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β -arrestin Interacting Domains on the Type II Gonadotropin-Releasing Hormone (GnRH) Receptor

by

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LIST OF ABBREVIATIONS

AT _{1A} R	Angiotensin type IA receptor
α ₂ AR	α ₂ -adrenergic receptor
β ₂ AR	β ₂ - adrenergic receptor
CKII	Casein kinase II
DAG	Diacylglycerol
DMEM	Dulbecco's modified medium
ECL	Extracellular loop
FCS	Fetal calf serum
FSH	Follicle-stimulating hormone
GFP	Green fluorescent protein
GnRH	Gonadotropin releasing hormone
GPCR	G-protein coupled receptor
GRK	G-protein coupled receptor kinase
HEPES	N-2-Hydroxyethylpiperazine-N-2'ethansulphonic acid
ICL	Intracellular loop
IP ₃	Inositol 1,4,5-trisphosphate
LH	Luteinising hormone
MAPK	Mitogen activated protein kinase
PCR	Polymerase chain reaction
PIP ₂	Phosphatidylinositol 4,5,-bisphosphate
PKA	Protein kinase A
PKC	Protein Kinase C
PLC	Phospholipase C
T1	Type I GnRH receptor
T2	Type II GnRH receptor
TM	Transmembrane domain
TRH	Thyrotropin-releasing hormone receptor

ABSTRACT

The mammalian type II GnRH receptor possesses an intracellular C-terminal tail that is known to play a role in desensitisation, internalisation and overall signalling in GPCRs. On the other hand, the mammalian type I GnRH receptor, which lacks a C-terminal tail, does not readily desensitise and undergoes slow internalisation compared to the mammalian type II GnRH receptor.

Over-expression of β -arrestin 1 in COS-1 cells revealed that the mammalian type II GnRH receptor can internalise in a β -arrestin dependent manner whereas the internalisation of the mammalian type I GnRH receptor is β -arrestin independent. To investigate which domains on the mammalian type II GnRH receptor are required for β -arrestin dependent internalisation, chimeric receptors were created. Firstly, a chimera in which the full length type II GnRH receptor C-terminal tail was added to the tail-less type I GnRH receptor (T1/T2tail) was created. This chimera internalised in a β -arrestin and GRK dependent manner, demonstrating that the type II GnRH receptor C-terminal tail confers β -arrestin/GRK dependent internalisation on the originally β -arrestin/GRK insensitive GnRH receptor. Mutating the putative GRK and casein kinase II phosphorylation sites (serines 338 and 339) on the C-terminal tail of T1/T2tail to alanine residues did not abolish β -arrestin dependent internalisation but eliminated GRK dependent internalisation, suggesting that other regions on the C-terminal tail are required for β -arrestin dependent internalisation.

A second chimera, in which the whole third intracellular loop of the type II GnRH receptor was replaced with that of the type I GnRH receptor (T2/T1ICL3), was created. This chimera could not utilise β -arrestin in its internalisation, indicating that the third intracellular loop of the type II GnRH receptor is required for β -arrestin dependent internalisation. An alignment of the amino acid sequences of the two mammalian GnRH receptor third intracellular loops identified a basic residue rich area (R234, R236 and K237) on the type II GnRH receptor that was absent on the type I GnRH receptor. Interestingly, the triple mutant (R234,236,K237A) still internalised in a β -arrestin dependent manner, however, truncation of the C-terminal tail of R234,236,K237A abolished the ability of the receptor to internalise in a β -arrestin dependent manner. This

result indicated that the C-terminal tail of the type II GnRH receptor was compensating for the absence of the three basic residues.

To summarise, this thesis demonstrates that the C-terminal tail of the type II GnRH receptor can confer β -arrestin dependent internalisation on the type I GnRH receptor. Furthermore, the third intracellular loop, and more specifically, basic residues R234, R236 and K237 on the mammalian type II GnRH receptor are required for β -arrestin dependent internalisation.

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1. INTRODUCTION

1.1 G protein-coupled receptors

G protein-coupled receptors (GPCRs) belong to a large family of plasma membrane spanning receptors that respond to different stimuli such as light, odour, taste, neurotransmitters and hormones (Ferguson 2001). Based on the analysis of their sequences, GPCRs are classified into three sub-families; the rhodopsin receptor family, the secretin/vasointestinal peptide receptor family and the metabotropic glutamate receptor family. The common characteristic between these receptors is their overall structure, consisting of seven transmembrane spanning domains (TMs), three intracellular loops (ICLs), three extracellular loops (ECLs), beginning with an extracellular amino terminus and ending with an intracellular carboxyl terminus. The secretin/vasointestinal receptors possess a long amino terminus that is involved in receptor-ligand interactions, while the metabotropic glutamate receptors are considerably much larger than the other receptors, being made up of 1199 amino acids (Strader *et al.* 1995).

The rhodopsin receptor family constitutes the majority of GPCRs identified to date, including receptors such as the β_2 -adrenergic receptor (β_2 AR), the Gonadotropin releasing hormone (GnRH) receptor and the thyrotropin releasing hormone (TRH) receptor (Strader *et al.* 1995). The tertiary structure of this family of receptors was revealed by using several approaches and the latest was by the identification of the crystal structure of the rhodopsin receptor (Palczewski *et al.* 2000). The sequence of the transmembrane domains of these GPCRs is more conserved than the ECL and ICL sequences. Conserved cysteine residues in ECL2 and ECL3 form disulphide bonds which help maintain receptor conformation (Dohlman *et al.* 1991). ICL2 and ICL3 and part of the carboxyl-terminal tail are important for G protein interaction (Strader *et al.* 1995).

GPCRs are coupled to heterotrimeric guanine nucleotide binding proteins (G proteins) which enable the receptor to transduce information received from external stimuli into

cell signals. Following its cognate G protein-dependent signalling, agonist activation of a GPCR leads to the uncoupling of the receptor from its G protein resulting in desensitisation, internalisation and G protein-independent signal transduction (Ferguson 2001).

1.1.2 GPCR signalling

The binding of an agonist to a GPCR causes the receptor to be stabilised in its active conformation, facilitating the interaction of the receptor with its coupled G protein. G proteins consist of three associated protein subunits, called α , β and γ (Hamm and Gilchrist 1996). In this active state, the receptor promotes the exchange of GDP for GTP on the G protein α -subunit, which is followed by the dissociation of the G protein α from the $\beta\gamma$ subunits (section 1.1.3: Fig. 1.1). Both α -GTP and $\beta\gamma$ can then bind to and regulate the activity of effector enzymes in various ways. For example, the activation of $G\alpha_{q/11}$ leads to the activation of Phospholipase C (PLC), the activation of the $G\alpha_s$ results in the stimulation of adenylyl cyclase, while activated $G\alpha_i$ proteins inhibit adenylyl cyclase (Gainetdinov *et al.* 2004). Subsequently, these effector enzymes generate second messengers such as Inositol 1,4,5- triphosphate (IP_3), Diacylglycerol (DAG) and adenosine-3', 5'- cyclic phosphate (cAMP) (Fig 1.1). Signal transduction is terminated when the GTP bound to the G protein α subunit is hydrolysed to GDP. GTP hydrolysis may occur via the intrinsic GTPase activity of the α -subunit or may be enhanced by regulators of G protein signalling (RGS) which increase the rate of hydrolysis of GTP on $G\alpha$ subunits, thereby dampening the G protein mediated signal (Siderovski *et al.* 1999). The dissociated $\beta\gamma$ subunits of the G protein are then re-united with the α -subunit, preparing for another G protein activation cycle (Ferguson *et al.* 2001).

GPCRs also mediate the activation of mitogen-activated protein kinases (MAPKs). For example, the activation of the GnRH receptor in α -T3 cells leads to the activation of PKC which results in the downstream activation of MAPKs (Levi *et al.* 1998; Bernard *et al.* 2001). Furthermore, the transactivation of the epidermal growth factor receptor (EGFR), by GPCRs, leads to the downstream activation of ERK1/2 (Luttrell *et al.* 1997). Other

kinases activated by GPCRs include the jun-N-terminal kinase (JNK) and the p38 MAPKs (Miller and Lefkowitz 2001).

1.1.3 GPCR desensitisation

Receptor desensitisation occurs within minutes of agonist exposure and is followed by internalisation, which in some cases can lead to the down-regulation of total cellular receptors. Desensitisation is characterised by a functional uncoupling of receptors from heterotrimeric G proteins and is typically a consequence of receptor phosphorylation. There are two ways in which receptors are phosphorylated; firstly, via agonist-dependent phosphorylation through G protein coupled receptor kinases (GRKs) and through second messenger-activated kinases such as protein kinase A (PKA), protein kinase C (PKC) (Ferguson 2001), casein kinase 1a and casein kinase II (CKII) (Budd *et al.* 2000; Hanyaloglu *et al.* 2001).

GRKs phosphorylate GPCRs at both serine and threonine residues within either the third intracellular loop or the carboxyl-terminal (C-terminal) tail domains (Fig. 1.1). Mutation of all the serine and threonine residues within either the C-terminal tail or the third intracellular loop of the m2 muscarinic receptor abolishes GRK-mediated phosphorylation of this receptor (Nakata *et al.* 1994), demonstrating that these sites are required for phosphorylation by GRK. Subsequently, arrestin binds the receptor leading to desensitisation and it is thought that the phosphorylation of serine and threonine residues on the C-terminal tail of most GPCRs may regulate the stability of the receptor/arrestin complexes (Oakley *et al.* 1999). Generally, the phosphorylation of the activated GPCR is the determining factor of β -arrestin interaction. When serine and threonine sites on the angiotensin type 1A receptor ($AT_{1A}R$) are mutated, phosphorylation of the agonist activated receptor is impaired, and β -arrestin binding is diminished (Qian *et al.* 2001). In addition, over-expression of GRKs promotes the recruitment of GFP (Green fluorescent protein)- β -arrestin to several receptors (Shenoy and Lefkowitz 2003). Receptor mutants that could not be phosphorylated by GRKs or

were not able to interact with β -arrestin displayed impaired internalisation (Ferguson *et al.* 1996), showing that GRKs facilitate the recruitment of β -arrestin.

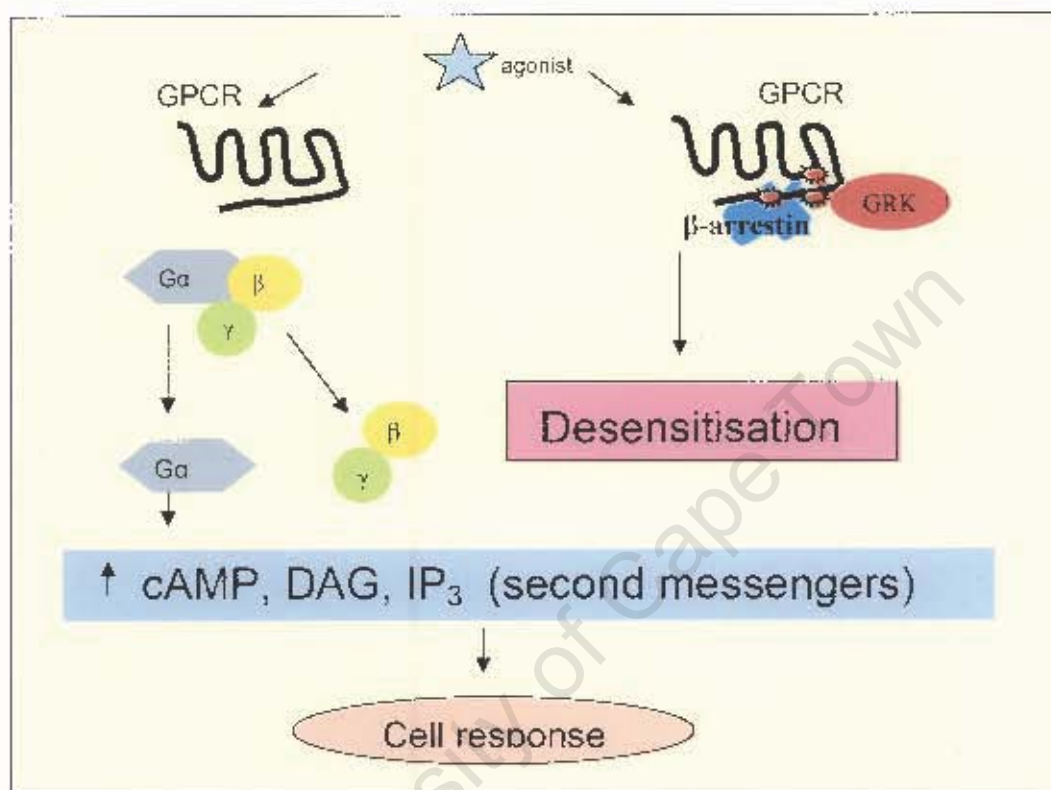


Fig. 1.1 G protein-coupled receptor signalling. The agonist-activated receptor stimulates heterotrimeric G proteins and is rapidly phosphorylated by G protein-coupled receptor kinases (GRKs) at serine/threonine residues in ICL3 or the C-terminal tail (red explosions), consequently leading to β -arrestin recruitment. This results in the desensitisation of the receptor.

1.2 Proteins involved in the desensitisation and internalisation of GPCRs

1.2.1 The G protein-coupled receptor kinases (GRKs)

The GRK family of kinases comprises seven family members (GRK1-GRK7). GRK1 and GRK 7 are retinal enzymes that phosphorylate opsins. GRK4 is exclusively expressed in the brain, kidney and testes (Shenoy and Lefkowitz 2003) while GRK2 and GRK3 (also known as β ARK1 and β ARK2, respectively), as well as GRK5 and GRK6 are ubiquitously distributed. Each of the GRKs shares a similar functional organisation, consisting of three distinct domains. The N-terminal region of GRKs is thought to be involved in substrate recognition and contains an RGS-like domain termed the RH (RGS homology) domain, suggesting that GRKs can also regulate GPCR signalling at the level of the G protein. The second domain on GRKs is the catalytic domain that is most similar to the PKA and PKC kinases. The third domain is the C-terminal region, which is more variable among the GRKs and contributes to the plasma membrane targeting of GRKs. Post-translational farnesylation of the C-terminal CAAX motif of GRK1 facilitates movement of the kinase to the plasma membrane, following the photoactivation of rhodopsin (Ferguson 2001).

GRK2 and GRK3 are not farnesylated and their translocation to the plasma membrane is partly regulated by their association with G protein $\beta\gamma$ -subunits, which bind to the C-terminal pleckstrin homology-like domain of the kinases. Furthermore, the targeting of GRK2 and GRK3 is also influenced by phosphatidyl inositol 4,5-bisphosphate (PIP₂) binding to the C-terminal pleckstrin homology domain of the kinases (Pitcher *et al.* 1998). GRK4, GRK5 and GRK6 are all localised to the plasma membrane, even in the absence of agonist activation. Plasma membrane localisation of GRK4 and GRK6 seemed to be a result of palmitoylation on their C-terminal cysteine residues (Stoffel *et al.* 1998). It is thought that the localisation of GRK5 to the plasma membrane is mediated by the electrostatic interaction between highly basic residues in the C-terminus of the kinase and phospholipids.

Unlike other kinases such as PKA, PKC and CKII, a consensus sequence for GRK phosphorylation has not been found. Nonetheless, GRKs have been shown to phosphorylate serine and threonine residues adjacent to a pair of acidic residues on their N-terminal side (Pitcher *et al.* 1998).

1.2.2 Arrestins

Cessation of G protein-mediated signalling is not achieved by GRK phosphorylation alone but other proteins are required for full inactivation of GPCRs. Several studies have revealed that proteins called arrestins are recruited to agonist-activated and phosphorylated receptors where they sterically uncouple the receptor from its G protein, leading to desensitisation (Lohse *et al.* 1992).

The first member of the arrestin family to be discovered, now called visual arrestin, was shown to bind light-activated rhodopsin. This 48-kDa protein was found to act in concert with rhodopsin kinase to terminate rhodopsin-mediated signalling (Ferguson *et al.* 2001). Subsequently, in (β_2 AR) studies, a non-visual form of arrestin, β -arrestin 1 (arrestin 2), was identified in the purification of GRK2. The inability of GRK2 preparations to desensitise β_2 AR suggested that a co-factor had been lost during purification and was required for GRK2-mediated β_2 AR desensitisation *in vitro* (Benovic *et al.* 1987). Later, β -arrestin 1 was cloned and it was found to share a sequence homology of 59% with visual arrestin (Ferguson 2001).

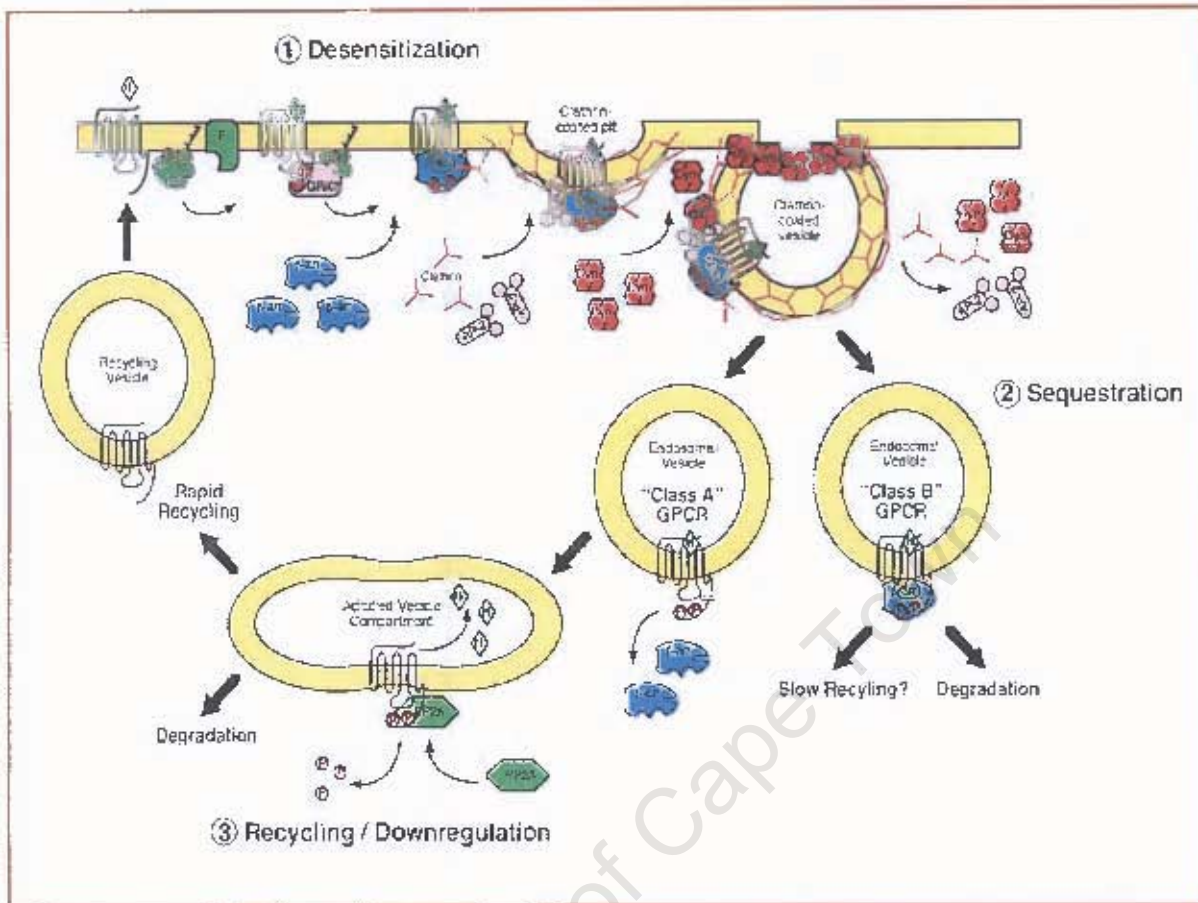
To date, four arrestin isoforms have been identified and according to sequence homology, function and tissue distribution, the members of the arrestin family can be divided into two groups. The first group comprises visual arrestin (S-antigen or 48K protein) and cone arrestin (X-arrestin or C-arrestin), while β -arrestin 1 (arrestin 2) and β -arrestin 2 (arrestin 3) belong to the second group (Ferguson 2001). Visual arrestin is distributed in rod outer segments and primarily localised to the retina. Cone arrestin is also found in the retina but mainly localised within cone photoreceptors. Both β -arrestin isoforms are ubiquitously

distributed outside the retina but are mainly found in neuronal tissues and spleen (Attramadal *et al.* 1992). β -arrestin 1 and 2 are structurally homologous sharing 78% amino acid identity (Krupnick and Benovic. 1998).

Interaction of the β -arrestins with GPCRs was demonstrated in vitro with purified β_2 AR (Luttrell *et al.* 1999), and visualisation of the recruitment of cytosolic β -arrestins to receptors at the plasma membrane after agonist stimulation was made possible by the development of GFP- β -arrestin fusion proteins (Barak *et al.* 1997).

Although, for many receptors, it has been observed that β -arrestin binding is preceded by phosphorylation, surprisingly, β -arrestins bind some receptors independent of receptor phosphorylation but requiring receptor activation. For example, the activated human lutropin receptor binds β -arrestin independent of receptor phosphorylation (Min *et al.* 2002).

Depending on how β -arrestin interacts with receptors after being targeted to the internalisation machinery, the receptors are classified into two classes; Class A and Class B receptors (section 1.2.2: Fig. 1.2). Class A receptors including β_2 AR, α_{1b} AR, μ -opioid receptor, endothelin ET_A receptor and dopamine D_{1A} receptors bind to β -arrestin 2 with a higher affinity compared with β -arrestin 1. β -arrestin seems to bind to these receptors transiently, since the β -arrestin dissociates from the receptor immediately after movement of the receptor into clathrin coated pits. Therefore, receptor-arrestin complexes do not co-localise in endosomes. Class B receptors include AT_{1A}R, thyrotropin-releasing hormone (TRH) receptor, neurotensin 1 receptor and neurokinin NK1 receptor bind both β -arrestin 1 and 2 with equal affinity (Oakley *et al.* 2000). With these receptors, the receptor-arrestin complex is more stable and the activated receptor and arrestin co-localise in endosomes for longer periods. Class A receptors were seen to resensitise and recycle faster than class B receptors. The co-localisation of β -arrestins with the activated receptors in endosomes (for class B receptors) is attributed to a set of triplet serine residues in the C-terminal tails of these receptors (Oakley *et al.* 2001).



Luttrell and Lefkowitz. *Journal of Cell Science*. 2002

Fig. 1.2 Role of β -arrestin in the desensitisation, and intracellular trafficking of GPCRs. β -arrestins (blue) are recruited to the phosphorylated receptor, leading to desensitisation (1). Receptor-bound β -arrestins also act as adapters, binding to clathrin and AP-2 and targeting the complex to clathrin coated vesicles. Sequestration (2) occurs as a consequence of dynamin (red) pinching off the clathrin coated vesicle from the plasma membrane. Once internalised, receptors exhibit two distinct patterns of β -arrestin interaction; Class A receptors rapidly dissociate from β -arrestin upon internalisation, while Class B receptors form stable receptor- β -arrestin complexes. Receptors accumulate in endosomes where they become dephosphorylated and are either targeted for degradation or recycled (3).

The consequence of β -arrestin binding to activated receptors is not only the disruption of the G protein-receptor complex, but also the targeting of receptors to the endocytic machinery, such as clathrin coated vesicles. Arrestins contain sites for interaction with clathrin and the clathrin associated adapter AP-2 (Goodman *et al.* 1996; Laporte *et al.* 1999) which play an important role in receptor internalisation.

1.2.3 Clathrin

Clathrin is a trimeric protein arranged as a triskelion when assembled and is the major structural protein of the characteristic polygonal lattice of the coated pit. These pits also contain the clathrin adaptor protein AP-2, which binds to clathrin and β -arrestin. β -arrestin binds to the heavy chain of clathrin and the β_2 -adapting subunit of AP-2 which is important for clathrin coat formation (Laporte *et al.* 2000).

The effect of β -arrestin in targeting GRK phosphorylated receptors to clathrin coated pits is believed to occur in two ways; either β -arrestin interacts with the β -subunit of AP-2 and clathrin to initiate the assembly of clathrin cages and formation of clathrin coated pits or the receptor/ β -arrestin complex is targeted to pre-existing clathrin coated pits where it interacts with AP-2 and clathrin (Claing *et al.* 2002). Dynamin, a GTPase then 'pinches' off the clathrin coated vesicles from the plasma membrane leading to receptor internalisation (Ferguson 2001).

1.2.4 Dynamin

Dynamin is a GTPase that plays a role in the internalisation of vesicles. Dynamin is targeted to clathrin coated pits in its GDP-bound form and is believed to be the key regulator of vesicle budding since it catalyses the budding off of clathrin-coated vesicles from the plasma membrane (Hill *et al.* 2001; Claing *et al.* 2002). Over-expression of the dominant negative mutant of dynamin (K44A) affected the internalisation of the β_2 AR (Zhang *et al.* 1996), demonstrating that vesicle fission requires dynamin.

For many years, dynamin was known as a 'pinchase' because it was thought that dynamin pinched the vesicle off the plasma membrane. However, it was later discovered that the internalisation of the vesicles occurs as a function of the hydrolysis of the GTP bound to dynamin which leads to shearing of the neck (reviewed in Claing *et al.* 2002) and consequently, internalisation.

1.3 Role of internalisation in the resensitisation and down-regulation of GPCRs

Much information on internalisation has been gathered from studies on the β_2 AR. Initial evidence of internalisation prototypical to the β_2 AR was observed in frog erythrocytes where the number of receptors at the plasma membrane decreased upon agonist exposure. Furthermore, studies using hydrophilic versus hydrophobic compounds demonstrated that subsequent to agonist activation, receptors were sequestered into specialised intracellular compartments, indicating that internalisation had occurred (Claing *et al.* 2002).

Interestingly, GPCR internalisation was originally deemed to be a primary mechanism of receptor desensitisation due to the uncoupling of the receptor from its effectors (Sibley and Lefkowitz 1985). However, it was later revealed that desensitisation proceeds much faster than internalisation. In addition, treatments that inhibited the internalisation of β_2 AR were observed not to affect the desensitisation of this receptor (Pippig *et al.* 1995), demonstrating that desensitisation is independent of internalisation.

The first visual example of the sequence of biochemical events in live cells was done on the neurokinin 1 receptor in HEK 293 cells using GFP conjugates of PKC, GRK 2 and β -arrestin 2. The translocation of PKC-GFP to the plasma membrane following agonist stimulation showed that the neurokinin 1 receptor becomes activated within a few seconds and desensitises within 30 seconds. The redistribution of GRK-GFP from the cytosol to the plasma membrane occurs simultaneously with desensitisation and is followed by the translocation of β -arrestin 2- GFP (Barak *et al.* 1999).

Prolonged or irreversible receptor desensitisation would leave a cell unable to respond appropriately to extracellular stimuli, therefore resensitisation of receptors is an important mechanism. Resensitisation enables desensitised receptors to be reactivated upon agonist binding. Treatments such as concanavalin A and hypertonic sucrose, that blocked receptor internalisation also blocked resensitisation without affecting either receptor G protein coupling or desensitisation (Pippig *et al.* 1995), demonstrating that resensitisation is

highly dependent on internalisation. Furthermore, internalisation defective-mutants were observed to desensitise but did not undergo resensitisation (Barak *et al.* 1994). The internalisation of agonist-activated receptors into an endosome, with GPCR-specific phosphatase activity, leads to the dephosphorylation of internalised receptor and subsequent recycling back of the receptor to the cell surface in an inactive form (Krueger *et al.* 1997; Pitcher *et al.* 1995). Interestingly, some receptors do not have to undergo internalisation in order to be dephosphorylated and resensitised. The D1 dopamine receptor is dephosphorylated at the cell surface without having been internalised (Gardner *et al.* 2001).

The consequence of receptor internalisation is not only resensitisation but also degradation of receptors. Instead of being recycled back to the cell surface, the receptor-containing endosome can fuse with a lysosome, leading to receptor degradation. Some GPCRs, such as the protease-activated receptors (PARS) are directly targeted to lysosomes for degradation (Trejo and Coughlin 1999). The degradation of receptors results in down-regulation of total receptor number per cell. Post-translational attachment of ubiquitin (Ub) to proteins, known as ubiquitination plays an important role in determining which receptors are to be degraded. The C-terminal glycine of Ub becomes covalently attached to the ϵ -amino group of the lysine residue in the receptor and the receptor is targeted to a proteasome which functions as a degradation compartment (Hartmann-Petersen *et al.* 2003). The importance of the lysine residue in ubiquitination and degradation was demonstrated by mutating lysine residues in the C-terminal tail region of the chemokine receptor CXCR4. Removal of these sites eliminated both ubiquitination and degradation, but not internalisation of this receptor (Marchese *et al.* 2001).

What is clear is that GPCR signalling is a highly regulated process that requires various proteins to be involved in signal termination, down-regulation of receptors and even resensitisation. Interestingly, for all the above processes (except desensitisation) to occur, receptors must first be concentrated to regions of membrane internalisation on the cell surface.

1.3.1 Mechanisms of internalisation

Different modes of GPCR internalisation exist, but the most common mechanism of GPCR internalisation is mediated by clathrin-coated pits (Shenoy and Lefkowitz 2003), which is also facilitated by arrestins and dynamin. The activated receptors become phosphorylated by GRKs, leading to the recruitment of β -arrestin and subsequent interaction of the receptor-arrestin complex with clathrin and the adapter protein AP-2. This leads to the redistribution of these complexes into clathrin-coated pits that get pinched off the membrane by the cytoplasmic GTPase, dynamin and become internalised (Sever 2002) either still associated with β -arrestins or without β -arrestins (Fig 1.2).

The mechanism of internalisation via clathrin-coated vesicles, as described above, is one that requires GRKs, β -arrestins and dynamin. Other mechanisms of receptor internalisation have been identified. These have been elucidated via over-expressing dominant-negative constructs of arrestin, dynamin and clathrin, which facilitate or inhibit the use of a particular pathway. Over-expression of the dominant-negative mutants of β -arrestin and dynamin does not block agonist-activated internalisation of the AT_{1A}R and m2 muscarinic receptor (Zhang *et al.* 1996; Vogler *et al.* 1999), showing that these receptors internalise in a manner independent of β -arrestin and dynamin.

The different pathways of internalisation that have been elucidated from dominant-negative mutants include the arrestin-independent, dynamin/clathrin-dependent and the arrestin-independent, dynamin/clathrin independent (Claing *et al.* 2000), as well as, arrestin-dependent, dynamin/clathrin independent mechanisms (Kohout *et al.* 2001).

Examples of receptors that internalise in an arrestin-independent but dynamin dependent pathway include the vasoactive intestinal peptide type I (VIP 1) receptor and the endothelin type B (ET_B) receptors. Over-expression of the dominant-negative mutant of β -arrestin does not affect the internalisation of these receptors whereas over-expression of the dominant-negative dynamin (K44A) significantly impairs receptor endocytosis

(Claing *et al.* 2000). It is clear from this result that these receptors internalise in a manner that is independent of β -arrestin.

Other receptors seem to require neither β -arrestin nor dynamin, such as the $AT_{1A}R$. The internalisation of this receptor is not affected by the over-expression of the dominant-negative mutants of β -arrestin and dynamin (Claing *et al.* 2000; Zhang *et al.* 1996). However, it has been recently found that the internalisation of $AT_{1A}R$ is regulated by β -arrestins. In β -arrestin 1 knockout cells, the internalisation of this receptor was slightly reduced, but remained unaffected in the β -arrestin 2 knockout cells. However, in the β -arrestin 1, 2 double knockout cells, the internalisation of $AT_{1A}R$ was impaired. The m2 muscarinic, the bradykinin type 2 and the N-formyl peptide receptors are more examples of receptors that internalise in a β -arrestin and dynamin independent manner (Claing *et al.* 2000).

1.4 GPCR domains that interact with β -arrestin

Many receptors have been shown to interact with β -arrestins either on their C-terminal tail or the third intracellular loop (ICL3). The delta opioid receptor demonstrated that the C-terminal tail and ICL3 are both sites of β -arrestin interaction, and bind β -arrestin on distinct sites (Cen *et al.* 2001). Neurokinin 1 receptor showed a similar result (Schmidlin *et al.* 2003). Studies on the chemokine receptor CXCR4 also showed that β -arrestin was able to bind efficiently in vitro to both the ICL3 and the C-terminal tail of this receptor (Cheng *et al.* 2000).

Furthermore, studies on the muscarinic and α_2 -adrenergic receptors also identified the ICL3 as the domain of β -arrestin binding on these receptors (Wu *et al.* 1997). Interaction of visual arrestin with rhodopsin can be blocked by synthetic peptides representing ICL1 and ICL3 (Krupnick *et al.* 1994). For the α_2 -adrenergic receptor it was further discovered that the presence of basic residues in ICL3 facilitated the binding of β -arrestin (DeGraff *et al.* 2002). Interestingly, for the LH/CG receptor it was an aspartic acid 564 in ICL3

that was found to be crucial for phosphorylation-independent interaction with β -arrestin (Mukherjee *et al.* 2002).

In many GPCRs, such as the α_2 AR, the third intracellular loop is quite large compared to the other intracellular loops and includes sites for GRK phosphorylation (Liggett *et al.* 1992) G protein activation (Wade *et al.* 1999) and binding of arrestin (Wu *et al.* 1997). It therefore seems likely that specific regions of the third intracellular loop might confer β -arrestin binding following receptor activation.

Interestingly, a recent study has suggested that the first half of the second intracellular loop, including the conserved DRY motif, could be a site for β -arrestin interaction common to rhodopsin-like GPCRs (Marion *et al.* 2006).

For other GPCRs, the C-terminal tail seems to play an important role in β -arrestin binding. Addition of C-terminal tails to several tail-less receptors appears to confer β -arrestin dependence. The rat type I GnRH receptor (that does not normally utilise β -arrestin in its internalisation) can internalise in a β -arrestin dependent manner on addition of a thyrotropin-releasing hormone (TRH) receptor C-terminal tail (Heding *et al.* 2000). Furthermore, truncation of a catfish type II GnRH receptor C-terminal reduced β -arrestin dependent internalisation of this receptor (Hanyaloglu *et al.* 2001).

1.5 Role of β -arrestin in desensitisation

Recombinant β -arrestin 1 and β -arrestin 2 proteins purified from COS cells were utilised to investigate the desensitisation function of β -arrestins. Both β -arrestin isoforms produced an 80% inhibition of the measured GTPase activity of the G protein stimulated by the β_2 AR, demonstrating that β -arrestin 1 and 2 are indeed involved in β_2 AR desensitisation (Attramadal *et al.* 1992). It has also been shown via siRNA (small interfering RNA) methods that reducing endogenous β -arrestin levels in HEK 293 cells results in enhanced cAMP accumulation following endogenous β_2 AR stimulation (Ahn *et*

al. 2003). Furthermore, the desensitisation of both the β_2 AR and the AT_{1A} R is impaired in mouse embryonic fibroblast (MEF) cell lines lacking both β -arrestins (Kohout *et al.* 2001).

On the other hand, the desensitisation of the PAR1 (proteinase-activated receptor 1) receptor is mediated by β -arrestin 1, not β -arrestin 2, since this receptor could not desensitise in β -arrestin 1 null MEF cell lines but could in the β -arrestin 2 null cell lines (Paing *et al.* 2002).

It has recently been demonstrated that β -arrestin recruits phosphodiesterases (PDEs) to the activated β_2 AR at the plasma membrane. PDEs are enzymes that degrade cAMP and regulate the levels of cAMP in the cell. Interestingly, PDE4D is not recruited to the membrane in cells that lack β -arrestins, suggesting that β -arrestins specifically scaffold these enzymes at the membrane. Furthermore, over-expression of a catalytically inactive PDE4D mutant, which can bind β -arrestin and competes with endogenous PDE4D, leads to lower rates of degradation of cAMP at the membrane. Hence, β -arrestin displays dual regulation in desensitising $G\alpha_s$ -coupled receptors such as the β_2 AR by simultaneously decreasing the rate of cAMP production (uncoupling of G protein) and increasing cAMP degradation by recruiting and scaffolding cellular PDEs (review: Shenoy and Lefkowitz 2003).

1.6 Role of β -arrestin in internalisation

The role of β -arrestin in internalisation became clearer with the finding that β -arrestin mutants inhibited β_2 AR internalisation and that over-expression of β -arrestin could rescue internalisation-impaired β_2 AR mutants (Ferguson *et al.* 1996). In addition, the discovery that β -arrestin interacts with clathrin confirmed that β -arrestin is involved in internalisation (Goodman *et al.* 1996), since activated β_2 ARs were found localised to clathrin-coated pits, which are known to be involved in internalisation (von Zastrow and Kobilka 1992). Thus, β -arrestins both desensitise agonist-activated GPCRs and by binding to clathrin, promote internalisation via clathrin-coated vesicles.

In addition to binding clathrin, β -arrestin also interacts with the β_2 subunit of the adapter protein AP-2 (Laporte *et al.* 2000). Two arginine residues (394 and 396) in β -arrestin are important for the interaction with AP-2. Mutation of these arginine residues in β -arrestin does not affect binding to the β_2 AR, but blocks the targeting of receptor- β -arrestin complexes to clathrin coated pits. Therefore, by binding to both clathrin and AP-2, β -arrestin acts as an adapter to link the receptors to the internalisation machinery (review: Shenoy and Lefkowitz. 2003).

1.7 Other roles of β -arrestin

In addition to the classical roles such as desensitisation and internalisation, β -arrestins have been shown to initiate signals from the same receptors that they 'desensitise' by binding to kinases and other regulatory proteins (Fig. 1.3). β -arrestins act as adapters for the Src-family kinases and as scaffolds for several Mitogen activated protein kinase (MAPKs), such as, ERK (extracellular signal-regulated kinase), JNK (c-Jun N-terminal kinase) and p38 MAPK modules (Shenoy and Lefkowitz. 2003).

MAPKs are the downstream terminal elements of highly conserved kinase cascades consisting of MAPKKKs, like Raf, or MAPKKs such as MEK, and MAPKs themselves. Each kinase is activated by a preceding kinase in the cascade (Morrison *et al.* 2003). GPCRs are connected to these MAPK signalling pathways by classical G protein-stimulated synthesis of second messengers. When MAPKs are activated they translocate from the cytosol to the nucleus, where they phosphorylate and activate transcription factors leading to responses, such as proliferation, differentiation, and many other cellular processes (Lefkowitz and Shenoy 2005).

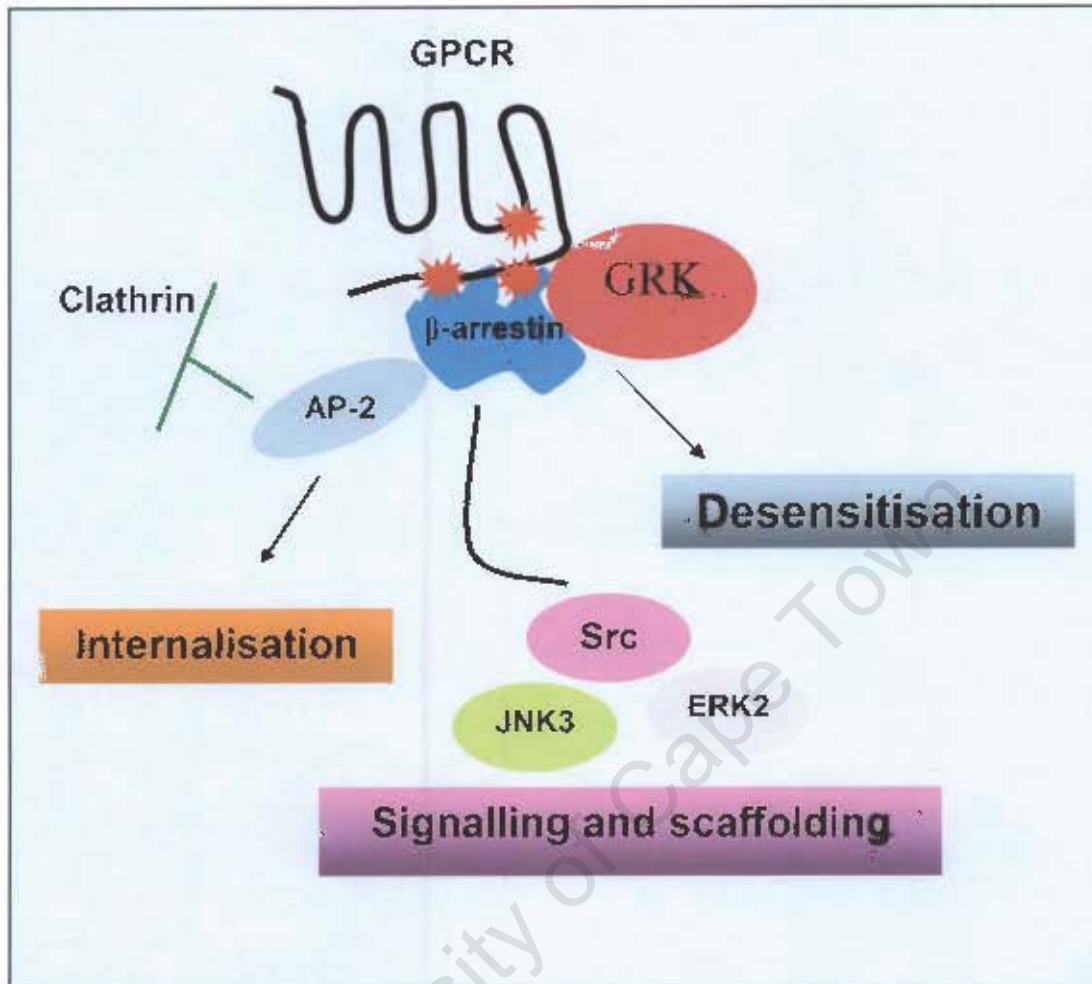


Fig. 1.3 Role of β -arrestins as signal transducers. β -arrestins not only mediate desensitisation of G protein–signalling but also act as signal transducers themselves. The binding of β -arrestin to agonist-activated GPCRs coincides with the recruitment and activation of several signalling molecules such as MAPKs, tyrosine kinases and PI3 kinases.

Studies on the β_2 AR demonstrated that β -arrestin forms a complex with c-Src, leading to the downstream activation of MAPK (Luttrell *et al.* 1999). Src has also been shown to phosphorylate dynamin after agonist binding to the β_2 AR (Ahn *et al.* 1999). In addition, expression of a catalytically inactive construct of Src blocks dynamin phosphorylation and impairs β_2 AR internalisation (Miller *et al.* 2000). Furthermore, agonist stimulation of

the neurokinin 1 receptor leads to a robust ERK activation at the membrane upon the formation of a complex of the receptor with β -arrestin, c-Src and ERK1/2 (Defea *et al.* 2000).

β -arrestins also play a critical role in the suppression of GPCR-mediated apoptosis. Signalling via the N-formyl peptide receptor (FPR), and other receptors such as the V2 vasopressin and angiotensin (type 1A) receptors initiates an apoptotic response that is blocked by β -arrestin (Revanker *et al.* 2004)

In addition, β -arrestin seems to be involved in chemotaxis mediated by the chemokine receptor CXCR4. The direct movement of lymphocytes towards chemokines is regulated specifically by GRK6-mediated phosphorylation and β -arrestin 2 binding to CXCR4 (Shenoy and Lefkowitz. 2003).

University of Cape Town

1.8 GnRH ligands and their receptors

1.8.1 GnRH ligands

GnRH I is a hypothalamic decapeptide that is responsible for regulating reproductive function. GnRH stimulates the pituitary secretion of luteinising hormone (LH) and follicle-stimulating hormone (FSH), which in turn regulate steroidogenesis and gametogenesis (Millar 2005). Apart from regulating reproduction, GnRH has diverse functions in vertebrates and protochordates. These include neuroendocrine (for, example growth hormone secretion in some fish species), paracrine (in gonads and placenta), autocrine (in GnRH neurons, immune cells, breast and prostatic cancer cells) and neurotransmitter/neuromodulatory roles in the central and peripheral nervous system (Millar 2005).

In addition to GnRH I, another form of GnRH designated GnRH II has been found in vertebrates. The peptide sequence of GnRH II differs from that of GnRH I at positions five, seven and eight (King and Millar, 1995; see alignment below).

GnRH-I (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂)

GnRH-II (pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH₂)

Fig. 1.4 Alignment of peptide sequences of mammalian GnRH I and GnRH II. The bold amino acids in blue highlight the variation between the peptide sequences of GnRH I and GnRH II.

GnRH II was identified from chicken brain (chicken GnRH II) and is ubiquitous and uniquely conserved in structure from bony fish to humans (Sherwood *et al.* 1993; Seaflon *et al.* 1997; Millar *et al.* 1987). The conservation of structure suggests that GnRH II has an important function which has selected against any change (Millar 2005).

Both GnRH I and GnRH II are present in the brains of most vertebrates (Millar 2005). The first evidence of this fact was observed in the musk shrew, where GnRH I and GnRH II were found in the mid brain of this placental mammal (Dellovade 1993). Unlike GnRH I, GnRH II is widely distributed in vertebrates, being identified in both hypothalamic and extra hypothalamic regions, such as the kidney, bone marrow (White *et al.* 1998), endometrium (Cheon *et al.* 2001), ovarian surface epithelial cells (Kang *et al.* 2003) and breast tissue (Chen *et al.* 2002). GnRH II is also widely distributed in the central and peripheral nervous systems suggesting a neurotransmitter/neuromodulatory role (Troskie *et al.* 1997). In addition, GnRH II may be involved in sexual arousal in rodents (Rissman *et al.* 1997) and reproductive behaviour in ring doves (King and Miller. 1997), song sparrows (Maney *et al.* 1997) and musk shrews (Temple *et al.* 2003). Interestingly, the cognate receptor for GnRH II, recently cloned in our laboratory from the marmoset, was distributed in areas of the primate brain associated with reproductive behaviour (Millar *et al.* 2001).

Both GnRH I and GnRH II interact with their cognate receptors, the type I and type II GnRH receptors, respectively.

1.8.2 GnRH receptors

The type I GnRH receptor belongs to a family of G protein-coupled receptors (GPCRs) and is expressed mainly in the gonadotropes of the anterior pituitary, where it interacts with GnRH I leading to the secretion of the two gonadotropins, LH and FSH. The mammalian type I GnRH receptor is the best characterised GnRH receptor and is a unique GPCR in that it lacks a C-terminal tail (see review; Millar 2005).

The sequence of the type I GnRH receptor was first realised for the mouse receptor cloned from the pituitary α T3-1 gonadotrope cell line (Tsutsumi *et al.* 1992). Subsequently, several type I GnRH receptor cDNAs have been cloned from different

species including human (Kakar *et al.* 1992; Chi *et al.* 1993) and sheep (Illing *et al.* 1993). The type I GnRH receptor has also been cloned from the cow and pig and all these mammalian GnRH receptors share an amino acid sequence homology of over 80% (reviewed in Millar 2005).

Mammalian type I GnRH receptors differ from non-mammalian type I GnRH receptors in that they do not possess a C-terminal tail. Furthermore, there is poor conservation of sequence of the type I receptor between mammalian species and amphibians compared to amphibians and bony fish which share closer homologies. This result suggests acceleration in evolutionary change in the mammals which could have been due to the loss of the C-terminal tail (Millar 2005). The absence of the C-terminal tail may serve an important role in the functioning of the mammalian GnRH receptor.

The type I GnRH receptors possess cysteine residues in ECL1 and ECL2 which form disulphide bonds. In the human type I GnRH receptor, two disulphide bridges were identified between Cys 101 (ECL1) and Cys 196 (ECL2) and between Cys 14 (N-terminus) and Cys 200 (ECL2). The disulphide bond between ECL1 and ECL2 has been shown to be important for receptor function (Davidson *et al.* 1997). Mammalian type I GnRH receptors have conserved residues in TM2 and TM7; an asparagine residue found in TM2 and an aspartate in TM7 (which is part of DPLIY motif that is equivalent to the NPXXY motif conserved in most GPCRs). These residues have been shown to play an important role in receptor expression, activation and G protein coupling (Flanagan *et al.* 1999).

Equivalent to the conserved DRY sequence in most GPCRs is the DRS sequence at the cytoplasmic side of TM3 in the mammalian type I GnRH receptors. This conserved sequence is involved in receptor activation (Arora *et al.* 1995). Ala 261, located in the ICL3 of the type I GnRH receptor is required for coupling to $G_{\alpha q/11}$ in order to stabilise receptor conformation necessary for G protein interaction (Myburgh *et al.* 1998). In addition, a BBXXB motif (where B is a basic amino acid) in the ICL2 of the type I GnRH receptor is required for the coupling to $G_{\alpha s}$ (Arora *et al.* 1998).

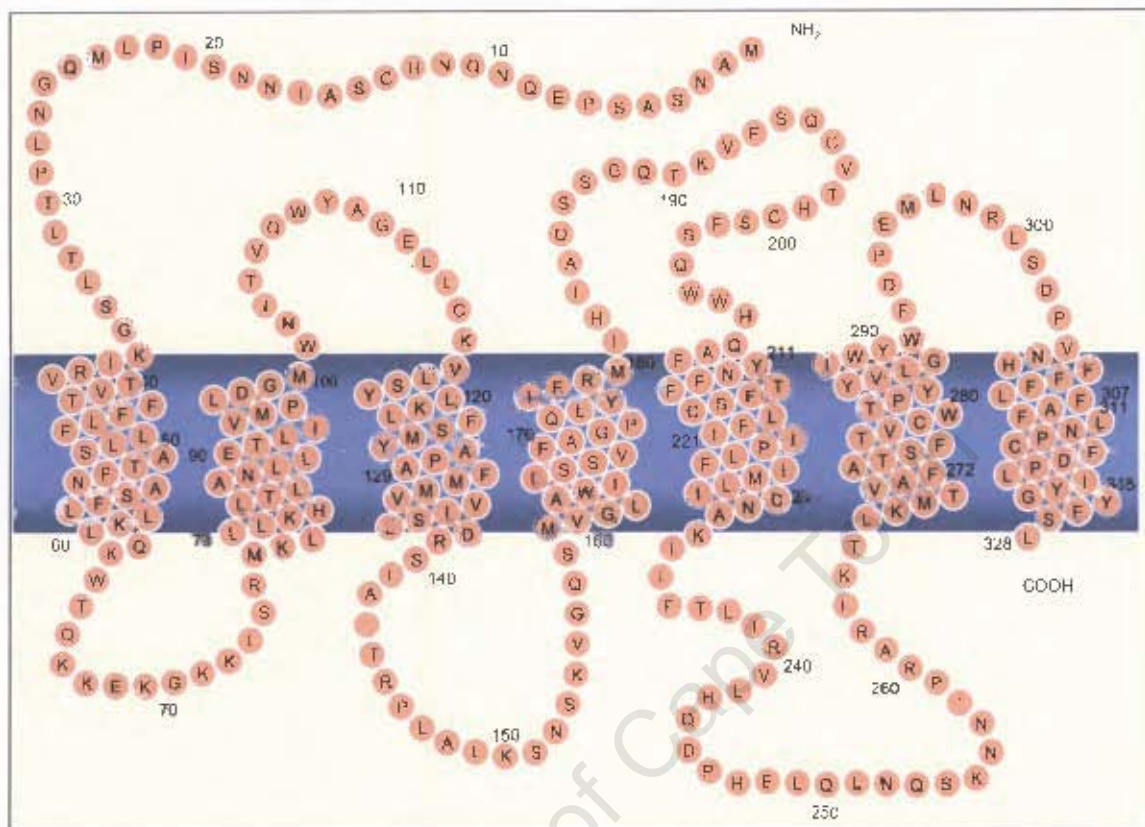


Fig. 1.5 Schematic representation of the human type I GnRH receptor. This receptor is a unique G protein-coupled receptor in that it lacks a carboxyl-terminal tail.

The type II GnRH receptor is also a G protein-coupled receptor; it was cloned and characterised from the marmoset monkey (*Callithrix jacchus*) in our laboratory (Millar *et al.* 2001). This receptor was also cloned from other species such as the rhesus monkey (*Macaca mulatta*), African green monkey (Neill *et al.* 2001), and more recently, the pig (reviewed in Neill JD *et al.* 2004). Human type II GnRH receptor transcripts have also been identified, however, these transcripts lack the ATG start codon and there is a stop codon within the reading frame, suggesting that the receptor is not functional in humans (Faurholm *et al.* 2001).

Unlike the type I GnRH receptor, the type II GnRH receptor has a C-terminal tail, which functions as a regulatory domain in most GPCRs. The C-terminal tail of the marmoset

type II GnRH receptors consists of 55 amino acid residues (Fig. 5). It has several serine and threonine residues which provide a consensus sequence for GRK-mediated phosphorylation. Other kinases that may phosphorylate the GnRH receptor on its C-terminal tail are PKA, PKC and casein kinase II.

The type II GnRH receptor has 41% sequence identity compared to the type I GnRH receptor and is highly selective for GnRH II. GnRH II binds the type II GnRH receptor with an affinity that is about 24 fold greater than the type I GnRH receptor. Similar to GnRH II, the type II GnRH receptor is expressed throughout the brain including areas associated with sexual arousal and in diverse non-neural and reproductive tissues (Millar *et al.* 2001).

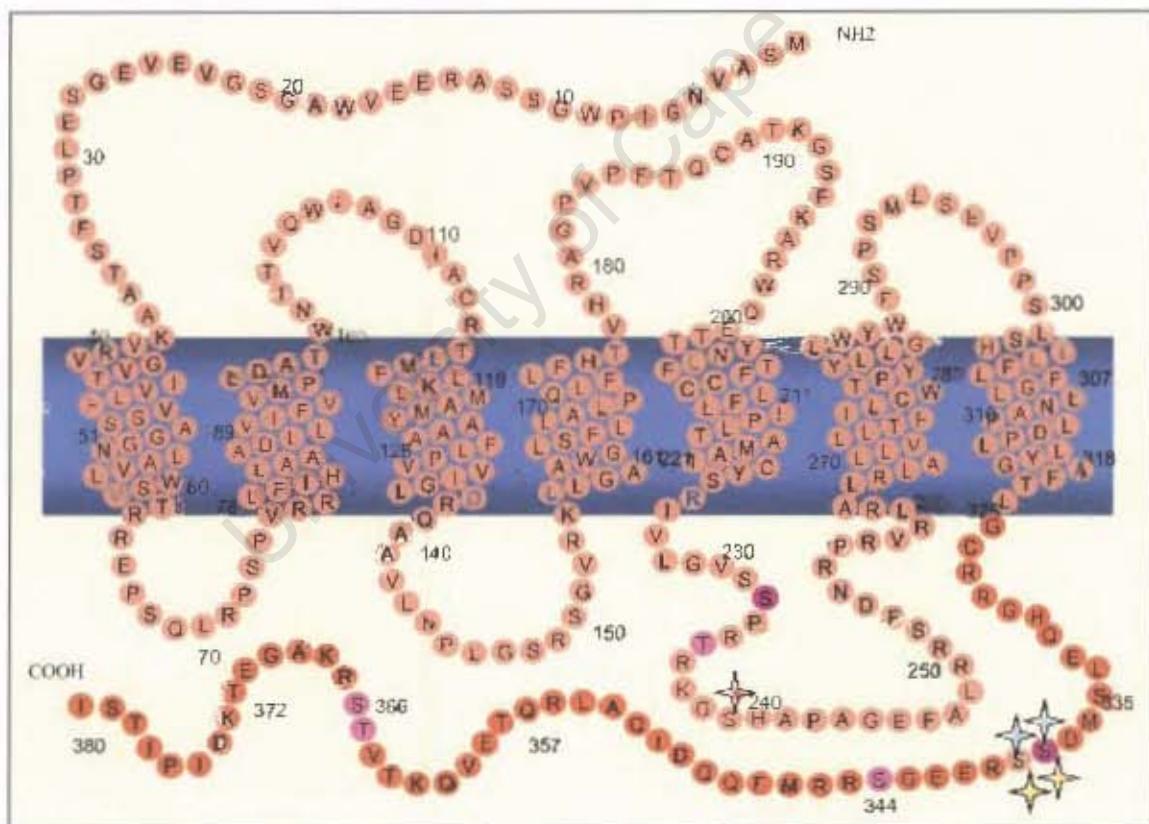


Fig. 1.6 Schematic representation of the marmoset type II GnRH receptor. Unlike the mammalian type I GnRH receptor, this receptor possesses a carboxyl-terminal tail. The purple residues indicate putative PKC phosphorylation sites and the red star indicates a putative PKA phosphorylation site. Putative CKII and GRK phosphorylation sites are indicated by the blue and yellow stars, respectively.

The type II GnRH receptors and the non-mammalian type I GnRH receptors have a conserved Asp/Asp motif in TM2 and TM7 (Millar 2005). Furthermore, the mammalian type II GnRH receptors also have a DRQ sequence, an equivalent to the highly conserved DRY in most GPCRs and DRS in the mammalian type I GnRH receptors in TM3 (Arora *et al.* 1995). Disulphide bridges for the type II GnRH receptor have not been demonstrated.

1.8.3 GnRH receptor signalling

GnRH receptors can signal via two main pathways which are G protein mediated signalling and MAPK signalling.

Upon exposure to agonist, both the type I and type II GnRH receptors predominantly activate $G\alpha_q$ and $G\alpha_{11}$ G proteins, leading to the stimulation of Phospholipase C β . Phospholipase C β hydrolyses phosphatidyl 4,5-bisphosphate (PIP₂) to Inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). IP₃ binds to IP₃ receptors on the endoplasmic reticulum (ER) membrane, leading to the mobilisation of Ca²⁺ from the ER into the cytoplasm and the activation of PKC via DAG (Fig. 1.7).

The type I GnRH receptor has been reported to activate the $G\alpha_s$ sub-family of G proteins. Activation of $G\alpha_s$ leads to the stimulation of adenylyl cyclase and PKC which subsequently increase cAMP levels (Arora *et al.* 1998).

It has been shown for these receptors that the G protein signalling pathway can converge with the MAPK signalling pathway at different levels. Ca²⁺ release can stimulate CaM kinase II, which phosphorylates and inactivates Ras-GTPase activating protein (Ras-GAP), leading to Ras and MAPK activation (Chen *et al.* 1998).

ERK activation via PKC has been identified for the type I GnRH receptor in pituitary cells (Reiss *et al.* 1997). JNK activation through the type I GnRH has also been shown to be PKC dependent. However, it seems that the role of PKC is less dominant in this pathway since PKC inhibitors only diminished PKC activity by 70% whereas protein tyrosine kinase inhibitors abolished JNK activity completely. Therefore, it emerges that

after GnRH activation the principle mediator for JNK activation is the non-receptor tyrosine kinase, Src (Levi *et al.* 1998). In view of the fact that the mammalian type I GnRH receptor does not utilise β -arrestin, the interaction of this receptor with Src may be occurring directly, as observed with the β_2 AR (Fan *et al.* 2001).

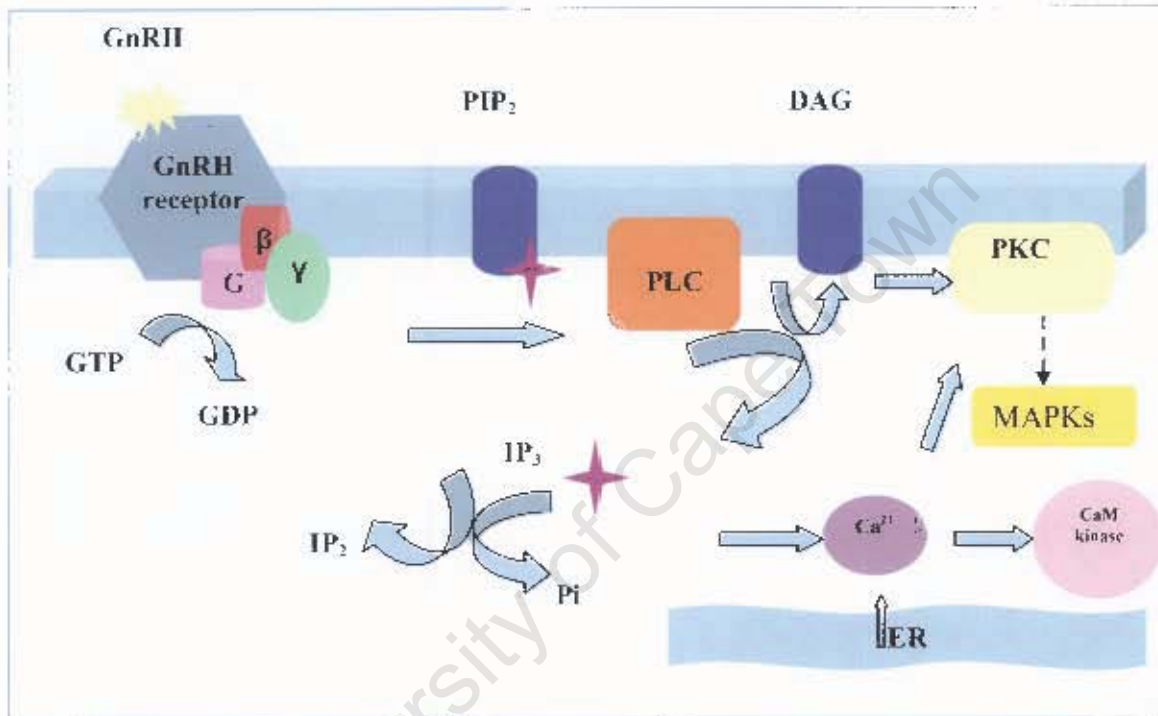


Fig. 1.7 Signalling of GnRH receptors. The agonist-activated GnRH receptor induces the activation of phospholipase C (PLC), leading to the cleavage of PIP₂ to DAG and IP₃. This is then followed by Ca²⁺ release (from endoplasmic reticulum stores) and the activation of PKC and CaM kinase. Activation of PKC may lead to the downstream activation of MAPKs.

The type II GnRH receptor can also activate ERK and p38, although the precise mechanisms are not yet known. In COS 7 cells, the activation of ERK by the type II GnRH receptor compared to the type I GnRH receptor was similar, however, it was observed that the type I GnRH receptor could not activate p38 MAPK (Millar *et al.*

2001). This could be cell-type dependent since in α -T3-1 cells, the type I GnRH receptor is able to activate p38 MAPK (Roberson *et al.* 1999).

1.8.4 GnRH receptor desensitisation and internalisation

Unlike most GPCRs, the mammalian type I GnRH receptor lacks the C-terminal tail that is believed to play an important role in desensitisation and internalisation. It is therefore no surprise that agonist-induced phosphorylation and β -arrestin binding have not been possible to demonstrate for the type I GnRH receptor. Furthermore, the mammalian type I GnRH receptors do not readily undergo desensitisation (Heding *et al.* 1998) and exhibit slow internalisation (Pawson *et al.* 1998; Vrecl *et al.* 1998; Willars *et al.* 1999).

Non-mammalian GnRH receptors do possess C-terminal tails and studies on these receptors including the catfish GnRH receptor (Heding *et al.* 1998) and the *Xenopus* GnRH receptor (Hislop *et al.* 2000) have shown that these receptors do undergo rapid desensitisation. The non-mammalian GnRH receptor C-terminal tails contain multiple serine and threonine sites which are potential phosphorylation sites by PKA, PKC and GRKs (McArdle *et al.* 1999). In addition, it was demonstrated that the catfish GnRH receptor could become phosphorylated on its C-terminal tail (Blomenrohr *et al.* 1999; Willars *et al.* 1999) and removal of these phosphorylation sites decreased internalisation (Blomenrohr *et al.* 1999). Interestingly, when the C-terminal tail of the catfish GnRH receptor was added onto the human type I GnRH receptor, this chimera demonstrated agonist-induced phosphorylation and rapid desensitisation (Willars *et al.* 1999).

Furthermore, the addition of the catfish GnRH C-terminal tail to the rat type I GnRH receptor, which does not rapidly desensitise, caused the chimera to desensitise within minutes of agonist stimulation. In addition, the rate of internalisation of this chimera was increased compared to the internalisation of the rat GnRH receptor (Heding *et al.* 1998). It was also shown via *in vivo* phosphorylation studies that GnRH does not induce phosphorylation of rat GnRH receptors but increases the phosphorylation of catfish

GnRH receptors, and of rat GnRH receptor chimeras with C-terminal tails of TRH receptors or catfish GnRH receptors (Willars *et al.* 1998). These results further demonstrate that the C-terminal tail is an important domain in receptor desensitisation and internalisation.

The C-terminal tail also plays an important role in the internalisation kinetics of the non-mammalian GnRH receptors which internalise more rapidly compared to the tail-less mammalian GnRH receptors (Heding *et al.* 1998). In COS cells, the chicken GnRH receptor internalises much faster than the tail-less human type I GnRH receptor. In addition, truncation of the chicken GnRH receptor C-terminal tail causes the receptor to internalise to the same extent as the human GnRH receptor (Pawson *et al.* 1998). Similarly, recent studies have shown that truncation of the marmoset type II GnRH receptor C-terminal tail at serine 335 markedly impairs the internalisation of this receptor (Ronacher *et al.* 2004). At early time points the internalisation of the receptor mutant was 75% lower than that of the wild type receptor. Interestingly, the receptor mutant truncated at serine 344 internalised like the wild type receptor, demonstrating that the region between serine 335 and serine 344 is critical for rapid internalisation. The serine residues at positions 338 and 339 fall in this region and were identified as critical for the rapid internalisation of the type II GnRH receptor. Mutation of these sites caused a decrease in internalisation equivalent to that caused by truncating the C-terminal tail at serine 335. Serines 338 and 339 were identified as putative GRK phosphorylation sites and are part of a consensus sequence for PKC and CKII, therefore these residues are important for receptor internalisation (Ronacher *et al.* 2004), as they undergo phosphorylation.

1.9 GnRH receptors and β -arrestin dependent internalisation

As previously discussed, for most GPCRs, β -arrestin binding to the receptor follows phosphorylation. For non-mammalian GnRH receptors, the presence of the C-terminal tail seems to be the determining factor for β -arrestin interaction. Agonist activation of the catfish and *Xenopus* GnRH receptors seems to mediate β -arrestin interaction with these

receptors and enhanced internalisation in the presence of β -arrestin (Blomenrohr *et al.* 1998; Heding *et al.* 2000; McArdle *et al.* 2002). The serine residue at position 363 of the catfish GnRH receptor and the last 12 residues of the C-terminal tail were shown to be important for β -arrestin dependent internalisation (Blomenrohr *et al.* 1999).

The tail-less GnRH receptors do not seem to interact with β -arrestin and do not show increased internalisation in the presence of β -arrestin (Vrecl *et al.* 1998; McArdle *et al.* 2002). However, addition of C-terminal tails to these GnRH receptors can cause them to internalise in a β -arrestin dependent manner. The rat type I GnRH receptor was able to internalise in a β -arrestin dependent manner on addition of a thyrotropin releasing hormone (TRH) receptor C-terminal tail (Heding *et al.* 2000; Hanyaloglu *et al.* 2001). Unlike the TRH C-terminal tail, the catfish GnRH C-terminal was unable to change the β -arrestin independent internalisation of the rat GnRH receptor (Hanyaloglu *et al.* 2001). A sequence comparison between the TRH receptor and the catfish GnRH receptor C-terminal tails revealed that both receptors contained consensus sites for PKC but there were no casein kinase II consensus sites on the catfish GnRH receptor C-terminal tail. Addition of the three casein kinase II consensus sites (found on the TRH receptor) to the C-terminal tail of the rat/catfish GnRH chimera enabled the chimera to internalise in a β -arrestin dependent manner (Hanyaloglu *et al.* 2001). These results indicate that phosphorylation by GRK is not the only way that β -arrestin can interact with the C-terminal tail of these receptors, but other kinases, such as casein kinase II can mediate β -arrestin binding.

The marmoset type II GnRH receptor internalises in a β -arrestin independent pathway in COS-1 cells but over-expression of β -arrestin 1 and 2 enhances the internalisation of this receptor, demonstrating that the type II GnRH receptor can internalise in a β -arrestin dependent manner. However, when the C-terminal tail of the type II GnRH receptor was truncated, receptor internalisation, in the presence of β -arrestin, decreased while β -arrestin dependency remained unchanged (Ronacher *et al.* 2004). The type II GnRH receptor could still utilise β -arrestin even without its C-terminal tail, indicating that there could be other domains involved in β -arrestin binding, such as the intracellular loops.

The investigation of the mammalian type II GnRH receptor with respect to β -arrestin has not been completed. What is known so far is that this receptor can utilise β -arrestin in the presence of exogenous β -arrestin in COS-1 cells and that the C-terminal tail is not required for β -arrestin dependent internalisation.

Therefore, the aim of this project is to identify the domains on the type II GnRH receptor that are required for β -arrestin dependent internalisation and to determine whether the type II GnRH receptor C-terminal tail can confer β -arrestin dependent internalisation to the tail-less mammalian type I GnRH receptor.

Since the mammalian type I GnRH receptor does not utilise β -arrestin in its internalisation, even in the presence of exogenous β -arrestin, whereas the type II GnRH receptor can utilise β -arrestin in its internalisation, chimeric receptor constructs of both mammalian GnRH receptors may provide insight into β -arrestin interaction domains on the type II GnRH receptor. Creating chimera constructs incorporating the type I and type II GnRH receptor domains may help in determining whether it is the absence of the C-terminal tail that causes the type I GnRH receptor to internalise in a β -arrestin independent manner. Furthermore, the domains that are responsible for β -arrestin interaction on the type II GnRH receptor may be identified by replacing specific regions of the receptor with corresponding regions from the type I GnRH receptor, and seeing how β -arrestin dependent internalisation is affected.

2. MATERIALS AND METHODS

2.1 Site-directed mutagenesis and chimera construction

Both the type I and the type II GnRH receptor were cloned in the expression vector pcDNA3.1(+) (see Appendix 6.3) between the Eco RI and Xba I restriction sites. The gene specific primers were designed in the laboratory and are complementary to sections of the type I and type II GnRH receptors. The vector specific primers are complementary to the pcDNA3.1(+) sequence. PCR was employed for the generation of mutants and chimeras.

PCR conditions included an initial 5 minutes at 94°C and 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 2 minutes at 72°C. The final extension was performed at 72°C for 10 minutes. The DNA polymerase, Deep vent® (New England Biolabs, UK) was used for normal PCR reactions while Taq polymerase was used for colony PCR. For bridge PCR, there was an initial cycle (without primers) of 1 minute denaturation at 94°C and 15 minutes of annealing at 50°C and 5 minutes of extension at 72°C to allow for adequate annealing of complementary sections of fragments to be joined. Following this cycle, a normal PCR reaction would be performed with conditions as mentioned above.

For the ligation of fragments into the vector, a FASTlink® DNA ligation kit (Epicentre Technologies, Madison, USA) was used and the ligation mixture was transformed into competent *E. coli* DH10B cells using a standard heat shock protocol. The transformed cells were plated out on 2xYT agar plates (see Appendix 6.5) and colonies were picked the next day. The colonies that were positive for the gene and mutation were identified via PCR. Plasmid DNA was isolated and purified by using the Nucleobond PC 500 maxi prep kit (Macherey-Nagel, Germany).

The DNA was quantified using a UV spectrophotometer. To confirm the sequences of the different constructs, the DNA was sent to a sequencing lab.

2.1.1 Addition of the type II GnRH receptor C-terminal to the type I GnRH receptor

To create the chimeric receptor of the human type I GnRH receptor incorporating the marmoset type II GnRH receptor C-terminal tail (T1/T2tail) (Fig. 2.3), a primer integrating both the receptor sequences was designed. The 71 base pair chimeric primer included the last C-terminal 52 bases of the type I GnRH receptor (T1) cDNA (933-984) and the first 19 bases of the marmoset type II GnRH receptor (T2) C-terminal tail (976-994) (see Appendix 6.1.2). This sense primer incorporated a Dra I site that was part of the T1 receptor sequence to facilitate cloning. A PCR reaction was performed using the chimeric sense primer and a vector specific antisense primer Bgh (see Appendix 6.1.1), with T2 in pcDNA3.1(+) as template (Fig. 2.1(a)).

The fragment obtained from the PCR amplification was cleaved at the Dra I and Xba I (Fig. 2.1 (b)) and the T1 in pcDNA3.1(+) was digested with Eco RI and Dra I (Fig. 2.1(c)). The digested PCR product and T1 fragment were ligated into the pcDNA3.1(+) expression vector which was digested with Eco RI and Xba I (Fig. 2.1 (d)).

The constructed chimera sequence was confirmed by direct sequencing.

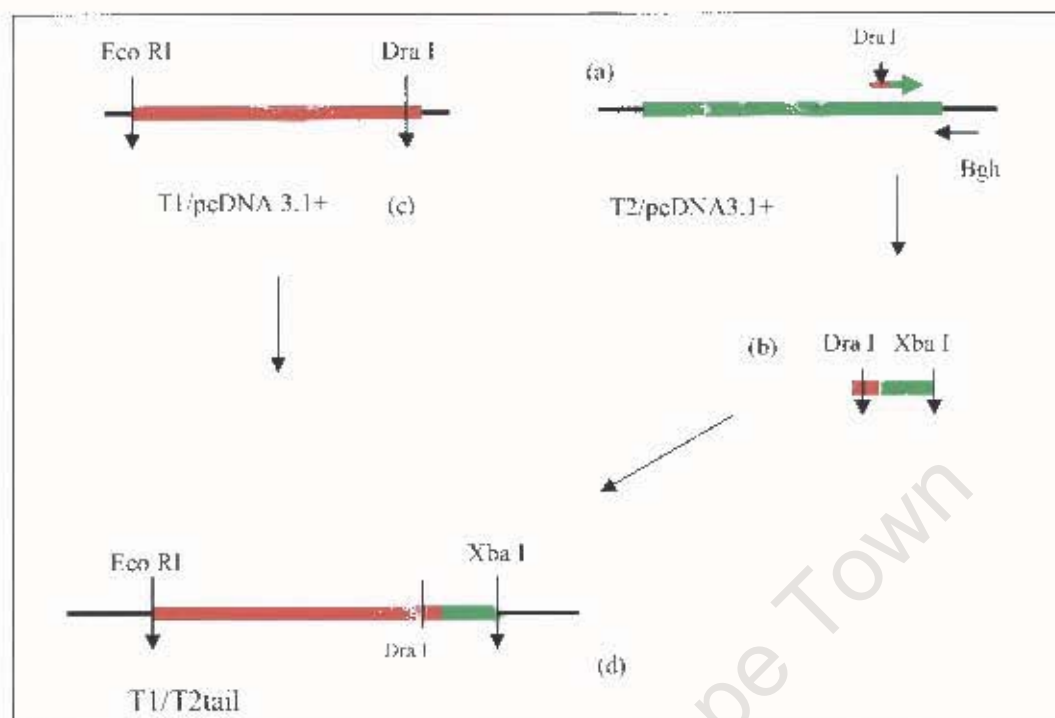


Fig. 2.1 Construction of the type I GnRH receptor (T1) incorporating the type II GnRH receptor (T2) C-terminal tail (T1/T2tail). The red lines represent the type I GnRH receptor, the green lines represent the type II GnRH receptor and the black lines represent the vector pcDNA3.1(+). (a) PCR amplification of T2 with a vector primer, Bgh, and the chimeric primer. (b) PCR fragment obtained from (a) cleaved at Dra I and Xba sites. (c) Digestion of T1 at Eco RI and Dra I to prepare for ligation. (d) three-piece ligation of fragment (b), (c) and the vector pcDNA3.1(+). (c) and the vector pcDNA3.1(+)

For the T1/T2tail chimera with serine residues at 338 and 339 (Fig. 2.4) mutated to alanine residues (T1/T2tailSA), the T1/T2tail chimera was used as a template and site directed mutagenesis was utilised to replace the two serine residues with alanine residues. Both sense and antisense primers (S338,339A s and S338,339A as) were previously designed in the laboratory (Ronacher *et al.* 2004) (see Appendix 6.1.3). These primers, together with the vector primers, T7 and Bgh were used in a PCR reaction to amplify both C-terminal and N-terminal ends of the receptor, yielding two fragments which contained the desired mutations. The resulting fragments were spliced by bridge PCR using the vector specific primers and again were digested with Eco RI and Xba I and sub

cloned into pcDNA3.1(+) between the respective sites. The sequence of the chimera with the serine sites mutated to alanine was confirmed by direct sequencing.

2.1.2 Construction of the type II GnRH receptor chimera incorporating the third intracellular loop of the type I GnRH receptor

For chimeric receptor of the type II GnRH receptor incorporating the full length of the type I GnRH receptor third intracellular loop (Fig. 2.3) (see Appendix 6.2 for amino acid sequence from TM5 to TM6), bridge PCR was again utilised. Primers integrating sequences of both the type I and type II GnRH receptor sequences were designed. For the full length exchange of loops, the type I GnRH receptor loop region was amplified by chimeric primers (see Appendix 6.1.4) in a PCR reaction. This process yielded a fragment that had the full type I GnRH receptor third intracellular loop flanked by sequences complementary to the type II GnRH receptor on either side (Fig. 2.2 (a)). The chimeric fragment obtained from the PCR reaction (ii) was then joined to the PCR generated sequences (i) and (iii) (Fig. 2.2(b)) of the type II GnRH receptor via a two step bridge PCR reaction (Fig. 2.2(b)). The complete chimeric receptor gene was then digested with restriction enzymes Eco RI and Xba I and sub-cloned into pcDNA3.1(+) at corresponding sites (Fig 2.2 (c)). The sequence of the PCR product was confirmed by direct sequencing.

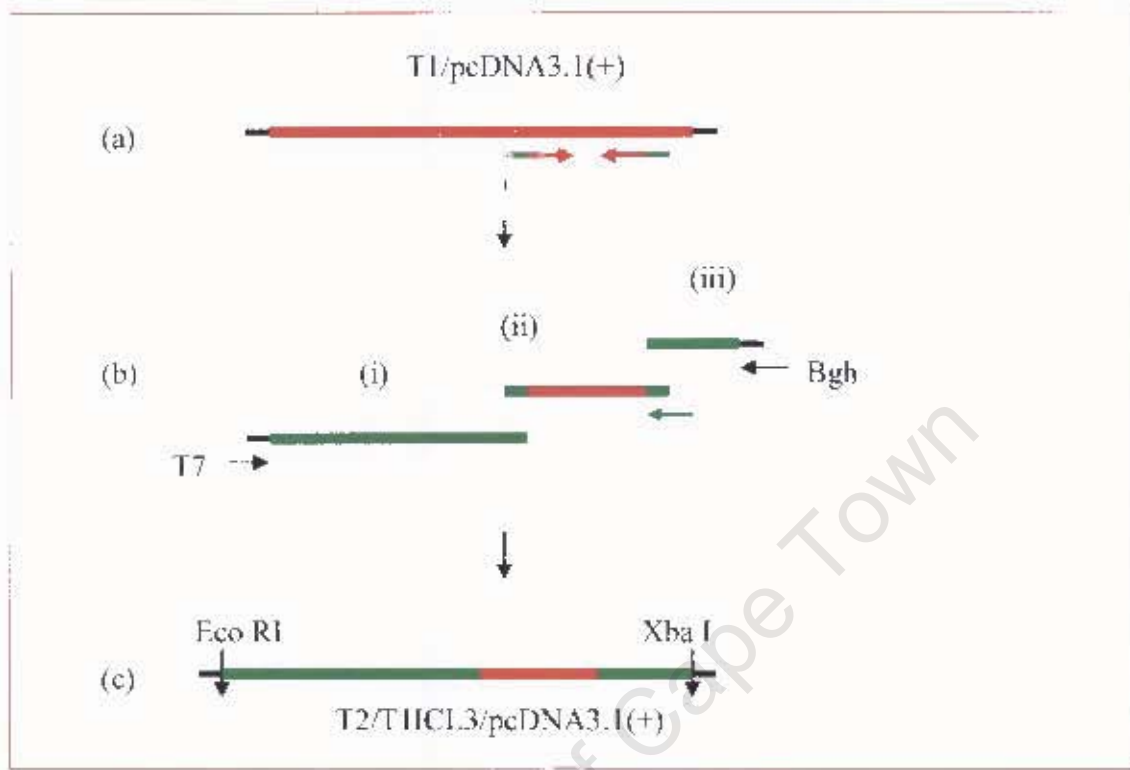


Fig. 2.2 Construction of the type II GnRH receptor chimera incorporating the third intracellular loop of the type I GnRH receptor (T2/T1ICL3). The red line represents the type I GnRH receptor, the green lines represent the type II GnRH receptor and the black lines represent the cloning vector pcDNA3.1(+).





RECEPTOR CONSTRUCTS	
	Type I GnRH receptor (T1)
	Type II GnRH receptor (T2)
	T1 with T2 C-terminal tail (T1/T2tail)
	T2 with T1 third intracellular loop (T2/T1ICL3)

Fig. 2.3 Schematic representation of the type I and type II GnRH receptors as well as the receptor chimeras constructed in this project.

2.1.3 Alanine substitutions of the basic residues in the third intracellular loop of the type II GnRH receptor

The three basic residues in the third intracellular loop of the type II GnRH receptor (R234, R236 and K237 (Fig. 2.3)) were mutated to alanine residues via site-directed mutagenesis and bridge PCR. Sense and antisense primers containing the desired mutation and silent restriction sites were designed (see Appendix 6.1.5). Vector primers, T7 and Bgh together with the gene specific primers amplified the specific fragments which were joined by bridge PCR. The individual and the double mutations (R234,R236A) were introduced on wild type receptor. The triple mutation (R234,R236,K237A), however, was achieved by using the double mutant (R234,R236A) as a template and introducing one more mutation (K237A). The resulting mutated type II

GnRH receptor genes were digested with Eco RI and Xba I and cloned into pcDNA3.1(+).

All the sequences of the mutants were confirmed by direct sequencing.

2.1.4 Truncation of C-terminal tail of the triple mutant R234,R236, K237A

For the C-terminal tail truncation of the mutant receptor (R234,R236,K237A), a previously designed (Ronacher *et al.* 2004) gene specific anti sense primer was used (see Appendix 6.1.6). This primer had the codon, serine 335 (see location in Fig. 2.3), replaced by a stop codon and followed by an Xba I site. The receptor was cleaved at the Xba I to generate a truncated receptor, having a C-terminal tail of only nine residues. A PCR reaction was performed with the anti sense primer and a vector specific sense primer (T7). The resulting fragment was cleaved at the newly introduced Xba I site and Eco RI, and cloned into pcDNA3.1(+) at the corresponding sites. The truncation of the tail was confirmed via sequence analysis.

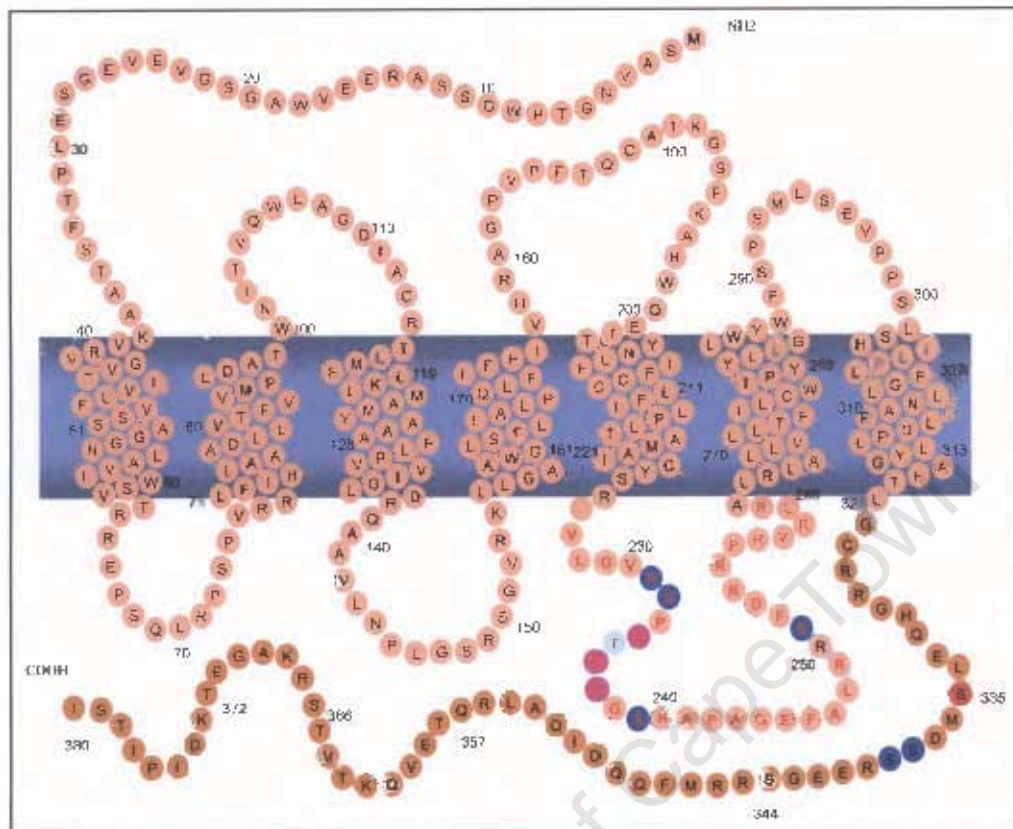


Fig. 2.4 Summary of the mutations introduced to the marmoset type II GnRH receptor. The amino acids represented as brown circles correspond to the C-terminal tail. The third intracellular loop is depicted by amino acid residues in red font. The basic residues (R234, R236, and K237) that were mutated to alanine residues in the third intracellular loop are shown in pink. The deep blue colour represents serine residues in both the third intracellular loop (S231, S232, S239, S251) and the C-terminal tail (S338, S339) that were mutated to alanine residues. The threonine residue (T235) in the third intracellular loop is shown in pale blue. The serine residue (S335) that was mutated to a stop codon (where the receptor was truncated) is shown in red.

2.2 Cell culture and transient transfections

COS-1 cells were grown in 25cm² flasks (Corning) in antibiotic-free low glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, San Diego, USA) containing 10% heat inactivated fetal calf serum (Delta Bioproducts, Kempton Park, SA). Cells were maintained in a 10% CO₂ incubator at 37°C.

The cells were passaged regularly; a minimum of twice a week when confluence was observed. For passaging, the cells were treated with 0.25% trypsin, 0.04% EDTA in phosphate buffered saline (PBS) to detach them from the flask.

For experiments, COS-1 cells were trypsinised and resuspended in DMEM/10%FCS with 1% P/S (2mg/ml streptomycin sulfate and 4000U/ml sodium benzyl penicillin). An aliquot of cells were counted using a hemocytometer.

A day before the DNA transfections, the cells were trypsinised and resuspended in DMEM containing 10% FCS and 1% P/S. The cells were then plated out on poly-D-lysine-coated 12 well plates (celstar) to a density of 2 X 10⁵ cells per well. The cells were placed in a CO₂ incubator at 37°C overnight. Twenty-four hours later the cells were transfected with 2µg/well of the indicated DNA constructs in serum free DMEM for 4 hours using a diethylaminoethyl (DEAE)-dextran method (Keown *et al*, 1990) as previously described (Millar *et al*, 1995). For co-transfections 1µg/well receptor DNA and 1µg/well β-arrestin or empty vector were added. In experiments that included the T1/T2tail chimeric receptor, the concentration of β-arrestin was increased to twice that of receptor DNA, to compensate for the increased expression of this chimera.

A 1:10 dilution of HBS/DEAE-dextran (see Appendix: 6.5) was added to DMEM with P/S and the DNA.

The incubation of cells in medium containing DEAE-dextran facilitates the formation of complexes that are taken up by the cells through endocytosis. After the incubation, the cells were incubated for another hour at 37°C in DMEM (with 2% FCS) containing chloroquine (200µM) (see Appendix 6.3). Chloroquine is included in order to inhibit lysosomal degradation of the transfected DNA.

The cells were then rinsed with a solution of HEPES/DMEM (10mM HEPES pH 7.2 buffered DMEM) containing P/S (1ml/well, after which they were transiently exposed to 10% dimethyl sulphoxide (DMSO) in DMEM for 2 minutes. This step serves to 'shock' the cells, facilitating DNA uptake. A solution of HEPES/DMEM was used to wash the cells before the growth medium (DMEM, 10% FCS, P/S) was added. Cells were grown at 37°C in a 10% incubator for 48 hours after the transfection, prior to being assayed.

2.3 Iodination of the GnRH agonist

The high affinity agonist [His⁵, D-Tyr⁶]-GnRH was radioactively labelled with NaI¹²⁵ (1mCi; Amersham, Aylesbury, UK) using the chloramine-T method as previously described (Flanagan *et al.* 1998). The iodinated peptide was purified using a G25 sephadex column.

2.4 Receptor radioligand binding assay

To measure the expression of the receptor constructs expressing the receptor constructs in COS-1 cells, a whole-cell competition binding assay was performed as previously described (Pawson A, 1998). Cells were trypsinised and plated onto 12 well plates to a density of 2×10^5 cells per well. The following day, cells were transfected with the receptor constructs (2µg per well) and incubated at 37°C in a 10% CO² incubator. The media was changed the next day. 48 hours after the transfection, the binding assay was performed. The cells were rinsed with HEPES-DMEM at 4°C, after which 0.5ml of media containing radiolabelled peptide (¹²⁵I-[His⁵, D-Tyr⁶]-GnRH) with total counts of 100000cpm/0.5ml together with unlabelled peptide was added to the cells. The unlabelled peptide (GnRH II and GnRH I) was diluted to different concentrations, from 10⁻⁵ M to 10⁻¹¹ M. The cells were incubated at 4°C for 5 hours, and subsequently washed with ice-cold PBS to separate unbound peptide from cell-surface bound peptide.

The cells were then solubilised with 0.5ml 1M NaOH for 10 minutes and transferred to counting tubes. Radioactive bound peptide was measured by counting tubes in a Gamma counter and the data was analysed using GRAPHPAD Prism[®] software.

2.5 Total inositol phosphates assay

COS-1 cells (2×10^5 cells/well) transfected with 2 μ g DNA of the different constructs, were 24 hours later labelled with 2 μ Ci/ml of 3H-myoinositol (Amersham, UK) in medium 199 (Invitrogen, San Diego, USA) supplemented with 2% FCS. The cells were then incubated at 37°C for 20 hours. Tritium tagged myo-inositol acts as a tracer for the amount of inositol produced during the assay as it gets incorporated into PIP₂. This assay was performed as previously described (Millar *et al.* 1995). The next day, the cells were washed with Buffer I (140mM NaCl, 4mM KCl, 20mM Hepes, 8mM glucose, 0.1% BSA, 1mM MgCl₂, 1mM CaCl₂, 10mM LiCl) for 15 minutes at 37°C, after which they were stimulated with different concentrations (10^{-11} – 10^{-6} M) of GnRH peptide in Buffer I. This stimulation step was performed for 60 minutes at 37°C. LiCl is included in the buffer because it inhibits the cleavage of inositol phosphates to inositol. The medium was then aspirated from the cells and 1ml of ice cold 10mM Formic acid was added to each well. The cells were placed at 4°C for 30 minutes, followed by loading of the samples onto washed Dowex 1x8-200 anion exchange columns (Sigma-Aldrich, Steinheim, Germany). The columns were washed with 10 ml distilled water followed by a rinse with 3ml of 3M ammonium formate with 0.1M formic acid. The resin was then washed again with 10 ml distilled water. After loading the samples, the columns were washed with 10 ml distilled water, followed by 5ml of 5mM myo-inositol with 0.1M formic acid. The inositol phosphates were eluted with 3ml of 1M ammonium formate with 0.1M formic acid into 16ml of scintillation fluid (Zinsser Analytical, Frankfurt, Germany) and the radioactivity was counted. EC₅₀ values were calculated using GRAPHPAD PRISM[®] software.

2.6 Internalisation assay

Receptor internalisation was measured by utilising a radiolabelled ligand. A high affinity agonist [His⁵, D-Tyr⁶]-GnRH (Flanagan *et al.* 1998) was labelled with a radioactive isotope of iodine (¹²⁵I) using the chloramine-T method as previously described (Millar *et al.* 1995). The internalisation assays were performed based on an acid-wash method developed in the laboratory (Pawson *et al.* 1998).

Cells plated in 12 well plates at a density of 2×10^5 cells/well were transfected with different receptor constructs. After 48 hours the cells were washed with ice cold PBS before being incubated with ¹²⁵I-[His⁵, D-Tyr⁶]-GnRH in HEPES-DMEM at 100000cpm/well and incubated at 4°C for 5 hours to allow binding. The cells were then transferred to 37°C for different time intervals (5, 10, 15, 30, 60 minutes) to allow for internalisation to take place. To terminate receptor internalisation and to remove unbound iodinated ligand, the cells were transferred to ice and washed twice with ice cold PBS. Cell-surface-bound labelled receptor was removed by incubating the cells in 0.5ml ice-cold acid solution (150mM NaCl, 50mM Acetic acid, pH 2.8) for 10 minutes. The acid wash was then collected and transferred to counting tubes. The cells were then solubilised in 0.5ml of 1M NaOH in order to determine acid resistant (internalised) labelled ligand. The solubilised cell extracts were transferred to counting tubes. Non-specific binding was determined by including mock transfected COS-1 cells in the assay and subtracting from total binding at each time point. The counting tubes were placed in a gamma counter which measures radiation emitted in counts per minute (cpm).

Internalisation of the radiolabelled analogue was expressed as the percentage of total cell surface-bound and acid-resistant ligand (internalised) at each time point. Data points were determined in duplicate and analysed using GRAPHPAD PRISM[®] software.

2.7 Statistical analysis

Statistical significance was determined using either a one-tailed or two-tailed t test (GRAPHPAD PRISM[®] software). The difference of means was considered significant at $P < 0.05$.

University of Cape Town

3. RESULTS

3.1 The type II GnRH receptor, in contrast to the type I GnRH receptor internalises in a β -arrestin dependent manner

Initial experiments in this project involved confirming the role of exogenous β -arrestin in the internalisation of the type I and type II GnRH receptors. Both the type I and type II GnRH receptors were co-expressed with β -arrestin 1 and the effect of β -arrestin 1 on receptor internalisation was investigated. The control was both the type I and type II GnRH receptors co-transfected with empty vector, in this case, pcDNA3.1(+). Internalisation was measured using a radiolabelled GnRH agonist as a tracer (section 2.6).

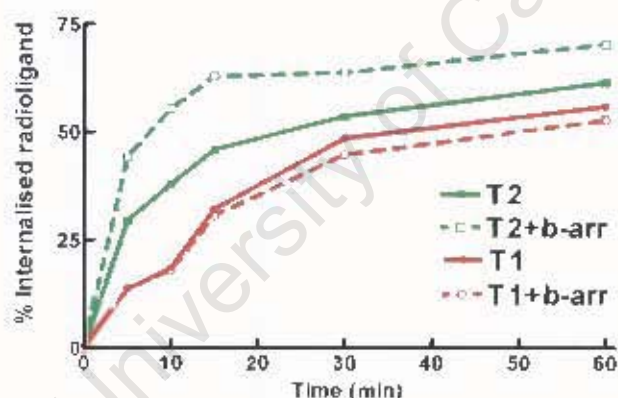


Fig. 3.1 Internalisation of the type I (T1) and type II (T2) GnRH receptors co-expressed with β -arrestin 1 in COS-1 cells. COS-1 cells were transiently transfected with $1\mu\text{g}$ receptor and $1\mu\text{g}$ β -arrestin per well. Internalisation is expressed as percentage internalised radioligand at different time points. Results are from a single representative experiment performed at least three times in duplicate. The range between duplicates in this experiment was small, therefore error bars are too small to be seen.

The internalisation of the type II GnRH receptor was significantly enhanced in the presence of β -arrestin compared to the type I GnRH receptor ($P < 0.05$; t test). Five

minutes after agonist stimulation, the internalisation of the type II GnRH receptor with β -arrestin was $50 \pm 5.4\%$ higher than the internalisation of the type II GnRH with empty vector (control) ($P < 0.05$). There was no significant change in internalisation for the type I GnRH receptor in the presence of β -arrestin, demonstrating that β -arrestin 1 is not involved in the internalisation of the type I GnRH receptor.

Since differences in the utilisation of β -arrestin in the internalisation of the type I and type II GnRH receptors could be due to the C-terminal tail, the next step was to investigate whether adding a C-terminal tail to the tail-less type I GnRH receptor would confer β -arrestin dependence.

3.2 The role of the type II GnRH receptor C-terminal tail in the internalisation of the type I GnRH receptor

3.2.1 The type II GnRH receptor C-terminal tail does confer β -arrestin dependency on the type I GnRH receptor in COS-1 cells

To investigate whether addition of the type II GnRH receptor C-terminal tail to the tail-less type I GnRH receptor confers β -arrestin dependent internalisation on the type I GnRH receptor, the type I GnRH receptor chimera incorporating the type II GnRH receptor C-terminal tail (T1/T2tail) was created using a PCR based method as detailed in section 2.1.1. The sequence of the PCR product was confirmed by direct sequencing.

Initially, COS-1 cells were co-transfected with the T1/T2tail chimera and β -arrestin 1 in a DNA ratio of 1:1. However, since this tail chimera was highly expressed compared to the wild type GnRH receptors (as determined from cell surface bound receptors in internalisation assays (section 2.6), the ratio of β -arrestin to receptor was increased from 1:1 to 2:1 to ensure that there were enough β -arrestin molecules to interact with the increased number of receptors. Increasing the ratio of β -arrestin to receptor did not alter the

internalisation of the type II GnRH receptor (positive control) or the type I GnRH receptor (negative control) compared to the original ratio of 1:1 (compare Fig. 3.1 (ratio 1:1) with Fig. 3.2 (ratio 2:1)).

As a control, instead of β -arrestin, receptors were co-transfected with an equivalent concentration of empty pcDNA3.1(+) vector.

In the presence of β -arrestin the internalisation of the T1/T2tail chimera showed a significant increase of $84\pm 20\%$ at 5 minutes and at 60 minutes, internalisation was $51\pm 19\%$ higher than T1/T2tail without β -arrestin ($P < 0.05$).

The extent of internalisation of the T1/T2tail chimera appears to be lower than both the wild type receptors because of increased expression of the chimera with the addition of the type II GnRH receptor C-terminal tail. Overall internalisation of the T1/T2tail chimera seems reduced, as a consequence of the method used to calculate percentage internalisation (Internalised receptor/ (Internalised receptor + Surface bound receptor)). Therefore, as cell surface bound receptor increases without necessarily increasing internalised receptor, the total percent of internalised receptor appears lower. As seen in the previous figure, the type I GnRH receptor, T1, does not internalise in a β -arrestin dependent manner whereas the type II GnRH receptor, T2, utilises β -arrestin in its internalisation in COS-1 cells. In the presence of β -arrestin 1, the internalisation of T2 was $107\pm 9.3\%$ higher at 5 minutes and at 60 minutes it was $40\pm 4.7\%$ higher than the internalisation of T2 with empty vector ($P < 0.05$).

In conclusion, the result demonstrates that the addition of the type II GnRH C-terminal tail to the type I GnRH receptor confers β -arrestin dependent internalisation to the type I GnRH receptor.

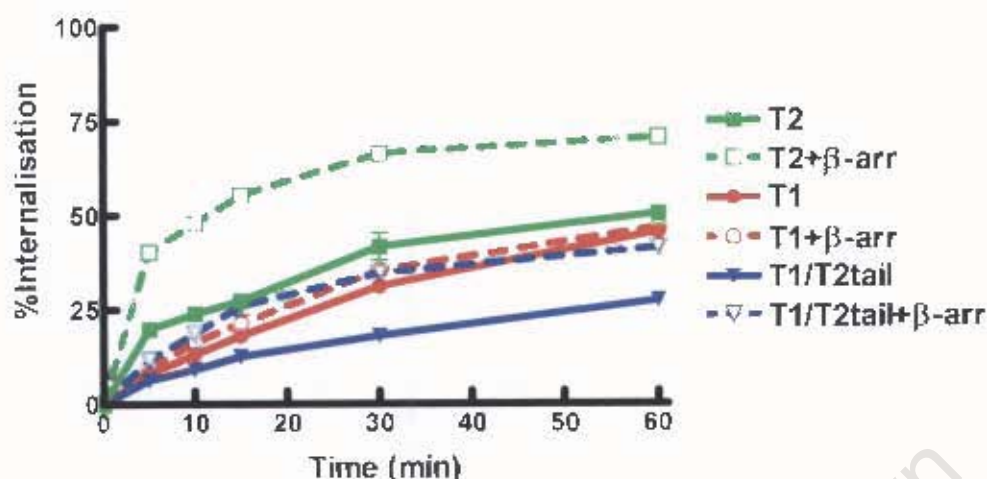


Fig. 3.2 Internalisation of the type I GnRH receptor chimera incorporating the type II GnRH receptor C-terminal tail (T1/T2tail). The internalisation of the chimera was performed in the presence of β -arrestin 1 and compared to the internalisation of the type I and type II GnRH receptors. Results are from a single representative experiment performed at least three times in duplicate.

3.2.2 The T1/T2tail chimera internalises in a GRK 2 dependent manner whereas the internalisation of the wild type receptor (T1) is GRK 2 independent

The type I GnRH receptor does not possess a C-terminal tail and it has been observed that this receptor is not phosphorylated by GRKs (Willars *et al.* 1999; Vrecl *et al.* 2000).

In order to determine whether the addition of the type II GnRH C-terminal tail to the type I GnRH receptor would cause this chimera to internalise in a GRK dependent manner, the T1/T2tail chimera was co-expressed with GRK2 in COS-1 cells. Internalisation assays were performed as seen in Fig. 3.3.

In the presence of GRK2, the internalisation of the chimera was significantly increased by $11 \pm 4.8\%$ after 5 minutes and by $51 \pm 4.3\%$ sixty minutes after agonist stimulation,

compared to the internalisation of the chimera with empty vector ($P < 0.05$). This result demonstrates that the tailed chimera internalises in a GRK dependent manner.

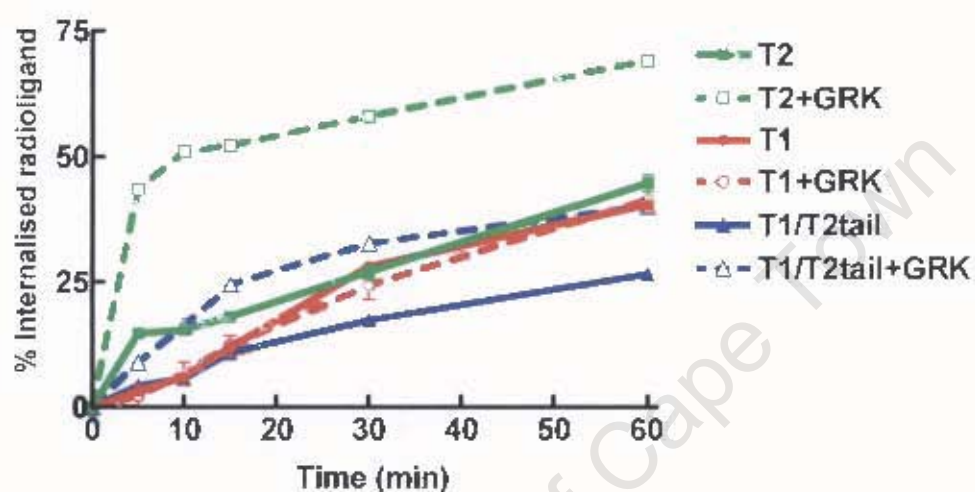


Fig. 3.3 Internalisation of the type I GnRH receptor chimera incorporating the type II GnRH receptor C-terminal tail (T1/T2tail) in the presence of GRK2, compared to the internalisation of the type I and type II GnRH receptors. COS-1 cells were co-transfected with receptor constructs and GRK2. Results are from a single representative experiment performed at least three times in duplicate.

The type II GnRH receptor, as expected, showed a significant increase in internalisation in the presence of GRK. At 5 minutes, the internalisation of the type II GnRH receptor in the presence of GRK2 was about two fold higher ($189 \pm 22\%$) and at 60 minutes, $55 \pm 16\%$ higher than the control ($P < 0.05$). As expected (Vreel *et al.* 2000), the type I GnRH receptor did not demonstrate an increase in internalisation in the presence of GRK, confirming that GRK 2 is not involved in the internalisation of the type I GnRH receptor.

It has been shown that β -arrestin binding to GPCRs is enhanced by agonist stimulation and subsequent phosphorylation of receptors (Lohse *et al.* 1992). Therefore, to

investigate whether putative phosphorylation sites, serines 338 and 339, on the type II GnRH C-terminal tail (Ronacher et al. 2004) were responsible for the β -arrestin dependent internalisation of the T1/T2tail chimera, a T1/T2tailSA mutant was created. The serine residues 338 and 339 in the C-terminal tail of the tailed chimera T1/T2tail were mutated to alanine residues, utilising a PCR method as detailed in section 2.1.1. Internalisation assays were performed on the T1/T2tailSA mutant in the presence and absence of β -arrestin 1 and GRK2.

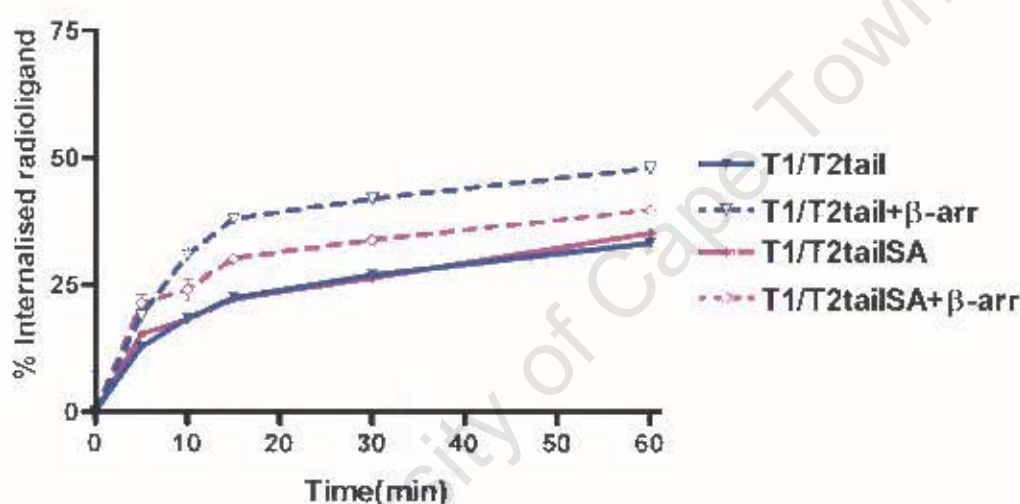


Fig. 3.4 Internalisation of the serine mutant T1/T2tail chimera, T1/T2tailSA, in the presence of β -arrestin 1 compared to the T1/T2tail chimera. Results shown are from a single representative experiment performed at least three times in duplicate.

The internalisation of T1/T2tailSA in the presence of β -arrestin was increased by $38 \pm 14\%$ at 5 minutes, and at 60 minutes was increased by $13 \pm 5.6\%$ compared to the internalisation of T1/T2tailSA without β -arrestin 1 (Fig. 3.4) ($P < 0.05$). The removal of the phosphorylation sites on the C-terminal tail of the T1/T2tail does not abolish β -arrestin dependent internalisation of the chimera. The fact that this mutant receptor still internalises in a β -arrestin dependent manner, albeit reduced, suggests that serine residues 338 and 339 are required but not necessary for β -arrestin dependency. Therefore there may be other

residues on the type II GnRH receptor C-terminal tail that confer β -arrestin dependent internalisation on the type I GnRH receptor. The internalisation of T1/T2tail in the presence of β -arrestin increased by $49\pm 3.3\%$ after 5 minutes and by $45\pm 9.8\%$ after 60 minutes, compared to the internalisation of T1/T2tail without β -arrestin ($P<0.05$).

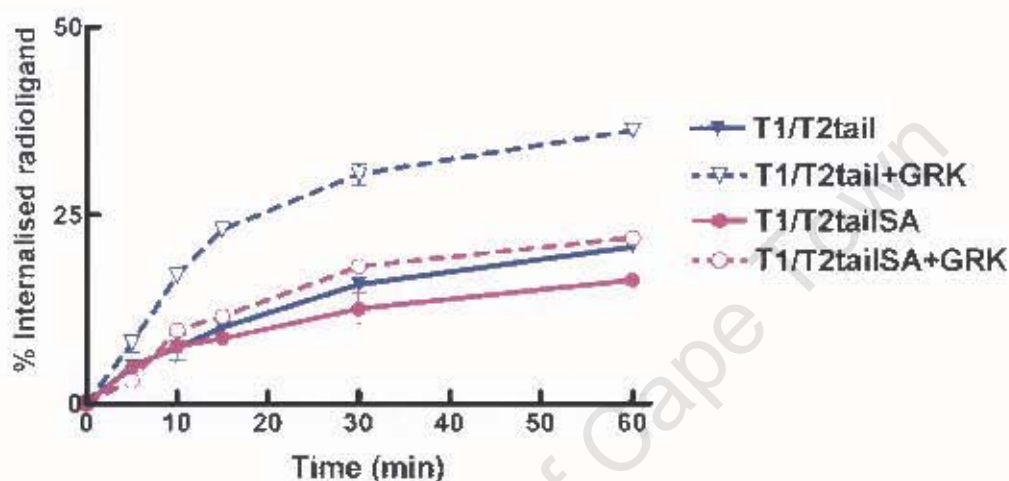


Fig. 3.5 Internalisation of the serine mutant T1/T2tail chimera, T1/T2tailSA, in the presence of β -arrestin 1 compared to the T1/T2tail chimera. Results shown are from a single representative experiment performed at least three times in duplicate.

The internalisation of T1/T2tailSA in the presence of GRK (Fig. 3.5) did not show an increase 5 minutes after agonist stimulation, however there was an increase of $34\pm 8\%$ after 60 minutes, compared to T1/T2tailSA without GRK, although this is not a significant result ($P>0.05$). In the presence of GRK2, the internalisation of T1/T2tail was increased by $17\pm 9.9\%$ after 5 minutes and $75\pm 10\%$ after 60 minutes, compared to the internalisation of T1/T2tail without GRK2 ($P<0.05$). Therefore, the removal of the putative phosphorylation sites, serine 338 and 339, did eliminate GRK dependent internalisation.

3.3 Identification of β -arrestin interacting domains on the type II GnRH receptor

For most GPCRs the third intracellular loop and the C-terminal tail of the receptor are key sites of β -arrestin interaction. Experiments involving ICL3 peptides corresponding to the ICL3 of muscarinic receptors and the $\alpha_{2A/D}$ -adrenergic receptors confirmed that β -arrestin does bind the third intracellular loop of these receptors (Wu *et al.* 1997). Furthermore, for the α_{2b} -adrenergic receptor, it was found that the third intracellular loop, and more specifically, the basic residues in this loop were required for β -arrestin interaction (DeGraff *et al.* 2002).

3.3.1 The third intracellular loop of the type II receptor is required for β -arrestin interaction

From truncation studies on the type II GnRH receptor, it was demonstrated that the C-terminal tail of this receptor is not required for β -arrestin binding (Ronacher *et al.* 2004). Therefore, other domains such as the intracellular loops are possible regions of β -arrestin interaction on the type II GnRH receptor.

To investigate whether the type II GnRH receptor third intracellular loop is the site of β -arrestin interaction, a chimera of the type II GnRH receptor incorporating the type I GnRH receptor third intracellular loop (T2/T1ICL3) was created, as detailed in section 2.1.2. Internalisation assays were performed on this chimera in the presence of β -arrestin 1, and compared with the internalisation of the type I and type II GnRH receptors, also co-expressed with β -arrestin 1.

Binding assays and inositol phosphate (IP) assays performed on this chimera showed reduced expression ($36.9 \pm 0.6\%$ of maximum binding of the type II GnRH receptor) and low IP response ($4.1 \pm 0.3\%$ of maximum IP response of the type II GnRH receptor) (see Table 6.1: Appendix 6.4), which could be due to reduced expression. However, the chimera

could still internalise, although to a lower extent compared to the type I and type II GnRH receptors.

The type II GnRH receptor ICL3 chimera, T2/T1ICL3, in the presence of β -arrestin 1 internalised to the same extent as T2/T1ICL3 without β -arrestin 1 ($P>0.05$) (Fig. 3.6). This result demonstrates that T2/T1ICL3 does not internalise in a β -arrestin dependent manner, indicating that the third intracellular loop of the type II GnRH receptor is required for β -arrestin dependent internalisation.

Again, as seen before, there is a distinct increase in the internalisation of the type II GnRH receptor in the presence of β -arrestin 1 ($P<0.05$). At 5 minutes the internalisation of T2 with β -arrestin is $99\pm 10\%$ higher and at 60 minutes, $34\pm 4.8\%$ higher than the internalisation of T2 without β -arrestin. Furthermore, there is no change in the internalisation of the type I GnRH receptor with or without β -arrestin 1.

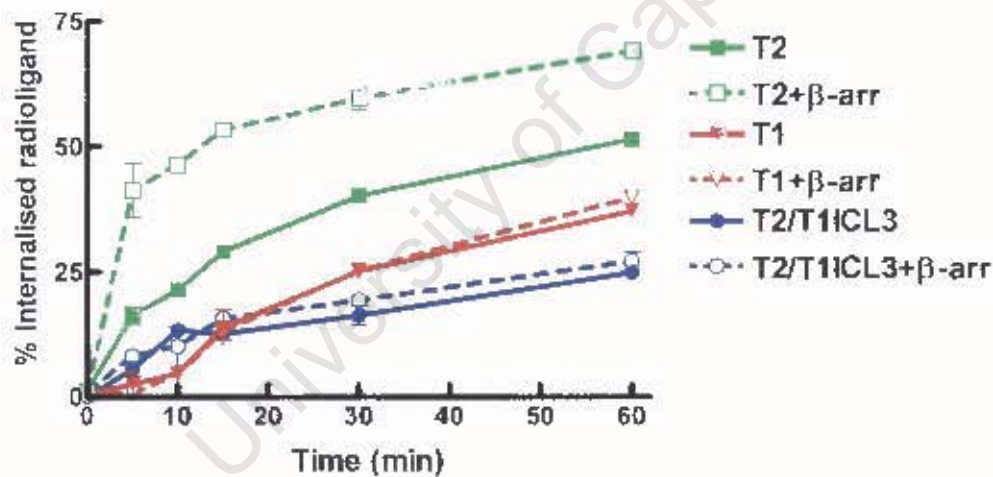


Fig. 3.6 Internalisation of the type II GnRH receptor chimera incorporating the type I third intracellular loop (T2/T1ICL3) in the presence of β -arrestin compared to the type I (T1) and type II (T2) GnRH receptors. COS-1 cells were co-transfected with receptor constructs and β -arrestin 1 and internalisation assays were performed.

These results suggest that the third intracellular loop is the domain of β -arrestin interaction on the type II GnRH receptor. In order to identify the specific region within the third

intracellular loop of the type II GnRH receptor, that is required for β -arrestin dependency, internalisation assays were performed on various mutants of the third intracellular loop.

Various studies had shown that basic residues in the third intracellular loops of different receptors were responsible for β -arrestin interaction (DeGraff *et al.* 2002). Interestingly, the alignment of the amino acid sequences of the type I and type II GnRH receptor third intracellular loops (Fig. 3.7) revealed that there is a region on the type II GnRH receptor ICL3 that is absent in the type I GnRH receptor ICL3. Three out of five residues in this area are basic residues R234, R236 and K237. In order to test their role in β -arrestin interaction these residues were mutated to alanine residues using site directed mutagenesis.

T2 ICL3	IVLGVSSP RT R K GSHAPAGEFALRR SFDNRPR
T1 ICL3	I IFT LTRV●●●●● LHQDPHELQLNQSKNN IPR

Fig. 3.7 Amino acid sequence alignment of the type II and type I GnRH receptors third intracellular loops. The basic residues R234, R236 and K237 are shown in blue. The symbol (●) represents the region on the type I GnRH third intracellular loop that has no similarity to the type II GnRH receptor third intracellular loop.

The mutations of the basic residues to alanine residues were either done individually (R234A, R236A, and K237A), in a pair (R234, R236A) or in triple (R234, R236, K237A). The assumption made was that a single mutation would show slight or even undetected reduction in β -arrestin dependent internalisation. Therefore, mutating the basic residues in pairs or in triplicate would amplify these changes, making differences between the internalisation of the wild type and mutant receptors in the presence of β -arrestin more significant.

Interestingly, all the basic residue mutants internalised in a β -arrestin dependent manner, comparable to that of the type II GnRH receptor (Fig. 3.8) ($P > 0.05$). This indicates that the basic residues are not important for β -arrestin interaction, therefore other residues may be involved in β -arrestin dependent internalisation.

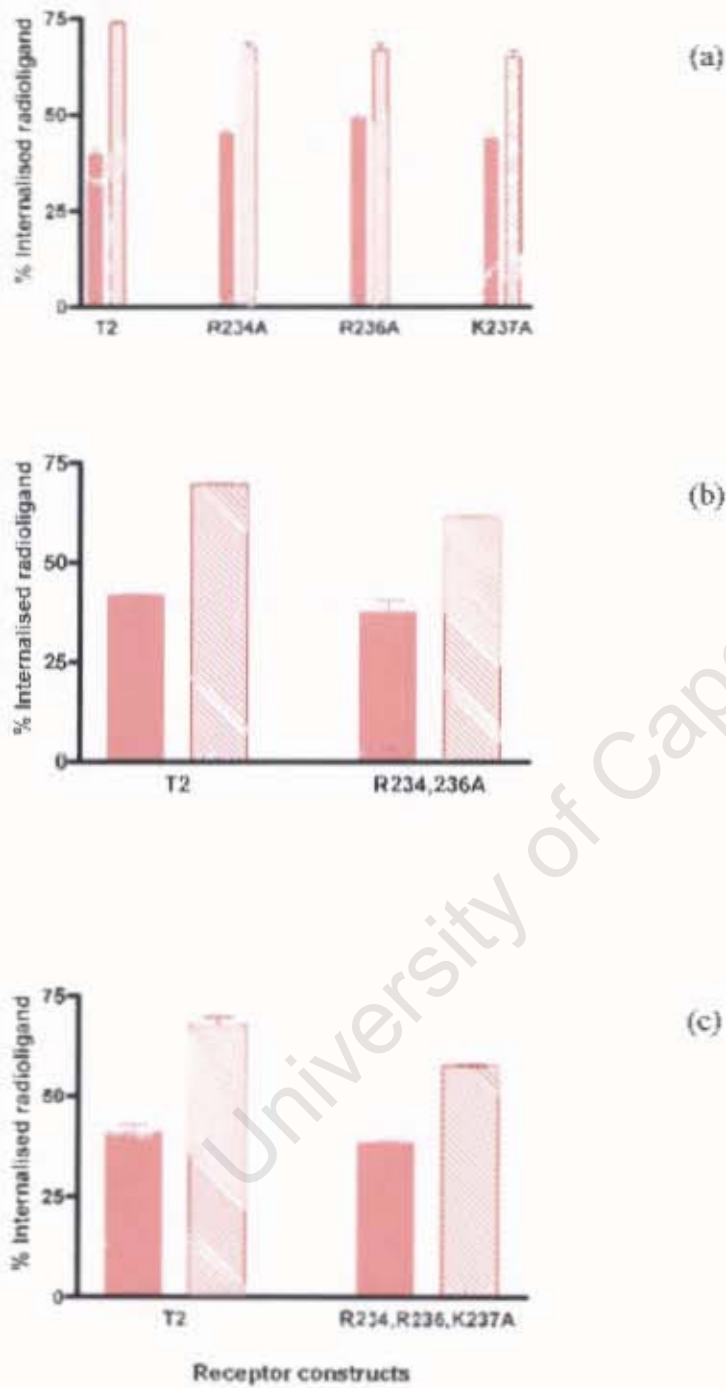


Fig. 3.8 Internalisation of the basic residue mutants R234A, R236A, K237A: (a) individual mutations, (b) double mutation and (c) triple mutation, in the presence (stripes) or absence (solid shade) of β -arrestin 1 compared to the type II GnRH receptor (T2). Bar graphs show percentage internalisation at 30 minutes.

Table 3.1 Summary of results from Fig 3.8. Percentage internalisation of the various mutants of the type II GnRH receptor compared to the internalisation of the type II GnRH receptor in the presence of β -arrestin 1. Results show the percentage increase in internalisation 30 minutes after agonist stimulation, in the presence of β -arrestin compared to constructs without β -arrestin.

CONSTRUCT	% increase in internalisation in the presence of β -arrestin
T2	72 \pm 16
R234A	49 \pm 8.3
R236A	36 \pm 2.7
K237A	48 \pm 0.4
R234,R236A	65 \pm 26
R234,R236,K237A	50 \pm 7.2

The next step was to test serine and threonine mutants of the third intracellular loop of the type II GnRH receptor. As already mentioned, serine and threonine residues are putative GRK phosphorylation sites and in some receptors, it was shown that these sites also play a role in β -arrestin binding. A threonine residue in the third intracellular loop of human lutropin receptor was found to promote β -arrestin 2 association with the receptor (Bhaskaran *et al.* 2003). Serine and threonine third intracellular loop mutants of the type II GnRH receptor were available in our laboratory as they had been used in previous experiments (Ronacher K. Ph.D. Thesis 2003).

Internalisation assays were performed on these mutants (Fig. 3.9).

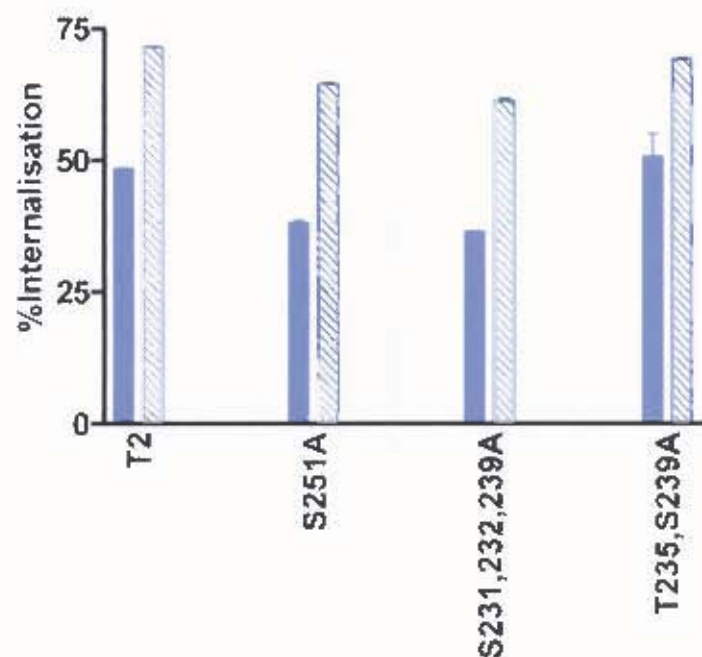


Fig. 3.9 Internalisation of type II GnRH ICL3 mutants in which serine and threonine residues have been mutated to alanine residues in the presence (stripes) or absence (solid shade) of β -arrestin. The bar graph shows percentage internalisation of these constructs at the 30 minutes.

All the serine and threonine mutants did not seem to alter β -arrestin dependent internalisation (Fig. 3.9). They all internalised in a manner comparable to the wild type receptor. In the presence of β -arrestin 1 ($P < 0.05$).

Table 3.2 Summary of results from Fig 3.9. Percentage internalisation of the various mutants of the type II GnRH receptor compared to the internalisation of the type II GnRH receptor in the presence of β -arrestin 1. Internalisation was measured 30 minutes after agonist stimulation.

CONSTRUCT	% increase in internalisation with β -arrestin 1
T2	48 ± 0.6
S251A	69 ± 5.5
S231,232A	68 ± 2.0
T235,S239A	37 ± 2.8

3.3.2 Truncation of the C-terminal tail of the triple mutant (R234,R236,K237A) abolishes β -arrestin dependent internalisation

The results have shown that the third intracellular loop is important for β -arrestin dependent internalisation and since the basic residues have no effect on β -arrestin dependent internalisation, it is possible that the C-terminal tail may be compensating for the loss of these residues.

In order to test this hypothesis, the C-terminal tail of the triple mutant (R234,R236,K237A) was truncated at serine 335 on the C-terminal tail (section 2.1.4). Binding assays and IP assays confirmed that the truncated mutant could still be expressed on the cell surface ($55 \pm 9.1\%$ of maximum binding of T2) and the receptor was still functional ($109 \pm 7.4\%$ of maximum IP response of T2) (see Table 6.2: Appendix 6.4). Internalisation assays were performed on this mutant in the presence of exogenous β -arrestin I.

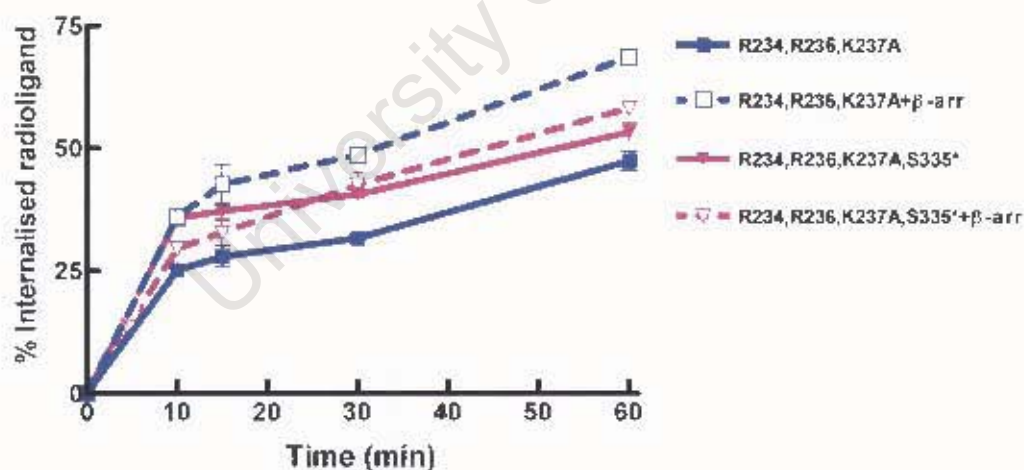


Fig. 3.10 Internalisation of the truncated mutant R234,R236,K237A,S335* in the presence and absence of β -arrestin I. Internalisation of R234,R236,K237A,S335* was compared to the internalisation of the triple mutant R234,R236,K237A in the presence and absence of β -arrestin I.

Over-expression of β -arrestin 1 caused a significant increase in internalisation of $43\pm 12\%$ at 10 minutes and $45\pm 13\%$ at 60 minutes for the triple mutant (R234,R236,K237A) ($P < 0.05$). However, there was no significant increase in the internalisation of the truncated triple mutant (R234,R236,K237A,S335*) in the presence of exogenous β -arrestin 1 ($P > 0.05$). At 10 minutes, the internalisation of R234,R236,K237A,S335* in the presence of β -arrestin showed a decrease and at 60 minutes there was a slight increase of $9.2\pm 4\%$ compared to the control (without β -arrestin) ($P > 0.05$). These results show that the C-terminal tail on the triple mutant R234,R236,K237A is important for β -arrestin dependent internalisation.

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4. DISCUSSION

4.1 The role of the C-terminal tail of the type II GnRH receptor in the internalisation of the type I GnRH receptor

To determine the role of the type II GnRH receptor C-terminal tail on the type I GnRH receptor and to investigate which domains on the type II GnRH receptor are required for β -arrestin dependent internalisation, chimeras of both the type I and type II GnRH receptors were constructed.

The type II GnRH receptor, in contrast to the type I GnRH receptor, possesses a C-terminal tail (Millar *et al.* 2001) that has been shown to mediate rapid internalisation (Pawson *et al.* 1998) and desensitisation (Heding *et al.* 1998).

Initial experiments in this project validated previous reports (Ronacher *et al.* 2004) and demonstrated that the type II GnRH receptor internalises in a β -arrestin dependent manner in the presence of exogenous β -arrestin in COS-1 cells (Fig. 3.1), whereas the type I GnRH receptor fails to utilise β -arrestin in its internalisation. Based on these results, a chimera of the type I GnRH receptor incorporating the full length of the type II GnRH receptor C-terminal tail (T1/T2tail) was constructed. This was to test whether the C-terminal tail of the type II GnRH receptor would confer β -arrestin dependent internalisation to the tail-less and β -arrestin insensitive type I GnRH receptor. Internalisation assays were performed in the presence of exogenous β -arrestin 1, which has a similar effect as β -arrestin 2 on the internalisation of the type II GnRH receptor (Ronacher *et al.* 2004).

Over-expression of β -arrestin in the internalisation of the T1/T2tail chimera enhanced internalisation by $84\pm 20\%$ after five minutes and by $51\pm 19\%$ after sixty minutes, compared to the control (without β -arrestin) (Fig. 3.2). This result demonstrates that the type II GnRH C-terminal tail can impart β -arrestin dependent internalisation to the type I GnRH receptor, even though for the type II GnRH receptor, the C-terminal tail is not required for β -arrestin dependent internalisation (Ronacher *et al.* 2004).

Several studies have also shown that C-terminal tails added to mammalian type I GnRH receptors can cause the originally tail-less and β -arrestin insensitive receptors to internalise in a β -arrestin dependent manner. For example, addition of the TRH receptor C-terminal tail to the rat type I GnRH receptor generated a chimera that could utilise β -arrestin in its internalisation (Heding *et al.* 2000; Hanyaloglu *et al.* 2001). Furthermore, Hislop *et al.* 2005 have demonstrated that the addition of the xenopus GnRH receptor C-terminal tail to the human type I GnRH receptor causes the chimeric receptor to internalise in a β -arrestin dependent manner. However, addition of the catfish GnRH receptor C-terminal tail to the rat GnRH receptor did not confer β -arrestin dependency (Heding *et al.* 2000), suggesting that not all C-terminal tails added to β -arrestin insensitive GnRH receptors are able confer β -arrestin dependent internalisation.

Not only did addition of the type II GnRH receptor C-terminal tail impart β -arrestin dependent internalisation to the type I GnRH receptor, it also enabled the receptor to utilise GRK in its internalisation. The internalisation of the type I GnRH receptor is not dependent on GRK (Fig. 3.3; Willars *et al.* 1999), but on addition of the type II GnRH receptor C-terminal tail, the chimeric GnRH receptor internalises in a GRK dependent manner. In the presence of exogenous GRK2, the internalisation of T1/T2tail was increased by $111 \pm 4.8\%$ after five minutes and $51 \pm 4.3\%$ after sixty minutes. From the results, it is evident that the presence of the type II GnRH C-terminal tail on the type I GnRH receptor enables the receptor to utilise GRK in its internalisation. The GRK and β -arrestin dependency of this chimera is consistent with the role of these proteins in receptor internalisation, where the receptor undergoes phosphorylation by GRK before β -arrestin binding (Ferguson *et al.* 2001).

The mechanism by which the type II GnRH C-terminal tail confers GRK and β -arrestin dependent internalisation on the type I GnRH is not known. It could be specific sites on the added C-terminal tail or a change in conformation on addition of the C-terminal tail that enables recognition of both GRK and β -arrestin.

Two serine residues at positions 338 and 339 in the C-terminal tail of the marmoset type II GnRH receptor were identified as putative GRK phosphorylation sites required for rapid receptor internalisation as the S338,339A mutant lacking these sites exhibited decreased internalisation compared to the wild type receptor. These serine residues were,

however, not required for β -arrestin dependent internalisation, since the S338,339A mutant still internalised in a β -arrestin dependent manner, comparable to the wild type GnRH receptor (Ronacher *et al.* 2004). To determine whether the two serine residues on the C-terminal tail of the T1/T2tail chimera were required for the observed β -arrestin/GRK dependent internalisation, serine 338 and 339 were mutated to alanine residues (T1/T2tailSA).

As expected, T1/T2tailSA did not internalise in a GRK dependent manner (Fig 3.5), supporting the observation that serine residues 338 and 339 on the type II GnRH receptor C-terminal tail are putative GRK phosphorylation sites (Ronacher *et al.* 2004).

Consistent with the type II GnRH receptor, mutating the serine residues did not abolish β -arrestin dependent internalisation (Fig. 3.4). However, compared to the internalisation of T1/T2tail with β -arrestin, the increase in percentage internalisation of T1/T2tailSA in the presence of β -arrestin was slightly lower, suggesting that the two serines might play a role in the β -arrestin dependent internalisation of the tailed chimera. In addition, mutating the serine residues in the T1/T2tail chimera did not lower overall internalisation (without β -arrestin), contrary to what was observed for the type II GnRH receptor mutant S338,339A, where overall internalisation was impaired. This observation indicates that serines 338 and 339 are not required in the internalisation of T1/T2tail without β -arrestin but do play a role in the β -arrestin dependent internalisation of this chimera. Therefore, in the tailed chimera, serines 338 and 339 may play a role that is different to the role they play in the type II GnRH receptor.

Serine residues 338 and 339 also form part of a consensus sequence for phosphorylation by casein kinase II (CKII) and it has been shown that CKII sites play an important role in the β -arrestin dependent internalisation of the rat GnRH receptor with the TRH receptor C-terminal tail (Hanyaloglu *et al.* 2001). However, since mutating the serine residues only reduced the percentage increase in internalisation but did not abolish β -arrestin dependent internalisation, another determinant, other than the two CKII sites is required for β -arrestin dependent internalisation of T1/T2tail.

A possibility is that the addition of the C-terminal tail to the type I GnRH receptor elicits a change in receptor conformation that facilitates β -arrestin interaction. Marion *et al.* 2006 have recently shown that the conserved proline and alanine residues located six

residues distal to the highly conserved DRY motif in rhodopsin-like GPCRs regulate β -arrestin interaction. Interestingly, the type I GnRH receptor also possesses the conserved proline and alanine residues, but does not internalise in a β -arrestin dependent manner. It could be that the conformation of the wild type receptor is such that it cannot interact with β -arrestin, hence, the addition of the C-terminal tail may stabilise the receptor in a conformation that is recognised by β -arrestin.

Why would the C-terminal tail be required to confer β -arrestin dependent internalisation on the tail-less GnRH receptor but does not seem important in the type II GnRH receptor? It could be due to redundancy in domains required for β -arrestin interaction such that even without its C-terminal tail, the type II GnRH receptor may utilise other domains and still internalise in a β -arrestin dependent manner.

Evidently, the primary region required for β -arrestin dependent internalisation of the type II GnRH receptor is not the C-terminal tail since truncation of the C-terminal tail does not abolish β -arrestin dependent internalisation. Therefore, it is clear that other domains on the type II GnRH receptor are required for β -arrestin interaction.

4.2 The type II GnRH receptor third intracellular loop is required for β -arrestin dependent internalisation

Since the C-terminal tail of the type II GnRH receptor is not required for β -arrestin dependent internalisation, it is likely that other domains in the receptor do play a role in β -arrestin dependency.

Another domain that has been shown to mediate β -arrestin interaction in GPCRs is the third intracellular loop. Interaction of β -arrestin with the third intracellular loops of various receptors has been shown for receptors such as the m2 and m3 muscarinic receptors (Wu *et al.* 1997), the α_{2A} AR (Wu *et al.* 1997), the α_{2b} AR (DeGraff *et al.* 2002) and the chemokine receptor CXCR4 (Cheng *et al.* 2000).

To investigate whether the type II GnRH receptor third intracellular loop is required for β -arrestin dependent internalisation, a chimera of the type II GnRH receptor with the third intracellular loop replaced by the third intracellular loop from the type I GnRH receptor was created. This mutant (T2/T1ICL3) exhibited very low binding and low IP response compared to the wild type GnRH receptors. The low IP response could be due to impaired coupling of the receptor with its cognate G protein leading to poor activation and overall signalling (Wade *et al.* 1999), as a consequence of replacing the whole third intracellular loop of the type II GnRH receptor. A change in receptor conformation can also explain the reduced binding of this chimera. Low expression, however, did not hinder measurement of β -arrestin dependent internalisation as internalisation is measured as a ratio of internalised receptors to total (internalised + cell-surface bound) receptors, and the absolute change in percentage internalisation in the presence of β -arrestin can still be demonstrated.

Replacing the third intracellular loop of the type II GnRH receptor with the type I GnRH receptor third intracellular loop reduced overall internalisation and abolished β -arrestin dependent internalisation (Fig. 3.6). This result indicates that the third intracellular loop of the type II GnRH receptor is required for internalisation in general, and more specifically, β -arrestin dependent internalisation.

An alignment of the amino acid sequences of the type I and type II GnRH receptors revealed a basic residue rich area in the type II GnRH receptor third intracellular loop that was absent in the type I GnRH receptor third intracellular loop. Three of the five residues in this area were basic residues R234, R236 and K237. Based on previous studies where basic residues in the third intracellular loop were shown to be important for β -arrestin interaction (DeGraff *et al.* 2002; Murkhejee *et al.* 1999), the three basic residues were mutated to alanine residues singly, in a pair and in triplicate. However, all the mutants internalised in a β -arrestin dependent manner, comparable to the wild type GnRH receptor (Fig. 3.8), demonstrating that the basic residues are not required for β -arrestin dependent internalisation of the type II GnRH receptor.

In addition to phosphorylating serine and threonine residues on the C-terminal tail of GPCRs, GRKs phosphorylate serine and threonine residues in the third intracellular loop, after which β -arrestin binds (Ferguson 2001). Therefore mutants of the third intracellular loop of the type II GnRH receptor lacking serine and threonine residues were tested to determine whether the serine and threonine residues are required for β -arrestin dependent internalisation (Fig. 3.9). These mutants still internalised in a β -arrestin dependent manner, comparable to the wild type receptor, indicating that the serine and threonine residues are not important for β -arrestin dependent internalisation.

The fact that the C-terminal tail of the type II GnRH receptor had been able to confer β -arrestin dependence to the type I GnRH receptor, suggested that the tail, even on the type II GnRH receptor could be contributing to β -arrestin dependent internalisation, albeit not absolutely necessary. From the results it has been demonstrated that the third intracellular loop is the primary domain required for β -arrestin dependent internalisation but it could be that the C-terminal tail compensates for the third intracellular loop if the conformation of the loop is slightly modified. Based on this assumption, the C-terminal tail of the basic residue triple mutant (R234,R236,K237A) was truncated at serine 335. The triple mutant was chosen to eliminate any redundancy that might occur in a double or single basic residue mutation. This mutant lost its ability to internalise in a β -arrestin dependent manner (Fig. 3.10), demonstrating that indeed the C-terminal tail of the mutant receptor

(R234,R236,K237A) was compensating for the mutated residues (R234, R236 and K237) in the third intracellular loop, necessary for β -arrestin dependent internalisation.

This result suggests that mutating the basic residues of the receptor interferes with β -arrestin interaction but in the presence of the C-terminal tail the receptor is stabilised in a conformation that facilitates β -arrestin interaction. Therefore, in this specific context, the C-terminal tail of the type II GnRH receptor is important for β -arrestin dependent internalisation.

The C-terminal tail could not, however, rescue β -arrestin dependent internalisation of the T2/T11CL3 chimera. Since the whole third intracellular loop of the type II GnRH receptor had been exchanged with that of the type I GnRH receptor, it is possible that a major conformational change occurred that altered overall receptor- β -arrestin interaction.

The C-terminal tail and the third intracellular loops are not the only domains in GPCRs that have been identified as important for β -arrestin dependent internalisation. It has been recently shown that the first half of the second intracellular loop of rhodopsin-like GPCRs could be a site influencing β -arrestin dependent internalisation (Marion *et al.* 2006). As mentioned earlier (Section 5.1), this could explain why the type I GnRH receptor can internalise in a β -arrestin dependent manner in the presence of the type II GnRH C-terminal tail. The tail might be stabilising a conformation that facilitates β -arrestin interaction, since the type I GnRH receptor also possesses the conserved residues in the second intracellular loop that are believed to regulate β -arrestin interaction.

In conclusion, this project has highlighted, via the use of chimeras, that the structural differences between the type I and type II GnRH receptors do play an important role in the utilisation of β -arrestin in internalisation. Even though the removal of the C-terminal tail from the type II GnRH receptor does not have an effect on β -arrestin dependent internalisation, the addition of the same tail to the type I GnRH receptor caused this receptor to utilise β -arrestin and GRK in its internalisation. Removal of the C-terminal tail from the R234,R236,K237A mutant abolished β -arrestin dependent internalisation of this mutant. The failure of the truncated triple mutant (R234,R236,K237A,S335*) to utilise β -arrestin whereas R234,R236,K237A could utilise β -arrestin shows that the C-terminal tail

of the type II GnRH receptor can rescue β -arrestin dependent internalisation even though the three basic residues in the primary site of β -arrestin interaction are removed.

The type II GnRH receptor C-terminal tail functions as the secondary domain involved in β -arrestin dependent internalisation since the receptor can still internalise in a β -arrestin dependent manner without its C-terminal tail. The third intracellular loop is the primary domain required for the β -arrestin dependent internalisation of the type II GnRH receptor, without which the receptor cannot internalise in a β -arrestin dependent manner. This explains why mutating the basic residues alone did not have an effect on β -arrestin dependent internalisation, whereas truncating the C-terminal tail of the triple mutant eliminated β -arrestin dependent internalisation of the type II GnRH receptor.

Evidently, multiple domains are involved in the β -arrestin dependent internalisation of the type II GnRH receptor. In this project, the C-terminal tail and the third intracellular loop have been identified as important for β -arrestin dependent internalisation, even though the presence of the C-terminal tail in the wild type GnRH receptor (type II) seems redundant. Based on recent findings (Marion *et al.* 2006), the second intracellular loop may also play a role in the β -arrestin dependent internalisation of the type II GnRH receptor.

Overall, these results indicate that the third intracellular loop and the C-terminal tail of the type II GnRH receptor are required for β -arrestin dependent internalisation. These domains either work in concert to induce a receptor conformation that is recognised by β -arrestin or they form a contact site for interaction with β -arrestin.

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6. APPENDIX

6.1 PRIMERS

6.1.1 pcDNA3.1(+) vector specific primers

T7 (anneals to the expression vector pcDNA3.1(+) 90 base pairs before the Eco RI site)

5' TTA ATA CGA CTC ACT ATA GGG 3'

Bgh (anneals to the expression vector pcDNA3.1(+) about 40 base pairs from the Apa I site on the vector)

5' TAG AAG GCA CAG TCG AGG 3'

6.1.2 Primer used in the addition of the type II GnRH C-terminal tail to the type I GnRH receptor (T1/T2tail)

The primer shown below is a sense primer that incorporates both sequences of the type I GnRH receptor (underlined) and the type II GnRH receptor (not underlined). The highlighted region represents the Dra I enzyme restriction site, which is part of the type I GnRH sequence. This primer was used in a PCR reaction with the vector primer Bgh.

5' TGC CTT TTT AAA CCC ATG CTT TGA TCC ACT TAT CTA TGG AIA TTT
TTC TCT GGG CTG CCG AAG AGG GCA CC 3'

6.1.3 Primers used to mutate serine residues 338 and 339 on the C-terminal tail of the T1/T2tail chimera.

The primers below were used together with the vector specific primers, T7 and Bgh to mutate the two serines on T1/T2tail chimera to alanine residues. The highlighted sequences represent a Cla I silent restriction site.

S338,339A_s

5' GGG CAC CAA GAA CTA TCG ATG GAC GCT GCT AGG GAA GAA GGG 3'

S338,339_as

5' CCC TTC TTC CCT AGC AGC GTC CAT CGA TAG TTC TTG GTG CCC 3'

6.1.4 Primers used to replace the third intracellular loop of the type II GnRH receptor with that of the type I GnRH receptor (T2/T1ICL3)

For the full exchange of the type II GnRH receptor ICL3 with that of the type I GnRH receptor, chimeric primers, together with type II GnRH receptor specific primers were utilised. These primers were used in a PCR reaction with the vector specific primers, T7 and Bgh.

H1/m2loop_sense

5' GAC TGC CAT GGT CAC CTG CTA TAG CCG CAT CAT CTT CAC CCT GAC ACG GGT CC 3'

h1/m2loop_antisense

5' CTC AGG GCC CGA AGA CGG ACT CTT GGT ATA TTG TTC TTG GAC TGA TTC AGT TG 3'

m2apa-sense

5' GTG TCC GTC TTC GGG CCC TGA GAC TGG 3'

Apa I

m2bst-antisense

5' TGA CTG CCA TGG TCA CCT GCT ATA GCC GCG 3'

BstEII

6.1.5 Mutations of the third intracellular loop

The primers shown below were used to mutate the basic residues in the third intracellular loop to alanine residues (underlined). The highlighted sequences represent the silent restriction enzyme sites introduced. For the single mutations, the restriction enzyme highlighted is *Acc III*, and for the double and triple mutations, *Eco RI*.

R234A_s

5' TCC AGC CCC GCG ACA AGG AAG GGG AGC 3'

R234_as

5' GCT CCC CTT CCT TGT CGC GGG GCT GGA 3'

R236A_s

5' TCC AGC CTC CGG ACA GCG AAG GGG AGC 3'

R236A_as

5' GCT CCC CTT CGC TGT CCG GAG GCT GGA 3'

K237A_s

5' TCC AGC CTC CGG ACA AGG GCG GGG AGC 3'

K237A_as

5' GCT CCC CCG CCT TGT CCG GAG GCT GGA 3'

(Eco RI silent restriction site highlighted for all sequences below)

R234,236A_s

5' AGC CCC GCG ACA GCG AAG GGG AGC CAT GCC CCT GCC GGG GAA TTC
GCC CTC 3'

R234,236A_as

5' GAG GGC GAA TTC CCC GGC AGG GGC ATG GCT CCC CTT CGC TGT CGC
GGG GCT 3'

R234,236A,K237A_s

5' AGC CCC GCG ACA GCG GCG GGG AGC CAT GCC CCT GCC GGG GAA TTC
GCC CTC 3'

R234,236A,K237A_as

5' GAG GGC GAA TTC CCC GGC AGG GGC ATG GCT CCC CCG CGC TGT CGC
GGG GCT 3'

6.1.6 Truncation of R234,R236,K237A

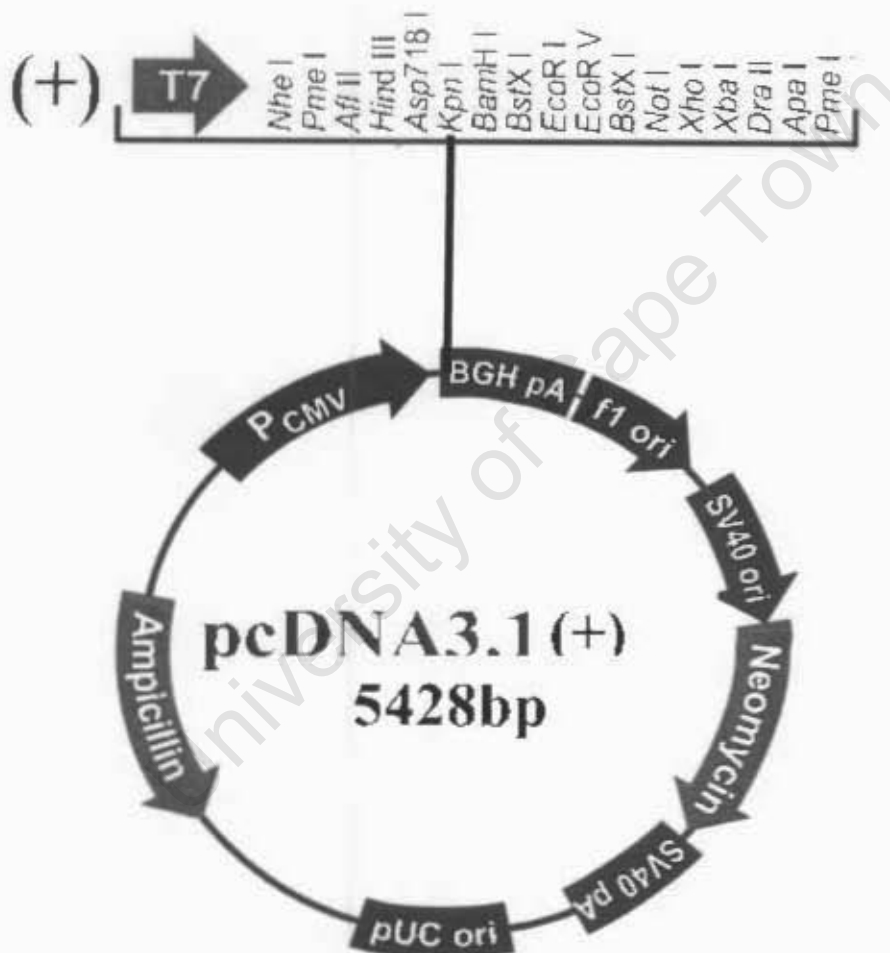
To truncate the triple mutant, a gene specific antisense primer was used together with T7 in a PCR reaction. This primer introduced an Xba I site.

S335stop

5' CCG ^{Xba I} TCT AGA TCA AAG TTC TTG GTG CCC TC 3'

6.3 Expression vector pcDNA 3.1(+), as supplied by Invitrogen, San Diego, USA

All the wild type receptors, chimeric receptors and mutants were cloned in the pcDNA3.1(+) expression vector between Eco RI and Xba I.



6.4 TABLES

Table 6.1 Percentage binding and Inositol phosphates released of the T2/T11CL3 chimera compared to the type II GnRH receptor

Construct	Maximum IP (% of T2)	EC ₅₀ (nM)	Maximum binding (% of T2)	IC ₅₀ (nM)
T2	100	0.6 ± 0.01	100	19 ± 7.2
T1	93 ± 9.9	9.6 ± 1.1	67 ± 5.1	9.5 ± 11
T2/T11CL3	4.1 ± 0.3	3 ± 4.04	36.9 ± 0.6	69 ± 22

Table 6.2 Percentage binding and Inositol phosphates released of the R234,R236,K237A and R234,R236,K237A,S335* mutants compared to the type II GnRH receptor

CONSTRUCT	Maximum IP (% of T2)	EC ₅₀ (nM)	Maximum binding (% of T2)	IC ₅₀ (nM)
T2	100	28 ± 32	100	3.2 ± 1.7
R234,R236,K237A	94 ± 38	45 ± 35	125 ± 43	3.9 ± 0.4
R234,R236,K237A,S335*	109 ± 7.4	52 ± 47	55 ± 9.1	2.2 ± 0.9

6.5 SOLUTIONS

2xYT agar plates, 1l

16g tryptone
10g yeast extract
5g NaCl
15g agar

HBS Buffer

137mM NaCl
5mM KCl
0.7mM NaH₂PO₄
20mM HEPES
pH to 7.4 with NaOH

HBS/DEAE-dextran

3mg/ml DEAE-dextran in HBS buffer
Filter-sterilise and store at 4°C.

CHLOROQUINE

In solution chloroquine is light sensitive:
10mM = 5.15mg/ml
make solution with sterile distilled water
Dilute solution 1:50 with DMEM/2%FCS/PS

The final concentration of chloroquine in solution is 200µm.