Members of this class have a large N-terminus region comprising of the 'Venus Fly-trap' domain. The domain comprises two lobes with a cleft in between which forms the ligand-binding site. Binding of a ligand leads to closure of this domain, resulting in activation of the receptor. For the majority of class C receptors the Venus Fly-trap domain is connected to the 7 TM bundle by a cysteine-rich region (Pin et al., 2004).

1.8.2.4 Classes D, E and F

There are a few minor groups of GPCRs which have no apparent sequence identity with the other classes, these are the pheromone receptors (class D) and the frizzled/smoothened receptors (class E) found in yeast as well as the cAMP receptors (class F) found in *Dictyostelium discoideum* (Bockaert and Pin, 1999).

1.8.3 GPCR crystal structure

Until recently, the only high-resolution structural information for GPCRs was limited to that of the rhodopsin receptor (determined by Palczewski et al., 2000), a naturally abundant receptor that is highly specialised for the detection of light (Kobilka and Schertler, 2008). GPCRs for diffusible hormones and neurotransmitters have been difficult to crystallise due to their inherent structural flexibility (Kobilka and Schertler, 2008). However, in 2007 the second GPCR crystal structure was published, that of the human β_2-adrenergic receptor (β_2AR) (Rosenbaum et al., 2007; Cherezov et al., 2007; Rasmussen et al., 2007), a receptor more typical of the GPCR superclass in the sense that its ligands, adrenaline and noradrenaline, are diffusible and the receptor is expressed at relatively low levels in native tissue (Cherezov et al., 2007). To stabilise the receptor structure β_2AR crystals were generated in two ways; with a monoclonal antibody (Fab 5) raised against the flexible third intracellular loop (Rasmussen et al., 2007) or with the third intracellular loop replaced with the small soluble protein T4 lysozyme (Rosenbaum et al., 2007; Cherezov et al., 2007). The receptor was also further stabilised by the binding of a diffusible ligand (the partial inverse agonist carazolol), which had the effect of stabilising the receptor in one conformation, aiding receptor crystallisation (Cherezov et al., 2007).

2008 saw the publication of the crystal structure of the bovine opsin receptor in complex with the carboxy terminus of the G protein transducin (Park et al., 2008), which is expected to represent the active state of the rhodopsin receptor (the rhodopsin receptor consists of the protein opsin and the covalently bound ligand molecule called 11-cis retinal which prevents the protein from signalling; Schwartz and Hubbell, 2008). 2008 also saw the publication of the crystal structure of the turkey β_1-adrenergic receptor in complex with the antagonist cyanopindolol (Warne et al., 2008) and the adenosine A_{2A} receptor in complex with the high-affinity antagonist ZM241385 (with the third intracellular loop replaced with T4 lysozyme and the C-terminus tail deleted; Jaakola et al., 2008). All these structures have provided new information about the structural organisation of GPCRs and the

potential mechanisms involved in receptor activation and will be discussed with respect to the GRLN-R.

1.8.4 GRLN-R structure

1.8.4.1 TM domains

The GRLN-R, like all GPCRs, is predicted to comprise of 7 transmembrane (TM)-spanning α-helical domains, connected by three intracellular loops (ICL) and three extracellular loops (ECL). This results in the N-terminus and the C-terminus being on different sides of the plasma membrane, with the N-terminus being located on the extracellular side and the C-terminus being located on the intracellular side (Figure 1-1). The membrane orientation of the GRLN-R has been confirmed by Feighner and colleagues who demonstrated, using indirect immunofluorescence, that an antibody raised against a predicted intracellular peptide (from the C-terminus of the GRLN-R) only reacted upon permeabilisation of HEK293 or COS-7 cells transfected to express the human GRLN-R. In contrast an antibody raised against a predicted extracellular peptide (part of ECL2) reacted without prior cell permeabilisation (Feighner et al., 1998).

Although to date no crystal structure is available for the GRLN-R, it is expected that the TM helices of the GRLN-R will be slightly 'kinked' due to the presence of glycine and proline residues and tilted from the plane of the membrane; a prediction based on the findings from the crystal structures of the rhodopsin receptor, the β_2AR, the β_1AR and the adenosine A_{2A} receptor (Palczewski et al.,2000; Cherezov et al., 2007; Rosenbaum et al., 2007; Rasmussen et al., 2007; Warne et al., 2008; Jaakola et al., 2008). These kinks are thought to enable the structural rearrangements required for G protein activation (Yohannan et al., 2004).

The GRLN-R contains the highly conserved E/DRY motif at the intracellular end of TMIII (conserved among 72 % of class A GPCRs; Kobilka and Deupi, 2007). The E/DRY sequence may be critical for G protein binding. For example, mutating $R136^{3.50}$ in the motilin receptor, GPR38, to either an alanine or a histidine residue inhibits the signalling of the receptor to motilin (as measured through calcium mobilisation) without affecting the affinity of motilin for the receptor (Tokunaga et al., 2007).

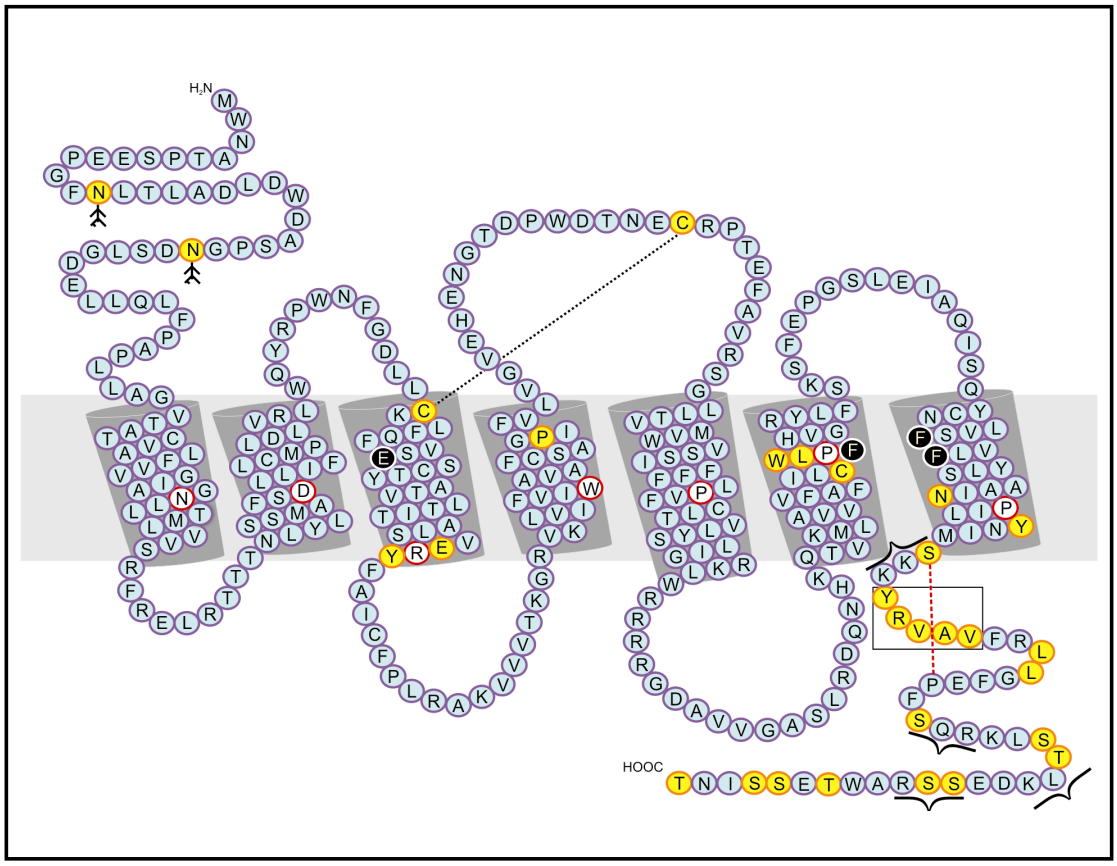

Figure 1-2 – Snake plot diagram of the GRLN-R. Residues that are conserved throughout class A GPCRs and used for Ballesteros-Weinstein numbering are shown outlined in red (N in TM1; D in TMII; R in TMIII and P in TM V-VII). Also shown (in yellow) are residues that are typically conserved throughout class A GPCRs. These include two asparagine residues in the N-terminus (occurring as part of the N-X-S/T motif) that are predicited to undergo *N*-linked glycosylation; two cysteine residues (top of TMIII and in ECL2) that are predicted to form a di-sulphide bond; E/DRY and NPXXY motifs in TMIII and TMVII, respectively, that are thought to be important in stabilising the inactive state of receptors and a CWLP fingerprint motif (TMVI) that is thought to act as a rotomer toggle switch important for receptor activation. Helix VIII is predicted to form between the serine residue (S327) and a proline residue (P342) in the C-terminus of the GRLN-R (marked by a red dotted line). Also shown are several serine and threonine residues within the C-terminus tail that are predicted to undergo phosphorylation by second messenger kinases and G protein receptor kinases. PKC phosphorylation sites (S/T-X-R/K) are marked with a bracket. A di-leucine motif (LL) and a tyrosine-based motif (YRVAV; boxed) are thought to be important in binding the adaptor protein AP-2, leading to internalisation of the receptor. Lastly, residues shown in black (a glutamic acid residue in TMIII and phenylalanine residues in TM VI-VII) are thought to be important in receptor activation (the glutamic acid residue forms a salt-bridge with the positively charged amine group of ghrelin and the growth hormone secretagogues; whilst the phenylalanine residues are thought to be important in maintaining the constitutive activity of the GRLN-R).

In the rhodopsin receptor an interaction between a glutamic acid residue 134 ($E^{3.49}$) and an arginine residue ($R^{3.50}$) in the E/DRY sequence with a glutamic acid residue ($E^{6.30}$) and a threonine residue ($T^{6.34}$) at the cytoplasmic end of TMVI, is predicted to stabilise the inactive state of the receptor by allowing the formation of an 'ionic lock'. Breakage of the ionic lock is thought to occur upon receptor activation. In support of this constitutively active mutants (CAMs) can be generated by the neutralisation of aspartic acid/glutamic acid charges at the cytoplasmic end of TMVI. This has been demonstrated for a number of receptors e.g. β_2AR (Rasmussen et al., 1999); rhodopsin (Cohen et al., 1993) and the muscarinic M_1 receptor (Lu et al., 1997). In the β_2AR the arginine residue in the E/DRY sequence ($R131^{3.50}$) is not close enough to $E268^{6.30}$ in TMVI to form a hydrogen bond, thus the ionic lock is not formed. It has been suggested that the lack of an ionic lock may be due to the receptor being partially constitutively active or signalling through interaction with scaffold proteins (Lefkowitz et al., 2008). However the salt bridge is lacking in the structure of the β_1AR and the A_{2A} receptor, receptors that lack any measurable constitutive activity, thus suggesting that the ionic lock is not an essential feature of maintaining class A GPCRs in their inactive state (Warne et al., 2008; Jaakola et al., 2008).

The GRLN-R contains a highly conserved class A motif, the NPXXY motif, in TMVII which is thought to be important in stabilising the inactive state of receptors by allowing TMVII to interact with TMVI. The GRLN-R also contains a CWLP fingerprint motif, which may play an important role in receptor activation acting as a rotomer toggle switch. The CWLP motif and residues that are involved in the maintenance of the constitutive activity of the GRLN-R are discussed in section 1.8.5.

1.8.4.2 Extracellular regions

As with the vast majority of class A receptors, the GRLN-R contains two cysteine residues that are predicted to form a disulphide bond. One cysteine residue is located at the top of TMIII ($C116^{3.25}$) whilst the other cysteine residue is located in ECL2 (C198). This disulphide bond appears to be important for correct protein folding of the GRLN-R as Feighner and colleagues have shown mutation of $C116^{3.25}$ to A116 disrupted cell surface trafficking of the GRLN-R (Feighner et al., 1998).

Virtually all GPCRs, from all classes, possess at least one *N*-linked glycosylation site (an oligosaccharide linked to the nitrogen in the side chain amide of an asparagine residue) in their extracellular regions. *N*-linked glycosylation occurs at the consensus sequence: N-X-S/T, where X can be any amino acid except proline or asparagine (Kristiansen, 2004).

Mutagenesis of the consensus sequence shows that glycosylation is vital for the cell-surface expression of some receptors e.g. the angiotensin 1 receptor (Lanctot et al., 2006) but has no significant influence on the distribution of others e.g. the muscarinic M2 receptor (van Koppen and Nathanson, 1990). There are two predicted *N*-linked glycosylation sites in the GRLN-R located in the N-terminus of the receptor at N13 and N27. Deletion of amino acids 1-35 in the N-terminus of the GRLN-R has no effect on the cell surface expression or the ligand-dependent and independent signalling of the receptor (Holst et al., 2009) thus it appears glycosylation has no effect on the cell surface expression of the GRLN-R.

The crystal structure of the β_2AR demonstrated that the ECL2 contains a helix that permits extracellular ligands access to the ligand-binding site (Cherezov et al., 2007), which is in contrast to the rhodopsin receptor that displays a buried β-sheet structure in this region (Palczewski et al., 2000) which effectively shields the binding site from the extracellular compartment (Lefkowitz et al., 2008). Basing the folding of the TM segments of the GRLN-R on the structure of the rhodopsin receptor and modelling the extra-membrane regions to different templates to favour local homologies, Pedretti and colleagues predict that, in the inactive state, ECL2 of the GRLN-R partially penetrates the TM domains forming the upper part of the polar ligand-binding pocket. However, in the closed state the receptor ECL2 forms a more folded conformation, which results in ECL2 moving away from the TM domains (Pedretti et al., 2007).

1.8.4.3 Intracellular regions

The crystal structures of the β_1AR and the A_{2A} receptor reveal that ICL2 forms a small helix which disrupts the formation of the ionic lock between the E/DRY sequence at the base of TMIII with residues in TMVI (Warne et al., 2008; Jaakola et al., 2008). However, the crystal structure of the constitutively active β_2AR reveals that ICL2 does not form a helix (Rosenbaum et al., 2007; Cherezov et al., 2007; Rasmussen et al., 2007). This suggests that ICL2 may act as a control switch, facilitating G protein interactions (Jaakola et al., 2008). The conformation of ICL2 in the GRLN-R has not yet been established.

In addition to the 7 TM spanning domains, crystal tructures of the rhodopsin receptor, the β_2AR and the β_1AR demonstrated the presence of a small helix, helix VIII, perpendicular to the membrane at the intracellular end of TMVII. Helix VIII is believed to be common to all class A GPCRs (Katragadda et al., 2004). In the rhodopsin receptor palmitoylation (the attachment of a palmitic acid via a thioester bond) of cysteine residues 322 and 323

anchored the helix to the membrane - effectively creating a fourth intracellular loop (Palczewski et al., 2000). Using the structure of the rhodopsin receptor as a template, Pedretti and Vistoli modelled the GRLN-R and predicted that helix VIII was formed from S327 to P342 and was located perpendicular to helix VII (Pedretti and Vistoli, 2007). Predicted helix VIII is rich in positively charged residues (K328; R329; R331; R336), which may anchor the helix to phospholipidic heads (Sakmar et al., 2002) making the C-terminal tail of the GRLN-R (after helix VIII) shorter than the average GPCR, consisting of less than 30 amino acids (Holliday et al., 2007). Interestingly putative Helix VIII in the GRLN-R has also been described to contain the proposed nuclear localisation sequence KKYR (Lee et al., 2004).

In addition, in the C-terminal tail of the GRLN-R multiple residues are found that are thought to be important in the termination of receptor signalling, including several serine and threonine residues, a di-leucine motif (LL) and four potential PKC phosphorylation sites (S/T-X-R/K) (see section 1.10.4 for further details).

1.8.5 Residues contributing to the constitutive activity of the GRLN- R

Previous studies have indicated that the activation of GPCRs involves an inward movement of TMVI and VII towards TMIII in the main ligand-binding pocket at the extracellular sides of these helices (Elling et al., 1999; Ghanouni et al., 2001). This led to the development of the global toggle-switch model for the activation of GPCRs (Elling et al., 2006) where TMVI (and to some degree TMVII), is predicted to perform a vertical see-saw movement around a pivot point located close to the middle of the membrane (where the conserved proline residue is located; Schwartz et al., 2006). Pivoting of TMVI around $P^{6.50}$ results in the extracellular side of TMVI tilting into the main TM bundle, whilst the intracellular end of the helix tilts away from the main TM bundle. In support of the global toggle-switch for receptor activation, the crystal structure of bovine opsin receptor shows that, compared to the inactive rhodopsin receptor structure, TMVI shows a large tilt, accompanied by a smaller motion of TMVII at the intracellular side of the receptor (Park et al., 2008; Schwartz and Hubbell, 2008).

Several micro-switches (residues highly conserved during evolution) that switch between substantially different conformations in the inactive versus active receptor states are believed to be integral components of the global toggle-switch model for GPCR activation (Nygaard et al., 2009). One of these micro-switches is $W^{6.48}$ in the conserved CWLP motif.

The GRLN-R contains the conserved CWLP fingerprint motif in TMVI. In the rhodopsin receptor inactive structure the indole side-chain of W265$^{6.48}$ is situated vertically at the interface between TMIII and TMVI, stabilised by an interaction with a structural water molecule, preventing the movement of the outer segment of TMVI (Shi et al., 2002; Schwartz et al., 2006). It has been suggested that W$^{6.48}$ functions as a rotomer toggle switch as upon receptor activation, the rotomeric state of the indole side-chain alters, allowing the indole side-chin of W$^{6.48}$ to interact with F$^{5.47}$ in TMV (Nygaard et al., 2009). This moves the side-chain away from the interface between TMIII and TMVI straightening the proline kink of TMVI (Schwartz and Rosenkilde, 1996; Schwartz et al., 2006).

In the GRLN-R the ligand-independent signalling of the receptor appears to be controlled by a cluster of aromatic amino acid residues located at the interface between TMIII, TMVI and TMVII. Using a systematic mutagenesis approach Holst and colleagues demonstrated that aromatic-aromatic interactions between residues F279$^{6.51}$ F309$^{7.39}$ and F312$^{7.42}$ could act as a tethered agonist, stabilising the receptor in the active conformation in the absence of agonist by stabilising the active, inward-bend conformation of TMVI obtaining constitutive activation of the rotamer toggle switch (Holst et al., 2004; Schwartz et al., 2006). The aromatic-aromatic interactions appeared to be important for maintaining the constitutive activity of the GRLN-R as constitutive activity of the GRLN-R could only be observed if the residue at position 312$^{7.42}$ was a phenylalanine residue or a tyrosine residue. By changing the size and hydrophobicity/aromaticity of the side chain of F279$^{6.51}$ in the presence of large hydrophobic residues at F309$^{7.39}$ and F312$^{7.42}$, the constitutive activity of the receptor could be ‘tuned’-up or down (Holst et al., 2004). Thus it has been suggested that these large aromatic side chains act as tethered agonists to obtain constitutive activation of the toggle switch (Holst et al., 2004; Schwartz et al., 2006).

Interestingly two naturally occurring missense mutations of the GRLN-R have been identified which appear to ablate the constitutive activity of the receptor. These mutations are A204 to E204 in ECL2 and F279$^{6.51}$ to L279$^{6.51}$ in TMVI (Wang et al., 2004). The F279L mutation is within the cluster of amino acids shown to be important for the constitutive activity of the GRLN-R. However, the A204E mutation is distinct from residues shown to be important for maintaining the constitutive activity of the GRLN-R, so the mechanism of how mutation of this residue affects the constitutive activity of the GRLN-R is unknown. Interestingly for the free fatty acid receptor 1 (FFA1) two glutamic acid residues in ECL2 are thought to form interactions with two arginine residues located in the TM domains (R183$^{5.39}$ and R258$^{7.35}$) to create two ionic locks. These ionic locks are broken upon agonist binding, whilst mutation of the glutamic acid residues to alanine

residues results in constitutive receptor activation (Sum et al., 2009). Thus it is conceivable that the alanine residue in ECL2 of the GRLN-R is required for constitutive activation of the receptor by disrupting the formation of an ionic lock between ECL2 and a residue in the TM domains.

1.8.6 Ligand-binding pocket of the GRLN-R

Interestingly comparison of the cDNA of the human GRLN-R to the teleost puffer fish GRLN-R reveals that there is 58 % homology between these two receptors. Furthermore the growth hormone secretagogues MK-677, GHRP-6 and L-163,540 were all shown to be capable of activating the puffer fish GRLN-R indicating that the ligand-binding pocket of the GRLN-R has been conserved for at least 400 million years (Smith et al., 1999).

Studies into ghrelin and the growth hormone secretagogues have shown that the presence of a basic amino acid group is important for their bioactivity (Momany et al., 1984; Smith et al., 1993; McDowell et al., 1995; Patchett et al., 1995). Mutagenesis studies have shown that a glutamic acid residue in TMIII ($E124^{3.33}$) of the GRLN-R appears to be a key residue for the binding of these ligands (Feighner et al., 1998) and is expected to be similarly disposed (but not identical) to $D113^{3.32}$ in the β_2AR which forms a salt bridge with the positively charged amine group of the catecholamines. Indeed, mutating $E124^{3.33}$ to $Q124^{3.33}$ (which alters the charge at this position) ablates GRLN-R signalling to ghrelin, MK-677, GHRP-6 and L-692,585. Restoration of function of the $E124Q^{3.33}$ mutant can be achieved by a complementary change in the MK-677 ligand through modification of its amine side-chain into the corresponding alcohol (Feighner et al., 1998). Mutating $E124^{3.33}$ to $D124^{3.33}$ (which conserves the charge at this position) has been demonstrated to be non-disruptive to ligand binding and receptor activation (Feighner et al., 1998). Thus $E124^{3.33}$ in the GRLN-R acts as a key charge-charge interaction point, stabilising the positive charge of the basic amino group in ghrelin and the growth hormone secretagogues. Interestingly this residue is also conserved in GPR38 ($E119^{3.33}$) where it has been shown to be a key charge-charge interaction for small molecule agonists of the receptor, such as erythromycin (Xu et al., 2005).

Besides $E124^{3.33}$, ghrelin and the growth hormone secretagogues GHRP-6, MK-677, L-692,429, SM-130686 and SM-157740 also appear to interact with residues in TMVI ($F279^{6.51}$, $R283^{6.55}$ and $F286^{6.58}$) and TMVII (Holst et al., 2009), thus the main ligand-binding pocket of the GRLN-R appears to be confined to the interface between the extracellular ends of TMIII, TMVI and TMVII (Holst et al., 2006).

Ghrelin also appears to make contact with residues in the ECLs as substitution of Q302 to a A302 in ECL3 and E197 to Q197 in ECL2 had a small effect on the potency of ghrelin (causing around a 5-6 fold decrease in the potency of ghrelin) (Holst et al., 2009); this fits well with the concept that larger, peptide ligands tend to exploit mainly residues at the most extracellular end of the helices and the extracellular domains (Schwartz and Holst, 2006).

The growth hormone secretagogues share overlapping, but not identical binding sites with ghrelin on the GRLN-R, with the differences mainly in the residues with which they interact with in TMVII. Here MK-677 and L-692,429 were shown to be affected by substitutions at N305$^{7.35}$ and F309$^{7.39}$, whilst SM-130686 and SM-157740 were affected by substitutions of (the more deeply located) F312$^{7.42}$ (Holst et al., 2009). In TMIV substitution of I178$^{4.60}$ affected the growth hormone secretagogues but to different extents, having a large impact for GHRP-6 (130-fold decrease in potency) but not so much on the response to MK-677 (5.3-fold decrease in potency). The same could be seen for mutation of D99$^{2.60}$ in TMII where the potency of MK-677 was decreased by greater than 1300-fold, whereas the potency of L-692,585 was actually increased by 6-fold (Holst et al., 2009).

Interestingly two naturally occurring missense mutations of the GRLN-R have been identified that appear to affect the signalling of the receptor, either ablating the response to ghrelin (I134$^{3.43}$ to T134$^{3.43}$) or by increasing the potency of ghrelin (V160$^{4.42}$ to M160$^{4.42}$) (Liu et al., 2007). The effect of these mutations on the signalling of the growth hormone secretagogues has not been studied to date.

1.8.7 Ligand-binding pocket for SPA

Like ghrelin and the growth hormone secretagogues, the inverse agonist SPA is positively charged but mutation of the glutamic acid residue E124$^{3.33}$ (E124Q) does not affect signalling of SPA as much as mutation of D99$^{2.60}$ to an N99$^{2.60}$. Indeed mutation of E124$^{3.33}$ only causes around a 14-fold decrease in SPA potency whereas mutation of D99$^{2.60}$ causes a 47-fold change in potency (Holst et al., 2006). Thus it appears that the SPA binding pocket extends towards TMII and that the negatively charged D99$^{2.60}$ makes a charge-charge interaction with the positively charged amino group of SPA (Holst et al., 2006). Indeed the SPA binding pocket also appears to extend towards TMVI with mutation of I178$^{4.60}$ to A178$^{4.60}$ causing a 22-fold decrease in the potency of SPA.

It is predicted that the central aromatic tripeptide of SPA (WFW) interacts with the aromatic cluster in TMVI and VII of the GRLN-R thus preventing the inward movement of TMVI and TMVII (Holst et al., 2006), thereby preventing the constitutive activation of the receptor.

1.9 Dimerisation

1.9.1 Introduction to dimerisation

Although GPCRs are often thought of as acting as monomers there is a wealth of evidence to suggest that many GPCRs can form dimers, or higher-order oligomers, that may be important for their function (Milligan et al., 2006). Dimerisation may occur between identical receptors (homo-dimerisation) or between non-identical receptors (hetero-dimerisation) and has been established in class A (e.g. the chemokine receptors CXCR1 and CXCR2 receptors; Wilson et al., 2005), class B (e.g. the secretin receptor; Harikumar et al., 2008) and class C GPCRs (e.g. the metabotropic glutamate receptors; Kniazeff et al., 2004).

Dimerisation of GPCRs in the endoplasmic reticulum (ER) has been shown to be required for the maturation and cell-surface expression of many GPCRs (Bulenger et al., 2005). A classical example of this is the $GABA_B$ receptor. The $GABA_B$ receptor was cloned in 1997, however, expression of the receptor in a heterologous system resulted in a discrepancy between the binding affinities of agonists for the receptor compared to those measured from the endogenous $GABA_B$ receptor expressed in the brain (Kaupmann et al., 1997). Within a year after the cloning of the $GABA_B$ receptor, three groups simultaneously published that the functional receptor is actually composed of a hetero-dimer between two receptor subunits: $GABA_{B1}$ and $GABA_{B2}$ (White et al., 1998; Kaupmann et al., 1998; Jones et al., 1998). The endogenous ligand GABA binds to $GABA_{B1}$ with the $GABA_{B2}$ subunit coupling to a G protein and acting as a chaperone. The $GABA_{B1}$ subunit contains an ER-retention motif within the C-terminal tail, the interaction of $GABA_{B1}$ with the C-terminal tail of $GABA_{B2}$ masks the ER-retention motif allowing the hetero-dimer to be exported from the ER and trafficked to the cell surface (Pagano et al., 2001; Margeta-Mitrovic et al., 2000).

As well as affecting receptor trafficking, dimerisation has also been shown to modify receptor signalling. For example, the orexin-1 antagonist SB-674042, which has no affinity for the cannabinoid receptor CB_1, was shown to decrease the potency of a CB_1 agonist (WIN 55,212-2) when the cannabinoid receptor CB_1 and the orexin receptor orexin-1 were co-expressed in HEK293 cells. This effect was dependent on the receptors forming a hetero-dimer and demonstrates that the formation of dimers allows a ligand at one receptor to modulate the function of a receptor for which it has no significant and inherent affinity (Ellis et al., 2006).

1.9.2 Dimerisation of the GRLN-R

Akin to the $GABA_B$ receptor, dimerisation of the GRLN-R has been suggested to be important for the correct trafficking of the receptor to the plasma membrane. The GRLN-R has been demonstrated to form a homo-dimer in two independent studies (Jiang et al., 2006; Leung et al., 2007). As the GRLN-R contains a potential nuclear localisation signal (KKYR) in putative helix VIII (Lee et al., 2004) the GRLN-R may need to exist as a dimer to mask the nuclear retention signal, to allow trafficking of the receptor to the plasma membrane (Leung et al., 2007).

As well as forming homo-dimers the GRLN-R has also been reported to form hetero-dimers; both with the GHs-R1b (Leung et al., 2007) and with the dopamine D1 receptor (Jiang et al., 2006). It is thought that hetero-dimerisation of the GRLN-R with GHs-R1b reduces the cell surface expression, and hence constitutive activity, of the GRLN-R by translocating the receptor to the nucleus (acting as a dominant negative mutant of the GRLN-R), although there needs to be a 13-fold excess of GHs-R1b to the GRLN-R for this to occur (Leung et al., 2007). Thus the formation of a GRLN-R/GHs-R1b hetero-dimer could fail to mask the nuclear localisation signal at the C-terminus of the GRLN-R causing nuclear retention of the dimer (Leung et al., 2007). Indeed mRNA for both the GRLN-R and the GHs-R1b have been described to co-exist in the same tissues (Gnanapavan et al., 2002) and in the same cells, for example, in a cell-line prepared from pancreatic adenocarcinoma cells, thus it is possible that GRLN-R/GHs-R1b hetero-dimers exist *in vivo* (Duxbury et al., 2003).

When the GRLN-R and the dopamine receptor D1R are co-expressed in the same cells, the addition of ghrelin appears to amplify dopamine signalling (Jiang et al., 2006). This mechanism involves dimerisation of the GRLN-R with the D1R (which has been demonstrated to occur in co-immunoprecipitation and saturation bioluminescence resonance energy transfer experiments). As the GRLN-R and D1R are co-expressed within the same neurones *in vivo* (and have also been shown to co-localise in a SK-N-SH neuronal cell line which endogenously expresses both receptors) (Jiang et al., 2006) it suggests that ghrelin can amplify the signalling of the D1R *in vivo*.

1.10 GPCR signalling

This section will focus on the G protein-dependent and independent signalling of the GRLN-R. Following this, the mechanisms that can modulate GRLN-R signalling (desensitisation/endocytosis; constitutive activation and allosteric modulation) will be discussed.

1.10.1 Introduction to G proteins

Ligand-induced activation of the GRLN-R causes a profound change in the TM helices which affects the conformation of ICLs and uncovers masked G protein binding sites allowing interaction with heterotrimeric G proteins (Camina, 2006). Heterotrimeric G proteins consist of three subunits α, β and γ and act to couple changes in receptor conformation into cellular responses within the cell via activation of second messenger pathways.

In its inactive state, a G protein has a molecule of guanosine 5' diphosphate (GDP) bound. Receptor activation (via ligand binding or constitutive activity) results in a conformational change that allows the receptor to associate more effectively with G proteins. The receptor acts as a guanine nucleotide exchange factor by promoting the release of the molecule of GDP from the G protein, which is exchanged for a molecule of guanosine 5' triphosphate (GTP). As GTP is present at a higher concentration within the cell than GDP this leads to fast binding of GTP. Binding of GTP leads to a conformational change causing the dissociation of the heterotrimer into two signalling subunits: an α subunit and a βγ complex. These subunits can then interact with their effectors to cause amplification in signal via second messenger pathways. The α subunit has intrinsic GTPase activity and so hydrolyses the terminal phosphate of GTP to form a molecule of GDP (+ P_i), which returns the α subunit into an inactive state and promotes the reassociation with the βγ complex – ceasing signalling (Figure 1-3).

The GTPase activity of the α subunit can be modified by the binding of proteins known as G protein Activity modifying Proteins (GAPs) including the Regulators of G protein Signalling (RGS) family of proteins. The RGS proteins reduce the signal transmitted by the α subunit by increasing the rate of GTP hydrolysis, returning G protein to the GDP-bound state in a shorter time scale (De et al., 2000).

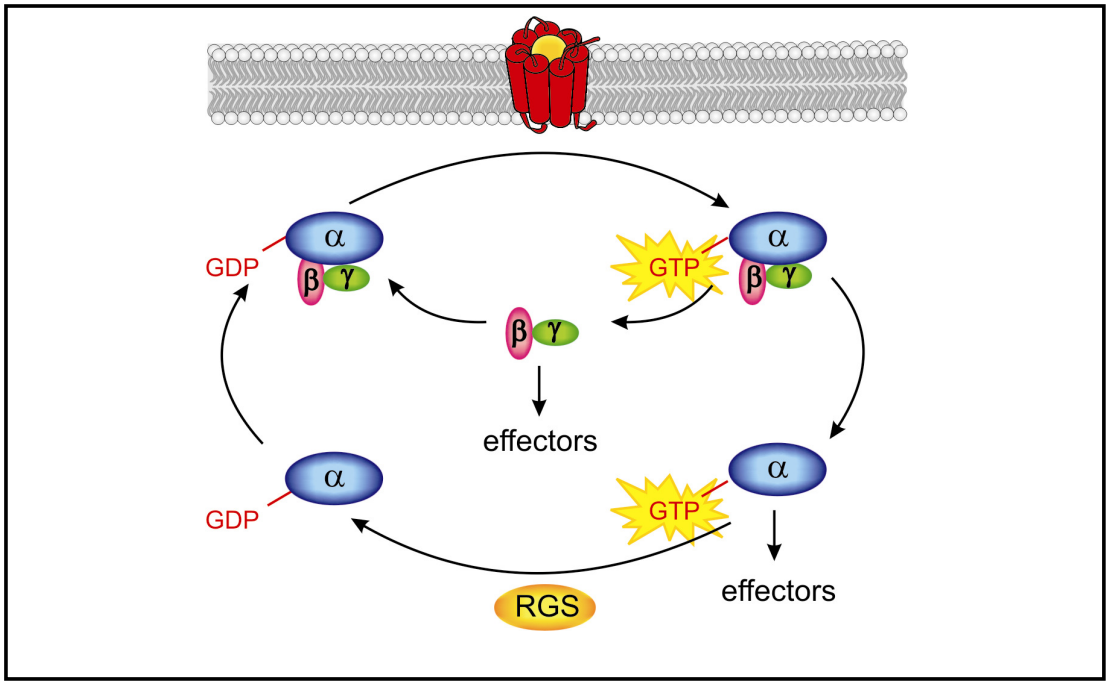

Figure 1-3 – The G protein activation cycle (adapted from Milligan and Kostenis, 2006). In the inactive state the α subunit of the G protein has a molecule of GDP bound and is able to associate with the βγ complex. Upon activation the GPCR undergoes a conformational change that results in an increase in affinity for the G protein. As a result the GDP molecule is exchanged for a GTP molecule on the α subunit. This reduces the affinity of the α subunit for the βγ complex, leading to dissociation into two signalling complexes (the α and the βγ complexes) which can interact with their effectors, transducing the external signal into an intracellular response. Signalling is halted by the hydrolysis of GTP to GDP (+ P_i) by the intrinsic activity of the α subunit. RGS proteins can increase the rate of GTP hydrolysis, returning the G protein to the GDP bound state in a shoter time scale.

Interestingly several studies have suggested that the G protein may not always dissociate into α and βγ complexes upon G protein activation; instead there may be a rearrangement in the conformation of these subunits (Bunemann et al., 2003; Frank et al., 2005). However this may be specific for the $G_{\alpha q/11}$ (Galés et al., 2005) and $G_{\alpha i/o}$ family (Bunemann et al., 2003) (see below) as activation of another G protein family ($G_{\alpha s}$) has been shown to be consistent with the classical model of G protein subunit dissociation (Janetopoulos et al., 2001).

1.10.2 G protein coupling of the GRLN-R

There are 21 G protein α subunits encoded for by 16 genes resulting in subunits that range in size from 39-52 kDa (Downes and Gautam, 1999). The α subunits can be classified, based on sequence homology, into four subfamilies: $G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q/11}$ and $G_{\alpha 12/13}$; with even the most diverse α subunits exhibiting 50 % sequence homology (Milligan and Kostenis, 2006).

1.10.2.1 $G_{\alpha q/11}$

The $G_{q/11}$ family consists of $G_{\alpha q}$, $G_{\alpha 11}$, $G_{\alpha 14}$, $G_{\alpha 15}$ and $G_{\alpha 16}$ with $G_{\alpha q/11}$ ubiquitously expressed and $G_{\alpha 15/16}$ expressed in hematopoietic cells (Milligan and Kostenis, 2006). This family activates phospholipase Cβ (PLCβ) isoforms which hydrolyse phosphatidyl inositol [4,5] bisphosphate (PIP_2) into 1,2 diacylglycerol (DAG) and inositol [1,4,5] trisphosphate (IP_3). IP_3 binds and activates IP_3-sensitive Ca^{2+} channels on the ER, releasing Ca^{2+} which causes an elevation in intracellular [Ca^{2+}] ([$Ca^{2+}]_i$), whilst DAG activates the downstream enzyme protein kinase C (PKC) (Figure 1-4). Activation of the $G_{\alpha q/11}$ G protein family can be specifically blocked by YM-254890 (isolated from *Chromobacterium* sp), which specifically blocks the exchange of GDP for GTP on the G protein α subunit (Takasaki et al., 2004).

Cloning of the GRLN-R revealed that the GRLN-R was coupled to the $G_{\alpha q/11}$ pathway; more specifically, the GRLN-R was found to couple to $G_{\alpha 11}$ but not $G_{\alpha q}$ (Howard et al., 1996). Since then, coupling of the GRLN-R to the $G_{\alpha q/11}$ pathway has been confirmed in numerous other studies, which have used calcium mobilisation and inositol phosphate assays to measure activation of the GRLN-R, both upon treatment with ghrelin and the growth hormone secretagogues (McKee et al., 1997; Kojima et al., 1999; Holst et al., 2004; Holliday et al., 2007; Holst et al., 2009).

Although the GRLN-R is expected to couple principally to $G_{\alpha q/11}$, there is also evidence that the GRLN-R is able to couple to, and activate, multiple G protein pathways, i.e. the receptor appears to be promiscuous. Promiscuity has been demonstrated for a number of other GPCRs, for example: the β_2AR activates both $G_{\alpha s}$ and $G_{\alpha i}$ (Kilts et al., 2000); the metabotropic glutamate 1a receptor can activate $G_{\alpha i}$ and $G_{\alpha q/11}$ (Hermans et al., 2000) and the muscarinic 1 receptor is preferentially coupled to $G_{\alpha q/11}$ but can also activate $G_{\alpha i/o}$ (Offermanns et al., 1994). Indeed, it appears that receptors may display multiple active conformations as situations in which a ligand acting at a promiscuous receptor can preferentially activate one G protein pathway over another have been described, a property termed 'functional selectivity' (Urban et al., 2007). This has been demonstrated for the serotonin receptor 5-HT_{2C} where the ligand DOI was shown to preferentially activate $G_{\alpha s}$ over $G_{\alpha q/11}$ (Berg et al., 1998).

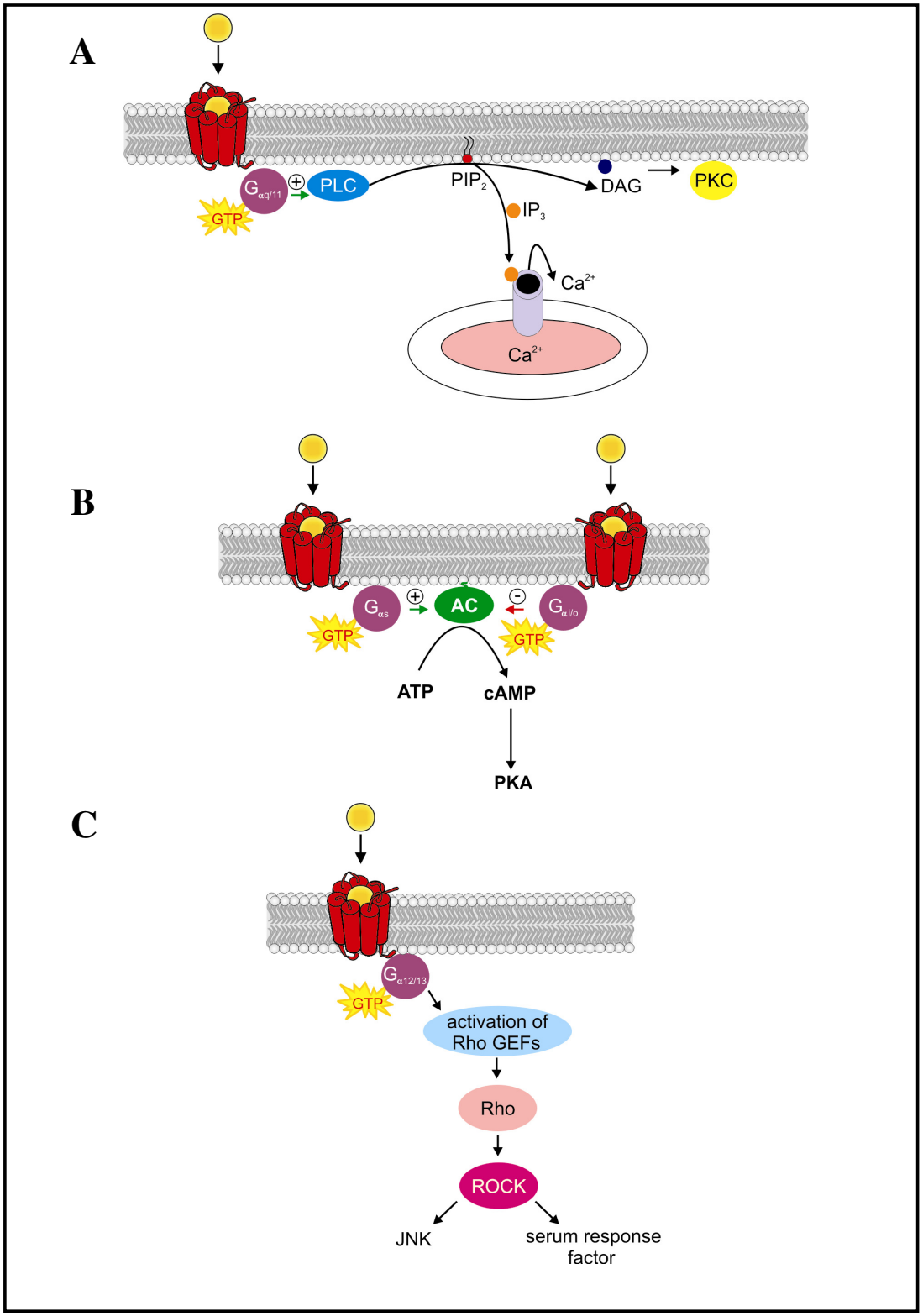

Figure 1-4 – Signalling pathways of the GRLN-R. A. Signalling through the $G_{\alpha q/11}$ pathway. Activation of the $G_{\alpha q/11}$ pathway activates PLCβ, which hydrolyses PIP_2 into IP_3 and DAG. DAG activates the downstream kinase PKC whilst IP_3 can activate IP_3 sensitive channels on the ER causing an increase in $[Ca^{2+}]_i$. **B.** Signalling through $G_{\alpha s}$ and $G_{\alpha i/o}$ pathways. Activation of the $G_{\alpha s}$ pathway stimulates adenylate cyclase, which generates cAMP from ATP. cAMP activates the downstream kinase protein kinase A (PKA). The $G_{\alpha i/o}$ pathway acts to inhibit adenylate cyclase activation. **C.** In the $G_{\alpha 12/13}$ pathway, G protein activation causes the activation of Rho guanine exchange factors (Rho GEFs), which are linked to the activation of Rho. Rho activates rho-dependent kinase (ROCK), which stimulates c-Jun N-terminal kinase (JNK) and the formation of stress fibres and in part, through these events, activates serum response factor transcription. Activation of $G_{\alpha s}$; $G_{\alpha i/o}$ and $G_{\alpha 12/13}$ remains little studied for the GRLN-R.

1.10.2.2 $G_{\alpha s}$

The $G_{\alpha s}$ family is named due to their ability to stimulate adenylate cyclase. The family consists of $G_{\alpha s(S)}$; $G_{\alpha s(L)}$; $G_{\alpha s(XL)}$ and $G_{\alpha olf}$ – with $G_{\alpha s}$ being ubiquitously expressed and $G_{\alpha olf}$ expression limited to olfactory neurones, certain central nervous system ganglia, digestive tract and urogenital tract (Milligan and Kostenis, 2006). The $G_{\alpha s}$ family acts to stimulate adenylate cyclase activity, which converts ATP to cAMP. The rise in cAMP in the cytosol activates protein kinase A (PKA) (Figure 1-4).

In vivo studies of the GRLN-R have suggested that the receptor may be able to couple to the $G_{\alpha s}$ G protein family. In rat NPY-containing neurones, ghrelin-induced calcium mobilisation has been proposed to occur through activation of the $G_{\alpha s}$ pathway as a protein kinase A inhibitor (H89) reduced the ghrelin-induced calcium response (Kohno et al., 2003). In ovine somatotrophs, growth hormone secretion in response to treatment with GHRP-6 and GHRP-2 has been suggested to be mediated through activation of the $G_{\alpha s}$ pathway (Wu et al., 1997). The ability of the human GRLN-R to couple to $G_{\alpha s}$ in recombinant systems remains controversial, with some studies suggesting the GRLN-R can couple to $G_{\alpha s}$ (Rossi et al., 2008; Caminos et al., 2005) whilst others suggest that it cannot (Cunha and Mayo, 2002; Casanueva et al., 2008).

1.10.2.3 $G_{\alpha i/o}$

The $G_{\alpha i/o}$ family is named due to their ability to inhibit adenylate cyclase activation (Figure 1-4) and consists of $G_{\alpha o1}$, $G_{\alpha o2}$, $G_{\alpha i1\text{-}i3}$, $G_{\alpha z}$, $G_{\alpha t1/2}$ and $G_{\alpha gust}$ subtypes. $G_{\alpha o1\text{-}2}$ is expressed in neurones, neuroendocrine cells, astroglia and the heart. $G_{\alpha i1\text{-}i3}$ is expressed in neurones, $G_{\alpha z}$ in platelets, neurones, adrenal chromaffin cells and neurosecretory cells, $G_{\alpha t1}$ in rod outer segments and taste buds, $G_{\alpha t2}$ in cone outer segments and $G_{\alpha gust}$ in the sweet and bitter taste buds and the chemoreceptor cells in the airways (Milligan and Kostenis, 2006). Pertussis toxin (PTx) can catalyse the ADP ribosylation of a cysteine residue 4 amino acids from the C-terminal which prevents functional interaction of the α subunit (apart from $G_{\alpha z}$) with the receptor.

As well as activating the $G_{\alpha s}$ pathway, the GRLN-R has been demonstrated to activate the $G_{\alpha i/o}$ pathway in cell lines endogenously expressing the GRLN-R (Kim et al., 2004; Maccarinelli et al., 2005). For example, ghrelin suppression of glucose-induced insulin release in rat pancreatic islet β cells was shown to be mediated through the G protein subunit $G_{\alpha i2}$, as ghrelin suppression of insulin release was sensitive to treatment with PTx

and completely abolished by treatment with the antisense oligonucleotide specific for the $G_{\alpha i2}$ subunit (but not by antisense oligonucleotides specific for $G_{\alpha i1}$ or $G_{\alpha i3}$) (Dezaki et al., 2007). Furthermore this suppression of glucose-induced insulin release was reduced by treatment with the GRLN-R antagonist [D-lys^3]-GHRP-6 (Dezaki et al., 2007) suggesting ghrelin was exerting its actions through the GRLN-R coupled to $G_{\alpha i2.}$ The ability of the GRLN-R to couple to the $G_{\alpha i/o}$ pathway has also been demonstrated in recombinant systems expressing the human GRLN-R (Camina et al., 2007) or co-expressing the human GRLN-R and $G_{\alpha i}$ (Bassil et al., 2007).

Although it may appear counter-productive for a receptor to be able to couple to both $G_{\alpha i/o}$ and $G_{\alpha s}$ the ability of the GRLN-R to couple to these proteins may depend on their local and subcellular concentration. Indeed there is considerable evidence to suggest that GPCR signalling components are organised together into lipid rafts, which can act to regulate GPCR signal transduction (Insel et al., 2005).

1.10.2.4 $G_{\alpha 12/13}$

The $G_{\alpha 12/13}$ family is the least understood G protein family; it is ubiquitously expressed and associated with communications between heterotrimeric G proteins and cellular reponses mediated by monomeric GTP-binding proteins (Rho), including morphology (e.g. actin reorganisation) and cell proliferation (Riobo and Manning, 2005) (Figure 1-4).

The GRLN-R has been proposed to activate the serum response element-mediated transcription mainly through activation of $G_{\alpha 13}$ (Holst et al., 2004) although this pathway may also be activated by various G protein systems such as $G_{\alpha q}$, $G_{\alpha i}$ and G protein $\beta\gamma$ subunits (Mao et al., 1998; Gruijthuijsen et al., 2002; Niu et al., 2003).

1.10.3 G protein-independent signalling

1.10.3.1 The mitogen-activated protein kinase cascade

Extracellular-regulated kinases (ERKs) belong to a large family of kinases collectively known as the mitogen-activated protein kinases (MAPKs) (Miller and Lefkowitz, 2001). The activation of ERK1/2 occurs via activation of the Ras-dependent cascade. Classically, activation of the Ras-dependent cascade occurs via G protein activation, which leads to activation of Raf-1, a MAPK kinase kinase, which phosphorylates the downstream MAPK kinases MEK1 and MEK2. MEK1 and MEK2 can then phosphorylate ERK1 and ERK2

(Miller and Lefkowitz, 2001). ERK1/2 activation leads to the translocation of the active cytoplasmic kinase to the nucleus where it activates numerous transcription factors involved in mitogenesis and proliferation (Miller and Lefkowitz, 2001). The β-arrestins serve as multifunctional adaptors, scaffolding and/or signal transducers that connect the activated receptor with diverse signalling molecules within the cell (Lefkowitz and Shenoy, 2005; Reiter and Lefkowitz, 2006) including members of the ERK-MAPK signal transduction pathway, such as the non-receptor tyrosine kinase Src (Luttrell et al., 1999).

For some GPCRs, such as the angiotensin II type 1A receptor (AT_{1A}), ERK1/2 activation has been demonstrated to occur via a G protein-independent pathway. In a study by Wei et al. (2003) β-arrestin was shown to act as a signal transducer entirely independent of G protein activation. Using a modified angiotensin II peptide ([Sar^1,Ile^4,II^8]-angiotensin II), which is unable to activate G protein, recruitment of β-arrestin 2 to the receptor led to activation of ERK1/2, although this was only to a level around 50 % of that achieved by wild-type angiotensin II (Wei et al., 2003). Further studies into ERK1/2 activation by angiotensin II (acting on the AT_{1A} receptor) have demonstrated that activation of ERK1/2 occurs in a biphasic manner, with a rapid and transient response mediated via G protein activation, and a slower, more prolonged response via β-arrestin activation (Ahn et al., 2004).

1.10.3.2 G protein-independent signalling of the GRLN-R

In addition to signalling via activation of G proteins, the GRLN-R has also been demonstrated to signal through a G protein-independent pathway, mediated by β-arrestin 1 and β-arrestin 2 (Camina et al., 2007). The activation of ERK1/2 occurs in a biphasic manner, with the rapid ERK stimulation occurring via a G protein-dependent mechanism (through the coupling to $G_{\alpha q/11}$ and $G_{\alpha i/o}$ pathways), with the slower, persistant activation of ERK1/2 being dependent on both β-arrestin 1 and 2. It is thought that the β-arrestin-dependent ERK1/2 activation occurs via a mechanism that involves entry of the GRLN-R into a multi-protein complex with Src-1, Raf-1, ERK1/2 and perhaps other components of the MAPK cascade (Camina et al., 2007).

1.10.4 Desensitisation and endocytosis

The signalling response mediated by receptor activation can be halted at the level of the receptor through the physical uncoupling of the receptor from the G protein, a process known as desensitisation. Desensitisation acts as a protective mechanism against receptor

over-stimulation allowing a fast attenuation (within seconds) of the signal after agonist exposure, allowing the cell to respond to several successive extracellular stimuli over time (Chuang et al., 1996). There are two mechanisms of desensitisation; heterologous and homologous desensitisation.

Heterologous desensitisation occurs when an activated GPCR causes desensitisation of surrounding receptors. Binding of an agonist causes activation of second messenger kinases, for example, PKA and PKC. Once activated, PKA and PKC can phosphorylate serine and threonine residues in the C-terminal tail and ICL3 of GPCRs, resulting in a 40-50 % loss of receptor function (Chuang et al., 1996). As they do not distinguish between activated and non-activated receptors, this can lead to the desensitisation of surrounding inactive receptors.

In contrast to heterologous desensitisation, homologous desensitisation is the specific desensitisation of active receptor. Following activation, the receptor is phosphorylated at serine and threonine residues in the C-terminus and ICL3 either by second messengers produced via G protein activation (such as PKA and PKC) or by G protein receptor kinases (GRKs). GRKs are proteins that are recruited to the receptor after receptor activation, at least partly by dissociated $G_{\beta\gamma}$ subunits (Kristiansen, 2004). GPCR phosphorylation causes minimal desensitisation, but increases the affinity of the receptor for the binding of cytosolic proteins named arrestins.

There are two main classes of arrestins – the visual and cone arrestins and the β-arrestins (which are ubiquitously expressed outside the retina, with their highest expression in neuronal tissue and in the spleen; Attramadal et al., 1992). Phosphorylation of active receptor by GRKs causes arrestin translocation from the cytosol to the plasma membrane where arrestin binds to the receptor, physically uncoupling the G protein-receptor complex.

Following desensitisation the receptor may become physically removed from the cell surface into intracellular membrane vesicles, a process known as endocytosis. The classical pathway in which an activated GPCR is endocytosed is through the formation of clathrin-coated pits. β-arrestins 1 and 2 contain two recognition sites which allow them to link GPCRs with two components of the endocytic machinery; clathrin and the β2-adaptin subunit of the clathrin adapter protein 2 (AP-2) complex (Goodman, Jr et al., 1996; Laporte et al., 1999). The AP-2 complex also binds to clathrin and dynamin (Kirchhausen, 1999). Dynamin is a GTPase that pinches off the vesicle to allow transport of the clathrin-coated vesicle into the cytosol (Hinshaw and Schmid, 1995; Takei et al., 1996). Once

internalised a receptor can then either be recycled back to the cell surface or targeted to the lysosome for degradation.

Interestingly, for the GRLN-R the process by which the receptor is internalised in response to ghrelin differs to that involved in the constitutive internalisation of the receptor.

1.10.4.1 Ghrelin-mediated GRLN-R desensitisation and endocytosis

The GRLN-R appears to undergo homologous desensitisation as, upon exposure to ghrelin, a rapid attenuation of the ghrelin response is observed, resulting from a combination of uncoupling of the receptor from G protein and internalisation of the receptor into intracellular compartments (Camina et al., 2006). Although there are several potential PKC phosphorylation sites in the C-terminal tail of the GRLN-R (see Figure 1-2), the homologous desensitisation of the GRLN-R does not appear to be mediated by PKC, as pre-treatment with the phorbol ester phorbal-12-myristate-13-acetate (PMA), which activates PKC, failed to reduce specific binding of [His[^{125}I]]-ghrelin to the GRLN-R (which shows the receptor is still located at plasma membrane) (Camina et al., 2004). However PKC was shown to cause heterologous desensitisation of surrounding receptors susceptible to regulation by PKC, such as the lysophosphatidic acid (LPA) receptor (Camina et al., 2004).

Two studies have suggested that ghrelin-activated GRLN-R is internalised principally by a clathrin-coated pit mechanism (Camina et al., 2004; Holliday et al., 2007). Ghrelin stimulation of the GRLN-R has been shown to cause receptor phosphorylation, recruiting β-arrestin 2 to the receptor. The receptor-β-arrestin 2 complex is then internalised via vesicles that co-localised with transferrin – a marker of clathrin-mediated endocytosis and recycling compartments. It appears that the clathrin-coated pits then become early endosomes by dropping their clathrin coat, as the GRLN-R co-localises with an early endosome marker ‘early endosome autoantigen 1’ (EEA1) (Camina et al., 2004) and the monomeric G protein Rab5a (which is essential for docking of EEA1 to the endosomal membrane; Patki et al., 1997; Simonsen et al., 1998; Mills et al., 1998; Christoforidis et al., 1999). It is suggested that ghrelin then dissociates from the receptor in the acidified endosomal compartments where it is subsequently degraded by the lysosomes (Figure 1-5).

Following internalisation, the GRLN-R appears to recycled back to the plasma membrane as, in one study, 360 min after ghrelin stimulation, in the presence of cyclohexamide

(which blocks *de novo* protein sysnthesis) the level of GRLN-R measured at the plasma membrane in radioligand binding studies was close to the level measured before ghrelin treatment (Camina et al., 2004). In support of this Holliday and colleagues demonstrated that the GRLN-R was found localised in Rab 11a-positive compartments, compartments that mediate slow recycling such as the perinuclear recycling compartment (PNRC) (Holliday et al., 2007).

Receptor re-sensitisation determines the frequency of ghrelin response (Camina et al., 2006). In a study by Camina et al. (2004) the maximal internalisation of the GRLN-R (in CHO and HEK293 cells transfected to express the GRLN-R) was observed within 20 min of the ghrelin stimulus whilst the whole recycling process was shown to take around 3-6 h. This fits in well with physiological studies, which have shown that growth hormone release in response to ghrelin is blunted if one pulse is given 60 min after an initial pulse, whereas the full GH response is seen if the pulses are separated by 180, 240 or 360 min (Tolle et al., 2001).

Depending on their association with β-arrestins, GPCRs can be classed into one of two classes; class A or class B. Class A receptors have a higher affinity for β-arrestin 2 than β-arrestin 1; whereas class B receptors have equally affinity for β-arrestins 1 and 2 (Oakley et al., 2000). As class A receptors bind to β-arrestin 2 in a transient manner the receptor-β-arrestin complex dissociates rapidly when the receptor is internalised (Pierce and Lefkowitz, 2001; Luttrell and Lefkowitz, 2002). In contrast class B receptors exhibit slower recycling kinetics as they form more stable receptor-β-arrestin complexes, resulting in internalisation of the whole complex into endosomes (Luttrell and Lefkowitz, 2002). Due to the extremely slow recycling kinetics of the GRLN-R compared to other GPCRs (Luttrell and Lefkowitz, 2002), it appears that the GRLN-R is internalised via a class B mechanism, however, this needs to be further explored.

1.10.4.2 Constitutive GRLN-R endocytosis

Although the ghrelin-induced internalisation of the GRLN-R was shown to be β-arrestin 2 mediated, the constitutive internalisation of the GRLN-R persisted in the presence of a dominant negative version of β-arrestin that competes for interaction with clathrin (Holliday et al., 2007). Indeed constitutive internalisation of the GRLN-R was also detected in mouse embryonic fibroblasts that lack both β-arrestin 1 and 2 (Kohout et al., 2001) that had been transfected with the GRLN-R (Holliday et al., 2007). However, constitutive internalisation of the GRLN-R was mediated through clathrin-coated pits (e.g.

the internalisation was dependent on both Rab 5 and Rab 11); thus it appears that the constitutive internalisation of the GRLN-R is β-arrestin-independent but may instead internalise through interaction with, for example, AP-2 (Holliday et al., 2007) (Figure 1-5). Indeed it is possible that AP-2 interacts directly with the GRLN-R, as the C-terminus of the GRLN-R contains a dileucine motif (LL) and a tyrosine-based motif (YRVAV) that can act as binding sites for AP-2 (Kirchausen et al., 1997; Holliday et al., 2007).

Upon treatment for 15-30 min with the inverse agonist SPA, the constitutive internalisation of the GRLN-R was ablated (Holliday et al., 2007; Holst et al., 2004). This suggests that the constitutive internalisation of the GRLN-R occurs at a faster rate than the 3-6 h measured for the ghrelin-induced internalisation, although more studies are needed to compare both ligand-dependent and ligand-independent receptor internalisation within the same study.

Interestingly, the C terminal tail of the GRLN-R has been shown to contain information that leads to the constitutive internalisation of the receptor. For example, Holliday and colleagues conducted a study with chimeric receptors, where the C terminal tail of the GRLN-R (from the NPXXY motif) was swapped with the C-terminal tail of the related (and constitutively active but not constitutively internalised) receptor GPR39 (and vice versa). Unlike the wild-type GRLN-R, which was predominantly located in intracellular regions of cells, the GRLN-R receptor with the C-terminal tail of GPR39 was localised primarily at the plasma membrane. The converse chimera (GPR39 with the GRLN-R C-terminal tail) showed reduced cell surface expression, but receptor that did reach the surface was constitutively internalised (Holliday et al., 2007). For the GRLN-R, the constitutive activity of the receptor appears to be necessary but not sufficient for GRLN-R endocytosis, as blocking the constitutive activity of the GRLN-R with SPA leads to a redistribution of the GRLN-R to the plasma membrane. However in the GRLN-R receptor chimera with the GPR39 tail constitutive endocytosis is greatly reduced suggesting that other regulatory elements in the C terminus are also needed (Holliday et al., 2007).

1.10.5 Constitutive activity

1.10.5.1 Introduction to constitutive activity

In 1989 Costa & Herz introduced the concept of constitutive activity (in which a receptor can form a spontaneously active conformation in the absence of ligand) after the discovery that the δ opioid receptor could signal in the absence of ligand (Costa and Herz, 1989).

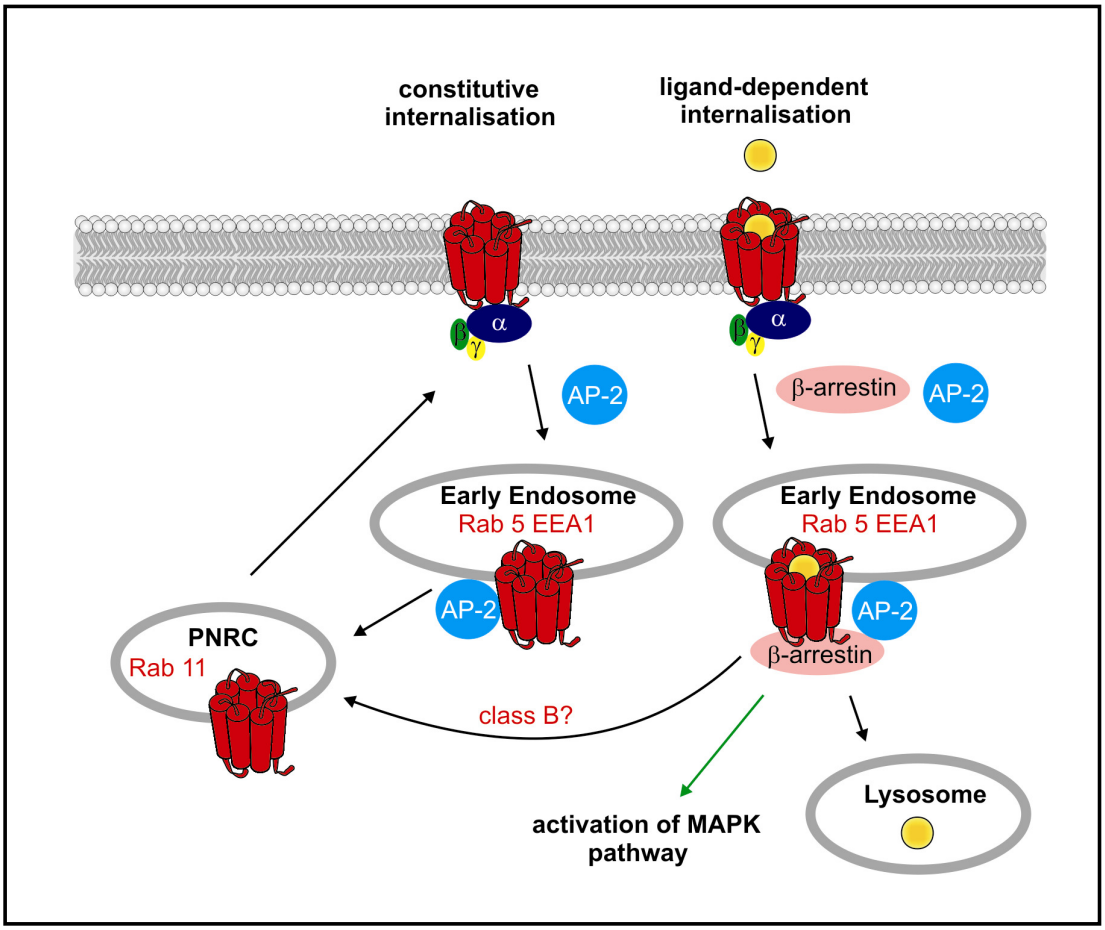

Figure 1-5 – Endocytosis of the GRLN-R (adapted from Holliday et al., 2007). Endocytosis of the GRLN-R occurs via two distinct pathways. Ligand-dependent internalisation occurs via interaction of the activated receptor with β-arrestin-2 (and presumably AP-2). The receptor is internalised via clathrin-coated pits, which discard their clathrin coats to become early endosomes (as demonstrated by co-localisation with the early endosome markers EEA1 and Rab 5). It is thought that ghrelin dissociates from the receptor in the early endosomes and is subsequently degraded in lysosomes. The internalised GRLN-R is expected to be able to signal via G protein-independent mechanism to activate the MAPK pathway. Receptor is then recycled back to the plasma membrane via recycling endosomes such as the peri-nuclear recycling compartment (PNRC) (where the GRLN-R co-localises with Rab 11). This cycle shows very slow kinetics (3-6 h), which suggests the receptor internalises via a class B mechanism. The constitutive internalisation of the GRLN-R does not require β-arrestins. Instead it has been suggested that AP-2 can bind to the C-terminus of the GRLN-R to internalise the GRLN-R via a clathrin-coated pit-mediated mechanism. Once again the clathrin-coated pits are expected to become early endosomes by shedding their clathrin coat and the receptor is recycled back to the plasma membrane via a Rab 11-positive compartment. The constitutive internalisation and recycling of the GRLN-R appears to be much faster (15-30 min) than the ligand-induced internalisation/recycling of the GRLN-R.

Since then many GPCRs, across all classes have been shown to be constitutively active including the class A β_2AR (Milano et al., 1994); the class B calcitonin receptor (Cohen et al., 1997) and the class C metabotropic glutamate receptors (Prezeau et al., 1996).

Naturally occurring mutations in receptors that cause ligand-independent activation have also been identified and linked to diseases such as congenital night blindness (in the case of the rhodopsin receptor; Rao et al., 1994). Indeed there is reason to believe that a degree of constitutive activity occurs in all GPCRs (the one exception being the highly-specialised rhodopsin receptor) (Bond and Ijzerman, 2006).

The methods used to study constitutive activity rely heavily on *in vitro* studies, which may lead to over-expression of the receptor and, consequently, constitutive receptor activity may be an artefact of the expression system (Seifert and Wenzel-Seifert, 2002). Nevertheless, studies of GPCRs in native systems (e.g. the β_2AR in turkey erythrocyte membranes; Mewes et al., 1993) and intact organs (β_2AR in normal heart tissue; Varma et al., 1999) have clearly demonstrated that constitutive activity occurs in native systems.

1.10.5.2 Receptor activation models and receptor theory

Various models have been proposed and modified to explain the activation of GPCRs as our knowledge of receptor activation has expanded (Rang, 2006). One of the simplest models is the two-state model, which states that a receptor exists in equilibrium between an inactive (R_i) and an active (R_a) state. In the absence of agonist, the majority of receptor exists as R_i, whilst binding of an agonist alters the equilibrium to favour formation of R_a. To incorporate binding of G protein, the extended ternary complex model was formed (Figure 1-6; Samama et al., 1993), which takes into account the interactions between receptor and G protein in addition to interactions between receptor and agonist, thus allowing a receptor to form a R_a state that can couple to G protein (and transduce a signal) in the absence of agonist (i.e. it incorporates constitutive receptor activation).

Ligands acting on GPCRs can be characterised by the nature of their effects, governed by affinity (how tightly a ligand binds to a receptor) and efficacy (the ability of a ligand, once bound, to elicit a response) (Kenakin, 2001). Thus ligands can be classified as agonists (full or partial), inverse agonists (full or partial) or antagonists. A full agonist (which displays high affinity for the R_a state) is able to produce a system maximal response, whereas a partial agonist would produce a measurable but submaximal response. An inverse agonist has higher affinity for the R_i state rather than R_a, thus it favours formation

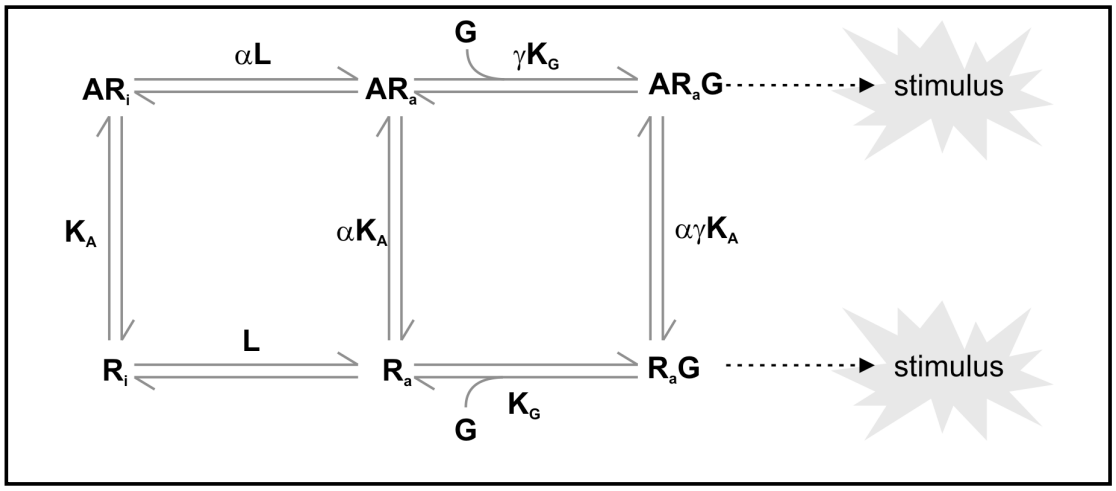

Figure 1-6 – The extended ternary complex model. The model describes the spontaneous formation of an active receptor state (R_a) from inactive receptor (R_i) according to the allosteric constant L. The active receptor can form a complex with G protein spontaneously (R_aG) or after agonist (A) activation (AR_aG). K_A and K_G are the equilibrium dissociation constants of the ligand-receptor and G protein-receptor complexes, respectively. The term α refers to differences in affinity for a ligand for R_a over R_i, whilst γ denotes the multiple differences in affinity of the receptor for G protein when ligand is bound to receptor.

of R_i, blocking constitutive receptor activation. Like agonists, inverse agonists can be classed either as full inverse agonists (fully able to block constitutive activity) or as partial inverse agonists (those which only partially block constitutive activity). An antagonist is a ligand which has equal affinity for R_i and R_a, thus it produces no change in the equilibrium between R_i and R_a (i.e. it has no efficacy), but can block the actions of an agonist either reversibly (a competitive antagonist) or irreversibly (non-competitive antagonist).

1.10.5.3 Constitutive activity of the GRLN-R

Before 2003 the majority of studies into the pharmacology of the GRLN-R focused on measuring calcium mobilisation as a read-out of receptor activation. In this assay the GRLN-R appeared to be devoid of any constitutive activity. However, in 2003 Holst and colleagues discovered that the GRLN-R was constitutively active in inositol phosphate accumulation assays (i.e. via activation of the $G_{\alpha q/11}$ pathway) and in assays measuring cAMP-responsive element (CRE) binding protein phosphorylation (which measures activation of both the $G_{\alpha q/11}$ and $G_{\alpha s}$ pathways) (Holst et al., 2003). Thus the lack of constitutive activity of the GRLN-R in the calcium mobilisation assays appeared to be due to the fact that calcium has to be kept within strict margins within cells (Holst et al., 2003). A year after the first reports of the constitutive activity of the GRLN-R, constitutive activity of the GRLN-R was also measured in the serum-response element-mediated transcription pathway, a pathway which is expected to be activated by $G_{\alpha 13}$ for the GRLN-

R (Holst et al., 2004) (but may also be activated by $G_{\alpha i}$ or $G_{\beta\gamma}$; Mao et al., 1998; Ponimaskin et al., 2002; Gruijthuijsen et al., 2002; Niu et al., 2003).

To investigate whether the receptor was truly constitutively active, gene-dosing experiments were performed showing that constitutive CRE-binding protein phosphorylation increased in COS-7 cells as GRLN-R cDNA was increased; although the same was not true for the motilin receptor, GPR38, in which the basal activity of the receptor remained the same (Holst et al., 2004). This clearly demonstrated that the constitutive activity of the GRLN-R was not an artefact of receptor over-expression. Furthermore as downregulation of CRE-mediated gene transcription was shown to be a major signal transduction pathway for the effects of the anoretic hormone leptin in NPY/AGRP-containing neurones *in vivo*, it suggests that constitutive activation of CRE-mediated gene transcription by the GRLN-R (Shimizu-Albergine et al., 2001) played an important role in appetite regulation (Holst & Schwartz, 2004).

Generally it is very difficult to determine the *in vivo* physiological importance of constitutive activity (Holst et al., 2003). However, rather interestingly, analyses of naturally occurring missense mutations at the GRLN-R (which ablate the ligand-independent activity of the GRLN-R) have been associated with the development of an obese/short stature phenotype *in vivo* (Wang et al., 2004; Pantel et al., 2006), thus demonstrating that constitutive activity appears to serve an important function *in vivo*. Whilst the finding that a high level of GH secretion could be measured in isolated pituitary cells that express the GRLN-R (Cervia et al., 2002) which, as cultured isolated cells should mean they are devoid of any hormonal tone, suggests the GH release is being mediated via the constitutive activity of the GRLN-R and this likely occurs *in vivo*.

It is worth noting that the GRLN-R does not appear to display any constitutive activity/constitutive internalisation when expressed in CHO cells (i.e Camina et al., 2004; Camina et al., 2006). This appears to be a cell-specific effect as constitutive activity has been measured in other recombinant expression systems such as HEK293 cells (Holliday et al., 2007) and COS-7 cells (Holst et al., 2003; Holst et al., 2004) and may reflect the absence of machinery required for signal generation or receptor internalisation. Furthermore the GRLN-R does not appear to constitutively activate the MAPK pathway (as measured through ERK1/2 phosphoryation) (Holst et al., 2004). The reason for this is not clear as constitutive activation of the MAPK pathway can be measured for other constitutively active receptors, such as the virally encoded receptor ORF-74 (Smit et al.,

2002). On the other hand, other regulatory mechanisms may be in place to stop excessive ERK activation, regulating constitutive signalling of the GRLN-R.

1.10.6 Allosterism

1.10.6.1 Introduction to allosterism

The primary binding site for the endogenous ligand on a GPCR is termed the orthosteric site, however ligands may also bind to a topographically distinct site on the receptor; a site termed the allosteric site. Although allosteric modulators (ligands that bind to the allosteric site) are well established as research tools and therapeutic agents of ion channels, they have not been a traditional focus of drug discovery efforts of GPCRs (Conn et al., 2009). Recently the potential of allosteric modulators as therapeutic targets of GPCRs has been proven with two allosteric modulators entering the market, cinacalcet, a modulator of the calcium-sensing receptor (Harrington and Fotsch, 2007) and maraviroc, a modulator of the chemokine receptor CCR5 (Dorr et al., 2005).

Allosteric modulators can act to modify the affinity of the orthosteric ligand for a receptor and/or modulate the efficacy of orthosteric ligands. For example, when both the orthosteric and allosteric sites are occupied by ligand, an allosteric interaction between the sites occurs allowing the allosteric modulator to increase or decrease the affinity of the orthosteric ligand for the receptor (i.e. the allosteric modulator shows positive or negative co-operativity, respectively). An allosteric effect on efficacy can be shown as changes in the potency and/or efficacy of the orthosteric ligand, as demonstrated by the action of the allosteric modulator CGP7930, which enhances both the potency and efficacy of GABA at the $GABA_B$ receptor (Urwyler et al., 2001).

In addition, 'allosteric agonists' have also been identified, which are ligands that express the property of agonism in the absence of bound orthosteric ligand (May et al., 2007). This was first demonstrated for the adenosine A_1 receptor, where the allosteric modulator PD 81723 was shown to be able to activate the $G_{\alpha i/o}$ pathway on its own but it was also shown to enhance the binding of the orthosteric radioligand [^{3}H]-cyclohexyladenosine when both ligands were present (Bruns and Fergus, 1990).

1.10.6.2 Detecting an allosteric effect

Ideally to confirm an allosteric mode of action three criteria need to be met. Firstly, the co-operativity between an allosteric ligand and an orthosteric ligand means that allosteric

interactions are proposed to have a saturable or 'ceiling'effect; whereby increasing concentrations of the allosteric modulator fail to shift the concentration-response curve of the orthosteric ligand any further. Thus one of the characteristics of an allosteric interaction is that there is saturability in effect. Secondly, allosteric effects are predicted to show 'probe-dependence'. This is because co-operativity can change with each ligand so that an allosteric modulator can inhibit the binding of one orthosteric ligand whilst having no effect on a second orthosteric ligand (Leach et al., 2007). This has been demonstrated for the chemokine receptor CCR5 where the allosteric modulator GSK873140 creates a receptor conformation that inhibits binding of the orthosteric ligand CCL3 without affecting binding of a second orthosteric ligand CCL5 (Watson et al., 2005). Thirdly, as dissociation of the orthosteric ligand is altered by the presence of an allosteric ligand (the binding of an allosteric modulator to a GPCR causes a conformational change that alters association or dissociation rate constants that govern binding of the orthosteric ligand) an alteration in the kinetics of binding of an orthosteric probe to the receptor in the presence of an allosteric modulator can be used as a determination of an allosteric effect.

1.10.6.3 Allosteric modulation of the GRLN-R

Holst and colleagues have suggested that L-692,429 and GHRP-6 are allosteric agonists of the GRLN-R, as co-administration of ghrelin with either L-692,429 or GHRP-6 acted to increase or decrease the potency of ghrelin as well as acting to increase the efficacy of ghrelin. However co-administration of ghrelin with MK-677 had no effect on the potency of ghrelin although MK-677 acted to increase the efficacy of ghrelin (Holst et al., 2005). Both GHRP-6 and L-692,429 failed to fully compete with binding of the orthosteric probe [His[^{125}I]]-ghrelin which suggested that these compounds were binding at a site on the receptor distinct from ghrelin, whereas MK-677 could fully compete for ghrelin binding (suggesting that MK-677 was a simple orthosteric ligand). Thus L-692,429 and GHRP-6 were termed 'ago-allosteric modulators'; a term coined to describe ligands that function both as agonists and as allosteric modulators of the potency and/or efficacy of the orthosteric ligand (Schwartz and Holst, 2006). Although in the study by Holst et al. (2005) none of the three hallmarks of an allosteric effect were investigated thoroughly.

However, allosteric modulation of the GRLN-R by L-692,429 and GHRP-6 is complicated by the finding that these ligands share an overlapping binding site with ghrelin on the receptor. Thus to account for this observation the GRLN-R was proposed to exist as a dimer where ghrelin could bind to one protomer of the dimer leaving the second protomer free to bind GHRP-6 or L-692,429 whereby they could exert their allosteric effects (Holst

et al., 2005). This model also allows MK-677 to co-bind with ghrelin accounting for the increase in efficacy of ghrelin in the presence of MK-677 (in the absence of any allosteric effects). Allosterism across a dimer has been demonstrated for the chemokine receptors. Using a hetero-dimer formed by CCR2 and CCR5, dissociation of [^{125}I]MCP-1 from CCR2 was shown to be strongly increased by the presence of unlabelled MIP-1β, a ligand for CCR5, suggesting that ligand binding in one monomer induced a conformational change in the partner, resulting in faster dissociation of the bound ligand (Springael et al., 2006).

1.11 Project aims

The GRLN-R has become an attractive therapeutic target, mainly due to its effects in stimulating GH release and its role in the regulation of appetite and energy homeostasis. Although the signalling of the GRLN-R through the activation of the $G_{\alpha q/11}$ pathway is well studied there is a lack of information about the signalling of the receptor through other pathways, such as $G_{\alpha i/o.}$

The aim of this project was to investigate activation of the $G_{\alpha i/o}$ pathway in a heterologous system. Specifically to:

1. Further investigate ago-allosteric modulation of the GRLN-R in the $G_{\alpha i/o}$ pathway.

2. Compare the signalling of the GRLN-R through activation of the $G_{\alpha q/11}$ pathway and the $G_{\alpha i/o}$ pathway to see if there is any difference in the potencies and/or efficacies of ghrelin and the growth hormone secretagogues in these pathways

3. Investigate whether naturally occurring mutations of the GRLN-R (A204E and I134T) affect the function of the GRLN-R through both the $G_{\alpha q/11}$ and $G_{\alpha i/o}$ pathways.

2 Materials and methods

2.1 General reagents, enzymes and kits

Alpha Biotech Ltd., London, UK

GF/C Glass fibre filters

BDH, Lutterworth, Leicestershire, UK

22 mm coverslips, acetic acid, formaldehyde, methanol, microscope slides, KOH, sodium formate

Biorad, Hemel Hempstead, UK

1 x 8 formate form 200-400 mesh size Dowex® resin

Fisher Scientific UK Ltd, Loughborough, Leicestershire, UK

$CaCl_2$, D-glucose, ethanol, ethylene glycol, isopropranolol, glacial acetic acid, glycerol, glycine, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), KCl, KH_2PO_4, methanol, 3-(N-Morpholino)propanesulfonic acid (MOPS), NaCl, sodium dodecyl sulphate (SDS), sucrose, $MnCl_2$.

Flowgen Biosciences Ltd., Nottingham, UK

Agarose

GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK

Full range Rainbow™ molecular weight markers, protein G, wheat germ agglutinin (WGA)-polystyrene LEADseeker imaging beads

Greiner Bio-One Ltd., Stonehouse, UK

96 deepwell blocks

Invitrogen Ltd., Paisley, UK

ER-Tracker™ red (glibenclamide BODIPY® TR), NuPage Novex pre-cast 4-12 % Bis-Tris gels, NuPage MOPS SDS running buffer, WGA-Alexa Fluor® 594 conjugate

Konica Europe, Hohenbrunn, Germany

X-ray film

Merck Chemicals Ltd., Beeston, Nottingham, UK

Luria-Bertani (LB)-agar, LB-bouillon, nonident P-40 (NP-40), pansorbin beads

Perkin Elmer Life and Analytical Science, Boston, MA

AlphaScreen cAMP assay kit, Ultima Gold XR liquid scintillation cocktail, Unifilter® 96 well GF/B filter block

Perbio Science UK Ltd., Cramlington, Northumberland, UK

Supersignal West Pico chemiluminescent substrate (ECL)

Promega UK Ltd., Southampton, UK

All restriction endonucleases, T4 DNA ligase, Pfu DNA polymerase, Wizard™ Plus SV miniprep kit

Qiagen, Crawley, West Sussex, UK

QiaQuick PCR purification kit, QiaQuick gel extraction kit, Qiafilter maxi-prep kit

Roche Diagnostics Ltd., Lewes, East Sussex, UK

Complete EDTA-free protease inhibitor tablets, DNA molecular weight marker X, Endo-β-acetylglucosaminidase H (Endo H), peptide-N^4-(acetyl-β-glucosaminyl)-asparagine amidase (PNGaseF)

Sigma-Aldrich Company Ltd., Poole, Dorset, UK

Ammonium formate, ampicillin, ascorbic acid, BCA solution A, bromophenol blue, bovine serum albumin (BSA), deoxycholic acid (sodium salt), CHAPS (3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-O,O'-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), ethidium bromide, ethylene glycol, Ficoll, forskolin, guanine diphosphate (GDP), glycerol, guanine triphosphate (GTP), guanosine 5'-O-(3-thio)-triphosphate (GTPγS), HCl, 3-isobutyl-1-methylxanthine (IBMX), lithium chloride, $MgCl_2$, orange G, perchloric acid, polyethylenimine (PEI), pluronic F-127, $RbCl_2$, Na_2HPO_4, di-sodium orthophosphate, di-sodium tetraborate, sodium acetate, sodium azide, NaCl, NaH_2PO_4, NaOH, Triton X-100, Tween-20, Tris-base, xylene cyanol.

Stratagene, Amsterdam, The Netherlands

QuikChange® site directed mutagenesis kit

Thermo Electron, Ulm, Germany

Immu-mount, all oligonucleotides used for PCR reactions

Whatman International Ltd., Maidstone, UK

Protran® nitrocellulose transfer membrane

2.2 Antibodies and antisera

Abcam, Cambridge, Cambridgeshire, UK

Rabbit anti-calnexin polyclonal antibody

Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, UK

Donkey anti-mouse IgG-horse radish peroxidase (HRP) conjugate, donkey anti-rabbit IgG-HRP conjugate

Roche Diagnostics Ltd., Lewes, East Sussex, UK

Monoclonal mouse anti-haemagglutinin (HA) antibody (clone 12CA5), anti-vesicular stomatitis virus glycoprotein (VSV-G) antibody

GFP antisera and all G protein antisera were produced in-house. G protein antisera were raised against peptides based on the last 10 amino acids of the C-terminal tail.

Sigma-Aldrich Company Ltd., Poole, Dorset, UK

Rabbit anti-goat IgG-HRP conjugate, rabbit anti-GFP N-terminus antibody.

2.3 Pharmacological compounds

Bachem (UK) Ltd., St. Helens, Merseyside, UK

Ghrelin (human), [D-Arg^{1},D-Phe^{5},D-Trp^{7,9},Leu^{11}]-substance P (SPA), (amino acid sequence: His-D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH_2)

GlaxoSmithKline, Harlow, Essex, UK

MK-677 (systemic name: 2-Amino-2-methyl-*N*-[1-(1-methylsulfonylspiro[2*H*-indole-3,4'-piperidine]-1'-yl)-1-oxo-3-(phenylmethoxy)propan-2-yl]propanamide) was kindly supplied by Alan Wise

Sigma-Aldrich Company Ltd., Poole, Dorset, UK

Growth hormone releasing peptide-6 (GHRP-6) (amino acid sequence: His-D-Trp-Ala-Trp-D-Phe-Lys-NH_2)

Tocris, Avonmouth, Bristol, UK

L-692,585 (systemic name: 3-[[(2R)-2-Hydroxypropyl]amino]-3-methyl-N-[(3R)-2,3,4, 5-tetrahydro-2-oxo-1-[[2'-(1H-tetrazol-5-yl)[1,1'-biphenyl]-4-yl]methyl]-1H-1-benzazepin-3-yl]-butanamide)

2.4 Radiochemicals

Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, UK

[^{35}S]GTPγS, [^{3}H]*myo*-inositol

Perkin Elmer life and analytical science, Boston, MA

[His[^{125}I]]-ghrelin

2.5 Tissue culture disposables and reagents

American Tissue Culture Collection, Rockville, USA

HEK293T cells

Clontech, Saint-Germain-en-Laye, France

pECFP-N1 vector

Costar, Cambridge, MA., USA

5 mL, 10 mL and 25 mL pipettes, 25 cm^2, 75 cm^2 and 125 cm^2 vented tissue culture flasks, 6 well plates, 10 cm^2 dishes

Invitrogen BV, Groningen, The Netherlands

Blasticidin, Dulbecco's modified Eagle's medium (DMEM) (- sodium pyruvate), Flp-In T-REx HEK293 cell line, foetal bovine serum (FBS), L-glutamine (200 mM), Hank's buffered saline solution (HBSS), Lipofectamine transfection reagent, minimal essential medium (MEM) non-essential amino acids, new born calf serum (NBCS), optimem, 100 x penicillin-streptomycin mix, Versene, zeocin

Roche Applied Science, Lewes, East Sussex, UK

Hygromycin B

Sigma-Aldrich Company Ltd., Poole, Dorset, UK

DMEM (- sodium pyruvate, + L-glutamine, + 4.5 g/L glucose) 0.25 % trypsin-EDTA, pertussis toxin, poly-D-lysine

2.6 Buffers and solutions

2.6.1 Molecular biology buffers and solutions

Buffer 1 (for the preparation of competent bacteria)

30 mM potassium acetate, 0.1 M $RbCl_2$, 10 mM $CaCl_2$, 50 mM $MnCl_2$ and 15 $\%_{(w/v)}$ glycerol; pH 5.8. The solution was filter sterilised (through a 0.2 μm filter) and stored at 4 °C.

Buffer 2 (for the preparation of competent bacteria)

10 mM MOPS (pH 6.5), 75 mM $CaCl_2$, 10 mM $RbCl_2$, 15 $\%_{(w/v)}$ glycerol; pH 6.5. The solution was filter sterilised (through a 0.2μM filter) and stored at 4 °C.

DNA loading buffer (3 x)

15 $\%_{(w/v)}$ Ficoll (dissolved in H_2O and autoclaved), 200 μL saturated bromophenol blue, 200 μL saturated orange G, 25 μL saturated xylene cyanosol.

Laemmli buffer (5 x)

0.4 M DTT, 0.17 M SDS, 50 mM Tris-base, 5 M urea, 0.01 $\%_{(w/v)}$ bromophenol blue. Buffer was stored at –20 ºC.

Phosphate buffered saline (PBS) (10 x)

137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 10.2 mM Na_2HPO_4; pH 7.4. 1 x PBS was prepared by diluting the stock 1:10 in H_2O.

20 % Pluronic F-127

A 20 $\%_{(w/v)}$ solution was prepared by dissolving pluronic F-127 in DMSO at 37 °C.

Tris acetate-EDTA (TAE) buffer (50 x)

40 mM Tris-base, 1 mM EDTA (pH 8), 5.71 $\%_{(v/v)}$ glacial acetic acid. 1 x TAE was prepared by diluting 1:50 prior to use.

Tris-EDTA (TE) buffer

10 mM Tris-base, 0.1 mM EDTA; pH7.4. Buffer was stored at 4 °C.

2.7 Molecular biology protocols

2.7.1 Preparation of LB plates

LB-agar was prepared as per manufacturer's instructions. The solution was autoclaved and allowed to cool to 50 °C before addition of the appropriate antibiotic: ampicillin (final concentration 50 μg/mL for pcDNA3, pcDNA3.1, pcDNA5 and pOG44 vectors; Invitrogen) or kanamycin (final concentration 30 μg/mL for pECFP-N1 vector; Clontech). The medium was gently mixed to ensure antibiotic was distributed equally before approximately 25 mL was poured into a Petri dish. The plates were left to set at room temperature before storage at 4 °C. Unused plates were discarded after 1 month.

2.7.2 Preparation of competent bacterial cells

XL1 blue cells (a strain of *Escherichia coli*) were streaked out onto an LB agar plate (in the absence of antibiotics) and incubated for 16 h (37 °C). A single colony was used to inoculate a 5 mL culture of LB media, which was incubated for 16 h in a shaking incubator (37 °C). This culture was added to 100 mL of LB media and grown in a shaking incubator (37 °C) until the optical density of 0.48 at 550 nm was reached. The culture was chilled on ice for 5 min, then centrifuged in sterile falcon tubes (3220 x *g*; 10 min; 4 °C) to pellet the bacteria. The supernatant was removed and the pellet re-suspended in 20 mL of competent bacteria buffer 1. The suspension was incubated on ice for 5 min prior to centrifugation (3220 x *g*; 10 min; 4 °C). The supernatant was carefully removed and the pellet re-suspended in 2 mL of competent bacteria buffer 2. After incubation on ice for 15 min the competent bacteria were aliquoted into sterile Eppendorf tubes and stored at –80 °C.

2.7.3 Transformation of competent bacteria

An aliquot of competent bacteria was allowed to thaw on ice; 1-10 ng of DNA was incubated with 50 µL of competent bacteria (15 min; 4 ºC). The bacteria were subjected to heat-shock (90 s; 42 ºC) and returned to ice for 2 min before the addition of 450 µL of LB medium. The cells were incubated in a shaking incubator (45 min; 37 ºC) to allow recovery. 100 µL of the reaction was spread onto an LB plate containing the appropriate antibiotic and incubated inverted overnight at 37 ^{o}C.

2.7.4 Preparation of plasmid DNA

2.7.4.1 Mini preps

DNA was purified using Wizard™ Plus SV miniprep kit. 1.5 mL of a bacterial culture was pelleted by centrifugation (16,000 x *g*; 5 min) and subsequently resuspended in 250 µL of re-suspension buffer (50 mM Tris-HCl, 10 mM EDTA, 100 µg/mL RNase A; pH 7.5) by gentle pipetting. Cells were lysed by addition of 250 µL of lysis buffer (200 mM NaOH, 1 $\%_{(w/v)}$ SDS) and 10 µL alkaline phosphatase. Lysis was terminated after 5 min by the addition of 350 µL of neutralisation buffer (4.09 M guanidine hydrochloride, 0.76 M potassium acetate, 2.12 M glacial acetic acid; pH 4.2). The cell lysates were centrifuged (16,000 x *g*; 10 min) and the supernatant loaded onto miniprep columns. The columns were centrifuged (16,000 x *g*; 1 min) and washed twice with 750 µL wash buffer (60 mM potassium acetate, 10 mM Tris, 60 $\%_{(v/v)}$ ethanol; pH 7.4). After washing, bound DNA was eluted from the column by addition of 100 µL sterile water by centrifugation (16,000 x *g*; 1 min) into sterile 1.5 mL Eppendorf tubes.

2.7.4.2 Maxi preps

The Qiagen Qiafilter kit was used to produce larger scale DNA samples. A 100 mL culture of transformed bacteria was pelleted by centrifugation (3220 x *g* ; 30 min; 4 ^{o}C). Media was carefully removed and the pellet resuspended in 10 mL of chilled buffer P1 (50 mM Tris-HCL, 10 mM EDTA, 100 µg/µL RNase A; pH 8.0). Cells were lysed at room temperature by the addition of 10 mL buffer P2 (200 mM NaOH, 1 $\%_{(w/v)}$ SDS). After 10 min incubation cell lysis was stopped and the solution neutralised by the addition of 10 mL of buffer P3 (3 M potassium acetate; pH 5.5). The solution was applied to a QIAfilter cartridge and left for 10 min at room temperature to settle. Meanwhile, a Qiagen tip 500 was equilibrated by the addition of buffer QBT (750 mM NaCl, 50 mM MOPS, 15 $\%_{(v/v)}$ isopropanol; pH 7.0). After 10 min the cell solution was added onto the equilibrated tip and

allowed to drain by gravity flow. The column was washed with 60 mL of buffer QC (1 M NaCl, 50 mM MOPS, 15 $\%_{(v/v)}$ isopropanol; pH 7.0) and the DNA eluted by the addition of 15 mL of buffer QF (1.25 M NaCl, 50 mM Tris-HCl, 15 $\%_{(v/v)}$ isopropanol; pH 8.5). The DNA was precipitated by the addition of 10.5 mL isopropranol and pelleted by centrifugation (12,000 x *g*; 30 min; 4 ºC). The DNA pellet was washed with 5 mL of room temperature 70 $\%_{(v/v)}$ ethanol and then centrifuged (12,000 x *g*; 15 min; 4 ^{o}C). The supernatant was carefully removed and the pellet allowed to air-dry prior to re-suspension in 1 mL of sterile H_2O.

2.7.5 Quantification of DNA

Quantification of DNA samples was performed by examining the absorbance of a 1:100 dilution of the sample at 260 nm. An A_{260} value of 1 unit was taken to correspond to 50 µg/mL of double stranded DNA. The A_{280} value of sample was also measured to assess the purity of the DNA solution. A DNA solution with A_{260}/A_{280} ratio of between 1.7 and 2.0 was considered pure enough for use.

2.7.6 DNA digestion with restriction endonucleases

Digests were prepared using the appropriate restriction enzyme (1-2 units), buffer (as specified by the manufacturer), and DNA, in a final volume of 20 µL. Control reactions were carried out using 1 µg DNA and preparative reactions (i.e. to prepare an insert for ligation) were carried out using 5 µg DNA. Reactions were incubated at 37 ^{o}C for a minimum of 2 h.

2.7.7 DNA gel electrophoresis

Digested DNA samples or PCR reactions were examined using gel electrophoresis. Samples were diluted in 3x DNA loading buffer. A 1 $\%_{(w/v)}$ agarose gel in 1x TAE was prepared; the mixture was gently heated to allow the agarose to fully dissolve before the addition of 2.5 mg/mL ethidium bromide. The mixture was poured into a horizontal gel tank and once set, immersed in 1x TAE. Samples were loaded and the gels run at 75 mA for 20-30 min. The DNA fragments were visualised using ultraviolet light. The size of each fragment was assessed by comparison with DNA molecular marker X.

2.7.8 DNA purification from agarose gels

The DNA fragments of interest were excised from the DNA electrophoresis gel and purified using the QiaQuick gel extraction kit as per the manufacturer's instructions. DNA was eluted from the purification column using 30 µL sterile H_2O.

2.7.9 Ligation of DNA

Digested PCR fragments were ligated into plasmid vector using T4 DNA ligase. A ratio of 1:3 and 1:6 vector to PCR product was incubated (16 h, 4 °C) with 1 unit of ligase and 1 µL 10 x ligase buffer, in a final volume of 10 µL. The ligation reactions were transformed as detailed in section 2.7.3.

2.7.10 Polymerase chain reaction

2.7.10.1 Standard PCR

The polymerase chain reaction (PCR) was used to amplify specific sections of DNA and introduce epitope tags and/or restriction sites. Pfu DNA polymerase was selected for its proofreading ability. All reactions were performed using sterile materials. The PCR reactions were set up as follows:

Pfu polymerase buffer (10 x)	5 µL
DMSO (optional)	5 µL
Deoxynucleotide tri-phosphates (dNTPs)	5 µL
(0.25 mM of each dATP, dCTP, dGTP, dTTP)	
Primer sense: 25 pmol/µL	1 µL
Primer antisense: 25 pmol/µL	1 µL
DNA template: 50 ng/µL	1 µL
Pfu enzyme: 1 unit	1 µL
dH_2O to a final volume of	50 µL

Reactions were carried out on an Eppendorf gradient Thermocycler. PCR cycles used were as follows (annealing temperatures were dependent on the melting temperatures [Tm] of the primers used):

1.	Preheating	95 ^{o}C	5 min
2.	Denaturation	95 ^{o}C	1 min
3.	Annealing	50-60 ^{o}C	1 min

4.	Extension	72 ^{o}C	3 min
5.	Repeat from step 2.		29 times
6.	Final extension	72 ^{o}C	10 min
7.	Hold	4 ^{o}C	

2.7.10.2 QuikChange mutagenesis PCR

The QuikChange site-directed mutagenesis method was used to make single point mutations in GRLN-R. Primer design and PCR conditions were performed according to the manufacturer's instructions.

PCR reactions were carried out using with the following components: 50 ng DNA template, 15 pM of both forward and reverse primers, a final concentration of 10 μM dNTP mix and 1 μL (2.5 U) Pfu DNA polymerase to a total volume of 50 μL with reaction buffer. Samples were cycled 30 times in an Eppendorf Thermocycler system under the following conditions:

Cycle 1:	95 ^{o}C for 30 secs
Cycles 2-30:	95 ^{o}C for 30 secs,
	50 ^{o}C for 1 min
	68 ^{o}C for 7 min

The product was incubated with 1 μL (10 U) *Dpn*I restriction enzyme (60 min; 37 ^{o}C) to allow the digestion of parental methylated dsDNA. The digested mutated DNA and appropriate controls were then transformed into XL1 blue cells following the protocol described in section 2.7.3

2.7.11 PCR purification

PCR products were purified using the QiaQuick PCR purification kit as per manufacturer's instructions. DNA was eluted from the purification column using 30 μL of sterile H_2O.

2.7.12 DNA sequencing

DNA sequencing was performed by 'The Sequencing Service' (School of Life Sciences, University of Dundee, Scotland) using Applied Biosystems Big-Dye Ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer. Sequences

obtained were compared with the sequence of the human GRLN-R (accession number Q92847).

2.7.13 Generation of the VSV-G-GRLN-R-eCFP construct

To generate the VSV-G-GRLN-R-eCFP construct, primers were designed to add restriction sites to GRLN-R, a vesicular stomatitis virus (VSV-G) tag at the N-terminus and to remove the stop codon at the C-terminus. Human GRLN-R cDNA in pcDNA3.1 was supplied by GlaxoSmithKline.

Sense primer to add a VSV-G tag and *Hin*dIII site:

5'-
CCTCACCT***AAGCTT***GCCACCATGTACACCGACATCGAAATGAACCGCCTTGGTAAGTGGAACGCGACGCCCAGCGAAGAG-3'

Anti-sense primer to add a *Kpn*I site and remove the stop codon:

5'-GTATGCGAT***GGTACC***TGTATTAATACTAGATTCTGTCCA-3'

The *Hin*dIII and *Kpn*I sites are shown in bold italics; the VSV-G tag is underlined. A vector (pcDNA3) containing eCFP between *Kpn*I and *Not*I sites and the amplified DNA fragment were both sequentially digested with *Hin*dIII and *Kpn*I. The amplified fragment was then ligated into the pcDNA3-eCFP vector in frame with eCFP.

2.7.14 Generation of the HA-GRLN-R-A204E-eCFP constuct

Primers were designed to alter a single base to change A204 to E204 using the Qiagen QuikChange mutagenesis kit (using the conditions described in section 2.7.10.2) with GRLN-R-pcDNA3.1 vector as a template.

Sense 5'- CACCGAGTTTG**A**GGTGCGCTCTG – 3'

Anti-sense 5' – CAGAGCGCACC**T**CAAACTCGGTG – 3'

The altered base is shown in bold. The template was digested with *Dpn*I to leave mutated plasmid. Sequencing was used to confirm the introduction of the mutation.

Primers were then used to add N- and C-terminal restriction sites, a HA tag at the N-terminus and to remove the STOP codon at the C terminus of GRLN-R-A204E-pcDNA3.1.

Sense primer to add a HA tag and a *Hin*dIII site –

5' -
CCTCACCT***AAGCTT***GCCACCATGTATCCTTACGACGTTCCTGACTACGCATGGA
ACGCGACGCCCAGCGAAGAG – 3'

Anti-sense to add *Kpn*I site and remove the stop codon

5' – GTATGCGAT***GGTACC***TGTATTAATACTAGATTCTGTCCA – 3'

The *Hin*dIII and *Kpn*I sites are shown in bold italics; the HA tag is underlined. The amplified fragment and vector containing eCFP (pECFP-N1) were sequentially digested with *Hin*dIII and *Kpn*I. The fragment was ligated in-frame with eCFP and transformed into competent XL1-Blue bacteria. Colonies were picked and the sequences of positive clones were verified using DNA sequencing.

2.7.15 Generation of the HA-GRLN-R-I134T-eCFP construct

The GRLN-R-I134T mutation was performed using a standard PCR-based mutagenesis technique. Two sets of primers were designed, a set to mutate I134 to T134 ('mutation primers') and a set to allow the amplification of the full length receptor incorporating restriction sites at the N- and C-terminals ('extreme primers'). A PCR reaction was set up using GRLN-R in pcDNA3.1 as the template (using the conditions outlined in section 2.7.10.1). One reaction contained the sense mutation primer and an anti-sense extreme primer designed to remove the STOP codon and add a *Kpn*I restriction site at the C-terminus of GRLN-R.

Sense 5' – CACGGTGCTCACCA**C**CACAGCGCTGAGCGTC- 3'

Anti-sense 5' – GTATGCGAT***GGTACC***TGTATTAATACTAGATTCTGTCCA

The mutated base is shown in bold and the *Kpn*I restriction site in bold italics.

In parallel, a second reaction containing a sense primer designed to add a HA tag and a *Hin*dIII restriction site and the anti-sense mutation primer was set up, using the conditions outlined in section 2.7.10.1.

Sense 5' – CCTCACCT***AAGCTT***GCCACCATGTATCCTTACGACGTTCCTGACTACGCATGGAACGCGACGCCCAGCGAAGAG – 3'

Anti-sense 5' – GACGCTCAGCGCTGTG**G**TGGTGAGCACCGTG – 3'

The *Hin*dIII restriction site is shown in bold italics, the HA tag is underlined and the mutated base shown in bold.

The products from both reactions were PCR purified (as in section 2.7.11), diluted 1:200 in sterile H_2O and 0.5 µL of each product used as a template for another PCR reaction. In this reaction the extreme primers were used to amplify the template. The product from this reaction was sequenced to confirm the presence of the mutation.

The amplified fragment and vector containing eCFP (pECFP-N1) were sequentially digested with *Hin*dIII and *Kpn*I. The fragment was ligated in-frame with eCFP and transformed into competent XL1-Blue bacteria. Colonies were picked and the sequences of positive clones were verified using DNA sequencing.

2.8 Cell culture protocols

2.8.1 Maintenance of HEK293T cells

Human embryonic kidney cells stably expressing the SV40 large T-antigen (HEK293T) were grown in DMEM (-sodium pyruvate) supplemented with 10 $\%_{(v/v)}$ NBCS and 2 mM L-glutamine. Cells were grown in a humidified incubator (95 % air/5 % CO_2; 37 ^oC).

2.8.2 Maintenance of Flp-In™ T-REx™ HEK293 cells

Cells were maintained in DMEM (with 4.5 g/L glucose, L-glutamine, - sodium pyruvate). The media was supplemented with 10 $\%_{(v/v)}$ FCS, 1 $\%_{(v/v)}$ penicillin/streptomycin mix, 1 $\%_{(v/v)}$ MEM non-essential amino acid mix and 10 µg/mL blasticidin. Cells were grown in a humidified incubator (95 % air/5 % CO_2; 37 °C).

2.8.3 Passage of cells

Confluent HEK293T or Flp-In™ T-REx™ HEK293 cells were washed once with sterile PBS to remove all traces of media. Sterile 0.25 % trypsin-EDTA solution was then added to the cells and flasks were gently rotated to cover the monolayer and placed in the incubator (for up to 5 min) until cells became detached. Once detached, 7 mL of fresh media was added to inactivate the trypsin and the cells were centrifuged at 288 x *g* (5 min; 25 ºC). The resulting pellet was resuspended in fresh media and the suspension split into flasks, dishes, plates or coverslips as required.

2.8.4 Transient transfection

Transfection of cells was performed when the cells had reached 60-70 % confluency. Briefly, for a 10 cm^2 dish, 5 µg of DNA was diluted in optimem to a final volume of 600 µL. To this 20 µL of Lipofectamine diluted in 580 µL optimem was added dropwise. The resulting mixture was incubated for 30 min (25 ºC). During this period cells were washed with optimem. After 30 min media was removed from the cells and the DNA/Lipofectamine/optimem mix added, made up to a volume of 5 mL with fresh optimem. After 5-7 h the transfection mixture and replaced with the appropriate growth media. The amount of DNA transfected was scaled up or down depending on the dimensions of the culture vessel.

2.8.5 Generation of a Flp-In™ T-REx™ HEK293 inducible cell line

Flp-In™ T-REx™ HEK293 cells were transfected with 0.8 µg of the GRLN-R construct (in pcDNA5/FRT/TO vector) and 7.2 µg pOG44 vector using Lipofectamine as described in section 2.8.4. After 48 h, the medium was changed to Flp-In™ T-REx™ HEK293 media (as detailed in section 2.8.2) with 200 µg/mL hygromycin to allow selection of stably transfected cells. A doxycycline titration and time course was performed to determine optimal time and doxycycline concentration required to initiate expression of receptor.

2.8.6 Cell harvesting

Transfected cells were harvested 24-48 h post-transfection. Stably transfected Flp-In T-REx HEK293 cells were harvested after 16 h doxycycline treatment. In both instances the media was removed and cells were washed three times in 5 mL ice cold PBS. Cells were scraped from the dish using a disposable cell scraper and transferred to a 15 mL centrifuge

tube and centrifuged (288 x *g*; 5 min; 4 ^{o}C). After discarding the supernatant, the cell pellet was frozen at –80 ^{o}C until required.

2.8.7 Pertussis toxin treatment

Cells were treated with 25 ng/mL *Bordella pertussis* toxin added to appropriate growth media. Cells were grown in a humidified incubator (95 % air/5 % CO_2; 37 °C) for 16 h prior to cell harvesting.

2.9 Biochemical assays and other methods of analysis

2.9.1 Preparation of cell membranes

Harvested pellets were thawed and re-suspended in Tris-EDTA buffer. The cells were homogenised (50 passes of a Teflon-in-glass homogeniser) and the resulting suspension centrifuged (288 x *g*; 10 min; 4 °C) to remove unbroken cells and nuclei. The supernatant was ultracentrifuged (50,000 x *g*; 30 min; 4 °C) in an Optima TLX Ultracentrifuge (Beckam Coulter, Palo Alto, CA). The resulting pellet was re-suspended in Tris-EDTA buffer and passed 10x through a 25-gauge needle. The protein concentration was determined as detailed in section 2.9.3 and the membranes stored at –80 ^{o}C until required.

2.9.2 Preparation of cell lysates

Cells were grown on 6-well sterile tissue culture plates. 1x radioimmunoprecipitation (RIPA) buffer was made fresh on the day of the assay by diluting 2 x RIPA (100 mM HEPES, 300 mM NaCl, 2 $\%_{(v/v)}$ Triton X-100, 1 $\%_{(w/v)}$ sodium deoxycholate, 0.2 $\%_{(w/v)}$ SDS; pH 7.4) buffer in H_2O – this was supplemented with 0.5 M sodium fluoride, 0.5 M EDTA, 0.2 M $NaPO_4$, 5 $\%_{(v/v)}$ ethylene glycol and 1 x EDTA-free protease cocktail inhibitor tablet. Cells were washed 3 x 5 min with PBS before the addition of 200-500 µL 1 x RIPA buffer. The mixture was rotated for 1 h at 4 ^{o}C on a rotating wheel before centrifugation (16,000 x *g*; 4 ºC; 30 min) to pellet cellular debris. Protein concentration was determined (as described in section 2.9.3) and the lysates diluted to the required concentration before the addition of Laemmli buffer. Lysates were stored at –20 ºC until required.

2.9.3 Bicinchoninic acid protein quantification

The protein concentration of samples was quantified using the BCA assay. This assay utilises bicinchoninic acid (BCA) and copper sulphate solutions. Protein reduces Cu(II) ions to Cu(I) ions in a concentration-dependent manner. Reduced Cu(I) can be bound by BCA which causes a colour change that has an absorption maximum of 562 nm. BSA of known concentrations (0.2-2.2 mg/mL) was used to construct a standard curve, which allows the concentrations of unknown samples to be established. A 1:50 ratio of reagent A (1 $\%_{(w/v)}$ BCA, 2 $\%_{(w/v)}$ Na_2C0_3, 0.16 $\%_{(w/v)}$ sodium tartrate, 0.4 $\%_{(w/v)}$ NaOH, 0.95 $\%_{(w/v)}$ $NaHCO_3$; pH 11.25) : reagent B (4 $\%_{(w/v)}$ $CuSO_4$) was mixed and 200 µL of this solution added to 10 µL of protein standard or unknown sample in a 96-well ELISA plate. The assay was incubated (30 min; 37 ^oC) before the absorbance was read at 600 nm.

2.9.4 Deglycosylation with Endo H or PNGaseF

To 30 µg of protein 1 unit (U) of PNGaseF or 1 mU of Endo H was added. Samples were digested overnight (4 ºC) before being resolved by sodium dodecyl sulphide polyacrylamide gel electrophoresis (SDS-PAGE; section 2.9.6) followed by western blotting (section 2.9.7).

2.9.5 Co-immunoprecipitation

Cells were grown in 6-welled plates and receptor expression induced by addition of doxycycline (6 ng/mL). Protein G beads (15 µL/sample) were washed twice in IPP500 (10 mM Tris-base, 500 mM NaCl, 0.1 $\%_{(v/v)}$ NP-40, 0.1 $\%_{(v/v)}$ Tween-20, 0.1 $\%_{(w/v)}$ CHAPS, 0.1 $\%_{(w/v)}$ BSA; pH 8.0) and incubated rotating with 2 µL anti-GFP antibody for 16 h at 4 °C. Cells were washed twice with PBS before the addition of 750 µL of IPP100 (10 mM Tris-base, 100 mM NaCl, 0.1 $\%_{(v/v)}$ NP-40, 0.1 $\%_{(v/v)}$ Tween-20, 0.1 $\%_{(w/v)}$ CHAPS and 0.1 $\%_{(w/v)}$ BSA; pH 8.0) supplemented with an EDTA-free protease inhibitor cocktail tablet, to lyse the cells. This mixture was rotated for 1 h at 25 °C before centrifugation (16,000 x *g*; 10 min; 4 °C). At this stage an aliquot of sample was removed to measure protein concentration (as detailed in section 2.9.3) and the protein concentrations of the samples were equalised. To investigate protein expression a 200 µL sample of lysate was reserved and to this 50 µL of 5x Laemmli buffer with was added. Protein G beads were washed two times in IPP500 then twice in IPP100. The remainder of the lysates were added to the washed protein G beads. After 16 h incubation on a rotating wheel (4 ˚C) the samples were centrifuged (800 x *g*; 1 min; 4 °C) and washed two times with IPP100, twice with IPP500 and once with 10 mM Tris pH 8.0 with EDTA-free protease inhibitor cocktail. Any

remaining liquid was removed using a 30G needle before the addition of 30 µL 2x Laemmli buffer. Both the immunoprecipitated samples and the cell lysates were then subjected to SDS-PAGE and western blotting.

2.9.6 Sodium dodecyl sulphide polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were resolved using SDS-PAGE. Precast NuPage Novex Bis-Tris gels with a 4-12 % acrylamide concentration were locked into an XCell Surelock mini-cell gel tank and covered in NuPage MOPS SDS buffer. To allow estimation of protein weight, samples were compared with the full range molecular weight Rainbow™ marker. The gel was run at 200 V until the dye front reached the base of the gel.

2.9.7 Western blotting

Following separation of samples by SDS-PAGE (as detailed in section 2.9.6) proteins were electrophoretically transferred onto nitrocellulose using the XCell II blot module. Proteins were transferred at 30 V (1 h) in transfer buffer (0.2 M glycine, 25 mM Tris and 20 $\%_{(v/v)}$ methanol). To block non-specific binding sites, the membrane was incubated on a rotating incubator for 2 h at room temperature in 5 $\%_{(w/v)}$ low-fat milk, PBS+ 0.1 $\%_{(v/v)}$ Tween 20 (PBS-Tween). The membrane was incubated (16 h; 4 °C) with primary antibody in 5 $\%_{(w/v)}$ low fat milk dissolved in PBS-Tween containing the required antibody dilution (Table 2-1). The membrane was washed three times for 5 min in PBS-Tween. Secondary antibody linked to horseradish peroxidase was diluted in 5 $\%_{(w/v)}$ low fat milk dissolved in PBS-Tween and incubated with the membrane at room temperature for 1 h. The membrane was washed three times for 5 min in PBS-Tween followed by incubation with ECL solution for 5 min and exposure to blue Kodak film as required.

Table 2-1 – Primary and secondary antibody dilutions used for western blotting

Primary antibody	Dilution factor	Secondary antibody	Dilution factor
Anti-GFP (raised in sheep)	1:10000	Anti-goat	1:20000
Anti-GFP	1:10000	Anti-rabbit	1:10000
Anti-HA	1:5000	Anti-mouse	1:10000
Anti-VSV-G	1:2000	Anti-rabbit	1:10000
Anti-calnexin	1:5000	Anti-rabbit	1:10000

2.9.8 [^{35}S]GTPγS binding assay with immunoprecipitation step

This assay was used to measure G protein activation via the $G_{\alpha q/11}$ pathway. [^{35}S]GTPγS binding experiments were initiated by the addition of 10 µg of membranes (prepared as described in section 2.9.1) to assay buffer (20 mM HEPES; 3 mM $MgCl_2$; 100 mM NaCl; 1 µM GDP; 0.2 mM ascorbic acid; pH 7.4) and 50 nCi [^{35}S]GTPγS containing the test compound. Non-specific binding was determined by addition of 100 µM cold (i.e. unlabelled) GTPγS. Reactions were incubated for 30 min at 30 ^{o}C before the reaction was terminated by the addition of 500 µL of ice-cold assay buffer supplemented with a protease-cocktail inhibitor tablet. Samples were centrifuged (16,000 x *g*; 10 min; 4 ^{o}C) and the resulting pellets were re-suspended in 50 µL solubilisation buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA, 1.25 $\%_{(v/v)}$ NP-40, 0.2 $\%_{(w/v)}$ SDS; pH 7.4). Samples were pre-cleared for 1 h by the addition of 60 µL of a mixture of pansorbin beads in bead buffer (2 % $BSA_{(w/v)}$, 0.1 $\%_{(w/v)}$ sodium azide; pH 7.4) i.e. 40 µL bead buffer and 20 µL pansorbin mix per sample. Samples were centrifuged (16,000 x *g*; 10 min; 4 ^{o}C) and the supernatant transferred to clean Eppendorf tubes. Immunoprecipitation was performed by addition of 50 µL of protein G beads with rabbit anti-$G_{\alpha q}$ anti-sera (approximately a 1:200 dilution; Mitchell et al., 1993) and subsequently rotated for 16 h at 4 ºC. The beads were centrifuged (16,000 x *g*; 5 min; 4 ^{o}C) and washed with 500 µL ice-cold solubilisation buffer. The remaining liquid was removed, 1 mL of liquid scintillation cocktail added and bound radioactivity was determined using a 5 min [^{35}S] counting programme on a LS 6500 scintillation counter (Beckman Coulter, Palo Alto, CA).

2.9.9 [^{35}S]GTPγS filtration binding assay

This assay was used to measure G protein activation via the $G_{\alpha i/o}$ pathway. Cell membranes (10 µg), 10 µM GDP and 0.1 nM [^{35}S]GTPγS and ligand were mixed in a final volume of 1 mL GTPγS filtration buffer (20 mM HEPES, 100 mM NaCl, 6 mM $MgCl_2$, 0.1 $\%_{(w/v)}$ BSA; pH 7.4). The assay was incubated for 30 min at 30 ºC before termination by rapid filtration through GF/C filters pre-soaked in ice-cold PBS using a Brandell cell harvester (Brandell, Gaithersberg, MA). The filters were washed three times in 3 mL ice-cold PBS and allowed to air-dry before transfer to tubes and the addition of 3 mL liquid scintillation cocktail. Bound radioactivity was determined using a 5 min [^{35}S] counting programme on a LS 6500 scintillation counter (Beckman Coulter, Palo Alto, CA).

2.9.10 [^{35}S]GTPγS scintillation proximity assay

This assay was used to measure [^{35}S]GTPγS binding in membranes prepared from a stable cell line expressing $G_{\alpha o1}$ and transiently transfected with GRLN-R (kindly supplied by GlaxoSmithKline). In this assay carbohydrate residues in cell membranes (prepared from cells expressing the receptor of interest) are bound to wheat germ agglutinin (WGA) on the outside of the LEAD-seeker imaging beads, which are filled with scintillant. These beads emit light when an emission from an isotope occurs in close proximity i.e. when [^{35}S]GTPγS binds to receptor.

The test compound (0.5 µL), diluted in DMSO, was stamped onto a 384-well Lumitrac 200 white plate, using the Biomek FX (Beckman Coulter, Palo Alto, CA). DMSO alone was used as a negative control. Cell membranes were defrosted, diluted to 50 µg/mL and added to scintillation proximity assay buffer (20 mM HEPES, 100 mM NaCl, 10 mM $MgCl_2$; pH 7.4) supplemented with 0.05 $\%_{(v/v)}$ BSA and 0.05 $\%_{(v/v)}$ pluronic F-127. WGA-polystyrene LEADseeker imaging beads and GDP were added to the membrane/assay buffer mixture at a concentration of 2 mg/mL and 8 µM, respectively, and pre-coupled under gentle shaking in the dark for 30 min at room temperature. [^{35}S]GTPγS was diluted to 1.2 nM in assay buffer. To each well of the 384-well plate 25 µL of the membrane/assay/GDP/bead mixture and 25 µL of diluted [^{35}S]GTPγS was added and the plate sealed and centrifuged (800 x *g*; 2 min; 25 °C). The assay was incubated in the dark for 80 min at 25 °C and read for 5 min in a Viewlux platereader (Perkin Elmer, Boston, MA) in luminescene mode at an emission of 613 nm and 6 x binning.

For competition [^{35}S]GTPγS experiments the assay was performed as above except that the compound that is kept at a fixed concentration was pre-incubated with the assay buffer/membrane/bead/GDP mixture at 2 x the final concentration.

2.9.11 [His[^{125}I]]-ghrelin association and dissociation binding assay

Radioligand binding assays were performed by addition of 5 µg cell membranes to ice-cold assay buffer (50 mM Tris-base, 2 mM EGTA, 0.1 $\%_{(w/v)}$ BSA; pH 7.3) in a final volume of 150 µL in cooled 96-well blocks. To determine non-specific binding, cold ghrelin was added to a final concentration of 1 µM. At various time points a final concentration of 83 pM [His[^{125}I]]-ghrelin was added to the wells. As a control to determine binding of radioactivity to filters, membranes were replaced with assay buffer. The assay was

terminated by rapid filtration through GF/B filters pre-soaked in 0.5 $\%_{(w/v)}$ PEI and the filters were washed three times in 1 mL ice-cold assay buffer. The filters were allowed to air-dry before the addition of 100 µL scintillation cocktail. The filters were read (1 min/well) on a Topcount NXT™ microplate scintillation counter (Packard Instrument Co, Downers Grove, IL).

Dissociation binding experiments were carried out as above. Association and dissociation was staggered so that the whole plate was harvested at the same time.

2.9.12 Two point dissociation assay

5 µg of cell membranes in ice-cold assay buffer (50 mM Tris-base, 2 mM EGTA, 0.1 $\%_{(w/v)}$ BSA; pH 7.3) was added to cooled 6 mL glass binding tubes. To determine non-specific binding, 30 µL of a final concentration of 1 µM cold ghrelin was added. Association of [His[^{125}I]]-ghrelin to the GRLN-R was initiated by the addition of a final concentration of 83 pM of radioligand. After 120 min, dissociation was initiated by the addition of 10 µL of a final concentration of 1 µM cold ghrelin plus 10 µL of buffer or varying concentrations of GHRP-6, L-692,585 or MK-677. After 60 min of dissociation, the experiment was terminated by rapid filtration through GF/C filters presoaked in 0.5 $\%_{(w/v)}$ PEI using a Brandel cell harvester (Brandell, Gaithersburg, MA). The filters were washed three times with 3 mL ice-cold assay buffer and counted using a 60 s [^{125}I] counting programme on a LS 6500 scintillation counter (Beckman Coulter, Palo Alto, CA).

Association and dissociation was staggered so the whole assay could be harvested at the same time.

2.9.13 [His[^{125}I]]-ghrelin competition binding assay

Competition assays were set up as described in section 2.9.11 with the addition of varying concentrations of cold ligands. After the addition of 83 pM [His[^{125}I]]-ghrelin the assay was incubated for 2 h at 4 ºC. The assay was terminated and results measured as described in section 2.9.11.

2.9.14 Total inositol phosphate accumulation assay

Cells were seeded onto poly-D-lysine-coated 12 well sterile tissue culture plates and grown to 80-90 % confluence on the day of the assay. Sixteen hours prior to the assay, the media was replaced with media supplemented with 1 µCi/mL [^{3}H]*myo*-inositol in the presence or

absence of 6 ng/mL doxycycline and the cells left in a humidified incubator for 16 h to allow incorporation of [^{3}H]*myo*-inositol. Subsequently, the medium was removed and cells washed twice with 1 mL Krebs-Ringer buffer (145 mM NaCl, 20 mM HEPES, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 5 mM KCl, 1.3 mM $CaCl_2$, 10 mM glucose; pH 7.4) supplemented with 10 mM LiCl. A further 1 mL of Krebs-Ringer buffer with LiCl was added to the cells and the cells were incubated for 30 min at 37 ºC in a humidified incubator. LiCl blocks the breakdown of inositol phosphates into *myo*-inositol, leading to accumulation of inositol phosphates within the cell. Inositol phosphate formation was stimulated by the addition of ligand in Krebs-Ringer buffer supplemented with 0.25 $\%_{(w/v)}$ BSA and 10 mM LiCl for 30 min (37 ºC; 5 % CO_2/95 % air). The assay was terminated by lysis of the cells by addition of 250 µL of ice-cold 1.5 M perchloric acid and incubation for 30 min. An aliquot (0.75 mL) of the mixture was removed and neutralised by the addition of 300 µL of 2 M KOH in 1 M Tris-base. The samples were centrifuged (800 x *g*; 5 min; 4 ºC) and a 0.75 mL sample of the supernatant was mixed with 2.5 mL of 5 mM disodium tetraborate containing 0.5 M EDTA (pH 7.2).

Total inositol phosphates were extracted by ion-exchange chromatography on formate-form Dowex columns according to the method of Berridge and Irvine (1989). Samples were applied to Dowex columns and allowed to drain by gravity flow, then washed four times with 2.5 mL 5 mM disodium tetraborate in 60 mM sodium formate to remove unbound [^{3}H]*myo*-inositol and [^{3}H]glycerolphosphoinositol. Columns were placed over 20 mL scintillation vials and total inositol phosphates eluted by the addition of 2x 2.5 mL of 1 M ammonium formate in 0.1 M formic acid, followed by addition of 10 mL scintillation fluid to the eluant. Radioactivity was determined using a 120 s [^{3}H] counting programme on a LS 6500 scintillation counter (Beckman Coulter, Palo Alto, CA).

After use the columns were reformed by 4 x 2.5 mL washes with 0.4 M formic acid, followed by 4 x 2.5 mL washes with H_2O. Columns were then capped and stored in 2.5 mL H_2O.

2.9.15 cAMP accumulation assay

The level of cAMP accumulation was measured using a AlphaScreen cAMP assay kit. Stimulation buffer (0.5 mM IBMX, 5 mM HEPES, 0.1 % BSA, diluted in 1 x HBSS; pH 7.4) and detection buffer (5 mM HEPES, 0.1 % BSA, 0.3 % Tween-20, pH 7.4) were prepared fresh on the day of assay. For 384-data points 1U/15 µL of donor beads and

biotinylated cAMP were added to detection buffer and the volume made up to 6 mL. This mixture was incubated with gentle agitation in the dark (30 min; 25 ºC).

Cells were seeded as to be 70-90 % confluent on the day of the assay and were treated with 6 ng/mL doxycycline (16 h) to induce receptor expression. Cells were subsequently detached by addition of 5 mL Versene (5 min; 37 ºC) and collected by centrifugation (275 x *g*; 5 min). The pellet was re-suspended in PBS and the cells counted using a haemocytometer. The cell mixture was centrifuged again (275 x *g*; 5 min) before supernatant was decanted and the pellet re-suspended to a final concentration of 10,000 cells/μL in PBS. An acceptor bead/cell mix was prepared by adding 0.2 U/μL anti-cAMP acceptor beads to 400 μL of the cell solution, which was subsequently made to 2 mL using stimulation buffer.

For $G_{\alpha i/o}$ assays forskolin was diluted to 0.4 μM in stimulation buffer and ligand was diluted to four times the final concentration required. A 1:1 ratio of ligand and forskolin was then prepared. For $G_{\alpha s}$ assays ligand was diluted to two times the final concentration required in stimulation buffer.

All assays were carried out in 384-well white opaque plates (Perkin Elmer, Boston, MA), 5 μL of acceptor bead/cell mix were added to each well followed by 5 μL of ligand or ligand/forskolin mix. Plates were sealed and incubated in a humidified incubator (37 ºC; 5 % CO_2/95 % air) for 30 min. After 30 min 15 μL of detection mixture was added per well and the plate incubated in the dark for 1 hr (25 ºC). The plate was read on an EnVision multilabel plate reader (Perkin Elmer, Boston, MA).

2.9.16 Membrane staining

Cells were grown on poly-D-lysine coated coverslips in 6-welled plates and receptor expression was induced with 6 ng/mL doxycycline (16 h), as required. Cells were washed 2x 5 min with 2 mL room-temperature PBS, before the cells were fixed by the addition of 2 mL 3.7 $\%_{(v/v)}$ formaldehyde for 10 min. Cells were washed three times for 5 min with PBS. WGA-Alexa Fluor® 594 conjugate was diluted in PBS to a concentration of 10 μg/mL, added to the plates and samples were incubated for 30 min in the dark. Coverslips were mounted onto microscope slides using Immu-mount and stored in the dark at 4 ºC before being imaged using epifluorescence microscopy (section 2.9.18)

2.9.17 Endoplasmic reticulum (ER) staining

Cells were grown on poly-D-lysine coated coverslips in 6-welled plates; receptor expression was induced by the addition of 6 ng/mL doxycycline for 16 h. Subsequently, cells were rinsed twice with 2 mL room temperature PBS before incubation in a humidified incubator with 1 μM ER-Tracker™ red (BODIPY glibenclamide which binds to sulphonylurea receptors of ATP-sensitive potassium channels prominenet on the ER) for 20 min at 37 ˚C. Cells were then washed two times for 5 min with 2 mL room-temperature PBS before samples were fixed for 10 min with 2 mL 3.7 $\%_{(v/v)}$ formaldehyde. Cells were washed three times for 5 min with PBS before coverslips were mounted onto microscope slides and stored as described in section 2.9.16.

2.9.18 Epifluorescence microscopy

Formaldehyde-fixed cells were imaged using an inverted Nikon TE2000-E microscope (Nikon Instruments, Melville, NY) equipped with a x 60 (NA_1.4) oil-immersion Plan Fluor Apochromat lens, a z axis linear encoder, and a cooled digital Cool Snap-HQ CCD camera (Roper Scientific/Photometrics, Tucson, AZ). Epifluorescence excitation light was generated by an ultrahigh point intensity 75-watt xenon arc Optosource lamp (Cairn Research, Faversham, Kent) coupled to a computer-controlled Optoscan monochromator (Cairn Research, Faversham, Kent). The monochromator was set to 436/12 nm for excitation of eCFP. eCFP excitation light was transmitted through the objective lens using a 455DCLP single pass dichroic. eCFP fluorescence emission was controlled via a high speed filter wheel device (Prior Instruments Ltd., Cambridge, Cambridgeshire) containing HQ480/40 nm emitter. Images were collected using a Cool Snap-HQ digital camera operated in 12-bit mode. Computer control of all electronic hardware and camera acquisition was achieved using Metamorph software (version 6.3.3; Molecular Devices Corp., Downing, PA).

2.9.19 Analysis of cell surface and intracellular receptors

Fluorescent microscopy was used to image fluorescently-tagged receptors. To quantitatively measure the fluorescence intensity, images were deconvoluted using an iterative and constrained algorithm (Autodeblur software, version 9.3.6, Autoquant Imaging, Watervliet, NY) to produce high-resolution images that were used to identify groups of adjacent pixels or ‘segments’ that corresponded to receptors located on the cell surface or intracellular vesicles. A manual segmentation method, using the object counting module of the Autoquant imaging software, was used to quantify the mean total

fluorescence intensity values corresponding to receptors located at the membrane surface and the cytoplasm of the cell. The total fluorescence pixel intensity measured from membrane and intracellular receptor segmented pixels was expressed as a percentage of the total eCFP fluorescent intensity.

2.9.20 Data analysis

2.9.20.1 General

Data was analysed using GraphPad Prism 4.0 (GraphPad software Inc., San Diego, CA). Data is expressed as the mean ± the standard error of the mean (S.E.M). Statistical and post-hoc analysis appropriate to the data were applied as indicated in individual chapters. Differences were considered to be statistically significant if $p<0.05$.

Unless otherwise stated, concentration-responses curves were analysed with non-linear regression (sigmoidal concentration-response curves with Hill slopes constrained to 1).

2.9.20.2 Global fit model

The global fit model (Motulsky and Christopoulos, 2004) was used to analyse Schild regression data using GraphPad Prism 4.0 software (GraphPad software Inc., San Diego, CA).

Equation 2-1 – Global fit model described by Motulsky and Christopoulos (2004).

$$Y = Bottom + \frac{(Top - Bottom)}{1 + \left(\frac{10^{\log EC_{50}} \left[1 + \left([B] / 10^{-pA_2} \right)^{s} \right]}{[A]} \right)^{n_H}}$$

In the global fit equation (Equation 2-1): Y = response, *Top* represents the maximal asymptote of the curves, *Bottom* represents the lowest asymptote (basal response) of the curves, log EC_{50} represents the logarithm of the agonist EC_{50} in the absence of antagonist/inverse agonist, [A] represents the concentration of the agonist, [B] represents the concentration of the antagonist/inverse agonist, n_H represents the Hill slope of the agonist curve, *s* represents the Schild slope for the antagonist, and pA_2 represents the

negative logarithm of the concentration of antagonist that shifts the agonist EC_{50} by a factor of 2 (Langmead et al., 2005).

To test whether the Schild slope was statistically different to unity an F-test was used, if the slope was not significantly different to unity the data was re-fitted with the Schild slope = 1 to allow determination of the pK_B. F-tests were also used to allow comparison of fit between two equations.

2.9.20.3 Modified operational model of allosterism

A modified version of the operational model of allosterism (formulated by Leach et al., 2007; modified by Dr. Christopher Langmead, GlaxoSmithKline) was used to determine whether data generated in Chapter 3 reflected an interaction between ghrelin and the growth hormone secretagogues that was allosteric or simply competitive in nature. Data was kindly analysed by Dr. Christopher Langmead using GraphPad Prism version 5.0 (GraphPad software Inc., San Diego, CA).

The [^{35}S]GTPγS binding datasets studying the effect of multiple, fixed concentrations of ghrelin on concentration-response curves to GHRP-6, MK-677 or L-692,585 were analysed globally according to a modified version of an operational model of allosterism which accounts for allosteric modulation of affinity, efficacy and allosteric agonism. The equation represents a simplified model whereby it is assumed that the concentration-response curve data is to a full agonist Equation 2-2:

Equation 2-2 – Modified operational model of allosterism (originally described by Leach et al., 2007)

$$Y = Basal + \frac{(E_M - Basal)\cdot(([A](K_B + \alpha\beta[B])) + \tau_B[B]\cdot EC_{50})^n}{(([A](K_B + \alpha\beta[B])) + \tau_B[B]\cdot EC_{50})^n + \left(EC_{50}{}^n \cdot (K_B[B])^n\right)}$$

Where Y = response, *Basal* is the response in the absence of ligand, [A] is the concentration of orthosteric ligand and [B] is the concentration of allosteric ligand, EC_{50} is the midpoint of the full agonist concentration-response curve, K_B is the equilibrium dissociation constant of the putative allosteric ligand, τ_B denotes the capacity of the putative allosteric ligand to exhibit agonism (a function of the intrinsic efficacy and receptor expression) and $\alpha\beta$ represents a net affinity/efficacy co-operativity parameter which describes the effect of the putative allosteric ligand on agonist function. The terms

E_M and n denote the maximal possible system response and the slope factor of the transducer function that links occupancy to response, respectively.

If the interaction between ghrelin and GHRP-6, MK-677 or L-692,585 were competitive, then the value of $\alpha\beta$ would be zero (because the value of the affinity cooperativity factor, α, would be zero) and Equation 2-2 would reduce to that for the interaction of a partial agonist and full agonist binding to the same site. Therefore, the datasets were analysed under two conditions – where the value of $\alpha\beta$ is left to float or constrained to zero. Comparison of the two fits was performed using Akaike's Information Criterion (AICc; Motulsky and Christopoulos, 2004) to determine which fit was most likely to be correct.

In order to further validate the results of the interaction studies, complementary experiments were performed in which the effects of multiple, fixed concentrations of GHRP-6, MK-677 or L-692,585 on concentration-response curves to ghrelin was examined. These data were analysed using a re-cast version of Equation 2-2 such that the concentration of ghrelin is the independent variable on the x-axis (i.e. full agonist versus partial agonist). As previously, the datasets were analysed under two conditions (where the value of $\alpha\beta$ is left to float or constrained to zero) and the fits compared using AICc.

3 Investigating ago-allosteric modulation of the GRLN-R via activation of the $G_{\alpha i/o}$ pathway

3.1 Introduction

Allosteric modulators are defined as ligands that act to increase or decrease the actions of an orthosteric agonist or antagonist (i.e. an agonist or antagonist which binds to the same site as the endogenous ligand), by combining with a distinct, or 'allosteric', site on a receptor macromolecule (Neubig et al., 2003). Within the Pharmaceutical industry, allosteric modulators have attracted a great deal of attention as they may have many advantages over orthosteric ligands. For example, because, by definition, allosteric modulators only modulate the effect of the existing orthosteric ligand, allosteric modulators display a saturability of effect. Thus large doses of modulators can be administered with a lower propensity towards target-based toxicity than orthosteric ligands (Conn et al. 2009). Indeed, allosteric modulators have proven to be clinically useful drugs with two GPCR modulators entering the market; maraviroc, a drug launched for the treatment of HIV infections, and cinacalcet, a drug licensed to treat disorders of the calcium-sensing receptor, such as hyperparathyroidism (Conn et al., 2009).

In 2005 a study published by Thue Schwartz's group demonstrated GHRP-6 and L-695,429 acted as allosteric regulators of the GRLN-R. The growth hormone secretagogues (GHRP-6, L-692,429 and MK-677) had been previously described as agonists of the GRLN-R (Howard et al., 1996). However, in Schwartz's study it was found that, when co-administered with ghrelin, the growth hormone secretagogues all acted to increase the maximum efficacy of ghrelin (Holst et al., 2005). Furthermore, co-administration of ghrelin with either L-692,429 or GHRP-6 either increased or decreased (respectively) the potency of ghrelin (Holst et al., 2005). Therefore GHRP-6 and L-692,429 could act both as agonists of the GRLN-R and as allosteric enhancers or allosteric antagonists of ghrelin function. To describe the effects of GHRP-6 and L-692,429 the term 'ago-allosteric modulator' was created; this defines a ligand that functions both as an (allosteric) agonist and as an allosteric modulator of the efficacy and/or the potency of an orthosteric ligand (Schwartz and Holst, 2006; Holst et al., 2005).

Although by definition the binding site of an allosteric modulator is distinct from the orthosteric site (Neubig et al., 2003), in general binding sites for allosteric modulators are not well defined and, for the muscarinic acetylcholine receptors at least, may be located

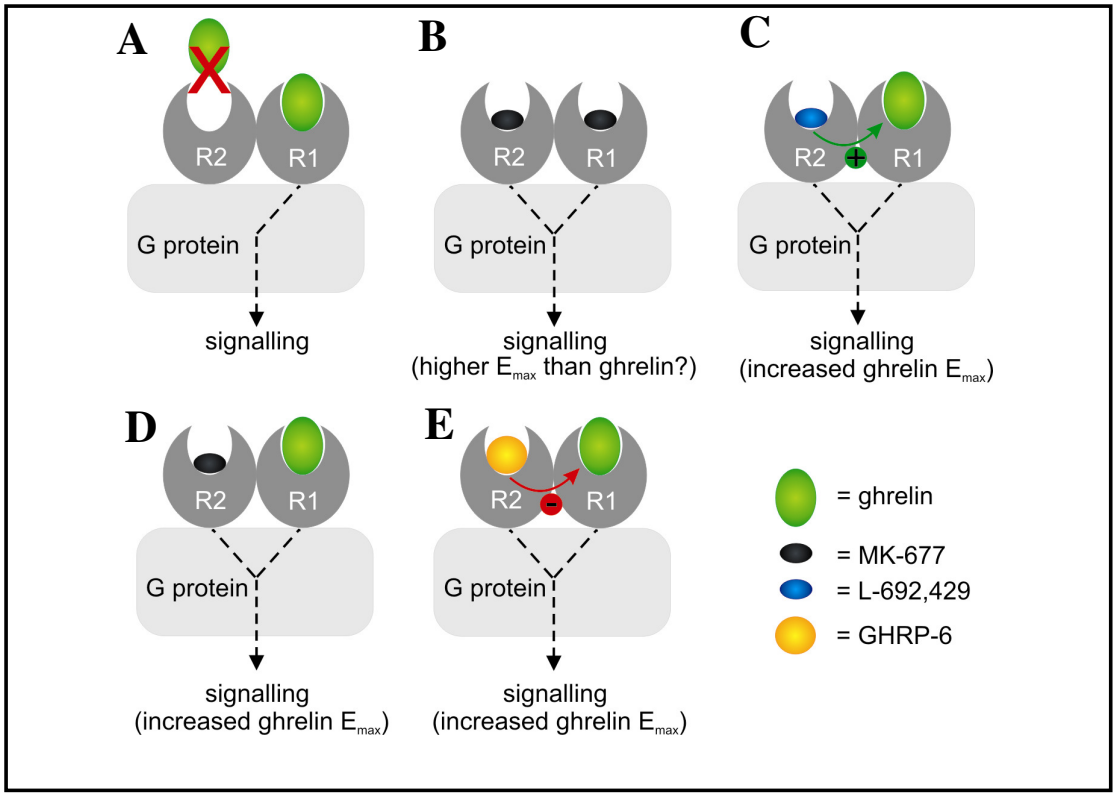

Figure 3-1 - Ago-allosteric modulation of the GRLN-R (adapted from Holst et al., 2005). A dimeric model was chosen by Holst et al. (2005) to explain the changes in ghrelin efficacy upon co-administration with GHRP-6, L-692,429 and MK-677, as well as to explain the positive and negative modulation of ghrelin signalling upon co-administration with L-692,429 and GHRP-6 (respectively). **A.** Ghrelin only binds to one protomer of the dimer. **B.** Due to their smaller size it is hypothesised that the growth hormone secretagogues could bind to both protomers leading to an increase in the observed maximum signalling compared to the maximum signalling achieved by ghrelin. **C.** Co-binding and co-activation of ghrelin with L-692,429 leads to an increase in the maximum signalling achieved by ghrelin alone, whilst shifting the ghrelin concentration-response curve to the left i.e. increasing the potency of ghrelin. **D.** Co-binding and co-activation of ghrelin with MK-677 leads to an increase in the maximum signalling achieved by ghrelin alone but has no effect on the potency of ghrelin. **E.** Co-binding and co-activation of ghrelin and GHRP-6 leads to an increase in the maximum signalling achieved by ghrelin alone, whilst shifting the concentration-response curve to ghrelin to the right, i.e. decreasing the potency of ghrelin.

close to the orthosteric site (Christopoulos and Kenakin, 2002; Birdsall and Lazareno, 2005). Indeed mutational studies of the GRLN-R have demonstrated that the binding sites for GHRP-6, L-692,429 and MK-677 overlap with, but are not identical to, the binding site for ghrelin (Feighner et al., 1998; Holst et al., 2009). Thus to describe the ago-allosteric properties of the growth hormone secretagogues a model was formed which relied on the GRLN-R existing as a homo-dimer (Figure 3-1; Holst et al., 2005). Ghrelin, due to its large size, was predicted to only bind to one protomer of the homo-dimer, leaving the second protomer free to bind the growth hormone secretagogues which acted as co-agonists (MK-677) and as positive (L-692,429) or negative (GHRP-6) modulators of ghrelin function (Holst et al., 2005).

To date, functional studies of allosterism at the GRLN-R have been limited to studying the activation of the $G_{\alpha q/11}$ pathway. However, there is a large body of evidence to suggest that many GPCRs interact with more than one type of G protein α subunit (Wess, 1998). Examples include receptors that can activate G proteins from the same subfamily, like the dopamine D_2 receptor that can activate $G_{\alpha i1\text{-}3}$ and $G_{\alpha o1}$ (Gazi et al., 2003), to receptors that can activate G proteins from all four distinct families such as the thyrotrophin receptor (Laugwitz et al., 1996). Indeed the GRLN-R has been previously described to couple to the $G_{\alpha q/11}$ (Howard et al., 1996; Kojima et al., 1999), $G_{\alpha 13}$ (Holst et al., 2005), $G_{\alpha s}$ (Kohno et al., 2003; Rossi et al., 2008) and $G_{\alpha i}$ (Bassil et al., 2007; Dezaki et al., 2007; Esler et al., 2007) pathways.

The aim of this study was to explore the allosteric modulation of the GRLN-R in the $G_{\alpha i/o}$ pathway by testing whether the growth hormone secretagogues could act both as co-agonists and as allosteric modulators of ghrelin function through activation of $G_{\alpha o1}$.

3.2 Results

3.2.1 The GRLN-R can activate the $G_{\alpha i/o}$ pathway

To measure activation of the $G_{\alpha i/o}$ pathway by the GRLN-R, [^{35}S]GTPγS binding assays were utilised. [^{35}S]GTPγS binding assays measure one of the earliest steps in GPCR signalling, the loss of GDP and the loading of GTP or, in this case, [^{35}S]GTPγS (a poorly hydrolysable analogue of GTP) onto the α-subunit of heterotrimeric G proteins. Thus incorporation of [^{35}S]GTPγS into G protein can be used as a measurement of receptor activation.

Membranes were prepared from HEK293 cells transfected to express $G_{\alpha o1}$, or to co-express $G_{\alpha o1}$ and the GRLN-R, and challenged with 1 μM ghrelin; a concentration of ghrelin that was shown to be maximally effective in stimulating $G_{\alpha q/11}$ responses, such as calcium mobilisation and inositol phosphate accumulation (Holst et al., 2005). The expression of $G_{\alpha o1}$ was confirmed using anti-$G_{\alpha o1}$ antisera (data not shown). After stimulation with ghrelin, the assay was terminated by rapid filtration through glass fibre filters, which allowed separation of G protein-bound [^{35}S]GTPγS from unbound [^{35}S]GTPγS. Terminating [^{35}S]GTPγS binding assays by the use of a filtration step is

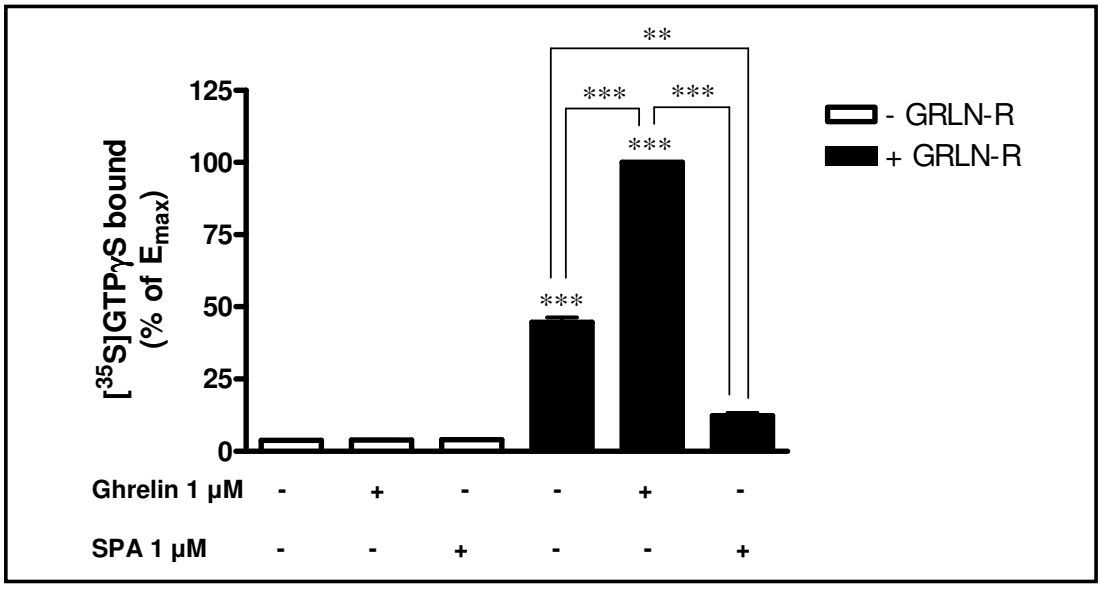

Figure 3-2 - Testing the ability of the GRLN-R to couple to $G_{\alpha i/o}$. Membranes were prepared from HEK293 cells expressing $G_{\alpha o1}$ (white bars) or co-expressing $G_{\alpha o1}$ and the GRLN-R (black bars). The ability of membranes to respond to 1 μM ghrelin or 1 μM SPA (20 min; 30 °C) was tested using a [^{35}S]GTPγS binding assay to measure G protein activation. G protein-bound [^{35}S]GTPγS was separated from the reaction mixture by rapid filtration through GF/C filters soaked in 1 x PBS and bound [^{35}S]GTPγS measured (for 5 min) using liquid-scintillation spectrometry. Data is expressed as the percentage of the maximum response achieved in each assay. Data points represent the mean ± S.E.M of three independent experiments performed in triplicate. ***p<0.001, **p<0.01 when compared using a one-way ANOVA with Tukey's multiple comparison test.

particularly suited to measure the activation of the $G_{\alpha i/o}$ family of G proteins due to a combination of their high rate of basal nucleotide exchange and relatively high expression levels in HEK293 cells (Milligan, 2003).

In this study a one-way ANOVA with Tukey's multiple comparison test confirmed that the addition of 1 μM ghrelin caused a significant ($p<0.001$) increase in bound [^{35}S]GTPγS in membranes co-expressing $G_{\alpha o1}$ and the GRLN-R (Figure 3-2). This was not observed in membranes of equivalent cells transfected to express $G_{\alpha o1}$ but not the GRLN-R, suggesting ghrelin was exerting its effect through activation of the GRLN-R. The lack of a ghrelin signal in the absence of the GRLN-R meant that HEK293 cells provided a useful null background in which to study the pharmacology of the GRLN-R.

In membranes from HEK293 cells co-expressing the GRLN-R and $G_{\alpha o1}$, a significantly higher level of basal [^{35}S]GTPγS loading was observed than in membranes from HEK293 cells transfected to express $G_{\alpha o1}$ but not the GRLN-R ($p<0.001$), thus indicating that the GRLN-R could constitutively activate the $G_{\alpha i/o}$ pathway. To further explore this, the response of membranes to 1 μM SPA was also tested. SPA has been described as an inverse agonist of the GRLN-R, with a concentration of 1 μM SPA being sufficient to fully inhibit the constitutive activity of the GRLN-R in the $G_{\alpha q/11}$ pathway (Holst et al., 2003).

Indeed in this study the addition of 1 μM SPA significantly ($p<0.01$) lowered the recovery of bound [^{35}S]GTPγS in membranes co-expressing the GRLN-R and $G_{\alpha o1}$ compared to values obtained in the absence of ligand (basal conditions) (Figure 3-2). However, in membranes expressing $G_{\alpha o1}$ but not the GRLN-R, SPA failed to alter the levels of [^{35}S]GTPγS binding compared to basal conditions. Together these results confirmed that the GRLN-R could constitutively activate the $G_{\alpha i/o}$ pathway.

3.2.2 The growth hormone secretagogues can activate the $G_{\alpha i/o}$ pathway

Previous studies have shown that GHRP-6, MK-677 and L-692,585 act as agonists at GRLN-R via activation of the $G_{\alpha q/11}$ pathway (Howard et al., 1996; Holst et al, 2005; Holst et al., 2009; Feighner et al., 1998). Whilst ghrelin has been demonstrated to activate $G_{\alpha i/o}$ (Bassil et al., 2007; Dezaki et al., 2007; Esler et al., 2007), there is currently a lack of data in the literature demonstrating the ability of the growth hormone secretagogues to activate this pathway. To test whether the growth hormone secretagogues could evoke a GRLN-R

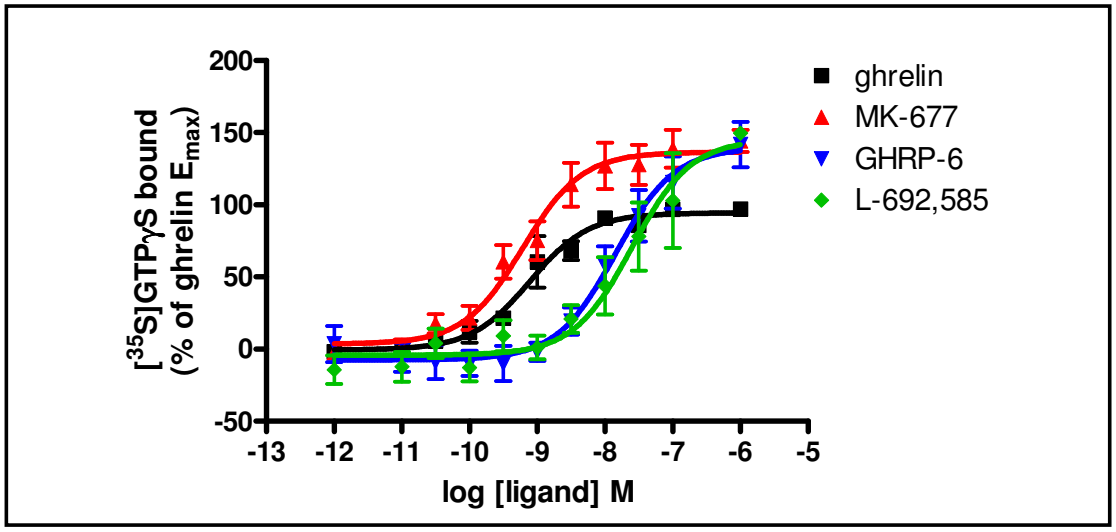

Figure 3-3 - Testing the response of the GRLN-R through activation of the $G_{\alpha i/o}$ pathway in response to ghrelin and the growth hormone secretagogues. A [^{35}S]GTPγS scintillation proximity assay was used to generate concentration-response curves to ghrelin, GHRP-6, L-692,585 and MK-677 by incubating membranes, prepared from HEK293 cells co-expressing $G_{\alpha o1}$ and the GRLN-R, with varying concentrations of ligand for 80 min (25 °C). Activation of the GRLN-R was assessed by measurement of G protein-bound [^{35}S]GTPγS (5 min/plate using a Viewlux platereader). Data are expressed as a percentage of the maximum response achieved by ghrelin. Data points represent the mean ± S.E.M of three individual experiments performed in triplicate.

response through $G_{\alpha i/o}$, membranes (kindly supplied by GlaxoSmithKline) prepared from HEK293 cells co-expressing both the GRLN-R and $G_{\alpha o1}$ were incubated with increasing concentrations of ghrelin, GHRP-6, L-692,585 (a commercially available derivative of L-692,429) and MK-677. The responses of the GRLN-R were measured using a [^{35}S]GTPγS scintillation proximity assay, which allowed measurement of [^{35}S]GTPγS binding in a high-throughput format.

In the scintillation proximity assay, beads filled with scintillant and coated with wheat-germ agglutinin are used to measure receptor activation. Briefly, carbohydrate residues present on cell membranes (prepared from cells expressing the receptor of study) bind to the wheat-germ agglutinin on the surface of the beads. When an emission from an isotope occurs in close proximity to the beads (i.e. when [^{35}S]GTPγS binds to G protein following receptor activation) the beads emit light. Measurement of emitted light allows quantification of [^{35}S]GTPγS binding.

The results of the [^{35}S]GTPγS binding assays revealed that ghrelin, GHRP-6, L-692,585 and MK-677 all caused a concentration-dependent increase in [^{35}S]GTPγS binding (Figure 3-3). Fitting the data to dose-response curves allowed the potency and efficacy values of each ligand to be obtained, as displayed in Table 3-1. Statistical tests revealed that GHRP-6 and L-692,585 acted with significant lower potencies than ghrelin ($p<0.01$; as measured using a one-way ANOVA with Dunnett's post-hoc test). Comparing the efficacies of MK-

677, GHRP-6 and L-692,585 to ghrelin revealed that the growth hormone secretagogues all acted with significantly higher efficacies than ghrelin ($p<0.05$; as measured using a one-way ANOVA with Dunnett's post-hoc test). Thus GHRP-6, L-692,585 and MK-677 could be classed as 'super-agonists' in activating the $G_{\alpha i/o}$ pathway.

Table 3-1 – Potency and efficacy of ghrelin and the growth hormone secretagogues as measured using a [^{35}S]GTPγS scintillation proximity assay.

Ligand	pEC_{50} ± S.E.M	E_{max} ± S.E.M
Ghrelin	9.11± 0.10	95.4 ± 3.4
MK-677	9.21 ± 0.12**	139.6 ± 9.9*
GHRP-6	7.85 ± 0.13	139.5 ± 5.4*
L-692,585	7.60 ± 0.17**	145.4 ± 14.0*

Data represents the mean ± S.E.M of three independent experiments performed in triplicate. *p<0.05, **p<0.01 as measured using a one-way ANOVA with Dunnett's post-hoc test. E_{max} values quoted in this Table (and all subsequent Tables) are the predicted top asymptotes of sigmoidal concentration-response curves fitted to the data.

3.2.3 Effect of ghrelin on the potency and efficacy of the growth hormone secretagogues

To further explore the effects of the ghrelin and the growth hormone secretagogues in the $G_{\alpha i/o}$ pathway and to investigate the suggestion that the growth hormone secretagogues could act as allosteric regulators of the ghrelin response (Holst et al., 2005), a series of [^{35}S]GTPγS binding studies were performed in membranes co-expressing the GRLN-R and $G_{\alpha o1}$. Concentration-response curves were generated to GHRP-6, MK-677 and L-692,585 in the presence of multiple, fixed concentrations of ghrelin. The concentrations of ghrelin chosen were 10 pM, 0.1 nM, 0.3 nM, 1 nM and 100 nM, which corresponded to 0, 20, 25, 60 and 100 % of the level of [^{35}S]GTPγS binding that could be achieved by maximally effective concentrations of ghrelin, as shown in Figure 3-3.

3.2.3.1 GHRP-6

The addition of increasing concentrations of ghrelin caused a concentration-dependent increase in the loading of [^{35}S]GTPγS at GHRP-6 concentrations of 1 pM to 10 nM (Figure 3-4A). Indeed compared to GHRP-6 acting alone, co-administration of 1 pM GHRP-6 with 0.3-100 nM ghrelin statistically increased [^{35}S]GTPγS binding ($p<0.05$ at 0.3 nM ghrelin; $p<0.001$ at 1 nM ghrelin and 100 nM ghrelin; as measured using a one-way ANOVA with

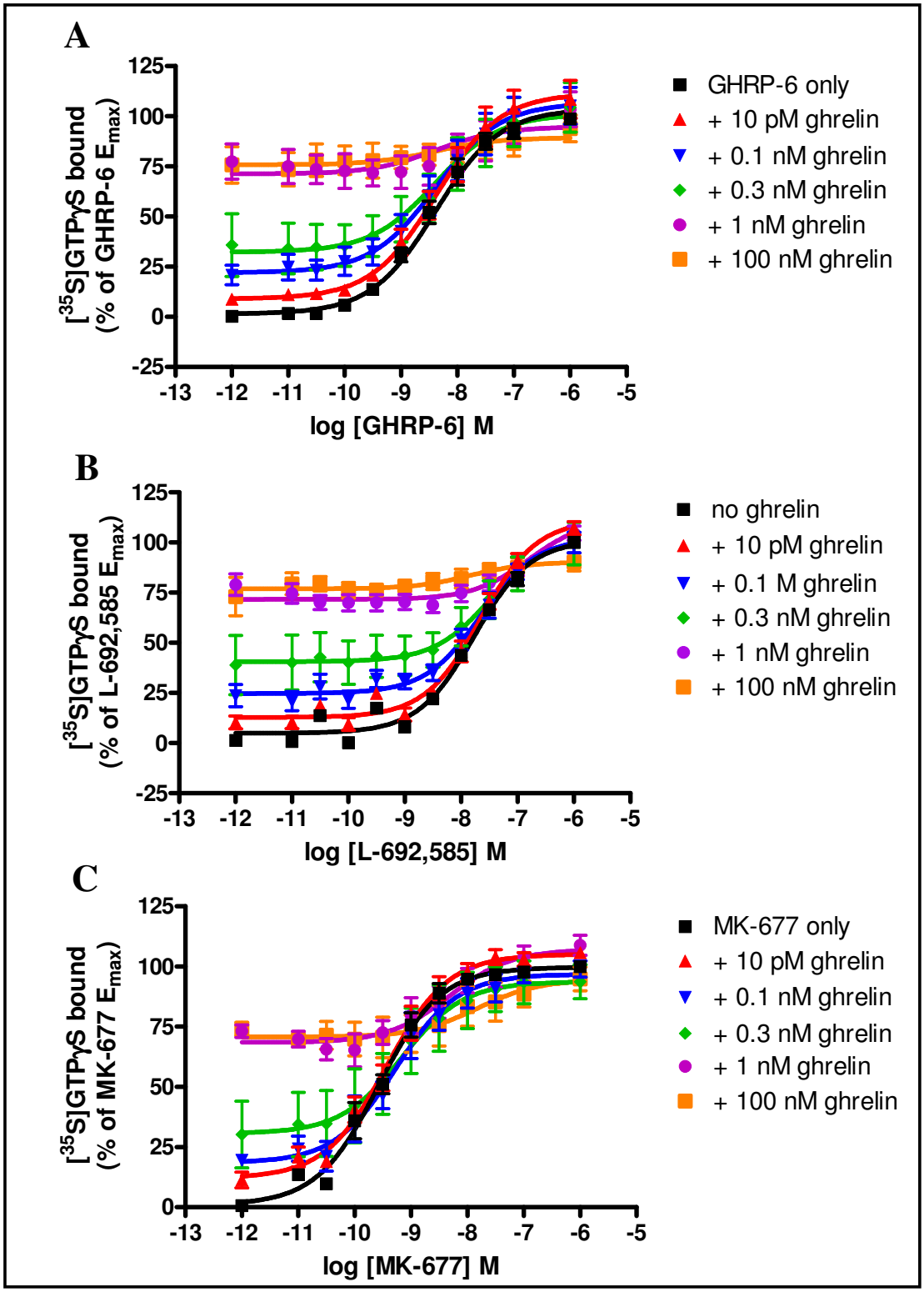

Figure 3-4 - Testing the effect of ghrelin on the response of the GRLN-R to GHRP-6, L-692,585 and MK-677. Using a [^{35}S]GTPγS scintillation proximity assay, concentration-response curves to **A.** GHRP-6, **B.** L-692,585 and **C.** MK-677 were generated in the presence of increasing concentrations of ghrelin (10 pM-100 nM, as indicated) in membranes prepared from HEK293 cells co-expressing $G_{\alpha o1}$ and the GRLN-R. The assay was incubated for 80 min (25 °C) before receptor activation was assessed by measurement of G protein-bound [^{35}S]GTPγS (5 min/plate using a Viewlux platereader). Datasets were fitted with sigmoidal concentration-response curves with Hill slopes shared across each dataset. Hill slopes (± S.E.M): GHRP-6 = 0.89 ± 0.09, L-692,585 = 0.87 ± 0.10; MK-677 = 0.78 ± 0.10. Data is expressed as a percentage of the maximum response achieved in the absence of ghrelin. Data points represent the mean ± S.E.M of three independent experiments performed in triplicate.

Dunnett's post-hoc test). In contrast, at higher concentrations of GHRP-6, ghrelin appeared to have little effect on the level of [^{35}S]GTPγS binding (possibly as at high concentrations of GHRP-6 [^{35}S]GTPγS levels reached the system maximal response) . Indeed, co-administration of 1 μM GHRP-6 with 1 pM-100 nM ghrelin had no effect on the efficacy of GHRP-6 ($p=0.87$; as measured using a one-way ANOVA with Dunnett's post hoc test). As Figure 3-4 shows, the co-administration of either 1 nM or 100 nM ghrelin significant reduced the potency of GHRP-6 ($p<0.01$; as measured using a one-way ANOVA with Dunnett's post-hoc test).

Table 3-2 – Potency and efficacy of GHRP-6 in the presence of increasing concentrations of ghrelin.

Condition	pEC_{50} ± S.E.M	E_{max} ± S.E.M
GHRP-6	8.55 ± 0.02	99.6 ± 1.0
+ 10 pM ghrelin	8.41 ± 0.05	110.6 ± 1.9
+ 0.1 nM ghrelin	8.40 ± 0.70	106.5 ± 2.4
+ 0.3 nM ghrelin	8.17 ± 0.13	105.0 ± 3.8
+ 1 nM ghrelin	7.31 ± 0.22**	106.5 ± 4.0
+ 100 nM ghrelin	7.55 ± 0.35**	93.4 ± 2.8

Efficacy is displayed as the percentage of the maximum efficacy to GHRP-6 in the absence of ghrelin. Data is expressed as the mean ± S.E.M of three individual experiments performed in triplicate. **p<0.01 as measured using a one-way ANOVA with Dunnett's post-hoc test.

3.2.3.2 L-692,585

Co-administration of ghrelin with L-692,585 produced results that were similar to those obtained for GHRP-6. For example, at low concentrations of L-692,585 (1 pM-30 nM) the co-administration of ghrelin caused a concentration-dependent increase in [^{35}S]GTPγS binding (Figure 3-4B). Indeed a one-way ANOVA with Dunnett's post-hoc test revealed that the level of [^{35}S]GTPγS loading after challenging membranes with 1 pM L-692,585 was significantly increased by the co-administration of 0.3-100 nM ghrelin ($p<0.05$ after co-administration of 1 pM L-692,585 and 0.3 nM ghrelin; $p<0.01$ after co-administration of 1 pM L-692,585 and 1 nM or 100 nM ghrelin). At high concentrations of L-692,585, ghrelin (up to a concentration of 1 nM), appeared to have little effect on [^{35}S]GTPγS binding. Indeed statistical tests revealed co-administration of 10 pM-1 nM ghrelin did not significantly alter the level of [^{35}S]GTPγS binding achieved after challenging membranes with 1 μM L-692,585 ($p>0.05$; as compared using a one-way ANOVA). However, co-

administration of L-692,585 with 100 nM ghrelin significantly ($p<0.05$) reduced the maximum efficacy of [^{35}S]GTPγS binding measured in response to L-692,585 alone.

Although there was a trend for the potency of L-692,585 to be reduced as ghrelin concentration increased, this was only deemed significant upon co-administration of 1 nM ghrelin ($p<0.01$; measured using a one-way ANOVA with Dunnett's post-hoc test) (Table 3-3).

Table 3-3 - Potency and efficacy of L-692,585 in the presence of increasing concentrations of ghrelin.

Condition	pEC_{50} ± S.E.M	E_{max} ± S.E.M
L-692,585	7.77 ± 0.03	101.3 ± 1.4
+ 10 pM ghrelin	7.65 ± 0.03	111.4 ± 1.7
+ 0.1 nM ghrelin	7.65 ± 0.05	102.3 ± 2.0
+ 0.3 nM ghrelin	7.50 ± 0.13	102.0 ± 4.4
+ 1 nM ghrelin	6.80 ± 0.12**	111.5 ± 3.0
+ 100 nM ghrelin	7.84 ± 0.25	90.3 ± 1.6*

Efficacy is displayed as a percentage of the maximum efficacy to L-692,585 in the absence of ghrelin. Data is expressed as the mean ± S.E.M of three individual experiments performed in triplicate. *p<0.05, **p<0.01 as measured using a one-way ANOVA with Dunnett's post-hoc test.

3.2.3.3 MK-677

The trend seen upon the co-administration of ghrelin with GHRP-6 or L-692,585 was continued for MK-677. At low concentrations of MK-677 (1 pM -1 nM) co-administration of ghrelin significantly increased [^{35}S]GTPγS binding ($p<0.05$ after the addition of 0.3 nM ghrelin; $p<0.01$ after the addition of 1 nM or 100 nM ghrelin; measured using a one-way ANOVA with Dunnett's post-hoc test) (Figure 3-4C). At higher MK-677 concentrations (1 nM -1 μM) ghrelin appeared to have no additional effect on [^{35}S]GTPγS binding. Indeed at a concentration of 1 μM MK-677, a one-way ANOVA revealed that, compared to MK-677 acting on its own, the addition of ghrelin had no significant effect on [^{35}S]GTPγS binding ($p=0.16$).

The results of the [^{35}S]GTPγS binding studies revealed that the co-administration of ghrelin with GHRP-6, L-692,585 or MK-677 caused an increase in the level of [^{35}S]GTPγS binding at low concentrations of the growth hormone secretagogues, at high concentrations of ghrelin (100 nM), co-administration of ghrelin and GHRP-6 had no effect on the

maximum [^{35}S]GTPγS binding compared to GHRP-6 acting alone ($p>0.05$), whilst co-administration of 100 nM ghrelin with either L-692,585 or MK-677 significantly decreased the maximum level of [^{35}S]GTPγS binding compared to L-692,585 or MK-677 acting alone ($p<0.05$). Ghrelin significantly decreased the potency GHRP-6, L-692,585 and MK-677 when high concentrations of ghrelin (1 nM and/or 100 nM) were co-administered with the growth hormone secretagogues. This suggested that ghrelin was exerting a negative allosteric effect on the signalling of GHRP-6, L-692,585 and MK-677, with the presence of ghrelin shifting the concentration-response curves to each of the growth hormone secretagogues to the right.

Table 3-4 - Potency and efficacy of MK-677 in the presence of increasing concentrations of ghrelin.

Condition	pEC_{50} ± S.E.M	E_{max} ± S.E.M
MK-677	9.58 ± 0.03	99.7 ± 0.8
+ 10 pM ghrelin	9.43 ± 0.03	105.1 ± 0.9
+ 0.1 nM ghrelin	9.27 ± 0.06	96.8 ± 1.3
+ 0.3 nM ghrelin	9.16 ± 0.13	93.7 ± 2.6
+ 1 nM ghrelin	8.32 ± 0.10**	107.1 ± 1.5
+ 100 nM ghrelin	7.82 ± 0.89**	94.6 ± 2.3*

Efficacy is displayed as a percentage of the maximum efficacy to MK-677 in the absence of ghrelin. Data is expressed as the mean ± S.E.M of three individual experiments performed in triplicate. *p<0.05, **p<0.01 when compared using a one-way ANOVA with Dunnett's post-hoc test.

To investigate if the effect that ghrelin had on the concentration-response curves to the growth hormone secretagogues was allosteric or competitive in nature; data was fitted to a modified operational model of allosterism originally described by Leach et al. (2007) (Equation 3-1; see Materials and methods, section 1.9.20.3 for details on the definitions used).

Equation 3-1 – Modified version of an operational model of allosterism (Leach et al., 2007).

$$Y = Basal + \frac{(E_M - Basal)\cdot(([A](K_B + \alpha\beta[B])) + \tau_B[B]\cdot EC_{50})^n}{(([A](K_B + \alpha\beta[B])) + \tau_B[B]\cdot EC_{50})^n + \left(EC_{50}{}^n \cdot (K_B[B])^n\right)}$$

If the interaction between ghrelin and the growth hormone secretagogues is competitive in nature, then the co-operativity factor, α (which denotes the magnitude by which the affinity of each ligand is modified by the concomitant binding of the other; Christopoulos et al., 2004), would be equal to zero. This means in the operational model of allosterism

the parameter $\alpha\beta$ (where β is used to describe any allosteric effects on ligand efficacy) would equal zero. If the interaction between ghrelin and the growth hormone secretagogues was allosteric in nature, then α could be greater than, less than or equal to one, which would describe situations in which the allosteric ligand imparted positive, negative or neutral effects (respectively) on the affinity of the orthosteric ligand.

The [^{35}S]GTPγS data was fitted to the modified operational model of allosterism (Leach et al., 2007) first with $\alpha\beta$ constrained to zero and then with $\alpha\beta$ left unconstrained (Figure 3-5). Comparison between the two fits was assessed using Akaike's Information Criterion (AICc; Motulsky and Christopoulos, 2004).

Akaike's Information Criterion is not a null hypothesis based test; therefore it does not measure 'significance'. Instead AICc indicates a preference for a model, for example, an AICc value of –0.5 would indicate a preference of 1.5-fold for one model over another model. The parameters determined by fitting the data from Figure 3-4 with the modified operational model of allosterism, as well as the AICc results, are listed in Table 3-5.

Table 3-5 – Parameters determined from fitting the [^{35}S]GTPγS data (which investigates the effect of increasing concentrations of ghrelin on concentration-response curves to the growth hormone secretagogues; Figure 3-4) with the modified model of allosterism.

Parameter	MK-677	GHRP-6	L-692,585
pEC_{50}	9.4 (9.3 – 9.6)	8.5 (8.4 – 8.6)	7.8 (7.7 – 7.9)
$pK_{ghrelin}$	8.8 (8.6 – 9.0)	8.8 (8.6 – 9.0)	8.9 (8.7 – 9.0)
Log $\tau_{ghrelin}$	0.48 (0.35 – 0.61)	0.60 (0.44 – 0.76)	0.58 (0.44 – 0.71)
E_m	98 (95 – 101)	102 (97 – 106)	103 (98 – 107)
$\alpha\beta$	0	0	0
Difference in AICc	N/A	-1.8	-0.6

pEC_{50} is the negative logarithm of the EC_{50} of the synthetic agonist to stimulate [^{35}S]GTPγS binding; $pK_{ghrelin}$ is the negative logarithm of the equilibrium dissociation constant for ghrelin binding to the ghrelin receptor; Log $\tau_{ghrelin}$ is the logarithm of the agonist operational efficacy parameter for ghrelin; E_m is the maximal system response level; $\alpha\beta$ is the net affinity and efficacy cooperativity factor governing the interaction between ghrelin and the synthetic agonists. The difference in AICc value quoted represents the preference for the data fit where the value of $\alpha\beta = 0$ compared to a fit where the value of $\alpha\beta$ was left unconstrained. Where 'N/A' is quoted, a comparison was not possible as the dataset failed to fit a model where $\alpha\beta$ was not constrained to zero. Numbers in paratheses represent 95 % confidence intervals.

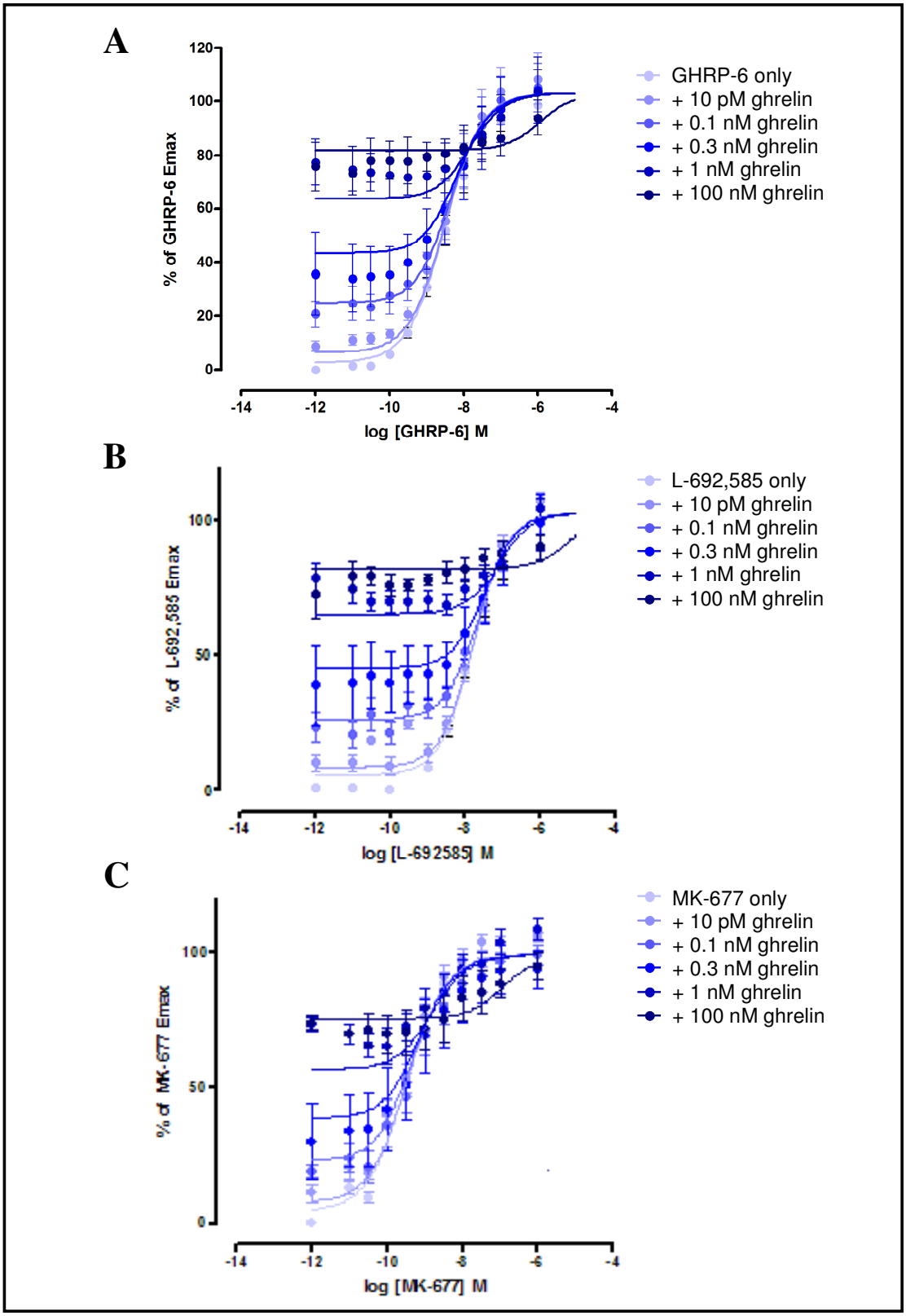

Figure 3-5 – Testing the effects of ghrelin on the response of the GRLN-R to A. GHRP-6 B.L-692,585 or C. MK-677 using a modified operational model of allosterism. Data generated in the [^{35}S]GTPγS binding studies (shown in Figure 3-4) was fitted by Dr Christopher Langmead (GlaxoSmithKline) to an modified operational model of allosterism (Equation 3-1) described by Leach et al. (2007). Data shown here is fitted with $\alpha\beta = 0$ to describe a situation where ghrelin and the growth hormone secretagogues compete in a competitive fashion. Data was also fitted with $\alpha\beta$ unconstrained (to describe an allosteric mode of interaction) however an F-test and Akaike's Information Criterion showed data was best fitted to describe a competitive interaction.

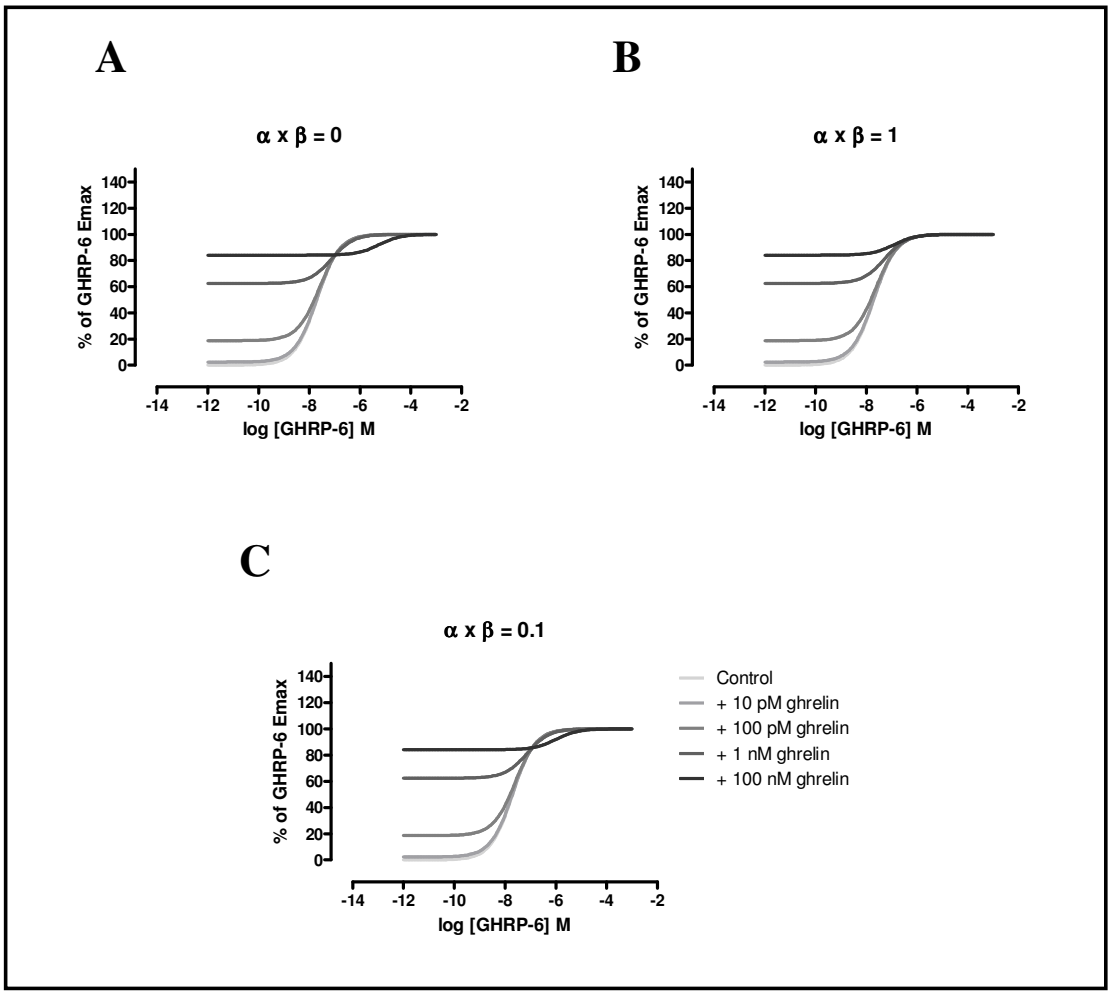

Figure 3-6 – Data simulated with an operational model of allosterism with the parameter $\alpha\beta$ constrained to describe competitive or allosteric modes of interaction. Taking the EC_{50} of GHRP-6 (determined in Figure 3-4) and the K_B and τ values for ghrelin, data was simulated with the modified version of the operational model of allosterism (Leach et al., 2007) by Dr Christopher Langmead (GlaxoSmithKline). The parameter $\alpha\beta$ was set to **A.** equal 0 (to describe a competitive mode of interaction) **B.** equal to 1 which describes a net neutral co-operativity between GHRP-6 and ghrelin, and **C.** equal to 0.1 which would describe a situation where ghrelin imparts a negative co-operative effect on GHRP-6 binding. The key curve is that generated in the presence of 100 nM ghrelin, which predicts a large shift in the curve if the interaction were competitive.

The use of AICc showed that in each instance data was best fit with the model where $\alpha\beta$ was constrained to zero (demonstrated in Figure 3-6); to further test this (as AICc does not measure significance) a comparison by extra sum of squares test was performed, this showed there was no significant preference for an allosteric mechanism for any dataset ($p>0.05$). Thus data from the [^{35}S]GTPγS binding studies did not provide evidence in favour of an allosteric mode of interaction between ghrelin and the growth hormone secretagogues, but favoured a competitive model. Thus ghrelin, which acts as a partial agonist with respect to the actions of the growth hormone secretagogues (see Figure 3-3), could be viewed as competing against the growth hormone secretagogues for binding to the orthosteric site on the GRLN-R.

3.2.4 Effect of the growth hormone secretagogues on the potency and efficacy of ghrelin

To further investigate the interaction between ghrelin and the growth hormone secretagogues the experimental protocol was reversed so that the effect of varying concentrations of the growth hormone secretagogues on ghrelin signalling could be assessed. Thus concentration-response curves to ghrelin were performed in the presence of multiple, fixed concentrations of GHRP-6, L-692,585 or MK-677. The responses of the GRLN-R in membranes prepared from HEK293 cells co-expressing the GRLN-R and $G_{\alpha o1}$ were measured using a [^{35}S]GTPγS scintillation proximity assay.

3.2.4.1 GHRP-6

Concentration-response curves to ghrelin were performed in the presence of 0.1, 1, 10 and 100 nM of GHRP-6. At these concentrations GHRP-6 produced a [^{35}S]GTPγS response around 0 % (at 0.1 and 1 nM GHRP-6), 60 % (10 nM GHRP-6) or 115 % (100 nM GHRP-6) of the maximum [^{35}S]GTPγS response achieved by ghrelin (Figure 3-3).

Fitting the data to concentration-response curves revealed that at low concentrations of ghrelin (1 pM-3 nM), co-administration of GHRP-6 increased [^{35}S]GTPγS binding in a concentration-dependent manner (Figure 3-7A). For example, statistical tests revealed that at 1 pM of ghrelin the addition of 1 nM, 10 nM or 100 nM of GHRP-6 significantly increased the level of [^{35}S]GTPγS binding achieved by ghrelin acting on its own ($p<0.01$; compared using a one-way ANOVA with Dunnett's post-hoc test). Indeed co-administration of 1 pM ghrelin with either 10 nM or 100 nM GHRP-6 actually caused an increase in [^{35}S]GTPγS binding that was above the level of binding achieved by incubation

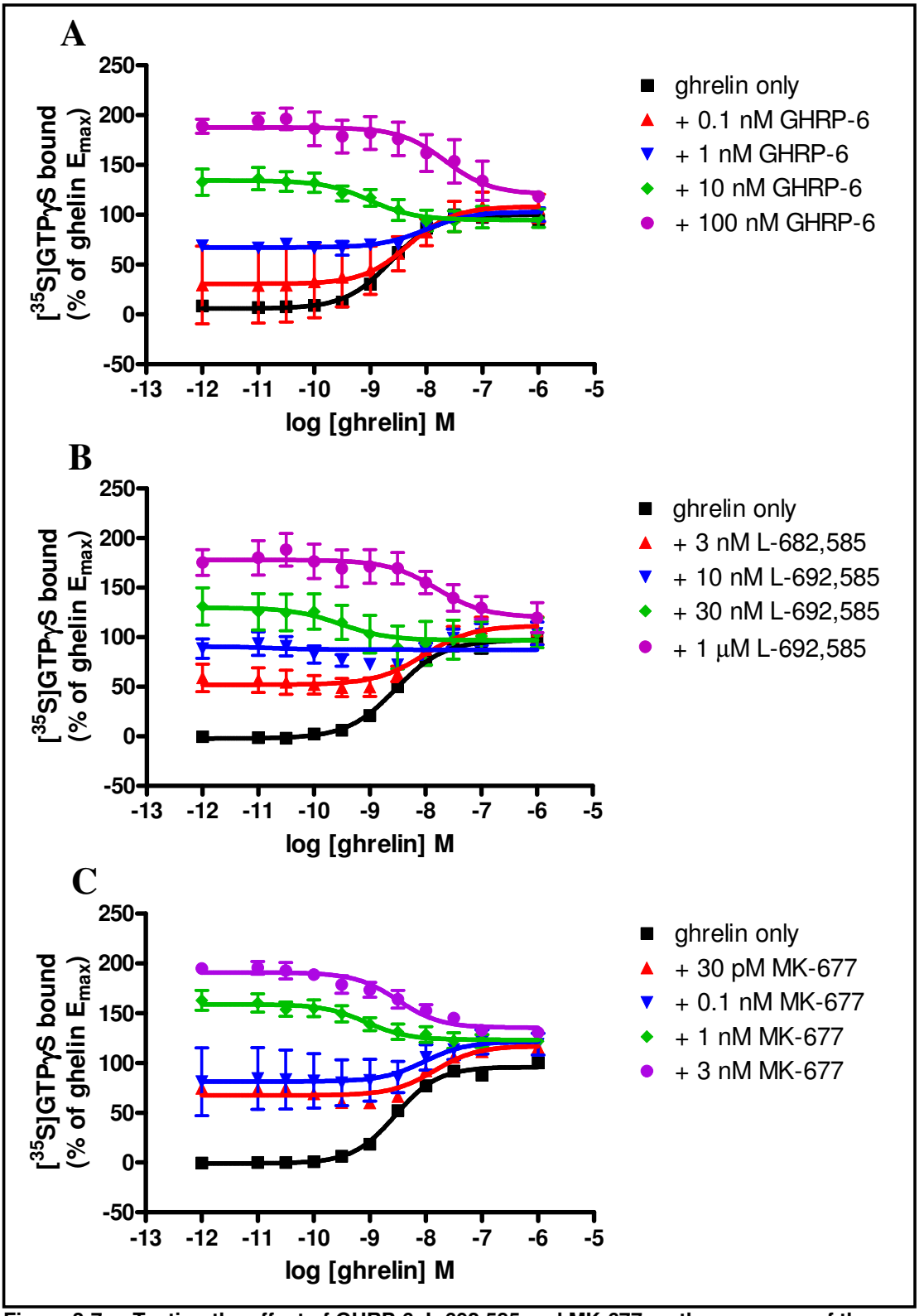

Figure 3-7 - Testing the effect of GHRP-6, L-692,585 and MK-677 on the response of the GRLN-R to ghrelin. Using a [^{35}S]GTPγS scintillation proximity assay, concentration-response curves to ghrelin were generated in the presence of increasing concentrations of **A.** GHRP-6 **B.** L-692,585 **C.** MK-677 in membranes prepared from HEK293 cells co-expressing $G_{\alpha o1}$ and the GRLN-R. The assay was incubated for 80 min (25 °C) before incorporation of [^{35}S]GTPγS into G protein was assessed by measurement of G protein-bound [^{35}S]GTPγS (5 min/plate using a Viewlux platereader). Datasets were fitted with sigmoidal concentration-response curves with Hill slopes shared across each dataset. Hill slopes (± S.E.M): GHRP-6 = constrained to 1, L-692,585 = 1.14 ± 0.28; MK-677 = 1.07 ± 0.31. Data is expressed as a percentage of the maximum response achieved by ghrelin in the absence of growth hormone secretagogues. Data points represent the mean ± S.E.M of three independent experiments performed in triplicate.

of membranes with a maximally effective concentration of ghrelin alone ($p<0.01$; as measured using a one-way ANOVA with Dunnett's post-hoc test).

Strikingly, as the concentration of ghrelin increased, the level of [^{35}S]GTPγS binding achieved by high concentrations of GHRP-6 (10 nM or 100 nM) decreased. Co-adminstration of 0.1 nM or 100 nM GHRP-6 significantly decreased the potency of ghrelin ($p<0.05$ and $p<0.01$, respectively: as compared using a one-way ANOVA with Dunnett's post-hoc test) (Table 3-6).

Table 3-6 - Potency and efficacy of ghrelin in the presence of increasing concentrations of GHRP-6.

Condition	pEC_{50} ± S.E.M	E_{max} ± S.E.M	Response at 1 pM ghrelin ± S.E.M
Ghrelin	8.64 ± 0.02	100.2 ± 0.7	8.5 ± 0.6
+ 0.1 nM GHRP-6	8.32 ± 0.21*	108.3 ± 7.6	29.5 ± 39.0
+ 1 nM GHRP-6	8.01 ± 0.08	103.1 ± 1.5	68.8 ± 5.5
+ 10 nM GHRP-6	9.01 ± 0.15	134.3 ± 2.2**	132.8 ± 13.1**
+ 100 nM GHRP-6	7.67 ± 0.15**	187.6 ± 2.5**	188.9 ± 7.03**

Efficacy is displayed as a percentage of the maximum efficacy to ghrelin in the absence of GHRP-6. Data is expressed as the mean ± S.E.M of three individual experiments performed in triplicate. *$p<0.05$; **$p<0.01$ when compared using a one-way ANOVA with Dunnett's post-hoc test.

3.2.4.2 L-692,585 and MK-677

Ghrelin concentration-response curves were also performed in the presence of 3 nM, 10 nM, 30 nM and 1 μM of L-692,585 (producing approximately 20, 45, 80 or 150 % of the maximum [^{35}S]GTPγS response produced by ghrelin), or 30 pM, 0.1 nM, 1 nM or 3 nM MK-677 (producing approximately 20, 75 or 115 % of the maximum [^{35}S]GTPγS response produced by ghrelin; shown in Figure 3-3).

Increasing the concentrations of both L-692,585 or MK-677 increased [^{35}S]GTPγS binding in the presence of low concentrations of ghrelin (Figure 3-7B and C). For example, a one-way ANOVA with Dunnett's post-hoc test revealed that at a ghrelin concentration of 1 pM, co-addition of GHRP-6 or MK-677 significantly increased [^{35}S]GTPγS binding ($p<0.05$ after addition of 3 nM L-692,585, $p<0.01$ after addition of 10 nM, 30 nM and 1 μM L-692,585 and $p<0.05$ after addition of 30 pM or 0.1 nM MK-677, $p<0.01$ after addition of 1 nM or 3 nM MK-677).

Co-administration of ghrelin with 30 nM and 1 μM L-692,585 or 0.3 nM – 3 nM MK-677 increased [^{35}S]GTPγS binding compared to the level achieved when challenging membranes with a maximally effective concentration of ghrelin. For example, a one-way ANOVA with Dunnett's post-hoc test revealed that there was a significant increase in the maximum [^{35}S]GTPγS binding achieved by ghrelin after co-administration of 1 μM L-692,585 and 0.3 nM – 3 nM MK-677 ($p<0.01$). A one-way ANOVA also revealed that co-addition of L-692,585 had no effect on the potency of ghrelin ($p=0.09$; Table 3-7) whereas the addition of 0.03 nM MK-677 significantly ($p<0.05$; as measured using a one-way ANOVA with Dunnett's post-hoc test) lowered the potency of ghrelin compared to ghrelin acting alone (Table 3-8).

Table 3-7 - Potency and efficacy of ghrelin in the presence of increasing concentrations of L-692,585.

Condition	pEC_{50} ± S.E.M	E_{max} ± S.E.M	Response at 1 pM ghrelin ± S.E.M
Ghrelin	8.54 ± 0.05	97.3 ± 1.8	0.3 ± 1.3
+ 3 nM L-692,585	8.03 ± 0.27	111.7 ± 7.7	59.0 ± 13.9
+ 10 nM L-692,585	Not fitted	Not fitted	88.7 ± 9.8**
+ 30 nM L-692,585	9.53 ± 0.78	129.8 ± 10.6*	131.1 ± 18.9**
+ 1 μM L-692,585	7.80 ± 0.39	177.9 ± 5.6**	175.3 ± 13.0**

Efficacy is displayed as a percentage of the maximum efficacy to ghrelin in the absence of L-692,585. Data is expressed as the mean ± S.E.M of three individual experiments performed in triplicate. **p<0.01 when compared using a one-way ANOVA with Dunnett's post-hoc test.

Table 3-8 - Potency and efficacy of ghrelin in the presence of increasing concentrations of MK-677.

Condition	pEC_{50} ± S.E.M	E_{max} ± S.E.M	Response at 1 pM ghrelin ± S.E.M
Ghrelin	8.54 ± 0.02	96.0 ± 0.8	0.5 ± 2.6
+ 0.03 nM MK-677	7.84 ± 0.06*	117.2 ± 1.8**	74.6 ± 2.9*
+ 0.1 nM MK-677	8.03 ± 0.31	121.5 ± 6.6**	81.2 ± 34.0*
+ 1 nM MK-677	9.07 ± 0.12	159.0 ± 1.7**	162.9 10.0**
+ 3 nM MK-677	8.50 ± 0.07	190.8 ± 1.3**	195.0 ± 3.8**

Efficacy is displayed as a percentage of the maximum efficacy to ghrelin in the absence of MK-677. Data is expressed as the mean ± S.E.M of three individual experiments performed in triplicate. *p<0.05; **p<0.01 when compared using a one-way ANOVA with Dunnett's post-hoc test.

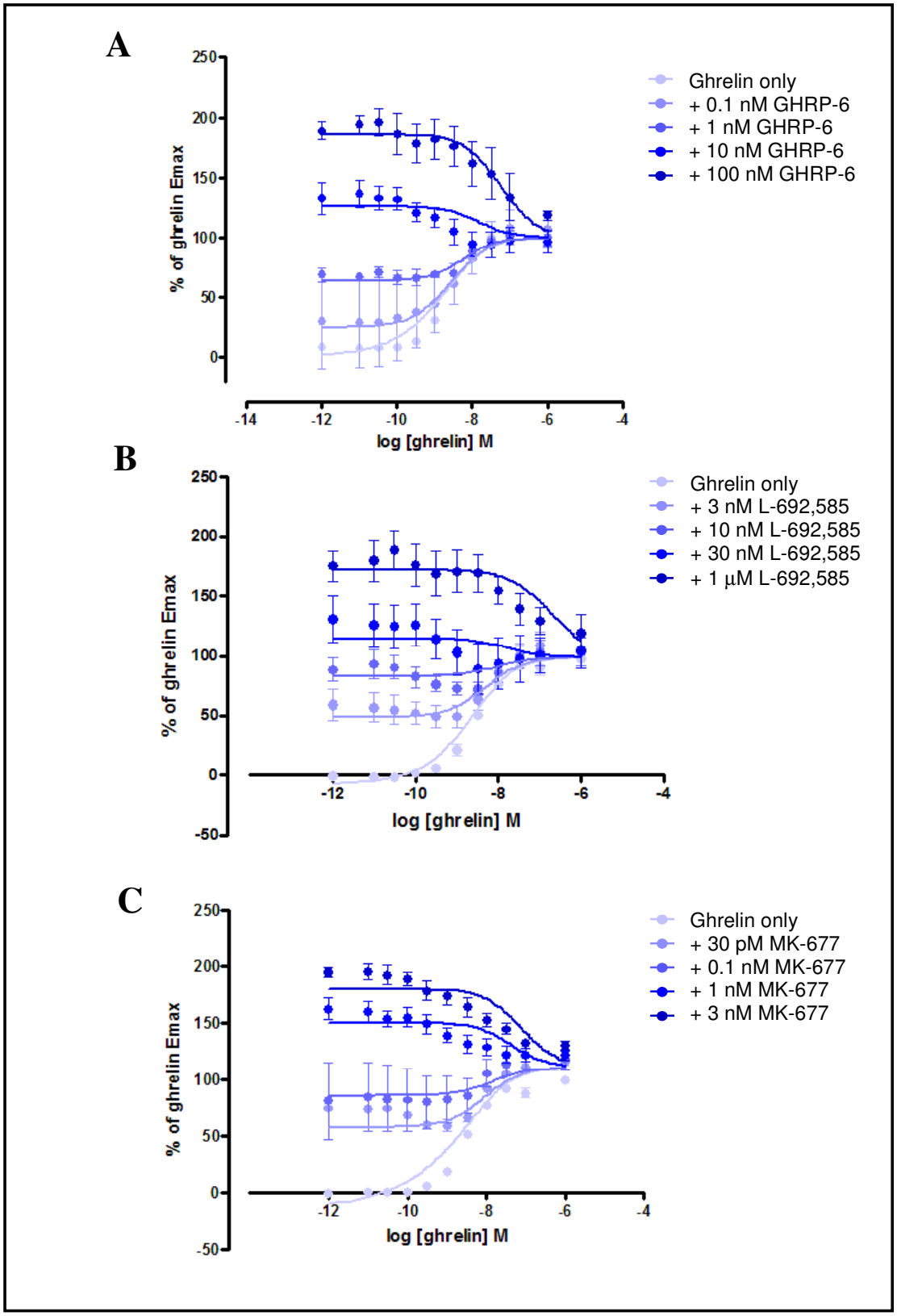

Figure 3-8 - Testing the effect of A. GHRP-6, B. L-692,585 and C. MK-677 on the response of the GRLN-R to ghrelin using a modified operational model of allosterism. Data generated in the [^{35}S]GTPγS binding studies (shown in Figure 3-7) was fitted by Dr Christopher Langmead (GlaxoSmithKline) to an modified operational model of allosterism (Equation 3-1) described by Leach et al. (2007). Data shown here is fitted with $\alpha\beta = 0$ to describe a situation where ghrelin and the growth hormone secretagogues compete in a competitive fashion. Data was also fitted with $\alpha\beta$ unconstrained (to describe an allosteric mode of interaction) however an F-test and Akaike's Information Criterion showed data was best fitted to describe a competitive interaction.

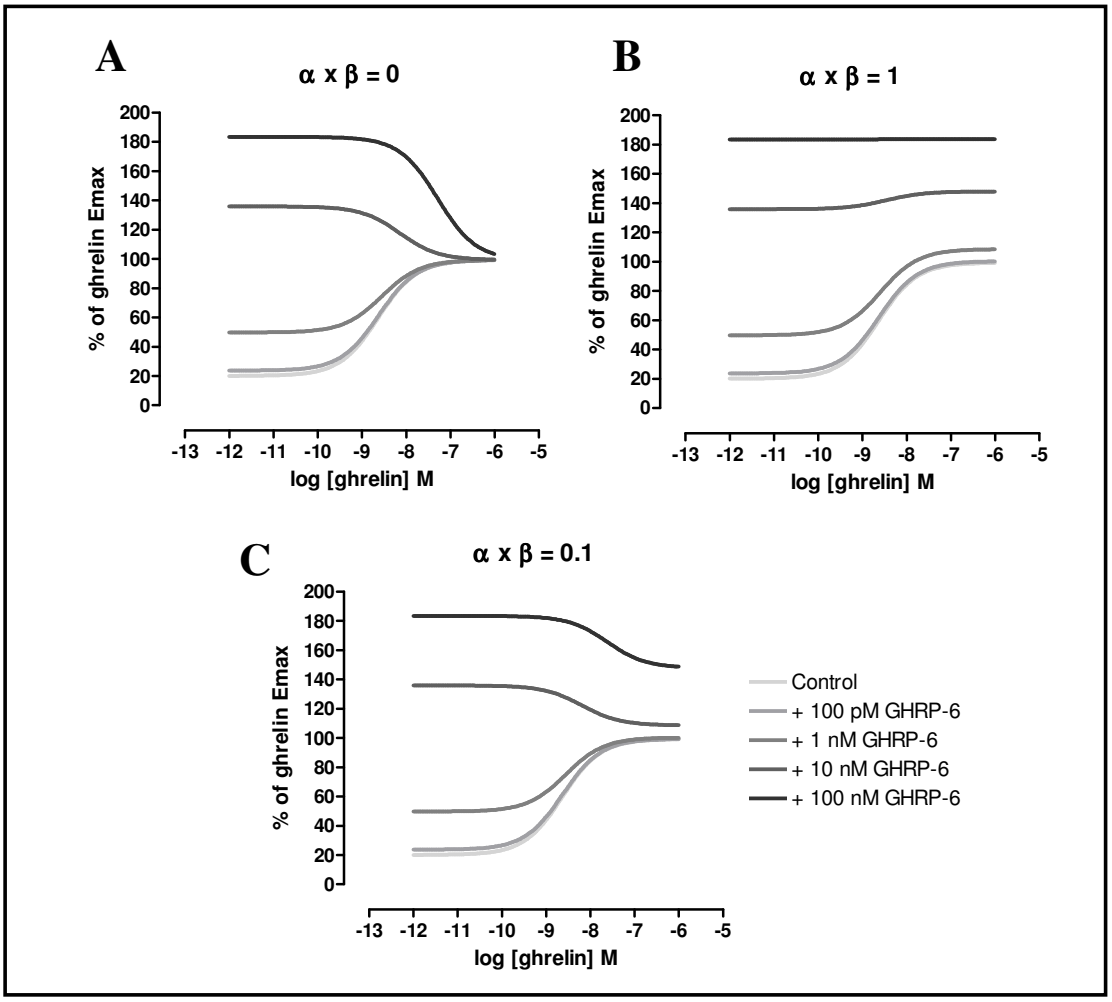

Figure 3-9 – Data simulated with an operational model of allosterism with the parameter $\alpha\beta$ constrained to describe competitive or allosteric modes of interaction. Taking the EC_{50} of ghrelin (determined in Figure 3-7) and the K_B and τ values for GHRP-6 (Table 3-9), data was simulated with the modified version of the operational model of allosterism (Leach et al., 2007) by Dr Christopher Langmead (GlaxoSmithKline). The parameter $\alpha\beta$ was set to **A.** equal 0 (to describe a competitive mode of interaction) **B.** equal to 1 which describes a net neutral co-operativity between GHRP-6 and ghrelin, and **C.** equal to 0.1 which would describe a situation where GHRP-6 imparts a negative co-operative effect on ghrelin binding. The key curve is that generated in the presence of 100 nM GHRP-6, which shows that the response to 1 μM ghrelin would be significantly greater in the presence of 100 nM GHRP-6 than that achieved in the absence of GHRP-6 if a negative or neutral allosteric interaction between ghrelin and GHRP-6 was occuring.

Once again data was modelled with the modified operational model of allosterism (Leach et al., 2007; Materials and methods section 1.9.20.3) to determine whether the interaction between ghrelin and the growth hormone secretagogues was allosteric or competitive in nature. Comparison of data fits to the operational model of allosterism (Equation 3-1) was assessed using AICc. However, the operational model of allosterism was re-cast such that ghrelin, the partial agonist, was the independent variable on the x-axis (parameters measured by fitting the data to the modified operational model of allosterism are displayed in Table 3-9). As previously, data was best fit to a competitive rather than an allosteric model of ligand interaction (Figure 3-8), as demonstrated in Figure 3-9. Thus ghrelin and the growth hormone secretagogues appeared to be competing for binding to the same site on the GRLN-R.

Table 3-9 - Parameters determined from fitting the [^{35}S]GTPγS data (which investigates the effect of increasing concentrations of the growth hormone secretagogues on the concentration-response curves to ghrelin; Figure 3-7) with the modified model of allosterism.

Parameter	MK-677	GHRP-6	L-692,585
pEC_{50}	10.0 (9.0 – 10.1)	8.3 (8.2 – 8.5)	7.9 (7.8 – 8.1)
$pK_{ghrelin}$	8.1 (7.8 – 8.3)	8.4 (8.1 – 8.6)	8.3 (8.0 – 8.5)
Log $\tau_{ghrelin}$	0.17 (0.07 – 0.28)	-0.06 (-0.17 – 0.04)	0.11 (0.01 – 0.21)
E_m	177 (169 - 185)	191 (179 – 203)	171 (162 – 179)
$\alpha\beta$	0	0	0
Difference in AICc	-0.5	N/A	-1.8

pEC_{50} is the negative logarithm of the EC_{50} of the synthetic agonist to stimulate [^{35}S]GTPγS binding; $pK_{ghrelin}$ is the negative logarithm of the equilibrium dissociation constant for ghrelin binding to the ghrelin receptor; Log $\tau_{ghrelin}$ is the logarithm of the agonist operational efficacy parameter for ghrelin; E_m is the maximal system response level; $\alpha\beta$ is the net affinity and efficacy cooperativity factor governing the interaction between ghrelin and the synthetic agonists. The difference in AICc value quoted represents the preference for the data fit where the value of $\alpha\beta = 0$ compared to a fit where the value of $\alpha\beta$ was left unconstrained. Where 'N/A' is quoted, a comparison was not possible as the dataset failed to fit a model where $\alpha\beta$ was not constrained to zero. Numbers in paratheses represent 95 % confidence intervals.

3.2.5 Kinetics of [His[^{125}I]]-ghrelin binding

Allosteric ligands are predicted to alter the kinetics of binding of orthosteric ligands to the receptor of study (Langmead and Christopoulos, 2006; May et al., 2007), an effect that can be monitored as a change in the dissociation rate of a radiolabelled orthosteric ligand in the

presence of an allosteric ligand (Kostenis and Mohr, 1996). Before the effect that GHRP-6, L-692,585 and MK-677 had on the dissociation of [His[^{125}I]]-ghrelin from the GRLN-R could be investigated, a radioligand binding assay had to be established and the kinetics of [His[^{125}I]]-ghrelin binding in the absence of the growth hormone secretagogues assessed.

To study the association of [His[^{125}I]]-ghrelin binding to the GRLN-R, membranes co-expressing $G_{\alpha o1}$ and the GRLN-R were incubated in ice-cold assay buffer with the inclusion of cold ghrelin, where appropriate, used to determine non-specific binding. The concentration of membranes chosen ensured that less than 10 % of the total radioactivity added bound to the GRLN-R. The assay was terminated by rapid filtration through GF/B plates pre-soaked in 0.5 $\%_{(w/v)}$ PEI. Pre-soaking the GF/B plates in 0.5 $\%_{(w/v)}$ PEI was found to prevent excessive binding of the radioligand to the plates (data not shown), as previously described by Muccioli et al. (2001).

Specific binding of [His[^{125}I]]-ghrelin to the GRLN-R was calculated by subtracting the values obtained in the presence of cold ghrelin (non-specific binding) from the values obtained in the absence of cold ghrelin (total binding). This showed that [His[^{125}I]]-ghrelin binding increased over time (Figure 3-10). The use of an F-test showed that the data was best fitted to a one-site exponential association curve, which revealed that the binding of [His[^{125}I]]-ghrelin increased until around 120 min where the response reached a plateau, yielding an observed association rate constant (K_{obs}) of 0.03 ± 0.005 min^{-1}.

To study the dissociation kinetics of [His[^{125}I]]-ghrelin from the GRLN-R, 83 pM of [His[^{125}I]]-ghrelin was incubated at 4 ºC with membranes co-expressing $G_{\alpha o1}$ and the GRLN-R. After 120 min, 10 µL of a final concentration of 1 µM ghrelin was added to the reaction mixture to prevent re-association of the radioligand (by competing against radiolabelled ghrelin for binding to the GRLN-R). Again, specific binding was calculated by the subtraction of the values of non-specific binding from total binding.

The addition of cold ghrelin caused the specific binding of [His[^{125}I]]-ghrelin to decrease with time. An F-test revealed that the dissociation data was best fitted with a one-phase exponential decay curve, which yielded a dissociation rate constant (K_{off}) of 0.02 ± 0.002 min^{-1} (or a K_{off} of 0.01 ± 0.002 min^{-1}, calculated by fitting data with a semi-log plot) (Figure 3-11). Using Equation 3-2 and Equation 3-3 allowed the determination of the kinetically derived equilibrium dissociation constant (K_D) of 2.53 x 10^{-10} M.

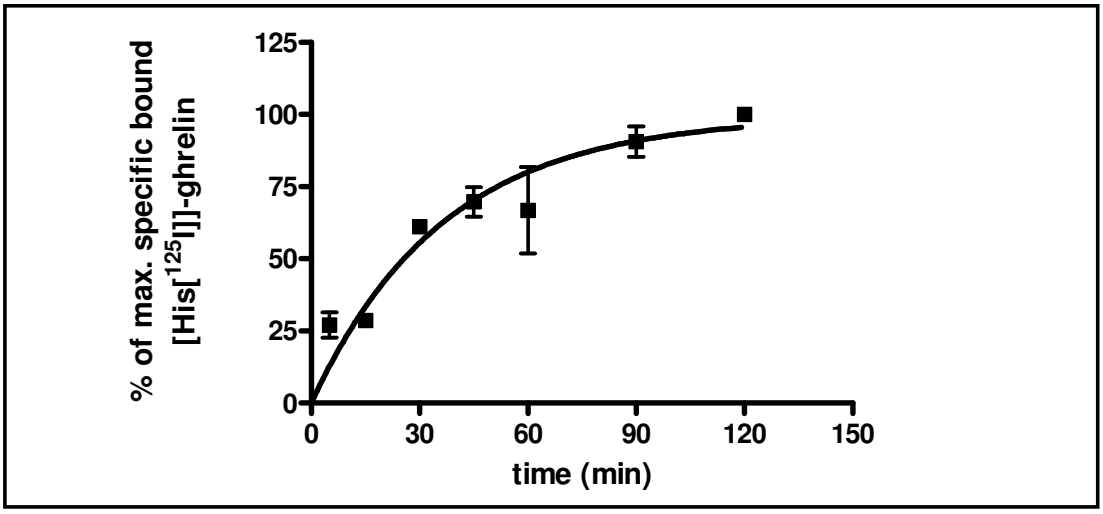

Figure 3-10 - Association of [His[^{125}I]]-ghrelin to the GRLN-R. The specific binding of [His[^{125}I]]-ghrelin to the GRLN-R was measured over varying time periods (0-120 min) in membranes co-expressing the GRLN-R and $G_{\alpha o1}$. Binding was carried out at 4 °C with non-specific binding determined by the inclusion of 1 µM cold ghrelin and the addition of a final concentration of 83 pM [His[^{125}I]]-ghrelin staggered so that the assay was terminated by rapid filtration through 96-well GF/B filter plates pre-soaked in 0.5 $\%_{(w/v)}$ PEI after 2 h. Filters were allowed to air-dry before the addition of 100 µL scintillation cocktail to each filter. Bound radioactivity was measured using a [^{125}I]counting programme on a Topcount NXT™ microplate scintillation counter. Data points represent the mean ± S.E.M of three independent experiments performed in triplicate. Data was expressed as the percentage of the maximum specific binding achieved and fitted to a one-site exponential association curve, yielding a K_{obs} value of 0.03 ± 0.005 min^{-1}.

Equation 3-2 – Determination of K_{on}.

$$K_{on} = (K_{obs} - K_{off})/[radioligand]$$

Where K_{on} is the association rate constant; K_{obs} is the observed association rate constant; K_{off} is the dissociation rate constant and [radioligand] is the final concentration of radioligand in M.

Equation 3-3 – Determination of K_D.

$$K_D = K_{off}/K_{on}$$

Where K_D is the equilibrium dissociation constant; K_{off} is the dissociation rate constant and K_{on} is the association rate constant.

3.2.6 Ghrelin and the growth hormone secretagogues compete for [His[^{125}I]]-ghrelin binding

The specific binding of [His[^{125}I]]-ghrelin to the GRLN-R was measured in membranes co-expressing the GRLN-R and $G_{\alpha o1}$ in the presence of increasing concentrations of ghrelin, GHRP-6, L-692,585 and MK-677. Ghrelin and the growth hormone secretagogues were all

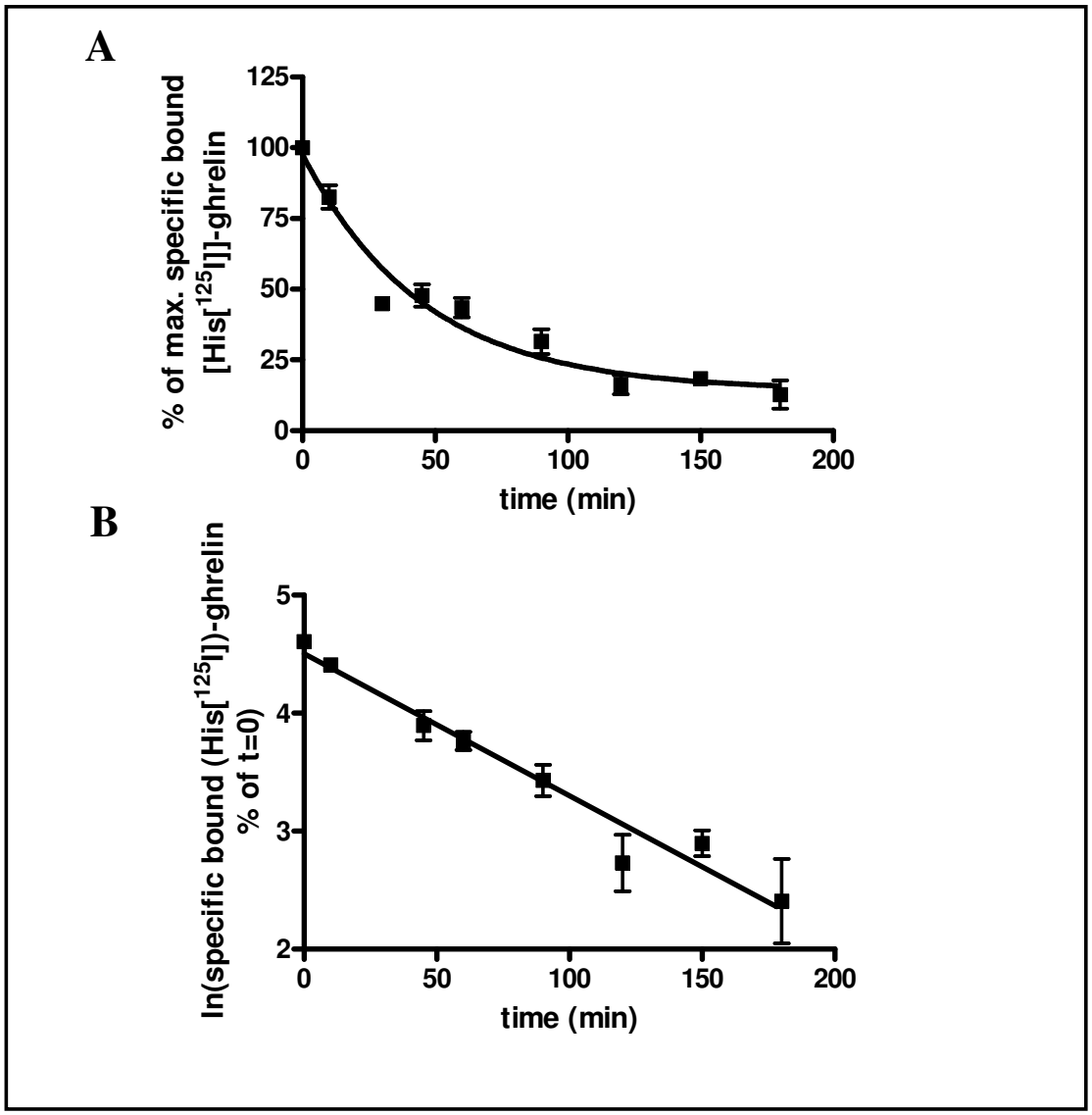

Figure 3-11 - Dissociation of [His[^{125}I]]-ghrelin from the GRLN-R. Following association (to equilibrium, 120 min), the dissociation of [His[^{125}I]]-ghrelin from the GRLN-R was measured over varying time periods in membranes co-expressing the GRLN-R and $G_{\alpha o1}$. Binding was carried out at 4 °C for 2 h with non-specific binding determined by the inclusion of 1 µM cold ghrelin before re-association of [His[^{125}I]]-ghrelin was inhibited by the addition of 1 µM cold ghrelin for varying amounts of time (0-180 min). The assay was terminated by rapid filtration through 96-well GF/B filter plates pre-soaked in 0.5 $\%_{(w/v)}$ PEI. Filters were allowed to air-dry before the addition of 100 µL scintillation cocktail to each filter. Bound radioactivity was measured using a [^{125}I]counting programme on a Topcount NXT™ microplate scintillation counter. Data points represent the mean ± S.E.M of three independent experiments performed in triplicate and are expressed as a percentage of the maximum specific binding achieved in each assay. **A.** Data is fitted to a one-site exponential decay curve, yielding a K_{off} of 0.02 ± 0.002 min^{-1} **B.** Semi-log plot of dissociation yielded a K_{off} of 0.01 ± 0.002 min^{-1}.

able to compete with [His[^{125}I]]-ghrelin in a concentration-dependent manner to limit the specific binding of ghrelin, although sufficiently high concentrations of L-692,585 could not be employed in these studies to allow adequate fitting of the data (Figure 3-12). The use of an F-test revealed that ghrelin, GHRP-6 and MK-677 were best fit to a monophasic competition curve with Hill slopes (determined from fitting the data to variable-slope dose-response curves) not significantly different from unity (Table 3-10). As the IC_{50} can change depending on experimental conditions used, the pK_i for each ligand was calculated. To determine the pK_i from the pIC_{50} values the Cheng-Prusoff equation was used (Equation 3-4; Cheng and Prusoff, 1973).

Equation 3-4 – Cheng-Prusoff equation for determining the K_i from the IC_{50}.

$$K_i = \frac{IC_{50}}{1 + \left[\frac{[\text{radioligand}]}{K_D}\right]}$$

Where IC_{50} is the concentration of ligand that blocks specific binding by 50 %, K_D is the affinity of the radioligand for the receptor and [radioligand] is the concentration of the radioligand in M. The equation was originally described by Cheng and Prusoff (1973).

Table 3-10 – Ghrelin and the growth hormone secretagogues compete for [His[^{125}I]-ghrelin binding.

Ligand	pIC_{50}	pKi	Hill slope
Ghrelin	9.06 ± 0.34	8.97 ± 0.27	-0.59 ± 0.41
GHRP-6	8.29 ± 0.60	7.51 ± 0.71	-0.52 ± 0.63
MK-677	8.12 ± 0.33	8.14 ± 0.08	-0.67 ± 0.49
L-692,585	< 5.00	< 6.00	Not fitted

Data is expressed as the mean ± S.E.M of three individual experiments performed in triplicate. F-test showed data was best fitted to a one-site competition model which allowed pIC_{50} and pKi values to be obtained. Hill slope values were obtained from fitting the data to a variable-slope sigmoidal dose-response curve. The use of an F-test revealed the Hill slope values obtained were not significantly different from unity.

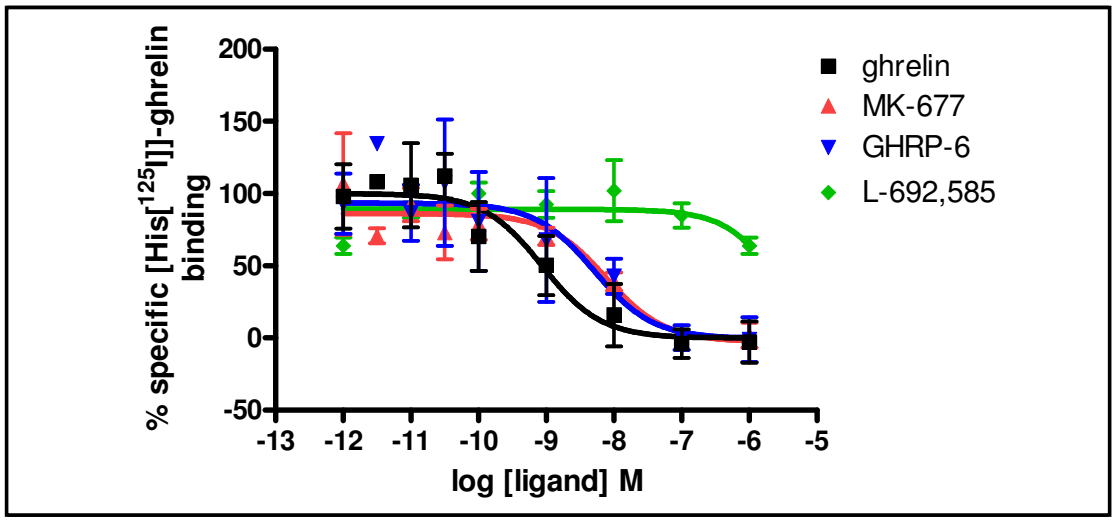

Figure 3-12 - The specific binding of [His[^{125}I]-ghrelin is inhibited by the presence of ghrelin and the growth hormone secretagogues. Binding of a final concentration of 83 pM [His[^{125}I]]-ghrelin to membranes co-expressing $G_{\alpha o1}$ and the GRLN-R was measured in the presence or absence of varying concentrations of ghrelin, MK-677, GHRP-6 and L-692,585. Binding was carried out at 4 °C for 2 h with non-specific binding determined by the inclusion of 1 µM cold ghrelin, before the assay was terminated by rapid filtration through 96-well GF/B filter plates pre-soaked in 0.5 $\%_{(w/v)}$ PEI. Filters were allowed to air-dry before the addition of 100 µL scintillation cocktail to each filter. Bound radioactivity was measured using a [^{125}I]counting programme on a Topcount NXT™ microplate scintillation counter. Data points represent the mean ± S.E.M of three independent experiments performed in triplicate. Data is expressed as a percentage of the maximum specific binding achieved in the absence of competing ligand and fitted to a one-site competition model.

3.2.7 The growth hormone secretagogues do not alter the time course of [His[^{125}I]]-ghrelin dissociation

The data from the competition binding experiments was consistent with ghrelin and the growth hormone secretagogues competing for a common binding site. To further test this the effect of ghrelin and the growth hormone secretagogues on the dissociation of [His[^{125}I]]-ghrelin was explored.

The dissociation of [His[^{125}I]]-ghrelin from the GRLN-R was measured in membranes co-expressing the GRLN-R and $G_{\alpha o1}$ (Figure 3-13). Re-association of [His[^{125}I]]-ghrelin was inhibited by the addition of cold ghrelin to the reaction mixture, in the presence or absence of 0.3 µM L-692,585. Data was best fitted to a one-phase exponential decay model (assessed using an F-test). This revealed that the K_{off} in the absence of L-692,585 was 0.02 ± 0.002 min^{-1} whilst the K_{off} in the presence of L-692,585 was 0.02 ± 0.004 min^{-1}, comparing the K_{off} values obtained using a un-paired two-tailed t-test revealed there was no significant differences in the values obtained (p=0.85). Thus L-692,585 had no effect on the dissociation rate of [His[^{125}I]]-ghrelin. To further test the effects of the growth hormone secretagogues on the dissociation of [His[^{125}I]]-ghrelin from the GRLN-R, two-point

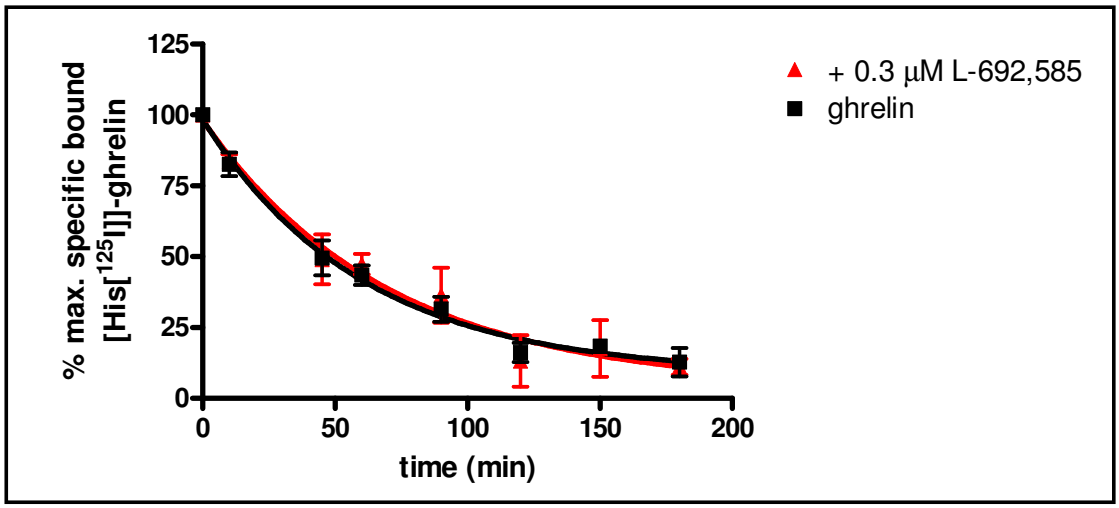

Figure 3-13 - L-692,585 does not affect the dissociation rate of [His[^{125}I]]-ghrelin from the GRLN-R. Following association (to equilibrium, 120 min), the dissociation of [His[^{125}I]]-ghrelin from the GRLN-R was measured over varying time periods in membranes co-expressing the GRLN-R and $G_{\alpha o1}$. Binding was carried out at 4 °C for 2 h with non-specific binding determined by the inclusion of 1 µM cold ghrelin before re-association of [His[^{125}I]]-ghrelin was inhibited by the addition of 1 µM cold ghrelin ± 0.3 µM L-692,585 for varying amounts of time (0-180 min). The assay was terminated by rapid filtration through 96-well GF/B filter plates pre-soaked in 0.5 $\%_{(w/v)}$ PEI. Filters were allowed to air-dry before the addition of 100 µL scintillation cocktail to each filter. Bound radioactivity was measured using a [^{125}I]counting programme on a Topcount NXT™ microplate scintillation counter. Data points represent the mean ± S.E.M of three independent experiments performed in triplicate. Data is expressed as the percentage of the maximum specific binding achieved in each assay and is fitted to a one-site exponential decay curve. The K_{off} of [His[^{125}I]]-ghrelin in the presence of L-692,585 was calculated as 0.02 ± 0.004 min^{-1}.

dissociation experiment were used. Two-point dissociation experiments are a simplified procedure for studying radioligand dissociation, where only the amount of radioligand bound at the start and after a fixed interval of dissociation are measured (Kostensis and Mohr, 1996), allowing the amount of radiolabel used in each experiment to be reduced. However, this method is only valid for use if full time-course of radioligand dissociation is monophasic both in the absence and presence of modulator (Kostenis and Mohr, 1996; Lazareno and Birdsall, 1995), which was the case in this study.

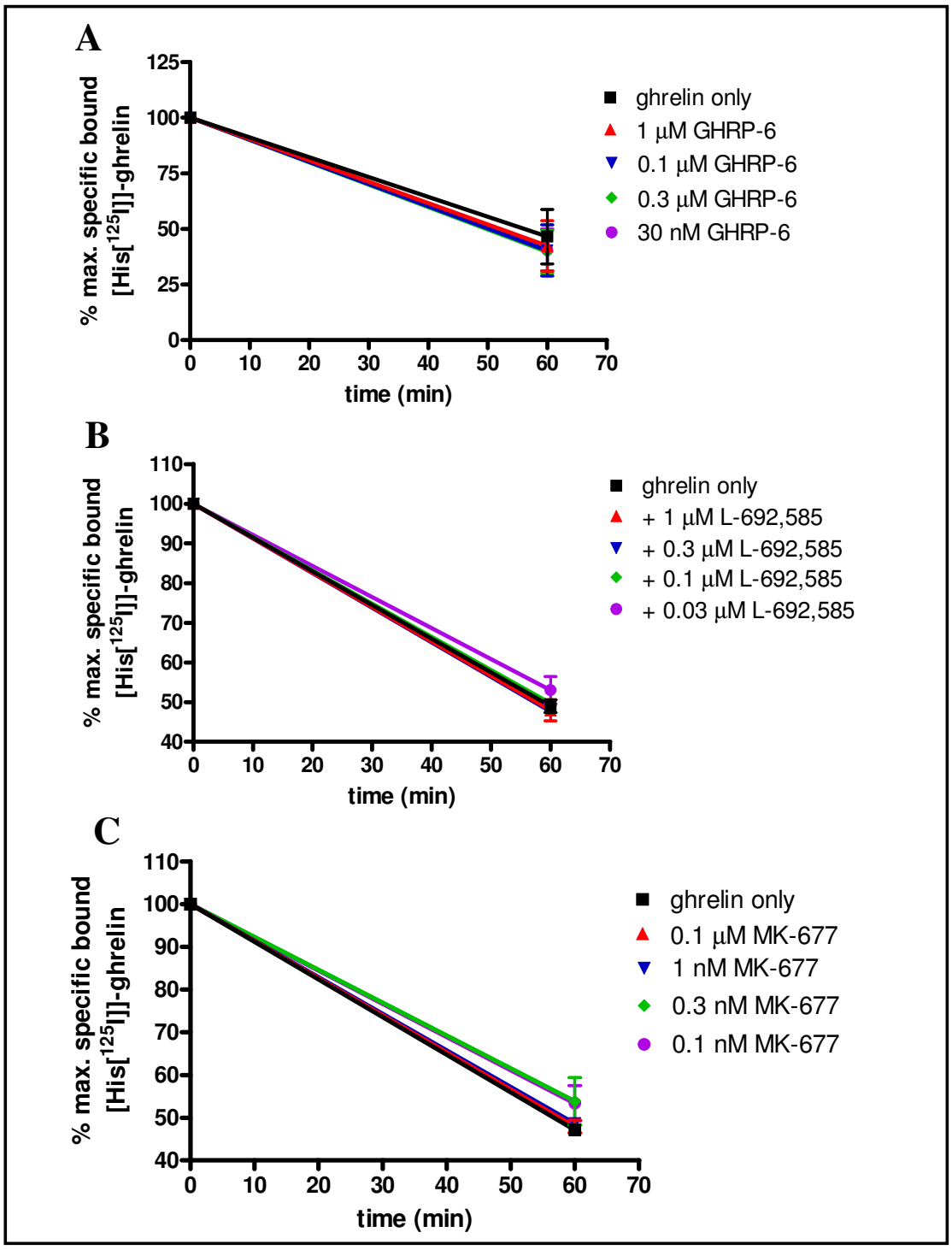

Figure 3-14 - GHRP-6, L-692,585 and MK-677 do not affect the dissociation rate of [His[^{125}I]]-ghrelin from the GRLN-R. Following association, the dissociation of [His[^{125}I]]-ghrelin from membranes co-expressing the GRLN-R and $G_{\alpha o1}$ was measured 0 min and 60 min after the addition of 1 µM ghrelin in the presence or absence of varying concentrations of **A.** GHRP-6, **B.** L-692,585 or **C.** MK-677. The assay was terminated by rapid filtration through GF/C filters pre-soaked in 0.5 $\%_{(w/v)}$ PEI. Filters were allowed to air-dry and bound [His[^{125}I]]-ghrelin measured for 60 s using a [^{125}I] counting programme on a LS 6500 scintillation counter. Data points represent the mean ± S.E.M of three independent experiments performed in triplicate. Data is expressed as the percentage of specific binding achieved in the absence of competitor and fitted with linear regression.

Specific binding of [His[^{125}I]]-ghrelin in membranes co-expressing the GRLN-R and $G_{\alpha o1}$ was measured 60 min after administration of cold ghrelin in the presence or absence of increasing concentrations of GHRP-6, MK-677 and L-692,585 and data fitted with linear-regression (Figure 3-14).

The use of a one-way ANOVA revealed that dissociation of [His[^{125}I]]-ghrelin was not altered by co-administration of ghrelin with either GHRP-6 (p=0.99), L-692,585 (p=0.45) or MK-677 (p=0.47). Thus even at maximally effective concentrations of the growth hormone secretagogues there was no change in the rate of dissociation of [His[^{125}I]]-ghrelin compared to that achieved by ghrelin acting on its own; data consistent with the lack of an allosteric effect of the growth hormone secretagogues on the binding of [His[^{125}I]]-ghrelin.

3.2.8 The inverse agonist SPA competes with ghrelin and the growth hormone secretagogues

To investigate whether ghrelin and the growth hormones did bind to similar sites on the GRLN-R, the effect of incubation of SPA on [^{35}S]GTPγS binding evoked by ghrelin, GHRP-6, L-692,585 and MK-677 was tested.

A [^{35}S]GTPγS scintillation proximity assay was used to measure the response of ghrelin or the growth hormone secretagogues at a concentration that evoked a response around 80 % of the maximum signalling achieved by each ligand in the [^{35}S]GTPγS binding assays (as measured in Figure 3-3). Concentration-response curves to SPA were then generated in the presence of EC_{80} concentrations of ghrelin, GHRP-6, L-692,585 and MK-677 (Figure 3-15). In all instances, SPA was able to decrease [^{35}S]GTPγS binding in a concentration-dependent manner, thus acting as an antagonist at the GRLN-R. Fitting dose-response curves to the data allowed determination of pIC_{50} values of SPA, as listed in Table 3-11.

Table 3-11 – Potency values of SPA in inhibiting an EC_{80} concentration of ghrelin, GHRP-6, L-692,585 or MK-677.

Ligand	pIC_{50} ± S.E.M
Ghrelin	5.98 ± 0.13
MK-677	6.85 ± 0.15
GHRP-6	6.62 ± 0.21
L-692,585	6.38 ± 0.12

Data is expressed as the mean ± S.E.M of three individual experiments performed in triplicate.

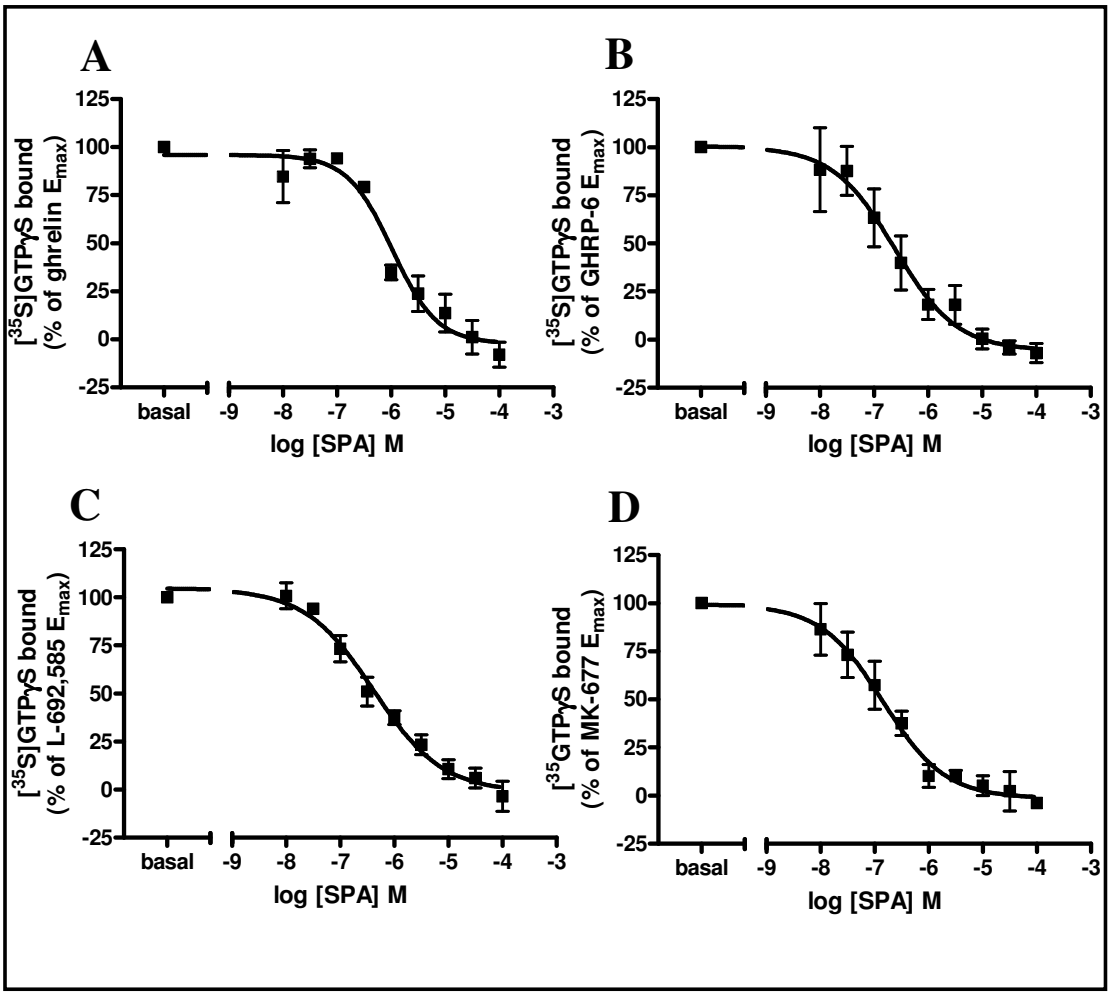

Figure 3-15 - SPA competes with ghrelin and the growth hormone secretagogues for binding to the GRLN-R. A [^{35}S]GTPγS scintillation proximity assay was used to measure the response of membranes co-expressing the GRLN-R and $G_{\alpha o1}$ to **A.** 3 nM ghrelin, **B.** 30 nM GHRP-6, **C.** 0.3 μM L-692,585 or **D.** 1 nM MK-677 in the presence of varying concentrations of the inverse agonist SPA (80 min; 25 °C). Activation of the GRLN-R was assessed by measurement of G protein-bound [^{35}S]GTPγS (5 min/plate using a Viewlux platereader).Data points represent the mean ± S.E.M of three individual experiments performed in triplicate. Data is normalised with 100 % being the response achieved by the EC_{80} concentration of growth hormone secretagogues in the absence of SPA and 0 % being the basal response of the membranes.

To determine whether SPA acted in a competitive or non-competitive manner Schild analysis was used; where concentration-response curves to ghrelin, GHRP-6, L-692,585 and MK-677 were performed in the presence of multiple, fixed concentrations of SPA. Data was globally fitted with the equation described by Motulsky and Christopoulos (2004) (Equation 3-5).

Equation 3-5 - Global-fit model described by Motulsky and Christopoulos (2004).

$$Y = Bottom + \frac{(Top - Bottom)}{1 + \left(\frac{10^{\log EC_{50}} \left[1 + \left([B] / 10^{-pA_2} \right)^{s} \right]}{[A]} \right)^{n_H}}$$

Definitions of the terms in Equation 3-4 are described in Materials and methods, section 1.9.20.2.

Fitting of the data to the global fit model revealed that SPA caused a concentration-dependent decrease in the baseline of the concentration-response curves to ghrelin and the growth hormone secretagogues. This is best demonstrated in Figure 3-16 which showed at a GHRP-6 concentration of 1 pM the addition of 0.3-10 μM of SPA caused a significant decrease in [^{35}S]GTPγS binding ($p<0.05$; as measured using a one-way ANOVA with Dunnett's post-hoc test). Thus, due to the inverse agonist effects of SPA, the data from the Schild analysis appeared poorly fitted to the global fit model (Figure 3-16), indeed re-analysing the data so that for each curve the basal values of [^{35}S]GTPγS were unconstrained led to a better fit of the data (measured using an F-test) and reduced the apparent affinity of SPA for the GRLN-R. However, as there was a distinct trend for the baseline of the curves to decrease (which breaks the rules of Schild analysis which requires that parallel shifts of the concentration-response curves should occur with no dimunition in maxima; Kenakin, 2006) the data was normalised to remove the basal level of [^{35}S]GTPγS binding. Once again this reduced the apparent affinity of SPA for the GRLN-R (Table 3-12; Figure 3-17).

Fitting the data to the global fit model resulted in Schild slopes that were not significantly different to 1 ($p>0.05$; measured using an F-test). Thus SPA could be seen to act as a competitive antagonist. Constraining the Schild slope to 1 allowed measurement of the affinity of SPA for the GRLN-R (pK_B). Comparing pK_B values using a one-way ANOVA with Tukey's multiple comparison test revealed that the affinity of SPA for the GRLN-R was significantly lower ($p<0.01$) in the presence of ghrelin than the affinity of SPA for the GRLN-R measured in the presence of either GHRP-6 or L-692,585.

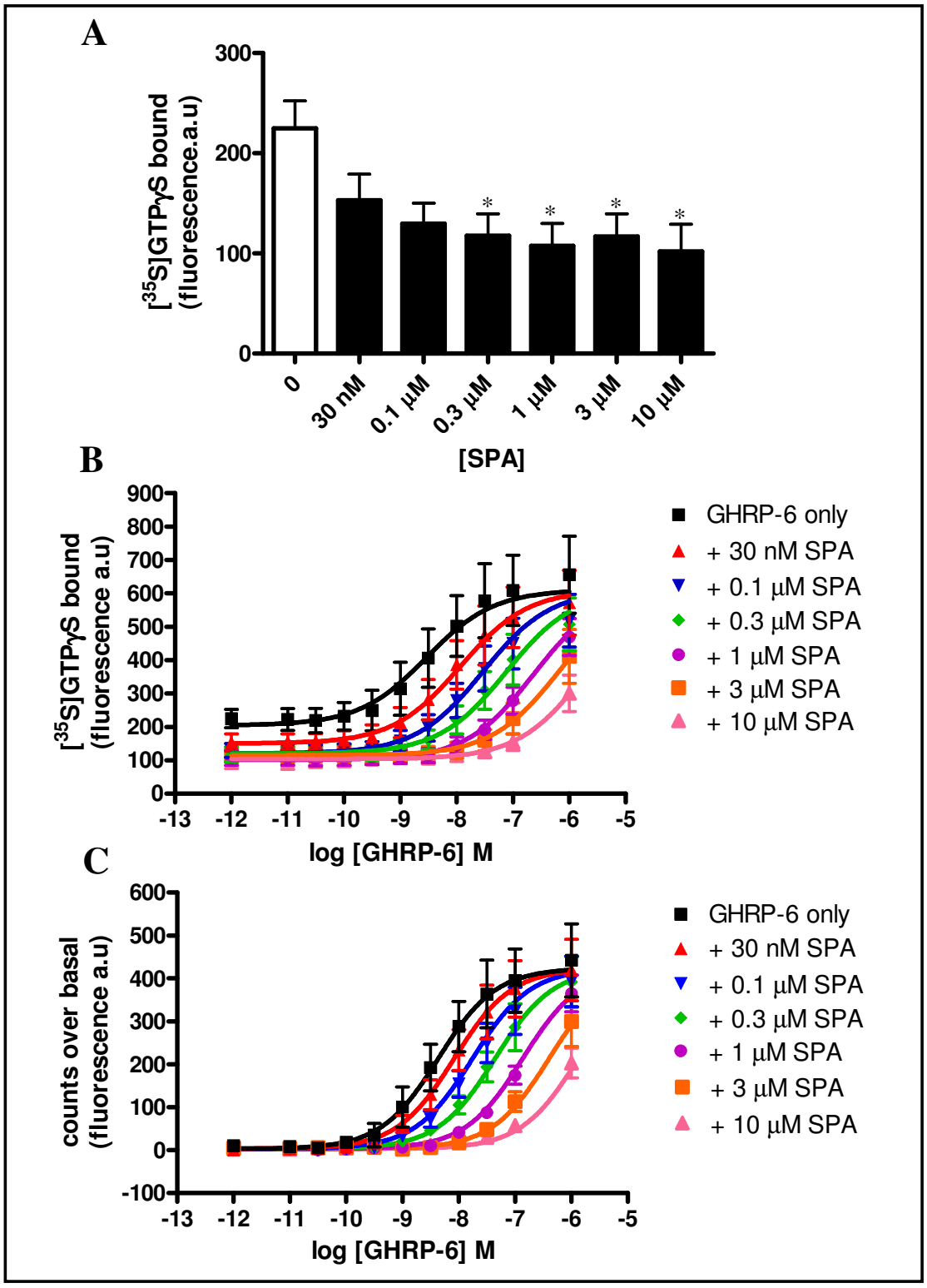

Figure 3-16 - SPA competes with GHRP-6 binding in a concentration-dependent manner. Using a [^{35}S]GTPγS scintillation proximity assay, concentration-response curves to GHRP-6 were performed in the presence of varying concentrations SPA, in membranes co-expressing the GRLN-R and $G_{\alpha o1}$. After incubation for 80 min (25 °C) G protein-bound [^{35}S]GTPγS was measured (5 min/plate using a Viewlux platereader). **A.** White bar shows the level of [^{35}S]GTPγS binding in response to 1 pM GHRP-6 in the absence of SPA. In the presence of SPA (black bars), binding of [^{35}S]GTPγS in response to 1 pM GHRP-6 is reduced in a concentration-dependent manner. *$p<0.05$; as measured using a one-way ANOVA with Dunnett's post-hoc test. **B.** Data was fitted with the global fit model (Motulsky and Christopoulos, 2004) **C.** Fitting the data to the global fit model with the baseline values removed revealed that SPA acts as a competitive antagonist. Data points represent the mean ± S.E.M of three independent experiments performed in triplicate.

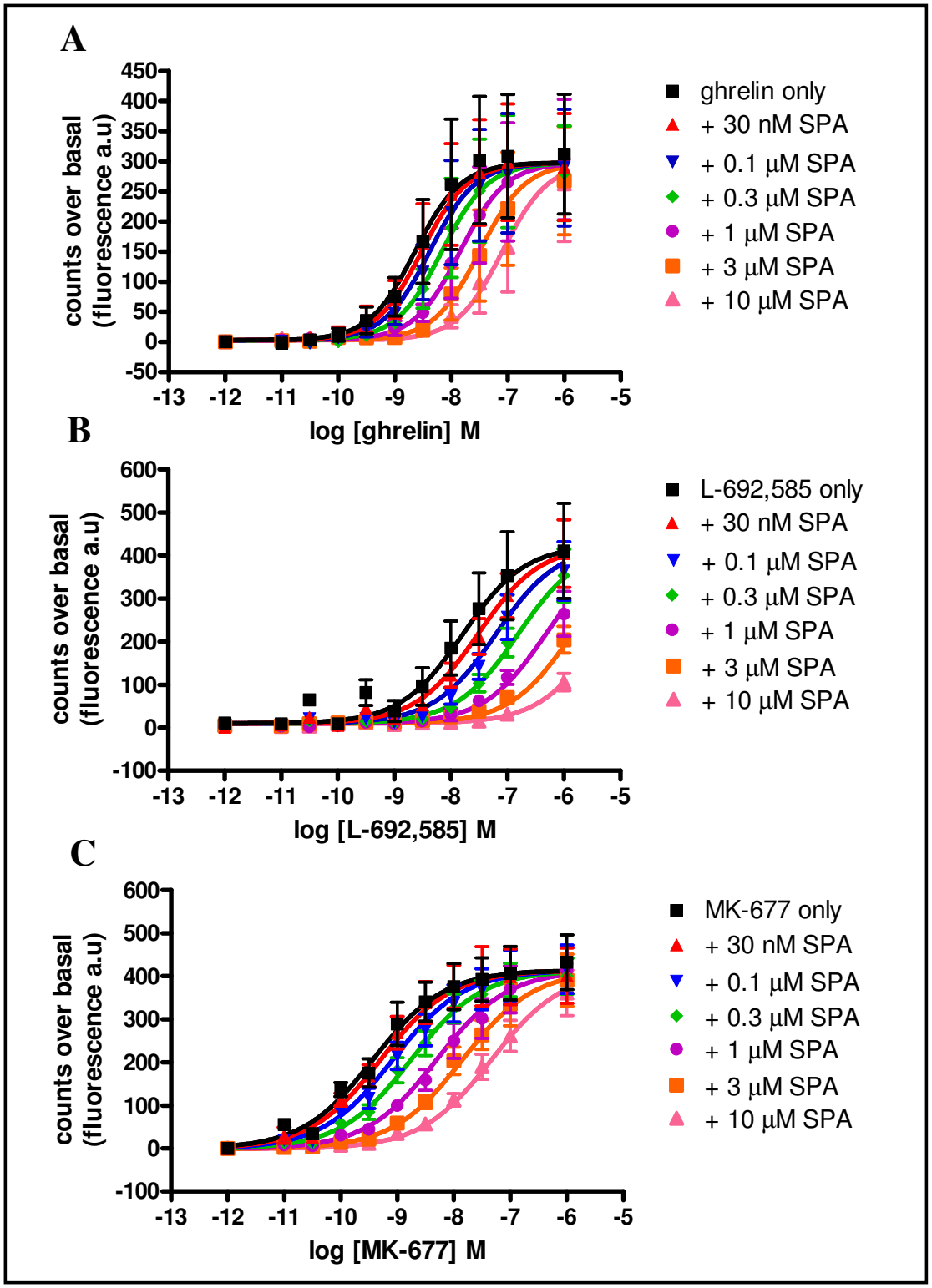

Figure 3-17 - SPA acts as a competitive antagonist in antagonising the effects of ghrelin, L-692,585 and MK-677. Using a [^{35}S]GTPγS scintillation proximity assay, concentration-response curves were generated to **A.** ghrelin, **B.** L-692,585 or **C.** MK-677 in the presence of varying concentrations of SPA, in membranes prepared from HEK293 cells co-expressing the GRLN-R and $G_{\alpha o1}$. The responses of the GRLN-R were assessed after 80 min incubation (25 °C) by measureing G-protein bound [^{35}S]GTPγS (5 min/plate, read on a Viewlux platereader). Data points represent the mean ± S.E.M of three independent experiments performed in triplicate. Data is expressed as fluorescence counts over basal and is fitted with a global fit model described by Motulsky and Christopoulos (2004).

Table 3-12 - Interaction between the inverse agonist SPA with ghrelin and the growth hormone secretagogues.

Ligand Raw data	pA_2 ± S.E.M	pK_B ± S.E.M	Slope significantly different to 1?
Ghrelin	8.05 ± 0.54	7.57 ± 0.29	No
MK-677	8.22 ± 0.24	8.09 ± 0.16	No
GHRP-6	8.86 ± 0.22	8.35 ± 0.15	No
L-692,585	8.51 ± 0.29	8.48 ± 0.17	No
Raw data unconstrained basal values			
Ghrelin	7.50 ± 0.76	7.01 ± 0.34	No
MK-677	7.93 ± 0.40	7.13 ± 0.05	No
GHRP-6	8.03 ± 0.29	7.89 ± 0.20	No
L-692,585	7.97 ± 0.36	7.75 ± 0.23	No
Normalised data			
Ghrelin	6.89 ± 0.43	6.58 ± 0.18	No
MK-677	7.21 ± 0.19	7.10 ± 0.10	No
GHRP-6	7.61 ± 0.15	7.49 ± 0.09	No
L-692,585	7.61 ± 0.17	7.45 ± 0.10	No

Concentration-response curves to ghrelin, MK-677, GHRP-6 and L-692,585 in the presence of increasing concentrations of the substance P analogue were generated and the data fitted with a global fit model described by Motulsky and Christopoulos (2004). Table lists the pA_2 value of the substance P analogue, whilst constraining the Schild slope to 1 allowed determination of the pK_B. F-tests were used to determine whether the Schild slope was significantly different from 1. Removing the base line values of [^{35}S]GTPγS binding in the presence of SPA (normalised data) led to a decrease in the apparent affinity of SPA.

3.3 Discussion

3.3.1 Ago-allosteric models

To explain the ago-allosteric effects of GHRP-6 and L-692,429 on the signalling of ghrelin, Holst and colleagues generated a complex model which relied on the GRLN-R existing as a homo-dimer (Holst et al., 2005). Although not studied by Holst et al. (2005), to date, two studies have demonstrated that the GRLN-R can form a homo-dimer (Jiang et al. 2006; Leung et al., 2007). Indeed, there have been multiple studies investigating receptor dimerisation that have described situations in which a ligand with a high affinity and/or potency for one GPCR have affected the pharmacology, function and/or cellular distribution of a second GPCR for which they have no direct affinity (Ellis et al., 2006; Parenty et al., 2008; Sohy et al., 2007; Milligan, 2008).

Given the evidence that GPCRs can form dimers, or higher oligomer structures, it is not inconceivable that allosteric modulation across the dimer interface may occur, a concept recently discussed by Milligan and Smith (2007) and Springael et al. (2007). Indeed, evidence to support allosteric modulation in receptor dimers has been supplied by studies into allosteric regulation of the $GABA_B$ receptor. The $GABA_B$ receptor is an obligatory hetero-dimeric receptor composed of two subunits; $GABA_{B1}$ and $GABA_{B2}$ (White et al., 1998; Kaupmann et al., 1998; Jones et al., 1998). Interestingly, Binet and colleagues demonstrated that the positive allosteric modulator CGP7930 activated a mutant receptor that consisted solely of the $GABA_{B2}$ subunit, as the endogenous ligand GABA binds solely to the $GABA_{B1}$ subunit, this data suggests CGP7930 could be imparting its allosteric effect across the dimer (Binet et al., 2004). However, it is much more challenging to investigate allosteric modulation across a homo-dimer. One approach that could be used is to generate an asymmetrical homo-dimer (or pseudo-hetero-dimer) between a wild-type receptor and a second receptor mutated to alter its affinity to ligand, allowing the effects of allosteric modulators to be explored in a receptor dimer that displays unique pharmacology at each of the protomers (Damian et al., 2006; Sartania et al., 2007).

Since the homo-dimeric model of ago-allosteric modulation was proposed, two further models have been developed to explain, in principle at least, how ago-allosteric modulation of a receptor may occur when the binding sites for the orthosteric and allosteric ligands overlap. In one model, binding of the allosteric ligand involves interaction of the ligand with residues that are part of the orthosteric binding site. When both the allosteric and orthosteric ligands are present, the orthosteric ligand occupies the binding site with high

affinity leaving the ago-allosteric modulator to adopt, at least partially, a different binding mode (Schwartz and Holst, 2007). In the second model, the orthosteric and allosteric ligands can bind to the receptor molecule at different points in time. The conformational interchanges of the receptor could then occur on a timescale that is relatively slow compared with the assay conditions at which receptor signalling is measured. Thus the ago-allosteric modulator could bias the receptor population for agonist binding and action – a process termed 'time-resolved allostery' (Schwartz and Holst, 2007).

Surprisingly, and in direct contrast to the original study by Holst et al. (2005), in this study no evidence was found to suggest that the growth hormone secretagogues acted as ago-allosteric modulators of the GRLN-R. Instead, data supported a much simpler model of interaction, where ligands of distinct efficacy competed for binding to the orthosteric site of the GRLN-R.

3.3.2 The growth hormone secretagogues act as super-agonists in the $G_{\alpha i/o}$ pathway

Previous studies exploring the activation of the GRLN-R in COS-7 cells transfected to express the human GRLN-R demonstrated that both GHRP-6 and MK-677 acted with higher efficacies than ghrelin (i.e. they acted as 'super-agonists'), either in increasing inositol phosphate accumulation (GHRP-6) or increasing serum-responsive element transcription or arrestin mobilisation (MK-677) (Holst et al., 2005). However, the study also revealed inconsistencies in the efficacies of the growth hormone secretagogues, depending on the assay end-point measured. For example, even though in the inositol phosphate accumulation assays GHRP-6 acted as a super-agonist, within the same pathway, calcium mobilisation studies showed GHRP-6, L-692,429 and MK-677 acted with efficacies equal to that of ghrelin (Holst et al., 2005). Discrepancies in the efficacies of the growth hormone secretagogues within the $G_{\alpha q/11}$ pathway may have resulted from the functional assays used to measure activation of the GRLN-R; as feedback, cross-regulation and even convergence of different effector systems may also be measured in downstream functional assays, complicating experimental results (Wess, 1998). To avoid this the actions of ghrelin, GHRP-6, L-692,585 and MK-677 in this study were explored at the level of receptor binding (via the use of radioligand binding studies) and at the level of G protein activation (with [^{35}S]GTPγS binding studies): thus allowing measurement of ligand efficacies and potencies in a response system close to receptor activation.

Initial [^{35}S]GTPγS binding assays revealed that the GRLN-R constitutively activated the $G_{\alpha i/o}$ pathway as expression of the GRLN-R led to a significant increase in [^{35}S]GTPγS loading, an increase that was inhibited by incubation with SPA. Indeed, SPA has previously been shown to act as an inverse agonist in the $G_{\alpha q/11}$ pathway as SPA acted to decrease inositol phosphate levels observed upon GRLN-R expression to the levels achieved in the absence of receptor (Holst et al., 2003). Although ghrelin has previously been demonstrated to activate $G_{\alpha i}$ (Bassil et al., 2007; Dezaki et al., 2007; Esler et al., 2007), to date there is a lack of data in the literature demonstrating the ability of the growth hormone secretagogues to activate the $G_{\alpha i/o}$ pathway. In this study, GHRP-6, L-692,585 and MK-677 were all able to evoke a concentration-dependent increase in [^{35}S]GTPγS binding in membranes co-expressing the human GRLN-R and the G protein α-subunit $G_{\alpha o1}$. Furthermore, GHRP-6, L-692,585 and MK-677 were all demonstrated to act as super-agonists; a finding that proved to be particularly important in the interpretation of the data gained from subsequent radioligand binding and functional studies.

3.3.3 GHRP-6, L-692,585 and MK-677 are not ago-allosteric modulators of the GRLN-R–evidence from binding studies

It is generally accepted that binding studies offer the most sensitive and direct means to detect the effect that an allosteric ligand imparts on binding of an orthosteric ligand. Thus radioligand binding studies have been widely used to investigate allosteric regulation of many GPCRs (May et al., 2003; Chan et al., 2008; Valant et al., 2008; Redka et al., 2008).

Competition binding studies used to explore [His[^{125}I]]-ghrelin binding to the GRLN-R revealed that ghrelin, GHRP-6 and MK-677 all fully competed with [His[^{125}I]]-ghrelin binding in a concentration-dependent manner, resulting in pIC_{50} and pK_i values similar to published values (see Table 3-11). A trend was also seen for the specific binding of [His[^{125}I]]-ghrelin to decrease upon treatment with 'cold' L-692,585 but sufficiently high concentrations could not be explored to allow accurate determination of the pIC_{50} or to determine whether L-692,585 fully competed with [His[^{125}I]]-ghrelin binding. Interestingly in functional studies the pEC_{50} of L-692,585 binding to the GRLN-R was shown to be 7.60 ± 0.17, so it would be expected that L-692,585 should fully inhibit [His[^{125}I]]-ghrelin binding at the concentrations tested. As potency is dependent on both affinity and efficacy of a ligand for a receptor, this suggests that L-692,585 has a low affinity for the GRLN-R but acts with high efficacy.

The finding that MK-677 and GHRP-6 fully competed with [His[^{125}I]]-ghrelin binding supported previous reports that ghrelin and the growth hormone secretagogues could bind to overlapping sites on the GRLN-R; mutagenesis studies found upon binding to the GRLN-R; ghrelin, GHRP-6, L-692,429, L-692,585 and MK-677 all made contact with a glutamic acid residue in TMIII (E124$^{3.33}$) (Feighner et al., 1998; Holst et al., 2009). Importantly mutation of E124$^{3.33}$ did not affect the cell surface expression or constitutive activity of the GRLN-R, suggesting the affect of this mutation on binding of ghrelin and the growth hormone secretagogues was not due to disturbance of the receptor structure (Holst et al., 2009).

Interestingly, the finding that GHRP-6 fully competed with [His[^{125}I]]-ghrelin binding contradicted with the results of the original study demonstrating the ago-allosteric properties of the growth hormone secretagogues (Holst et al., 2005). However, the effects of GHRP-6 on [His[^{125}I]]-ghrelin binding agreed both with the findings of Ma et al. (2007) (Table 3-13) and with the apparently overlapping binding sites of ghrelin and GHRP-6 (Feighner et al., 1998; Holst et al., 2009).

By establishing association and dissociation kinetics of [His[^{125}I]]-ghrelin binding to the GRLN-R an estimate of the equilibrium dissociation constant (K_D) for [His[^{125}I]]-ghrelin binding was calculated, yielding a K_D of 2.53 x 10^{-10} M. This K_D value was close to both kinetically derived K_D values (1x10^{-10} M for [His[^{125}I]]-ghrelin binding to human heart; Katugampola et al., 2001) and K_D values obtained from saturation binding experiments (4.4x10^{-10} and 4.1x10^{-10} M for [^{125}I-Tyr^4]-ghrelin binding to hypothalamus or pituitary, respectively; Muccioli et al., 2001 and 5.1x10^{-10} for the binding of [^{125}I-Tyr^4]-ghrelin to guinea pig ventricle; Bedendi et al., 2003).

Binding of an allosteric ligand to a GPCR results in the formation of a new structure due to the conformational change that ensues (May et al., 2007); thus binding of an allosteric ligand is predicted to alter the kinetics of binding of an orthosteric agonist. As it is difficult to determine whether an inhibitive effect on radioligand association is allosteric or competitive in nature (Kostensis and Mohr, 1996) the most common way to investigate an allosteric effect in binding assays is to measure the dissociation of an orthosteric ligand, as the only way dissociation of a pre-bound receptor-orthosteric ligand complex can be modified is by concomitant binding of a modulator to a topographically distinct and vacant site (May et al., 2007). Thus in addition to allowing the calculation of the K_D for ghrelin binding, determination of the kinetics of [His[^{125}I]]-ghrelin binding to the GRLN-R

Table 3-13 – pIC_{50} and pK_i values of ghrelin, MK-677 and GHRP-6 competing for binding against radiolabelled ghrelin.

Ligand	pIC_{50} ± S.E.M	pK_i ± S.E.M
Ghrelin	In this study: 9.06 ± 0.34 Ghrelin competing with [^{125}I]-Tyr^4 ghrelin for binding in guinea pig ventricle[1]: 8.09 ± 0.90 Ghrelin competing with [^{125}I]-ghrelin binding to human GRLN-R transfected into CHO cells[4]: 9.15 Ghrelin competing for [^{125}I]-ghrelin binding to human GRLN-R transfected into COS-7 cells[5]: 9.55	In this study: 8.97 ± 0.27 Ghrelin competing for [^{125}I]-ghrelin binding to human GRLN-R transfected into COS-7 cells[2]: 8.6
MK-677	In this study: 8.29 ± 0.60 MK-677 competing for [^{125}I]-ghrelin binding to human GRLN-R transfected into CHO cells[4]: 9.05	In this study: 8.14 ± 0.67 MK-677 competing for [^{125}I]-ghrelin binding to human GRLN-R transfected into COS-7 cells[2]: 8.18
GHRP-6	In this study: 8.12 ± 0.33 GHRP-6 fully competed for [^{125}I]-ghrelin binding to intact HEK293 cells transfected to express human ghrelin expression; no pIC_{50} or pK_i values given[6]	In this study: 7.51 ± 0.52 GHRP-6 competing for [^{125}I]-ghrelin binding to human GRLN-R transfected into NIH-3T3 cells[3]: 8.6 ± 0.0 GHRP-6 did not compete for [^{125}I]-ghrelin binding to human GRLN-R transfected into COS-7 cells[2]

Values shown correspond to results from this study and to results recorded in the literature (some studies failed to quote S.E.M values). References: [1]Bedendi et al. (2003), [2]Holst et al. (2005), [3]Ma et al., (2007), [4]Mousseaux et al. (2006), [5]Traebert et al. (2002), [6]Camina et al., 2004.

allowed the effects of the growth hormone secretagogues on the dissociation of [His[^{125}I]]-ghrelin to be explored.

Two-point kinetic experiments showed the growth hormone secretagogues had no effect on the dissociation of [His[^{125}I]]-ghrelin from the GRLN-R, as co-administration of ghrelin with increasing concentrations of GHRP-6, L-692,585 and MK-677 had no effect on the observed dissociation kinetics of [His[^{125}I]]-ghrelin; even at growth hormone secretagogue concentrations that were shown to be maximally effective in stimulating [^{35}S]GTPγS binding. This inferred that ghrelin and the growth hormone secretagogues bound to the same site on the GRLN-R. Furthermore, as ligand affinity is defined as the ratio of ligand association rates to dissociation rates, the results of this study suggests that co-administration of the growth hormone secretagogues may have no effects on the affinity of ghrelin for the GRLN-R.

3.3.4 GHRP-6, L-692,585 and MK-677 are not ago-allosteric modulators of the GRLN-R–evidence from functional studies

The use of binding assays means that only changes that affect the affinity of a radiolabelled ligand for a receptor will be detected (Kenakin, 2008). However, it is now recognised that in addition to effects on affinity, allosteric ligands can also modulate agonist efficacy and even act as agonists in their own rights (Langmead and Christopoulos, 2006). For example, the allosteric antagonist of the metabotropic glutamate receptor $mGluR_1$, 7-hydroxyiminocyclopropan[b]chromen-1a-carboxylic acid ethyl ester (CPCCOEt), has no effect on [^{3}H]-glutamate binding but decreases the maximum efficacy of glutamate in a concentration-dependent fashion (Litschig et al., 1999).

In the original study by Holst and colleagues, inositol phosphate accumulation assays showed co-administration of GHRP-6 or L-692,429 resulted in either a rightwards or a leftwards shift in the concentration-response curve to ghrelin, demonstrating either a decrease or an increase (respectively) in the potency of ghrelin (Holst et al., 2005). Thus to explore potential allosteric effects of the growth hormone secretagogues on ghrelin signalling, [^{35}S]GTPγS binding studies were utilised. Concentration-response curves to ghrelin generated in the presence of increasing concentrations of the growth hormone secretagogues revealed that there was a trend for the growth hormone secretagogues to decrease the potency of ghrelin, although the potency of ghrelin was not significantly reduced at all concentrations tested (likely due to the large variance in some of the datasets); appearing to support an allosteric interaction between ghrelin and the growth

hormone secretagogues. However, a more complex effect on the efficacy of ghrelin was revealed.

Concentration-response curves to ghrelin showed that at relatively low concentrations of ghrelin, co-administration of increasing concentrations of the growth hormone secretagogues caused a concentration-dependent increase in [^{35}S]GTPγS binding, as the growth hormone secretagogues were acting as agonists in their own right. At the highest concentrations of the growth hormone secretagogues tested, [^{35}S]GTPγS binding was increased over and above that achieved by ghrelin alone. Indeed, at the highest concentrations of the growth hormone secretagogues, increasing concentrations of ghrelin actually inhibited maximum [^{35}S]GTPγS binding, bringing [^{35}S]GTPγS levels down to around those observed for a maximally effective concentration of ghrelin in the absence of the growth hormone secretagogues.

Reversing the experimental protocol allowed the potencies and efficacies of the growth hormone secretagogues to be measured in the presence of increasing concentrations of ghrelin. However, as ghrelin acts as a high efficacy partial agonist in respect to the growth hormone secretagogues, the window in which to examine the interaction was limited. At relatively low concentrations of ghrelin, ghrelin had no effect on the potencies of the growth hormone secretagogues, but due to its agonist effects increased the level of [^{35}S]GTPγS binding in a concentration-dependent manner. At high concentrations of ghrelin there was a significant reduction in the potencies (pEC_{50}) of the growth hormone secretagogues, which appeared to support an allosteric interaction between the ligands. However, the trend towards decreasing potencies of the growth hormone secretagogues could also be explained by the finding that ghrelin acts as a partial agonist in regards to the growth hormone secretagogues. In a situation when both a full agonist and partial agonist are present, a partial agonist can compete with the full agonist for receptor occupancy, producing a net decrease in the activation of the receptor achieved by the full agonist alone, thus functionally the partial agonist acts as a competitive antagonist. This has been demonstrated at the dopamine D2 receptor where co-administration of the endogenous agonist dopamine with the partial agonist aripiprazole, resulted in aripiprazole acting to functionally antagonise dopamine signalling (Burris et al., 2002).

Many models have been formulated to describe allosteric modulation (Lazareno and Birdsall, 1995; Ehlert, 1988; Hall, 2000; Christopoulos and Kenakin, 2002); the simplest of these is the ternary complex model (Ehlert, 1988; see Figure 3-18), which describes allosteric effects in terms of changes in ligand-binding affinities only (Langmead and

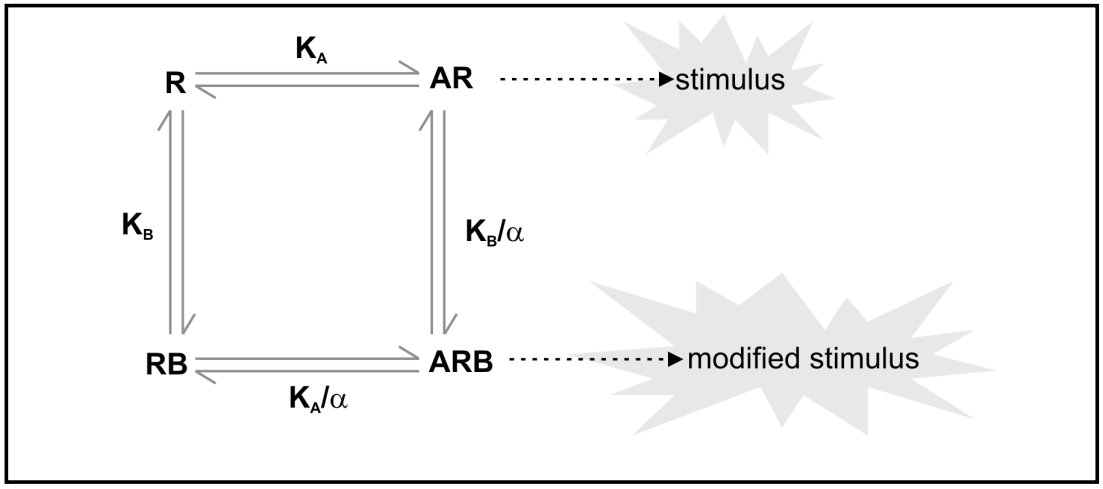

Figure 3-18 – The allosteric ternary complex model. The allosteric ternary complex model (Ehlert, 1988) describes the interaction between an orthosteric (A) and allosteric (B) ligand on binding to a receptor (R) (in terms of changes in affinity only). The interaction is described in terms of the orthosteric and allosteric equilibrium dissociation constants (K_A and K_B) and the co-operativity factor α, which describes the interaction between the two binding sites.

Christopoulos, 2006). The magnitude and direction of the allosteric effect on ligand-binding affinity is quantified by the co-operativity factor, α. An $\alpha>1$ would describe a situation of positive co-operativity (i.e. the allosteric modulator is capable of binding to the receptor where it acts to increase the affinity of the orthosteric ligand for the receptor); $\alpha<1$ would describe negative co-operativity (where the allosteric modulator decreases the affinity of the orthosteric ligand for the receptor) and $\alpha=1$ would describe a neutral effect (i.e. unaltered ligand affinity at equilibrium). An α of zero describes the situation of simple competition between the two ligands for binding to the orthosteric site of a receptor. Since the allosteric ternary complex model does not take into account any allosteric effects on efficacy, the model has been extended to include the co-operativity factors β and γ, which determines the efficacy of the orthosteric and allosteric ligand, respectively (Langmead and Christopoulos, 2006). Although these models are useful in understanding how allosteric modulation occurs, it is generally very difficult to fit such models to experimental data, thus 'operational models of allosterism' have been developed to incorporate both mechanistic and empirical factors for quantification of allosteric properties (Conn et al., 2009).

To determine whether the results from the [^{35}S]GTPγS binding experiments described an allosteric or competitive interaction between ghrelin and the growth hormone secretagogues, data was analysed using a modified version of the operational model of allosterism proposed by Leach et al. (2007). Data was fitted both when the co-operativity factor α was set to zero (a situation which would describe a simple competitive interaction) and when α was left to 'float' (to allow quantification of α). In all instances data was best

fitted to describe a competitive effect, providing further evidence to support a situation where ghrelin and the growth hormone secretagogues competed for binding to the orthosteric site of the GRLN-R.

3.3.5 SPA acts as a competitive antagonist at the GRLN-R

If ghrelin and the growth hormone secretagogues did bind to overlapping binding sites on the GRLN-R, then a ligand that acts as a competitive antagonist of the ghrelin response would be expected to antagonise the responses to the growth hormone secretagogues. To further investigate this a [^{35}S]GTPγS assay was used to test the effect of the inverse agonist, SPA, on the response of the GRLN-R to ghrelin, GHRP-6, L-692,585 and MK-677. This revealed that SPA competed with ghrelin and the growth hormone secretagogues in a concentration-dependent manner to fully inhibit [^{35}S]GTPγS binding achieved by EC_{80} concentrations of ghrelin and the growth hormone secretagogues. Schild analysis demonstrated that this antagonistic effect was competitive in nature.

The binding pocket of SPA on the GRLN-R has previously been shown to overlap with that of ghrelin (Holst et al., 2006), making contact with the key glutamic acid residue ($E124^{3.33}$) in TMIII, shown to be important for binding of ghrelin and the growth hormone secretagogues. However, as SPA also makes contact with residues outside the ghrelin-binding pocket, extending the SPA binding pocket towards TMII and TMIV of the GRLN-R (Holst et al., 2006) it cannot be determined whether ghrelin and the growth hormone secretagogues bind to the same site on the GRLN-R. Indeed, it is impossible to determine from data gained from Schild analysis alone if ligand binding sites overlap (Neubig et al., 2003), thus the results from this experiment can only confirm that binding of ghrelin or the growth hormone secretagogues is mutually exclusive of the binding of SPA.

The finding that SPA acts as a competitive antagonist contrasts with a previous report which described SPA as acting as a non-competitive antagonist of ghrelin function, tested at the human GRLN-R expressed in COS-7 cells (Holst et al., 2003). However, the data supplied by Holst et al. (2003) appeared to demonstrate that SPA acted as a competitive antagonist, as there was a clear leftwards shift of the ghrelin concentration-response curve in the presence of 1 μM SPA.

Interestingly, due to the inverse agonist effects of SPA, there was a distinct trend for the baseline of the concentration-response curves to ghrelin and the growth hormone secretagogues to be lowered in a concentration-dependent manner by co-administration

with SPA. However, concentration-response curves reflecting inverse agonism do not conform to the strict requirements of Schild analysis (i.e. that parallel shifts of concentration-response curve will occur with no dimunition in maxima) (Kenakin, 2006). Indeed, fitting data to the global fit model (Motulsky and Christopoulos, 2004) showed that due to the inverse agonists effects of SPA, the control curve (i.e. to agonist in the absence of SPA) was poorly fitted. To correct for the inverse agonist effects data was fitted to the global fit model (Motulsky and Christopoulos, 2004) after removal of baseline values of [^{35}S]GTPγS binding. This resulted in the lowering of estimates of the affinity of SPA for the GRLN-R.

Comparing the pK_B values of SPA binding to the GRLN-R revealed that SPA acted with a lower affinity for the GRLN-R when ghrelin was present (compared to when SPA was co-administered with either GHRP-6 or L-692,585). The nature of the agonist should not alter pK_B values, thus agonist-dependent antagonist pK_B values may be a sign of allosteric interaction (Christopoulos and Kenakin, 2002). Alternatively agonist-dependent pK_B values may demonstrate the formation of a unique receptor conformation, leading to an alteration in system-dependent activity manifest as differential antagonist affinities in the presence of different agonists (Hay et al., 2005). The data from the experiments performed in this Chapter support a simple competitive mode of interaction between ghrelin and the growth hormone secretagogues, thus the lower affinity of SPA for the GRLN-R in the presence of ghrelin compared to the growth hormone secretagogues suggest that ghrelin could be binding and stabilising a receptor state which exhibits a different conformation than that achieved by binding of the growth hormone secretagogues to the orthosteric site. This is supported by the study by Holst et al. (2009) who demonstrated that ghrelin and the growth hormone secretagogues have overlapping but not identical binding sites on the GRLN-R, indeed as ghrelin is a large peptide it would be expected to interact with residues outside the main ligand pocket (i.e. within ECL2) thus it is conceivable that this could alter the active receptor conformation achieved by binding of ghrelin compared to the growth hormone secretagogues.

3.4 Summary

Studies by Holst et al. (2008) and Feighner et al. (1998) demonstrated that the binding sites for ghrelin and the growth hormone secretagogues overlap - a finding supported in this study by the results from competition binding studies and dissociation studies. Initial [^{35}S]GTPγS binding studies provided evidence that ghrelin acted as a partial agonist in respect to the growth hormone secretagogues; this was an important finding for explaining

the changes in efficacy observed upon co-administration of ghrelin with the growth hormone secretagogues. Although subsequent [^{35}S]GTPγS studies showed a trend towards the potencies of the growth hormones decreasing in the presence of increasing concentrations of ghrelin, this could be explained by ghrelin acting functionally as a competitive antagonist, a finding supported by analysis of the data with a modified operational model of allosterism (Leach et al., 2007).

The results from this study do not attempt to replicate the model system used by Holst and colleagues (Holst et al., 2005), nor do they reject the conclusions that the growth hormone secretagogues act as ago-allosteric modulators of ghrelin function, at least in the $G_{\alpha q/11}$ pathway. However, in this study, the apparent allosteric actions of the growth hormone secretagogues in activating $G_{\alpha o1}$ can be fully attributed to features of ligand efficacy.

4 Construction of a Flp-In™ T-REx™ HEK293 cell line expressing the GRLN-R

4.1 Introduction

Antibodies can be useful for detecting GPCR expression, function and movement, however they may not be available for the receptor being studied or may be unsuitable for use due to low sensitivity and selectivity. To combat this epitope tags are commonly placed at the N- or C-terminus of GPCRs. Epitope tags are small peptides to which commercially available antibodies are readily obtainable and include the eleven amino acid, virally encoded transmembrane protein, vesicular stomatitis virus glycoprotein (VSV-G) (Kreis, 1986). In addition to these small tags, GPCRs may be linked to larger reporter molecules such as green fluorescent protein (GFP). GFP is a relatively small (27 kDa) protein originally isolated from the jellyfish *Aequorea victoria* (Morise et al., 1974), which possesses the ability to fold into a functional protein in a variety of expression systems without the need for substrate or accessory protein (Giepmans et al., 2006). Upon excitation with blue (395 nm) light GFP autofluoresces, emitting green light (with a wavelength of 508 nM), allowing the localisation of GFP, and any protein that is linked to it, to be visualised within a cell.

The introduction of point mutations into GFP has led to the development of a wide range of GFP variants that display distinct spectral properties. For example, altering six amino acids in GFP leads to the formation of enhanced cyan fluorescent protein (eCFP), a protein that fluoresces upon excitation with 430 nm light (Heim and Tsien, 1996). Due to improved efficiency of protein folding, eCFP displays enhanced brightness and improved solubility compared to GFP (Heim and Tsien, 1996; Cormack et al., 1996; Heim et al., 1995).

Although epitope tags and fluorescent fusion proteins are used widely in biochemical and pharmacological research, several considerations must be addressed to check that the addition of these tags/proteins to GPCRs do not alter receptor function. These include confirmation that modifying the receptor does not alter receptor expression and/or localisation, responsiveness to ligands or coupling to G proteins.

Ghrelin is a large peptide hormone that, as well as making contact with residues in the TM domains, is expected to bind to residues in the external domains of the GRLN-R, thus

tagging the receptor at the N-terminus could alter ligand binding properties. The C-terminus of the GRLN-R contains multiple residues that are thought to be important in terminating both the ligand-dependent and ligand-independent signalling of the GRLN-R (Camina et al., 2006; Holliday et al., 2007); hence the addition of eCFP to the C-terminus of the GRLN-R could potentially interfere with the pharmacology of the receptor.

The aims of this study were to:

1. Create a GRLN-R construct tagged at the N-terminus with VSV-G and at the C-terminus with eCFP.

2. Create a cell line expressing the VSV-G-GRLN-R-eCFP construct to aid pharmacological studies of the GRLN-R and test whether tagging the GRLN-R alters receptor localisation and function. It was decided to create a Flp-In™ T-REx™ HEK293 cell line, which places the gene of interest under the control of a tetracycline operon so receptor expression only occurs on the addition of tetracycline, to aid studies into the constitutive activity of the receptor. This would allow comparison of [^{35}S]GTPγS binding in the absence and presence of receptor expression within the same cell line.

4.2 Results

4.2.1 Generation of an inducible cell line expressing GRLN-R

To allow detection of the GRLN-R, a VSV-G tag (YTDIEMNRLGK) was added directly after the initiating methionine residue at the N-terminus of the receptor and an eCFP protein fused to the C-terminus using PCR (Figure 4-1). The inclusion of the VSV-G tag and eCFP fusion protein were confirmed using DNA sequencing. To construct a Flp-In™ T-REx™ HEK293 cell line expressing the GRLN-R, VSV-G-GRLN-R-eCFP was cloned into the inducible expression vector pcDNA5/FRT/TO placing VSV-G-GRLN-R-eCFP gene expression under the control of a cytomegalovirus (CMV) promoter. The CMV promoter contains two copies of a tetracycline operon system ($TeTO_2$), thus in the absence of tetracycline (or the related antibiotic doxycycline), two molecules of tetracycline repressor, expressed by the host cell line, can bind to each copy of the $TeTO_2$; inhibiting gene expression (Figure 4-2).

The pcDNA5/FRT/TO vector containing VSV-G-GRLN-R-eCFP was co-transfected with a pOG44 (a Flp-recombinase expression plasmid) into the Flp-In™ T-REx™ HEK293 cell line allowing homologous recombination to occur between the Flp recombinase target (FRT) site in the host cell line and the Flp-recombinase in the pcDNA5/FRT/TO vector (Figure 4-2). This placed VSV-G-GRLN-R-eCFP under the control of the tetracycline operon and ensured that gene expression only occurred from a single, defined chromosomal locus.

To select cells in which VSV-G-GRLN-R-eCFP had been stably integrated, hygromycin B was added to the media 48 h after transfection. Only successfully integrated

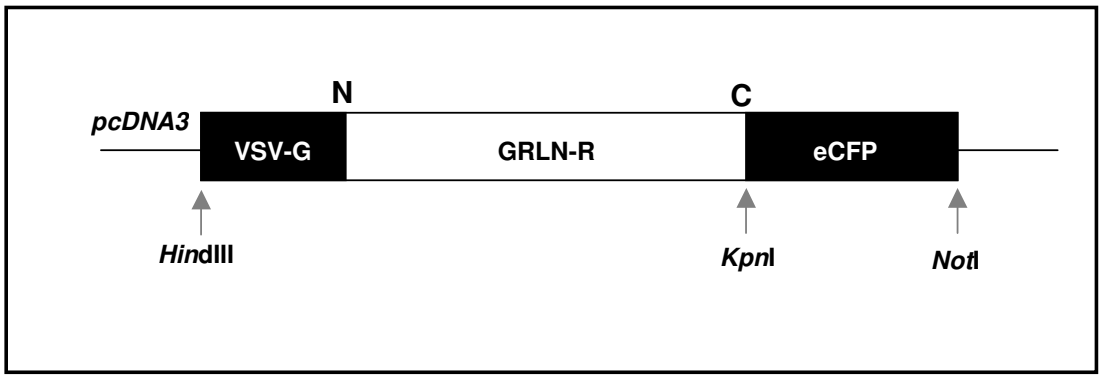

Figure 4-1 - Schematic diagram of the GRLN-R construct. PCR was used to add a *Hin*dIII site and a VSV-G tag at the N-terminus as well as to add a *Kpn*I restriction site and remove the STOP codon at the C-terminus of the GRLN-R. The resulting PCR product and vector (pcDNA3) containing eCFP were sequentially digest with *Hin*dIII and *Kpn*I, before VSV-G-GRLN-R was ligated into the vector in-frame with eCFP.

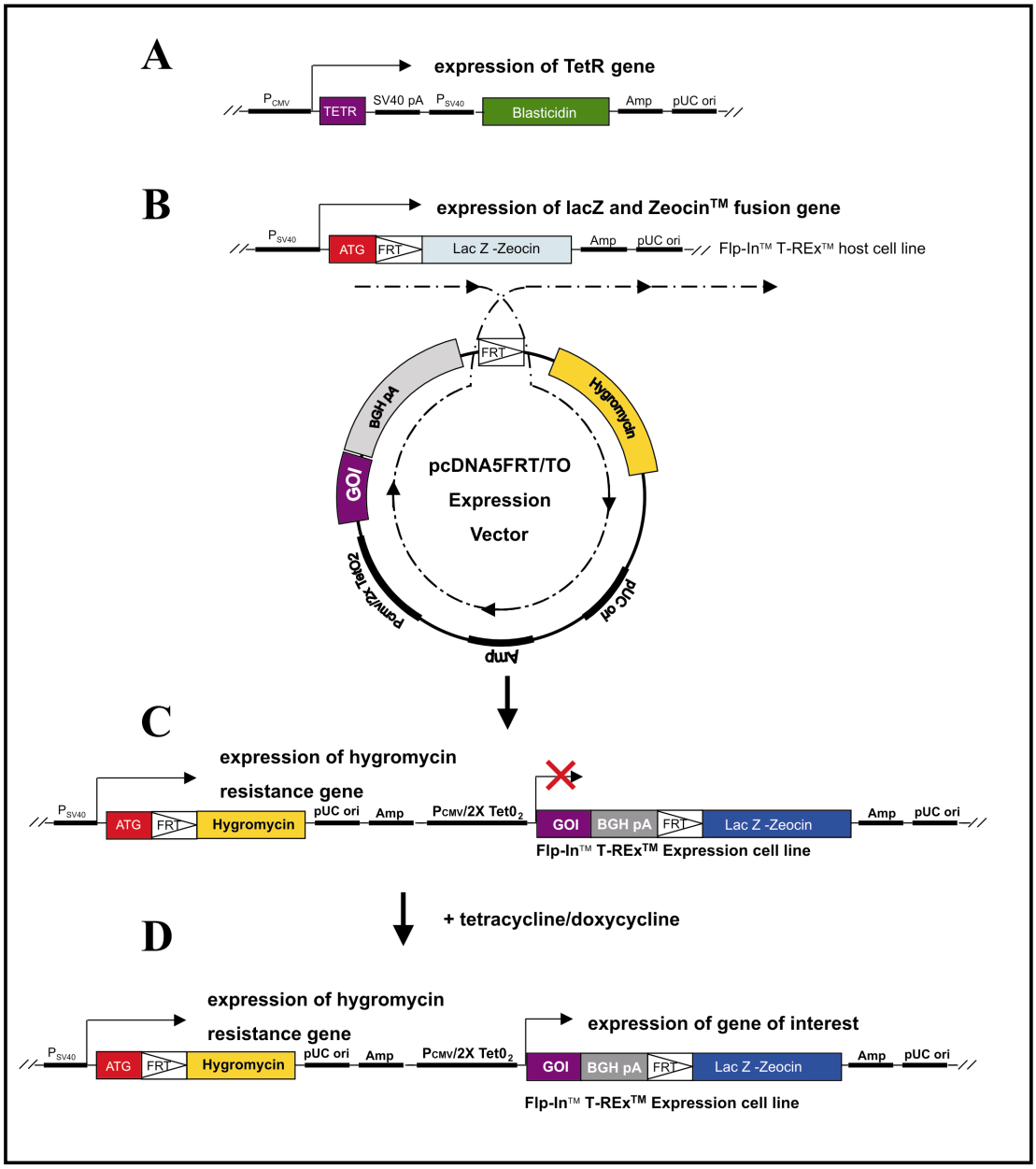

Figure 4-2 - Schematic diagram illustrating the construction of the Flp-In™ T-REx™ HEK293 cell line (adapted from Invitrogen's Flp-In™ T-REx™ HEK293 cell line product guide). A. VSV-G-GRLN-R-eCFP was ligated into a pcDNA5/FRT/TO plasmid which contained a hygromycin resistance gene lacking a promoter and START codon. This placed VSV-G-GRLN-R-eCFP under the control of a CMV promoter containing two $TetO_2$ sequences. Binding of two molecules of tetracycline (Tet) repressor produced by the Flp-In T-REx HEK293 cells to each of these sequences suppressed gene expression. **B**. Upon co-transfection of the pcDNA5/FRT/TO with pOG44 the Flp-recombinase in pOG44 mediated a homologous recombination event between the FRT sites in the host cells and pcDNA5/FRT/TO vector - integrating VSV-G-GRLN-R-eCFP into the genome at the FRT site. **C**. Integration of pcDNA5/FRT/TO bought the SV40 promoter and START codon (from the lac Z zeocin gene) into proximity to the hygromycin resistance gene conferring cells hygromycin resistance. **D**. To induce gene expression either tetracycline or doxycycline (used in these studies) can be added to the cells, this binds with high affinity to the Tet repressor causing a conformational change in the repressor rendering it unable to bind to the Tet operator. The dissociation of the doxycycline:repressor from the complex allows induction of transcription of the specific gene of interest, in this case, the GRLN-R gene.

pcDNA5/FRT/TO vector confers hygromycin resistance to the cells, as the hygromycin resistance gene lacks a promoter and start codon until successful integration into the parental Flp-In™ T-REx™ HEK293 cell line (where it is brought into close proximity to a SV40 promoter and ATG initiation codon).

4.2.2 Testing VSV-G-GRLN-R-eCFP protein expression

To test protein expression Flp-In™ T-REx™ HEK293 cells transfected with VSV-G-GRLN-R-eCFP (hereafter referred to as GRLN-R cells) were grown in media supplemented with 1 μg/mL doxycycline for 72 h. Membranes were prepared and immunoblotted using anti-GFP antiserum (antiserum that can detect eCFP; Lopez-Gimenez et al., 2007) (Figure 4-3). Upon doxycycline treatment receptor expression was detected with an apparent molecular mass of around 105-160 kDa. As the mobility of the species was larger than expected, samples were treated with peptide-N^4-(acetyl-β-glucosaminyl)-asparagine amidase (PNGaseF) – an enzyme that cleaves most common glycan moieties. PNGaseF treatment decreased the apparent molecular mass of the 105-160 kDa species to around 50-70 kDa.

Strikingly, treating GRLN-R cells with doxycycline (1 μg/mL; 72 h) led to considerable cell detachment with the remaining cells appearing rounded compared to uninduced cells (Figure 4-4). As uninduced cells appeared healthy and as, within our laboratory, Flp-In™ T-REx™ cell lines created with other receptors (for example, the orexin 1 receptor; Ellis et al., 2006) did not show the same phenotype, it appeared that the effect was mediated by expression of the receptor construct. Thus to optimise doxycycline treatment a time course of doxycycline induction was assessed using lysates prepared from GRLN-R cells treated with 1 μg/mL doxycycline for 0-12 h. Samples were resolved by SDS-PAGE followed by

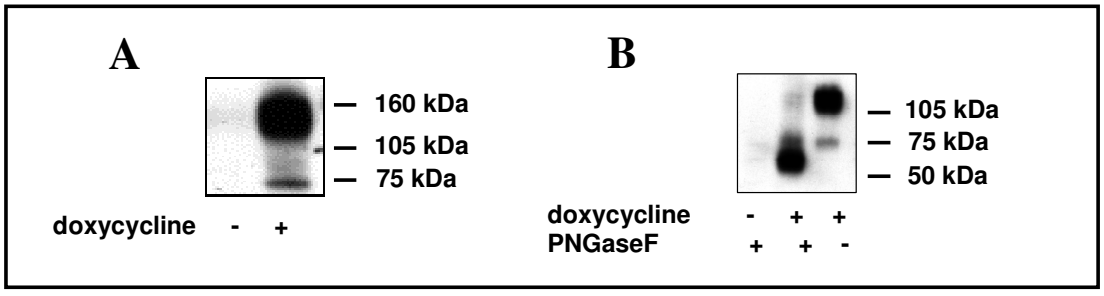

Figure 4-3 - Testing doxycycline induction in the Flp-In™ T-REx™ HEK293 cell line expressing VSV-G-GRLN-R-eCFP. Cells were incubated in media with (+) or without (-) 1 μg/mL doxycycline (as indicated). **A.** After 72 h cells were harvested, membranes prepared and resolved by SDS-PAGE followed by immunoblotting with anti-GFP antiserum. **B**. After 72 h of doxycycline treatment membranes were prepared and 30 μg of sample treated with 1 U PNGaseF (16 h; 4 °C; as indicated). Samples were resolved by SDS-PAGE followed by immunoblotting with sheep anti-GFP antiserum. Immunoblots shown are representative of three individual experiments.

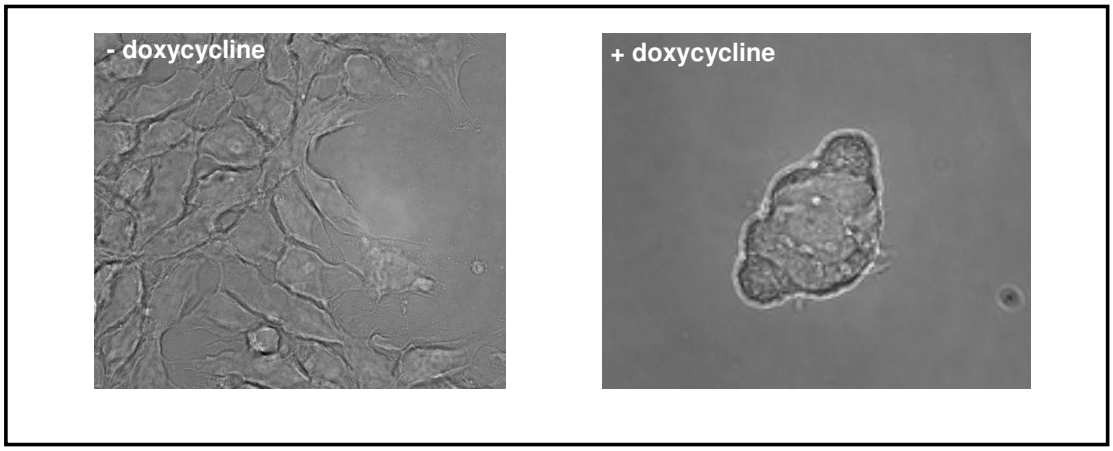

Figure 4-4 - Brightfield microscopy images of GRLN-R cells in their basal state and upon receptor expression. Cells were grown on coverslips and were treated with or without 1 µg/mL doxycycline for 72 h (37 °C). Cells were fixed using formaldehyde (3.7 $\%_{(v/v)}$; 10 min; 25 °C), mounted onto microscope slides and visualised using brightfield microscopy. Inducing GRLN-R expression led to rounding of the cells. Images shown are representative of three individual experiments.

immunoblotting using anti-GFP antiserum (Figure 4-5). This showed expression of the GRLN-R construct increased in a time-dependent manner, with a large proportion of mature receptor detected at all time points beyond 6 h.

Lysates were also prepared from GRLN-R cells that had been grown in media supplemented with varying concentrations of doxycycline (0–50 ng/mL) for 16 h. The time of 16 h was chosen for ease of receptor induction for future experiments. Lysates were resolved by SDS-PAGE and immunoblotted with anti-GFP antiserum alongside control lysates prepared from parental Flp-In™ T-REx™ HEK293 cells. Incubation of GRLN-R cells with 3-50 ng/mL doxycycline, led to a concentration-dependent increase in the intensity of two diffuse signals; one with an apparent molecular mass of around 105 kDa and another with an apparent molecular mass of around 70 kDa (Figure 4-5).

To confirm that increasing the concentration of doxycycline led to an increase in protein expression and to test that the addition of N- and C-terminal tags to the GRLN-R did not affect the ability of the receptor to signal to ghrelin, a functional assay was utilised (Figure 4-5). Membranes prepared from GRLN-R cells, transfected with $G_{\alpha o1}$ and induced with 0-25 ng/mL doxycycline for 16 h, were tested for their ability to produce a response in a [^{35}S]GTPγS binding assay. A one-way ANOVA with Dunnett's post-hoc test showed that at concentrations of 6 and 25 ng/mL doxycycline there was a significant increase in [^{35}S]GTPγS binding in response to ghrelin ($p<0.01$). Interestingly, only at a concentration of 25 ng/mL doxycycline was the GRLN-R inverse agonist, SPA, able to reduce [^{35}S]GTPγS binding to below the level obtained in the absence of ligand ($p<0.05$).

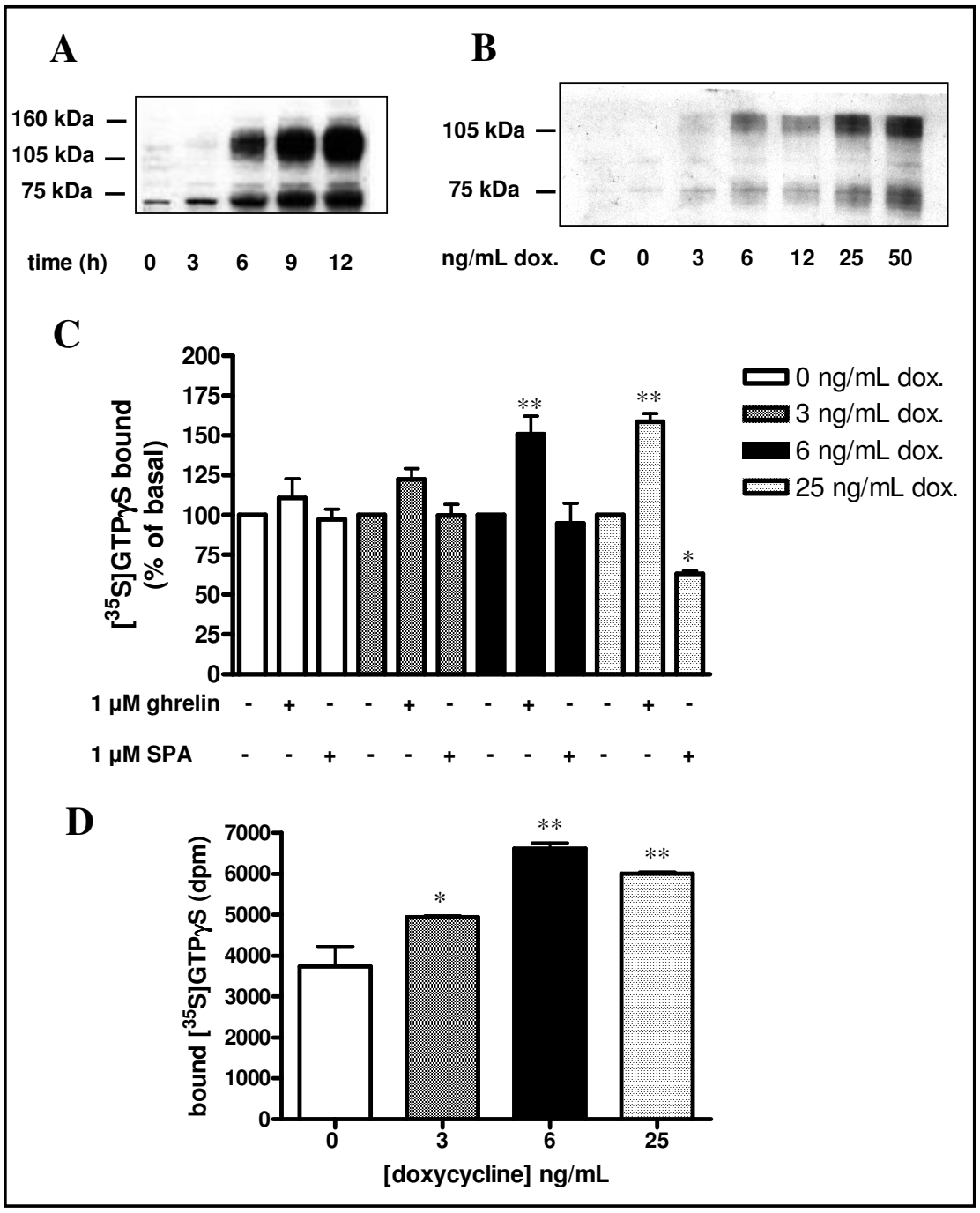

Figure 4-5 - Optimising the time and concentration of doxycycline required for GRLN-R expression. GRLN-R cells were treated with A.1 µg/mL doxycycline for varying amounts of time (0-12 h) or **B.** with varying concentrations of doxycycline (0-50 ng/mL) for 16 h. Cells were harvested and lysates prepared before samples were resolved by SDS-PAGE and immunoblotted with sheep anti-GFP antiserum (C = control lysates prepared from parental Flp-In™ T-Rex™ HEK293 cells). **C.** GRLN-R cells were treated with varying concentrations of doxycycline (dox.) (0-25 ng/mL) for 16 h. Cells were harvested and membranes prepared. A [^{35}S]GTPγS binding assay was used to measure the response of membranes to 1 µM ghrelin or 1 µM SPA (20 min; 30 °C). G protein-bound [^{35}S]GTPγS was separated from the reaction mixture by rapid filtration through GF/C filters soaked in 1 x PBS and bound [^{35}S]GTPγS measured (for 5 min) using liquid-scintillation spectrometry. Data points represent mean ± S.E.M for three independent experiments performed in triplicate, data is expressed as the percentage of [^{35}S]GTPγS bound at basal conditions at each concentration. *p<0.05, **p<0.01 when compared using a one-way ANOVA with a Dunnett's post-hoc test. **D.** Raw data of [^{35}S]GTPγS binding in membranes prepared from GRLN-R cells treated with 0-25 ng/mL doxycycline. *p<0.05, **p<0.01 when compared using a one-way ANOVA with a Dunnett's post-hoc test. Data shown is from one experiment performed in triplicate and is representative of the results from three separate experiments.

Analysis of the raw data from the experiments revealed that basal [^{35}S]GTPγS binding was significantly increased after addition of only 3 ng/mL doxycycline ($p<0.05$ for 3 ng/mL doxycycline; $p<0.01$ for 6 ng/mL and 25 ng/mL doxycycline; measured using a one-way ANOVA with Dunnett's post-hoc test).

As treatment with 6 ng/mL doxycycline for 16 h lead to a statistically significant increase in [^{35}S]GTPγS binding in response to ghrelin, this time and concentration was chosen for receptor induction in all future experiments. Using these conditions GRLN-R cells also appeared less rounded (data not shown).

4.2.3 Localisation of VSV-G-GRLN-R-eCFP

To test for proper protein folding and expression, as well as to examine receptor localisation, eCFP at the C-terminus of the GRLN-R was viewed by epifluorescence microscopy. In the absence of doxycycline no fluorescence signal was observed (Figure 4-6). Upon doxycycline induction eCFP fluorescence, and hence receptor, was observed both at the plasma membrane and in vesicles within the cell, a distribution potentially consistent with the receptor being constitutively active and internalised. The addition of ghrelin (1 μM; 30 min; 37 ºC) prior to formaldehyde-fixation led to a redistribution of receptor with a loss of cell surface expression and an increase in the appearance of vesicles within the cell.

4.2.4 Effect of the ghrelin, the growth hormone secretagogues and the inverse agonist, SPA, on GRLN-R localisation

To assess whether treatment with maximally effective concentrations (1 μM) of ghrelin, GHRP-6, L-692,585 or MK-677 could internalise the GRLN-R, cells were induced with doxycycline and stimulated for 30 min (37 ºC), prior to formaldehyde-fixation. The plasma membrane was visualised by staining carbohydrate residues with WGA-Alexa Fluor®594 conjugate prior to fixation. To view GRLN-R and plasma membrane localisation, eCFP was excited with 436/12 nm and Alexa Fluor®594 with 575/12 nm light (Figure 4-7). Upon the addition of doxcycline GRLN-R was localised both at the plasma membrane and in vesicles within the cell. Treatment with ghrelin and the growth hormone secretagogues resulted in an increase in the appearance of large punctuate vesicles inside the cell, with a corresponding loss of eCFP fluorescence at the plasma membrane.

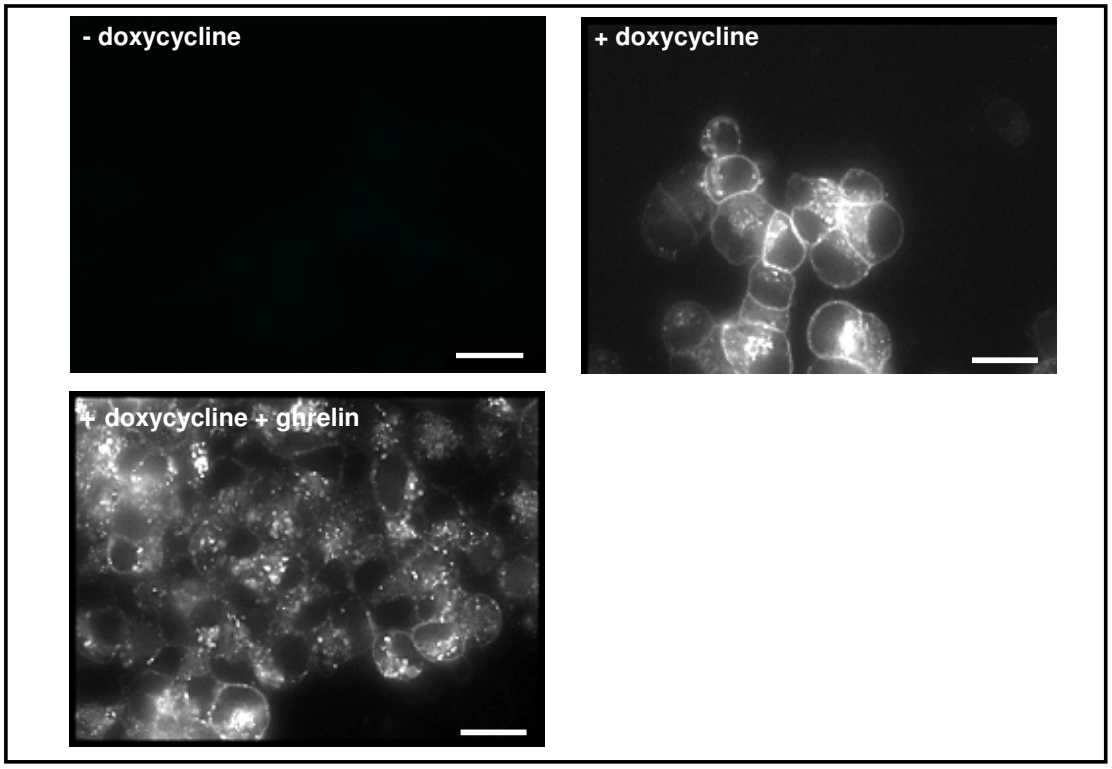

Figure 4-6 - Epifluorescence microscopy of GRLN-R cells in their basal state (- doxycycline) and after induction with doxycycline. VSV-G-GRLN-R-eCFP receptor expression was viewed by excitation of eCFP with 436/12 nm light. In the absence of doxycycline a fluorescent signal could not be detected. Upon doxycycline induction the GRLN-R could be detected both in vesicles throughout the cell and at the plasma membrane. Upon stimulation with 1 µM ghrelin (30 min; 37 °C) a redistribution of receptor was seen with an increase in punctate vesicles within the cells with a corresponding loss of cell surface receptor. Scale bar width represents 20 µm. Images are representative of three individual experiments.

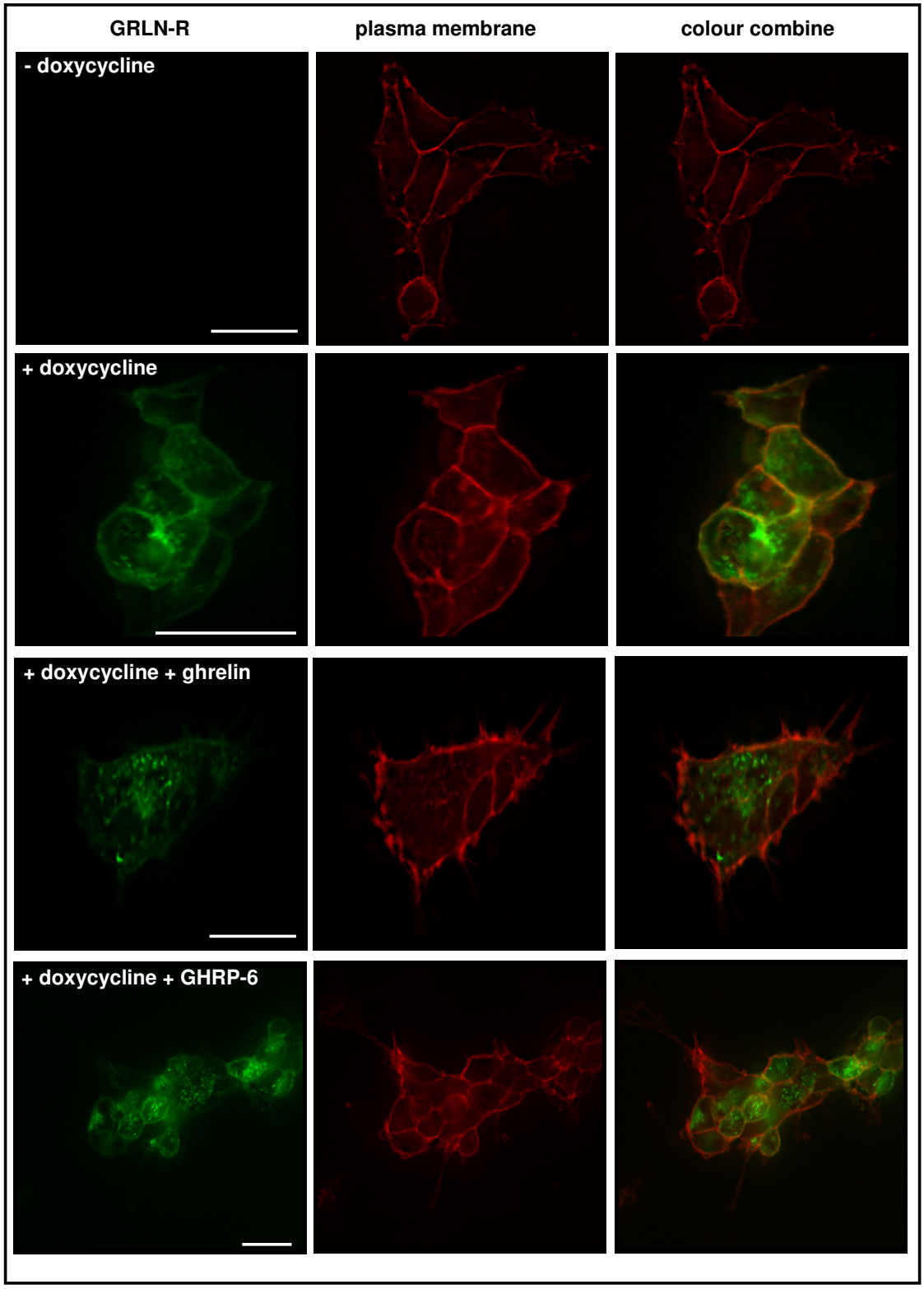

Figure 4-7 - Localisation of GRLN-R after treatment with ghrelin, GHRP-6, L-692,585, MK-677 or SPA (cont'd overleaf). Induced GRLN-R cells were treated with a maximally effective concentration of agonist (1 μM; 30 min) or inverse agonist (1 μM; 30 min or 16 h). Cells were formaldehyde-fixed before the plasma membrane was stained with WGA-Alexa Fluor® -594 conjugate. Cells were mounted onto slides and visualised using epifluorescence microscopy. The images of GRLN-R (psuedo-coloured green) and plasma membrane (red) were aligned (as seen in colour combine column) with overlapping regions shown as a yellow colour. Images are representative of three individual experiments. Scale bar width = 20 μm

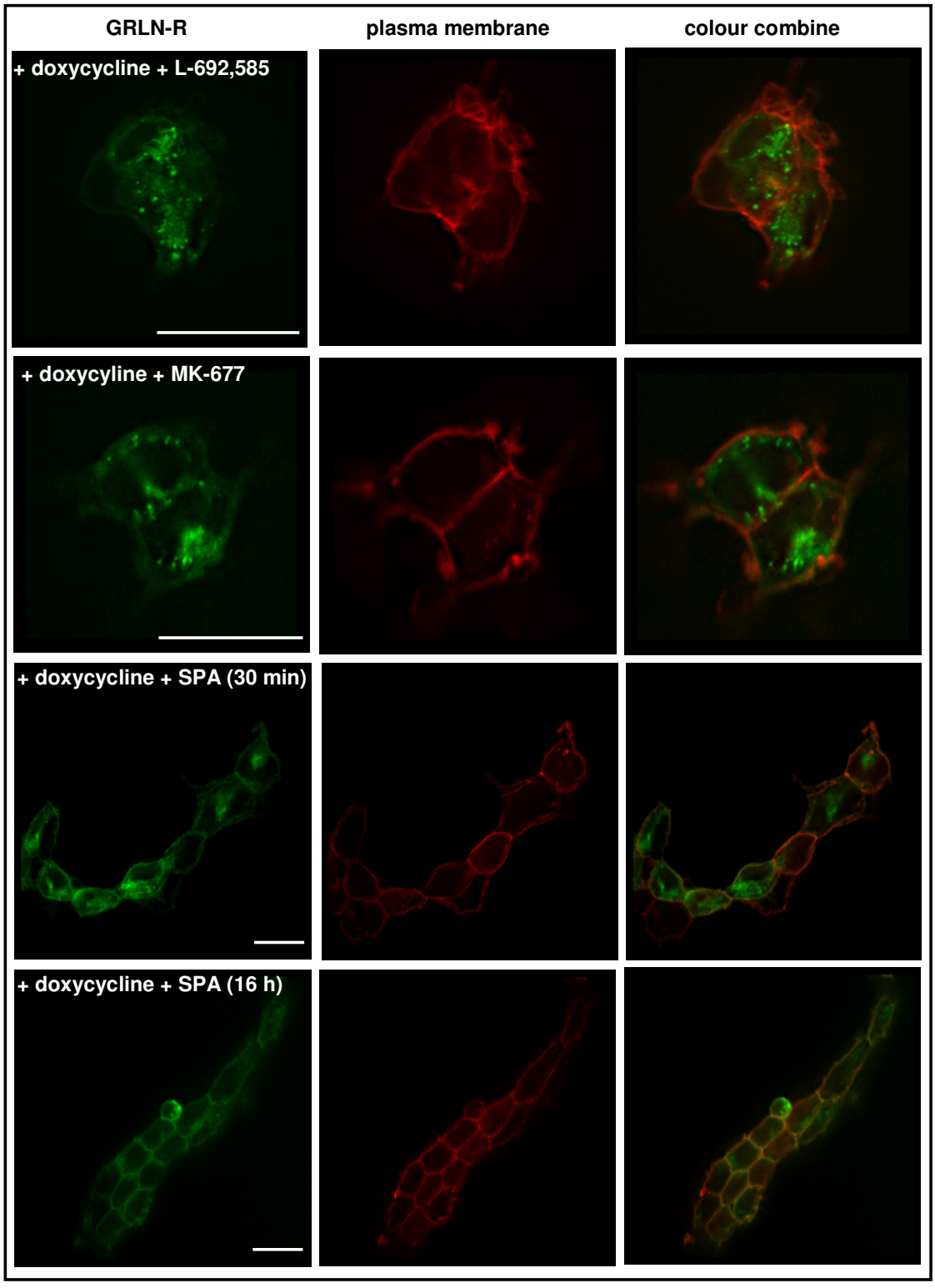

Figure 4-7 - Localisation of GRLN-R after treatment with ghrelin, GHRP-6, L-692,585, MK-677 or SPA (cont'd). Induced GRLN-R cells were treated with a maximally effective concentration of agonist (1 µM; 30 min) or inverse agonist (1 µM; 30 min or 16 h). Cells were formaldehyde-fixed before the plasma membrane was stained with WGA-Alexa Fluor® -594 conjugate. Cells were mounted onto slides and visualised using epifluorescence microscopy. The images of GRLN-R (psuedo-coloured green) and plasma membrane (red) were aligned (as seen in colour combine column) with overlapping regions shown as a yellow colour. Images are representative of three individual experiments. Scale bar width = 20 µm

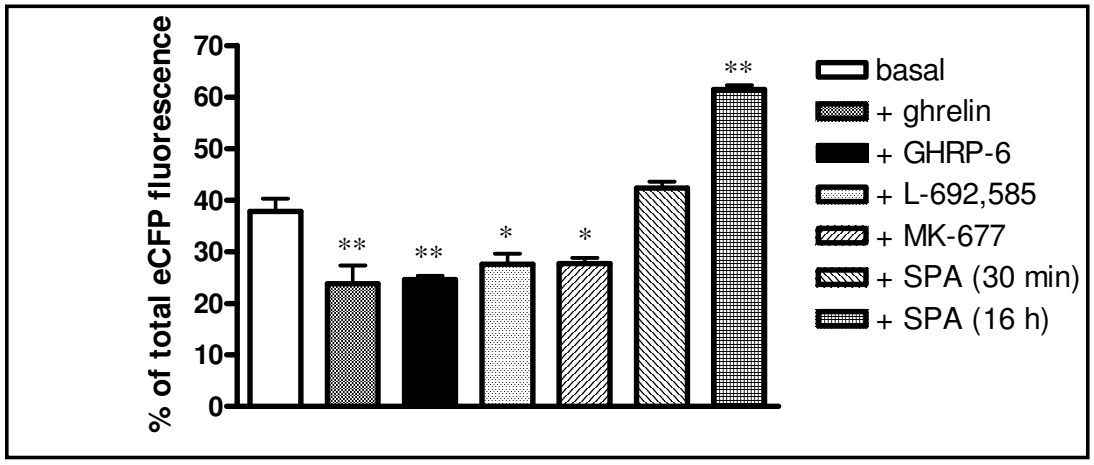

Figure 4-8 - Proportion of GRLN-R detected at the plasma membrane after the treatment of GRLN-R cells with ghrelin and the growth hormone secretagogues. Autoquant software was used to determine the proportion of receptor both at the plasma membrane and in intracellular regions of cells after treatment with 1 μM ghrelin, GHRP-6, L-692,585 MK-677 (30 min) or SPA (30 min and 16 h). Data points represent the mean ± S.E.M of the quantification of three cells.*p<0.05 **p<0.01 when compared to basal using a one-way ANOVA with a Dunnett's post-hoc test.

As SPA is an inverse agonist of the GRLN-R it would be expected to ablate the constitutive activity and internalisation of the GRLN-R. To test whether SPA altered GRLN-R localisation, cells were induced with doxycycline and treated with 1 μM SPA for either 30 min or 16 h. As SPA is a peptide ligand it is expected to be membrane-impermeable, therefore, SPA should only bind to the GRLN-R located at the plasma membrane. If receptor internalisation and recycling took longer than 30 min, than GRLN-R would still be visible as vesicles within the cell. After 30 min of SPA treatment, GRLN-R was still detected within intracellular regions of the cells, whereas after 16 h treatment the majority of receptor was located at the plasma membrane.

Internalisation was quantified using Autoquant software to determine the proportion of eCFP fluorescence at the plasma membrane and inside the cell (Figure 4-8). Using a one-way ANOVA with Dunnett's post-hoc test it was shown that there was a significant decrease in plasma membrane localised receptor upon stimulation with ghrelin, GHRP-6 ($p<0.01$), L-692,585 and MK-677 ($p<0.05$). Treatment with 1 μM SPA for 30 min did not alter the proportion of eCFP expressed at the plasma membrane, however, treatment with 1 μM SPA for 16 h significantly increased the amount of plasma membrane located receptor ($p<0.01$; one-way ANOVA with Dunnett's post-hoc test).

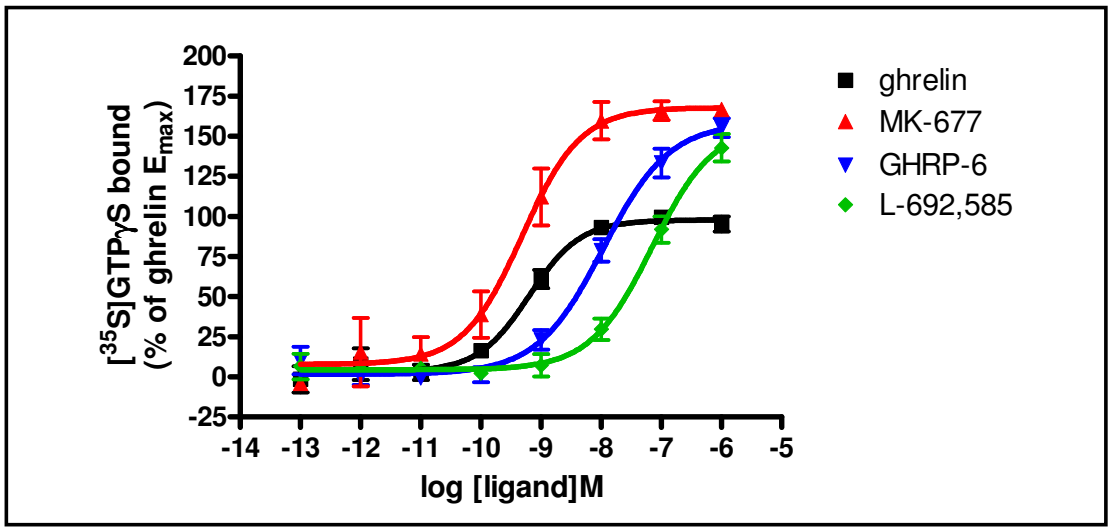

Figure 4-9 – Testing the ability of ghrelin and growth-hormone secretagogues to evoke a [^{35}S]GTPγS response in GRLN-R cells transfected to express $G_{\alpha o1}$. Concentration-response curves to ghrelin, MK-677, GHRP-6 and L-692,585 (20 min; 30 °C) were performed membranes from induced GRLN-R cells transfected with $G_{\alpha o1}$. G protein-bound [^{35}S]GTPγS was separated from the reaction mixture by rapid filtration through GF/C filters soaked in 1 x PBS and bound [^{35}S]GTPγS measured (for 5 min) using liquid-scintillation spectrometry. Data points represent mean ± S.E.M of three independent experiments performed in triplicate.

4.2.5 Response of GRLN-R through $G_{\alpha i/o}$ activation

In Chapter 3 the ability of GRLN-R to activate $G_{\alpha i/o}$ was assessed. In order to determine whether the pharmacology of GRLN-R had been altered by the addition of epitope tags concentration-response curves to ghrelin, GHRP-6, L-692,585 and MK-677 were constructed using a [^{35}S]GTPγS binding assay in membranes prepared from GRLN-R cells transfected with $G_{\alpha o1}$ (Figure 4-9). The efficacy and potency values obtained are displayed in Table 4-1.

The efficacy and potency of the growth hormone secretagogues were compared to the values obtained for ghrelin using a one-way ANOVA with Dunnett's post-hoc test. As before, GHRP-6, L-692,585 and MK-677 acted as super-agonists having a significantly higher efficacy than ghrelin ($p<0.01$). There were no differences in the values obtained for the potencies or efficacies of ghrelin and the growth hormone secretagogues acting at the wild-type (untagged) receptor compared to the epitope-tagged (tagged) receptor.

Table 4-1 - Potency and efficacy values of ligands acting at GRLN-R via $G_{\alpha i/o}$ activation as measured using a [^{35}S]GTPγS binding assay.

Ligand	pEC_{50} ± S.E.M		Max. efficacy ± S.E.M	
	Tagged receptor	Untagged receptor	Tagged receptor	Untagged receptor
Ghrelin	9.19 ± 0.16	9.11 ± 0.10	98.1 ± 1.9	95.4 ± 3.4
MK-677	9.28 ± 0.24	9.21 ± 0.12	167.8 ± 8.4	139.6 ± 9.9
GHRP-6	8.01 ± 0.07	7.85 ± 0.13	152.7 ± 6.5	139.5 ± 5.4
L-692,585	7.20 ± 0.03	7.60 ± 0.17	150.5 ± 8.0	145.4 ± 14.0

Efficacy values are displayed as the percentage of the maximum response obtained to ghrelin. Datasets were fitted with sigmoidal concentration-response curves with a Hill slopes constrained to 1. Comparing pEC_{50} values between the tagged and untagged receptor showed there was no significant differences in the potencies or efficacies of ghrelin and the growth hormone secretagogues acting at the tagged or untagged receptor. Values shown represent the mean ± S.E.M of three independent experiments performed in triplicate and compared using an un-paired, two-tailed t-test.

4.3 Discussion

4.3.1 The Flp-In™ T-REx™ HEK293 cell line allows control of GRLN-R expression

Tagging the GRLN-R at the N-terminus with VSV-G and at the C- terminus with eCFP allowed the receptor to be detected in immunoblotting and epifluorescence microscopy experiments. Using the VSV-G-GRLN-R-eCFP construct a Flp-In™ T-REx™ HEK293 cell line expressing the receptor construct under the control of a tetracycline operon was created. The use of the GRLN-R cell line in immunoblotting, epifluorescence microscopy and functional studies demonstrated that tagging the receptor had no effect on the localisation and signalling of the GRLN-R. Furthermore, in the absence of doxycycline the VSV-G-GRLN-R-eCFP construct could not be detected, demonstrating that expression of the GRLN-R gene occurred in a doxycycline-dependent manner.

4.3.2 GRLN-R is modified by N-linked glycosylation

Upon GRLN-R expression, immunoblotting of membranes prepared from GRLN-R cells revealed that the VSV-G-GRLN-R-eCFP construct existed with an apparent molecular mass that was greater than predicted (with a diffuse signal being observed running at 105 kDa instead of the predicted 70 kDa). However, during migration through the ER and the Golgi apparatus, GPCRs undergo post-translational modifications to achieve their mature status, with common modifications including the formation of disulphide bonds and the addition of oligosaccharides to the side-chain of asparagine residues (*N*-linked glycosylation). Two asparagine residues in the N-terminus of the GRLN-R are predicted to undergo *N*-linked glycosylation as they occur as part of a N-X-S/T motif. Indeed, in this study, the GRLN-R was shown to undergo *N*-linked glycosylation as treatment of membrane samples with PNGaseF, an enzyme that hydrolyses all common types of *N*-linked glycan chains, decreased the molecular mass of the 105 kDa species to around 50-70 kDa. The diffuse nature of the 105 kDa signal detected in immunoblotting was likely due to the effect of glycan residues on the movement of protein through the separating gel during SDS-PAGE as carbohydrate residues can interfere with peptide-SDS interactions (Wheatley and Hawtin, 1999).

PNGaseF cleaves all common glycan moieties and so does not distinguish between high-mannose and complex glycosylated receptor forms. Upon receptor induction immunoblotting revealed the presence of two species – one form with an apparent molecular mass of 70 kDa and a larger form with an apparent molecular mass of around

105 kDa. Although not tested in this study the lower molecular mass species is expected to correspond to immature receptor and the larger form to mature receptor.

N-linked glycosylation has been demonstrated to be vital for the correct cell-surface expression of some GPCRs, such as the angiotensin receptor AT_1 (Lanctot et al., 2006). However, this is not universally true as for other receptors disruption of *N*-linked glycosylation has no effect on receptor distribution (van Koppen and Nathanson, 1990). Indeed truncation of the N-terminus of the GRLN-R (from residues 1-35 which includes the asparagine residues that are predicted to undergo glycosylation) had no effect on the distribution of the GRLN-R (Holst et al., 2009); thus suggesting that glycosylation is not essential for the trafficking of the GRLN-R to the cell surface.

4.3.3 GRLN-R expression can be altered by time or concentration of doxycycline induction

By altering the time and concentration of doxycycline treatment in GRLN-R cells the level of receptor expression could be altered. For example, there was a time-dependent increase in GRLN-R expression in GRLN-R cells treated with 1 μg/mL doxycycline for 0-12 h, observed as an increase in intensity of 105 kDa and 70 kDa signals. After only 6 h of doxycycline treatment, the majority of GRLN-R existed with an apparent molecular mass of 105 kDa suggesting the formation of glycosylated, mature GRLN-R, which would indicate that the receptor had been synthesised, folded and transported to the plasma membrane. Immunoblotting of membranes prepared from cells induced with varying concentrations of doxycycline (0-50 ng/mL) showed that, at doxycycline concentrations of 6 ng/mL and above, the majority of the GRLN-R existed as a form with an apparent molecular mass of 105 kDa. As functional assays showed that 6 ng/mL was the lowest concentration of doxycycline to which a significant response to ghrelin was measured, treatment of GRLN-R cells for 16 h with 6 ng/mL doxycycline was deemed optimal to stimulate receptor expression.

The use of recombinant systems to express GPCRs may lead to receptors being over-expressed compared to the receptor level observed *in vivo*. Although in this study care was taken to optimise receptor expression, allowing the lowest possible level of GRLN-R expression to be used for these and future studies, even at this level receptor over-expression is likely due to the extremely low levels of endogenous GRLN-R *in vivo*. For example, GRLN-R expression levels have been reported as being 8 fmol/mg in human left

ventricle (Katugampola et al., 2001) and 6 fmol/mg in rat pituitary gland (McKee et al., 1997).

4.3.4 GRLN-R displays constitutive activity and internalisation

The GRLN-R has been reported to display a high degree of constitutive activity in the $G_{\alpha q/11}$ pathway (Holst et al., 2003). Early studies of constitutive activity by Costa and Herz showed high levels of receptor expression could uncover the existence of spontaneously active receptors (Costa and Herz, 1989). In this study, the constitutive activity of GRLN-R was shown to be linked to the level of receptor expression, as binding of [^{35}S]GTPγS was increased in a doxycycline-concentration-dependent fashion in membranes prepared from GRLN-R cells. However, the response of the GRLN-R to the inverse agonist SPA only reached statistical significance upon induction with 25 ng/mL doxycycline.

In Chapter 3 the basal [^{35}S]GTPγS loading observed upon expression of the GRLN-R was significantly reduced by the addition of 1 μM SPA. The membrane preparations used in Chapter 3 (supplied by GlaxoSmithKline) were prepared from HEK293 cells that had been transfected to express the GRLN-R using a Bacman virus, which, due to a higher transfection efficiency, would likely result in higher levels of GRLN-R expression. Thus the differences in GRLN-R expression levels may explain why SPA had a more marked effect in Chapter 3 than demonstrated here (Figure 4-5).

Interestingly, for some GPCRs, the perceived constitutive activity has been demonstrated to be due to the presence of an endogenous agonist in the cell line used to express the receptor. For example, the free fatty acid 1 receptor showed a high degree of basal [^{35}S]GTPγS loading in HEK293 cells, however, the addition of fatty acid-free BSA to the assay media reduced the apparent constitutive activity of the receptor, suggesting the presence of an endogenous fatty acid that is stripped away upon addition of fatty acid-free BSA (Stoddart et al., 2007). Other GPCRs, such as the protease-activated receptors, even have their own ligand encoded within their N-termini. Upon cleavage by thrombin or trypsin the ligand is exposed thus enabling it to activate the receptor. Furthermore, the N-terminal domain of the melanocortin-4 receptor (a receptor involved in appetite suppression) has been demonstrated to function as a tethered ligand that maintains the 'constitutive' activity of this receptor (Srinivasan et al., 2004). In the GRLN-R, the presence of phenylalanine residues in TMVI and TMVII (F279$^{6.51}$, F309$^{7.39}$ and F312$^{7.42}$) have been suggested to act as tethered agonists, maintaining the receptor in an active conformation (Schwartz and Holst, 2007). This study has made no attempt to rule out the

possibility that an agonist expressed in HEK293 cells (or the phenylalanine residues in TMVI and TMVII) cause the apparent constitutive activity of the GRLN-R, but it does confirm that tagging of the GRLN-R does not ablate the constitutive activity of the receptor.

Epifluorescence photographs showed that upon induction with 6 ng/mL doxycycline the GRLN-R was located both at the plasma membranes and in vesicles within the cell - a localisation consistent with a constitutively internalising receptor. Other studies, in both recombinant systems (Holliday et al., 2007) and in cells endogenously expressing the GRLN-R (Abizaid et al., 2006; Dass et al., 2003; Kleinz et al., 2006; Dixit et al., 2006) agree with this. In contrast, a study in CHO cells stably expressing the GRLN-R modified by the addition of a GFP tag to the C-terminus showed the GRLN-R to be plasma membrane localised (Camina et al., 2004). As the ability to observe constitutive activity is not just dependent on intrinsic receptor properties but on the specific cellular background, the apparent constitutive activity of a given GPCR may vary in different expression systems (Seifert and Wenzel-Seifert, 2002). For example, at similar expression levels, low constitutive activity of the μ-opioid receptor was seen when this receptor was transfected into CHO cells while, conversely, expression in HEK293 cells led to detection of moderate levels of activity (Burford et al., 2000; Selley et al., 2000). Indeed, although constitutive activity of the GRLN-R can be measured both in HEK293 and COS-7 cells (Holliday et al., 2007; Holst et al., 2003; Holst et al., 2004) to date constitutive activity and constitutive internalisation of the GRLN-R has not been detected in CHO cells (Camina et al., 2004; Camina et al., 2006), suggesting the absence of machinery required for constitutive signal generation and receptor internalisation in CHO cells (although the GRLN-R can still internalise upon stimulation with ghrelin; Camina et al., 2004).

In the epifluorescence microscopy experiments, only upon addition of 1 μM SPA for 16 h was the GRLN-R demonstrated to be enriched at the plasma membrane. In contrast, 30 min of SPA treatment had no significant effect on GRLN-R localisation, although this time was sufficient for SPA to reduce constitutive activation of the GRLN-R in the [^{35}S]GTPγS binding assays. As SPA is a peptide ligand it is expected that it is cell-impermeable; therefore it should only bind to receptors expressed at the cell surface. This suggests that the receptor internalisation and recycling process takes over 30 min, however, as only two time points were tested the exact kinetics of constitutive receptor internalisation could not be determined. Ligand-independent internalisation and recycling of the GRLN-R has been shown to take around 15-30 min in both COS-7 and HEK293 cells (Holliday et al., 2007; Holst et al., 2004), although this time scale is very rapid compared to the time-course of

receptor internalisation and recycling upon ghrelin treatment (3-6 h in CHO cells; Camina et al., 2004). To allow comparison of the ligand-dependent and ligand-independent internalisation of the GRLN-R, further experiments are needed to measure constitutive and agonist-induced internalisation, both within the same study and expression system.

4.3.5 Constitutive activity of GRLN-R appears to be detrimental to HEK293 cells

Upon the addition of 1 μg/mL doxycycline for 72 h, cells appeared rounded compared to cells uninduced with doxycycline, furthermore there was a considerable level of cell detachment. This effect was receptor mediated as reduction in receptor expression led to less prominent cell changes. Although not investigated further in this study, the cell rounding phenotype is likely to be mediated by the constitutive activity of the GRLN-R. This is supported by a study conducted in Kostenis's group where the expression of the constitutively active sphingosine 1-phosphate-5 receptor ($S1P_5$) led to a similar receptor-dependent cell rounding and detaching phenotype as observed here. This effect was cell line-independent as it was seen after receptor was expressed in HEK293, NIH-3T3, COS-7 and RH7777 cells (Niedernberg et al., 2003).

The cell rounding phenotype shown in this study may be due to constitutive activation of the $G_{\alpha q/11}$ pathway, which could induce calcium release from the endoplasmic reticulum and cause an influx of exogenous calcium into the cell. Calcium is an important signalling molecule that needs to be buffered within stringent margins. Constitutive release of calcium could, therefore, trigger the cell to undergo apoptosis. Surprisingly, GRLN-R has actually been shown to have an anti-apoptotic effect in primary cultured rat cortical neurones – an effect abolished by treatment with the GRLN-R antagonist/inverse agonist [D-lys-3]-GHRP-6 (Chung et al., 2008).

Actin cytoskeleton and cell rounding responses are frequently mediated by the $G_{\alpha 12/13}$ pathway to which the GRLN-R may also couple (Holst et al., 2005) therefore it is possible that the receptor is constitutively activating the $G_{\alpha 12/13}$ pathway, leading to the changes in cell morphology; a suggestion that warrants further investigation.

4.3.6 Ghrelin and the growth hormone secretagogues can internalise the GRLN-R

The eCFP tag provided an ideal way to monitor the localisation of GRLN-R in intact cells, allowing the internalisation of receptor to various ligands to be assessed. Maximally

effective concentrations of ghrelin, GHRP-6, L-692,585 and MK-677 all caused a marked redistribution in the localisation of the GRLN-R. This was demonstrated by a significant loss of fluorescent signal at the plasma membrane with a corresponding increase in the appearance of large punctate vesicles within the cell, characteristic of receptor endocytosis. The GRLN-R has previously been shown to internalise to treatment with ghrelin and GHRP-6 (Camina et al., 2004) however to the best of my knowledge the effect of GHRP-6 and L-692,585 on receptor distribution has not previously been studied.

The ability of the growth hormone secretagogues to internalise GRLN-R could not be predicted from their ability to activate the $G_{\alpha i/o}$ pathway as not all agonists are able to internalise GPCRs. For example, the μ-opioid receptor could be internalised upon treatment with etorphine but not by morphine even though both ligands promote a $G_{\alpha i/o}$-mediated response (Zhang et al., 1998). Internalisation is not inherently dependent on ligand potency or efficacy. Clinical trials on the GPR38 agonist ABT-229 showed that although ABT-229 was less potent than motilin (the endogenous agonist) it was more potent at inducing desensitisation and internalisation of GPR38 (Thielemans et al., 1995).

4.3.7 Tagging the GRLN-R does not affect receptor pharmacology

Comparing the potencies and efficacies of ghrelin and the growth hormone secretagogues acting on the wild-type (untagged) and modified (tagged) receptor showed that there was no differences in the responses of these receptors. Thus the [^{35}S]GTPγS data shows that even with the N- and C-terminal modifications, ligands can still bind to the GRLN-R and the GRLN-R can still bind to (and activate) G protein. This has also been demonstrated for other receptors such as the α_{1A} adrenoceptor (Stanasila et al., 2003).

4.3.8 Summary

The results of this chapter have shown that modifying the GRLN-R with both N- and C-terminal epitope tags does not affect receptor localisation and pharmacology. Furthermore, the Flp-In™ T-Rex™ HEK293 cell line expressing the VSV-G-GRLN-R-eCFP construct proved to be an extremely useful tool in studying both the ligand-dependent and ligand-independent activation of the GRLN-R.

5 Exploring the promiscuous G protein coupling of the GRLN-R

5.1 Introduction

It is now appreciated that instead of exhibiting just a single active state, GPCRs may display multiple active conformations. This has been elegently demonstrated in studies on the purified β_2 adrenoceptor labelled with a fluorescent probe (fluorescein malemide) at an environmentally sensitive cysteine residue (C265) located in the third intracellular loop at the cytoplasmic end of TMVI (Ghanouni et al., 2001; Swaminath et al., 2004). Using fluorescent lifetime spectroscopy to detect changes in the fluorophore, and therefore changes in the conformational state of the β_2 adrenoceptor, it could be seen that binding of the full agonist isoproterenol resulted in the display of two distinct conformational states of the receptor. However, binding of the partial agonist dobutamine to the β_2 adrenoceptor led to a significant change in the fluorescent lifetime of one of these conformational states, demonstrating that different agonists stabilise different, or 'agonist-specific', receptor states (Ghanouni et al., 2001; Kobilka, 2002).

Further evidence for agonist-specific receptor states has come from the observation that changes in the potencies and/or efficacies of a panel of ligands acting on a single receptor type can be measured upon activation of different pathways, a property that can be described by the term 'functional selectivity' (Urban et al., 2007). Functional selectivity has been demonstrated at the pituitary adenylate cyclase-activating peptide (PACAP) receptor, where the agonists $PACAP_{1-27}$ and $PACAP_{1-38}$ could stimulate PACAP receptor-mediated adenylate cyclase activation with equal potencies, but only $PACAP_{1-38}$ could evoke an increase in inositol phosphate levels through activation of phosholipase Cβ (Spengler et al., 1993). Functional selectivity, as demonstrated by differences in ligand efficacies, has been shown at the serotonin type 2C receptor ($5\text{-}HT_{2C}$) where the agonists (±)-2.5-Dimethoxy-4-iodoamphetamine (DOI) and 5-hydroxytryptamine (5-HT) displayed equal efficacies in activation of the arachidonic acid pathway, whilst DOI elicited only 60 % of the 5-HT-induced increase in inositol phosphate accumulation (Berg et al., 1998).

The GRLN-R has been described as a promiscuous receptor being able to couple to $G_{\alpha q/11}$ (Howard et al., 1996), $G_{\alpha i/o}$ (Bassil et al., 2007; Esler et al., 2007), $G_{\alpha s}$ (Rossi et al., 2008; Kohno et al., 2003) and $G_{\alpha 13}$ (Holst et al., 2005). The aim of this chapter was to further explore the promiscuous coupling of the GRLN-R, by comparing the activation of $G_{\alpha q/11}$

and $G_{\alpha i/o}$ pathways in response to ghrelin and the growth hormone secretagogues. To achieve this responses of the GRLN-R were measured both at the level of G protein activation, by the use of [^{35}S]GTPγS binding assays, and in whole cell second messenger signalling assays.

5.2 Results

5.2.1 Testing $G_{\alpha i/o}$ coupling to GRLN-R

[^{35}S]GTPγS filtration binding and scintillation proximity assays (shown in Chapters 3 and 4) are generally thought to reflect $G_{\alpha i/o}$ signalling, thus it was assumed that the responses in the [^{35}S]GTPγS filtration binding assays were mediated solely through $G_{\alpha o}$ and endogenous $G_{\alpha i/o}$ expressed in the Flp-In™ T-REx™ HEK293 cells. In this chapter I sought to confirm that this signalling was indeed $G_{\alpha i/o}$ mediated by the use of selective pharmacological inhibitors. The interaction between a receptor and the $G_{\alpha i/o}$ class of G proteins can be abolished by *Bordella pertussis* toxin (PTx)-mediated ADP-ribosylation of a cysteine residue four residues from the extreme C-terminus. Thus GRLN-R cells were co-incubated with 6 ng/mL doxycycline and 25 ng/mL PTx for 16 h. Following cell harvesting, membranes were prepared and the response to treatment with 1 μM ghrelin measured. To confirm that the response was not mediated through the $G_{\alpha q/11}$ family, membranes were challenged with 1μM ghrelin in the presence of 0.1 μM YM-254890 (Figure 5-1). YM-254890 is a toxin isolated from *Chromobacterium* species that specifically blocks the exchange of GDP for GTP on the α-subunit of the $G_{\alpha q/11}$ family of G proteins (Takasaki et al., 2004).

A one-way ANOVA with Tukey's multiple comparison test revealed that treating membranes from uninduced GRLN-R cells with 1 μM ghrelin did not increase [^{35}S]GTPγS binding compared to basal values ($p>0.05$). Inducing GRLN-R expression led to a significant increase in binding ($p<0.01$) in response to ghrelin stimulation. Indeed, binding of [^{35}S]GTPγS in membranes co-incubated with YM-254890 and ghrelin also showed a significant increase over basal levels ($p<0.01$) – an effect that was not statistically different to that obtained in the absence of YM-254890 ($p>0.05$). Treatment of membranes with PTx or a combination of PTx and YM-254890 attenuated the response to ghrelin ($p>0.05$; compared to basal values in uninduced cells). Thus it appears that the filtration method indeed measured predominantly $G_{\alpha i/o}$-mediated [^{35}S]GTPγS binding in this system.

To assess if PTx and YM-254890 were having an effect on the basal levels of [^{35}S]GTPγS binding, a one-way ANOVA was performed on the combined raw data obtained from these experiments. There was no significant difference in levels of basal [^{35}S]GTPγS binding ($p=0.75$).

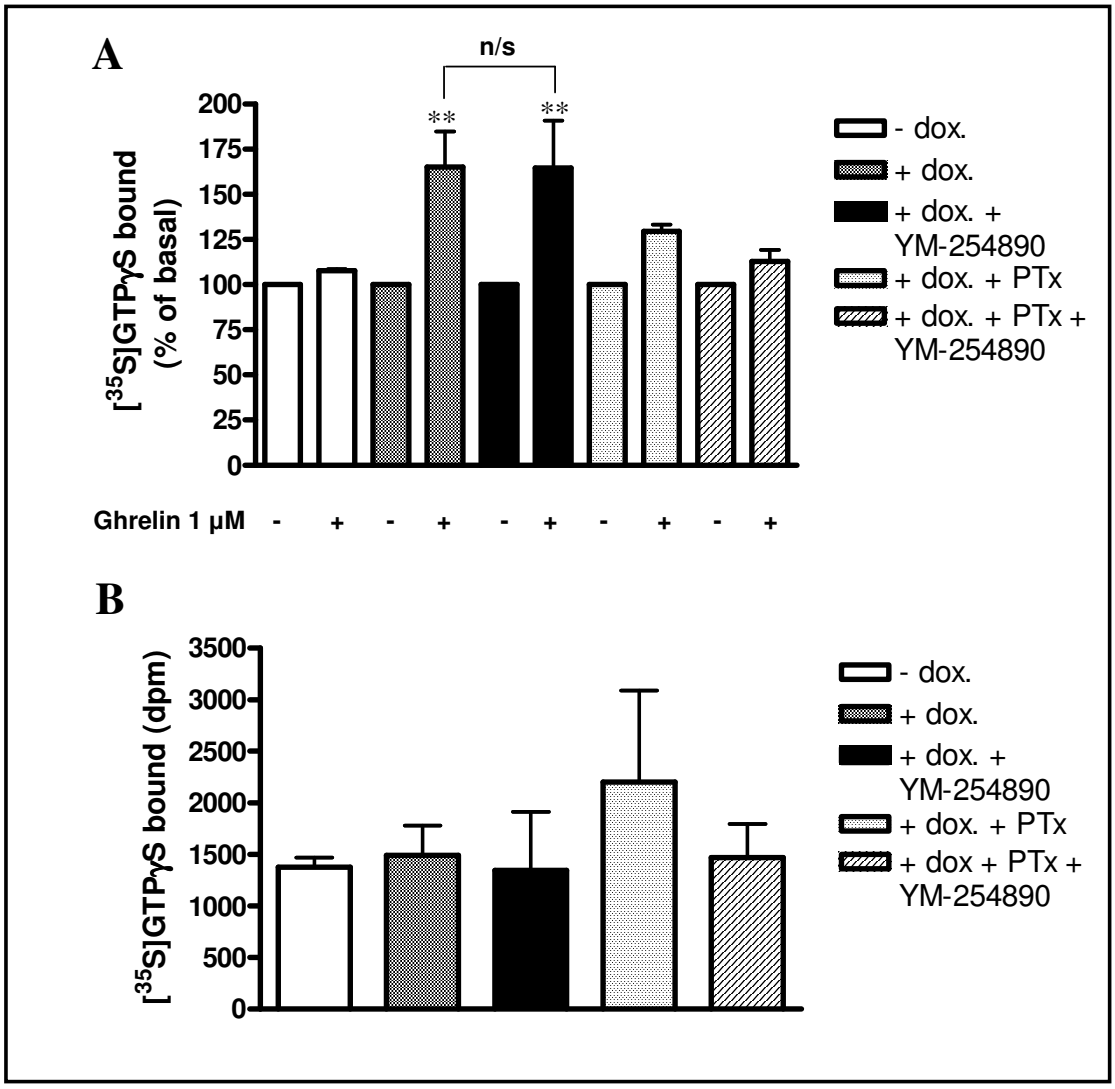

Figure 5-1 – The GRLN-R can couple to the $G_{\alpha i/o}$ pathway. GRLN-R cells were transfected with $G_{\alpha i/o}$ and receptor expression induced, as required, with 6 ng/mL doxycycline (dox.) for 16 h. **A.** To test receptor coupling to $G_{\alpha i/o}$ membranes were prepared from GRLN-R cells treated with 25 ng/mL PTx (16 h) and the response to 1 μM ghrelin (20 min; 30 ºC) measured. To test receptor coupling to $G_{\alpha q/11}$ the response to 1 μM ghrelin was measured after co-administration with 0.1 μM YM-254890 (20 min; 30 °C). G protein-bound [^{35}S]GTPγS was separated from the reaction mixture by rapid filtration through GF/C filters soaked in 1 x PBS and bound [^{35}S]GTPγS measured (for 5 min) using liquid-scintillation spectrometry. Data points represent the mean ± S.E.M of four independent experiments performed in triplicate. Data is expressed as the percentage of the response over basal values obtained in each of the conditions outlined above. N/s = non-significant, **p<0.01 when compared using a one-way ANOVA with Tukey's multiple comparison test. **B.** Raw data obtained for the basal levels of [^{35}S]GTPγS loading after treatment with YM-254890, PTx or a combination of the two. Data points represent the mean ± S.E.M of four independent experiments performed in triplicate.

In Chapter 4, I demonstrated that, in membranes from GRLN-R cells enriched with $G_{\alpha o1}$, the GRLN-R could increase [^{35}S]GTPγS binding upon treatment with ghrelin and the growth hormone secretagogues. However, transfection of extra G protein into the cells creates a stimulus-bias which could force GRLN-R to couple to non-preferred G protein species. Thus to test whether the GRLN-R could couple to $G_{\alpha i/o}$ in GRLN-R cells, a [^{35}S]GTPγS filtration assay was conducted in the absence of exogenous G protein (Figure

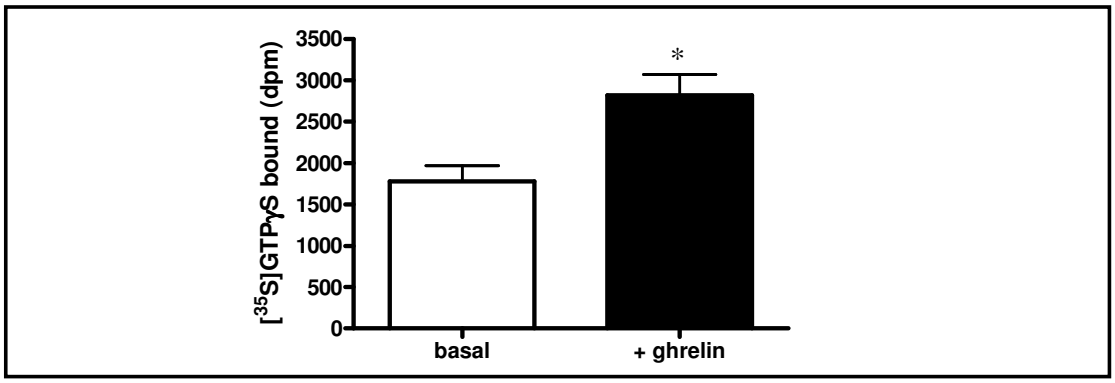

Figure 5-2 - The GRLN-R can respond to ghrelin in the absence of exogenous G protein. GRLN-R cells were induced with 6 ng/mL doxycycline (16 h) and membranes prepared. A [^{35}S]GTPγS filtration binding assay was used to measure [^{35}S]GTPγS binding at basal levels and after treatment with 1 μM ghrelin (20 min; 30 °C). G protein-bound [^{35}S]GTPγS was separated from the reaction mixture by rapid filtration through GF/C filters soaked in 1 x PBS and bound [^{35}S]GTPγS measured (for 5 min) using liquid-scintillation spectrometry. Data points represent the mean ± S.E.M of three independent experiments performed in duplicate. * $p<0.05$ as measured using an unpaired, two-tailed t-test.

5-2). An unpaired, two-tailed t-test revealed a significant increase in [^{35}S]GTPγS binding upon treatment with 1 μM ghrelin ($p<0.05$). Overall there was around a 1.2-fold increase in signal upon stimulation with ghrelin, compared to around a 2-fold increase observed upon the transfection of additional G protein.

5.2.2 Response of GRLN-R through $G_{\alpha q/11}$ activation

The [^{35}S]GTPγS filtration assay is limited to measuring the activation of the $G_{\alpha i/o}$ family of G proteins due to a combination of the high rate of basal guanine nucleotide exchange of $G_{\alpha i/o}$ and their relatively high expression levels in HEK293 cells (Milligan, 2003). To measure activation of other G proteins, [^{35}S]GTPγS binding assays are commonly employed, using an immunoprecipitation step to separate G protein-bound [^{35}S]GTPγS from unbound [^{35}S]GTPγS (e.g Stoddart et al., 2008; Parenty et al., 2008). Thus in this study, to measure activation of $G_{\alpha q/11}$, a [^{35}S]GTPγS binding assay with an immunoprecipitation step was performed in membranes prepared from induced GRLN-R cells transfected with $G_{\alpha q}$ (Figure 5-3), where [^{35}S]GTPγS-bound $G_{\alpha q}$ was measured following immunoprecipitation with anti-$G_{\alpha q}$ antisera.

To investigate whether the GRLN-R constitutively activated the $G_{\alpha q}$ pathway, $G_{\alpha q}$ was transfected into GRLN-R cells and receptor expression was induced with 6 ng/mL doxycycline (16 h). Receptor expression led to a significant increase in basal loading of [^{35}S]GTPγS ($p<0.001$), a response 17.6 ± 2.6 % over the basal response measured in the absence of the GRLN-R. Ghrelin stimulation significantly increased [^{35}S]GTPγS binding in

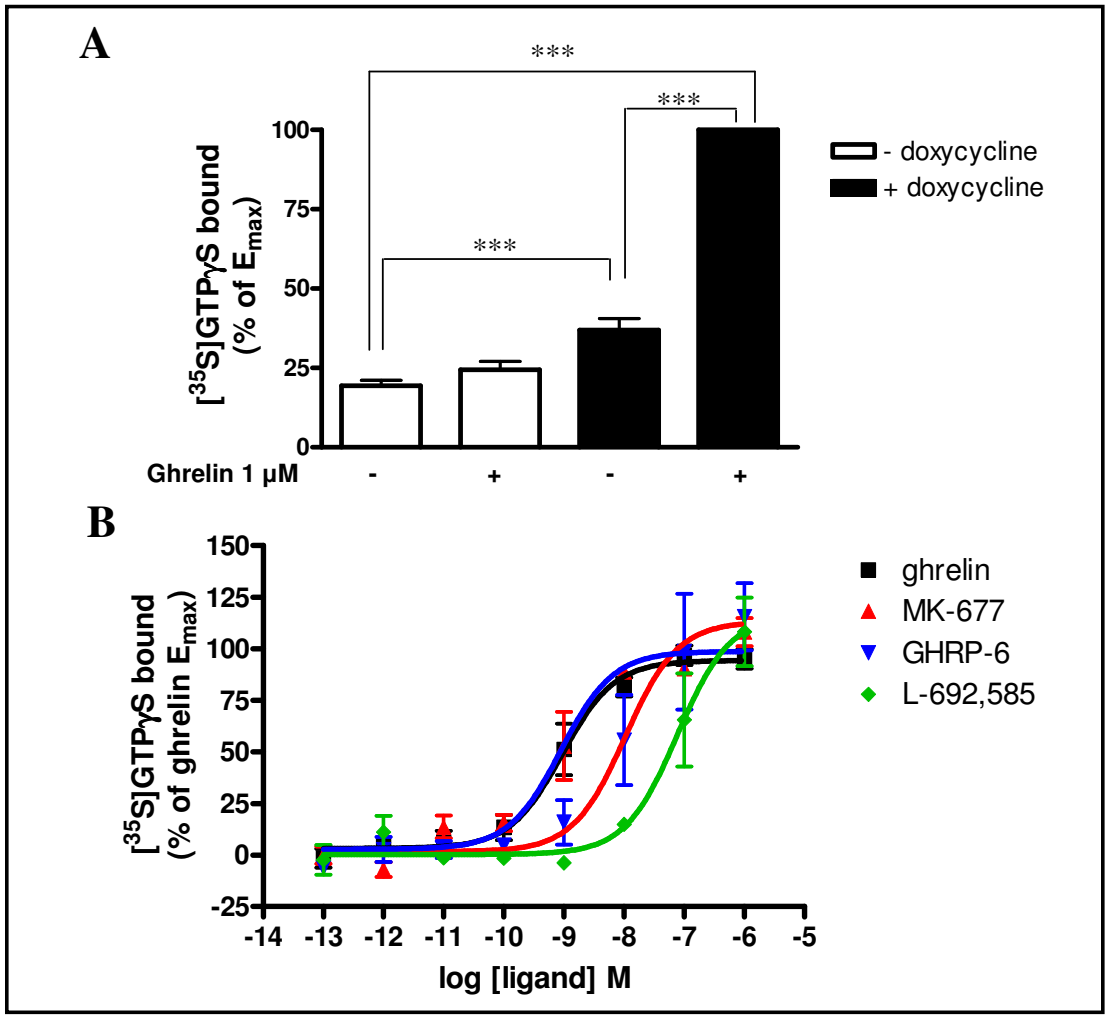

Figure 5-3 - Ghrelin and the growth hormone secretagogues can increase [^{35}S]GTPγS binding via activation of the $G_{\alpha q/11}$ pathway in membranes prepared from GRLN-R cells. A. To test whether GRLN-R was constitutively active in the $G_{\alpha q}$ pathway, membranes were prepared from GRLN-R cells transfected with $G_{\alpha q}$ and receptor expression either induced with 6ng/mL doxycycline (16 h) (black bars) or left uninduced (white bars). [^{35}S]GTPγS binding was measured at basal levels and after stimulation with 1µM ghrelin (30 min; 30 °C), as indicated. $G_{\alpha q}$ was immunoprecipitated from the reaction mixture using anti-$G_{\alpha q}$ antiserum and bound [^{35}S]GTPγS measured using liquid scintillation spectrometry (5 min/sample). Data is expressed as the percentage of the maximum response to ghrelin in doxycycline-induced cells. Data points represent the mean ± S.E.M of five independent experiments performed in triplicate. ***p <0.001 as measured using a one-way ANOVA with Tukey's multiple comparison test. **B**. Concentration-response curves to ghrelin, MK-677, GHRP-6 and L-692,585 (30 min; 30 °C) were performed on membranes prepared from induced GRLN-R cells transfected with $G_{\alpha q}$. Data points represent the mean ± S.E.M of three independent experiments performed in triplicate.

membranes prepared from induced GRLN-R cells ($p<0.001$), but had no significant affect in membranes prepared from uninduced GRLN-R cells ($p>0.05$).

To obtain potency and efficacy values for ghrelin and the growth hormone secretagogues through $G_{\alpha q/11}$ activation, concentration-response curves were generated (Figure 5-3) using a [^{35}S]GTPγS binding assay with an immunoprecipitation step. Table 5-1 displays the values obtained. A one-way ANOVA with Dunnett's post-hoc test showed that L-692,585 was significantly less potent than ghrelin in inducing a $G_{\alpha q/11}$ response ($p<0.01$). There was

no statistical difference in the efficacy of the ligands compared to ghrelin as determined using a one-way ANOVA (p=0.51).

Table 5-1 – Potency and efficacy values of ligands acting at GRLN-R via $G_{\alpha q}$ activation as measured using a [^{35}S]GTPγS binding assay with immunoprecipitation step.

Ligand	pEC_{50} ± S.E.M	E_{max} ± S.E.M
Ghrelin	9.03 ± 0.13	94.3 ± 4.0
MK-677	9.05 ± 0.15	98.7 ± 4.8
GHRP-6	7.98 ± 0.23	113.2 ± 10.9
L-692,585	7.11 ± 0.20**	116.5 ± 12.7

Data is expressed as mean ± S.E.M of three-seven individual experiments performed in triplicate. E_{max} is the maximum response of ligand expressed as the percentage of the maximum response to ghrelin. **p<0.01 when responses were compared to ghrelin using a one-way ANOVA with Dunnett's post-hoc test.

Sensitivity of the [^{35}S]GTPγS signal to YM-254890 was tested to confirm that the response to ghrelin measured in the [^{35}S]GTPγS binding assay with immunoprecipitation step was solely mediated through $G_{\alpha q}$. As it was shown in Chapter 3 and 4 (and Figure 5-1 of this chapter) that the GRLN-R could couple to $G_{\alpha i/o}$ the effect of PTx (25 ng/mL; 16 h) on [^{35}S]GTPγS binding was also investigated. Cells were transfected with $G_{\alpha q}$, receptor expression induced with 6 ng/mL doxycycline (16 h; as required) and challenged with either 1 μM ghrelin or 1 μM SPA. As Figure 5-4 demonstrates, no significant effect on [^{35}S]GTPγS binding could be detected in membranes prepared from uninduced GRLN-R cells treated with either ghrelin or SPA. The addition of 1 μM ghrelin caused a significant increase in bound [^{35}S]GTPγS, both in membranes prepared from induced cells and induced cells treated with PTx ($p<0.001$). This effect was abolished upon treatment with YM-254890 or upon co-incubation with YM-254890 and PTx.

To assess if PTx and YM-254890 altered basal levels of [^{35}S]GTPγS binding a one-way ANOVA with Tukey's multiple comparison test was performed on the combined raw data. The effect of 1 μM of the inverse agonist SPA was also assessed. This revealed that receptor expression led to a significant increase in basal loading of [^{35}S]GTPγS in membranes from doxycycline-induced cells and doxycycline-induced cells treated with PTx ($p<0.001$ and $p<0.01$, respectively). Incubation with 1 μM SPA caused a significant reduction in binding of [^{35}S]GTPγS in membranes prepared from doxycycline-induced GRLN-R cells although this was not significant in membranes prepared from PTx treated

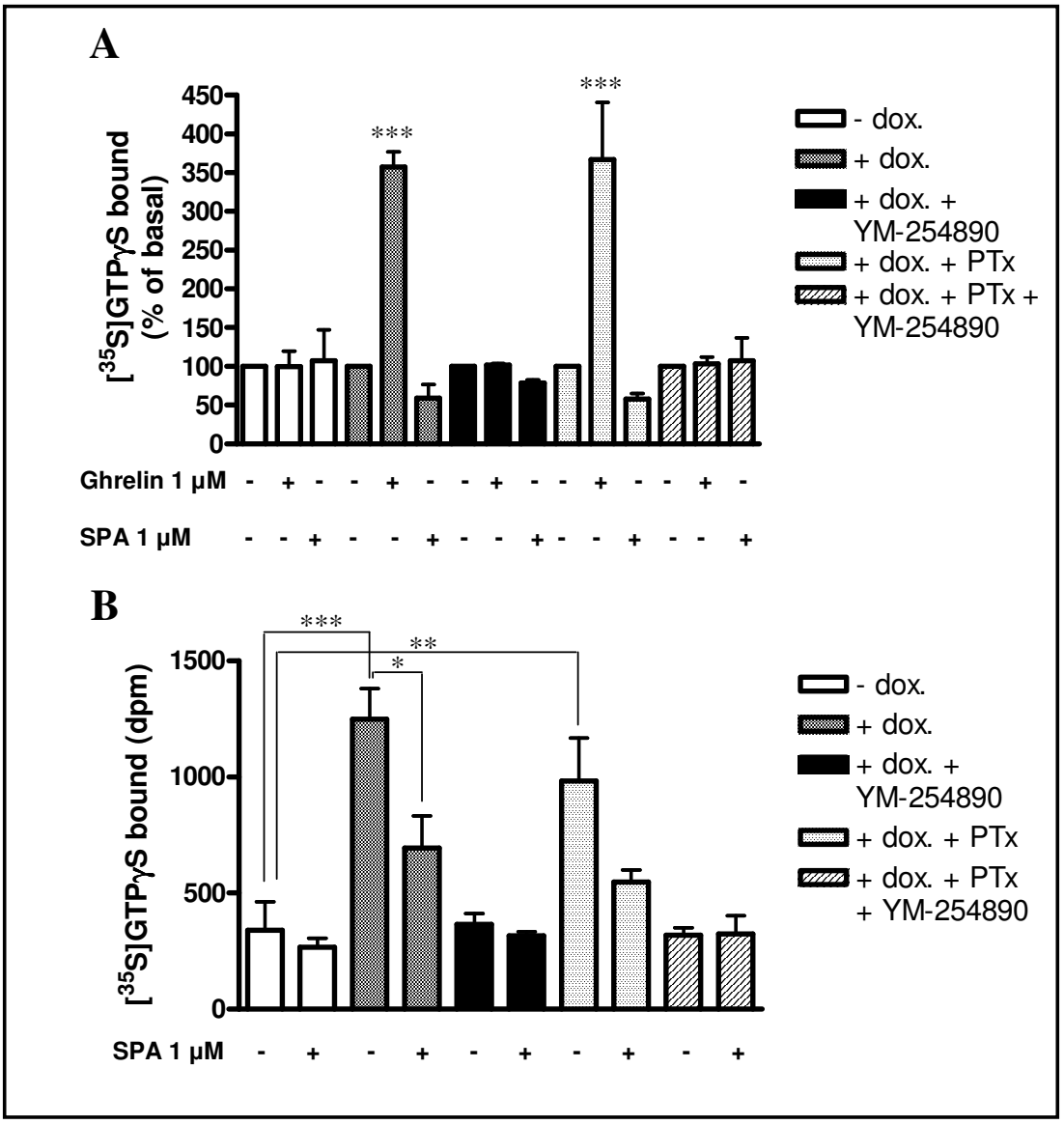

Figure 5-4 – The [^{35}S]GTPγS binding assay with immunoprecipitation step solely measures activation of the $G_{\alpha q/11}$ pathway. GRLN-R cells were transfected with $G_{\alpha q/11}$ and induced with 6 ng/mL doxycycline (16 h). **A.** To test receptor coupling to $G_{\alpha i/o}$, membranes were prepared from GRLN-R cells treated with 25 ng/mL PTx (16 h) and the response to 1 µM ghrelin and 1 µM SPA measured (30 min; 30 °C). To test receptor coupling to $G_{\alpha q/11}$ the response to 1 µM ghrelin and 1 µM SPA was measured after co-administration with 0.1 µM YM-254890 (30 min; 30 ºC), as indicated. $G_{\alpha q}$ was immunoprecipitated from the reaction mixture using anti-$G_{\alpha q}$ antiserum and bound [^{35}S]GTPγS measured using liquid scintillation spectrometry (5 min/sample). Data points represent the mean ± S.E.M of four independent experiments performed in triplicate. Data is expressed as the percentage of the response over basal values obtained in each of the conditions outlined above. ***p<0.001 when compared using a one-way ANOVA with Tukey's multiple comparison test. **B**. Raw data obtained for the basal levels of [^{35}S]GTPγS loading after treatment with YM-254890, PTx or a combination of the two. Data points represent the mean ± S.E.M of three independent experiments performed in triplicate. * p<0.05, **p<0.01 and ***p<0.001 when measured using a one-way ANOVA with Tukey's multiple comparison test

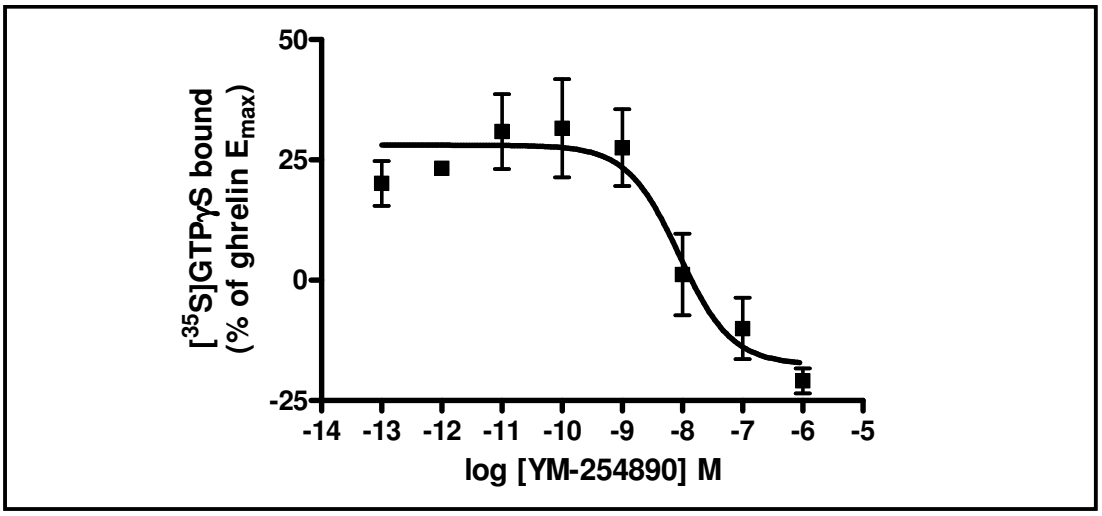

Figure 5-5 – The $G_{\alpha q/11}$ inhibitor YM-254890 inhibits the response of the GRLN-R to ghrelin in [^{35}S]GTPγS binding assays performed using an immunoprecipitation step. GRLN-R cells were transfected with $G_{\alpha q/11}$ and receptor expression induced with 6 ng/mL doxycycline (16 h). Membranes were prepared and incubated with 1 μM ghrelin and varying concentrations of YM-254890 (30 min; 30 °C). $G_{\alpha q}$ was immunoprecipitated from the reaction mixture using anti-$G_{\alpha q}$ antiserum and bound [^{35}S]GTPγS measured using liquid scintillation spectrometry (5 min/sample). Data is expressed as the percentage of ghrelin E_{max}, with 100 % set as the signal to ghrelin in the absence of YM-254890 treatment and 0 % set as the signal obtained in the absence of ghrelin or YM-254890 treatment. Data points represent the mean ± S.E.M of one experiment performed in triplicate.

cells. Treatment with YM-254890 abolished the increase in basal binding of [^{35}S]GTPγS that was seen upon GRLN-R expression. Indeed the level of [^{35}S]GTPγS binding achieved upon YM-254890 treatment was not significantly different to that achieved upon incubation with SPA (Figure 5-4).

To further test the response of the GRLN-R in the $G_{\alpha q/11}$ pathway the sensitivity of the response to ghrelin was measured in the presence of the $G_{\alpha q/11}$ inhibitor YM-254890 (Figure 5-5). A concentration-response curve was generated to YM-254890 (n = 1) in the presence of 1 μM ghrelin. Fitting of the data with a concentration-response curve yielded a pEC_{50} value of 8.05 ± 0.32 (n = 1). Data was normalised so that 100 % equalled the level of bound [^{35}S]GTPγS observed upon treatment with 1 μM ghrelin in the absence of YM-254890 and 0 % equalled the level of bound [^{35}S]GTPγS obtained upon GRLN-R expression in the absence of ghrelin and YM-254890. Normalisation of the data revealed that YM-254890 could lower the level of [^{35}S]GTPγS binding to below that observed upon GRLN-R expression, suggesting that YM-254890 was reducing the constitutive activity of the GRLN-R. At low concentrations of YM-254890 it appeared that YM-254890 could compete with ghrelin for binding to the GRLN-R so that only around 25 % of the maximum ghrelin signal could be obtained.

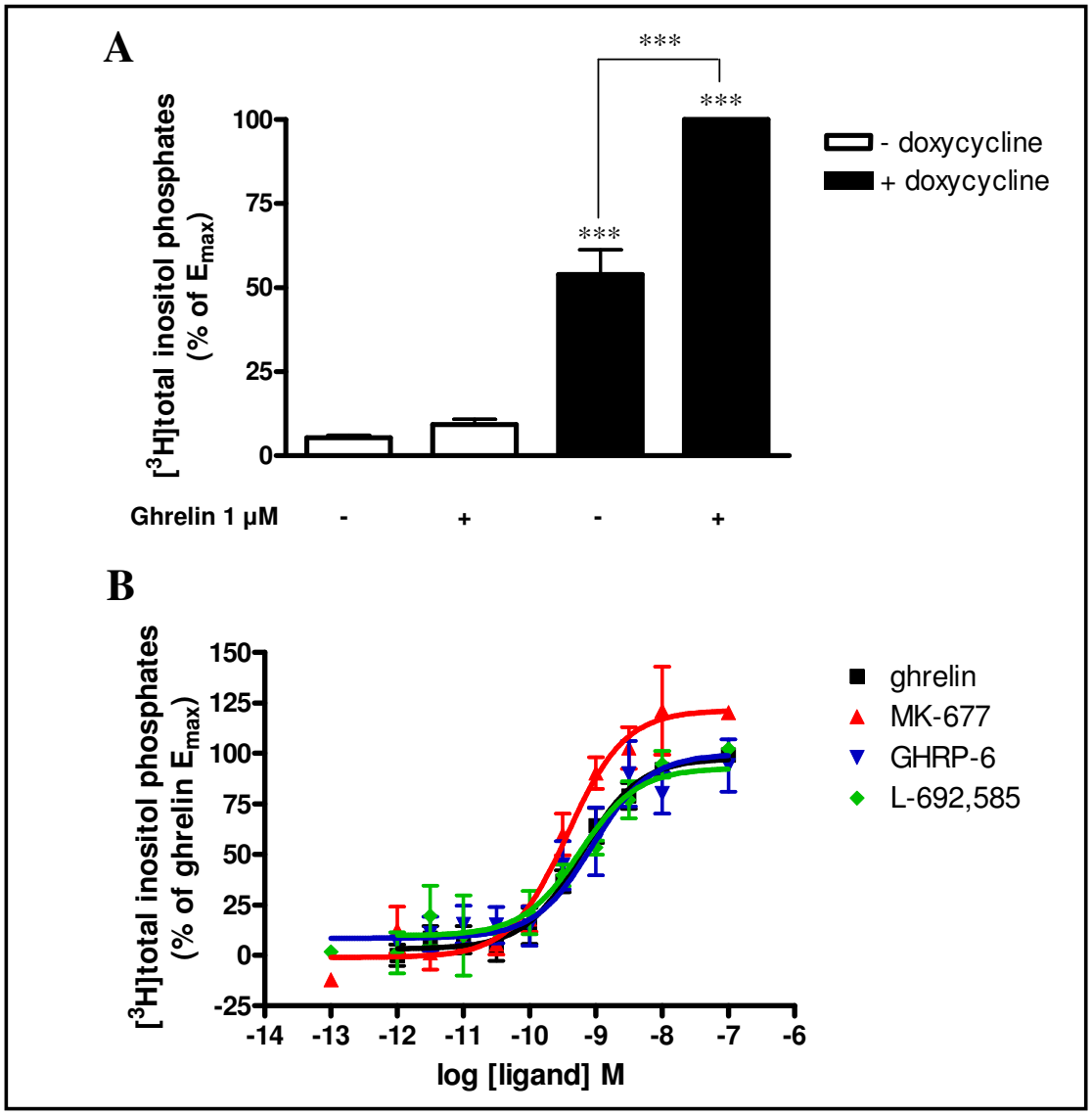

Figure 5-6 – Ghrelin and the growth hormone secretagogues can evoke a response in inositol phosphate accumulation assays. GRLN-R expression was induced as required with 6 ng/mL doxycycline (16 h) and media supplemented with 1 µCi/mL [^{3}H]*myo*-inositol (16 h). Prior to the assay, media was replaced with Krebs-Ringer buffer supplemented with 10 mM lithium chloride to block the breakdown of inositol phosphates into *myo*-inositol. **A.** Cells were stimulated with 1 µM ghrelin (30 min; 37 °C) as indicated, in the absence (white bars) or presence (black bars) of the GRLN-R. After ligand-stimulation cells were lysed with perchloric acid and [^{3}H]total inositol phosphates isolated from the mixture using ion-exchange chromatography. The amount of [^{3}H]total inositol phosphates accumulated were measured using liquid scintillation spectrometry (120 s/sample). Data points represent the mean ± S.E.M of four experiments performed in duplicate. ***p<0.001; **p< 0.01, *p<0.05 as measured using a one-way ANOVA with Tukey's multiple comparison test. **B**. Concentration-response curves to ghrelin, MK-677, GHRP-6 and L-692,585 were performed (as described above) in induced GRLN-R cells (30 min; 37 °C). Data points represent the mean ± S.E.M of three-six experiments performed in duplicate.

5.2.3 Whole cell assays to measure $G_{\alpha q/11}$ response

[^{35}S]GTPγS experiments are useful for measuring one of the first steps in signal transduction; activation of G proteins. However, these assays need to be performed in membranes or detergent-treated cells as [^{35}S]GTPγS is membrane-impermeable. As

GRLN-R couples to $G_{\alpha q/11}$ an inositol phosphate accumulation assay was established to measure functional responses to ghrelin and the growth hormone secretagogues in an intact cell system (using native $G_{\alpha q/11}$ expressed in HEK293 cells). This assay measures the accumulation of [^{3}H]total inositol phosphates, synthesised from [^{3}H]*myo*-inositol by the cell. Breakdown of [^{3}H]total inositol phosphates back into [^{3}H]*myo*-inositol was inhibited by the addition of 10 mM lithium chloride to the assay buffer which was added to the cells 30 min prior to stimulation with ligand. Subsequently, ligand was diluted in assay buffer containing LiCl and cells stimulated with this mixture for 30 min.

A one-way ANOVA with Tukey's multiple comparison test revealed that inducing receptor expression led to a significant increase in the generation of [^{3}H]total inositol phosphates ($p<0.01$), which could be further increased by incubation with 1 μM ghrelin ($p<0.001$). The level of basal [^{3}H]total inositol phosphates accumulated after receptor induction was 48.5 ± 5.6 % above that obtained in the absence of the GRLN-R. In the absence of the GRLN-R, ghrelin treatment had no effect on the level of inositol phosphate accumulation that was observed in basal conditions (Figure 5-6).

Concentration-response curves were generated to ghrelin, GHRP-6, L-692,585 and MK-677 (Figure 5-6) using an inositol phosphate accumulation assay, which allowed the measurement of the potency and efficacy of each of the compounds in activating the $G_{\alpha q/11}$ pathway (Table 5-2). A one-way ANOVA with Dunnett's post-hoc test showed there was no significant difference in potencies ($p=0.14$) or efficacies ($p=0.16$) of GHRP-6, L-692,585 or MK-677 compared to ghrelin.

Table 5-2 Potency and efficacy values of ligands acting at GRLN-R as measured using an inositol phosphate accumulation assay.

Ligand	pEC_{50} ± S.E.M	E_{max} ± S.E.M
Ghrelin	9.22 ± 0.11	97.5 ± 4.8
MK-677	9.43 ± 0.13	121.4 ± 7.3
GHRP-6	9.05 ± 0.19	99.1 ± 9.5
L-692,585	9.24 ± 0.22	92.7 ± 7.9

Data is expressed as mean ± S.E.M of three-four individual experiments performed in triplicate. E_{max} is the maximum response of ligand expressed as the percentage of the maximum response to ghrelin.

To investigate the effect of YM-254890 on the constitutive activity of the GRLN-R, GRLN-R cells were induced with doxycycline and concentration-response curves to YM-254890 and SPA were generated (Figure 5-7). Data was normalised so that 0 % was the

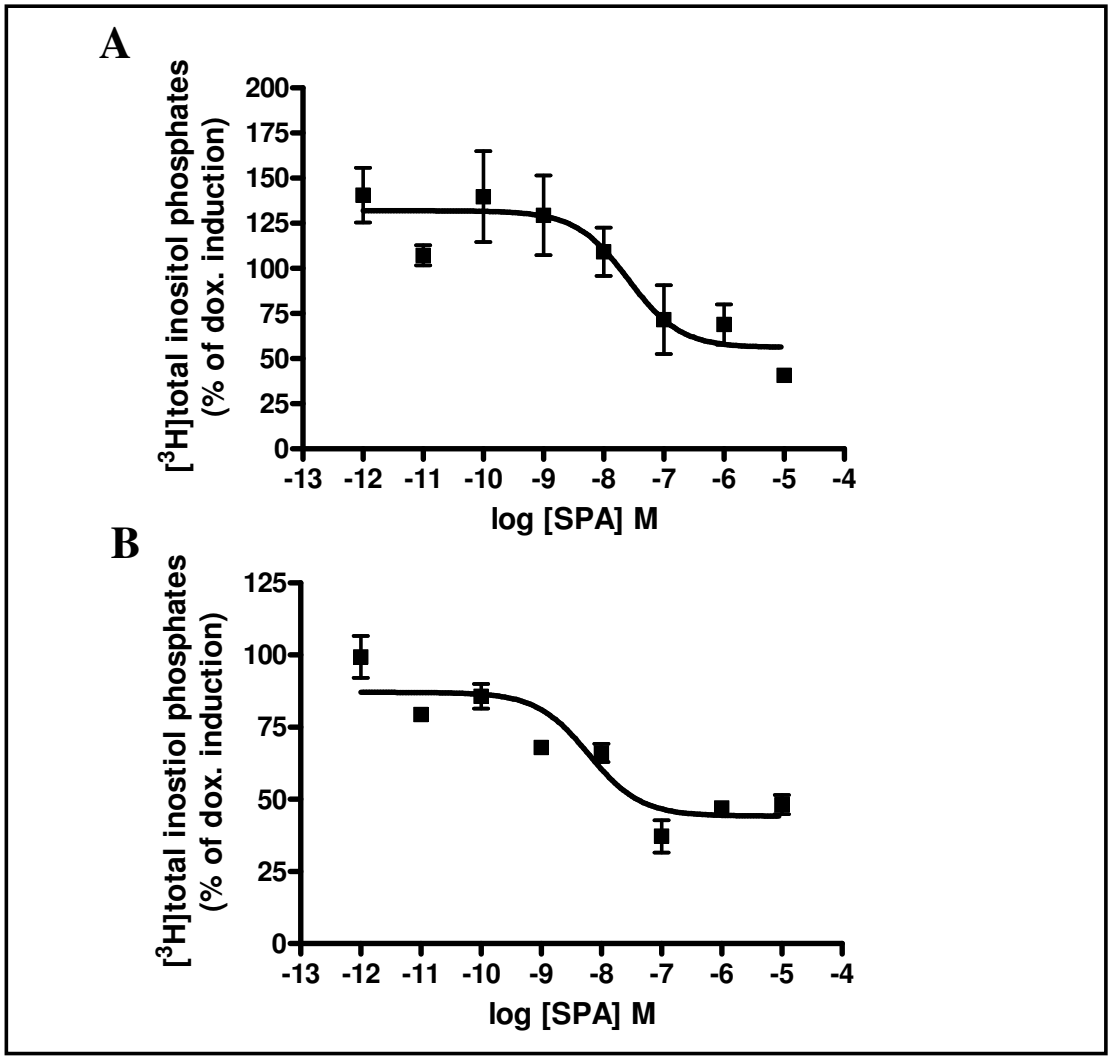

Figure 5-7 – Both YM-254890 and SPA can reduce the constitutive activity of the GRLN-R in inositol phosphate accumulation experiments. Cells were incubated with 6 ng/mL doxycycline (16 h) to induce GRLN-R expression, as required, and media supplemented with 1 µCi/mL [^{3}H]*myo*-inositol (16 h). Prior to the assay, media was replaced with Krebs-Ringer buffer supplemented with 10 mM lithium chloride to block the breakdown of inositol phosphates into *myo*-inositol. Subsequently, concentration-response curves to **A.** SPA or **B**. YM-254890 were generated (30 min; 37 °C). After ligand-stimulation cells were lysed with perchloric acid and [^{3}H]total inositol phosphates isolated from the mixture using ion-exchange chromatography. The amount of [^{3}H]total inositol phosphates accumulated were measured using liquid scintillation spectrometry (120 s/sample). Data is expressed as percentage of signal obtained by doxycycline induction, with 0% set as the signal obtained in the absence of GRLN-R. For the SPA concentration-response curve the data points represent the mean ± S.E.M of three independent experiments performed in duplicate. For the YM-254890 concentration-response curve data points represent the mean ± S.E.M of one experiment performed in triplicate.

level of inositol phosphates accumulated in the absence of the GRLN-R and 100 % was the level of inositol phosphates obtained in the presence of the GRLN-R. This yielded pEC_{50} values of 8.21 ± 0.32 for YM-254890 (n = 1) and 7.59 ± 0.45 for SPA (n = 3). Normalisation of the data showed that at maximally effective concentration of YM-254890 and SPA there was a 56.7 ± 6.9 % and 49.1 ± 20.5 % reduction, respectively, in the level of inositol phosphates accumulated. These results suggest that, in the inositol phosphate

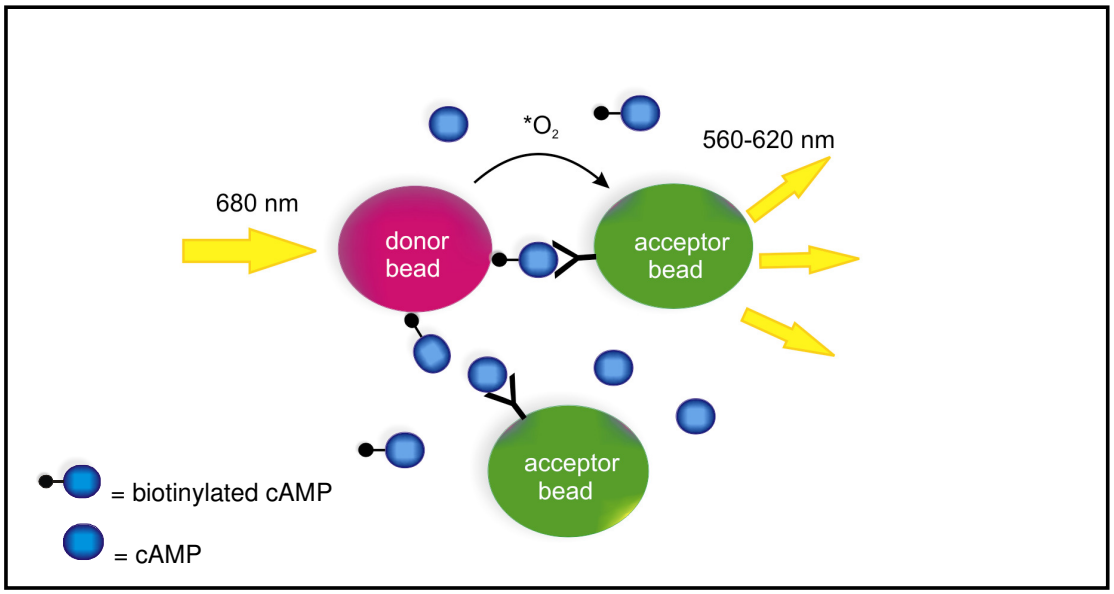

Figure 5-8 – Illustration of the AlphaScreen cAMP technology (adapted from the cAMP AlphaScreen product guide, Perkin Elmer). The AlphaScreen cAMP assay utilises a Tr-FRET based technology which measures energy transfer from 'donor beads' to 'acceptor beads' that are provided in the AlphaScreen kit. Briefly, donor beads are coated in streptavidin to which biotinylated cAMP can bind, whilst the acceptor beads are coated in an anti-cAMP antibody. If biotinylated cAMP binds to the anti-cAMP antibody it brings the donor bead in close proximity to the acceptor bead. Upon excitation of the donor bead with a laser at 680 nm, singlet oxygen energy ($*O_2$) produced by the donor bead is transferred to fluorescent acceptors within the acceptor bead. This generates a signal with a wavelength of around 560-620 nm. cAMP synthesised within cells can compete with biotinylated cAMP, binding to the antibody on the acceptor beads; in this situation energy transfer between the donor and acceptor beads does not occur as the beads are not in close enough proximity.

accumulation assay, neither YM-254890 or SPA were fully inhibiting the constitutive activity of the GRLN-R.

5.2.4 Whole cell assay to measure $G_{\alpha i/o}$ activation

An AlphaScreen cAMP accumulation assay was used to measure $G_{\alpha i/o}$ responses in intact GRLN-R cells. This assay utilises time-resolved fluorescence resonance energy transfer (Tr-FRET) technology to measure changes in cAMP levels within cells. Briefly, a donor bead is coated in streptavidin to which biotinylated cAMP molecules can bind via their biotin moiety. The acceptor beads are coated with an anti-cAMP antibody to which the biotinylated cAMP also binds, this time via the cAMP part of the molecule. Excitation with light at 680 nm causes the donor bead to convert ambient oxygen to single state oxygen, which can diffuse up to 250 nm before decaying. If the donor bead is in close proximity to the acceptor bead, the energy from the oxygen singlet is passed to fluorescent acceptors in the acceptor bead. This leads to a fluorescent signal of 520-620 nm being generated

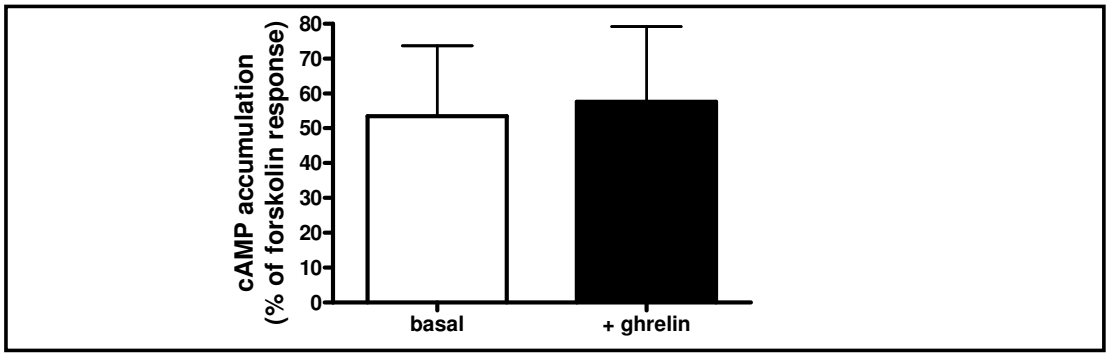

Figure 5-9 - The GRLN-R does not signal through the $G_{\alpha i/o}$ pathway in an intact cell assay. Cells were induced with 6 ng/mL doxycycline (16 h) and the response to 1 µM ghrelin (30 min; 37 °C) measured using an AlphaScreen cAMP accumulation assay. Data is expressed as the percentage of the response compared to that achieved by 0.1 µM forskolin. Data points represent the mean ± S.E.M of three independent experiments performed in triplicate. Data is expressed as the percentage of the cAMP response achieved by 0.1 µM forskolin.

(Figure 5-8). Synthesised cAMP from within a cell competes with the biotinylated cAMP on the acceptor bead – thus decreasing the amount of FRET occurring proportionally to amount of cAMP produced. Consequently, a $G_{\alpha s}$ response is seen as a decrease in the FRET signal. To obtain a $G_{\alpha i/o}$ response, the level of cAMP within a cell needs to be increased by activation of the enzyme adenylate cyclase, this is most commonly achieved by addition of forskolin. Upon activation of the $G_{\alpha i/o}$ family of G proteins, the activity of adenylate cyclase is inhibited, leading to a decrease in cAMP levels, thus increasing the Tr-FRET signal obtained. For ease of interpretation all cAMP AlphaScreen results presented in this chapter are expressed as the percentage of the response of cells to 0.1 µM forskolin.

GRLN-R cells were treated with doxycycline to induce receptor expression. A standard curve of forskolin stimulation was constructed (data not shown) and a concentration of 0.1 µM forskolin, which produced a response around the EC_{50}, was chosen to activate adenylate cyclase. To test if ghrelin could evoke a response via $G_{\alpha i/o}$ activation, cells were co-incubated for 30 min at 37 ºC with 0.1 µM forskolin and 1 µM ghrelin (Figure 5-9). There was no significant response to ghrelin in this assay ($p=0.95$), suggesting that either the GRLN-R could not couple to the $G_{\alpha i/o}$ pathway in intact GRLN-R cells or that it is not possible to measure cAMP inhibition in response to GRLN-R activation in these cells.

5.2.5 Whole cell assay to measure $G_{\alpha s}$ activation

Further investigation revealed that an increase in cAMP levels (therefore a $G_{\alpha s}$-mediated response) could be observed in response to ghrelin in doxycycline-induced GRLN-R cells. Thus concentration-response curves were generated to ghrelin and the growth hormone secretagogues using the AlphaScreen cAMP accumulation assay (Figure 5-10). The

potency and efficacy values are obtained in Table 5-3. Comparing the potency and efficacy values obtained for GHRP-6, L-692,585 and MK-677 to ghrelin using a one-way ANOVA with Dunnett's post-hoc test revealed that L-692,585 exhibited a significantly lower potency and efficacy ($p<0.05$) than ghrelin.

Table 5-3 – Potency and efficacy values of ligands acting at GRLN-R as measured using a cAMP accumulation assay.

Ligand	pEC_{50} ± S.E.M	E_{max} ± S.E.M
Ghrelin	8.78 ± 0.15	93.2 ± 5.0
MK-677	8.66 ± 0.21	85.0 ± 6.6
GHRP-6	7.85 ± 0.19	91.9 ± 7.3
L-692,585	7.97 ± 0.21*	70.0 ± 5.8*

Data is expressed as mean ± S.E.M of three individual experiments performed in triplicate. E_{max} is the maximum response to ligand expressed as the percentage of the maximum response to ghrelin. * $p<0.05$ when responses were compared to ghrelin using a one-way ANOVA with Dunnett's post-hoc test.

To test whether ghrelin and the growth hormone secretagogues could evoke a cAMP response in the absence of GRLN-R expression, GRLN-R cells were incubated with 1 μM ghrelin, GHRP-6, L-692,585 or MK-677 and the response measured using an AlphaScreen cAMP accumulation assay. There was no significant response to ghrelin and the growth hormone secretagogues ($p=0.06$; tested using a one-way ANOVA), thus showing GRLN-R expression was required for a $G_{\alpha s}$-mediated response in GRLN-R cells.

Finally, the ability of the GRLN-R to constitutively activate the $G_{\alpha s}$ pathway was tested. Doxycycline-induced GRLN-R cells were treated with 1 μM SPA and the level of accumulated cAMP was compared to the level of cAMP obtained in basal conditions using an unpaired, two-tailed t-test (Figure 5-11). SPA had no significant effect on cAMP accumulation compared to that observed in basal conditions ($p=0.35$). Furthermore, there was no significant difference in cAMP accumulation measured in the absence and presence of GRLN-R expression ($p=0.10$), thus further demonstrating that there was no measurable constitutive activation of the GRLN-R via activation of the $G_{\alpha s}$ pathway

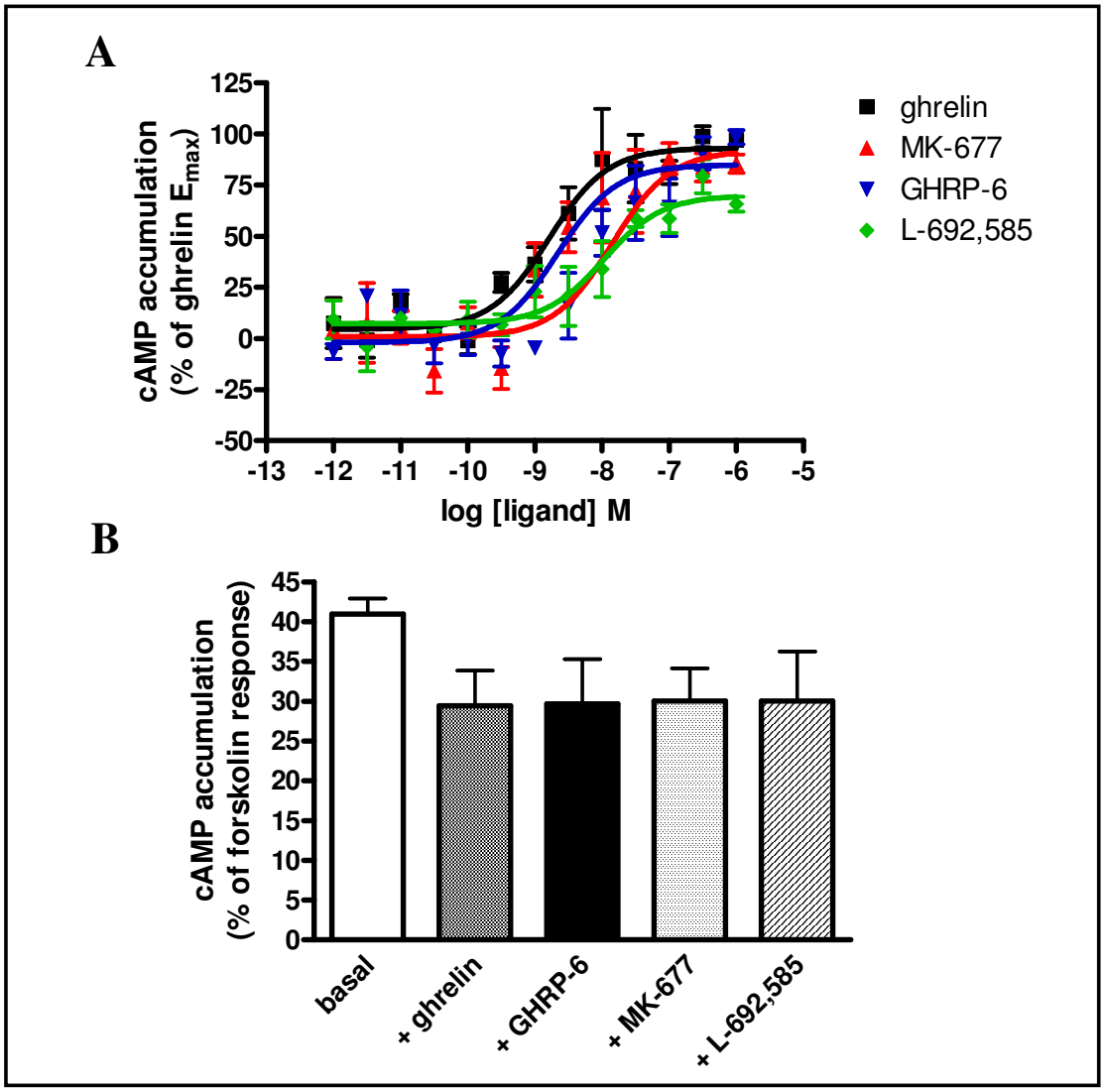

Figure 5-10 – The GRLN-R can signal through the $G_{\alpha s}$ pathway after challenge with ghrelin and the growth hormone secretagogues in an intact cell assay. A. GRLN-R expression was induced by treatment of GRLN-R cells with 6 ng/mL doxycycline (16 h). Concentration-response curves were generated to ghrelin, GHRP-6, MK-677 and L-692,585 (30 min; 37 °C) in intact GRLN-R cells using an AlphaScreen cAMP accumulation assay. Data points represent the mean ± S.E.M of three independent experiments performed in triplicate. **B.** Testing the ability of ghrelin and the growth hormone secretagogues to evoke a response in uninduced GRLN-R cells. Uninduced GRLN-R cells were incubated with 1 μM ghrelin, GHRP-6, MK-677 or L-692,585 (30 min; 37 °C) and the response measured using an AlphaScreen cAMP accumulation assay. Data points represent mean ± S.E.M of three independent experiments performed in triplicate. Data is expressed as the percentage of the cAMP response achieved by 0.1 μM forskolin.

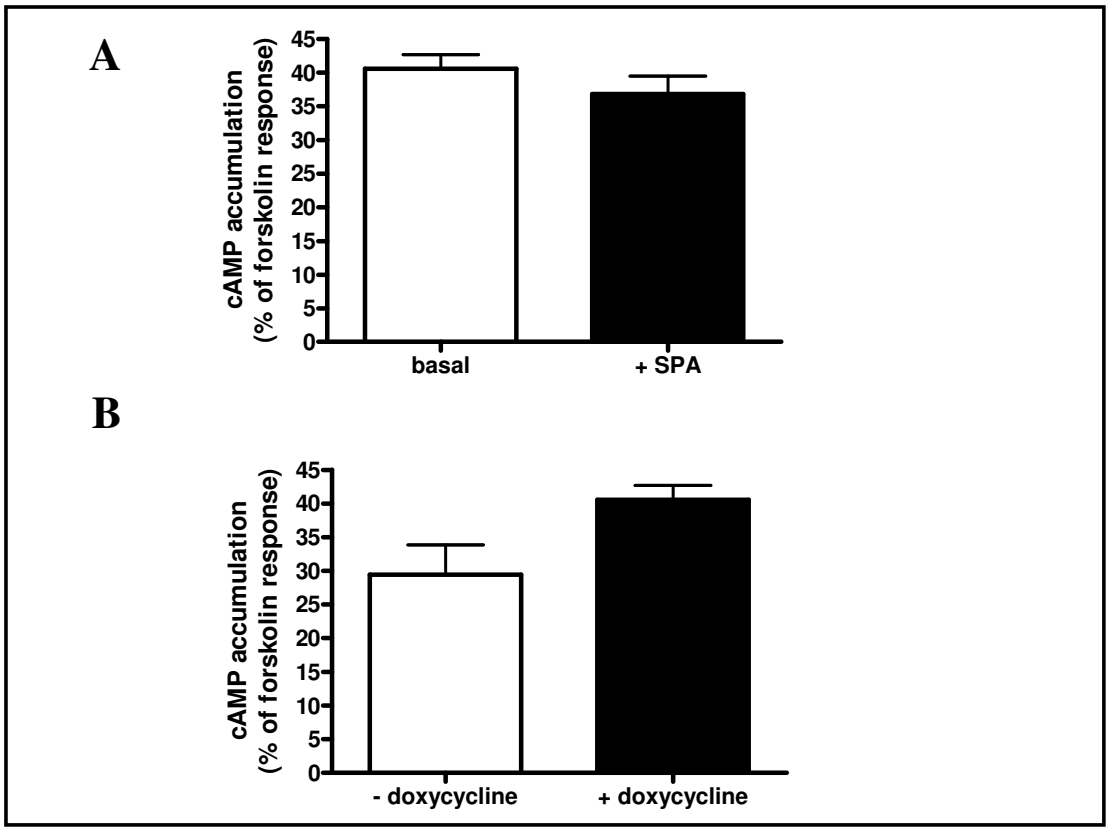

Figure 5-11 – The GRLN-R does not appear to constitutively activate the $G_{\alpha s}$ pathway in an intact cell assay. GRLN-R expression was induced by the treatment of GRLN-R cells with 6 ng/mL doxycycline (16 h).**A.**The effect of treatment with 1 µM SPA (30 min; 37 °C) on the level of cAMP accumulated upon GRLN-R expression was measured. **B.** The difference in the levels of cAMP accumulation in uninduced (white bar) and induced (black bar) GRLN-R cells were measured. In both experiments data points represent the mean ± S.E.M of three individual experiments performed in triplicate. Data is expressed as the percentage of the cAMP response achieved by 0.1 µM forskolin**.**

5.3 Discussion

5.3.1 Comparison of signaling of the GRLN-R as measured using $G_{\alpha q/11}$ and $G_{\alpha i/o}$ [^{35}S]GTPγS binding assays

Although some studies have used [^{35}S]GTPγS binding assays to measure ghrelin-induced activation of the $G_{\alpha i/o}$ pathway (i.e. both Bassil et al., 2007 and Dezaki et al., 2007 have demonstrated a response to ghrelin through activation of $G_{\alpha i}$) none of these studies have been extended to investigate the effects that the growth hormone secretagogues have on [^{35}S]GTPγS binding. Furthermore, there is a total lack of [^{35}S]GTPγS binding data investigating $G_{\alpha q/11}$-mediated signalling of the GRLN-R in response to either ghrelin or the growth hormone secretagogues.

In this study [^{35}S]GTPγS binding assays proved extremely useful tools for studying the pharmacology of the GRLN-R. For example, although mammalian cells express a number of endogenous G proteins to which the GRLN-R may couple, the use of selective G protein inhibitors revealed that the [^{35}S]GTPγS filtration binding assays measured a ghrelin response solely mediated through a PTx-sensitive ($G_{\alpha i/o}$) family of G proteins, whilst the [^{35}S]GTPγS binding assay with immunoprecipitation step exclusively measured a YM-254890-sensitive ($G_{\alpha q/11}$) response. Furthermore, as it is best to measure maximum efficacy of a ligand in a response system close to the receptor, where fewer compounds would reach the maximum system response (Strange, 2008); [^{35}S]GTPγS binding assays provided an excellent system for allowing comparison of the potencies and efficacies of ghrelin and the growth hormone secretagogues measured through the activation of $G_{\alpha q/11}$ and $G_{\alpha i/o}$ pathways.

Comparison of the $G_{\alpha q/11}$ and $G_{\alpha i/o}$ [^{35}S]GTPγS binding assays revealed that, although there was little difference in the potencies of ghrelin and the growth hormone secretagogues (Table 5-4), in the $G_{\alpha i/o}$ pathway the growth hormone secretagogues all acted as super-agonists compared to the response obtained upon ghrelin treatment (see Chapter 4, Figure 4-9 or Table 5-5). In contrast, measurement of the $G_{\alpha q/11}$ pathway showed that ghrelin and the growth hormone secretagogues acted with equal efficacies. These findings demonstrate that the GRLN-R exhibits functional selectivity. Furthermore, as the potencies of ghrelin and the growth hormone secretagogues do not alter between the $G_{\alpha q/11}$ and $G_{\alpha i/o}$ signalling pathways it can be extrapolated that the growth hormone secretagogues stabilise a receptor structure that favours coupling to $G_{\alpha i/o}$.

5.3.2 The GRLN-R can activate $G_{\alpha q}$

Interestingly, in this study ghrelin and the growth hormone secretagogues could evoke a $G_{\alpha q}$-mediated [^{35}S]GTPγS response. Studies into the activation of the GRLN-R though activation of the $G_{\alpha q/11}$ pathway have focused on measuring inositol phosphate accumulation or calcium mobilisation. Only one study, the initial cloning of the GRLN-R, has investigated to which G protein in the $G_{\alpha q/11}$ family the GRLN-R couples, reporting that in *Xenopus oocytes* the receptor can activate $G_{\alpha 11}$ but not $G_{\alpha q}$ (Howard et al., 1996). The differences in the expression systems (mammalian HEK293 cells versus amphibian *Xenopus oocytes*) could account for the discrepancies between these studies. As the level of $G_{\alpha q}$ transfected in the study by Howard et al. (1996) was not reported, it is possible that the G protein concentration in the *Xenopus oocytes* may not have been high enough to measure activation of $G_{\alpha q}$ as the GRLN-R could preferentially to $G_{\alpha 11}$. It would be interesting to investigate this further.

5.3.3 Inositol phosphate accumulation assays

In contrast to the [^{35}S]GTPγS binding assays, there have been a number of studies that have used inositol phosphate accumulation assays (Holst et al., 2003; Holst et al., 2004; Holst et al., 2007; Liu et al., 2007; Van Craenenbroeck, et al., 2004) or cAMP accumulation assays (Rossi et al., 2008; Caminos et al., 2005) to measure activation of the GRLN-R.

Generation of concentration-response curves in the inositol phosphate accumulation assay revealed that the potencies of ghrelin, GHRP-6 and MK-677 in this study were similar to those previously published (Holst et al., 2003). However, in contrast to Schwartz's group who found that GHRP-6 acted as a super-agonist (Holst et al., 2003), in this study, and consistent with the $G_{\alpha q/11}$ [^{35}S]GTPγS binding results, GHRP-6 acted with efficacy equal to that of ghrelin. Indeed in this study the efficacies of ghrelin and the growth hormone secretagogues and the potencies of ghrelin and MK-677, measured in the inositol phosphate accumulation assays were all similar to those measured in the $G_{\alpha q/11}$ [^{35}S]GTPγS binding assays (Tables 5-4 and 5-5). However, GHRP-6 and L-692,585 acted with significantly higher potencies in the inositol phosphate accumulation assay compared to the [^{35}S]GTPγS binding assays, which may reflect a difference in receptor reserve between [^{35}S]GTPγS binding assays and inositol phosphate assays.

Nucleotide exchange is one of the earliest measurable stages in the stimulus-response cascade, therefore the potency of compounds tested in the [^{35}S]GTPγS binding assays would be expected to be lower than responses measured in further downstream assays, which are subjected to a degree of amplification. Such response amplification has been observed in receptors such as the chemokine receptor CXCR2 (Hall et al., 1999) and the dopamine D_2 receptor (Gardner et al., 1997). In contrast, a lack of signal amplification may reflect interaction with a receptor that is only poorly coupled to the G protein of study. As the potencies of both ghrelin and MK-677 are not significantly greater in the inositol phosphate accumulation assay than the potencies measured in the $G_{\alpha q/11}$ [^{35}S]GTPγS binding assay, this may reflect poor coupling of the GRLN-R to $G_{\alpha q/11}$. However, the low levels of amplification of the MK-677 and ghrelin responses could be an artefact of the system due to the enrichment of G protein in the [^{35}S]GTPγS binding assays that could alter coupling efficiency (which depends on the ratio of G protein to receptor; Kenakin, 1997).

It is important to bear in mind that the inositol phosphate accumulation assays were performed in intact cells; consequently the response to ghrelin and the growth hormone secretagogues could include activation of other G protein-dependent or even G-protein-independent pathways. For example, Gierschik and colleagues demonstrated that PLCβ activity could be stimulated by G protein βγ-subunits released via activation of $G_{\alpha i/o}$ family of G proteins (Camps et al., 1992). Thus, activation of the $G_{\alpha i/o}$ family of G proteins by ghrelin or the growth hormone secretagogues could lead to activation of PLCβ, thereby increasing inositol phosphate levels within the cells. Thus activation of $G_{\alpha i/o}$ may be responsible for the apparent increase in potency of GHRP-6 and L-692,585 in the inositol phosphate accumulation assay. This could be assessed by measurement of inositol phosphate accumulation in the presence of PTx – although such studies were beyond the scope of this thesis.

5.3.4 cAMP accumulation assays

In Chapters 3 and 4 the GRLN-R was shown to couple to the $G_{\alpha i/o}$ family of G proteins in a [^{35}S]GTPγS filtration binding assay, yet in this chapter it was found that ghrelin could not decrease the level of cAMP accumulation achieved by incubation of GRLN-R cells with an EC_{50} concentration of forskolin. Further experiments are needed to test whether the GRLN-R does not couple to $G_{\alpha o1}$ in intact GRLN-R cells or whether the absence of a decrease in cAMP accumulation was because AC was not inhibited in these experiments.

This could be achieved by reproducing the experiment with a positive control (i.e. by activating $G_{\alpha i}$–coupled bradykinin 2 receptors which are endogenously expressed in HEK293 cells; Kramarenko et al., 2009). If bradykinin 2 receptors mediate a $G_{\alpha i/o}$ response in the GRLN-R this could suggest that the GRLN-R does not couple to $G_{\alpha i/o}$. Is is also possible that the GRLN-R may not couple to the $G_{\alpha i/o}$ pathway in HEK293 cells as the receptor may preferentially couple to endogenous $G_{\alpha q/11}$ or even $G_{\alpha s}$.

Initial [^{35}S]GTPγS binding experiments were performed in the presence of exogenous $G_{\alpha o1,}$ yet coupling of the receptor to $G_{\alpha o1}$ in HEK293 cells may be expected to have no effect on cAMP accumulation. The $G_{\alpha o}$ subfamily of G proteins inhibits the adenylyl cyclase isoform AC1, which is only expressed in the brain adrenal medulla (Sunahara and Taussig, 2002). Although $G_{\alpha o}$ can inhibit the adenylyl cyclase isoforms AC5 (expressed in heart, brain, kidneys, liver, lung, uterus and brown adipose tissue; Haber et al., 1994) and AC6 (ubiquitously expressed; Haber et al., 1994) it does so less potently than the $G_{\alpha i}$ G protein subfamily (Sunahara and Taussig, 2002). Furthermore, the significant response to ghrelin in [^{35}S]GTPγS binding studies in the absence of exogenous G protein confirms that coupling of GRLN-R to $G_{\alpha i/o}$ was not an artefact due to the enrichment of cells with $G_{\alpha o1.}$ This suggests that in this system the GRLN-R can couple to $G_{\alpha o1}$ but not to $G_{\alpha i}$. Indeed, the expression pattern of the ghrelin receptor and $G_{\alpha o1}$ overlap, as $G_{\alpha o1}$ is the most abundantly expressed G protein in the nervous system (Offermanns, 2001).

In a study by Bassil et al. (2007), ghrelin was able to increase [^{35}S]GTPγS binding in HEK293 cells co-expressing the GRLN-R and $G_{\alpha i}$, suggesting that the GRLN-R could signal through $G_{\alpha i}$ activation. Thus further studies are required to further elucidate whether the GRLN-R can couple to $G_{\alpha i1\text{-}3}$ and $G_{\alpha o1}$. This could be achieved by testing the response to ghrelin in PTx-treated GRLN-R cells transfected with PTx-insensitive versions of $G_{\alpha i1,}$ $G_{\alpha i2,}G_{\alpha i3}$ and $G_{\alpha o1}$, a system previously used by our group to study the promiscuous coupling of the dopamine D_2 receptor (Lane et al., 2007).

In the absence of forskolin, ghrelin could evoke an increase in cAMP accumulation, therefore demonstrating coupling of the GRLN-R to $G_{\alpha s}$, which is in agreement with other recombinant studies into the pharmacology of the GRLN-R (Rossi et al., 2008; Caminos et al., 2005). Indeed, ghrelin increased cAMP accumulation with a pEC_{50} value of 8.78 ± 0.15 which is in close agreement to the pEC_{50} value published by Rossi et al. (pEC_{50} 8.28; Rossi et al. 2008). The concentration-response curves generated using the cAMP accumulation

assay revealed that ghrelin and the growth hormone secretagogues all acted with similar potencies as measured in the [^{35}S]GTPγS binding assays, with MK-677 and GHRP-6 exhibiting similar efficacies to those measured in the $G_{\alpha q/11}$ [^{35}S]GTPγS binding assays and inositol phosphate accumulation assays. However, in the cAMP accumulation assay, L-692,585 acted as a partial agonist, having a significantly lower efficacy and potency than ghrelin in the $G_{\alpha s}$ pathway, thus suggesting that L-692,585 only weakly couples to the $G_{\alpha s}$ pathway.

It is well known that overexpression of a receptor can lead to an increase in receptor reserve and in this condition a partial agonist may appear to have full intrinsic activity (Adham et al., 1993; Watts et al., 1995; Pohjanoksa et al., 1997). Therefore it is hard to compare the efficacies in downstream effector systems such as the inositol phosphate accumulation and cAMP pathways, which may exhibit different degrees of receptor reserve. Furthermore, negative or positive feedback, cross-regulation and even convergence of different effector systems may also be contribute to measures of downstream functional assays complicating experimental results (Wess 1998). Thus further experiments are needed to measure $G_{\alpha s}$ activation in the [^{35}S]GTPγS binding assay to allow comparison of the responses to the $G_{\alpha i/o}$ and $G_{\alpha q/11}$ pathways. For example, use of the [^{35}S]GTPγS binding assay with immunoprecipitation of $G_{\alpha s}$ would allow a direct comparison to activation of the $G_{\alpha q/11}$ pathway and of the responses obtained in the intact cell $G_{\alpha s}$ assay.

5.3.5 The GRLN-R may be promiscuous in vivo

The findings from this study have clearly demonstrated that the GRLN-R is a promiscuous receptor, coupling to $G_{\alpha i/o,}$ $G_{\alpha q/11}$ and $G_{\alpha s}$ in the GRLN-R cell line. However, receptor coupling to G protein in the native cell environment not only depends on the relative affinities of the receptor for different G proteins but also on the relative proportions and availability of receptors and G proteins. Indeed, it is well established that altering the G protein to GPCR ratio can alter ligand function and receptor pharmacology (Milligan, 2000). Nevertheless, promiscuity cannot be seen as purely an artefact of recombinant systems as there are numerous examples of it occurring *in vivo* or in cell lines endogenously expressing the receptor of study. For example, the δ opioid receptor, endogenously expressed in NG108-115 cells, has been shown to activate $G_{\alpha i2-3}$ and $G_{\alpha o1-2}$ in response to the receptor agonist DADLE (Prather et al., 1994). Conversely, the presence of multiple G protein families does not necessarily result in receptor promiscuity. For

example, use of a recombinant system to study the dopamine D2 receptor ligands *p*-tyramine and S-(-)-3PPP showed these ligands were agonists at $G_{\alpha o1}$ but could not elicit a response via activation of $G_{\alpha i1\text{-}3}$ (Lane et al., 2007).

In support of the findings from this study, are several examples of ghrelin activating $G_{\alpha s}$, $G_{\alpha q/11}$ and $G_{\alpha i/o}$ pathways in cell lines endogenously expressing the GRLN-R. The GRLN-R has been reported to couple to $G_{\alpha q/11}$ in a cell line from a rat pituitary adenoma endogenously expressing GRLN-R (Falls et al., 2006) and in the AR42J cell line from rat pancreas (Lai et al., 2005). Evidence for a $G_{\alpha i/o}$ interaction *in vivo* is demonstrated in a study by Dezaki et al., (2007) which showed that GRLN-R activates $G_{\alpha i2}$ in rat pancreatic islet β-cells. In 3T3-L1 preadipocytes the mitogenic effect of ghrelin is mediated via PI3K/Akt and MAP kinase signalling via a PTx-sensitive G protein (Kim et al., 2004).

The ability of the GRLN-R to couple to $G_{\alpha s}$ is more controversial. In NPY neurones the cAMP pathway has been implicated in ghrelin-induced calcium mobilisation as a protein kinase A inhibitor reduced the calcium response, indicating the $G_{\alpha q/11}$ pathway is not solely responsible for the calcium signal observed (Kohno et al., 2003). In cultured porcine pituitary cells, GRLN-R activation led to an increase in cAMP comparable to that obtained by activation of the $G_{\alpha s}$ coupled growth hormone releasing hormone (GHRH) receptor. Furthermore, ghrelin acted additively in increasing cAMP levels in porcine somatotrophs when co-administered with GHRH (Malagon et al., 2003). However, studies in recombinant systems suggest that $G_{\alpha s}$ signalling may not be mediated solely through GRLN-R activation. In one study using HeLa-T4 cells, GRLN-R could not increase cAMP levels in response to ghrelin. Meanwhile, co-activation of GRLN-R and GHRH produced an increase in the cAMP response compared to GHRH activation alone. This response was not altered upon inhibition of signalling molecules associated with GRLN-R activation e.g. phospholipase Cβ. A possible explanation for the potentiation of signalling was the formation of heterodimers of GRLN-R and GHRH (Cunha and Mayo, 2002). Interestingly, GHRH has been described as an allosteric modulator of the GRLN-R, acting as an agonist in calcium mobilisation assays but when co-administered with ghrelin, GHRH decreases the potency of ghrelin (Casanueva et al., 2008). However, the Casanueva study found that neither ghrelin nor GHRH could induce a significant effect on cAMP levels in HEK293 cells (Casanueva et al., 2008). Thus the ability of the GRLN-R to couple to $G_{\alpha s}$ *in vivo* remains inconclusive.

5.3.6 Constitutive activity of the GRLN-R in the $G_{\alpha q/11}$ and $G_{\alpha s}$ pathways

The GRLN-R is recognised as a highly constitutively active receptor, based upon the receptor exhibiting a high degree of constitutive activity in inositol phosphate accumulation assays (Holst et al., 2003). However, it cannot be assumed that the GRLN-R would constitutively activate every G protein pathway to which it is coupled. For example, it is possible that a promiscuous receptor can constitutively activate one pathway but be silent in another, as seen by the α_{1b} adrenoceptor mutated at C128 (C128F) that is able to constitutively activate $G_{\alpha q/11}$ pathway but not the $G_{\alpha s}$ pathway (Perez et al., 1996).

In agreement with multiple studies (e.g. Holst et al., 2003; Holst et al., 2004; Holst and Schwartz, 2004; Liu et al., 2007) the GRLN-R was shown to constitutively activate the $G_{\alpha q}$ pathway. This was demonstrated by the increase in basal loading of [^{35}S]GTPγS upon expression of the GRLN-R, which was fully inhibited by incubation with either YM-254890 or SPA. Indeed YM-254890 was shown to inhibit [^{35}S]GTPγS binding to a level below that achieved by expression of the GRLN-R, demonstrating YM-254890 could inhibit the constitutive activity of the GRLN-R.

Interestingly, SPA, at maximally effective concentrations, decreased inositol phosphate accumulation to a level that was still 50.9 % above that observed in the absence of GRLN-R expression. This suggests that other receptors, or other proteins for example, insulin-like growth factor (El-Shewy et al., 2006) were activating $G_{\alpha q/11.}$ However, treatment with YM-254890 alone was still not sufficient to return inositol phosphate accumulation to the level obtained in the absence of the GRLN-R. Even at maximally effective concentrations, the response was still 43.3 % above that measured in the absence of receptor. Thus the residual level of inositol phosphates appeared not to be generated by either activation of the GRLN-R or the $G_{\alpha q/11}$ pathway, but by non-$G_{\alpha q/11}$ mediated pathways, such as from cross-talk with other endogenous receptors expressed in HEK293 cells. It is possible that the continued accumulation of inositol phosphates observed could be due to signalling of GRLN-R through $G_{\alpha q/11}$ activation from within intracellular compartments. Indeed $G_{\alpha q/11}$ is distributed both on the plasma membrane and in intracellular compartments (Hughes et al., 2001) and an endosomal G protein signalling pathway has previously been described in yeast (Slessareva et al., 2006). As the GRLN-R is continually internalising and recycling, the cell-impermeable compounds SPA and YM-254890 would be unable to bind to and inhibit the signalling of internalised GRLN-R. However, by trapping the GRLN-R in endosomes, Holliday et al. (2007) demonstrated a selective inhibition of the constitutive

inositol phosphate accumulation mediated by the GRLN-R, suggesting that the GRLN-R does not signal upon internalisation through the $G_{\alpha q/11}$ pathway.

The results of this study are of contrast to those of Holst et al. (2003) who have previously shown that the inverse agonist SPA is a full inverse agonist, fully inhibiting the constitutive accumulation of inositol phosphates mediated by the GRLN-R (Holst et al., 2003). The differences in the ability of SPA to fully inhibit the constitutive accumulation of inositol phosphates in this study and in Schwartz's group may be solely due to the expression system used; HEK293 cells in this study and COS-7 cells in the study by Holst et al. (2003), although no attempt was made to further investigate this in the present study. The pIC_{50} values of SPA acting to decrease the constitutive activity of the GRLN-R were in close agreement in this study (8.21 ± 0.13) with the study by Holst et al. (2003) (8.3 ± 0.7).

Comparing the constitutive activity in the $G_{\alpha q/11}$ assays revealed that the constitutive activity of the GRLN-R was significantly higher in the inositol phosphate accumulation assays than the [^{35}S]GTPγS binding assays. This was presumably due to amplification of the GRLN-R-mediated signal, as the response measured in the inositol phosphate accumulation assay is further down the stimulus-response pathway than the response measured in the [^{35}S]GTPγS binding assay.

In contrast to the $G_{\alpha q/11}$ pathway, the GRLN-R did not appear to constitutively activate the $G_{\alpha s}$ pathway, as seen by the absence of a response to SPA and the lack of an increase in basal cAMP accumulation upon receptor expression. This may be a consequence of a weaker coupling of the GRLN-R to the $G_{\alpha s}$ pathway than the $G_{\alpha i/o}$ and $G_{\alpha q/11}$ pathways. Also once $G_{\alpha s}$ is activated it can dissociate from the membrane to the cytosol (Wedegaertner et al., 1994; Ransnas et al., 1989; Witte et al., 1999), therefore to evoke a further response another G protein must be found before the next G protein cycle can be activated. In Chapter 4 the constitutive activation of the $G_{\alpha i/o}$ pathway through GRLN-R activation was only revealed upon inducing receptor expression at a higher doxycycline concentration than used in this study (i.e. 25 ng/mL not 6 ng/mL used in this study). Thus increasing the GRLN-R expression level may also reveal that the GRLN-R can constitutively activate the $G_{\alpha s}$ pathway, a suggestion that warrants further investigation.

As the apparent constitutive activity of a GPCR may vary substantially in various systems (Seifert and Wenzel-Seifert, 2002) it is impossible to compare the constitutive activities

achieved in the $G_{\alpha q/11}$, $G_{\alpha i/o}$ and $G_{\alpha s}$ pathways. The use of a [^{35}S]GTPγS binding assay followed by immunoprecipitation of $G_{\alpha q}$, $G_{\alpha s}$ and $G_{\alpha o}$ from the reaction mixture using G protein specific antiserum could allow a direct comparison of the constitutive activity of these pathways, but the variables of G protein expression and relative affinities of the G protein antibodies must be taken into account.

5.4 Summary

In this study [^{35}S]GTPγS binding studies revealed that the growth hormone secretagogues act with a higher efficacy in activation of the $G_{\alpha i/o}$ pathway than ghrelin, but act with similar efficacies in the $G_{\alpha q}$ pathway, thus for the first time demonstrating functional selectivity of the GRLN-R. Furthermore, it has been shown that GRLN-R can couple to $G_{\alpha s}$, $G_{\alpha o}$ and $G_{\alpha q}$ pathways. Certainly, it is possible that GRLN-R may couple to all four G protein families as a study by Schwartz's group has suggested the GRLN-R stimulate serum response element-mediated transcriptional activity via the activation of $G_{\alpha 13}$ (Holst et al., 2005).

Table 5-4 – Potency of ghrelin and the growth hormone secretagogues as measured in the [^{35}S]GTPγS binding, inositol phosphate accumulation and cAMP accumulation assays.

Ligand	pEC_{50} ± S.E.M			
	$G_{\alpha i/o}$ [^{35}S]GTPγS filtration binding assay	$G_{\alpha q}$ [^{35}S]GTPγS binding assay with immunoprecipitation step	Inositol phosphate accumulation assay ($G_{\alpha q/11}$)	cAMP accumulation assay ($G_{\alpha s}$)
Ghrelin	9.19 ± 0.16	9.03 ± 0.13	9.22 ± 0.11	8.78 ± 0.15
MK-677	9.28 ± 0.24	9.05 ± 0.15	9.43 ± 0.13	8.66 ± 0.21
GHRP-6	8.01 ± 0.07	7.98 ± 0.23	9.05 ± 0.19 #	7.85 ± 0.19
L-692,585	7.20 ± 0.03	7.11 ± 0.20	9.24 ± 0.22 †	7.97 ± 0.21

GHRP-6 acts with a significantly higher potency in the inositol phosphate accumulation assay than in the $G_{\alpha i/o}$ filtration binding assay ($p<0.05$), the $G_{\alpha q/11}$ binding assay with immunoprecipitation step ($p<0.05$) and the cAMP accumulation assay ($p<0.01$).

† L-692,585 acts with a significantly higher potency in the inositol phosphate accumulation assay than the $G_{\alpha i/o}$ filtration binding assay ($p<0.01$), the $G_{\alpha q/11}$ binding assay with immunoprecipitation step ($p<0.01$) and the cAMP accumulation assay ($p<0.01$).

Data was compared using a one-way ANOVA with Turkey's multiple comparison test. Data is expressed as the mean ± S.E.M of at least three individual experiments.

Table 5-5 – Efficacy of of ghrelin and the growth hormone secretagogues as measured via [^{35}S]GTPγS binding, inositol phosphate accumulation and cAMP accumulation assays.

Ligand	E_{max} ± S.E.M			
	$G_{\alpha i/o}$ [^{35}S]GTPγS filtration binding assay	$G_{\alpha q}$ [^{35}S]GTPγS binding assay with immunoprecipitation step	Inositol phosphate accumulation assay ($G_{\alpha q/11}$)	cAMP accumulation assay ($G_{\alpha s}$)
Ghrelin	98.1 ± 1.9	94.3 ± 4.0	97.5 ± 4.8	93.2 ± 5.0
MK-677	167.8 ± 8.4#	98.7 ± 4.8	121.4 ± 7.3	85.0 ± 6.6
GHRP-6	152.7 ± 6.5†	113.2 ± 10.9	99.1 ± 9.5	91.9 ± 7.3
L-692,585	150.5 ± 8.0‡	116.5 ± 12.7	92.7 ± 7.9	70.0 ± 5.8

MK-677 acts with a significantly higher efficacy in the $G_{\alpha i/o}$ filtration binding assay than in the $G_{\alpha q/11}$ binding assay with immunoprecipitation step ($p<0.001$), the inositol phosphate accumulation assay ($p<0.01$) and the cAMP accumulation assay ($p<0.001$).

† GHRP-6 acts with a significantly higher efficacy in the $G_{\alpha i/o}$ filtration binding assay than measured in the inositol phosphate accumulation assay ($p<0.05$) and the cAMP accumulation assay.

‡ L-692,585 acts with a significantly higher efficacy in the $G_{\alpha i/o}$ filtration binding assay than measured in the inositol phosphate accumulation assay ($p<0.01$) and the cAMP accumulation assay ($p<0.001$). L-692,585 also acts with a significantly higher efficacy in the $G_{\alpha q/11}$ binding assay with immunoprecipitation step than measured in the cAMP accumulation assay ($p<0.05$).

Data is expressed as the percentage of the maximum response achieved to ghrelin in each assay (mean ± S.E.M). Data was compared using a one-way ANOVA with Turkey's multiple comparison test. Data is expressed as the mean ± S.E.M of at least three individual experiments.

6 Effect of A204E and I134T mutations on the function of the GRLN-R

6.1 Introduction

Mutations within GPCR genes may affect receptor function and have been linked to the development of a number of human diseases such as: female infertility (Huhtaniemi et al., 2006); nephrogenic *diabetes insipidus* (Rosenthal et al., 1992; Van den Ouweland et al., 1992) *retinitis pigmentosa* (Kennan et al., 2005) and obesity (Vaisse et al., 1998, Vaisse et al., 2000; Sina et al., 1999). The latter involves the melanocortin-4 receptor, in which over 50 different obesity-associated mutations have been described, most of which are missense mutations (point mutations that result in the substitution of one amino acid for another) that reduce the ability of the receptor to signal to its endogenous ligand, α-melanocyte-stimulating hormone (Farooqi et al., 2003; Lubrano-Berthelier et al., 2003; Vaisse et al., 1998; Vaisse et al., 2000; Nijenhuis et al., 2003).

As well as affecting receptor pharmacology, mutations within GPCRs can disrupt the normal trafficking of receptors to the cell surface as cells employ a quality control system in the ER to prevent misfolded membrane proteins from being trafficked to the plasma membrane. Any proteins that do not attain their native conformation are retained in the ER to undergo several rounds of protein folding. If the native conformation fails to be reached, they are unfolded, transported to the cytosol and degraded by the proteasome (Ma & Hendershot, 2001).

Four naturally occurring missense mutations of the GRLN-R have been identified that result in decreased cell surface expression and distinct pharmacological abnormalities of the GRLN-R (Liu et al., 2007, Wang et al., 2004; Holst and Schwartz, 2006; Pantel et al., 2006). The I134T ($I^{3.43}$), V160M ($V^{4.42}$) and F279L ($F^{6.51}$) mutations are present in the third, fourth and sixth transmembrane spanning domains, respectively, whilst the A204E mutation is located in the second extracellular loop (Figure 6-1). The results of studies that have investigated the effects of these mutations on the GRLN-R are summarised in Table 6-1.

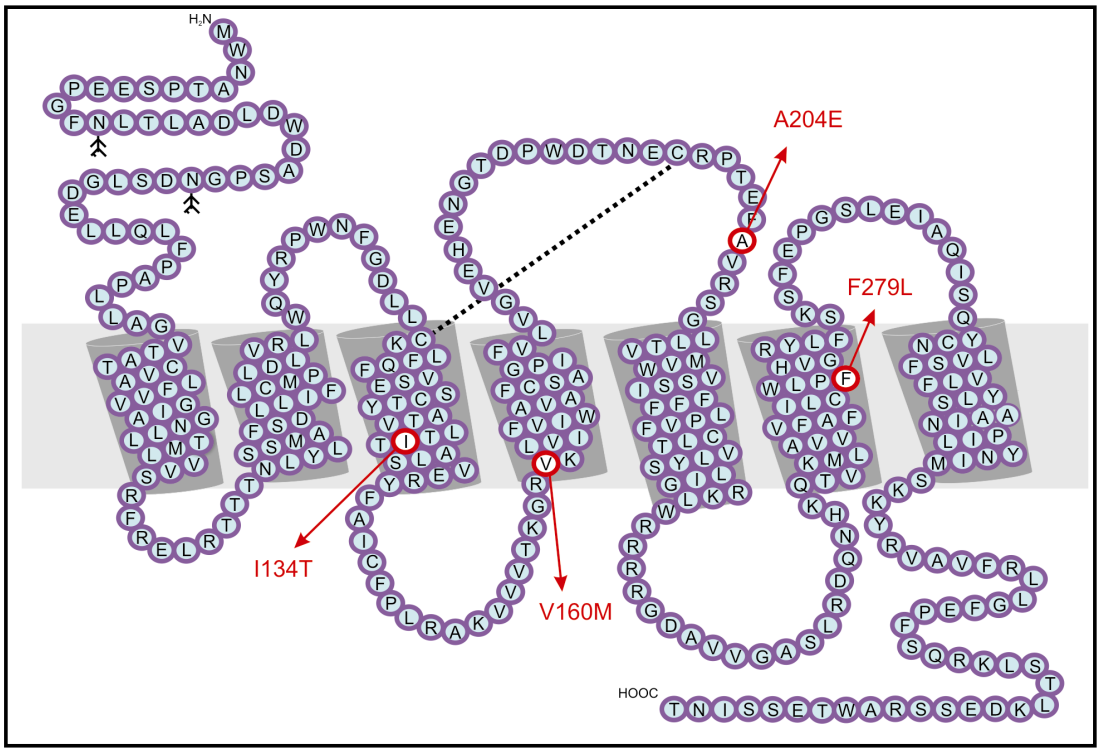

Figure 6-1 – Structure of the GRLN-R identifying the known missense mutations of the GRLN-R. Missense mutations of the GRLN-R are shown in red. A cysteine residue in the extracellular end of TMIII is predicted to form a disulphide bond with a cysteine residue in ECL2 (shown as a dotted line). Residues that are predicted to undergo *N*-linked glycosylation are marked with Y.

Interestingly, the A204E and F279L mutations have been associated with the development of a short stature/obese phenotype *in vivo* (Pantel et al., 2006). Indeed, it has been suggested that the development of this phenotype provides evidence that constitutive activity of the GRLN-R is important *in vivo* (Holst & Schwartz, 2006). However, the F279L mutation has been described in one study as reducing, but not completely ablating, the constitutive activity of the mutated GRLN-R (Liu et al., 2007). Thus the reduced cell-surface expression of the GRLN-R, caused by these mutations (Liu et al., 2007) may be important in the development of the short stature/obese phenotype. To date no studies have investigated whether the decreased cell-surface expression of the GRLN-R caused by the A204E is due to ER-retention of the receptor.

The I134T mutation has been shown to ablate the signalling of the GRLN-R to ghrelin, whilst having no effect on the constitutive activity of the receptor (Liu et al., 2007). However, the effect of this mutation on the signalling to the growth hormone secretagogues has not been tested. Indeed as ghrelin and the growth hormone secretagogues have overlapping, but not identical binding sites, on the GRLN-R (Holst et al., 2009; Feighner et al., 1998), it is possible that the mutated GRLN-R retains its ability to signal upon activation by the growth hormone secretagogues.

Table 6-1 – Effect of the A204E, F279L, I134T and V160M mutations on the pharmacology of the GRLN-R.

Mutation	Effect *in vitro*	Phenotype *in vivo**
A204E	No effect on ghrelin affinity and efficacy[1]. Ablated the constitutive activity of the GRLN-R[1,3,4]. May decrease cell surface expression of the GRLN-R[3,4]	Short stature and obese phenotype[4]
F279L	One study showed the F279L mutation ablated receptor constitutive activity[2], another showed a reduced but nonetheless measurable constitutive activity[3]. One study showed the F279L mutation had no affect on ghrelin potency[3], whilst another study showed the mutation decreased the potency of ghrelin[7]. The mutation has also been reported to decrease the cell surface expression of the GRLN-R[3] and decrease the specific binding of MK-677[6]	Short stature and obese phenotype[5]
I134T	Ablates signalling to ghrelin, no effect on the constitutive activity of the GRLN-R, decreased cell surface expression[3]	Not reported
V160M	Increased ghrelin potency by some 20-fold, reduced the constitutive activity of the GRLN-R, decreased cell surface expression[3]	Not reported

[1]Holliday et al., 2007, [2]Holst et al., 2004, [3]Liu et al., 2007,[4]Pantel et al., 2006, [5]Wang et al., 2004, [6]Feighner et al., 1998, [7]Holst et al., 2009. * Due to the rarity of these mutations, a direct link between the A204E and F279L mutations and the phenotype observed *in vivo* cannot be established.

The aim of this study was to explore the effects of the A204E and I134T mutations on the localisation and signalling of the GRLN-R; in particular, to establish whether these mutations caused ER-retention of the GRLN-R and to test whether receptor containing the I134T mutation retained its ability to signal to the growth hormone secretagogues.

6.2 Results

6.2.1 Construction of a Flp-In™ T-REx™ cell line expressing HA-GRLN-R-I134T-eCFP or HA-GRLN-R-A204E-eCFP

Epitope tags were added to the GRLN-R containing either the A204E or the I134T missense mutations, to allow detection of the receptor by immunoblotting and epifluorescence microscopy. The 9 amino acid HA tag (YPYNVPDYA), which originates from the full length haemagglutinin protein, a glycoprotein required for infectivity of the human influenza virus (Wilson et al., 1984), was fused at the N-terminus (directly after the initiating methionine residue) and a eCFP reporter molecule at the C-terminus (Figure 6-2). The HA tag was added to the mutant receptors rather than a VSV-G tag added to the wild-type receptor (i.e. the un-mutated GRLN-R tagged at the N-terminus with VSV-G and at the C-terminus with eCFP; described in Chapter 4) to aid future studies into the dimerisation of the mutated receptors with the wild-type GRLN-R, which would require differential tagging of the receptors.

The HA-GRLN-R-I134T-eCFP and the HA-GRLN-R-A204E-eCFP constructs were ligated into pcDNA5 and co-transfected with the Flp-recombinase expression plasmid pOG44 into Flp-In™ T-REx™ HEK293 cells. Flp-In™ T-REx™ HEK293 cells transfected with the HA-GRLN-R-I134T-eCFP construct (hereafter referred to as GRLN-R-I134T cells) or the HA-GRLN-R-A204E-eCFP construct (GRLN-R-A204E cells) were

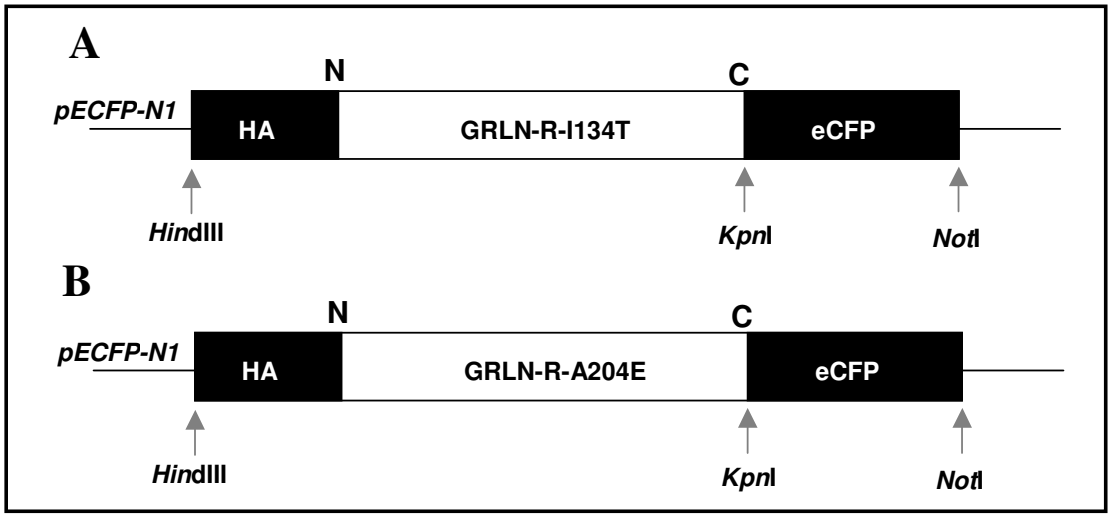

Figure 6-2 - Schematic diagrams of the HA-GRLN-R-I134T-eCFP and HA-GRLN-R-A204E-eCFP constructs. A. PCR was used to mutate $I134^{3.43}$ to $T134^{3.43}$ or **B.** to mutate A204 to E204. Subsequently PCR was used to add a *Hin*dIII site and an HA tag at the N terminus as well as to add a *Kpn*I restriction site and to remove the STOP codon at the C-terminus of the mutated receptors. The resulting PCR products and vector (pECFP-N1) were sequentially digested with *Hin*dIII and *Kpn*I, before the constructs were ligated into the vector in-frame with eCFP.

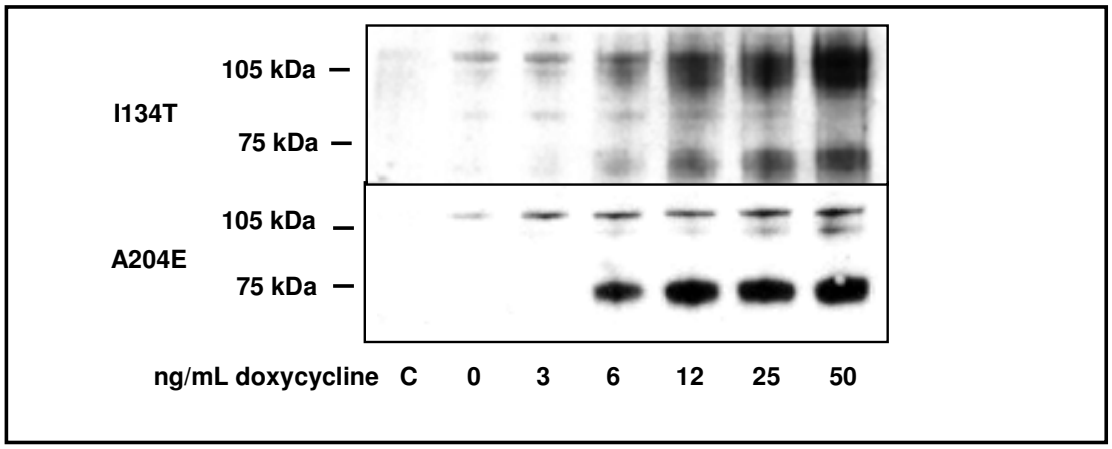

Figure 6-3 - Testing doxycycline induction in GRLN-R-I134T and GRLN-R-A204E cells. Cells were treated with varying concentrations of doxycycline (0-50 ng/mL) for 16 h. Cells were harvested and lysates prepared. As a control lysates were also prepared from parental Flp-In™ T-REx™ HEK293 cells (marked 'C'). Samples were resolved using SDS-PAGE followed by western blotting using sheep anti-GFP antiserum (which cross-reacts with eCFP). The top panel is the resulting western blot of lysates from GRLN-R-I134T cells and the bottom panel from GRLN-R-A204E cells. Immunoblot is representative of the results from three individual experiments.

tested for the successful integration of the expression cassette into the host cell line. To test whether receptor expression could be detected, cells were grown for 16 h in media supplemented with increasing concentrations (0-50 ng/mL) of doxycycline; the cells were subsequently lysed and the samples analysed by immunoblotting.

In the absence of doxycycline, both the GRLN-R-A204E and GRLN-R-I134T cells showed similar protein banding to parental Flp-In™ T-REx™ HEK293 cells, indicating that no receptor-specific signals were detected (Figure 6-3). In lysates prepared from GRLN-R-I134T cells the addition of doxycycline led to the detection of two receptor species; one with a molecular mass of approximately 70 kDa and another with an apparent molecular mass of 105 kDa (the mass of the construct is expected to be around 69 kDa). At doxycycline concentrations of 6-50 ng/mL, the largest proportion of signal was expressed as the 105 kDa form. In contrast, immunoblotting of lysates prepared from GRLN-R-A204E cells showed that, despite a doxycycline-dependent increase in receptor expression, even at the highest doxycycline concentrations tested, the majority of receptor detected existed as a species with an apparent molecular mass of 70 kDa.

As Flp-In™ T-REx™ cells ensure integration of the gene occurs at only one locus, protein expression becomes solely the property of the gene of interest, thus construction of the cell lines expressing the GRLN-R-I134T or the GRLN-R-A204E allowed comparison of the pharmacology and localisation of the mutant receptors with the wild-type GRLN-R. As treatment with 6 ng/mL doxycycline (16 h) was sufficient to induce expression of the

GRLN-R, GRLN-R-I134T and GRLN-R-A204E, this concentration was chosen to induce receptor expression in all further studies.

6.2.2 Glycosylation states of the GRLN-R, GRLN-R-I134T and GRLN-R-A204E

Glycosylated membrane proteins change from a high mannose glycosylated state to a complex glycosylated state in the medial-*trans*-Golgi compartment (Sadeghi et al., 1997). Addition of complex glycosylation renders protein insensitive to digestion with Endo-β-acetylglucosamindase H (Endo H) whilst retaining the sensitivity of the protein to peptide-N^4-(acetyl-β-glucosaminyl)-asparagine amidase (PNGaseF). Thus digestion of membrane proteins with Endo H and/or PNGaseF has been widely used to determine whether a mutant membrane protein resides in the ER-*cis*-Golgi complex (Sadeghi et al., 1997). To determine the glycosylation states of both wild-type and mutated GRLN-R, lysates were prepared from doxycycline-treated GRLN-R, GRLN-R-A204E and GRLN-R-I134T cells and digested with either PNGaseF or Endo H and, as a control, samples were prepared without the addition of enzyme (Figure 6-4). Immunoblotting of these cell lysates with anti-GFP antiserum revealed that for both the GRLN-R and the GRLN-R-I134T, in the abscence of treatment with PNGaseF or Endo H, a large proportion of total receptor existed with a mass of around 105 kDa; although a small proportion of receptor with an apparent mass of 70 kDa was also detected. Treatment of the cell lysates with Endo H did not reduce the apparent mass of the 105 kDa species, however Endo H reduced the apparent mass of the 70 kDa receptor pool to around 50 kDa. Treatment of the cell lysates with PNGaseF reduced the mass of both receptor species, resulting in the detection of a species with a molecular mass around 50-60 kDa.

Immunoblotting cell lysates produced from GRLN-R-A204E cells showed that, in the absence of treatment with PNGaseF or Endo H, only one receptor specific species with a mass of around 70 kDa was detected. This 70 kDa signal was reduced to a mass of around 50 kDa by treatment with either PNGaseF or Endo H. Interestingly, treatment with PNGaseF led to an increase in intensity of the 50 kDa signal (corresponding to unglycosylated receptor), compared to that obtained after Endo H treatment.

The results from these experiments demonstrate that the majority of GRLN-R and GRLN-R-I134T exist as mature (complex-glycosylated) receptor. In contrast, the largest

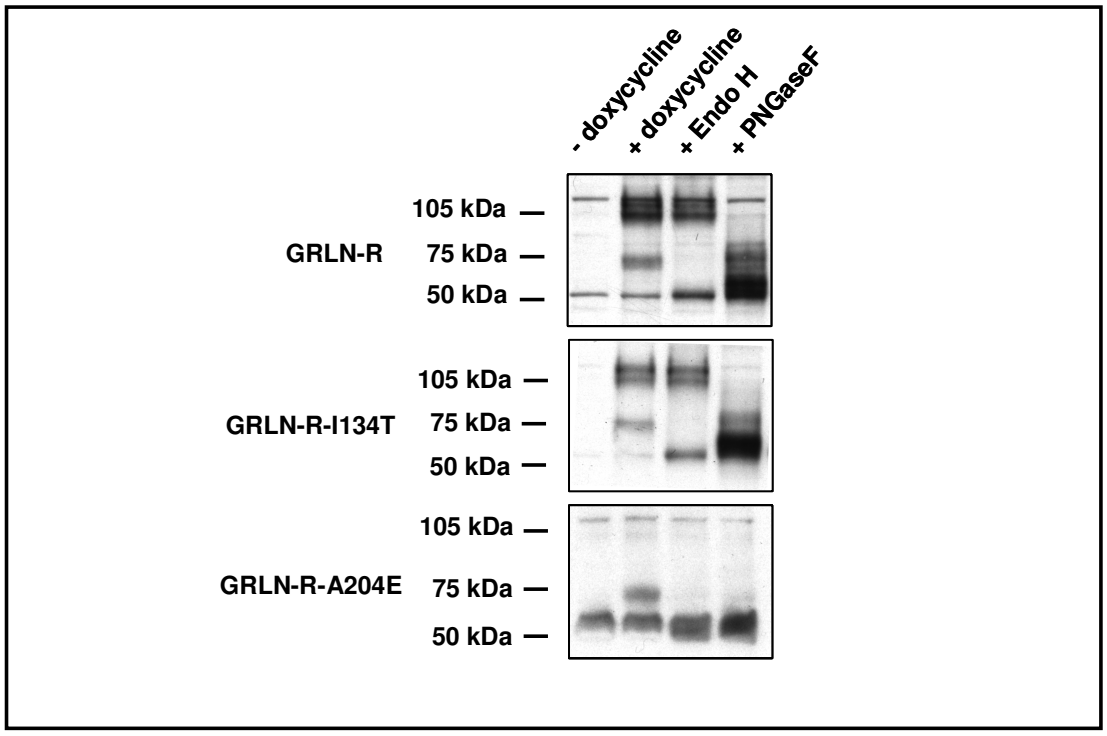

Figure 6-4 - Effect of deglycosylation with Endo H or PNGaseF on the molecular mass of GRLN-R, GRLN-R-I134T and GRLN-R-A204E. GRLN-R. GRLN-R-I134T and GRLN-R-A204E cells were incubated in media supplemented, as required, with 6 ng/mL doxycycline (16 h). Cells were harvested, lysates prepared and to 30 µg of protein 1 unit (U) of PNGaseF or 1 mU of Endo H was added. Samples were digested for 16 h at 4 °C before being resolved by SDS-PAGE followed by immunoblotting with sheep anti-GFP antiserum. Immunoblots are representative of the results from three individual experiments.

proportion of GRLN-R-A204E exists as immature (high-mannose glycosylated) receptor, suggesting that the GRLN-R-A204E is being retained within the pre-*trans*-Golgi compartment. However, the results of the deglycosylation experiments suggest that some mature GRLN-R-A204E is formed, as digestion with PNGaseF appeared to lead to an increase in the intensity of the species with an apparent mass of 50 kDa (corresponding to unglycosylated receptor) compared to that obtained after digestion with Endo H.

6.2.3 Localisation of GRLN-R-I134T and GRLN-R-A204E

The results of the deglycosylation experiments (Figure 6-4) suggested that the GRLN-R-A204E might be retained within the ER. Thus the localisation of the wild-type GRLN-R and its mutants were examined to test whether the mutations had any effect on the receptors' subcellular distribution. Receptor localisation was visualised by exciting the eCFP tag located at the receptor's C-terminus. In both GRLN-R-I134T and GRLN-R-A204E cell lines, no fluorescent signal was observed in the absence of doxycycline (Figure 6-5). In GRLN-R-I134T cells, eCFP expression was detected both at the plasma membrane and in vesicles within the cell, a distribution similar to that observed for wild-type GRLN-

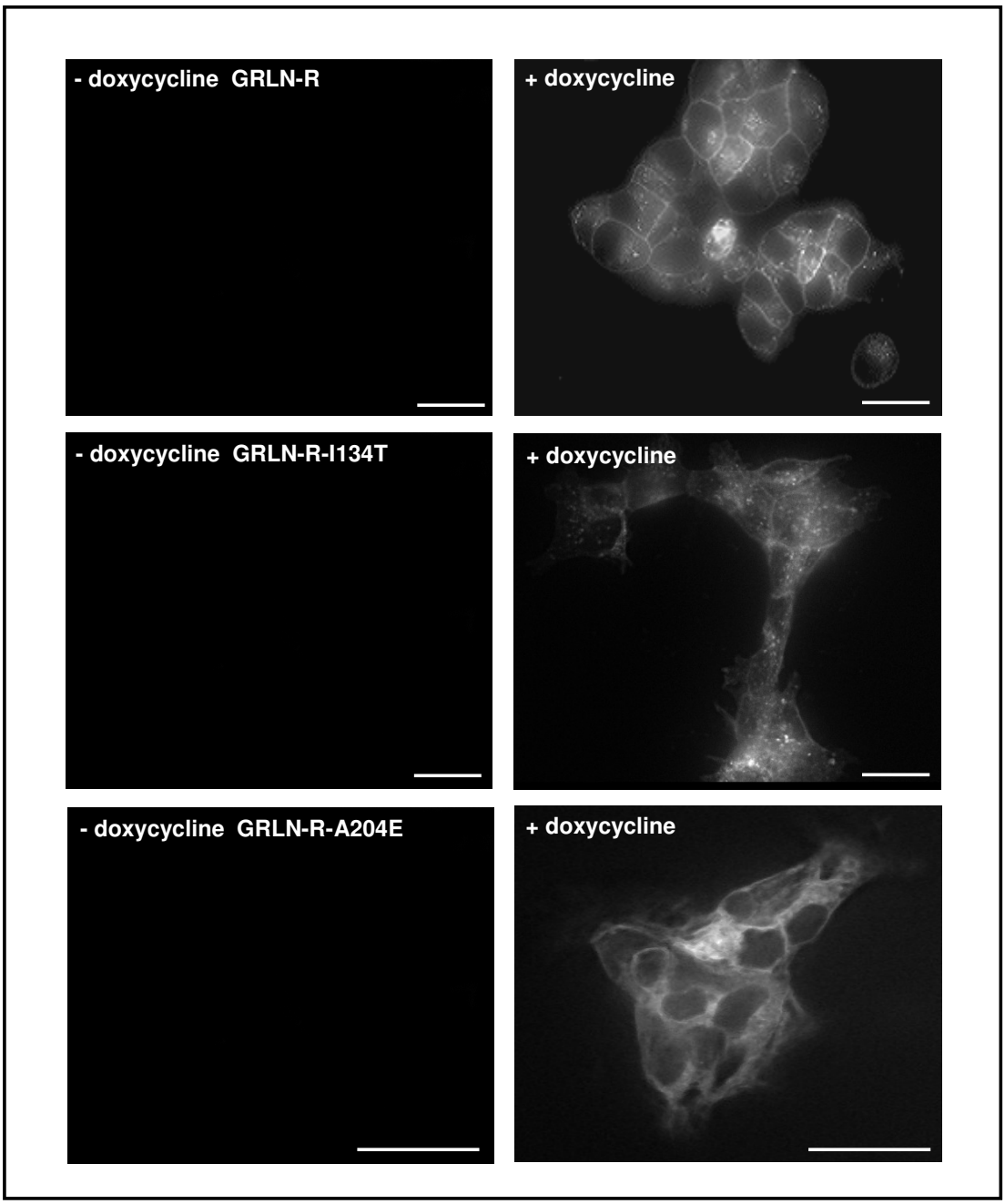

Figure 6-5 - Epifluorescence microscopy images showing the localisation of the wild-type GRLN-R, the GRLN-R-I134T and the GRLN-R-A204E. Wild-type GRLN-R cells (top panel), GRLN-R-I134T cells (middle panel) and GRLN-R-A204E cells (bottom panel) were grown on coverslipes in media supplemented, where indicated, with 6 ng/mL doxycycline (16 h). Subsquently cells were formaldehyde-fixed (3.7 $\%_{(v/v)}$ formaldehyde; 10 min; 25 °C) and receptor expression viewed by excitation of the C-terminal eCFP tag with 436/12 nm light. In the absence of doxycycline, eCFP fluorescence could not be observed. Upon doxycycline induction, in the wild-type GRLN-R and GRLN-R-I134T cells, eCFP linked to the receptor could be seen both at the plasma membrane and in vesicles throughout the cell. In the GRLN-R-A204E cells the eCFP-linked receptor appeared to be retained in cytoplasmic regions of the cells. Scale bar width represents 20 µm. Images are representative of three individual experiments.

R cells (see Chapter 4, Figure 4-6). In contrast, in the GRLN-R-A204E cells, eCFP expression appeared to be distributed evenly throughout the inside of the cell.

To further investigate the subcellular distribution of the receptors, an ER-Tracker™ red dye was used to stain the ER and co-localisation with eCFP fluorescence assessed. Analysis of GRLN-R, GRLN-R-I134T and GRLN-R-A204E cells revealed weak co-localisation of the GRLN-R and GRLN-R-I134T with the ER marker, with the eCFP signal also detected in punctuate vesicles within the cell that did not co-localise with the ER marker. In contrast, the GRLN-R-A204E showed a high degree of co-localisation with the ER marker, demonstrating that the receptor variant was trapped within the early secretory pathway (Figure 6-6).

6.2.4 Testing the interaction of GRLN-R, GRLN-R-I134T and GRLN-R-A204E with calnexin

Calnexin is an ER chaperone protein that is involved in ensuring the proper folding of many transmembrane glycoproteins (Parodi, 2000), making prolonged interaction of a protein with calnexin indicative of protein misfolding. The results of the previous experiments had provided evidence that the A204E mutation caused the GRLN-R to be retained within the ER, possibly due to protein misfolding. Thus to test whether the GRLN-R-A204E associated more stably with calnexin than the GRLN-R or the GRLN-I134T, a co-immunoprecipitation assay was utilised.

Lysates from GRLN-R, GRLN-R-A204E and GRLN-R-I134T cells were prepared, immunoprecipitated using anti-GFP antiserum and analysed by western blotting. Analysis of the total lysates demonstrated that all samples were equally loaded and there was no up-regulation of calnexin following receptor expression (Figure 6-7). Analysis of the total lysates for the receptor's eCFP tag showed receptor expression was only observed upon induction with 6 ng/mL doxycycline. Despite equal loading of the lysates, the GRLN-R and GRLN-R-I134T were expressed at substantially higher levels than the intracellularly retained GRLN-A204E.

Analysis of the immunoprecipitation samples showed that each of the receptors interacted with calnexin, although to different extents. When taking into account for the differences in receptor expression level, the GRLN-R-A204E appeared to show a more stable interaction with calnexin compared to both the GRLN-R and the GRLN-R-I134T whilst both the GRLN-R and the GRLN-R-I134T showed similar interactions with calnexin. Thus

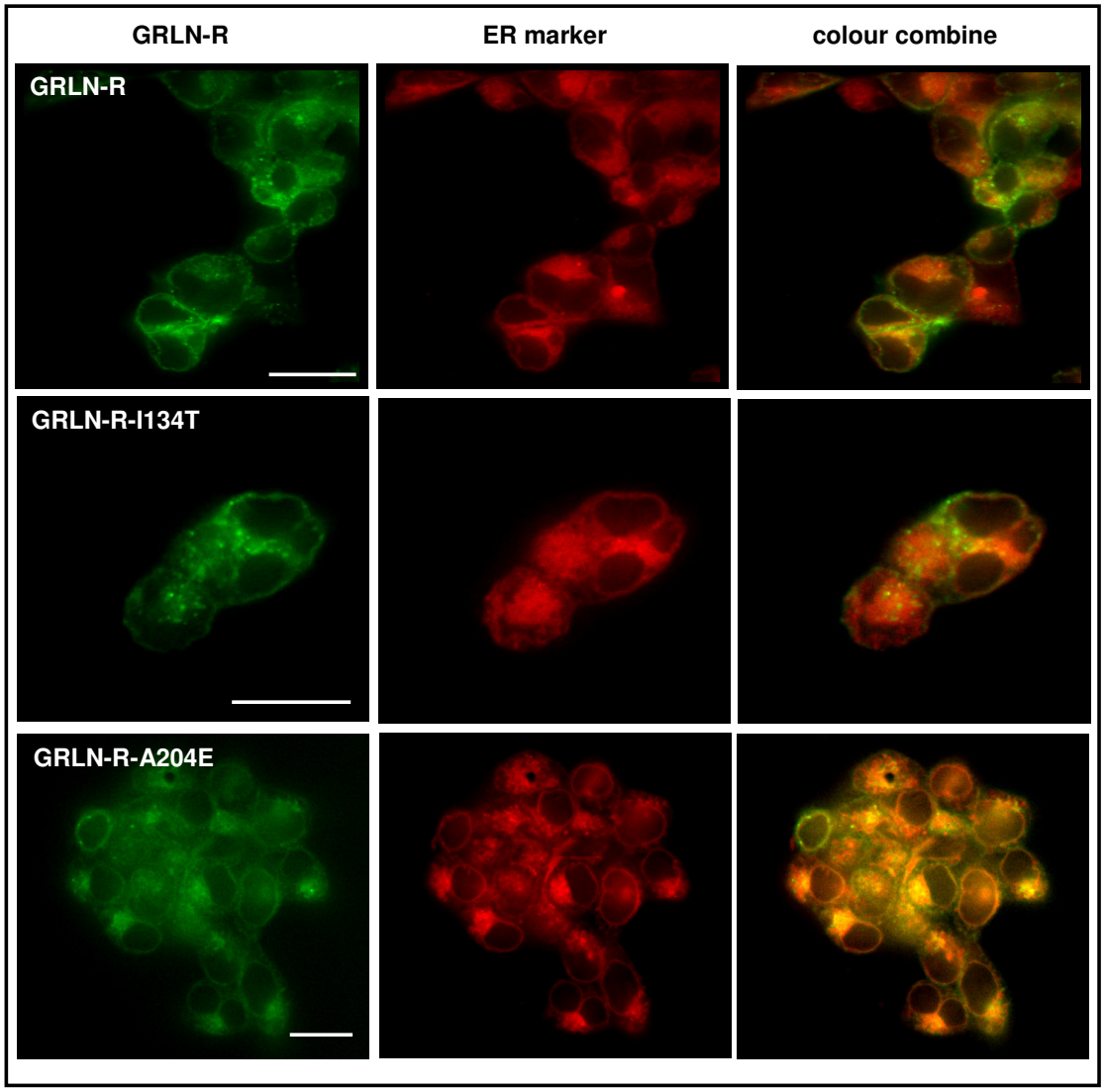

Figure 6-6 - Co-localisation of GRLN-R, GRLN-R-I134T and GRLN-R-A204E with a marker of the ER. In each cell line receptor expression was induced with 6 ng/mL doxycycline (16 h). Cells were rinsed with PBS before incubation in a humidified incubator (37 °C; 5 % CO_2/ 95 % air) with 1 µM ER-tracker™ red (20 min). Cells were subsequently formaldehyde-fixed and mounted onto microscope slides. The images of receptor (pseudo-coloured green) and ER marker (red) were aligned (colour combine column) with overlapping regions shown as a yellow colour. Scale bar width equals 20 µm. Images are representative of two individual experiments.

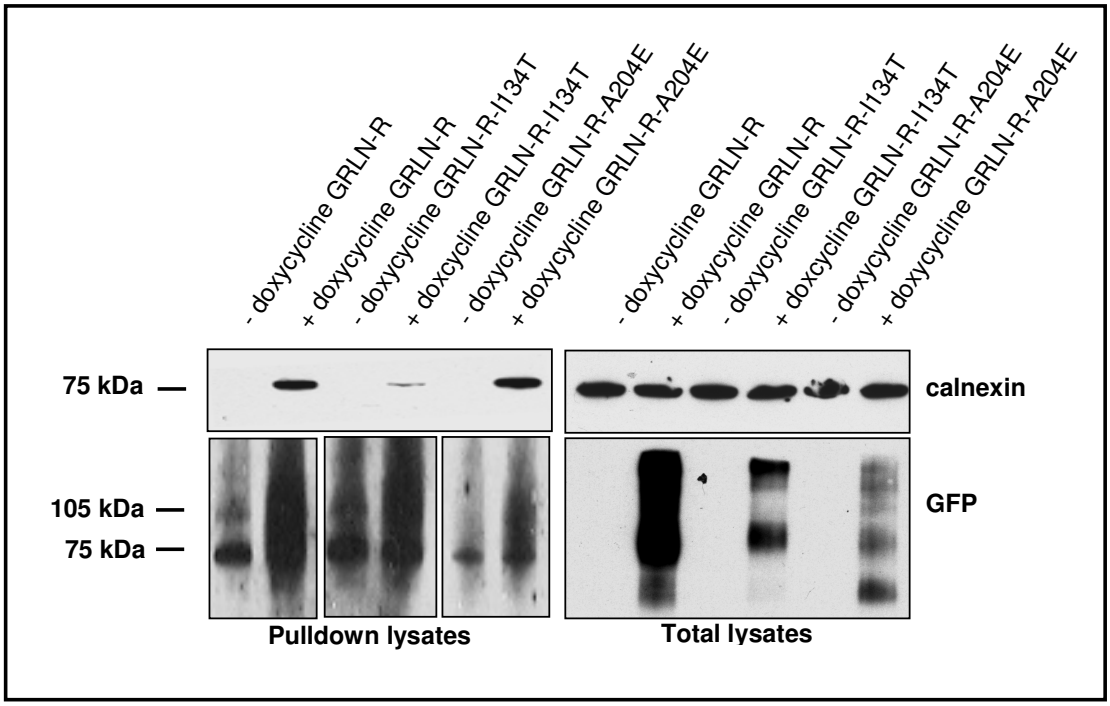

Figure 6-7 - Interaction of GRLN-R, GRLN-R-I134T and GRLN-R-A204E with the ER chaperone protein calnexin. Expression of GRLN-R, GRLN-R-I134T or GRLN-R-A204E was induced by the addition of 6 ng/mL doxycycline (16 h) in the respective cell lines. Cells were lysed and proteins immunoprecipitated using sheep anti-GFP antiserum. Subsequently, total lysates and immunoprecipitated (pulldown) lysates were resolved by SDS-PAGE followed by western blotting with either a rabbit anti-GFP antibody or an anti-calnexin antibody. Results shown are from one experiment (n =1).

these results are consistent with the previous findings, which suggest that the A204E mutation leads to intracellular retention of the GRLN-R.

6.2.5 Time course of GRLN-R-A204E folding

Next, to determine whether the GRLN-R-A204E failed to achieve maturation, or whether it exhibited slower folding kinetics than the GRLN-R and GRLN-R-I134T to reach its native conformation, a time course of induction of protein expression was performed.

Lysates were prepared from GRLN-R-A204E cells that had been treated with doxycycline for 0-72 h, lysates were subsequently deglycosylated (with either Endo H or PNGaseF) and analysed by immunoblotting. In the absence of doxycycline, no signal could be detected (Figure 6-8), whereas after 9 h induction, a faint 70 kDa signal was detected that was reduced to 50 kDa by treatment with Endo H or PNGaseF. The intensity of the signal for immature receptor increased as time of doxycycline-induction increased. Interestingly, a faint signal for the 105 kDa mature receptor was also detected, although this could

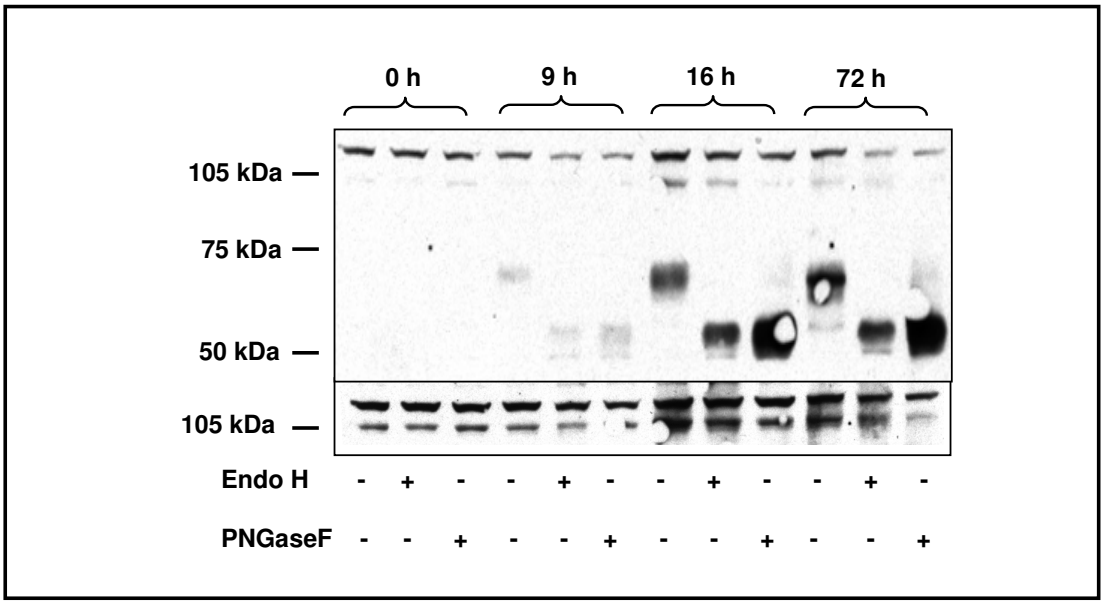

Figure 6-8 - Time course of folding of GRLN-R-A204E mutant. Lysates were prepared from GRLN-R-A204E cells treated with 1 µg/mL doxycycline for 0 –72 h. Samples were deglycosylated for 16 h (4 °C) with 1 mU Endo H or 1 U PNGaseF (as indicated) before being resolved by SDS-PAGE followed by western blotting with anti-GFP antiserum. The lower panel shows the presence of a signal at 105 kDa after overexposure of the blot. Immunoblots representative of an n=1.

only be seen after prolonged exposure of the immunoblot (lower panel of Figure 6-8). Treatment with PNGaseF reduced the intensity of 105 kDa band and increased the signal for the unglycosylated 50 kDa form. Thus, this demonstrates that some, albeit a small, proportion of GRLN-R-A204E protein underwent complex glycosylation.

6.2.6 Signalling of the GRLN-R-A204E in intact cells

To test whether mature receptors were being formed and whether these receptors were fully functional, an intact cell assay was utilised. As ghrelin is a peptide ligand it is expected to be cell-impermeable, as such, any response to ghrelin must be mediated via activation of receptors expressed at the plasma membrane. Using an inositol phosphate accumulation assay, non-induced control cells showed no response to treatment with 1 µM ghrelin or 1 µM of the inverse agonist SPA (Figure 6-9). Upon induction of GRLN-R-A204E expression, a significant response to ghrelin could be obtained ($p<0.001$; one-way ANOVA with Tukey's multiple comparison test), demonstrating that some functional GRLN-R-A204E was expressed at the plasma membrane. Inducing receptor expression had no effect on the level of basal inositol phosphate accumulation compared to non-induced cells, suggesting that unlike the wild-type GRLN-R, this receptor mutant was not

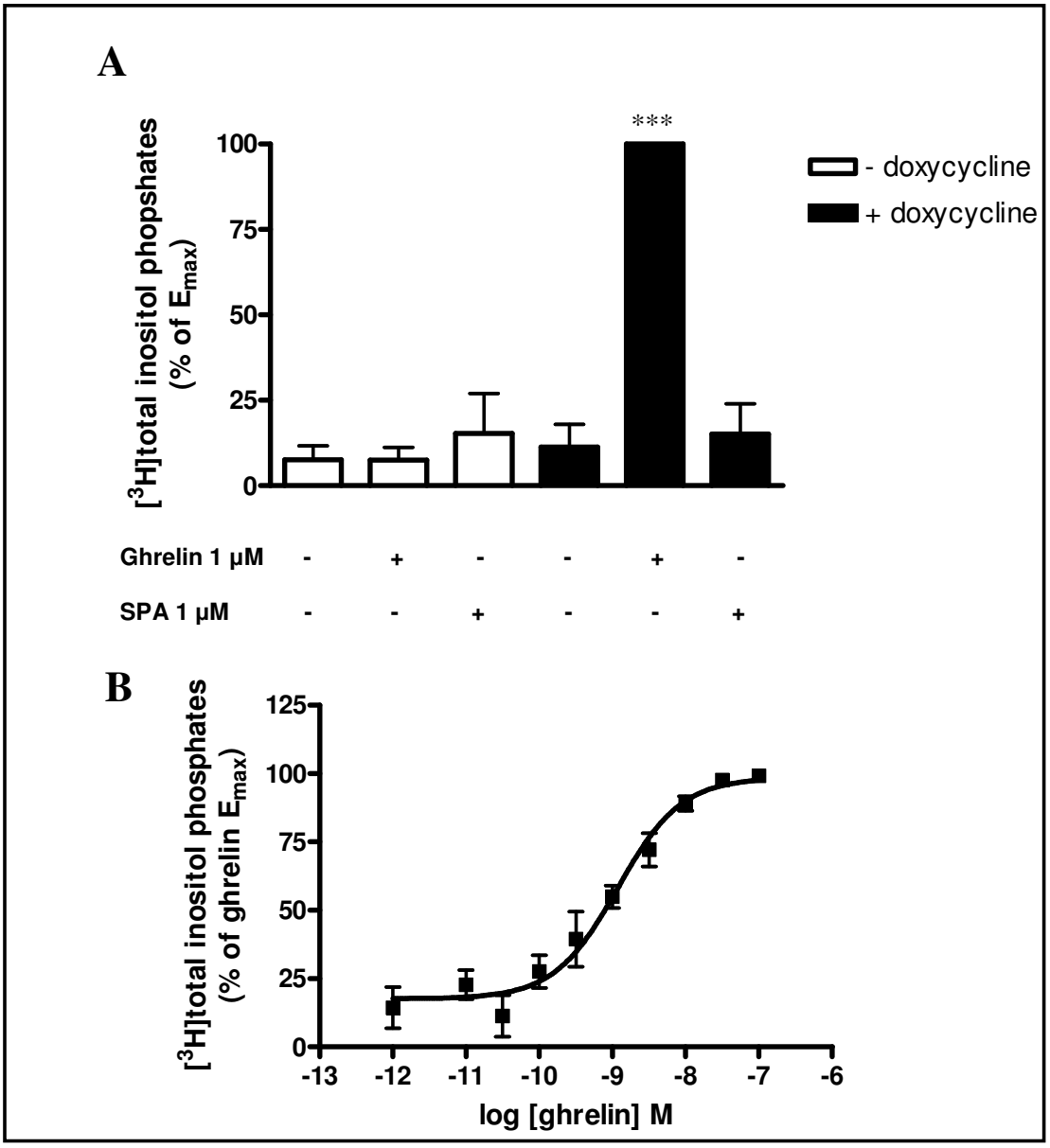

Figure 6-9 - Response of GRLN-R-A204E to ghrelin and SPA as measured using an inositol phosphate accumulation assay. Cells were incubated with 1 µCi/mL [^{3}H]*myo*-inositol and receptor expression induced as required. Prior to the assay media was replaced with Krebs-Ringer buffer supplemented with 10 mM lithium chloride to block the breakdown of inositol phosphates into *myo*-inositol. **A.** Cells were stimulated with 1 µM ghrelin or 1 µM SPA for 30 min (37 °C), as indicated. White bars indicate the responses obtained in the absence of receptor expression, whilst black bars indicate the responses obtained upon expression of GRLN-R-A204E. After ligand-stimulation cells were lysed with perchloric acid and [^{3}H]total inositol phosphates isolated from the mixture using ion-exchange chromatography. The amount of [^{3}H]total inositol phosphates accumulated were measured using liquid scintillation spectrometry (120 s/sample). Data points represent the mean ± S.E.M of three independent experiments performed in duplicate. ***$p<0.001$ as measured using a one-way ANOVA with Tukey's multiple comparison test. **B.** A concentration-response curve to ghrelin was generated in induced GRLN-R-A204E cells. Data points represent the mean ± S.E.M of three independent experiments performed in duplicate.

constitutively active. This was confirmed by treatment of cells with 1 μM SPA, which had no effect on the level of inositol phosphate accumulation in the absence of ghrelin.

To determine the potency of ghrelin signalling on GRLN-R-A204E at the plasma membrane, the inositol phosphate accumulation assay was used to generate a concentration-response curve to ghrelin. This demonstrated that the level of inositol phosphate accumulation increased in a concentration-dependent manner (Figure 6-9), yielding a pEC_{50} value of 8.93 ± 0.12 a value that was shown to not be significantly different (p=0.14) to the wild-type receptor (pEC_{50} = 9.22 ± 0.11) when compared using an unpaired, two-tailed t-test.

6.2.7 Signalling of the GRLN-R-A204E through the $G_{\alpha i/o}$ pathway

As the inositol phosphate accumulation assays demonstrated that the GRLN-R-A204E retained its ability to signal through activation of the $G_{\alpha q/11}$ pathway, the ability of the receptor to activate the $G_{\alpha i/o}$ pathway was tested. The wild-type GRLN-R has been described as a promiscuous receptor, able to activate the $G_{\alpha i/o}$ pathway in response to stimulation with ghrelin (Bassil et al., 2007; Esler et al., 2007; Dezaki et al., 2007; Chapters 3 and 4).

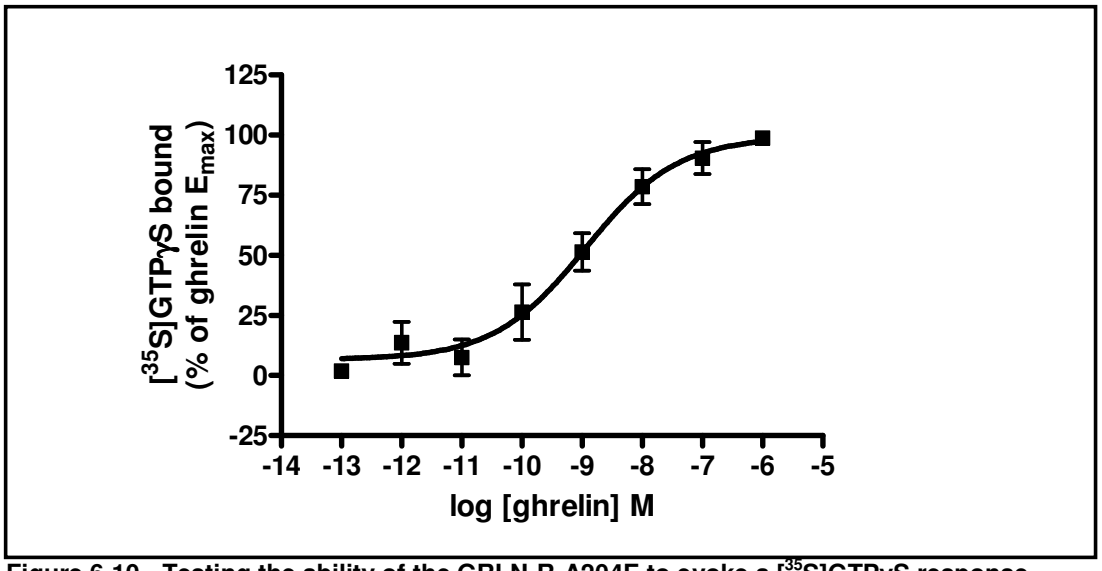

Figure 6-10 - Testing the ability of the GRLN-R-A204E to evoke a [^{35}S]GTPγS response through activation of $G_{\alpha i/o}$ pathway. GRLN-R-A204E cells were transfected with $G_{\alpha o1}$ and receptor expression was induced by the addition of 6 ng/mL doxycycline (16 h). Membranes were prepared and tested for their ability to increase [^{35}S]GTPγS binding in response to stimulation with increasing concentrations of ghrelin (20 min; 30 °C). G protein-bound [^{35}S]GTPγS was separated from the reaction mixture by rapid filtration through GF/C filters soaked in 1 x PBS and bound [^{35}S]GTPγS measured (for 5 min) using liquid-scintillation spectrometry. Data points represent the mean ± S.E.M of three independent experiments performed in triplicate.

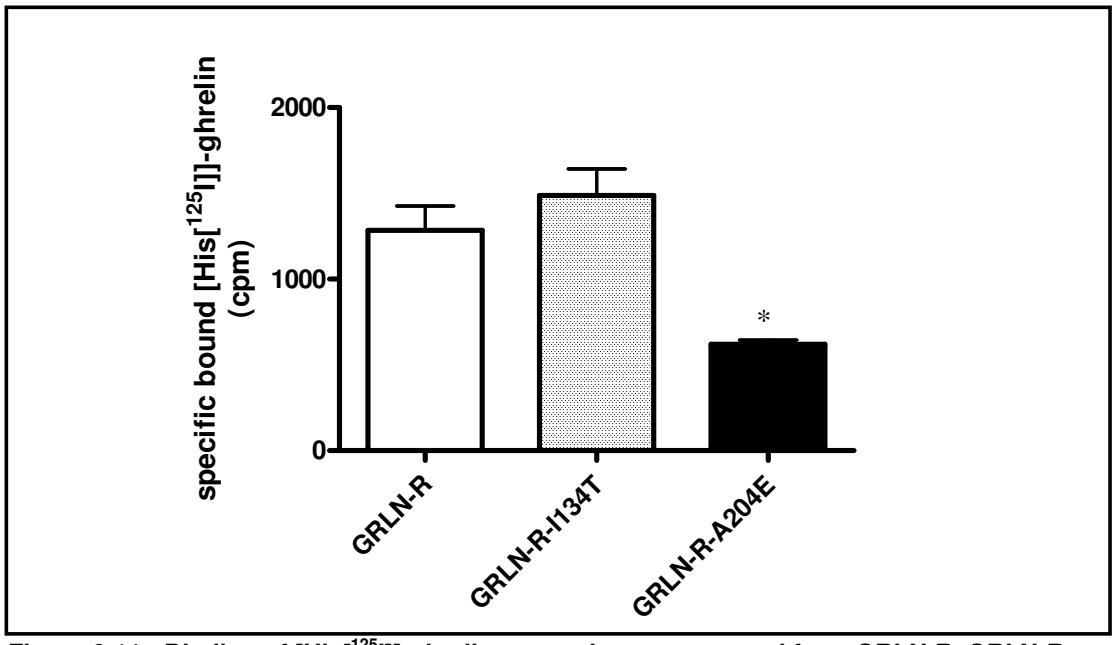

Figure 6-11 - Binding of [His[^{125}I]]-ghrelin to membranes prepared from GRLN-R, GRLN-R-I134T and GRLN-R-A204E cells. GRLN-R, GRLN-R-I134T, and GRLN-R-A204E cells were induced with 6 ng/mL doxycycline (16 h); subsequently cells were harvested and membranes prepared. Binding of [His[^{125}I]]-ghrelin was determined by addition of a final concentration of 83 pM of [His[^{125}I]]ghrelin to 5 µg of cell membranes, ± 1 µM cold ghrelin to label non-specific binding, in a final volume of 150 µL. Binding was carried out for 120 min at 4 °C before being terminated by rapid filtration through GF/B soaked in 0.5 %$_{(w/v)}$ PEI. Specific binding of [His[^{125}I]]ghrelin to membranes from GRLN-R cells and GRLN-R-I134T cells was determined by subtracting total [His[^{125}I]]-ghrelin binding from non-specific binding. Data points represent the mean ± S.E.M of three independent experiments performed in duplicate. *p<0.05 as compared using a one-way ANOVA with Dunnett's post-hoc test.

GRLN-R-A204E cells were transfected to express $G_{\alpha o1}$ and receptor expression was induced by the addition of doxycycline. Membranes were prepared and, using a [^{35}S]GTPγS binding assay, a concentration-response curve to ghrelin was generated, allowing determination of the potency of ghrelin (pEC_{50} = 8.94 ± 0.44), a value that was not significantly different (p=0.63) to that of the wild-type GRLN-R (pEC_{50} = 9.19 ± 0.16) when compared using an unpaired, two-tailed t-test (Figure 6-10). Thus the results from both this experiment and the inositol phosphate accumulation experiments demonstrate that the A204E mutation does not alter the potency of ghrelin via activation of the $G_{\alpha q/11}$ and $G_{\alpha i/o}$ pathway, respectively. Furthermore it demonstrates that, like the wild-type GRLN-R, the GRLN-R-A204E is able to promiscuously couple to multiple G protein pathways.

6.2.8 Binding of [His[^{125}I]]-ghrelin to membranes prepared from GRLN-R, GRLN-R-I134T and GRLN-R-A204E cells

To assess whether the A204E or I134T mutations affect the expression levels of the GRLN-R, a radioligand binding assay was utilised. Membranes were prepared from wild-type GRLN-R, GRLN-R-I134T and GRLN-R-A204E cells and the specific binding of

[His[^{125}I]]-ghrelin to the membranes measured. (Figure 6-11). Specific binding of [His[^{125}I]]-ghrelin to membranes prepared from GRLN-R-A204E cells was significantly lower than in membranes prepared from wild-type GRLN-R cells ($p<0.05$; one-way ANOVA with Dunnett's post-hoc test). In contrast, the I134T mutation had no significant effect on the level of specific [His[^{125}I]]-ghrelin binding compared to the wild-type GRLN-R, indicating that this mutation did not affect the mutant's expression level. Moreover, in contrast to published data (Liu et al., 2007), this data demonstrates that the I134T mutation does not ablate ghrelin binding to the GRLN-R.

6.2.9 Testing the response to GRLN-R-I134T in an intact cell assay

As the GRLN-R-I134T was shown to be able to bind [His[^{125}I]]-ghrelin (section 6.2.8), the ability of the mutant receptor to signal in response to ghrelin was tested using the intact-cell inositol phosphate assay. In the absence of receptor, no significant ($p>0.05$; one-way ANOVA with Tukey's multiple comparison test) response of the cells to 1 μM ghrelin or to 1 μM SPA was observed (Figure 6-12). Receptor expression led to a significant increase ($p<0.05$) in basal inositol phosphate accumulation, a response around 6.5-fold above that measured in the absence of receptor expression. The increase in the basal levels of inositol phosphate accumulation was similar to the 6.3-fold increase in inositol phosphate levels measured upon expression of the wild-type GRLN-R shown in Chapter 5 (Figure 5-6). However, for the GRLN-R-I134T, incubation with 1 μM SPA did not significantly reduce the level of inositol phosphate accumulation to that obtained in the absence of receptor expression, although the level of inositol phosphate accumulated in the absence of receptor expression was not statistically different compared to SPA-treated cells. These results suggest that the efficacy and/or affinity of SPA for the GRLN-R may have been lowered by the I134T mutation.

In line with the radioligand binding data (section 6.2.8), a significant response to 1 μM ghrelin was obtained in cells expressing GRLN-R-I134T ($p<0.001$). Moreover, GRLN-R-I134T responded to ghrelin in a concentration-dependent manner yielding a pEC_{50} value of 8.76 ± 0.13 (Figure 6-12). This value was not significantly different ($p=0.05$) to the potency of ghrelin acting at the GRLN-R (pEC_{50} = 9.22 ± 0.11; Chapter 5; Table 5-3; compared using an unpaired, two tail t-test). Taken together, these findings suggest that

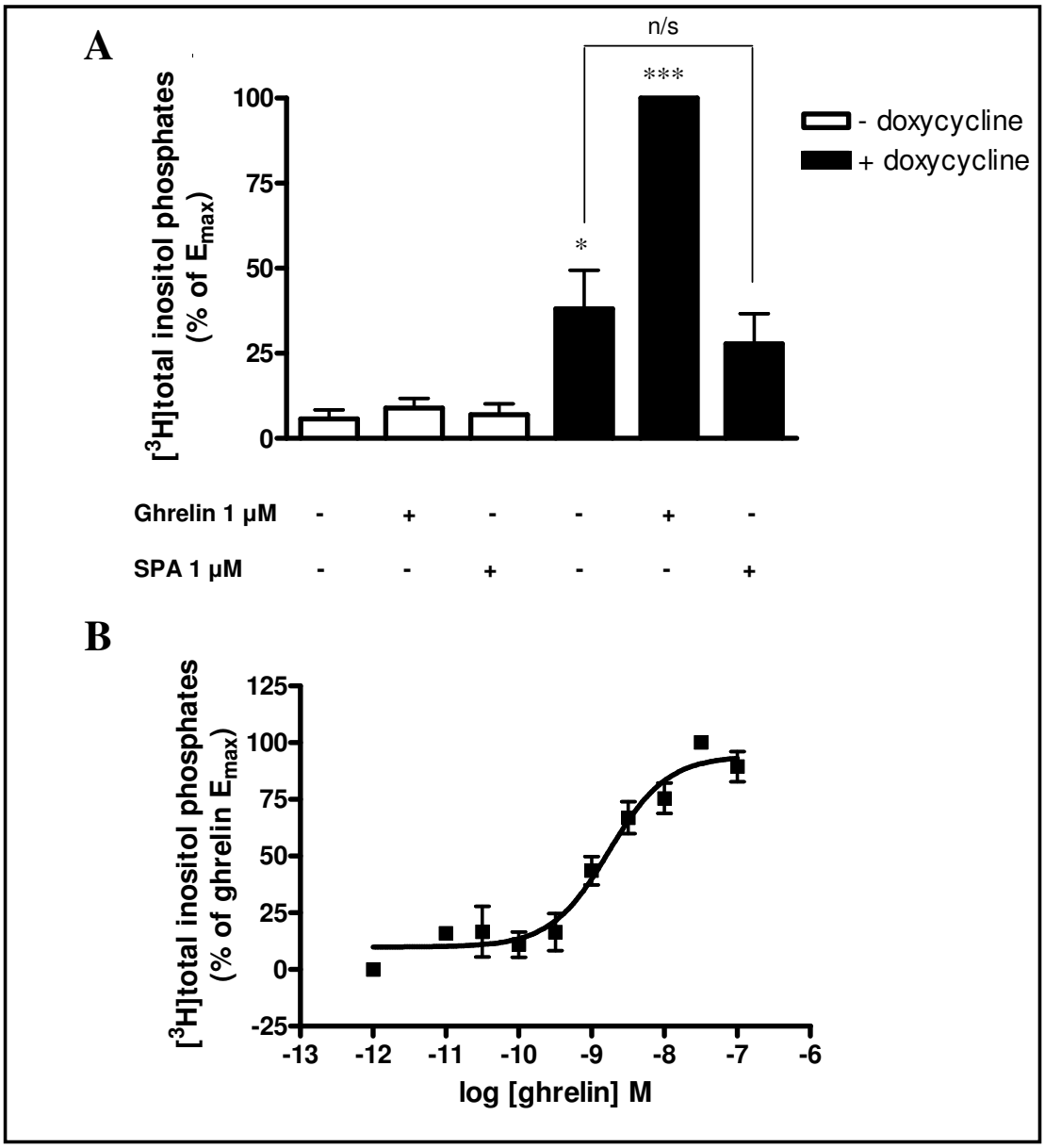

Figure 6-12 - Response of GRLN-R-I134T to ghrelin and SPA as measured using an inositol phosphate accumulation assay. Cells were incubated with 1 µCi/mL [^{3}H]*myo*-inositol and receptor expression induced as required. Prior to the assay, media was replaced with Krebs-Ringer buffer supplemented with 10 mM lithium chloride to block the breakdown of inositol phosphates into *myo*-inositol. **A**. Cells were stimulated with 1 µM ghrelin or 1 µM SPA for 30 min (37 °C), as indicated. White bars indicate the responses obtained in the absence of receptor expression, whilst black bars indicate the responses obtained upon expression of GRLN-R-I134T. After ligand-stimulation cells were lysed with perchloric acid and [^{3}H]total inositol phosphates isolated from the mixture using ion-exchange chromatography. The amount of [^{3}H]total inositol phosphates accumulated were measured using liquid scintillation spectrometry (120 s/sample).Data points represent the mean ± S.E.M of three independent experiments performed in duplicate. ***$p<0.001$; * $p<0.05$, as measured using a one-way ANOVA with Tukey's multiple comparison test. **B**. A concentration-response curve to ghrelin was generated in induced GRLN-R-I134T cells. Data points represent the mean ± S.E.M of three independent experiments performed in duplicate.

the GRLN-R-I134T was constitutively active, and could signal in response to ghrelin.

6.2.10 Signalling of GRLN-R-I134T through $G_{\alpha q/11}$ and $G_{\alpha i/o}$ pathways

As the wild-type GRLN-R was shown to signal through the $G_{\alpha q/11}$ and $G_{\alpha i/o}$ pathways, the signaling of the GRLN-R-I134T through these pathways was tested using [^{35}S]GTPγS binding assays. As the I134T mutation has been shown to ablate the signaling of the GRLN-R to ghrelin (Liu et al., 2007), the ability of the GRLN-R-I134T to signal to the growth hormone secretagogues was also tested.

The ability of the GRLN-R-I134T to couple to $G_{\alpha q/11}$ can be shown as an increase in inositol phosphate accumulation in response to ghrelin. To assess whether a maximally effective concentration of either ghrelin or the growth hormone secretagogues could increase [^{35}S]GTPγS binding via activation of $G_{\alpha q}$, a [^{35}S]GTPγS binding assay with immunoprecipitation step was used. Expression of GRLN-R-I134T was induced by the addition of doxycycline to GRLN-R-I134T cells transfected with $G_{\alpha q}$; subsequently

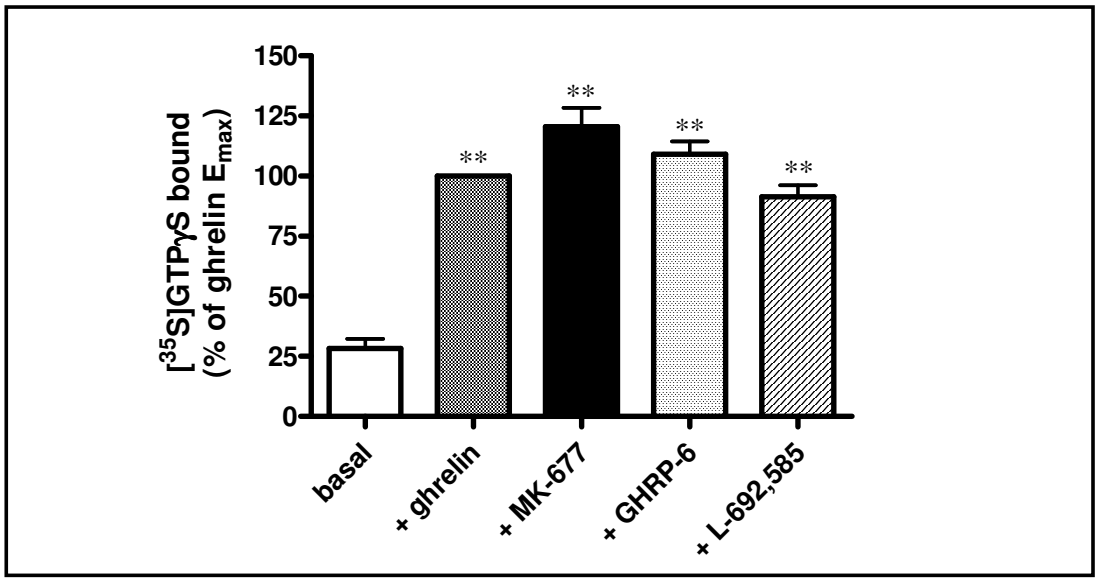

Figure 6-13 - Testing the ability of ghrelin and the growth hormone secretagogues to evoke a [^{35}S]GTPγS response in GRLN-R-I134T cells transfected to express $G_{\alpha q}$. GRLN-R-I134T cells were transfected to express $G_{\alpha q}$ and receptor expression was induced by the addition of 6 ng/mL doxycycline (16 h). Membranes were prepared and tested for their ability to increase [^{35}S]GTPγS binding in response to stimulation with 1 μM ghrelin, MK-677, GHRP-6 or L-692,585 (30 min; 37 °C). $G_{\alpha q}$ was immunoprecipitated from the reaction mixture using anti-$G_{\alpha q}$ antiserum and bound [^{35}S]GTPγS measured using liquid scintillation spectrometry (5 min/sample). Data points represent the mean ± S.E.M of three independent experiments performed in triplicate. **p<0.01 when compared to basal using a one-way ANOVA with Dunnett's post-hoc test.

membranes were prepared and stimulated with 1 μM ghrelin, GHRP-6, L-692,585 or MK-677 and inositol phosphate accumulation was measured (Figure 6-12). Membranes from GRLN-R-I134T cells showed significantly increased ($p<0.001$; one-way ANOVA with Dunnett's post-hoc test) [^{35}S]GTPγS binding in response to incubation with ghrelin and the growth hormone secretagogues, compared to membranes from unstimulated GRLN-R-I134T cells (Figure 6-13)

Next, to test whether the GRLN-R-I134T could activate the $G_{\alpha i/o}$ pathway, cells were transfected with $G_{\alpha o1}$ and the ability to increase [^{35}S]GTPγS binding measured (Figure 6-14). In the absence of receptor expression, treatment with maximally effective concentrations of ghrelin and the growth hormone secretagogues failed to significantly increase the levels of [^{35}S]GTPγS binding compared to the absence of ligand ($p=0.49$; one-way ANOVA). Upon induction of GRLN-R-I134T expression, ghrelin, GHRP-6, L-692,585 and MK-677 increased [^{35}S]GTPγS binding in a concentration-dependent manner. The potency and efficacy values of ghrelin and the growth hormone secretagogues are listed in (Table 6-2).

Table 6-2 - Potency and efficacy values of ligands acting at GRLN-R-I134T via $G_{\alpha i/o}$ activation, measured using a [^{35}S]GTPγS binding assay.

Ligand	pEC_{50} ± S.E.M	E_{max} ± S.E.M
Ghrelin	9.72 ± 0.18	89.6 ± 5.1
MK-677	9.77 ± 0.22	147.2 ± 10.3
GHRP-6	7.63 ± 0.17	145.3 ± 8.5
L-692,585	7.37 ± 0.34	150.4 ± 20.8

Data is expressed as the mean ± S.E.M of two (MK-677, GHRP-6, L-692,585) or three (ghrelin) individual experiments performed in triplicate. E_{max} is displayed as the maximum response of ligand expressed as the percentage of the maximum response achieved by ghrelin.

The potency values obtained for ghrelin acting at the GRLN-R-I134T were compared to ghrelin acting at the wild-type GRLN-R (obtained in Chapter 4; Table 4-1) with an unpaired, two-tailed t-test. This showed there was no significant difference in the potency of ghrelin acting at the wild-type GRLN-R and the GRLN-R-I134T ($p=0.09$). The potencies and efficacies of the growth hormone secretagogues acting at the GRLN-R-I134T appeared to be similar to those measured at the wild-type GRLN-R (obtained in Chapter 4; Table 4-1), however, due to the number of experiments performed ($n=2$) statistical tests could not be used to compare the data.

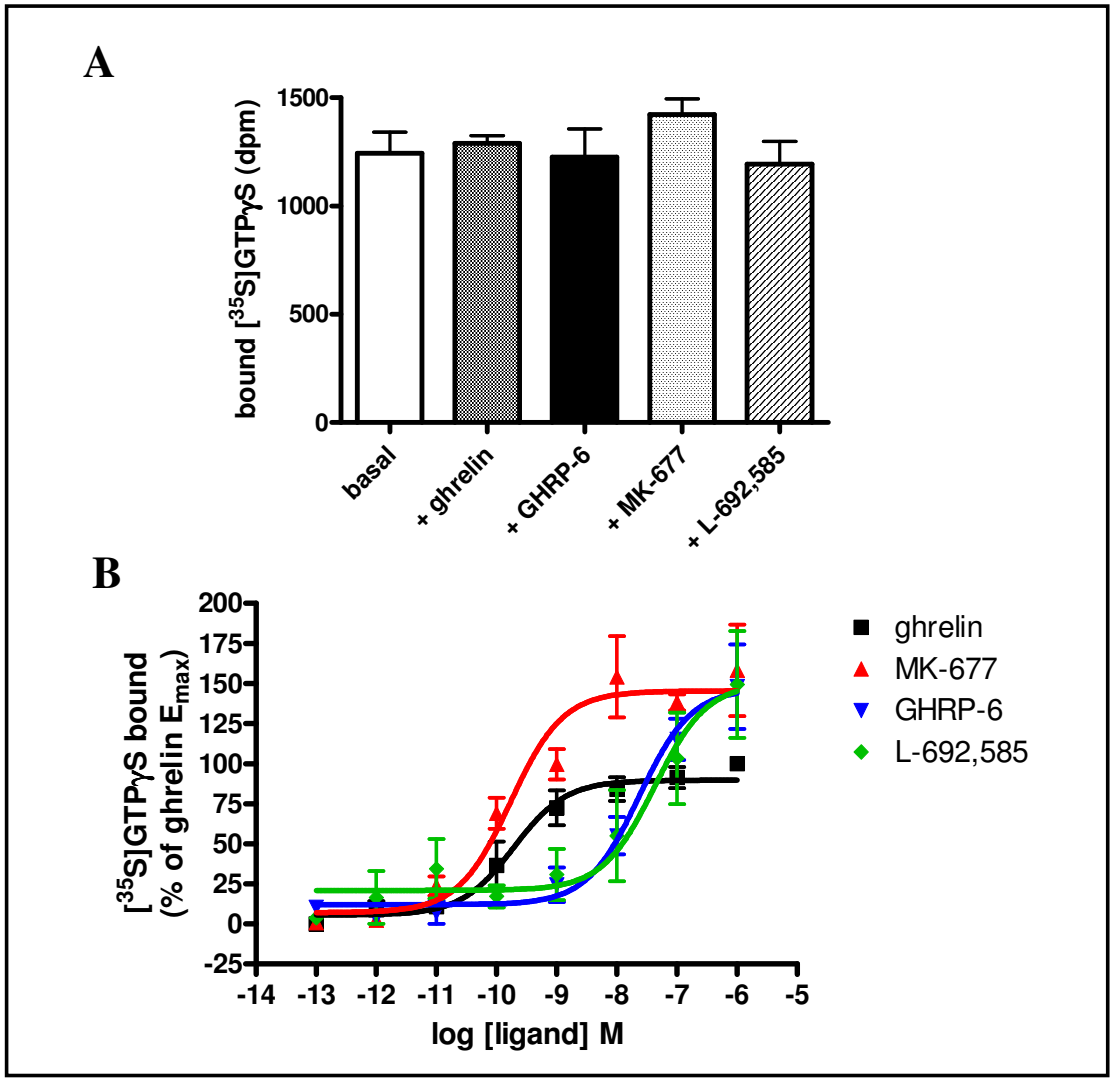

Figure 6-14 - Response of the GRLN-R-I134T via activation of the $G_{\alpha i/o}$ pathway as measured using a [^{35}S]GTPγS binding assay. A. Membranes prepared from uninduced GRLN-R-I134T cells transfected with $G_{\alpha o1}$ were tested for their ability to increase levels of [^{35}S]GTPγS binding in response to 1 µM ghrelin, GHRP-6, L-692,585 and MK-677 (20 min; 30 °C). G protein-bound [^{35}S]GTPγS was separated from the reaction mixture by rapid filtration through GF/C filters soaked in 1 x PBS and bound [^{35}S]GTPγS measured (for 5 min) using liquid-scintillation spectrometry. Data points represent the mean ± S.E.M of three independent experiments performed in triplicate. **B.** Concentration-response curves to ghrelin, GHRP-6, MK-677 and L-692,585 were generated in membranes prepared from GRLN-R-I134T cells transfected with $G_{\alpha o1}$, in which receptor expression had been induced by treatment with doxycycline (6 ng/mL; 16 h). Data points represent the mean ± S.E.M of three independent (for ghrelin) or two independent experiments (for GHRP-6, MK-677 and L-692,585) performed in triplicate.

6.2.11 Effect of ghrelin and the growth hormone secretagogues on the localisation of GRLN-R-I134T and GRLN-R-A204E

Finally, as both the GRLN-R-A204E and the GRLN-R-I134T responded to ghrelin the effect of ghrelin and the growth hormone secretagogues on the localisation of the receptors was tested. In Chapter 4, epifluorescence microscopy showed that the GRLN-R appeared to internalise upon treatment with maximally effective concentrations of ghrelin and the growth hormone secretagogues. In contrast, treatment with the inverse agonist SPA was shown to trap the GRLN-R at the plasma membrane leading to a loss of punctate vesicles within intracellular regions of the cells.

In the absence of ligand, GRLN-R-A204E was found inside the cell to a large extent, but the receptor also showed overlap with the plasma membrane marker wheat germ agglutinin (Figure 6-15). The addition of maximally effective concentrations of ghrelin, GHRP-6, MK-677, or L-692,585 (1 μM; 30 min) shifted the localisation of the receptor to punctuate vesicles within the cell (Figure 6-15). Quantification of the images showed that the formation of vesicles was accompanied by a significant decrease of the eCFP fluorescence intensity at the plasma membrane for ghrelin and all growth hormone secretagogues ($p<0.01$; one-way ANOVA with Dunnett's post-hoc test) (Figure 6-16). Surprisingly, the addition of the inverse agonist SPA (1 μM; 16 h) also decreased the amount of cell surface receptors ($p<0.05$), although this was not accompanied by the formation of intracellular vesicles.

In the GRLN-R-I134T cells, the addition of maximally effective concentrations of ghrelin and the growth hormone secretagogues also increased receptor internalisation from the plasma membrane into large punctuate vesicles within the cell (Figure 6-16). Quantification of the fluorescent signals showed that there was no significant decrease in plasma membrane-localised receptors upon treatment with ghrelin or the growth hormone secretagogues compared to the basal condition (Figure 6-17). The addition of SPA caused a significant ($p<0.01$) redistribution of the GRLN-R-I134T to plasma membrane, indicating both that SPA binds to the GRLN-R-I134T and that it acts as an inverse agonist, increasing the proportion of GRLN-R-I134T localised at the plasma membrane.

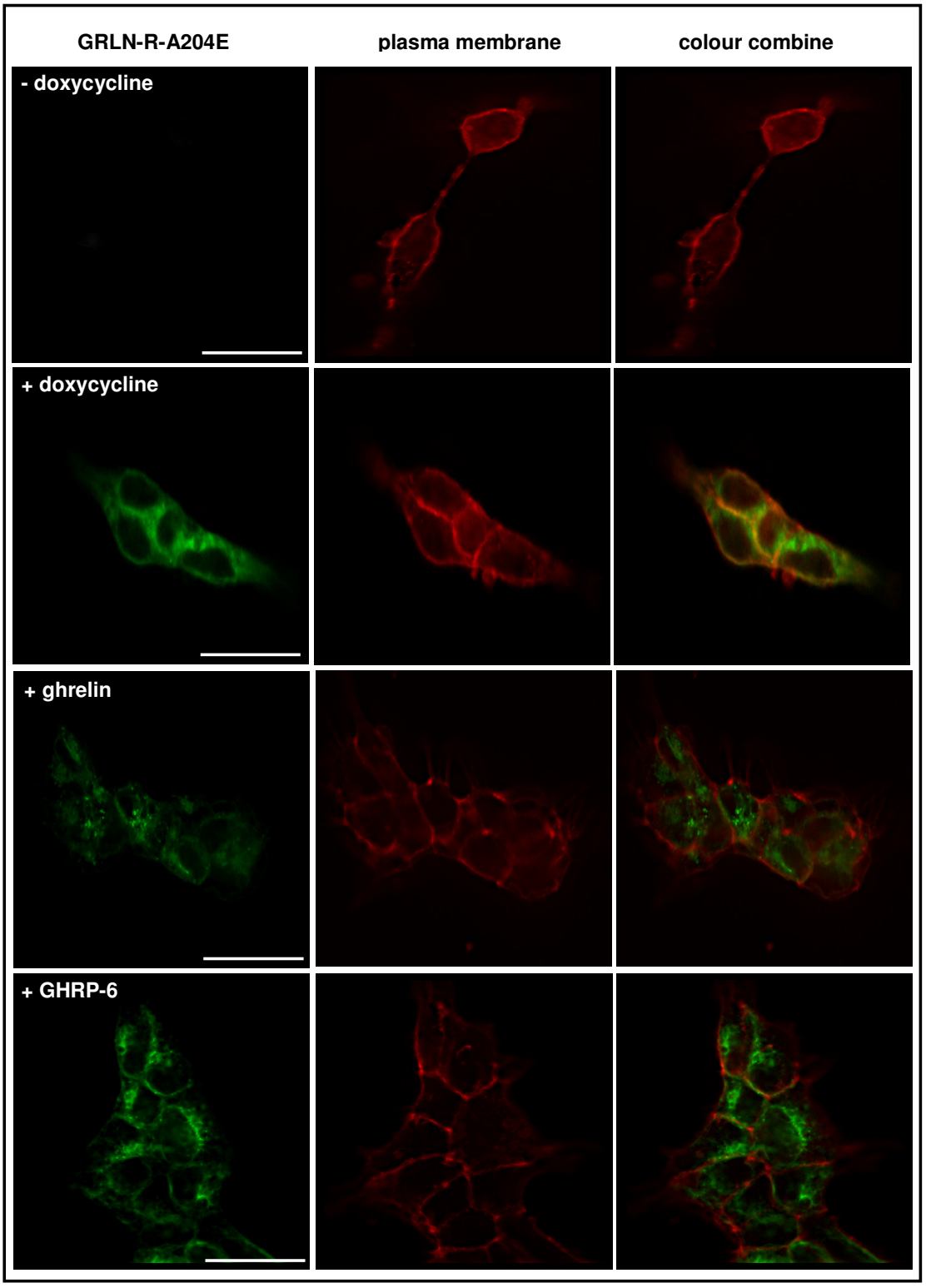

Figure 6-15 - Localisation of GRLN-R-A204E after treatment with ghrelin, GHRP-6, L-692,585, MK-677 or SPA (cont'd overleaf). Receptor was induced as required and treated with a maximally effective concentration of agonist (1 µM; 30 min for ghrelin, GHRP-6, L-692,585 and MK-677 and 1 µM for 16 h for SPA; 37 °C). Cells were formaldehyde-fixed before the plasma membrane was stained with WGA-Alexa Fluor®-594 conjugate. Cells were mounted onto coverslips, eCFP visualized using 436/12 nm light and the WGA-Alexa Fluor®-594 conjugate visualized at 575/12 nm. The images of GRLN-R-A204E (pseudo-coloured green) and the plasma membrane (red) were aligned (colour combine column) with overlapping regions shown as a yellow colour. Scale bar width represents 20 µm.

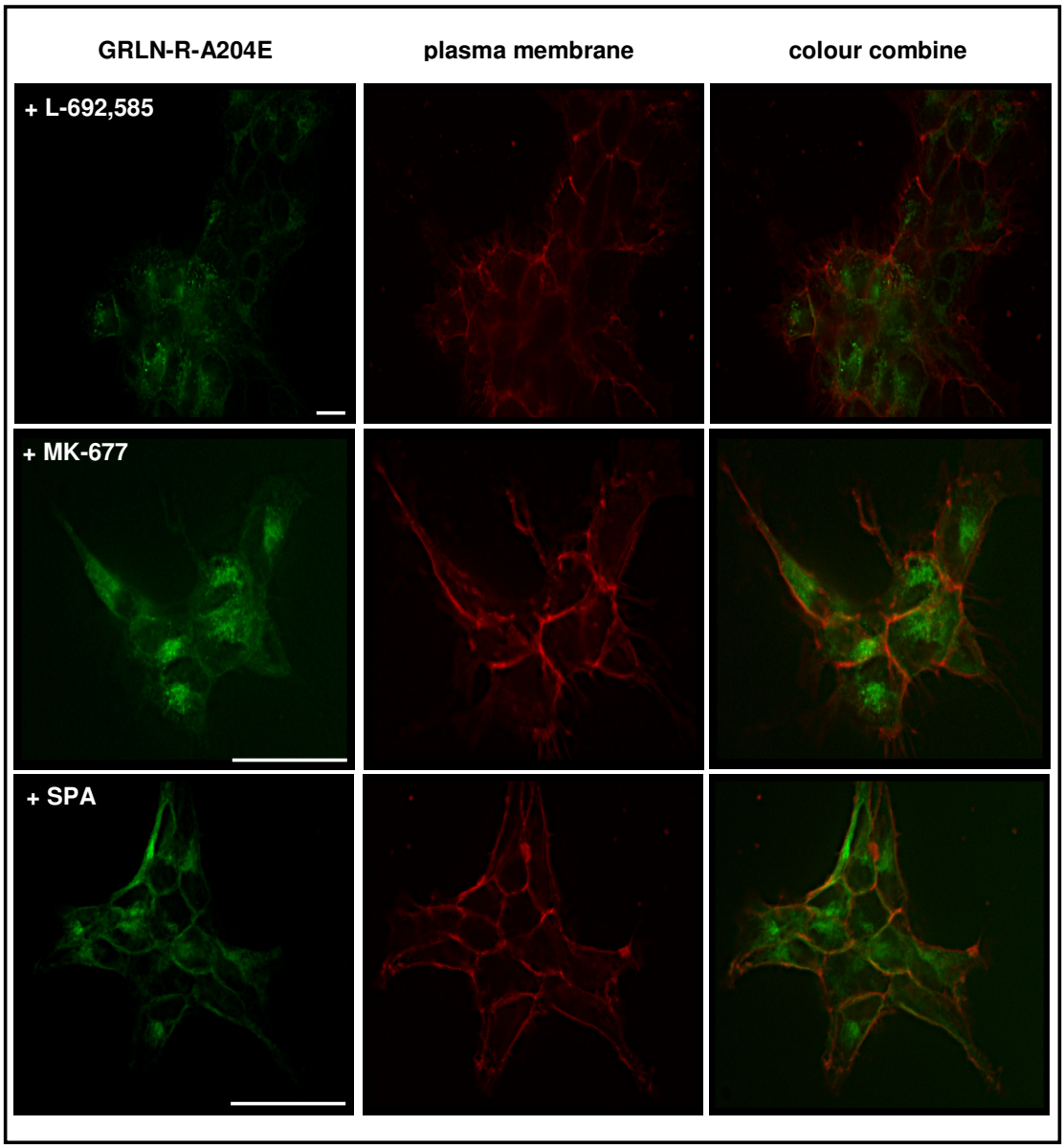

Figure 6-15 (cont'd) - Localisation of GRLN-R-A204E after treatment with ghrelin, GHRP-6, L-692,585, MK-677 or SPA. Receptor was induced as required and treated with a maximally effective concentration of agonist (1 μM; 30 min for ghrelin, GHRP-6, L-692,585 and MK-677 and 1 μM for 16 h for SPA; 37 °C). Cells were formaldehyde-fixed before the plasma membrane was stained with WGA-Alexa Fluor®-594 conjugate. Cells were mounted onto coverslips, eCFP visualized using 436/12 nm light and the WGA-Alexa Fluor®-594 conjugate visualized at 575/12 nm. The images of GRLN-R-A204E (pseudo-coloured green) and the plasma membrane (red) were aligned (colour combine column) with overlapping regions shown as a yellow colour. Scale bar width represents 20 μm.

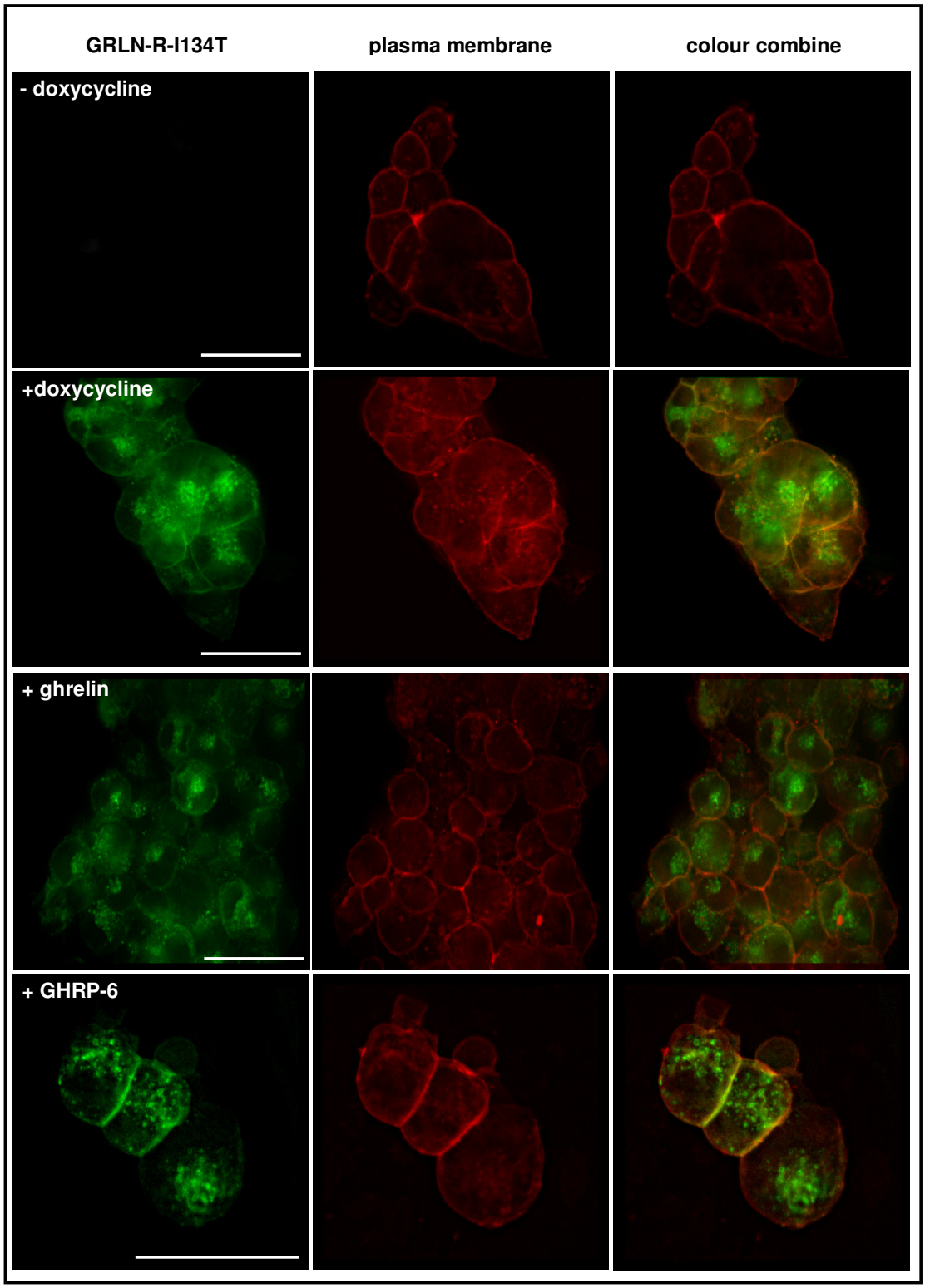

Figure 6-16 - Localisation of GRLN-R-I134T after treatment with ghrelin, GHRP-6, L-692,585, MK-677 or SPA (cont'd overleaf). Receptor was induced as required and treated with a maximally effective concentration of agonist (1 µM; 30 min for ghrelin, GHRP-6, L-692,585 and MK-677 and 1 µM for 16 h for SPA; 37 °C). Cells were formaldehyde-fixed before the plasma membrane was stained with WGA-Alexa Fluor®-594 conjugate. Cells were mounted onto coverslips, eCFP visualized using 436/12 nm light and the WGA-Alexa Fluor®-594 conjugate visualized at 575/12 nm. The images of GRLN-R-I134T (pseudo-coloured green) and the plasma membrane (red) were aligned (colour combine column) with overlapping regions shown as a yellow colour. Scale bar width represents 20 µm.

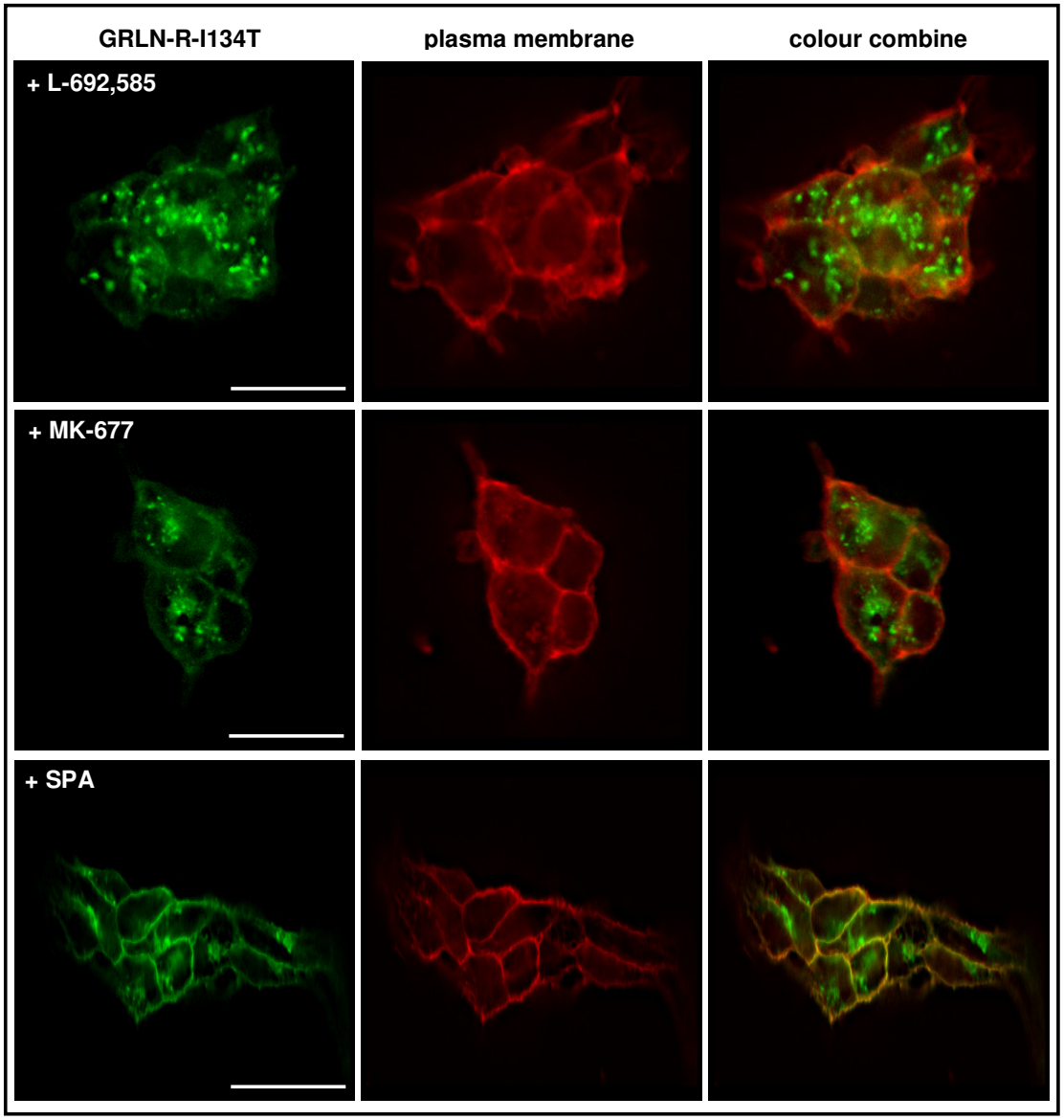

Figure 6-16 (cont'd) – Localisation of GRLN-R-I134T after treatment with ghrelin, GHRP-6, L-692,585, MK-677 or SPA. Receptor was induced as required and treated with a maximally effective concentration of agonist (1 μM; 30 min for ghrelin, GHRP-6, L-692,585 and MK-677 and 1 μM for 16 h for SPA; 37 °C). Cells were formaldehyde-fixed before the plasma membrane was stained with WGA-Alexa Fluor®-594 conjugate. Cells were mounted onto coverslips, eCFP visualized using 436/12 nm light and the WGA-Alexa Fluor®-594 conjugate visualized at 575/12 nm. The images of GRLN-R-I134T (pseudo-coloured green) and the plasma membrane (red) were aligned (colour combine column) with overlapping regions shown as a yellow colour. Scale bar width represents 20 μm.

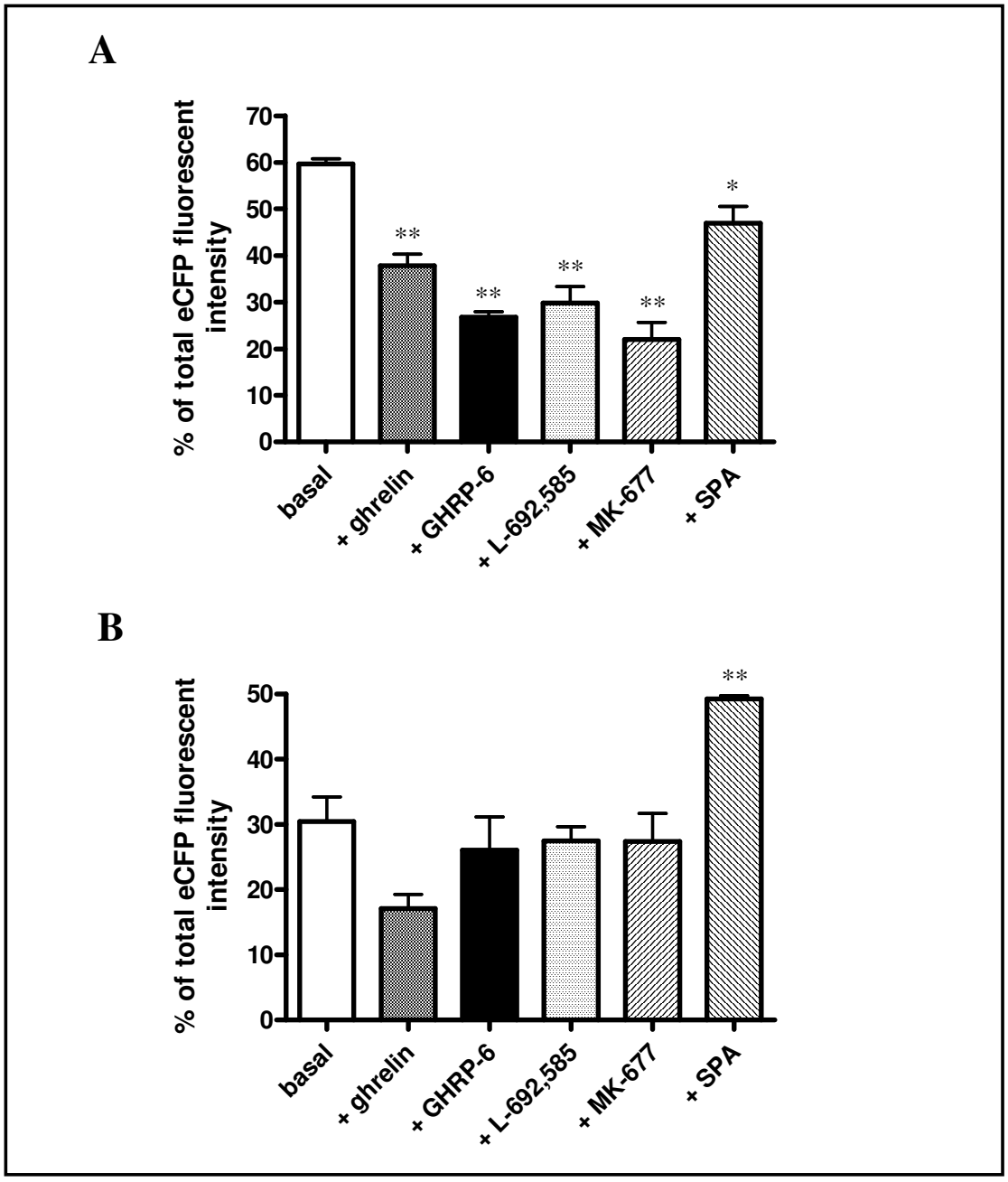

Figure 6-17 - Proportion of GRLN-R-A204E and GRLN-R-I134T detected at the plasma membrane after treatment with ghrelin, the growth hormone secretagogues or the inverse agonist SPA. Autoquant software was used to determine the proportion of total eCFP fluorescence located at the plasma membrane after treatment with 1 μM ghrelin, GHRP-6, L-692,595 or MK-677 for 30 min or 1 μM SPA for 16 h (37 °C). Data points represent the mean ± S.E.M of the quantification of **A.** three GRLN-R-A204E cells or **B.** three GRLN-R-I134T cells. *p<0.05, **p<0.001 when compared to basal using a one-way ANOVA with Dunnett's post-hoc test.

6.3 Discussion

Mutations within genes encoding GPCRs may affect receptor function and, for the GRLN-R, two missense mutations (A204E and F279L) have been associated with the development of a short-stature/obese phenotype *in vivo* (Pantel et al., 2006; Wang et al., 2004). The aim of this study was to further investigate two missense mutations of the GRLN-R, A204E and I134T, which have been reported to have altered pharmacology.

To aid studies into the effects of missense mutations on the function of the GRLN-R, receptors were engineered to contain either the A204E or I134T missense mutations and were subsequently tagged at the N-terminus with a HA tag and at the C-terminus with eCFP. In Chapter 4 the addition of an N-terminal epitope tag (VSV-G) and a C-terminal eCFP tag to the GRLN-R was investigated and shown to have no effect on the pharmacology and localisation of the receptor. The addition of a HA tag at the N-terminus of the GRLN-R has previously been used to study the pharmacology of the wild-type GRLN-R (Liu et al., 2007; Pantel et al., 2006). Importantly, these studies showed that the addition of the HA tag to the wild-type receptor did not affect the constitutive activity of the receptor (Pantel et al., 2006). Furthermore, the affinity of ghrelin for the GRLN-R was similar to the affinity values obtained for the untagged receptor and similar to the affinity values reported by Holst et al. (2005).

Using the HA-GRLN-R-A204E-eCFP and the HA-GRLN-R-I134T-eCFP constructs two inducible cell lines were created which proved to be extremely useful for investigating the effect of the A204E and I134T mutations on the pharmacology and localisation of the GRLN-R.

6.3.1 The GRLN-R-A204E is partially ER-retained

Whilst studies investigating the affects of the A204E mutation agree that this mutation ablates the constitutive activity of the GRLN-R (Holliday et al., 2007; Liu et al., 2007; Pantel et al., 2006), conflicting reports have been published about the effect of the A204E mutation on receptor localisation. If the A204E mutation only interferes with constitutive activity of the GRLN-R, the receptor would be expected to be plasma membrane delineated, as constitutive activity of the receptor has been shown to be a key requirement for constitutive endocytosis (Holliday et al., 2007). Indeed the distribution of the GRLN-R-A204E has been shown to be plasma membrane delineated in a study by Holliday et al. (2007). However, in this study, epifluorescence microscopy data showed that the GRLN-

R-A204E was trapped within the cell, a finding in agreement with that of Pantel et al. (2006). Furthermore immunoblotting of lysates prepared from GRLN-R-A204E cells revealed that, in contrast to the GRLN-R, the largest proportion of the GRLN-R-A204E existed as immature, high mannose glycosylated receptor – a glycosylation state indicative of ER retention of a GPCR (Sadeghi et al., 1997). Co-immunoprecipitation assays also revealed that, compared to the amount of protein recovered, the GRLN-R-A204E interacted more stably with the ER-resident molecular chaperone calnexin than the GRLN-R. Together, these experiments demonstrated that a large fraction of GRLN-A204E was being retained within the ER.

The insertion or removal of charged amino acids by missense mutations are well known to affect protein structure and folding. For example, a single change in the net charge of the gonadotrophin releasing hormone receptor (the alteration of a alanine residue, A129$^{3.40}$, to an aspartic acid residue) is sufficient to favour ER retention of the receptor, decreasing the amount of receptor available at the cell membrane, leading to the disorder hypergonadotropic hypogonadism (Conn et al., 2007). Similarly, the V206D mutation in the type 2 vasopressin receptor has been reported to induce ER retention of the receptor (Robben et al., 2006), leading to the heritable disorder nephrogenic *diabetes insipidus*. In line with these studies, the A204E mutation in the GRLN-R that introduces a negatively charged glutamic acid residue in place of a neutral alanine residue causes ER retention of the GRLN-R-A204E. However, in this study, immunoblotting experiments revealed that a small proportion of receptor was complex glycosylated as it was insensitive to Endo H, but could be digested by PNGaseF. Furthermore, ghrelin was shown to increase the level of inositol phosphate accumulation in a concentration-dependent manner in an intact cell assay; as ghrelin is membrane-impermeable the observed increase in inositol phosphate levels is most likely derived from GRLN-R-A204E localised at the cell-surface membrane. Therefore, even though a large proportion of the GRLN-R-A204E was ER retained, these data clearly demonstrated that some mature GRLN-R-A204E was expressed at the cell-surface; a finding in agreement with other groups who have used intact cell inositol phosphate accumulation assays to measure activation of the GRLN-R-A204E in response to ghrelin (Pantel et al., 2006; Liu et al., 2007; Holliday et al., 2007).

The difference between the localisation of the GRLN-R-A204E in this study compared to that of Holliday et al. (2007) may be due to the methods used to detect the receptor. Holliday et al. (2007) used immunocytochemistry with an antibody raised against a FLAG epitope tag (added to the N-terminus of the GRLN-R-A204E) to detect the receptor expressed in (permeabilised) HEK293 cells, perhaps the structure of the ER-retained

receptor was altered in such a way that the antibody was unable to recognise the FLAG tag.

6.3.2 Effect of the A204E mutation on the cell-surface expression of the GRLN-R-A204E

Interestingly, although data from this experiment clearly showed that the GRLN-R-A204E was partially ER retained, the data from the epifluorescence microscopy experiments seemed to contradict this, showing that the GRLN-R-A204E was expressed at the cell-surface at a higher level than the wild-type GRLN-R (measured in Chapter 4; Figure 4-8). Although it could be interpreted that the A204E mutation increased the level of surface expression of the GRLN-R-A204E compared to the wild-type receptor, quantification of epifluorescence microscopy images is a crude measurement of cell-surface expression and contradicts the findings from the more sensitive binding and immunoblotting experiments. Indeed the radioligand binding experiments demonstrated that specific binding of [His[^{125}I]]-ghrelin to the GRLN-R-A204E was significantly lower than for the wild-type GRLN-R; whilst immunoblotting experiments showed that the majority of the GRLN-R-A204E existed as an immature form.

6.3.3 The A204E mutation ablates the constitutive activity of the GRLN-R

The A204E mutation has been described to ablate the constitutive activity of the GRLN-R-A204E (Holliday et al., 2007; Liu et al., 2007; Pantel et al., 2006). Indeed, in this study the GRLN-R-A204E appeared to lack any measurable constitutive activity. The inositol phosphate accumulation studies (shown in Figure 6-9) revealed that, in contrast to the wild-type GRLN-R, induction of the expression of the GRLN-R-A204E did not alter the level of inositol phosphates observed compared to cells lacking receptor expression. In addition, incubation of GRLN-R-A204E cells with the inverse agonist SPA had no effect on the accumulation of inositol phosphates measured in the absence of ligand treatment. However, the results of the epifluorescence microscopy experiments appeared to show an inconsistency with the other data.

In a steady-state situation, the wild-type GRLN-R is localised at the plasma membrane and in intracellular vesicles, whilst treatment with the inverse agonist SPA promotes its accumulation at the plasma membrane; likely through blocking the constitutive activity, and thereby constitutive internalisation, of the GRLN-R (Holliday et al., 2007). As treatment of the GRLN-R-A204E cells with SPA had no effect on signalling of the

receptor in the inositol phosphate accumulation experiments, SPA was predicted to have little effect on the localisation of the GRLN-R-A204E. However, quantification of the epifluorescence microscopy experiments (shown in Figure 6-17) showed treatment with SPA actually led to a significant reduction in the cell surface expression of the GRLN-R-A204E.

The serotonin 5-HT_{2C} receptor is an example of a constitutively active receptor whose expression can be down-regulated, both *in vitro* and *in vivo* by chronic inverse agonist exposure (>24 h) (Devlin et al., 2004). In this study the GRLN-R-A204E was incubated with inverse agonist for 16 h. Although 16 h incubation with SPA was shown to increase the level of both wild-type GRLN-R and GRLN-R-I134T compared to basal conditions, this was likely due to retarding constitutive receptor activity which would trap the receptor at the plasma membrane. Thus the down-regulation of the GRLN-R may only become apparent in a situation where the receptor lacks any constitutive activity. However, further studies are needed to investigate this, as 16 h of inverse agonist exposure may be too short a time for receptor down-regulation to occur.

6.3.4 The GRLN-R-A204E responds to ghrelin with a potency similar to that of the GRLN-R

As many peptide ligands tend to exploit residues in the extracellular end of TM helices and in the extracellular domains upon receptor binding (Schwartz et al., 2006), the A204E mutation could have affected the binding of ghrelin or any of the growth hormone secretagogues. Indeed, another mutation in the second extracellular loop of the GRLN-R (E197G) was found to decrease the potency of ghrelin (Holst et al., 2009). However, in this study and in keeping with the findings from other studies (Liu et al., 2007; Pantel et al., 2006), the A204E mutation appeared to have no effect on the signalling of the receptor to ghrelin. The GRLN-R-A204E responded to ghrelin in the inositol phosphate accumulation assay with a potency (pEC_{50}) that was not statistically different to that obtained by the wild-type GRLN-R. Furthermore, the A204E mutation had no effect on the promiscuous coupling of the GRLN-R, as in GRLN-R-A204E cells the potency of ghrelin in the $G_{\alpha i/o}$ pathway was also similar to that obtained for the GRLN-R. In addition, maximally effective concentrations of ghrelin and the growth hormone secretagogues were all able to internalise the GRLN-R-A204E suggesting that the receptor could also bind and signal to GHRP-6, MK-677 and L-692,585 at the cell surface.

The effect of the A204E mutation on the structure of the GRLN-R has not been determined in this or any other study. However, another missense mutation of the GRLN-R, the F279L mutation in TMVI, has also been reported to affect the constitutive activity of the GRLN-R (Liu et al., 2007; Holst et al., 2004). The F279L mutation occurs in a cluster of amino acids in TMVI and TMVII that were shown in mutagenesis studies to be critical in maintaining the constitutive activity of the GRLN-R (Holst et al., 2004). Although the A204E mutation is distinct from this region and is distinct from residues known to participate in the ghrelin-binding pocket (Feighner et al., 1998, Holst et al., 2009) the data presented here suggest the A204E mutation may alter receptor folding, disrupting the structural domain responsible for the constitutive activity of the receptor. The concept of altered receptor folding is also supported by the findings that most of the GRLN-R-A204E exists as an immature form and that the receptor is partially ER-retained.

A recent study on the free fatty acid 1 receptor has shown that a glutamic acid to alanine mutation at two positions in ECL2 results in constitutive activation of the receptor due to the disruption of a ionic-lock formed between the two glutamic acid residues and two arginine residues located in the TM domains (TMV and TMVII) (Sum et al., 2009). Thus the A204E mutation in the GRLN-R may result in the ability of the glutamic acid to form an ionic-lock with residues in the TM domains, ablating the constitutive activity of the receptor, although clearly this would need to be further explored.

Interestingly, in the melanocortin-4 receptor, mutations within the extracellular domain of the receptor, which selectively impair the constitutive activity of the receptor, have been linked to the development of obesity (Srinivasan et al., 2004). Although it is tempting to link this lack of constitutive activity of the GRLN-R-A204E to the obese phenotype observed in subjects harbouring the A204E mutation, the results from this study demonstrate that the GRLN-R-A204E undergoes a higher degree of ER retention than the wild-type GRLN-R. Thus, the observed phenotype may be solely due to lower cell-surface expression of GRLN-R-A204E. As the expression of the GRLN-R has been reported to be extremely low *in vivo* (Katugampola et al., 2001), ER retention of the GRLN-R-A204E could potentially have a severe impact on receptor signalling. The short/obese phenotype has also been observed in subjects displaying the F279L mutation (Wang et al., 2004). In one study the F279L mutation was shown to reduce cell-surface expression of the GRLN-R, whilst reducing, but not completely inhibiting, the constitutive activity of the mutated GRLN-R (Liu et al., 2007). Thus, the decreased cell surface expression of the F279L or A204E mutants, rather than their reduced/lack of constitutive activity, may be the main

determinant for the development of the short/obese phenotype observed *in vivo*. However, this question requires further investigation.

6.3.5 The GRLN-R-I134T is localised both at the cell-surface membrane and in vesicles within the cell

In contrast to the A204E mutation, the I134T mutation has been reported to ablate the response of the GRLN-R to ghrelin whilst having no effect on the constitutive activity of the receptor (Liu et al., 2007). As the growth hormone secretagogues have been shown to have overlapping but not identical binding sites on the GRLN-R (Feighner et al., 1998; Holst et al., 2009), this study sought to investigate the effect of the growth hormone secretagogues on the I134T mutant. The I134T mutation causes a change of a non-polar, neutral isoleucine residue to a polar, neutral threonine residue. Therefore, although the mutation is within the structurally important transmembrane domains, it may not have such a pronounced effect on the GRLN-R as the A204E mutation.

In studies by Liu and colleagues (2007) the I134T mutation was demonstrated to decrease the cell-surface expression of the GRLN-R-I134T by around 50 %. In this study the co-immunoprecipitation experiments appeared to show that the GRLN-R-I134T indeed was expressed at a lower level than wild-type GRLN-R. In contrast, radioligand binding studies performed in membrane samples revealed that the specific binding of [His[^{125}I]]-ghrelin to GRLN-R-I134T was not significantly different to that of the GRLN-R, although the use of membrane samples means that, potentially, [His[^{125}I]]-ghrelin may also be binding to receptor expressed in the ER. However, immunoblotting experiments indicated that the largest proportion of receptor appeared to be mature, complex glycosylated protein, indicative of a localisation beyond the *cis*-Golgi complex. Moreover, epifluorescence microscopy data demonstrated that the GRLN-R-I134T was located both at the plasma membrane and in small vesicles within the cell, similar to the wild-type GRLN-R. However, studies by Liu et al. (2007) suggest that the I134T mutation may decrease the cell-surface expression of the GRLN-R-I134T, which needs to be confirmed by intact cell binding studies.

Interestingly, both the GRLN-R-I134T and the wild-type GRLN-R showed weak co-localisation with a marker of the ER. Moreover, co-immunoprecipitation studies showed that both the GRLN-R-I134T and the wild-type GRLN-R interact with calnexin, although this interaction was weaker compared to that observed for GRLN-R-A204E. Likely, these ER-localised GRLN-R and GRLN-R-I134T represent pools of newly-synthesised receptor

that are being folded in the ER. Alternatively, receptor overexpression may saturate the N-terminal glycosylation and/or protein folding system of the early secretory pathway, resulting in intracellular accumulation of these receptors. This could be investigated by assessing the interaction of these receptors with calnexin upon combined inhibition of protein synthesis (i.e. with cyclohexamide) and proteasomal degradation (i.e. using Bortezomib), which would discriminate between a protein that stayed retained within the ER and a protein that temporarily resides there whilst being folded.

6.3.6 The GRLN-R-I134T is constitutively active

Since the GRLN-R-I134T shows a similar localisation as the wild-type GRLN-R, and the vesicular distribution of the wild-type receptor was the result of its constitutive activity, the constitutive activity of the GRLN-R-I134T was assessed. Epifluorescence microscopy data demonstrated that the inverse agonist SPA caused a significant increase in the amount of GRLN-R-I134T located at the plasma membrane, a finding consistent with inhibition of the constitutive activity and hence, constitutive internalisation of the GRLN-R-I134T.

In inositol phosphate accumulation studies, expression of the GRLN-R-I134T increased accumulation of inositol phosphates, indicating that GRLN-R-I134T was constitutively active, at least in the $G_{\alpha q/11}$ pathway. Indeed the increase in inositol phosphate levels upon expression of the GRLN-R-I134T was similar to that observed upon expression of the wild-type GRLN-R. Interestingly, incubation of the cells with the inverse agonist SPA reduced inositol phosphate levels slightly, but did not cause a significant reduction to the level observed in the absence of GRLN-R-I134T expression. This suggested that, although the receptor was constitutively active, the I134T mutation might reduce the efficacy of SPA on the GRLN-R, perhaps by reducing the affinity of SPA for the receptor.

The results from the inositol phosphate and epifluorescence experiments are in agreement with the study by Liu and colleagues, who found that despite conserved inverse agonist function, both the efficacy and potency of SPA was reduced at the GRLN-R-I134T (Liu et al., 2007).

6.3.7 The GRLN-R-I134T is activated by ghrelin and growth hormone secretagogues

In contrast to previously published results (Liu et al., 2007), this study has shown that the GRLN-R-I134T responded to ghrelin in a concentration-dependent manner. In the inositol phosphate accumulation assay this yielded a response with a pEC_{50} of 8.76 ± 0.13, a

response not significantly different to ghrelin activating the wild-type receptor. Thus the GRLN-R-I134T, like the wild-type GRLN-R, was capable of activating more than one G protein family. Liu et al. (2007) used inositol phosphate experiments conducted in COS-7 cells to try to measure activation of the GRLN-R-I134T by ghrelin, however, in this study inositol phosphate accumulation experiments were performed in HEK293 cells, thus the discrepancies between the results of these studies could reflect differences in the folding of the GRLN-R-I134T in the two expression systems. Indeed, mutagenesis studies have identified several residues that are critical for binding of both ghrelin and the growth hormone secretagogues (Feighner et al., 1998; Holst et al., 2009). Although the residue $I134^{3.43}$ was not analysed in these studies, it appears that this residue is too deep within the TM domain to be critical for ligand binding, although it cannot be ruled out that alteration of this residue causes a disturbance in receptor structure.

In the $G_{\alpha i/o}$ pathway the growth hormone secretagogues all acted with potencies and efficacies at the GRLN-R-I134T that were not significantly different to that observed upon activation of the GRLN-R. Ghrelin and the growth hormone-secretagogues were also shown to be able to increase [^{35}S]GTPγS binding through activation of the $G_{\alpha q/11}$ pathway, but further studies are needed to test whether there were any significant changes in the potencies of the growth hormone secretagogues through this pathway. Further evidence that ghrelin and the growth hormone secretagogues bind GRLN-R-I134T came from epifluoresence microscopy experiments that showed GRLN-R-I134T was internalised upon addition of maximally effective concentrations of ghrelin, GHRP-6, L-692,585 and MK-677 into large intracellular vesicles. However, quantification of these images showed that receptor localisation at the plasma membrane upon agonist treatment was not significantly reduced compared to control cells. This suggests that for the GRLN-R-I134T, ghrelin and the growth hormone secretagogues have a reduced ability to internalise the receptor. However, as semi-quantification of epifluorescence data introduces relatively large errors (especially when there is so little receptor co-localised at the plasma membrane in basal conditions) whole-cell binding studies or cell surface biotinylation experiments will likely allow a more accurate quantification of receptor levels at the plasma membrane after treatment with ghrelin, the growth hormone secretagogues or SPA.

6.3.8 Classification of the A204E and I134T mutations

A classification scheme to define melanocortin-4 receptor inactivating mutations has been proposed in which receptors could be classed in one of five groups (Tao & Segaloff, 2003) according to their cell-biological and/or pharmacological effects. These are: defective

biosynthesis (class I); impaired trafficking to the cell surface (class II); defective ligand binding (class III); defective receptor activation (class IV) or no defects (class V). According to this system, the data from this study suggests that the A204E mutation leads to both class II and class IV defects, as the receptor is ER-retained (although its partial localisation at the plasma membrane makes it not a full class II mutant) and the constitutive activity of the receptor is ablated. SPA acting at the GRLN-R-I134T appears to be less efficacious in lowering accumulated levels of inositol phosphates upon expression of the GRLN-R-I134T compared to the wild-type GRLN-R. Thus the I134T mutation could cause a class III (defective ligand binding) and/or a class IV (defective receptor activation) mutation, as more studies are needed to determine whether ghrelin and SPA bind to the GRLN-R-I134T with the same affinity as the wild-type GRLN-R (which, if they did, would rule out a class III mutation).

6.4 Summary and conclusions

The results of this study have demonstrated for the first time that the A204E mutation causes partial ER retention of the GRLN-R. This may have detrimental effects *in vivo* as the reduced expression of the GRLN-R-A204E and its low level of plasma membrane localisation could have a serious impact on receptor function. Moreover, expression of mutant GPCRs may interfere with the cell surface expression of their corresponding wild-type counterparts through their association in the ER and intracellular entrapment of the resulting complex (Zhu and Wess, 1998; Brothers et al., 2004; Gehret et al., 2006).

In contrast to the A204E mutation, the I134T mutation does not cause ER-retention of the GRLN-R-I134T, but instead shows a subcellular localisation similar to the wild-type GRLN-R. However, the efficacy of the inverse agonist SPA appears reduced by the I134T mutation. Interestingly, in the melanocortin-4 receptor the S127L ($S^{3.30}$) mutation, a missense mutation linked to the development of obesity, decreases the potency of α-melanocyte-stimulating hormone some 30-fold but it has no effect on the constitutive activity of this receptor (Govaerts et al., 2005). Thus it will be interesting to see if the I134T mutation is linked to the development of disease *in vivo*.

7 Final discussion

Ghrelin and the GRLN-R have been shown to be involved in a number of physiological processes, such as: GH release, sleep regulation, energy homeostasis and appetite stimulation. This makes the ghrelin/GRLN-R system an attractive therapeutic target for the treatment of disorders such as cachexia, GH deficiency, obesity and ageing (i.e. by reversing the natural decline of GH which can lead to frailty and loss of independence). Although the GRLN-R has been suggested to couple to multiple G protein pathways ($G_{\alpha q/11}$, $G_{\alpha i/o,}$ $G_{\alpha s}$ and $G_{\alpha 12/13}$), little is known about the signalling of the GRLN-R to ghrelin and the growth hormone secretagogues in all but the $G_{\alpha q/11}$ pathway. The aim of this thesis was to further investigate the activation of the GRLN-R through the $G_{\alpha i/o}$ pathway.

7.1 Ligand-induced activation of the GRLN-R

In 2005 the growth hormone secretagogues GHRP-6 and L-692,429 were described to act as ago-allosteric modulators of the GRLN-R (Holst et al., 2005). Co-administration of the growth hormone secretagogues with ghrelin led to an increase in ghrelin's efficacy (as the growth hormone secretagogues acted as co-agonists), whilst L-692,429 and GHRP-6 acted either to increase or decrease (respectively) the potency of ghrelin (Holst et al., 2005). As the binding sites for ghrelin and the growth hormone secretagogues were known to overlap, to describe their results Holst and colleagues developed a necessarily complex model that relied on the GRLN-R existing as a homo-dimer (Holst et al., 2005). Ghrelin, due to its large size, was predicted to only bind to one protomer leaving the second protomer free to bind a growth hormone secretagogue. However, the study by Holst and colleagues was not designed to fully investigate allosteric regulation of the GRLN-R and thus did not examine any of the hallmarks of an allosteric interaction i.e. that the effect was saturable, probe-dependent or that the dissociation kinetics of a radiolabelled version of ghrelin was altered by co-administration with GHRP-6 or L-692,429. Nevertheless, up until now L-692,429 and GHRP-6 have been universally accepted as allosteric modulators of the GRLN-R (May et al., 2007; Leach et al., 2007; Langmead and Christopoulos, 2006).

In this thesis, the effects of co-administration of ghrelin with either GHRP-6, L-692,585 (a commercially available analogue of L-692,429) or MK-677 was measured through activation of the $G_{\alpha i/o}$ pathway. [^{35}S]GTPγS assays were chosen to study activation of the $G_{\alpha i/o}$ pathway to allow measurement of ligand efficacies in a system where fewer compounds would reach the maximum system response (Strange, 2008) and to avoid the

measurement of other G protein-dependent or independent pathways activated by the GRLN-R. Interestingly, in this system, GHRP-6, L-692,585 and MK-677 were shown not to be ago-allosteric modulators of the GRLN-R but simple orthosteric ligands.

Concentration-response curves to ghrelin in the presence of multiple, fixed concentrations of the growth hormone secretagogues led to an increase in the efficacy of ghrelin, most likely because each of the growth hormone secretagogues tested acted with a significantly higher efficacy than ghrelin i.e. they acted as super-agonists. In the reverse experiment (where concentration-response curves were generated to the growth hormone secretagogues in the presence of varying, fixed concentrations of ghrelin), ghrelin led to a concentration-dependent increase in the efficacy of the growth hormone secretagogues at low growth hormone secretagogues concentrations, whilst at high concentrations, ghrelin had no effect on efficacy.

In the presence of increasing ghrelin concentrations the growth hormone secretagogues showed a trend towards a decreased potency, but this could be due to ghrelin (which acts as a partial agonist in regards to the growth hormone secretagogues) competing with the growth hormone secretagogues for binding to the GRLN-R. Indeed, fitting the experimental data to a modified operational model of allosterism (originally described by Leach et al., 2007 and modified by Dr Christopher Langmead at GlaxoSmithKline), showed that the data was best fitted to a model which described a competitive, rather than an allosteric, interaction. Furthermore, binding studies demonstrated that the dissociation of [His[^{125}I]]-ghrelin from the GRLN-R was not altered by administration of the growth hormone secretagogues, thus demonstrating a lack of allosterism.

The model generated by Holst et al (2005) relied on the GRLN-R existing as a homo-dimer. In this study no attempt was made to determine whether the GRLN-R existed as a homo-dimer, although this has been previously described (Jiang et al., 2006; Leung et al., 2007). This opens up the possibility that the super-agonist effects of the growth hormone secretagogues could be due to their ability to bind to both protomers of a GRLN-R homo-dimer, creating a receptor structure which signals with higher efficacy than that produced by binding of ghrelin to one protomer of the dimer. To investigate this further an asymmetrical homo-dimer could be formed between a GRLN-R mutated at E124$^{3.33}$ to Q124$^{3.33}$ (a mutation which has been shown to ablate the binding of ghrelin and the growth hormone secretagogues; Feighner et al., 1998; Holst et al., 2009) and a GRLN-R mutated in the ERY sequence (i.e. at arginine R141$^{3.50}$) to inhibit G protein coupling. This would result in a receptor that could only signal to ghrelin and the growth hormone secretagogues

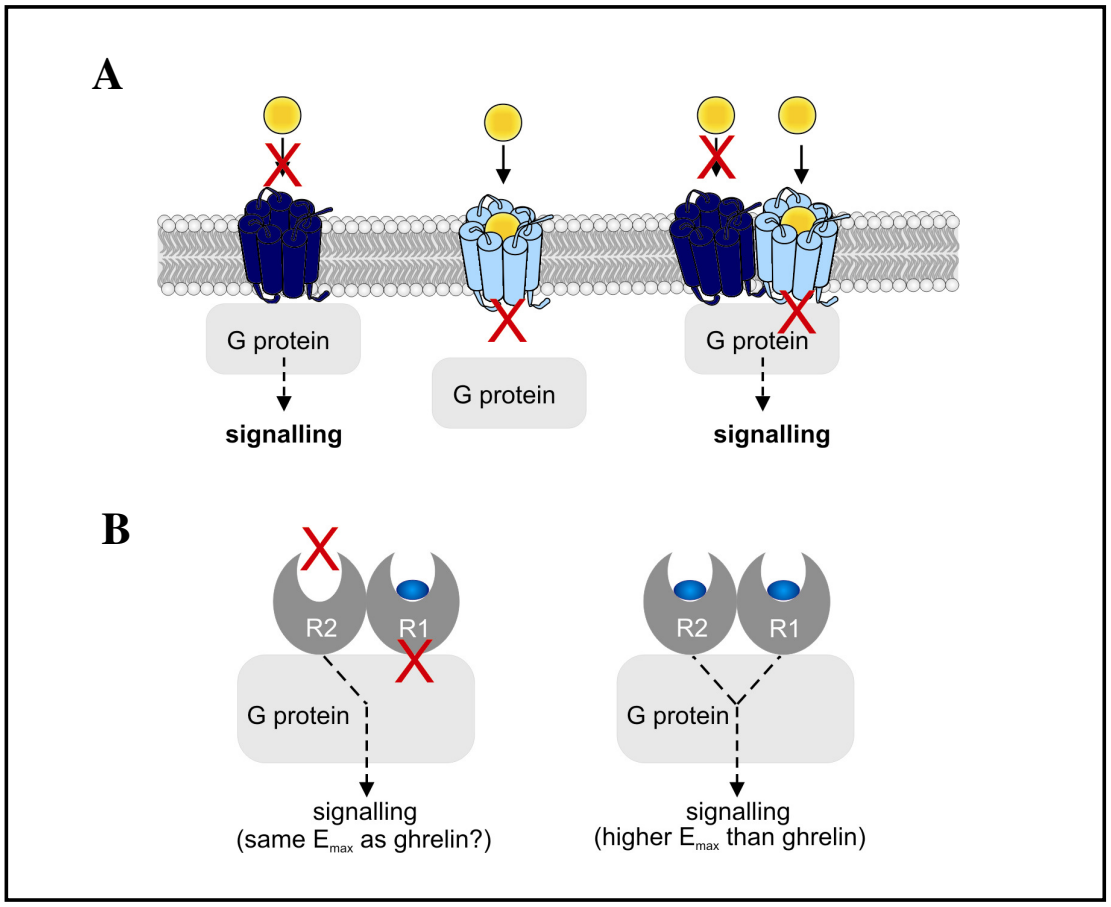

Figure 7-1 – Use of an asymmetrical homo-dimer to investigate the pharmacology of the GRLN-R. A. An asymmetrical homo-dimer could be created to investigate the pharmacology of the GRLN-R by mutating E124$^{3.33}$ (for example, to Q124$^{3.33}$) in one receptor (to ablate ligand binding; dark blue receptor) and mutating the arginine residue (R141$^{3.50}$) in the ERY sequence to inhibit G protein binding to a second receptor (light-blue receptor). Ghrelin and the growth hormone secretagogues should only evoke a G protein-mediated response if both the mutated receptors come together to form a 'homo-dimer'. **B.** In the model by Holst et al. (2005) the growth hormone secretagogues were predicted to be able to bind to both protomers of a GRLN-R homo-dimer, leading to the receptor signalling with a higher efficacy than that observed for ghrelin (which because of its size is predicted to be unable to bind to both protomers simultaneously). The asymmetrical homo-dimer could be used to investigate whether the growth hormone secretagogues signal with an efficacy equal to that of ghrelin when one protomer of the dimer is unable to bind ligand.

if the two mutated receptors formed a 'homo-dimer', thus allowing the measurement of growth hormone secretagogues efficacy in a system where ligand could only bind to one protomer of the dimer (Figure 1-1). It is worth noting that the receptor that is mutated to inhibit ligand binding would still be able signal in the absence of ligand as the Q124$^{3.33}$ does not affect the constitutive activity of the receptor. Similar approaches have been used successfully in the past for other GPCRs (e.g. the metabotropic glutamate receptors; Goudet et al., 2005 and the leukotriene B_4 receptor; Damian et al., 2008) to investigate 'trans' signalling across dimers.

To allow further investigation into the pharmacology of the GRLN-R a Flp-In™ T-REx™ HEK293 cell line was created expressing the receptor tagged at the N-terminus with VSV-G and at the C-terminus with an eCFP fusion protein. Tagging the GRLN-R had no effect on the pharmacology or localisation of the receptor and allowed confirmation that the GRLN-R was modified by *N*-linked glycosylation, most likely at two asparagine residues at the N-terminus which occur as part of a N-X-S/T motif.

[^{35}S]GTPγS binding experiments used to measure G protein activation in membranes prepared from GRLN-R cells (either transfected with $G_{\alpha q}$ or $G_{\alpha o1}$) revealed that GHRP-6, L-692,585 and MK-677 all acted as super-agonists in the $G_{\alpha i/o}$ pathway but as full agonists in the $G_{\alpha q/11}$ pathway, thus demonstrating functional selectivity at the GRLN-R. This is the first time that functional selectivity at the GRLN-R has been described. This finding could potentially have implications *in vivo*; as the growth hormone secretagogues appear to stabilise a receptor conformation that favours coupling to $G_{\alpha i/o}$, the efficacy of a response could differ from ghrelin depending on the local concentrations of G protein in different cells.

Whole-cell second messenger pathways were also used to measure activation of the $G_{\alpha q/11}$ and $G_{\alpha i/o}$ pathways. Inositol phosphate accumulation studies demonstrated that ghrelin and the growth hormone secretagogues acted with efficacies that were similar to those measured in [^{35}S]GTPγS binding assays performed using an immunoprecipitation step to separate [^{35}S]GTPγS-bound $G_{\alpha q}$ from unbound $G_{\alpha q}$. However, GHRP-6 and L-692,585 acted with significantly higher potencies in the inositol phosphate accumulation assays than the [^{35}S]GTPγS binding assays, most likely due to response amplification or a difference in receptor reserve between these two systems. In contrast to the [^{35}S]GTPγS studies, ghrelin and the growth hormone secretagogues failed to induce a response in cAMP accumulation assays. Although coupling of the GRLN-R to $G_{\alpha o1}$ could be viewed as an artefact created by the addition of exogenous G protein, it was demonstrated that [^{35}S]GTPγS binding assays were also able to measure a response to ghrelin in the absence of exogenous G protein. The [^{35}S]GTPγS filtration binding assay measured a response to ghrelin that was sensitive to PTx, confirming coupling of the GRLN-R to the $G_{\alpha i/o}$ pathway. Thus the results were interpreted as reflecting the inability of the GRLN-R to couple to $G_{\alpha i1\text{-}3}$, as inhibition of AC by $G_{\alpha o1\text{-}2}$ is less potent than by $G_{\alpha i1\text{-}3}$, although previous studies have demonstrated that the GRLN-R can couple to $G_{\alpha i2}$ in cells endogenously expressing the GRLN-R (Bassil et al., 2007).

Although studies *in vivo* and in cell lines endogenously expressing the GRLN-R have suggested that the GRLN-R can couple to $G_{\alpha s}$ (Kohno et al., 2003; Malagon et al., 2003), data from recombinant studies remains contradictory, with some studies supporting $G_{\alpha s}$ coupling (Rossi et al., 2008; Caminos et al., 2005) and others disagreeing with this (Cunha and Mayo, 2002; Casanueva et al., 2008). Interestingly, in this thesis ghrelin, GHRP-6, L-692,585 and MK-677 were all shown to be able to evoke a $G_{\alpha s}$ response in cAMP accumulation assays, with ghrelin and the growth hormone secretagogues displaying similar potencies as measured in the [^{35}S]GTPγS binding experiments. However, L-692,585 acted as a partial agonist with respect to ghrelin, suggesting L-692,585 induces a receptor conformation which only weakly couples to the $G_{\alpha s}$ pathway.

The results of these studies confirm that the GRLN-R is a promiscuous receptor being able to couple to $G_{\alpha q/11}$, $G_{\alpha i/o}$ and $G_{\alpha s}$ pathways. However, it is clear that further experiments, aimed at deducing the specific isoforms within each G protein family the GRLN-R can couple, are needed to aid understanding of the pharmacology of the GRLN-R. To achieve this [^{35}S]GTPγS binding assays could be performed in membranes prepared from GRLN-R cells transfected with a panel of G protein subunits, followed by immunoprecipitation of [^{35}S]GTPγS-bound G protein from the reaction mixture. If full concentration-response curves to ghrelin and the growth hormone secretagogues were performed then the potencies at each of the G proteins could be measured, allowing a rank-order of G protein coupling to the GRLN-R to be compiled.

7.2 Constitutive activity of the GRLN-R

The expression of GRLN-R in the Flp-In™ T-REx™ HEK293 cell line resulted in the HEK293 cells appearing rounded compared to un-induced cells, a response that was accompanied by a high level of cell detachment. This effect appeared to be mediated by the GRLN-R as reducing the levels of receptor expression led to a reduction in severity of the cell-rounding phenotype. In contrast, HEK293 cells expressing the GRLN-R-A204E (a mutant receptor that does not display any constitutive activity) did not display the cell-rounding phenotype (data not shown), supporting the hypothesis that constitutive activity was responsible for the changed morphology of the wild-type GRLN-R cells.

The cell-rounding phenotype has been reported for another constitutively active GPCR, the sphingosine 1-phosphate-5 receptor (Niedernberg et al., 2003). It would be interesting to investigate whether the phenotype mediated by GRLN-R expression is the result of constitutive activation of the $G_{\alpha 12/13}$ pathway, a pathway that can alter cell morphology i.e.

by actin re-organisation (Riobo and Manning, 2005). This could be achieved by over-expressing $G_{\alpha 13}$ (to see if this increased the severity of the cell-rounding phenotype) or by using small interfering ribonucleic acid (siRNA) to silence $G_{\alpha 13}$ expression.

The GRLN-R has been reported to constitutively activate the $G_{\alpha q/11}$ pathway (Holliday et al., 2007; Holst et al., 2004; Holst et al., 2003) being able to evoke a response that is up to 50 % of the maximal signal achieved upon ghrelin stimulation (Holst et al., 2003). However, it is clear that the constitutive activity of the GRLN-R is not a result of receptor over-expression as gene-dosing experiments performed by Holst et al. (2004) showed that, at similar expression levels, the related receptor GPR38 remained silent in the absence of ligand.

A doxycycline concentration of 6 ng/mL was deemed optimal to stimulate GRLN-R expression in the GRLN-R Flp-In™ T-REx™ cell line, as this was the lowest concentration of doxycycline tested that allowed a significant response of the GRLN-R to ghrelin to be detected in [^{35}S]GTPγS binding assays. At this expression level constitutive activity of the GRLN-R through activation of $G_{\alpha q}$ could be measured and the constitutive activity significantly reduced by addition of the inverse agonist SPA. However, in the $G_{\alpha i/o}$ pathway, although the addition of 6 ng/mL doxycycline led to a significant increase in [^{35}S]GTPγS loading, there was no significant decrease in [^{35}S]GTPγS binding levels upon addition of the inverse agonist SPA. Thus further experiments are needed to determine whether the GRLN-R is less effective at constitutively activating the $G_{\alpha i/o}$ pathway or whether this is just an artefact created by comparing the response of the GRLN-R in two different experimental systems (one using immunoprecipitation to recover [^{35}S]GTPγS-G protein and another using a filtration step to separate [^{35}S]GTPγS-bound G protein from unbound G protein). Indeed, the level of constitutive activity of the GRLN-R measured upon addition of exogenous $G_{\alpha q}$, $G_{\alpha o1}$, $G_{\alpha s}$ and $G_{\alpha 13}$ could be compared, using, for example, a [^{35}S]GTPγS assay with immunoprecipitation step to establish whether the GRLN-R can constitutively activate every G protein pathway to which it is coupled (although the variables of G protein expression and relative affinities of the G protein antibodies must be taken into account).

7.3 GRLN-R mutants

As well as studying the pharmacology of the wild-type GRLN-R, the effect of A204E or I134T missense mutations on the localisation and pharmacology of the GRLN-R was investigated. The A204E missense mutation occurs in ECL2, whilst the I134T mutation

occurs in TM3. Interestingly the A204E mutation (which has been associated with the development of an obese/short stature phenotype *in vivo*) was shown to cause partial retention of the GRLN-R within the ER-*cis*-Golgi complex, whilst receptor expressed at the cell-surface was shown to be devoid of any constitutive activity. The lack of constitutive activity was in agreement with other studies into the A204E mutation (Pantel et al., 2006; Liu et al., 2007; Holliday et al., 2007). Although the A204E mutation has been reported in some studies to lower cell-surface expression of the GRLN-R (Pantel et al., 2006: Liu et al., 2007), this is the first study to provide evidence to suggest this is due to partial retention of the receptor within the ER, most probably due to altered receptor folding.

Interestingly, a recent study by Sum et al. (2009) have shown that two glutamic residues in ECL2 of the free fatty acid receptor FFA1 form an ionic lock with two arginine residues in TMV and TMVII. Mutating the glutamic acid residues to alanine residues results in constitutive activation of the FFA1. Thus the A204E mutation may inhibit the constitutive activity of the GRLN-R, for example, by forming an ionic lock with a residue within the TM domains (such as $R283^{6.45}$ at the top of TMVI). This could be investigated further by introducing point mutations into the TM domains to see if the constitutive activity of the GRLN-R could be ablated, for example, by mutating $R283^{6.45}$ to $A283^{6.45}$.

The I134T mutation has been suggested to ablate the response of the GRLN-R to ghrelin, however, in this thesis the GRLN-R-I134T was shown to be able to bind [His[^{125}I]]-ghrelin and retained the ability to signal to ghrelin and the growth hormone secretagogues in both the $G_{\alpha q/11}$ and $G_{\alpha i/o}$ pathways. The efficacy of SPA at the GRLN-R-I134T was shown to be reduced in a study by Liu et al. (2007); results of the inositol phosphate accumulation experiments appeared to agree with this, as SPA failed to return the inositol phosphate levels accumulated upon receptor expression to those seen in the absence of receptor.

Missense mutations within GPCRs often result in protein misfolding, which commonly results in the retention of a misfolded receptor in the ER by molecular chaperones and quality control machinery. However, the expression of misfolded GPCRs at the plasma membrane can be greatly increased by treatment with chemical or pharmacological chaperones (Brown et al., 1996; Sato et al., 1996; Robben et al., 2005). Chemical chaperones include osmolytes such as glycerol, DMSO and trimethyl-amine-*N*-oxide (Sato et al., 1996; Tamarappoo et al., 1999; Yang et al., 1999; Song and Chuang, 2001) that may stabilise the receptor conformation or trigger a stress response that leads to upregulation of chaperone proteins (Welch and Brown, 1996; Diamant et al., 2001). Pharmacological

chaperones include non-peptide receptor antagonists that stabilise ER-retained mutated protein – as seen for the vasopressin 2 receptor (Morello et al., 2000; Robben et al., 2006). Indeed non-peptide antagonists have been used successfully *in vivo* to relieve nephrogenic *diabetes insipidus* (Bernier et al., 2006) demonstrating that rescuing ER-retained mutated GPCRs offers a clinical treatment that may be used to cure a number of debilitating diseases. Due to the lack of commercially available non-peptide GRLN-R antagonists the effects of pharmacological chaperones in altering GRLN-R-A204E localisation could not be investigated. However the effects of chemical chaperones (e.g. DMSO) on the localisation of the GRLN-R-A204E warrants further investigation.

7.4 Conclusion

This study has led to a fuller understanding of the pharmacology of the GRLN-R, showing that the growth hormone secretagogues do not act as allosteric modulators of the GRLN-R, although they do act as super-agonists in the $G_{\alpha i/o}$ pathway (favouring coupling of the GRLN-R to $G_{\alpha i/o}$). The development of GRLN-R cell lines has provided extremely useful tools for studying the GRLN-R and the two mutated receptors (GRLN-R-I134T and GRLN-R-A204E) and will enable further investigations into the G protein coupling and constitutive activation of the GRLN-R.

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