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Internalisation of the Type II Gonadotropin-Releasing Hormone Receptor of Marmoset Monkey

by

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In memory of T.E.

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List of Abbreviations

APS	Ammonium persulfate
AP-2	Adapter protein 2
ARF	ADP-ribosylation factor
Ask 1	Apoptosis signal-regulating kinase
AT receptor	Angiotensin receptor
β_2-AR	β_2-adrenergic receptor
BSA	Bovine serum albumin
CaM kinase II	Ca^{2+}/calmodulin-dependent protein kinase II
cAMP	Adenosine-3',5'- cyclic phosphate
cGMP	Guanosine-3',5'-cyclic phosphate
CKII	Casein kinase 2
DAG	Diacylglycerol
DMEM	Dulbeccos Modified Eagle's Medium
DNA	Deoxyribonucleic acid
ECL	Extracellular loop
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal regulated kinase
ET receptor	endothelin receptor
FAK	Focal adhesion kinase
FSH	Follicle stimulating hormone
GAP	GTPase activating protein
GnRH	Gonadotropin-releasing hormone
GnRH-RI	Type I GnRH receptor
GnRH-RII	Type II GnRH receptor
GDP	Guanosine diphosphate
GIT	GRK interactor
GPCR	G protein-coupled receptor
Grb	growth factor receptor binding protein
GRK	G protein-coupled receptor kinase
GRP	GPCR specific phosphatase
GTP	guanosine triphosphate

HEPES	N-2-Hydroxyethylpiperazine-N-2'ethansulphonic acid
5-HT receptor	5-Hydroxytryptamine receptor
ICL	Intracellular loop
IP_3	Inositol 1,4,5-triphosphate
JNK	jun-N-terminal kinase
LH	Luteinising hormone
mAchR	muscarinic acetylcholine receptor
MAPK	Mitogen activated protein kinase
NHERF	Na^+/H^+ exchange regulatory factor
NSF	N-ethylmaleimide-sensitive fusion protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIP_2	phosphatidyl inositol 4,5-biphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
Pyk-2	proline-rich tyrosine kinase 2
RGS	regulators of G protein signaling
RTK	Receptor tyrosine kinase
Shc	Src homology collagen-like protein
SDS	Sodium dodecylsulfate
TBS	Tris buffered saline
TEMED	N,N,N',N'-Tetramethylethylenediamine
TM	Transmembrane domain
TRH-R	Thyrotropin releasing hormone receptor
VIP receptor	vasoactive intestinal peptide receptor

Abstract

The mammalian type II GnRH receptor has a C-terminal tail unlike the mammalian type I GnRH receptor, which uniquely lacks the cytoplasmic C-terminal domain. Internalisation of a mammalian type II GnRH receptor has never been investigated, therefore this thesis studies the internalisation pathway of the type II GnRH receptor. As the C-terminal tail mediates rapid internalisation of many G protein-coupled receptors this research investigates the functional role of the C-terminal tail and intracellular loop in receptor internalisation.

The internalisation pathway of the type II GnRH receptor in COS-1 cells was investigated by co expressing dominant negative mutants and wild-type constructs of G protein-coupled receptor kinases (GRKs), dynamin-1 and β-arrestin 1 and 2 with the type II GnRH receptor. The results show that internalisation of the receptor requires GRK 2 and dynamin but does not require β-arrestin 1 and 2. Furthermore, inhibitors to both the caveolae pathway as well as the clathrin coated vesicle endocytosis abolished receptor internalisation indicating that both structures are involved in internalisation of the receptor. Even though in COS-1 cells the type II GnRH receptor internalises in a β-arrestin independent manner, internalisation of this receptor can be enhanced by over-expression of wild type β-arrestin. This indicates that the type II GnRH receptor is able to utilise a β-arrestin mediated internalisation pathway if high levels of β-arrestin are present in the cell.

The mammalian type II GnRH receptor internalises with enhanced rate and extent compared to the tail-less human type I GnRH receptor. The role of the C-terminal tail of the type II GnRH receptor in internalisation was investigated by measuring internalisation of C-terminally truncated mutants. It was found that the region between Gly 343 and Ser 335 within the C-terminal domain is important for receptor internalisation. Substitution of putative phosphorylation sites within this region revealed that Ser 338 and Ser 339 are critical for rapid receptor internalisation. Furthermore a serine residue in intracellular loop three (Ser 251) was shown to play a role in signalling as well as in internalisation. Since dominant negative GRK 2 could not inhibit

internalisation of a mutant lacking all three serine residues, but could reduce internalisation of the wild-type receptor, we suggest that Ser 251, 338 and 339 are target of phosphorylation by GRK. However these phosphorylation sites as well as the C-terminal tail are not necessary for β-arrestin dependent internalisation.

Taken together this thesis elucidates the internalisation pathway of a mammalian type II GnRH receptor and identified residues within the C-terminal tail and intracellular loop three that are critical for rapid internalisation.

Chapter 1: Introduction

1.1 G protein-coupled receptors

G protein-coupled receptors (GPCRs) form the largest group of plasma membrane receptors and account for approximately 1% of the genes in the human genome (Devi 2001). GPCRs play an important role in the central nervous system and immune system as well as in regulating physiological functions such as vision, smell and taste. Due to the wide variety of GPCR actions an understanding of this receptor family is essential for drug development. In fact more than 50 % of the prescription drugs currently on the market are targeted towards GPCRs (Marinissen and Gutkind 2001). The GPCR superfamily can be divided into six different subfamilies (Bockaert and Pin 1999), the rhodopsin receptor subfamily, the secretin/vasointestinal peptide receptor subfamily, the metabotropic glutamate receptor subfamily, the cAMP receptor subfamily, the pheromone receptor subfamily and the receptors involved in embryonic development. The Gonadotropin releasing hormone receptor (GnRH-R) belongs to the largest subfamily of GPCRs, the rhodopsin receptor subfamily. Therefore throughout this thesis rhodopsin and the β_2-AR will often be used as prototypes.

1.1.1 Structure

Even though GPCRs bind different ligands and have many different functions one of their common characteristics is their structure. Recently the crystal structure of rhodopsin was identified, which shed light on the tertiary structure of GPCRs in general (Palczewski *et al.* 2000).

All members of this protein family possess seven hydrophobic transmembrane domains (TMs) consisting of 20-25 amino acids. The TMs form α-helices, which connect three extracellular loops (ECLs) and three intracellular loops (ICLs) alternating, as well as an extracellular N-terminus and an intracellular C-terminus. Between different GPCRs the sequence of the TMs are more conserved than the ECL and ICL sequences. Proline residues are often found within the TM helices (Probst *et al.* 1992). These conserved proline residues are able to kink the helix and are involved in formation of a ligand-binding pocket (Gether 2000). The crystal structure of rhodopsin revealed that this GPCR has Pro-kinks in helix one, four, six and seven (Palczewski *et al.* 2000). However proline residues in TMs do not always induce a kink into the helical structure. The proline containing TM five does not display a Pro-kink due to the influence of other amino acids in this area. On the other hand TM two, which does not contain any proline residues, shows a typical Pro-kink structure. In this region two Thr residues are able to stabilise a bent conformation (Palczewski *et al.* 2000).

TM 7 of many GPCRs contains a conserved amino acid motif (N-P-X-X-Y) that has been implicated in receptor activation and G-protein coupling (Gether 2000). The crystal structure of rhodopsin showed that the Asn of this motif is able to interact with an Asp in TM two therefore allowing interhelical interaction (Palczewski *et al.* 2000). At the bottom side of TM 3 most GPCRs have the highly conserved motif E/D-R-Y, which is implicated in receptor activation (Gether 2000).

At the cytoplasmic side of the TMs most GPCRs have basic residues, which are thought to interact with the negatively charged phospholipid headgroups of the membrane.

In contrast to the TMs the ECLs and ICLs of GPCRs show low sequence homology. In rhodopsin the extracellular domains form a compact structure (Palczewski *et al.* 2000). The N-terminus is located below ECL 3 and might directly interact with ECL three through a proline residue. ECL 1 and two are on the periphery of the extracellular side whereas part of ECL 2 folds into the core of the helix bundle forming part of the binding site (Palczewski *et al.* 2000). The extracellular N-terminus and/or ECL 2 of all GPCRs found to date contain a consensus sequence for N-glycosylation (Asn-X-Ser/Thr) (Strader *et al.* 1995). Studies on the β_2-AR showed that inhibition of receptor glycosylation reduces receptor expression on the cell surface, but does not influence ligand binding and receptor function (Rands *et al.* 1990).

Another feature that is found amongst all GPCRs is the presence of highly conserved cysteine residues in ECL 2 and ECL 3, which are thought to form a disulfide bond. Mutation of these cysteines leads to destabilisation of receptor conformation of the β_2-AR (Dohlman *et al.* 1991).

Generally the ECLs as well as the TMs of GPCRs are involved in ligand binding. However some GPCRs use exclusively the TM domains for ligand binding (e.g. rhodopsin, dopamine receptors) or exclusively the N-terminal/ECL domains (glycoprotein receptors) (Gether 2000). The GnRH receptor binds its ligand, GnRH, partially in the ECL and the TM regions (Sealfon *et al.* 1997). A description of GnRH receptor/ligand interaction is given in section 1.6.3.

According to the crystal structure of rhodopsin ICL 1 and ICL 3 are in close contact with the C-terminal tail (Palczewski *et al.* 2000). ICL 2 has a distinct L-shaped structure. ICL 2, ICL 3 and the membrane proximal segment of the C-terminal tail are critical for G-protein interaction (Strader *et al.* 1995). Receptor chimera studies showed that the most important domain for G-protein specific interaction is ICL 3 (Wess 1998). ICL 2 is more important for the efficiency of G-protein activation and not the specificity.

The cytoplasmic C-terminal domain of some GPCRs contains a cysteine residue. In case of the α_{2A}-adrenergic receptor and rhodopsin the cytoplasmic cysteine residue is palmitoylated and therefore acts as an additional membrane anker. Substitution of the palmitoylated cysteine had no effect on rhodopsin (Karnik *et al.* 1988), but reduced coupling of the β_2-AR to its G protein (O'Dowd *et al.* 1989), indicating that the palmitoylation is essential for receptor function. In other cases, e.g. V_2 vasopressin, δ-opioid receptor and the CCR5 receptor, mutation of the palmitoylation sites hindered receptor maturation and the receptor mutants were trapped in the endoplasmic reticulum (reviewed in (Qanbar and Bouvier 2003)).

Most GPCRs possess serine and threonine residues within the ICLs and the C-terminal tail, which become phosphorylated by either G protein coupled receptor kinases (GRKs) or second messenger kinases during a process called desensitisation (see section 1.1.3).

1.1.2 Signalling

Apart from structural similarities another feature shared amongst GPCRs is ligand-induced activation of heterotrimeric G proteins.

The GPCR ligands vary considerably and range from small cations then amino acids and peptides to large glycoproteins. Ligand binding leads to stabilisation of the active conformation of the GPCR. In case of rhodopsin absorption of a photon by the 11-cis-retinal causes isomerisation to trans retinal and leads to stabilisation of the receptor in the active conformation. Once the GPCR is in its active state, it can interact with a G protein and an agonist-receptor-G protein complex is formed. In the active state the GPCR induces a GDP/GTP exchange at the α subunit of the heterotrimeric G protein followed by dissociation of the G protein into the α subunit and the $\beta\gamma$ heterodimer. The GTP bound α subunit as well as the $\beta\gamma$ subunits are responsible for activation of effector proteins. The type of G protein determines which effector is activated. Effectors include enzymes and ion channels. Phospholipase C (PLC) becomes activated by G proteins belonging to the $G\alpha_{q/11}$ subfamily as well as $\beta\gamma$ subunits. Adenylyl cyclase is activated by G proteins of the $G\alpha s$ subfamily. The $G_{i/o}$ subfamily of G-proteins can regulate ion channels as well as reduce the intracellular levels of cAMP, whereas activation of transducin, the G-protein for rhodopsin, leads to activation of the cGMP phosphodiesterase. The effector enzymes generate second messengers (e.g. IP_3, cAMP) that induce a variety of intracellular changes ranging from protein phosphorylation and gene transcription to membrane depolarisation. Termination of the signal takes place as soon as the GTP bound to the α subunit of the G protein is hydrolysed to GDP. The α subunit then re-associates with the $\beta\gamma$ subunits and the cycle of G protein activation through a receptor can start again.

The classical G protein mediated second messenger generating pathways alone cannot explain the wide range of effects of GPCRs. In recent years a number of alternative signalling pathways have been described for GPCRs. One major pathway is the MAPK signalling pathway, which can in some cases also be activated through G protein activation (see section 1.6.4). One G protein independent pathway leading to extracellular signal regulated kinase 1/2 (ERK 1/2) activation is initiated by transactivation of the epidermal growth factor receptor (EGFR) by GPCRs (Luttrell *et al.* 1997). Activation of some GPCR, e.g. the endothelin receptor or the m_1 muscarinic receptor, leads to the activation of a metalloprotease, which sheds the EGFR ligand, heparin binding EGF, from the membrane and enables EGF binding to the EGFR (Prenzel *et al.* 1999). Following agonist stimulation the EGFR, a receptor tyrosine kinase (RTK), dimerises and becomes autophosphorylated on tyrosine residues within the cytoplasmic domains. Phosphorylation of the EGFR leads to the recruitment of the adapter protein Src homology collagen-like protein (Shc) and association of Shc with growth factor receptor binding protein 2 (Grb 2). Grb 2 is bound to the Ras-guanine nucleotide exchange factor Sos 1. Sos 1 mediated GTP/GDP exchange on the monomeric G-protein, Ras, leads to Ras activation. In turn Ras activation results in activation of Raf, a MAPKKK. This process initiates the MAPK phosphorylation cascade in which Raf phosphorylates the MAPKK MEK and MEK finally phospohorylates and activates the MAPK ERK 1/2. However MAPK can also be activated in a Ras independent manner (see section 1.5.4).

Apart from RTKs, integrins, single transmembrane spanning proteins without tyrosine kinase activity, play an important role in GPCR mediated MAPK activation. After GPCR activation non-receptor tyrosine kinases such as proline-rich tyrosine kinase 2 (PYK 2) and focal adhesion kinase (FAK) are recruited to integrin dimers and autophosphorylated (Pierce *et al.* 2001). The following signalling cascade is analogous to the RTK pathway.

An alternative to both the RTK and the PYK 2/FAK mediated ERK activation is direct phosphorylation of Shc by the non-receptor tyrosine kinase Src. The β_2AR recruits Src indirectly via the adapter protein β-arrestin, which is involved in β_2-AR internalisation (Luttrell *et al.* 1999). The β_3-AR internalises in a β-arrestin independent manner and therefore interacts directly with Src (Cao *et al.* 2000). The SH 3 domain of Src binds to a proline rich motif (PXXP) within ICL3 and the C-terminal tail of the β_3-AR. After being recruited to the membrane Src phophorylates Shc and the pathway converges with the classical RTK pathway to finally activate ERK 1/2.

GPCRs not only activate ERK 1/2, but also two other classes of kinases, the jun-N-terminal kinase (JNK) and the p38 MAPKs. However the signalling pathways leading to JNK and p38 activation are less understood than the pathways leading to ERK1/2 activation. It is known that β-arrestins play a central role for JNK activation similarly to ERK 1/2 activation. Each of these MAPK are phosphorylated and activated by their MAPKK, which are phosphorylated themselves by MAPKKK. JNK 3 and ERK can bind directly to β-arrestin and therefore β-arrestin can act as a scaffold for MAPK activation (DeFea *et al.* 2000; McDonald *et al.* 2000).

Furthermore β-arrestin can interact with the upstream MAPKKK of ERK1/2 Raf and the upstream MAPKKK of JNK 3 apoptosis signal-regulating kinase 1 (Ask 1). Since β-arrestin plays an important role as an adaptor in assembly of a MAPK module, MAPK activation is often tightly linked to receptor internalisation. Inhibitors of endocytosis can in some cases not only block internalisation, but also MAPK signalling. This is the case for GPCRs like. the β_2-AR (Daaka *et al.* 1998), the LPA receptor, thrombin receptor and bombesin receptor (Luttrell *et al.* 1997). Internalisation, a process that has previously only been associated with signal termination, has now been found to play a key role in signal initiation.

1.1.3 Desensitisation, Resensitisation and Down-regulation

Prolonged exposure of GPCRs to agonists leads to attenuation of G protein signalling, a mechanism called desensitisation. GPCR signalling can be primarily terminated on the level of receptor or on the level of the G protein, but also on effector level.

Desensitisation on receptor level involves mostly receptor phosphorylation of serine and threonine residues within the intracellular loops and the C-terminal tail of GPCRs (for review see (Ferguson 2001)). Receptors can be phosphorylated by second-messenger-dependent protein kinases (e.g. PKA, PKC) as well as G protein coupled receptor kinases (GRKs). Generally GRKs mediate homologous desensitisation and only phosphorylate a GPCR that has been activated by its agonist. β-arrestins then bind stochiometrically to GRK phosphorylated receptors and sterically uncouple the receptors from their G proteins, resulting in signal termination. In contrast second-messenger-dependent kinases can phosphorylate GPCRs independent of ligand exposure. The second messenger dependent kinases can be activated by a different agonist stimulated receptor and are therefore involved in heterologous desensitisation. Recently it has been found that other kinases, casein kinase 1a and casein kinase 2 (CK II), play a role in receptor desensitisation and internalisation (Budd *et al.* 2000; Hanyaloglu *et al.* 2001).

On the level of the G protein, desensitisation is mediated by regulators of G protein signalling (RGS). RGS proteins are able to bind to G proteins and to increase the rate of hydrolysis of GTP on Gα subunits (for reviews see(Dohlman and Thorner 1997; Kozasa 2001)). Accelerated GTP hydrolysis leads to dampening of the G protein mediated signal. However some effectors of G proteins (e.g. PLCβ) can also act as GTPase activating proteins.

Receptor desensitisation occurs after seconds to minutes of agonist exposure and is usually followed by receptor internalisation, which last from minutes to hours. The different endocytic pathways, which will be discussed in detail in section 1.2, have been described. Once the receptor is internalised into vesicles, the vesicles fuse with endosomes. At this stage two different events determine the fate of the GPCR. On the one hand the GPCR can be dephosphorylated by a GPCR specific phosphatase (GRP)

(Pitcher *et al.* 1995) and resensitised. Receptor resensitisation is complete when the receptor has been recycled back to the plasma membrane. However there is new evidence of an alternative pathway of resensitisation. Internalisation is not a prerequisite for dephosphorylation and resensitisation. Some GPCRs (eg. the D1 dopamine receptor) are dephosphorylated at the cell surface without having been internalised (Gardner *et al.* 2001). The phosphatase responsible for the novel dephosphorylation mechanism is not a GRP and has not yet been identified.

On the other hand the receptor containing endosome can fuse with a lysosome, leading to degradation of the receptor. This event results in down-regulation of total receptor number per cell. Recently it has been shown that degradation of mammalian GPCRs is determined by receptor ubiquitination. Generally ubiquitination of proteins is the result of the action of three different enzymes, E 1, E 2 and E 3 and marks proteins for degradation (for reviews see (Hershko and Ciechanover 1998; Yamao 1999)). Down-regulation of the ubiquinated proteins can be achieved either by lysosomal degradation or degradation by the 26S proteasome a multicatalytic particle consisting of two 19S subunits and one 20S subunit.

Agonist stimulation of the β_2-AR not only leads to ubiquitination of the receptor, but also of the receptor associated β-arrestin (Shenoy *et al.* 2001). In fact, receptor ubiquitination requires β-arrestin, since this protein has been shown to be associated with the E 3 ubiquitin ligase Mdm 2. Degradation of the β_2-AR is mediated by the 26S proteasome (Shenoy *et al.* 2001). However ubiquitination can also target a GPCR for lysosomal degradation instead of degradation by the 26S proteasome. Agonist promoted ubiquitination of the CXCR 4 receptor regulates lysosomal sorting and the CXCR 4 receptor degradation is unaffected by 26S proteasome inhibitors (Marchese and Benovic 2001)

1.2 Biological role of G protein coupled receptor endocytosis

Originally GPCR endocytosis was thought to be a process for receptor desensitisation and therefore signal termination (Sibley *et al.* 1986). However it was soon recognised that receptor desensitisation occurs within seconds to minutes after agonist stimulation whereas receptor internalisation takes place within minutes to hours after stimulation. Furthermore treatment of agonist-stimulated cells with inhibitors of internalisation did not affect receptor desensitisation (Yu *et al.* 1993; Pippig *et al.* 1995). Therefore desensitisation is independent of internalisation and precedes internalisation.

It was observed that internalisation plays an important role in receptor resensitisation. Once GPCRs are internalised they can become dephosphorylated and can be recycled back to the plasma membrane. The rate at which GPCRs recycle back to the cell surface varies from receptor to receptor, but takes at least several minutes. Generally it can be distinguished between fast and slowly recycling receptors. The β_2-AR is immediately dephosphorylated and recycled back to the plasma membrane whereas the AT_{1A} receptor and the V2 vasopressin receptor stay longer in endocytic vesicles and recycles slowly (Oakley *et al.* 1999; Anborgh *et al.* 2000). Receptors that recycle slowly form a stable complex with β-arrestin in endocytic vesicles. In contrast the β_2-AR dissociates from β-arrestin when it is still at the cell membrane (Anborgh *et al.* 2000). Similarly to desensitisation, receptor sequestration, degradation and resensitisation are often determined by the C-terminal tail of GPCRs (Oakley *et al.* 1999; Trejo and Coughlin 1999; Anborgh *et al.* 2000; Gray and Roth 2002).

The necessity of internalisation for resensitisation has been demonstrated with internalisation blocking agents for a number of GPCRs, including the β_2-AR (Pippig *et al.* 1995), the m3 muscarinic acetylcholine receptor (mAchR) (Edwardson and Szekeres 1999), the neurokinin 1 receptor (Garland *et al.* 1996), the δ opioid receptor (Hasbi *et al.* 2000), the μ opioid receptor (Wolf *et al.* 1999), the endothelin A (ET_A) receptor

(Bremnes *et al.* 2000). However for the dopamine receptor one mechanism of receptor resensitisation has been described that does not require internalisation (Gardner *et al.* 2001). This receptor undergoes dephosphorylation directly at the plasma membrane.

After being internalised some GPCRs are not resensitised and recycled back to the plasma membrane, instead these receptors are degraded. The ET_B receptor and the protease-activated receptors (PARs) are directly targeted to lysosomes for degradation (Trejo and Coughlin 1999; Bremnes *et al.* 2000). Therefore another role of receptor endocytosis is receptor degradation and down regulation. Since PARs are activated by proteolytical cleavage of the N-terminal sequence, resensitisation of PAR mediated signalling is only achieved by de novo synthesis of new receptors and mobilisation of a reserve pool of nascent receptors being stored in intracellular compartments (Shapiro and Coughlin 1998). The intracellular pool of nascent PARs is maintained by agonist independent internalisation and constitutive exchange of cell surface receptors and intracellular receptors.

Finally the importance of internalisation in signal initiation has been recognised in recent years. As discussed in 1.1.2 internalisation can be a prerequisite for MAPK activation. MAPK activation mediated by the β_2-AR, the LPA receptor, the thrombin receptor and the bombesin receptor can be abolished by inhibitors of internalisation (Daaka *et al.* 1998) (Luttrell *et al.* 1997). Nevertheless the need of receptor internalisation for MAPK activation is still under debate since other receptors (e.g. AT receptor) do not require internalisation for MAPK activation (Shah *et al.* 2002).

In summary receptor endocytosis is important for resensitisation, recycling, degradation and down regulation of GPCRs, as well as for GPCR mediated MAPK signalling.

1.3 Receptor domains involved in internalisation

Different receptor domains contribute to the rate and extent of receptor internalisation. Numerous studies have examined the importance of the C-terminal tail of GPCR in endocytosis. Serine and threonine residues within the C-terminal tail are often target phosphorylation sites for GRKs. Examples of receptors with phosphorylation sites within their C-terminal tails are the β_2-AR (Fredericks *et al.* 1996) and the AT_{1A} receptor (Thomas *et al.* 1998). However the significance of the C-terminal tail with GRK phosphorylation sites varies from receptor to receptor. Whereas the phosphorylation sites and the C-terminal tail of the AT_{1A} receptor are required for receptor internalisation (Thomas *et al.* 1998) a C-terminally truncated β_2-AR or a β_2-AR receptor mutant lacking phosphorylation sites shows only slight reduction in internalisation compared to wild-type (Ferguson *et al.* 1995; Ferguson *et al.* 1996).

Another receptor domain that is often targeted by GRKs is ICL 3. The m_2 muscarinic receptor (Nakata *et al.* 1994) for example is phosphorylated in this region and it was shown that deletion of the third intracellular loop inhibits internalisation of this receptor indicating that GRK phosphorylation is necessary for internalisation (Tsuga *et al.* 1998). In addition the conserved DRY motif within ICL 2 is a determinant for internalisation in some GPCRs. The GnRH receptor possesses a DRS sequence and mutation of the serine residue to a tyrosine significantly enhanced internalisation (Arora *et al.* 1995). However this domain also influences ligand binding, G-protein coupling and receptor stability (Arora *et al.* 1995) and therefore it is difficult to distinguish whether the increase in internalisation is not a secondary effect. The same is true for mutations of the NPXXY motif found at the intracellular side of TM 7. Mutation of the tyrosine residue in the β_2-AR resulted in a receptor with reduced internalisation compared to wild type (Barak *et al.* 1994). However this domain plays a crucial role in receptor conformation and interaction of the receptor with its ligand, its G-protein and also GRKs (Barak *et al.* 1995; Ferguson *et al.* 1995).

Taken together the determinants for receptor endocytosis involve multiple receptor domains and the requirement for GRK phosphorylation sites varies with each individual receptor.

1.4 Internalisation pathways of G protein-coupled receptors

A variation of internalisation pathways is used by different receptors. To date at least three distinct internalisation pathways have been identified. The best-characterised internalisation pathway is endocytosis via clathrin-coated vesicles. This form of endocytosis requires, amongst other proteins, β-arrestins and the GTPase dynamin. Even though the alternative pathways to the clathrin coated vesicle internalisation are still focus of controversy, it has become evident that there are at least two other internalisation mechanisms. One pathway, often referred to as caveolae pathway, does not involve β-arrestin, but requires dynamin. Finally a β-arrestin and dynamin independent pathway has been described. Not only do pathways vary between different GPCRs, but the same GPCR can also internalise through different endocytotic pathways depending on the cell type, in which it is expressed. Furthermore inhibition of one internalisation pathway can lead to endocytosis of the receptor through an alternative pathway indicating the plasticity of receptor endocytosis.

1.4.1 β-arrestin and dynamin dependent receptor internalisation

β-arrestin and dynamin dependent internalisation is also referred to as clathrin coated vesicle internalisation. The first steps of clathrin mediated endocytosis involves proteins that also play a central role in receptor desensitisation, namely GRKs.

GRKs phosphorylate agonist activated GPCRs on serine and threonine residues. Some receptors, like rhodopsin and the β_2-AR, become phosphorylated within the C-terminal tail, while other receptors such as the

α_2-AR and the m2 muscarinic receptor become phosphorylated in ICL 3 (Ferguson 2001). Receptor phosphorylation was observed for the first time on the β_2-AR (Sibley *et al.* 1986), but initially it was not thought to be important for receptor sequestration. As mentioned above, the phosphorylation takes place on serine and threonine residues within the C-terminal tail. Mutation of all serine and threonine residues in the C-terminal domain abolished receptor phosphorylation (Bouvier *et al.* 1988). It was demonstrated that a phosphorylation deficient mutant of the β_2-AR (Y326A) showed reduced internalisation and over-expression of a dominant negative form of GRK 2 (K220M) significantly impaired internalisation of the wild-type β_2-AR (Ferguson *et al.* 1995). Additionaly mutation of the GRK phosphorylation sites also reduced receptor internalisation (Seibold *et al.* 2000) indicating that GRK phosphorylation is required for internalisation.

Once the GPCR is phosphorylated the endocytic adapter protein, β-arrestin, can bind to the receptor. Interaction of the β_2-AR with β-arrestin is essential for internalisation. Dominant negative β-arrestins, β-arrestin 1 (V53D) or β-arrestin 2 (V54D), inhibit internalisation of the β_2-AR (Ferguson *et al.* 1995). Even though GRK phosphorylation sites are located in the C-terminal tail, it has been shown that the C-terminal domain is not absolutely required for β-arrestin binding (Ferguson *et al.* 1996). Taken together, GRK phosphorylation of GPCRs allows subsequent binding of β-arrestin and is the first step in clathrin coated vesicle internalisation. Nevertheless there are some exceptions. Receptor phosphorylation has been thought to be crucial for β-arrestin interaction with the receptor, however very recent studies on the LH and FSH receptors show that β-arrestins can interact with GPCRs in a phosphorylation independent manner (Kishi *et al.* 2002; Min *et al.* 2002; Mukherjee *et al.* 2002). On the other hand other seven transmembrane receptors become phosphorylated by GRKs but do not interact with β-arrestins thereafter. The adenosine A3 receptor (Ferguson *et al.* 2000), the dopamine D2 receptor (Ito *et al.* 1999) and the m2 muscarinic receptor (Tsuga *et al.* 1994) all require GRK2, however these receptors can internalise in a β-arrestin independent manner.

After β-arrestins have been translocated to the receptor, they recruit proteins that are necessary for the formation of clathrin-coated pits. β-arrestin 1 and 2 bind to the β-subunit (β_2-adaptin) of the clathrin associated AP-2 complex and forms a functional assembly with the β_2-AR and β-arrestin upon agonist stimulation of the receptor (Laporte *et al.* 1999; Laporte *et al.* 2000). Furthermore β-arrestin 1 and β-arrestin 2, but not visual arrestin, have been shown to directly interact with the heavy chain of clathrin (Goodman *et al.* 1996). It has been suggested that the interaction of β-arrestin with clathrin and the AP-2 complex with clathrin are cooperative (Laporte *et al.* 2000).

After the coated pits, which now contain the ligand-bound, phosphorylated receptor and β-arrestin, have been formed, the GTPase dynamin is responsible for vesicle detachment from the membrane. Dynamin forms a ring around the neck of the coated pit and separates the vesicle from the membrane. It has been suggested that dynamin mechanically squeezes the connection between the vesicle and the plasma membrane (Schmid *et al.* 1998), however in recent years different mechanism for dynamin action have been proposed (Thompson and McNiven 2001; Claing *et al.* 2002) (see section 1.5.4). The importance of dynamin in β_2-AR internalisation was demonstrated by co-expression of a dominant negative mutant of dynamin (K44A), which reduced β_2-AR internalisation (Zhang *et al.* 1996). This dominant negative dynamin does not affect coated pit assembly and invagination, but dynamin K44A is deficient in its GTPase activity and therefore prevents budding of the coated vesicles from the membrane.

Once internalised the vesicle looses its clathrin coat. Depending on the type of GPCR that is internalised, β-arrestin can either stay associated with the receptor or dissociate from the vesicle. Fast recycling receptors like the β_2-AR loose the interaction with β-arrestin shortly after internalisation (Anborgh *et al.* 2000). These receptors can then quickly become dephosphorylated by GRP and recycled back to the plasma membrane. GPCRs that recycle slowly (e.g. the AT_{1A} receptor or the V2 vasopressin receptor) or receptors that are targeted towards degradation stay associated with β-arrestin (Anborgh *et al.* 2000).

Taken together, GPCR endocytosis via clathrin-coated vesicles is a process that requires GRKs, β-arrestins and dynamin. Therefore dominant negative mutants of GRKs, β-arrestins and dynamin block the clathrin coated vesicle pathway. They are useful tools in characterising the internalisation pathway of a receptor as is sucrose, which prevents clathrin polymerisation and therefore clathrin mediated internalisation (Heuser and Anderson 1989). (For a model showing endocytosis via clathrin coated vesicles see Fig. 1.1 in section 1.5)

1.4.2 β-arrestin independent, but dynamin dependent receptor internalisation

Even though clathrin coated vesicle endocytosis is the predominant pathway for GPCR internalisation the existence of alternative pathways has become evident. However these alternative pathways have not been well characterised so far and are still poorly understood.

With the use of dominant negative mutants some receptors have been found to internalise in a β-arrestin independent, but dynamin dependent manner. Over-expression of dominant negative β-arrestin does not affect internalisation of the vasoactive intestinal peptide 1 (VIP1) receptor and the ET_B receptor, while over-expression of dynamin K44A significantly impairs receptor endocytosis of both receptors (Claing *et al.* 2000). Receptor phosphorylation has been shown for the VIP1 receptor (Shetzline *et al.* 2002) and the endothelin receptor (Freedman *et al.* 1997). For both the VIP 1 and the ET_B receptor a caveolae mediated internalisation pathway has been suggested (Claing *et al.* 2000). In fact involvement of caveolae in ET_B receptor internalisation has been shown in an earlier study (Teixeira *et al.* 1999). Caveolae are flask shaped membrane invaginations believed to be involved in endocytic processes.

Little is known about this internalisation pathway besides the requirement of cholesterol and a 22 k Da integrated membrane protein, called caveolin (Claing *et al.* 2002). Dynamin is found at the neck of caveolae structures and is required for vesicle fission from the plasma membrane (Henley *et al.* 1998). Therefore dominant negative dynamin as well as cholesterol binding drugs can be used to inhibit caveolae internalisation. One of these cholesterol-binding drugs is filipin, a polyene antibiotic, which binds to cholesterol and removes it from the membrane, causing reversible caveolae disassembly (Schnitzer *et al.* 1994). The ET_A receptor, another type of endothelin receptor, has also been shown to utilise the caveolae pathway. Internalisation of the ET_A receptor was inhibited by two disruptors of the caveolae pathway, filipin and nystatin, but was unaffected by hypertonic sucrose, an inhibitor of the clathrin coated vesicle pathway (Okamoto *et al.* 2000). Other receptors internalising in a β-arrestin independent but dynamin dependent way are the 5-hydroxytryptamine 2A ($5\text{-}HT_{2A}$) receptor (Bhatnagar *et al.* 2001) and the prostacyclin (IP) receptor (Smyth *et al.* 2000). Recently a dynamin independent internalisation mechanis via caveolae has also been described (Lamb *et al.* 2001) as discussed in the following section.

1.4.3 β-arrestin and dynamin independent receptor internalisation

Some receptors appear to be internalised through a third endocytic pathway that does not require β-arrestin or dynamin.

The secretin receptor is phosphorylated by GRKs upon agonist stimulation and even recruits β-arrestin to the cell membrane, however dominant negative β-arrestin and dynamin mutants have no effect on internalisation (Walker *et al.* 1999). Similarly internalisation of the m_2 muscarinic receptor, the bradykinin type 2 and the N-formyl peptide receptor cannot be inhibited by dominant negative β-arrestin and dynamin K44A (Claing *et al.* 2000; Gilbert *et al.* 2001; Lamb *et al.* 2001; Roseberry and Hosey 2001). However the endocytic machinery involved in internalisation of these receptors is unknown and remains to be identified.

For the bradykinin receptor a β-arrestin and dynamin independent internalisation pathway via caveolae has been suggested (Lamb *et al.* 2001).

1.5 Proteins involved in G protein coupled receptor endocytosis

The proteins involved in GPCR endocytosis that have been mentioned in 1.2 will be discussed in more detail in this section. As explained earlier the clathrin-mediated endocytosis is not only the predominant but also the best-studied internalisation pathway. Therefore this chapter is dedicated to proteins important for internalisation via clathrin-coated vesicles.

1.5.1 G protein coupled receptor kinases

G protein coupled receptor kinases (GRKs) are serine and threonine kinases that are critical for receptor desensitisation and internalisation. In contrast to other kinases such as PKC, PKA or CKII a consensus sequence for GRK phosphorylation has not been identified. However GRKs seem to prefer Ser and Thr residues, which have a pair of acidic residues on their N-terminal side (for review see (Pitcher *et al.* 1998)).

The family of GRKs consists of seven members, GRK 1 (rhodopsin kinase), GRK 2 (β-adrenergic receptor kinase 1), GRK 3 (β-adrenergic receptor kinase 2), GRK4 (IT-11), GRK 5, GRK 6 and GRK 7. GRK 1 and GRK 7 are only expressed in retina and GRK 4 is only found in testes, while the other GRKs are ubiquitously expressed (Pitcher *et al.* 1998; Bunemann and Hosey 1999; Ferguson 2001). All GRKs possess a central catalytic domain and a structurally homologous N-terminus, which is thought to be important for substrate recognition. The N-terminus also contains a RGS like domain. The variable C-terminus contributes to plasma membrane targeting of the GRK. GRK 2 and GRK 3 contain in their C termini a pleckstrin homology (PH)-like domain, a module of approximately

100 amino acids with six blocks of conserved hydrophobic residues. PH domains are found in other serine/threonine and tyrosine kinases as well as in the mammalian phospholipase C. PH domains are thought to be involved in protein-protein interactions and protein membrane interactions. Ligands for PH domains are PKC, PIP_2, $\beta\gamma$ subunits of G proteins and IP_3 (Pitcher *et al.* 1998).

GRK 1-3 are found in the cytoplasm and only translocate to the cell membrane after agonist stimulation of their substrates. In contrast to GRK 1, GRK 2 and GRK 3 are not farnesylated and their recruitment to the plasma membrane is regulated by interaction with the $\beta\gamma$ subunit of heterotrimeric G proteins with the PH-domain of the kinase (Boekhoff *et al.* 1994). GRK 2 and GRK 3 targeting to the plasma membrane is also mediated through PIP_2 binding to the PH-like domain. However GRK 4 and 6 are constitutively membrane localised even in absence of agonist due to a palmitoylation on a C-terminal cystein residue (Stoffel *et al.* 1997). Membrane association of GRK 5 is mediated by electrostatic interactions between highly basic residues in the C-terminus of the kinase and the membrane phospholipids and a PIP_2 specific site located in the N-terminus.

As mentioned earlier, GRKs are involved in two important processes of GPCR regulation, desensitisation and internalisation. GRKs are responsible for phosphorylation of serine and threonine residues within the C-terminal tail as is the case for rhodopsin (McDowell *et al.* 1993) and the β_2-AR (Fredericks *et al.* 1996) or in ICL 3 as is the case for the m_2 muscarinic receptor (Nakata *et al.* 1994) and the α_2-AR (Eason *et al.* 1995). This phosphorylation enables β-arrestin binding to the receptor. β-arrestins then sterically inhibit interaction of the receptor with its G protein and therefore G protein signalling is terminated. However GRKs can desensitise the signal directly at the level of the G protein since they possess an RGS box and can therefore enhance GTP hydrolysis on the G protein (Sallese *et al.* 2000). Previously it has been thought that GRKs play an important role in internalisation only through recruitment of β-arrestins. However proteins belonging to the GIT (GRK interactor) family have been shown to interact with GRKs (Premont *et al.* 1998). The best-characterised member of the GIT family is GIT 1, a GTPase activating protein (GAP) for the ADP-ribosylation factor (ARF) family of small GTP binding proteins (see also section 1.4.2). GIT1 binding to GRKs is necessary for its membrane translocation. Over-expression of GIT1 has been shown to significantly impair clathrin-mediated endocytosis of the β_2-AR (Premont *et al.* 1998) and other receptors such as the β_1-AR, the μ opioid and the adenosine 2B receptors (Claing *et al.* 2000).

1.5.2 β-arrestins

Like GRKs, β-arrestins are important for desensitisation and internalisation. However β-arrestins are not only involved in terminating G protein signalling, but also in initiating MAPK signalling.

The first member of the arrestin family, now called visual arrestin, was identified in rod outer segments and was found to bind activated rhodopsin (Pfister *et al.* 1985). Later it was shown that a visual arrestin like protein is required for GRK mediated desensitisation of the β_2-AR (Benovic *et al.* 1987). So far four different β-arrestin isoforms have been identified, visual arrestin and cone arrestin on the one hand and the β-arrestins (β-arrestin 1 and 2) on the other hand. In contrast to visual arrestin and cone arrestin, the β-arrestins are ubiquitously expressed outside the retina. Arrestin consists of multiple domains, a N-terminal receptor activation domain, a central phosphate sensor domain, a receptor binding domain and a C-terminal clathrin and β-adaptin binding domain (reviewed in (Ferguson 2001)).

The affinity for β-arrestin 1 and β-arrestin 2 varies from receptor to receptor and accordingly two different classes of receptors can be distinguished (Oakley *et al.* 2000). The class A receptors (e.g. β_2-AR, μ-opioid receptor, ET_A receptor, dopamine type I and α_{1B}-AR) have higher affinity to β-arrestin 2 than to β-arrestin 1. The class B receptors (e.g. AT_{1A} receptor, vasopressin type 2 receptor, neurotensin receptor, type 1 TRH-R, NK1 receptor) have similarly high affinities to β-arrestin 1 and β-arrestin 2.

The first direct proof that β-arrestins link GPCRs to the internalisation machinery was established when direct interaction of β-arrestins with clathrin was shown (Goodman *et al.* 1996). Interestingly both β-arrestin 1 and β-arrestin 2, but not visual arrestin, have high affinity (in the nM range) for clathrin in vitro. In vivo, receptors, arrestins and clathrin were found to co localise after agonist stimulation (Goodman *et al.* 1996). In both β-arrestins the domain required for clathrin binding is the C-terminal region (Krupnick *et al.* 1997) and therefore a mini construct of β-arrestin 1 (319-418) containing the clathrin box is widely used as dominant negative β-arrestin. β-arrestin 1 (319-418) is still able to bind to clathrin, but lacks the ability to interact with the receptor. This mini-construct, which is constitutively localised in clathrin coated pits, competes with wild-type β-arrestin for clathrin and therefore acts as a dominant negative inhibitor for internalisation. Other dominant negative β-arrestins (β-arrestin 1 (V53D) and β-arrestin 2 (V54D)) interact better with clathrin than the wild type β-arrestin. They display however decreased affinity for phosphorylated receptors (Krupnick *et al.* 1997) and act as dominant negative mutants in competing with wild-type β-arrestin for the receptor. A β-arrestin 2 mini construct lacking the clathrin domain was still able to inhibit internalisation of the β_2-AR indicating that this construct can bind to another factor involved in clathrin mediated endocytosis (Laporte *et al.* 1999). In a yeast two-hybrid screen the factor was identified as the clathrin adapter protein AP-2. This β-arrestin/AP-2 association was later confirmed in cells. An arginine residue in the C-terminal region of β-arrestin downstream of the clathrin box is important for interaction with the β-adaptin of the AP-2. Mutation of this arginine to an alanine residue results in an arrestin molecule that cannot bind to AP-2 and is not translocated to clathrin coated pits (Laporte *et al.* 2000).

Other binding partners for β-arrestins are the small GTP binding protein ADP ribosylation factor six (ARF 6) and the ARF nucleotide exchange factor ARNO (Claing *et al.* 2001). Upon agonist stimulation of the receptor, β-arrestin binds to ARF 6. In contrast ARNO interaction with β-arrestin is ligand independent. Once ARNO and the GDP form of ARF are brought together by β-arrestin, ARNO is responsible for the GDP/GTP exchange and activation of ARF 6. ARF 6 then dissociates from β-arrestin and is able to induce the endocytic process by a mechanism, which has not yet been elucidated. Over-expression of ARNO, the ARF activator, enhances internalisation of the β_2-AR and over-expression of GIT 1, an ARF GAP, inhibits internalisation. Excess GIT 1 might lead to rapid inactivation of ARF 6 and inhibition of vesicle formation, whereas excess of ARNO might lead to vesicle formation following ARF 6 activation. Like GIT 1, ARNO, GRKs and β-arrestins are able to interact with PIP_2 and IP_3.

Other β-arrestin interacting proteins are NSF and Src, which will be discussed later, as well as proteins involved in MAPK signalling Raf, ASK 1, ERK and JNK 3.

1.5.3 Clathrin and the AP-2 complex

Clathrin coated vesicles exist in all nucleated cells, from yeast cells to mammalian cells. This form of vesicle transport is not only used for endocytosis of proteins (e.g. a receptor and its ligand) from the plasma membrane, but also for transport of proteins and lipids from the trans-Golgi network (TGN) to the endosome etc.

The clathrin molecule is the key element of clathrin mediated endocytosis and consists of three heavy chains (190 kDa each) and one light chain (25 kDa). Clathrin forms a spider like structure, called triskelion, with three legs radiating from a central joint. The clathrin triskelions form a lattice of pentagons and hexagons, which appears like a ball-like cage around the lipid bilayer (Kirchhausen 2000). Clathrin itself however does not interact with the lipid bilayer and requires the assistance of other proteins to be recruited to the membrane. These proteins are for example β-arrestin and the adapter protein (AP) complex. In mammalian cells there are four different AP complexes, AP-1 to AP-4. Each AP complex consists of an α-type large chain, a β-type large chain and μ and σ chains. The AP complex required for clathrin-mediated endocytosis is AP-2 (α, β_2, μ_2, σ_2). The other AP complexes are involved in intracellular trafficking. The heterotrimeric adapter proteins, AP-1 and AP-2, associate with clathrin through a domain situated in the β-chain of the adapter. While AP 1 is found in coated vesicles associated with the trans-golgi network, AP-2 is found in coated vesicles derived from the plasma membrane. The clathrin box in the APs is similar to the clathrin-binding domain in β-arrestin, indicating that these proteins interact at the same site with clathrin (Kirchhausen 2000). The consensus sequence for the clathrin box, which has recently also been found in GRK 2 (Shiina *et al.* 2001), is *L L p L (-)* (L denotes a leucine, p and (-) denote a polar and a negatively charged residue).

The AP-2 complex and other proteins containing a clathrin box recruit clathrin to the plasma membrane, however AP-complexes need to be membrane localised themselves with the help of small G proteins. In the case of AP-1 the small G protein ARF 1 is essential for targeting to the TGN membranes (Zhu *et al.* 1999). For AP-2 an equivalent protein has not been found yet, but it is likely that ARF 6 takes over the role of ARF 1, since ARF 6 has recently been shown to form a complex with β-arrestin and the β_2-AR after ligand stimulation (Claing *et al.* 2001). Furthermore, another possibility for membrane location of the AP-2 complex is the ability to directly interact with the conserved NPXXY motifs of some GPCRs (Kirchhausen *et al.* 1997).

Recruitment of clathrin and the AP-2 to the membrane leads to invagination of the membrane and formation of a receptor/β-arrestin containing clathrin-coated pit. The clathrin-coated pit is then pinched off by dynamin and the vesicle is internalised.

1.5.4 Dynamin

Dynamin is the GTPase responsible for fission of the clathrin-coated vesicle from the plasma membrane.

Initially dynamin molecules are targeted to coated pits in its GDP bound or unbound form followed by distribution randomly throughout the clathrin lattice. Binding of GTP and GDP/GTP exchange respectively induces movement of the molecules towards the neck of the coated pits forming a ring around them. It is thought that dynamin assembly leads to hydrolysis of GTP inducing a conformational change in dynamin, which is required for vesicle budding (Schmid *et al.* 1998). The GDP bound dynamin molecules are then disassembled and recycled. The exact mechanism of dynamin mediated vesicle fission is however still controversial. It has been argued that dynamin acts as a molecular "switch" or as a pinchase (Thompson and McNiven 2001). Switch-like GTPases have very high affinity for GTP and a rather slow rate of GTP hydrolysis. These enzymes are "switched on" when bound to GTP and "switched off" when bound to GDP. In contrast mechanoenzymes (e.g. dynein) bind ATP with low affinity but have a high hydrolysis rate. Even though dynamin binds GTP and not ATP, it has low affinity for the nucleotide, high hydrolysis rate and it tends to release GDP easily. Interestingly mutations in the GTPase domain of dynamin that keep the enzyme in a GTP bound state inhibited endocytosis, indicating that GTP alone is not sufficient. Dynamin also needs to undergo conformational change induced by GTP hydrolysis, which suggests that dynamin is actually a mechanoenzyme (Thompson and McNiven 2001).

However other studies suggest that oligomerisation and extension of the dynamin ring leads to fission of the vesicle (Stowell *et al.* 1999). According to this model dynamin molecules are tightly packed when associated with GTP and expand after GTP hydrolysis leading to shearing of the neck of the vesicle. In this study dynamin was described to be a "poppase" rather than a "pinchase".

In mammalian cells there are three different isoforms of dynamin. Dynamin 1 (Dyn 1) is expressed in neurons and dynamin 3 (Dyn 3) is mainly expressed in testes, whereas dynamin 2 (Dyn 2) is found ubiquitously. Dynamin is a 100 kDa protein consisting of multiple domains. The C-terminus contains a pleckstrin homology (PH) domain and a proline/arginine rich domain (PRD). Between these two domains, which are required for protein-protein and protein-lipid interactions, is a region necessary for high rates of GTP hydrolysis, the so-called GTPase effector domain (GED). The PRD domain has been shown to be important for dynamin assembly, since deletion of this region results in a dynamin isoform that cannot accumulate around the neck of clathrin coated pits (Okamoto *et al.* 1997). Within the PRD domain there is a SH3 binding motif, which allows proteins possessing a SH3 domain to interact with dynamin. A major SH3 binding partner for dynamin is amphiphysin. Amphiphysin itself has been shown to bind to clathrin and the AP-2 complex. Amphiphysin is thought to provide a physical link between dynamin and the clathrin lattice thus being responsible for recruiting dynamin to the plasma membrane (Wang *et al.* 1995; Ramjaun and McPherson 1998). Another SH3 binding partner for dynamin is endophilin. Endophilin has been suggested to be an effector of dynamin responsible for membrane invagination and is essential for the formation of vesicles (reviewed in (Claing *et al.* 2002).

Other SH3 domain containing proteins that have been found to bind to dynamin include Grb2, phospholipase Cγ, the p85 subunit of phosphatidyl inositol 3-kinase, Src and cortactin (Gout *et al.* 1993; Foster-Barber and Bishop 1998). Phosphorylation of dynamin at two tyrosine residues by the non-receptor tyrosine kinase Src has been shown to be critical for β_2-AR internalisation (Ahn *et al.* 1999). Mutation of these residues not only abolishes receptor internalisation but also receptor mediated MAPK signalling.

The PH domain of dynamin is required to bind membrane phosphoinositides and to bind the $\beta\gamma$ subunits of heterotrimeric G proteins.

There is indication that βγ subunits might be directly involved in internalisation, the exact mechanism is however unclear (Lin *et al.* 1998).

Dynamin is not only responsible for clathrin coated vesicle fission, but also for fission of caveolae, however partners associated with the caveolae pathway have not been identified yet.

1.5.5 Src

Src is a non-receptor tyrosine kinase involved in Ras mediated MAPK signalling, but recent evidence also suggests an important role for Src in receptor internalisation.

Src has been shown to phosphorylate dynamin after ligand stimulation of the β_2-AR (Ahn *et al.* 1999). Phosphorylation deficient dynamin (Y231F, Y597F) or blocking dynamin phosphorylation using a Src specific inhibitor significantly reduced β_2-AR internalisation. Furthermore expression of a catalytically inactive mini construct of Src containing only the SH1 domain blocks dynamin phosphorylation and β_2-AR internalisation (Miller *et al.* 2000).

In order to be able to phosphorylate dynamin, Src must first be translocated to the plasma membrane, which is either achieved by interaction with the adaptor β-arrestin (Luttrell *et al.* 1999; Miller *et al.* 2000) or by direct interaction with the receptor (Cao *et al.* 2000). In unstimulated cells Src is associated with intracellular vesicle membranes and β-arrestin is found in the cytosol. After stimulation of the β_2-AR, β-arrestin moves to the plasma membrane and recruits Src. Later on β-arrestin, Src and the receptor co-localise in clathrin-coated pits.

The question arises how receptors internalising in a β-arrestin independent manner are able to attract Src. In the case of the β_3-AR, the receptor itself recruits and binds Src, which has been shown to be essential for β_3-AR mediated MAPK activation (Cao *et al.* 2000).

1.5.6 NSF

Another protein that is recruited by β-arrestin is N-ethylmaleimide-sensitive fusion protein (NSF). NSF is an ATPase essential for membrane fusion (Block *et al.* 1988).

NSF interacts with SNAPs (soluble NSF attachment proteins), which themselves form a complex with SNAP receptors (SNAREs). The complex of NSF, SNAPs and SNAREs is called the 20S particle and forms a critical component of the machinery necessary for membrane fusion (Brunger 2000). Initially NSF was identified as a β-arrestin binding partner in a yeast two hybrid screen. Thereafter experiments in HEK 293 cells showed that over-expression of NSF dramatically enhanced internalisation of the β_2-AR (McDonald *et al.* 1999).

Similarly to Src, a β arrestin independent direct interaction of NSF with the receptor has been observed. NSF can also directly bind to the C-terminal amino acids of the β_2-AR (Cong *et al.* 2001). Internalisation and recycling of the β_2-AR could be enhanced by over-expression of NSF. Internalisation and recycling of a mutant receptor lacking the C-terminal amino acids however was unaffected by NSF (Cong *et al.* 2001). Interestingly the NSF binding site at the end of the C-terminal tail of the β_2-AR overlaps with the binding site for a proton pump called NHERF (Na^+/H^+ exchanger regulatory factor).

In contrast to NSF, NHERF binds to the β_2-AR via a PDZ domain. The NHERF/β_2-AR interaction regulates the endocytic sorting of the receptor. (Cao *et al.* 1999). Prevention of NHERF/β_2-AR interaction changes the receptor fate from recycling to degradation. It is thought that NSF interaction with the β_2-AR is also involved in regulation of receptor trafficking (Cong *et al.* 2001).

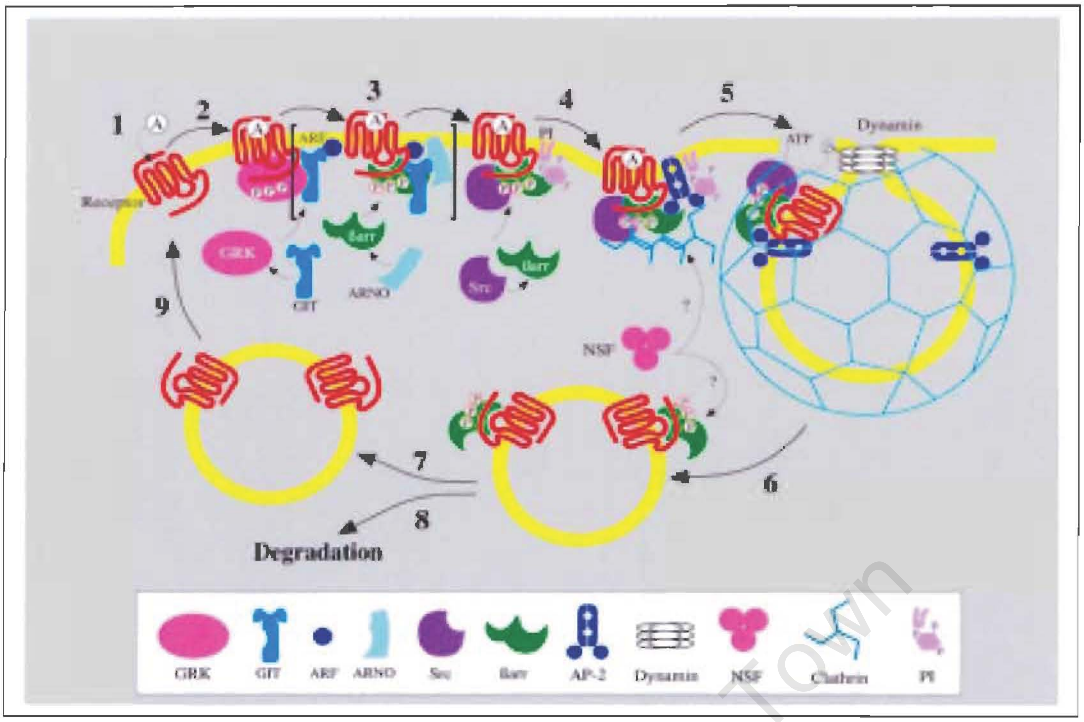

Fig. 1.1 Overview of proteins involved in clathrin coated vesicle internalisation from (Claing *et al.* 2002). GRKs phosphorylate agonist activated receptor followed by binding of β-arrestins. β-arrestin interacts with AP-2 and clathrin, which leads to formation of a clathrin coated pit. GRKs interact with GIT proteins and β-arrestins interact with ARF6 and ARNO. The exact events regulated by these interactions remain unclear.β-arrrestin can bind to Src, which regulates dynamin phosphorylation. Dynamin is responsible for vesicle fission. Once internalised the receptors can either become recycled or degraded.

1.6 The Gonadotropin-releasing hormone receptor

1.6.1 The type I GnRH receptor

The type I GnRH receptor is a central regulator of reproduction in vertebrates and protochordates. The GnRH receptor belongs to the subfamily of rhodopsin-like GPCRs and is mainly expressed in the gonadotropes of the anterior pituitary, where it interacts with its ligand, GnRH I. GnRH I is a decapeptide and the amino acid sequence for mammalian GnRH I is pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2.

GnRH itself is produced in neurones of the medial basal hypothalamus by cleavage of a larger precursor. It is then secreted in a pulsatile manner into the hypophyseal portal vasculature. Upon binding of GnRH I to the type I GnRH receptor in the gonadotropes of the anterior pituitary, the receptor undergoes conformational change and initiates a complex signal transduction cascade, which finally results in the biosynthesis and secretion of two gonadotropins, luteinising hormone (LH) and follicle stimulating hormone (FSH). LH and FSH are both large glycoprotein hormones and are transported to the gonads, where they regulate steroidogenesis and gametogenesis (for review, see (Stojilkovic *et al.* 1994)).

The first type I GnRH receptor was cloned from the mouse gonadotrope cell line αT3-1 (Tsutsumi *et al.* 1992), but cloning of GnRH receptor cDNAs from other species including human followed soon thereafter (Kakar *et al.* 1992; Reinhart *et al.* 1992; Chi *et al.* 1993; Illing *et al.* 1993; Knox *et al.* 1994; Hauser *et al.* 1998; Byrne *et al.* 1999; Illing *et al.* 1999; Troskie *et al.* 2000; Robison *et al.* 2001; Sun *et al.* 2001; Wang *et al.* 2001).

Most vertebrate species have one or two other forms of GnRH hormones. The GnRH II peptide is highly conserved from fish to man (Sherwood *et al.* 1993) and differs from GnRH I in positions five, seven and eight (pGlu-His-Trp-Ser-**His**-Gly-**Trp-Tyr**-Pro-Gly-NH_2). The structure of the GnRH genes, suggests that the existence of these two distinct peptides is a result of an early gene duplication. In contrast to GnRH I, GnRH II is not only widely distributed throughout the brain but also found in kidney, bone marrow, prostate, ovarian epithelial cells, endometrium and breast tissue (White *et al.* 1998; Cheon *et al.* 2001; Choi *et al.* 2001; Chen *et al.* 2002).

The human type I GnRH receptor has a 10 fold lower affinity for the type II peptide compared to the type I peptide (Millar *et al.* 2001). The wide distribution of GnRH II and the lower affinity towards the type I GnRH receptor compared to GnRH I indicates the presence of a second GnRH receptor.

1.6.2 The type II GnRH receptor

The type II GnRH receptor has recently been cloned from marmoset monkey (Millar *et al.* 2001), from African green monkey and rhesus monkey (Neill *et al.* 2001), as well as from bullfrog (Wang *et al.* 2001) and *Xenopus laevis* (Troskie *et al.*, unpublished).

Human type II GnRH receptor transcripts have been found in a wide range of tissues, but they have a frame shift mutation shortly after the methionine start codon and they have a stop codon in exon 2, and therefore raise doubt as to their functionality (Faurholm *et al.* 2001; Morgan *et al.* 2003). These transcripts originate from a genomic locus on chromosome one. In the antisense orientation this locus encodes for the RNA-binding motif protein 8 (RBM8A) overlapping with the gene for the type II GnRH receptor. A second locus for the GnRH receptor is situated on chromosome 14, however this locus contains pseudogenes for both RBM8A and the type II GnRH receptor, which might have originated by retro transposition of a cDNA.

The type II GnRH receptor has only 41 % identity with the type I GnRH receptor and is highly selective for GnRH II. GnRH II has 24 fold greater affinity for the type II GnRH receptor compared to the type I GnRH receptor (Millar *et al.* 2001). In contrast to the type I receptor the type II receptor is widely distributed and, like the GnRH II peptide, is expressed throughout the brain including the gonadotropes of the pituitary (Millar *et al.* 2001). Receptor expression is high in reproductive tissues (testis, prostate, mammary glands), in adrenal, thyroid, heart and pancreas.

The function of the type II GnRH receptor has not yet been fully established (Millar 2002). However the presence of both the type II and the type I receptor on the gonadotropes of the anterior pituitary suggests that these receptors might regulate LH and FSH biosynthesis and release together. Studies on sheep revealed that GnRH I was generally more potent in mediating LH and FSH secretion compared to GnRH II, but the ratio of FSH versus LH release was significantly higher when treated with GnRH II (Millar *et al.* 2001; Millar 2002). These results indicate that the type II GnRH receptor preferentially mediates FSH release and that GnRH II could be a specific FSH releasing factor. Apart from changes in GnRH I pulse frequency and feedback mechanisms from gonadal steroid hormones, the existence of an FSH releasing factor would explain the differential release of FSH and LH throughout the mammalian ovarian cycle.

Furthermore GnRH II is present in sympathetic ganglia of amphibia and binds to sympathetic ganglion receptors. Type II GnRH receptor activation in sympathetic ganglia inhibits M-type K^+ channels (Troskie *et al.* 1997). Inhibition of these K^+ channels facilitates excitatory transmission by neurotransmitters. This mechanism, that still has to be elucidated in detail, might represent a general role for the type II GnRH receptor and GnRH II as a neuromodulator in the central nervous system (Millar *et al.* 2001; Millar 2002).

The type II GnRH receptor could possibly also play a role in sexual arousal (Millar *et al.* 2001; Millar 2002). It has been shown that GnRH II is much more effective than GnRH I in stimulating mating behaviour in song sparrows and musk shrew (Maney *et al.* 1997; Rissman *et al.* 1997). Consistent with this observation lesions of brain areas in which also the type II GnRH receptor is expressed effect sexual interest and reproductive behaviour (Slimp *et al.* 1978)

1.6.3 GnRH receptor structure

Type I GnRH receptor belongs to the subfamily of rhodopsin-like GPCRs. The protein structure of the GnRH receptor consists, as is characteristic for GPCRs in general, of an extracellular N-terminus followed by seven TM helices, which are connected by alternating extracellular and intracellular loops. However the mammalian type I GnRH receptor is unique amongst GPCRs in that it lacks a cytoplasmic C-terminal tail. In contrast the non-mammalian type I GnRH receptors and all type II GnRH receptors possess C-terminal tails (for review, see (Sealfon and Millar 1995)) (Millar *et al.* 2001; Neill *et al.* 2001; Wang *et al.* 2001)(Troskie *et al.*, unpublished).

Within the extracellular N-terminus of all GnRH receptors one or more consensus sequences for N-glycosylations (Asn-X-Ser/Thr) is present. The mouse type I GnRH receptor has three putative glycosylation sites of which two are indeed glycosylated (Davidson *et al.* 1995), most other GnRH receptors including the type II receptor have only one glycosylation site. GnRH receptor glycosylation has been shown to have no influence on ligand affinity, but mutation of the asparagine within the glycosylation consensus sequence significantly decreased receptor expression (Davidson *et al.* 1995).

The GnRH receptors possess cysteine residues in ECL 1 and ECL 2, which form disulfide bonds. For the human type I GnRH receptor one disulfide bridge was demonstrated between Cys 104 and Cys 196 and a second one between Cys 14 and Cys 200 (Davidson *et al.* 1997). The disulfide bridge between the N-terminus (Cys 14) and ECL 2 (Cys 200) is not critical for receptor function, the second disulfide bond between ECL 1 and ECL 2 however is required for receptor function. Disulfide bridges for the type II GnRH receptor have not yet been demonstrated, however Cys 113 in ECL 1 and Cys 188 in ECL 2 are thought to form a disulfide bond.

Usually GPCRs have a highly conserved aspartate residue in TM 2 and a highly conserved asparagine in TM7. In the mammalian type I GnRH receptors these two residues are interchanged. The asparagine is found in TM 2 and the aspartate is found in TM 7 as part the motif DPLIY, which is the NPXXY motif in most other GPCRs. Both residues have been shown to be important for receptor expression, receptor activation and G protein coupling (Flanagan *et al.* 1999). Mutation of the asparagine to aspartate eliminated receptor function, however additional mutation of the aspartate in TM 7 to asparagine restored receptor function, suggesting that the two residues have complementary roles in maintaining receptor structure (Zhou *et al.* 1994).

Interestingly the type II GnRH receptor and the non-mammalian type I GnRH receptors have an Asp/Asp motif in this position, different to mammalian type I GnRH receptors as well as most other GPCRs. However the *Drosophila* GnRH receptor possesses the usual Asp/Asn motif (Hauser *et al.* 1998). It has been suggested that an initial mutation from an Asp/Asn motif (*Drosophila)* to an Asp/Asp motif (type II receptors and non-mammalian type I receptors) might have been followed by a second mutation to Asn/Asp (mammalian type I receptors) (Millar 2002). The recent crystallisation of rhodopsin (Palczewski *et al.* 2000) showed that in the tertiary structure transmembrane helix two and helix seven are interacting with each other. The distance between the asparagine in TM 7 and the aspartate in TM 2 is too long to form a direct hydrogen bond, but a water molecule near the aspartate seems to be able to interact with the side chain of the asparagine (Palczewski *et al.* 2000). Based on the crystal structure of rhodopsin (Palczewski *et al.* 2000) one can assume that the seven transmembrane helices of the GnRH receptor are arranged in a bundle forming a hydrophilic ligand-binding pocket.

The among GPCRs highly conserved DRY sequence at the cytoplasmic side of TM 3 is DRS in the mammalian type I GnRH receptors and DRQ in the mammalian type II GnRH receptors. Similar to other GPCRs this motif is involved in receptor activation. For the GnRH receptor it has been shown that substitution of the serine residue by a tyrosine did not affect G-protein coupling, but increased ligand affinity (Arora *et al.* 1995).

The ligand-binding site for GnRH is located in the extracellular and TM domains of the GnRH receptor. The region in GnRH receptors important for ligand selectivity is ECL 3. In this area the mammalian type I GnRH receptors have a LSD/EP motif and the glutamate/aspartate within this sequence determines the selectivity for GnRH ligands containing an arginine in position 8 (e.g. GnRH I) (Sealfon *et al.* 1997; Fromme *et al.* 2001). This motif is replaced by VPPS in mammalian and reptile type II GnRH receptors and by VPPV in amphibian type II GnRH receptors (Millar *et al.* 2001; Wang *et al.* 2001). The difference in ECL 3 between the type I and the type II GnRH receptors might determine the selectivity for GnRH I, which possesses an arginine in position 8, and GnRH II, which possesses a tyrosine in position 8.

The binding pocket of the type I GnRH receptor is formed by residues in TM 2 and TM 3 (Sealfon *et al.* 1997). TM 3 contains Lys 121, which is conserved in all cloned GnRH receptors and which is important for agonist as well as antagonist binding (Sealfon *et al.* 1997). Asp 98 in TM 2 interacts with and therefore confers specificity for His 2 in GnRH (Flanagan *et al.* 2000). Asn 102 at the extracellular side of TM 2 is proposed to interact with the glycine amide of GnRH. The residues that have been shown to play a role in ligand interaction of the mammalian type I GnRH receptor with GnRH I are also found in the type II GnRH receptor (Lys 120, Asp 97 and Asn 101), but their role in binding GnRH II still has to be elucidated.

The C-terminal domain of ICL 3 contains a residue that is required for coupling of the type I GnRH receptor to $G_{q/11}$. Ala 261 is necessary to stabilise a receptor conformation required for G-protein interaction (Myburgh *et al.* 1998). The type I GnRH receptor can also couple to G_s via a BBXXB (B = basic amino acid) motif in ICL 2 (Arora *et al.* 1998). For the type II GnRH receptor G-protein binding sites have not yet been identified.

1.6.4 GnRH receptor signalling pathways

Like other GPCRs, the GnRH receptors can signal through two major pathways, G protein/effector signalling and MAPK signalling. The type II GnRH receptor differs however in the signalling cascades compared to the type I GnRH receptor.

Both receptors primarily activate G proteins of the $G_{q/11}$ subfamily. $G_{q/11}$ proteins activate phospholipase Cβ (Fig. 1.2), which hydrolyses phosphatidylinositol 4,5-biphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 binds to IP_3 receptors on the membrane of the endoplasmic reticulum (ER), which leads Ca^{2+} efflux from the ER into the cytosol. GnRH receptor activation also results in opening of voltage-gated Ca^{2+} channels of the plasma membrane. DAG, still associated with the plasma membrane, activates PKC in a Ca^{2+} dependent manner.

The type I GnRH receptor can also activate G_S, which stimulates adenylate cyclase and PKA following increase in cAMP levels (Arora *et al.* 1998).

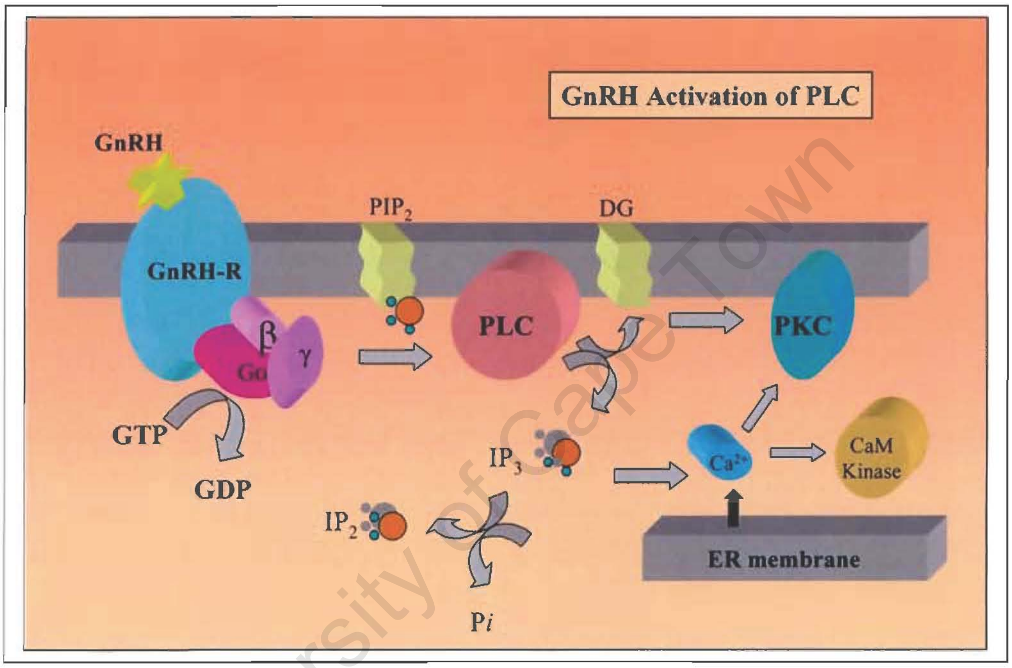

Fig. 1.2 GnRH induced activation of PLC and subsequent IP_3 production, Ca^{2+} release and PKC and CaM kinase activation

The G protein signalling pathway can converge with the MAPK pathway at several different levels. The free intracellular Ca^{2+} can activate Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II), which then can phosphorylate and inactivate Ras-GTPase activating protein (Ras-GAP) and induce Ras and MAPK activation (Chen *et al.* 1998). For the GnRH receptor intracellular calcium is required for ERK kinase activation, however direct involvement of CaM kinase has not been shown yet. Type I GnRH receptor mediated ERK activation in pituitary cells was shown to be PKC dependent (Reiss *et al.* 1997). Previously it had been thought that in case of the GnRH receptor ERK activation uses a Ras independent mechanism, through direct activation of Raf by PKC (Naor *et al.* 2000).

However a second pathway leading to Raf activation via Src activated Ras has been identified (Fig. 1.3) (Kraus *et al.* 2001). PKC is both required and sufficient for GnRH mediated ERK activation, in contrast intracellular Ca^{2+} is required, but on its own not sufficient for ERK activation (Naor *et al.* 2000).

As for ERK activation, JNK activation through the type I GnRH receptor is PKC dependent (Levi *et al.* 1998). Usage of PKC inhibitors diminished JNK activation by 70% whereas protein tyrosine kinase inhibitors abolished JNK activation completely, indicating a less predominant role for PKC in this pathway. After GnRH stimulation the main mediator for JNK activation has been shown to be the non-receptor tyrosine kinase Src (Levi *et al.* 1998). However the type I GnRH receptor does not interact with β-arrestin, which recruits Src in case of the β_2-AR (Luttrell *et al.* 1999; Miller *et al.* 2000).

Therefore the GnRH receptor could possibly directly interact with Src as direct interaction between Src and GPCRs has also been reported (Cao *et al.* 2000; Fan *et al.* 2001). Nevertheless neither direct nor indirect interaction of Src with the GnRH receptor has been shown to date. The type I GnRH receptor also activates BMK and P38 kinase, but the exact mechanism of activation of these two MAPKs still has to be elucidated (for review see (Kraus *et al.* 2001)).

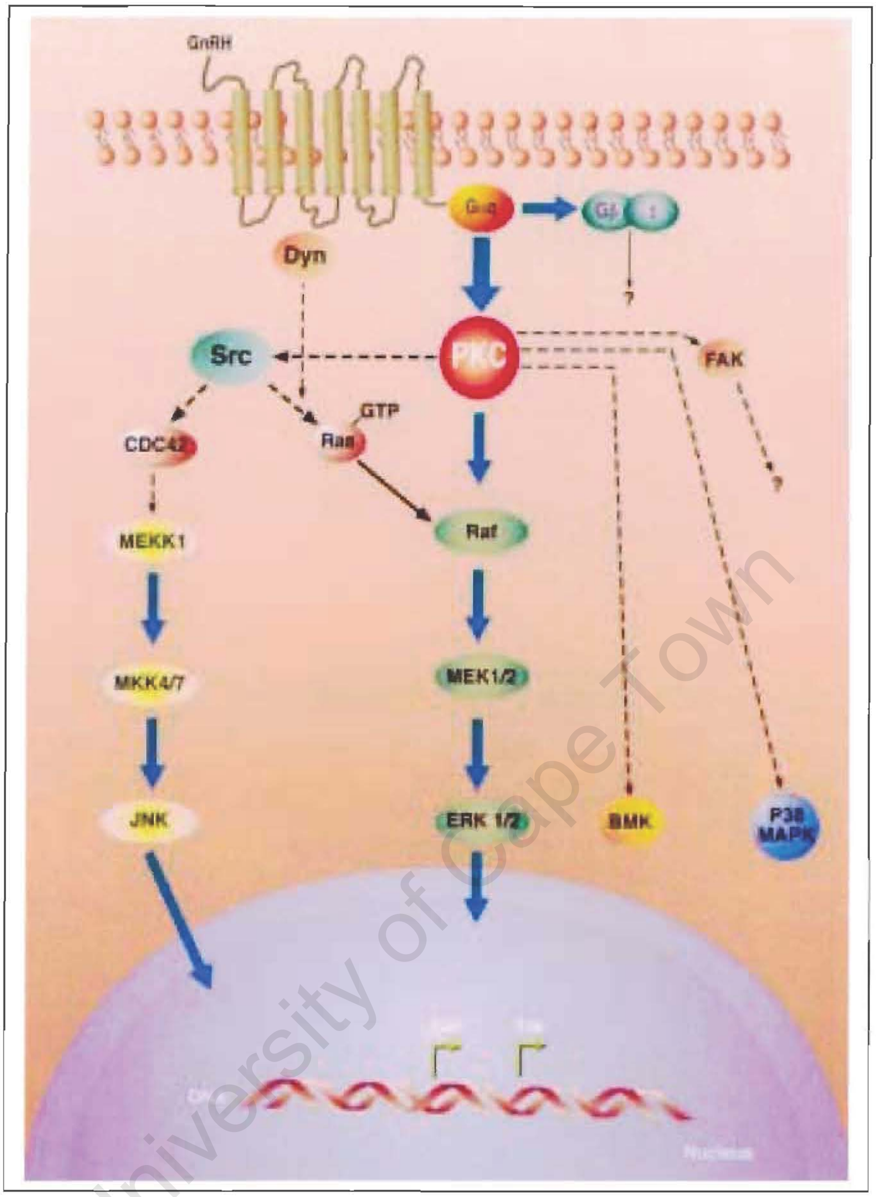

Fig. 1.3 Model of MAPK activation by GnRH in the mouse gonadotrope α-T3-1 cell line from (Kraus *et al.* 2001).

The novel type II GnRH receptor can activate ERK as well as p38 kinase, the exact mechanisms have however not yet been elucidated (Millar *et al.* 2001). Millar et al. reported that in COS-7 cells both the type I and the type II GnRH receptor activate ERK to a similar extent, but on a time course ERK activation mediated by the type II GnRH receptor was more protracted. In the same cell type the type II GnRH receptor could activate p38 MAPK while the type I GnRH receptor could not (Millar *et al.*

2001). Nevertheless other groups have reported p38 MAPK activation by the type I GnRH receptor in α-T3-1 cells (Roberson *et al.* 1999). The different results are possibly due to different cell types used for these experiments.

The distinct differences in signalling by the type I and the type II GnRH receptor suggest different effects especially in cells that express both proteins eg. gonadotropes.

1.6.5 GnRH receptor desensitisation and internalisation

One of the most interesting features of the mammalian type I GnRH receptor is the lack of a C-terminal tail, which is generally thought to be important for rapid desensitisation and internalisation of GPCRs.

Indeed the tail-less mammalian GnRH receptors are not phosphorylated in response to agonist and do not desensitise rapidly compared to other receptors (Davidson *et al.* 1994; Willars *et al.* 1999). However the two non-mammalian GnRH receptors investigated to date, the catfish (Heding *et al.* 1998) and the *Xenopus* GnRH receptor (Hislop *et al.* 2000), do desensitise rapidly, which was attributed to the presence of a C-terminal tail in these receptors. Furthermore for the catfish GnRH receptor agonist-induced phosphorylation of the C-terminal tail could be demonstrated (Blomenrohr *et al.* 1999; Willars *et al.* 1999). When the C-terminal tail of the catfish GnRH receptor was added to the human receptor, this chimeric receptor displayed agonist induced phosphorylation and rapid desensitisation (Willars *et al.* 1999).

The C-terminal tail of GPCRs not only plays an important role in receptor desensitisation, but also in internalisation. Mutation of the phosphorylation site of the catfish GnRH receptor decreased internalisation (Blomenrohr *et al.* 1999). The tail-less mammalian type I GnRH receptors, such as the human (Pawson *et al.* 1998; Hislop *et al.* 2000) and rat GnRH receptor (Vrecl *et al.* 1998), internalise slower than the non-mammalian receptors such as the chicken receptor (Pawson *et al.* 1998), the catfish

(Blomenrohr *et al.* 1999; Heding *et al.* 2000; Vrecl *et al.* 2000) or the *Xenopus* GnRH receptor (Hislop *et al.* 2000). Studies on the chicken GnRH receptor showed that truncation of the C-terminal tail leads to a receptor that internalises to the same extent than the tail-less human GnRH receptor (Pawson *et al.* 1998). On the other hand addition of the C-terminal tail of the TRH receptor or the C-terminal tail of the catfish GnRH receptor to the rat GnRH receptor augmented endocytosis of the GnRH/TRH-R chimera (Heding *et al.* 1998), while the internalisation of the rat GnRH/catfish GnRH receptor tail chimera was not enhanced compared to wild type rat GnRH receptor internalisation (Willars *et al.* 1999; Hanyaloglu *et al.* 2001). Addition of the *Xenopus* C-terminal tail to the human GnRH receptor even reduced internalisation (McArdle *et al.* 2002, 84th Annual Meeting of the Endocrine Society, San Fransciso, USA).

For many GPCRs agonist-induced receptor phosphorylation is followed by β-arrestin binding. Interaction of the catfish and the *Xenopus* receptor with β-arrestin has been shown either by agonist mediated translocation of β-arrestin or enhanced internalisation kinetics in presence of β-arrestin (Blomenrohr *et al.* 1999; Heding *et al.* 2000; McArdle *et al.* 2002). In contrast the tail-less GnRH receptors are not able to translocate β-arrestin and the internalisation is unaffected by co-expression of β-arrestin (Vrecl *et al.* 1998; McArdle *et al.* 2002). Nevertheless addition of the C-terminal tail of the TRH receptor to the rat GnRH receptor changes the internalisation from a β-arrestin independent to a β-arrestin dependent pathway (Heding *et al.* 2000). However addition of the catfish C-terminal domain did not change β-arrestin independent internalisation (Hanyaloglu *et al.* 2001). Nevertheless truncation of the C-terminal tail of the catfish receptor did affect β-arrestin mediated internalisation, suggesting the presence of a β-arrestin binding site in this area (Blomenrohr *et al.* 1999). Interestingly the TRH-R C-terminal tail has three consensus sequences for CKII whereas the C-terminal tail of the catfish GnRH receptor does not. Recently the role of CKII sites in determining β-arrestin dependent internalisation was investigated (Hanyaloglu *et al.* 2001). Introduction of three CKII site in the catfish C-terminal tail of a catfish/mammalian GnRH receptor chimera shifted the internalisation from a β-arrestin independent to a β-arrestin dependent pathway. Consistent with this mutation of the CK II sites of the TRH-R C-terminal tail resulted in a receptor internalising in a β-arrestin independent manner. However mutation of all three CK II sites was necessary to abolish β-arrestin dependency (Hanyaloglu *et al.* 2001). Therefore the determinants for β-arrestin dependency seem to be more complex than simply a GRK phosphorylated C-terminal tail.

The co-expression of dominant negative dynamin K44A inhibited internalisation of the non-mammalian GnRH receptors (Heding *et al.* 2000; Hislop *et al.* 2001). Dynamin dependency was also shown for the tail-less rat GnRH receptor (Heding *et al.* 2000), although co-expression of dynamin K44A resulted in inhibition of only 20% of internalisation compared to the control, but internalisation of the human GnRH receptor was not significantly reduced by dominant negative dynamin (Hislop *et al.* 2001). However the differences in dynamin dependency between the rat and the human GnRH receptor might have occurred because of the very different experimental conditions used by the two research groups. Surprisingly addition of the *Xenopus* C-terminal tail to the human GnRH receptor resulted in a GPCR that internalises, like the wild-type GnRH receptor, in a dynamin dependent manner (McArdle *et al.* 2002, 84th Annual Meeting of the Endocrine Society, San Fransciso, USA).

Taken together these observations indicate that mammalian type I GnRH receptors do not become phosphorylated by GRKs after agonist stimulation and utilise a β-arrestin independent internalisation mechanism. In contrast non-mammalian type I GnRH receptors become phosphorylated and internalise in a β-arrestin dependent manner.

Little is known about the internalisation of the type II GnRH receptors. The non-mammalian type II GnRH receptors of bullfrog (Acharjee *et al.* 2002) and *Xenopus* (Troskie *et al.*, unpublished data) internalise rapidly.

The bullfrog type II GnRH receptor internalises in a dynamin dependent, but β-arrestin independent pathway, whereas the type I GnRH receptor of the same species requires β-arrestin and dynamin similarly to other non-mammalian type I GnRH receptors. Internalisation of a mammalian type II GnRH receptor is not known.

To this date internalisation of a mammalian type II GnRH receptor has not been described. This thesis will investigate the internalisation pathway of the type II GnRH receptor of marmoset monkey. In contrast to the mammalian type I GnRH receptor this receptor possesses a C-terminal tail, therefore the role of the C-terminal domain in internalisation will be identified. Furthermore the importance of residues in ICL 3 will be elucidated.

Chapter 2: Materials and Methods

2.1 Site Directed Mutagenesis

2.1.1 Truncations of the cytoplasmic C-terminal tail of the type II GnRH receptor

The type II GnRH receptor of marmoset monkey was cloned into the pcDNA 3.1+ expression vector (Invitrogen, San Diego, USA) using EcoRI and XbaI (Millar *et al.* 2001). Site directed mutagenesis was performed using a PCR based method with primers containing the desired mutations. For the C-terminally truncated mutants T372stop, S366stop, Q357stop, S344stop, S335stop and G326stop anti sense primers were used, which had the codons for each of the amino acids (Thr 372, Ser 366, Gln 357, Ser 344, Ser 335 and Gly 326) replaced by a stop codon followed by a XbaI restriction site (for primer details see appendix 9.1). PCR was performed with the anti sense primers mentioned above and a vector specific sense primer (T7) using pfu polymerase (Promega, Madison, USA). All PCRs were performed under the same conditions of denaturation (3 minutes at 95°C), 35 cycles of 30 seconds at 95°C, 30 seconds at 55°C and 1 minute at 72°C, followed by 10 minutes of final extension at 72°C. The PCR products were digested with EcoRI and XbaI, ligated into the pcDNA 3.1+ expression vector using a fast-link DNA ligation kit (Epicentre Technologies, Madison, USA) and transformed into competent *E. coli DH10B* using a standard heat shock protocol. Plasmid DNA was prepared using the Nucleobond PC 500 kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. The mutations were confirmed either by manual DNA sequencing (Epicentre Technologies, Madison, USA) or by automated sequencing using the MegaBACE 500 automated capillary DNA sequencing system and the DYEnamic ET Dye terminator cycle sequencing kit (Amersham, Little Chalfont, UK).

2.1.2 Mutations of putative phosphorylation sites

For mutations of putative phosphorylation sites in ICL 3 and/or the C-terminal tail of the type II GnRH receptor serine and threonine residues were mutated to alanine residues. Site directed mutagenesis was performed using a bridge PCR method with vector primers (T7 and BGH) and sense and anti sense primers containing the desired mutation as well as a silent restriction site. Different silent restrictions sites were used for each individual mutation in order to be able to identify the mutant receptors for list of silent sites see appendix 9.1). The PCR products were digested with EcoRI and XbaI and cloned into the pcDNA 3.1+ expression vector (Invitrogen, San Diego, USA). Using the described approach three receptor mutants lacking putative phosphorylation sites in ICL 3 (S231,232A and T235A,S239A and S251A) and one mutant was lacking a putative phosphorylation site in the cytoplasmic tail (S338,339A) were created. Furthermore mutant receptors that contained combinations of ICL 3 mutations and mutations of the cytoplasmic tail (S231,232,338,339A and T235,S239,338,339A and S251,338,339A) were made. Therefore the receptor containing the mutation in the C-terminal tail was digested with ApaI and XbaI and the TM 7 and C-terminal tail fragment was ligated into the constructs containing the ICL 3 mutations. For FLAG tagged receptor constructs the wild type receptor and the mutants S338,339A and S251,338,339A were cloned into the N-Terminal pFLAG-CMV expression vector (Invitrogen, San Diego, USA) via EcoRI and XbaI. The DNA sequence of all constructs was confirmed either by manual DNA sequencing (Epicentre Technologies, Madison, USA) or by automated sequencing using the MegaBACE 500 automated capillary DNA sequencing system and the DYEnamic ET Dye terminator cycle sequencing kit (Amersham, Little Chalfont, UK).

All GRK, β-arrestin and dynamin as well as FLAG-tagged β_2-AR constructs were received from M.G. Caron (Duke University Medical Center, Durham, NC), S. Maudsley (Human Reproductive Sciences Unit, MRC, Edinburgh, UK) and Anna Aragay (University of Bergen, Norway).

2.2 Cell Culture and Transient Transfections

COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, San Diego, USA) containing 10 % heat inactivated fetal calf serum (Delta Bioproducts, Kempton Park, South Africa) in a 10 % CO_2 incubator at 37 °C. During experiments 2 mg/ml streptomycin sulfate and 4000 U/ml sodium benzylpenicillin was added to the medium. For inositol phosphates and internalisation assays cells were plated out on poly-D-lysine coated 12 well plates (2×10^5 cells/well) and transfected with 2μg of DNA/well using a modified DEAE-Dextran method (Millar *et al.* 1995). For whole cell phosphorylation assays cells were plated out on 10 cm dishes, transfected 8 μg of DNA using *Lipofectamine* (Invitrogen, San Diego, USA) and 24 hours after transfection replated onto uncoated 6 well plates (10^6 cells/well).

2.3 GnRH analogues

[His5,D-Tyr6]-GnRH was synthesised on a Beckman System 990 peptide synthesizer by a former member (R. Milton) of the laboratory in Cape Town using conventional solid phase methodology and purified by preparative C-18 reverse phase HPLC. GnRH II was purchased from Bachem, Switzerland.

2.4 Receptor Internalisation Assays

Receptor internalization was measured using a ^{125}I labelled GnRH analogue as tracer. The high affinity agonist [His^5,D-Tyr^6]-GnRH was radioiodinated by the chloramine-T method as previously described (Flanagan *et al.* 1998). The internalization assays were based on an acid-wash method developed in the laboratory (Pawson *et al.* 1998). Transfected cells were washed once with ice cold DMEM buffered with 10 mM HEPES pH 7.2 (HEPES/DMEM) and incubated with 2×10^5 cpm/well ^{125}I-[His^5,D-Tyr^6]-GnRH in 0.5 ml HEPES/DMEM for 5 hours at 4 °C. Afterwards cells were rapidly warmed to 37 °C in a waterbath for different periods of time (ranging from five minutes to 90 minutes) and internalization was stopped by washing the cells twice with ice cold PBS (100 mM NaCl, 10 mM Na_2HPO_4, 0.5 mM EDTA, pH 7.4). Surface bound radioligand was removed by adding 1 ml ice cold acid solution (50mM acetic acid, 150 mM NaCl, pH 2.8) and counted in a gamma counter. Cells were lysed with 1 ml 0.1 M NaOH and acid resistant (internalized) ligand was counted. Internalized radioligand was expressed as a percent of total cell-associated radioligand at each time point.

2.5 Total Inositol Phosphates Assays

Total inositol phosphates assays were performed as previously described (Millar *et al.* 1995). 24 hours after transfection COS-1 cells were incubated overnight with 1 µCi/well of myo-[2-^{3}H] inositol (Amersham, Little Chalfont, UK) in 0.5 ml Medium 199 (Invitrogen, San Diego, USA). After another 24 hours cells were washed twice with 1 ml of buffer I (140 mM NaCl, 4 mM KCl, 20 mM Hepes, 8 mM glucose, 0.1 % BSA, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.4) and stimulated with various concentrations of GnRH II in buffer I containing 10 mM LiCl for 1 hour at 37 °C. Basal IP accumulation was measured in absence of ligand. Finally the medium was taken off, 1 ml of 10 mM formic acid was added and the cells were incubated at 4 °C. The total inositol phosphates were separated from the cell extracts on 1 ml 1X8-200 DOWEX-1 ion exchange columns (Sigma-Aldrich, Steinheim, Germany). Before loading the sample onto the columns, the resin was saturated with 3 ml 3 M ammonium formate with 0.1 M formic acid and washed with 10 ml dH_2O. After loading the sample the columns were washed with 10 ml dH_2O, followed by 5 ml 5 mM myo-inositol with 0.1 M formic acid. The inositol phosphates were eluted with 3 ml of 1 M ammonium formate with 0.1 M formic acid into 18 ml of scintillation fluid (Zinsser Analytical, Frankfurt, Germany) and the radioactivity was counted.

2.6 Receptor Phosphorylation Assays

Receptor phosphorylation assays were performed according to a previously described method (Oppermann *et al.* 1996; Thomas *et al.* 1998). COS-1 cells were transfected with FLAG tagged wild type and mutant GnRH receptors as well as with β_2-AR in 10 cm dishes, trypsinised the following day and plated onto 6 well plates (10^6 cells/well). The cells were washed with phosphate free DMEM (Invitrogen, San Diego, USA) and incubated with 200 µCi/well ^{32}P-orthophosphate (Amersham, Little Chalfont Heights, UK) in phosphate free DMEM for 2 hours in a 10 % CO_2 incubator at 37 °C. 10 minutes before stimulating the cells with 1 µM GnRH or 0.1 mM epinephrine II for 15 minutes, okadaic acid (0.2 µM) was added to the wells. After stimulation with ligand the cells were washed twice with ice cold PBS and lysed in lysis buffer (50 mM Tris-HCl pH 7.6, 200 mM NaCl, 1% NP-40, 0.1 % sodium deoxycholate, 0.05 % SDS, 0.5 mM EDTA, 1 mM PMSF, 10 µg/ml leupeptin, 10 mM sodium fluoride, 10 mM tetra sodium pyrophosphate, 1 mM sodium orthovanadate, 0.5 µM okadaic acid). The cell lysate was centrifuged and the supernatant was incubated with anti-FLAG (M2) agarose beads (Sigma-Aldrich, Steinheim, Germany) overnight at 4 °C. The beads were washed three times with lysis buffer, resuspended in Laemmli buffer (125 mM Tris-HCl pH 6.8, 10 % glycerol, 5 % SDS, 12.5 µg/ml bromophenol blue, 2.5 % β-mercaptoethanol) and incubated at 95°C for 2 minutes. The immunoprecipitated proteins were loaded onto an SDS-polyacrylamide gel.

2.7 SDS-Polyacrylamide gel electrophoresis

All SDS-polyacrylamide gels consisted of a 10% resolving gel and a 5% stacking gel and were casted in the Mini PROTEAN II multicasting chamber (Biorad, Hercules, CA, USA). The gels were run in 1 x SDS-running buffer (24.8 mM Tris, 192 mM Glycine, 1 % SDS) in the Mini PROTEAN II gel electrophoresis tank (Biorad, Hercules, CA, USA) at 70 volt until the dye reached the interface between the stacking gel and the resolving gel. Thereafter the voltage was increased to 200 volt until the dye reached the bottom of the gel.

2.9 Western Blot

After separation 10 % SDS-PAGE the proteins were blotted onto a Hybond P (PVDF) membrane (Amersham, Little Chalfont, UK) using the Mini PROTEAN II Western Blotting System from Biorad (Biorad, Hercules, CA, USA). The blotting took place in 1 x transfer buffer (20 mM Tris, 150 mM Glycine, 20 % methanol) at 100 volt for 1 hour. Afterwards the non-specific binding sites were blocked in 5 % milk powder in TBS for 30 minutes. For detection of the FLAG-tagged proteins the membrane was incubated with anti-FLAG M2 antibody (Sigma-Aldrich, Steinheim, Germany) as a dilution of 1:400 in TBS for 45 minutes. The blot was washed 3 x 5 minutes with TBS + 0.1 % Tween before being incubated with the anti-mouse HRP conjugated secondary antibody (Amersham, Little Chalfont, UK). For detection of the proteins the ECL plus detection system (Amersham, Little Chalfont, UK) was used.

2.10 Immunofluorescence

COS-1 cells were grown on cover slips and transfected with 2µg of FLAG-tagged GnRH receptor DNA using the Lipofectamine method. 24 hours after the cells were stimulated with 1μM GnRH for 10 minutes before being washed twice with PBS (100 mM NaCl, 10 mM Na_2HPO_4, 0.5 mM EDTA, pH 7.4). Afterwards the COS-1 cells were fixed in 3% para formaldehyde in PBS for 15 minutes, quenched in 50 mM NH_4Cl in PBS for 10 minutes and washed 3 times in PBS. The cells were permeabilised in 0.1% Triton-X-100 in PBS for 30 seconds and again washed for 3 times in PBS. The cells were blocked with 3% BSA in PBS for 30 minutes and then incubated for 1 hour with the primary antibody , a 1:400 dilution of the anti-FLAG M2 antibody from mouse (Sigma-Aldrich, Steinheim, Germany) in PBS with 1% BSA. The cells were washed 3 times with PBS + 0.05 % Triton-X-100 and afterwards incubated with a 1:2000 dilution of Cy3-conjugated anti mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA) in PBS. Finally the coverslips were washed 3 times with PBS before being mounted with mounting medium form Sigma Aldrich (Steinheim, Germany). For IF microscopy a Leica SP DM IRBE microscope was used.

2.11 Data Analysis

For IP assays four-parameter non-linear curve fitting (Prism, Version 3, GraphPad Software Inc., San Diego, USA) was used to estimate the peptide concentrations required for stimulation of half-maximal IP production (EC_{50}). Data for internalisation assays were calculated according to the formula:

% internalised radioligand = [internalised ligand (NaOH fraction)/ total cell associated ligand (NaOH fraction + Acid wash fraction)] x 100.

Chapter 3: Identification of the Internalisation Pathway of the type II GnRH Receptor in COS-1 cells

3.1 Introduction

As discussed in section 1.4 there are three main pathways of GPCR endocytosis. Firstly, there is a β-arrestin and dynamin dependent pathway, which involves internalisation via clathrin coated vesicles. The majority of GPCRs internalise through this pathway, which is the best studied GPCR endocytosis. Secondly there is a β-arrestin independent but dynamin dependent pathway, which is thought to involve caveolae, and thirdly, there is an endocytic pathway that neither requires β-arrestin nor dynamin. Very little is known about the third internalisation pathway.

One objective of this research was to elucidate which endocytic pathway is used by a mammalian type II GnRH receptor and to identify whether the type II GnRH receptor requires β-arrestin and dynamin for its internalisation. β-arrestin, dynamin and also GRK 2 dependency in COS-1 cells were tested using dominant negative mutants of these proteins. Although COS cells have been reported to have low levels of β-arrestins (Menard *et al.* 1997) for some GPCRs such as the TRH receptor, the PAFR and the AT_1 receptor a β-arrestin-dependent internalisation pathway has been demonstrated in this cell line using dominant negative constructs (Vrecl *et al.* 1998; Gaborik *et al.* 2001; Chen *et al.* 2002).

Furthermore wild type β-arrestins, dynamin and GRKs were coexpressed with the type II GnRH receptor and their effect on receptor internalisation was investigated. However before the internalisation pathway was characterised, extent and kinetics of the internalisation of the mammalian type II GnRH receptor was determined and compared to type I GnRH receptors. It has been previously reported that non-mammalian type I GnRH receptor with a C-terminal tail generally internalise faster and to greater extent than the tail-less mammalian type I receptors (Heding *et al.* 1998; Pawson *et al.* 1998). Furthermore truncation of the C-terminal tail of the chicken GnRH receptor resulted in decreased internalisation comparable to the human GnRH receptor (Pawson *et al.* 1998). The C-terminal tails of GPCRs often contain serine and threonine residues that are target for phosphorylation by GRKs. Agonist induced phosphorylation could be demonstrated for the non-mammalian catfish GnRH receptor (Blomenrohr *et al.* 1999; Willars *et al.* 1999), however the tail-less rat type I GnRH receptor does not undergo phosphorylation (Willars *et al.* 1999).

Therefore the extent of internalisation, the internalisation pathway and the role of the C-terminal tail and ICL 3 of a mammalian type II GnRH receptor was studied.

3.2 Results

3.2.1 The type II GnRH receptor internalises more rapidly than the type I GnRH receptor

Firstly the extent of internalisation of the type II GnRH receptor was determined. The quantitative internalisation assay used in our laboratory measures receptor internalisation indirectly, since this method detects the uptake of radiolabelled GnRH (see section 2.4).

To show internalisation of FLAG-tagged type II GnRH receptor directly COS-1 cells were grown on cover slips and transfected using Lipofectamine. Twenty four hours after transfection one set of cover slips was stimulated with 1 μM GnRH II for 10 minutes and immunofluorescence was performed as described in section 2.10. Fig. 3.1 shows endocytosis of the type II GnRH receptor after stimulation with ligand, whereas unstimulated cells show GnRH receptor expression almost exclusively on the membrane. The negative controls, mock transfected cells with either only primary antibody or primary and secondary antibody did not show fluorescence (data not shown).

For quantitative analysis of internalisation of different receptors the indirect method of measuring internalisation has to be used.

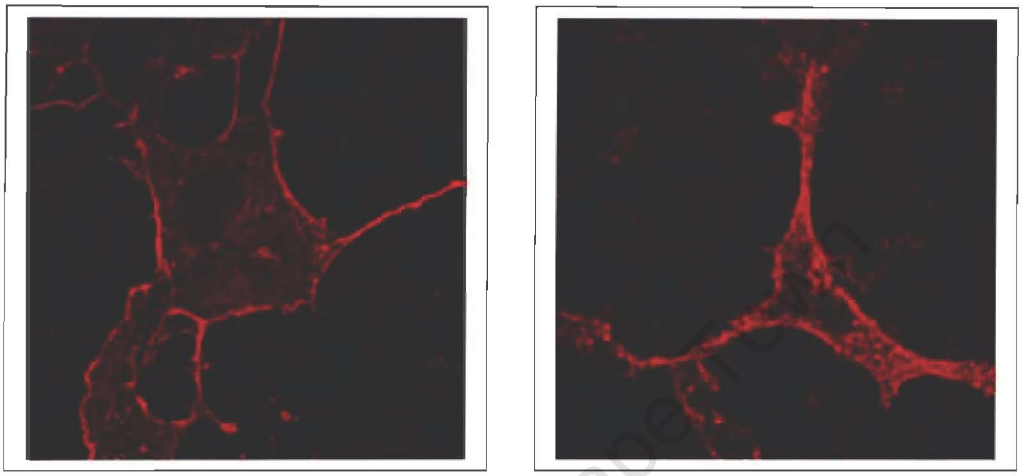

Fig. 3.1 Immunofluorescence with FLAG-tagged type II GnRH receptor. COS-1 cells were grown on cover slips and transfected with 2 μg of receptor DNA. Immunofluorescence was performed as described in Materials and Methods. Left picture shows an unstimulated cell expressing the receptor on the cell surface. Right picture shows a cell that had been stimulated with 1 μM of GnRHII for 10 minutes with endocytosed receptors.

To investigate the internalisation of the type II GnRH receptor and compare it with the type I GnRH receptor COS-1 cells were transfected with 2 μg of type I or type II GnRH receptor DNA using the DEAE/Dextran method. An internalisation assay was performed as described in Materials and Methods (see section 2.4).

It was observed that the type II GnRH receptor internalises more rapidly and to a greater extent compared to the human type I GnRH receptor (Fig. 3.2). Fifteen minutes after agonist stimulation 25 % of radiolabelled agonist bound to receptors on the cell surface was internalised into the cell, sixty minutes after stimulation the percentage of endocytosed radiolabel was 53 %. In comparison the tail-less human type I

GnRH receptor internalised only 7 % in fifteen minutes and 25 % in sixty minutes, which is similar to what has previously been reported for this receptor (Pawson *et al.* 1998).

Differences in internalisation kinetics between the type I and the type II GnRH receptors can on one hand be due to the absence or presence of a C-terminal tail. The role of the C-terminal tail of the type II GnRH receptor will be investigated in chapter four. On the other hand receptors can internalise through different internalisation pathways or use the same pathway with different efficiency. Therefore the proteins that are required for internalisation of the type II GnRH receptor were identified

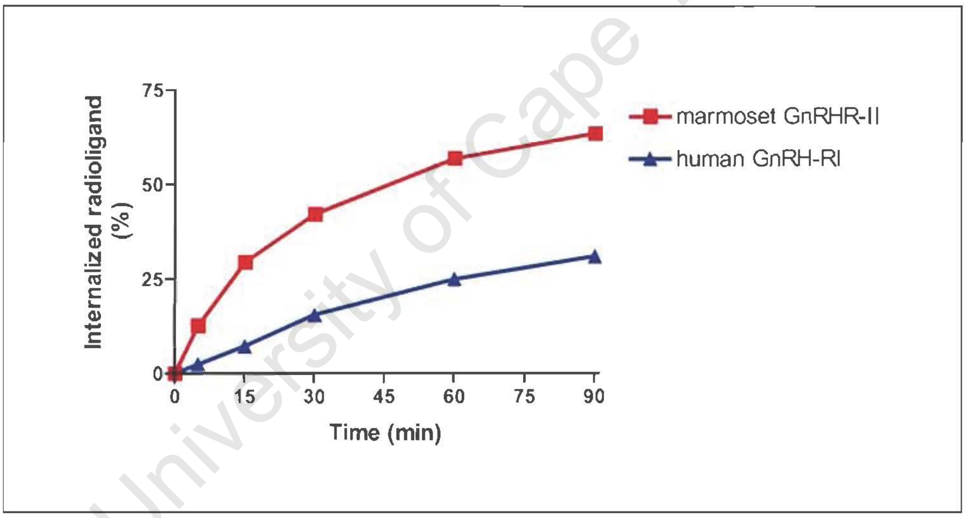

Fig. 3.2 Comparison of the internalisation of type II GnRH receptor of marmoset monkey with the human type I GnRH receptor. COS-1 cells were transfected with 2 μg of marmoset GnRH-RII or human GnRH-RI DNA and the internalisation assay was performed as described in 2.4. The result represents one out of three independent experiments each performed in duplicate.

3.2.2 The type II GnRH receptor internalises via a GRK dependent pathway in COS-1 cells

In order to test whether the type II GnRH receptor requires GRK for its internalisation in COS-1 cells receptor internalisation was measured in presence of dominant negative GRK 2 (K220R) as well as wild-type GRK 2 and GRK 1.

COS-7 cells were transfected with 1 µg of type II GnRH receptor DNA and 1 µg of GRK 1 and GRK 2 respectively per well using the DEAE/Dextran method. The internalisation assay was performed as described in 2.4. As control 1 µg of receptor DNA was cotransfected with 1 µg pcDNA 3.1. Co-expression of wild type GRK 1 and GRK 2 enhanced internalisation of the type II GnRH receptor. The internalisation in presence of GRK 1 or GRK 2 was 84 % higher at fifteen minutes and by 30 % higher at sixty minutes compared to control (Fig. 3.3 and Table 3.1). Possibly the exogenous GRKs are able to phosphorylate the receptor above normal levels, target the receptor for internalisation and therefore enhance internalisation kinetics.

This increase in receptor internalisation due to GRK 2 could be abolished by co-transfection of 2 µg of a dominant negative mutant of GRK 2 (K220R) indicating the specificity of the GRK 2 effect (Fig. 3.3 and Table 3.1). Nevertheless co-expression of dominant negative GRK 2 with the type II GnRH receptor reduced internalisation of the receptor by 44 % at fifteen minutes and 28 % at sixty minutes. Therefore the endogenous levels of GRK 2 in COS-1 cells play an important role in type II GnRH receptor internalisation. GRK 2 probably phosphorylates agonist-stimulated receptor and targets it for internalisation. However GRK (K220R) did not completely abolish internalisation. This could be either due to low efficiency of the dominant negative mutant in competing with the endogenous GRK 2 or alternatively the receptor could utilise a second GRK 2 independent pathway.

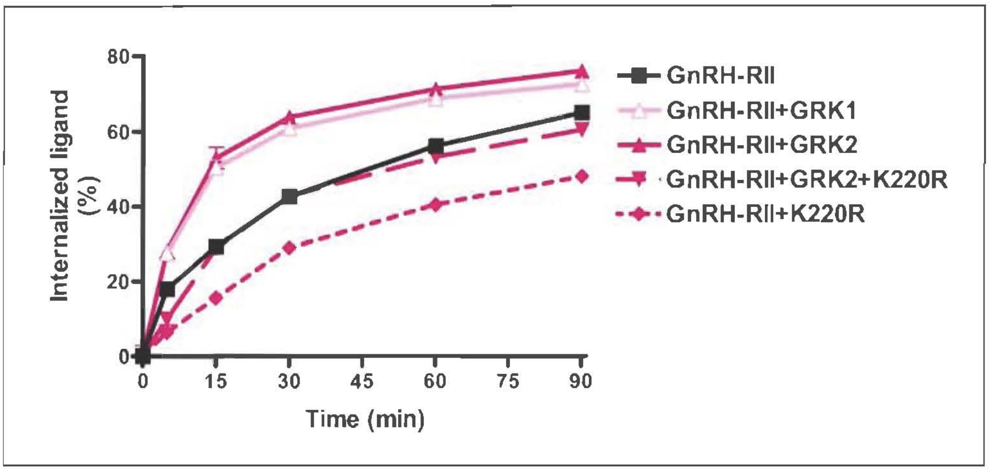

Fig.. 3.3 Effect of co-expression of dominant negative GRK 2 K220R, wild type GRK 1 and GRK 2 and a combination of wild type GRK 2 and dominant negative GRK 2 K220R on the internalisation of the type II GnRH receptor. Data shown are from a single representative experiment. Each experiment was performed at least three times in duplicate.

3.2.3 The type II GnRH receptor internalises via a β-arrestin independent pathway in COS-1 cells

Since the type II GnRH receptor requires GRK for internalisation, the receptor is possibly phosphorylated after agonist occupancy. This suggests that β-arrestin is involved in internalisation. Therefore the role of β-arrestin in endocytosis of the type II GnRH receptor was studied.

1 μg of receptor DNA was cotransfected with 2 μg dominant negative β-arrestin 1 constructs into COS-1 cells using the DEAE/Dextran method and receptor internalisation was measured as described in Materials and Methods. None of the two dominant negative mutants of β-arrestin 1 (V53D) and (318-419), had an effect on receptor internalisation (Fig. 3.4a and Table 3.1). Similarly the presence of a dominant negative mutant of β-arrestin 2 (V54D) did not inhibit internalisation kinetics and extent (Fig. 3.4b and Table 3.1). Therefore in COS-1 cells the type II GnRH receptor internalises in a β-arrestin 1 and β-arrestin 2 independent manner. However co-expression of wild-type β-arrestin 1 or β-arrestin 2 enhanced internalisation of the type II GnRH receptor by 87 % or 76 % respectively at fifteen minutes and by 29 % or 25 % respectively at sixty minutes. A

combination of wild type β-arrestin and dominant negative β-arrestin reduced β-arrestin mediated augmentation of internalisation to control level.

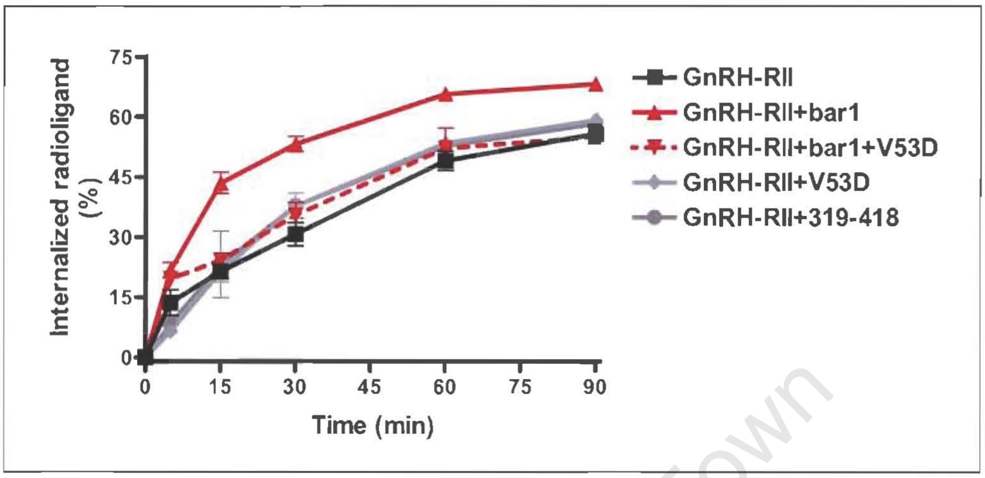

Fig. 3.4a Internalisation of type II GnRH receptor in presence of β-arrestin 1 and dominant negative mutants of β-arrestin 1V53D and 319-418. Data shown are from a single representative experiment. Each experiment was performed at least three times in duplicate.

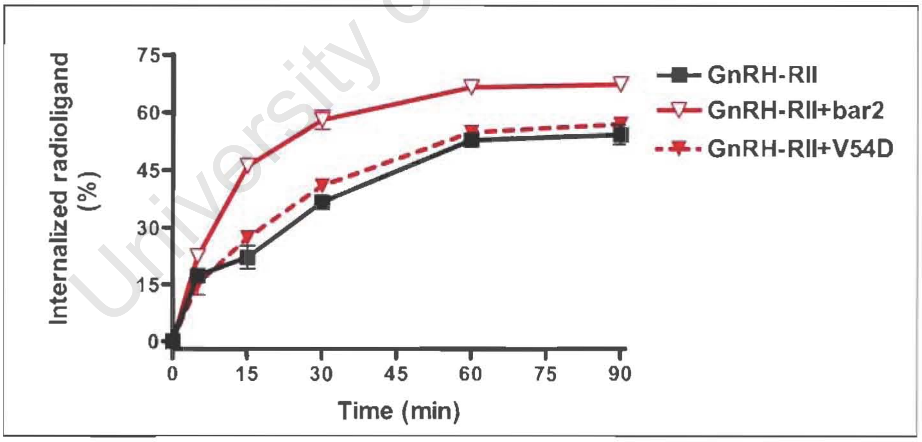

Fig. 3.4b Internalisation of the type II GnRH receptor in presence of β-arrestin 2 and a dominant negative mutant of β-arrestin 2. Results shown are from a single representative experiment. Each experiment was performed at least three times in duplicate.

3.2.4 The type II GnRH receptor internalises via a dynamin dependent pathway in COS-1 cells

To date three internalisation pathways have been described (see section 1.4). The clathrin coated vesicle internalisation involves β-arrestin and dynamin, the caveolae mediated internalisation pathway is β-arrestin independent, but requires dynamin and the third identified GPCR endocytosis does neither involve the β-arrestin nor the GTPase dynamin.

To investigate whether the type II GnRH receptor internalises in a dynamin dependent manner COS-1 cells were transfected with the type II GnRH receptor and either the dominant negative mutant of dynamin 1 K44A or wild-type dynamin. Co-expression of wild-type dynamin had no effect on the internalisation kinetics of the type II GnRH receptor. In presence of the dominant negative dynamin 1 (K44A) the receptor showed decreased internalisation (Fig. 3.5 and Table 3.1). Compared to control the reduction in internalisation was 38 % at fifteen minutes and 20 % at sixty minutes of agonist stimulation.

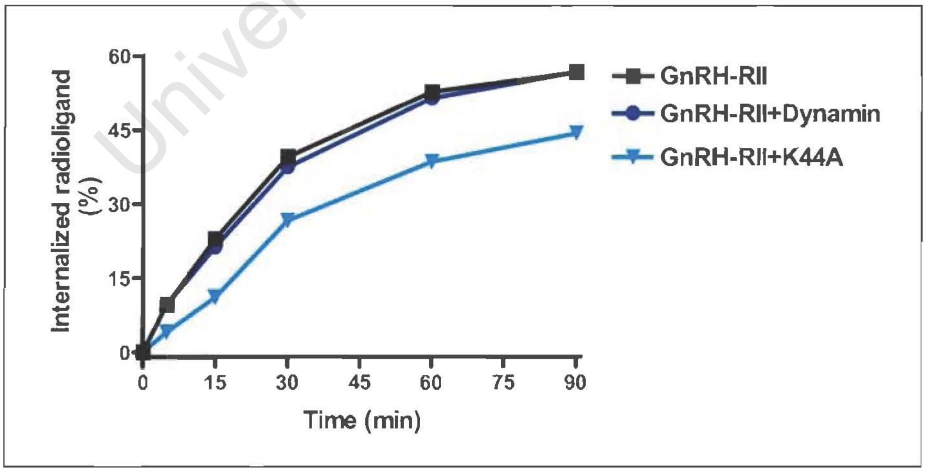

Fig. 3.5 Internalisation of the type II GnRH receptor in presence of wild-type dynamin and dominant negative dynamin respectively. Results shown are from a single representative experiment. Each experiment was performed at least three times in duplicate.

Construct	15 minutes	60 minutes
GRK 2	(+) 84 ± 24 %	(+) 30 ± 5 %
GRK 2 (K220R)	(-) 44 ± 6 %	(-) 28 ± 5 %
β-arrestin 1	(+) 87 ± 17 %	(+) 29 ± 7 %
β-arrestin 1 (V53D)	0	0
β-arrestin 1 (319-418)	0	0
β-arrestin 2	(+) 76 ± 29 %	(+) 25 ± 11 %
β-arrestin 2 (V54D)	0	0
Dynamin 1	0	0
Dynamin 1 (K44A)	(-) 38 ± 14 %	(-) 19 ± 13 %

Table 3.1 Effect of co-expression of GRK, β-arrestin, dynamin and their dominant negative mutants on internalisation of the type II GnRH receptor after 15 and 60 minutes of agonist stimulation. Increase in internalisation compared to cells expressing receptor only is indicated by (+), decrease is indicated by (-). Data represent the mean SEM for at least three independent assays performed in duplicate.

3.2.5 Internalisation of the type II GnRH Receptor can be blocked by an inhibitor of caveolae as well as by an inhibitor of clathrin coated vesicles

The finding that internalisation of the type II GnRH receptor in COS-1 cells is β-arrestin independent but requires dynamin suggests that the receptor internalises via caveolae in this cell line. In order to test this possibility, we measured internalisation of the type II GnRH receptor in the presence of filipin. Filipin is a polyene antibiotic, which is able to bind cholesterol and is therefore considered to inhibit formation of cholesterol rich structures such as caveolae (Schnitzer *et al.* 1994). COS-1 cells were transfected with 2 μg of receptor DNA using the DEAE/Dextran method and internalisation was performed in presence of 5μg/ml of filipin. Filipin caused a dramatic reduction in internalisation of 97 % compared to control (Fig. 3.6). When the experiment was performed in 0.45 M sucrose a similar reduction of 89

% was observed. Sucrose has been reported to be an inhibitor of clathrin coated vesicle internalisation, because it induces abnormal clathrin polymerisation (Heuser and Anderson 1989). Since both sucrose and filipin abolished internalisation of the type II GnRH receptor it is likely that internalisation of this receptor in COS cells proceeds via both structures, caveolae and clathrin coated vesicles.

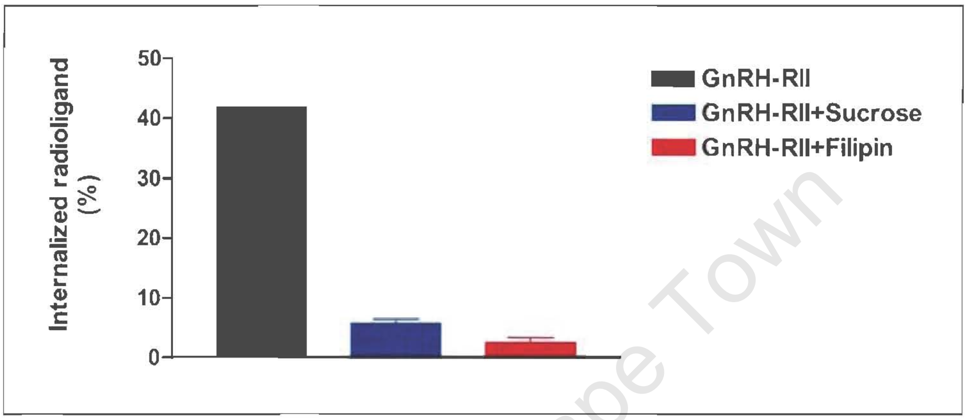

Fig. 3.6 Internalisation of type II GnRH receptor in COS-1 cells in presence of sucrose (0.45M) or filipin (5µg/ml) after 60 minutes of agonist stimulation. Data shown are from a single representative experiment. Each experiment was performed at least three times in duplicate.

Chapter 4: Identification of Residues within the C-terminal Tail and ICL 3 of the Type II GnRH receptor critical for rapid internalisation

4.1 Introduction

As mentioned in 3.2.1 the type II GnRH receptor of marmoset monkey internalises more rapidly than the human type I GnRH receptor. Slow internalisation kinetics of the type I GnRH receptor has been attributed to the receptor's lack of a C-terminal tail and to the lack in receptor phosphorylation (Heding *et al.* 1998; Pawson *et al.* 1998; Willars *et al.* 1999).

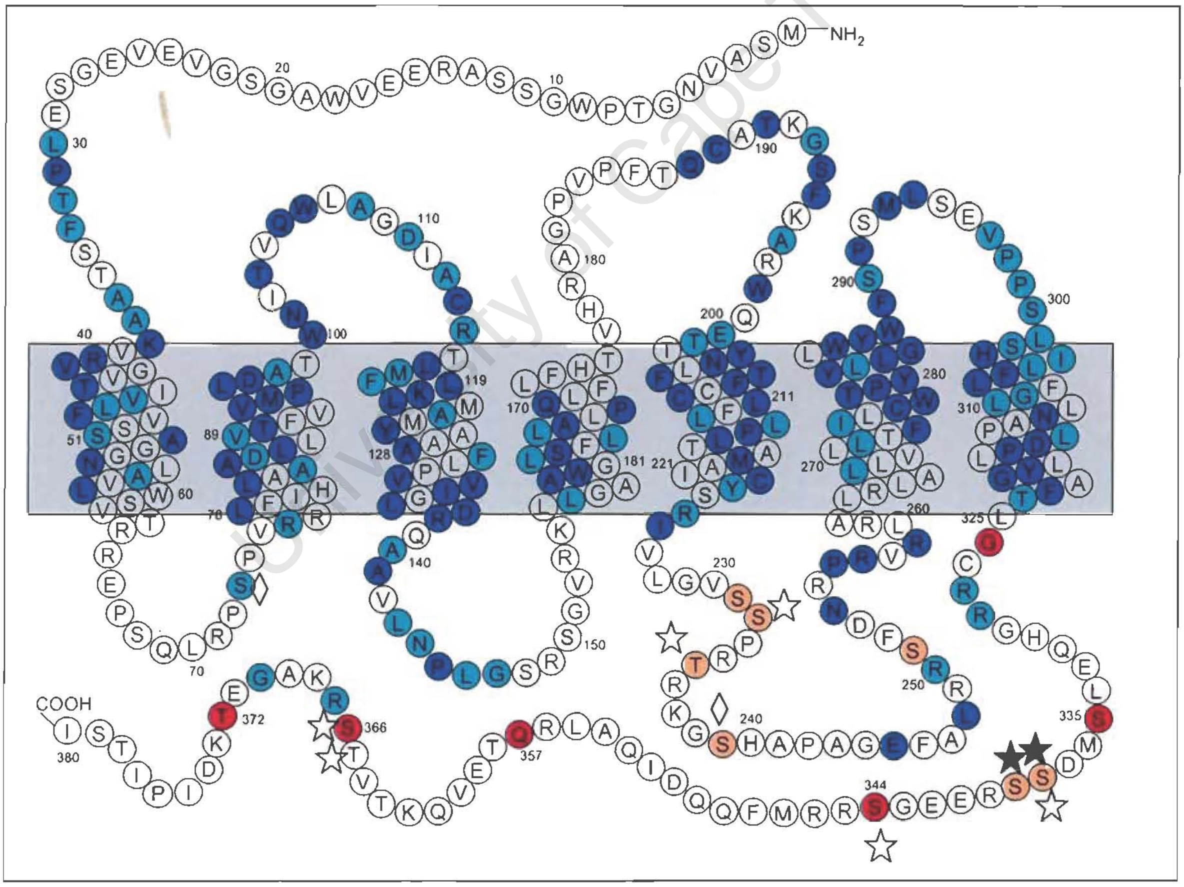

Fig. 4.1 Schematic diagram of the type II GnRH receptor of marmoset monkey. Green residues indicate amino acids conserved between all type II GnRH receptors cloned to date, blue residues indicate amino acids conserved between the type II GnRH receptors and the human type I GnRH receptor. Codons for the red residues were mutated to stop codons and the orange amino acids were mutated to alanine residues. Putative PKC phosphorylation sites are indicated by open stars, putative CKII phosphorylations site by closed stars and putative PKA phosphorylation sites by open diamonds.

Serine and threonine residues within the C-terminal tail of GPCRs are often a target for phosphorylation by GRKs, which subsequently allows arrestin binding and facilitates receptor desensitisation and internalisation. However in the literature there are reports of GPCRs that require GRK for internalisation, but are able to internalise in a β-arrestin independent manner (Tsuga *et al.* 1998; Ito *et al.* 1999; Ferguson *et al.* 2000). In the previous chapter it was shown that the type II GnRH receptor internalises in a β-arrestin independent but GRK dependent pathway.

The type II GnRH receptor internalises faster and to greater extent than the tail-less type I GnRH receptor. It has previously been reported that the C-terminal tail mediates rapid internlisation kinetics (Heding *et al.* 1998; Pawson *et al.* 1998). The C-terminal tail of the type II GnRH receptor contains several serine and threonine residues that are putative GRK phosphorylation sites. Therefore it was of particular interest to investigate whether the C-terminal tail of the type II GnRH receptor plays a central role in receptor internalisation. Firstly progressively C-terminally truncated mutants of the type II GnRH receptor were created (red residues in Fig. 4.1) and the effect of the truncation on internalisation was measured. Furthermore to actually pinpoint the important amino acids within the C-terminal tail putative phosphorylation sites were mutated to alanine residues. Secondly due to the fact that GRKs also phosphorylate serine and threonine residues within ICL 3 putative phosphorylation sites within ICL 3 were also mutated to alanines and the effect of these mutations was investigated.

4.2 Results

4.2.1 The region from Ser 335 to Gly 343 within the C-terminal tail of the type II GnRH receptor is critical for rapid internalisation

To investigate the function of the C-terminal tail of the type II GnRH receptor in mediating rapid agonist induced internalisation, the internalisation of progressively truncated mutants was measured. The truncated constructs were created by a PCR based method as described in section 2.1. Substitution of the codons for the C-terminal tail residues Thr 372, Ser 366, Gln 357, Ser 344, Ser 355 and Gly 326 respectively by a stop codon lead to the truncated receptor constructs T372stop, S366stop, Q357stop, S344stop, S355stop and G326stop (Fig. 4.1).

All truncated receptors were functional in signalling as determined by GnRH-induced inositol phosphates production. The EC_{50} values of all the truncated (up to S335) mutant receptors were similar to the EC_{50} value of the wild type receptor (Table 4.1). Interestingly, a receptor lacking the entire C-terminal tail (G326stop) was also found to be active. However the G326stop mutant showed only 57 % of maximum IP (IPmax) produced by the wild type receptor. This mutant also showed a 92.5 % reduction in ligand binding (data not shown) suggesting that the decreased level of receptor expression is responsible for the reduced IPmax. Due to the very low ligand binding it was not possible to measure internalisation of this mutant.

Construct	IPmax (% of wild type)	EC_{50} (nM), GnRH II
GnRH-RII	100	0.29 ± 0.3
T372stop	96 ± 5	0.30 ± 0.3
S366stop	98 ± 8	0.46 ± 0.4
Q357stop	90 ± 6	0.26 ± 0.2
S344stop	95 ± 16	0.26 ± 0.2
S335stop	92 ± 11	0.29 ± 0.4
G326stop	57 ± 11	0.97 ± 0.3
S338,339A	108 ± 12	0.07 ± 0.04
S251A	33 ± 8	3.3 ± 0.6
S251,338,339A	48 ± 6	1.2 ± 0.6
S231,232A	105 ± 41	0.22 ± 0.05
S231,232,338,339A	109 ± 31	0.09 ± 0.05
T235A,S239A	99 ± 5	0.54 ± 0.06
T235A,S239,338,339A	123 ± 8	0.30 ± 0.1

Table 4.1 Summary of maximum inositol phosphates production (IPmax) and EC_{50} for wild type and mutant type II GnRH receptors. COS-1 cells were transfected with 2μg of DNA of each construct using the DEAE/Dextran method. Total inositol phosphates production was determined as described in *Materials and Methods*. Data represent the mean SEM for three independent assays performed in duplicate.

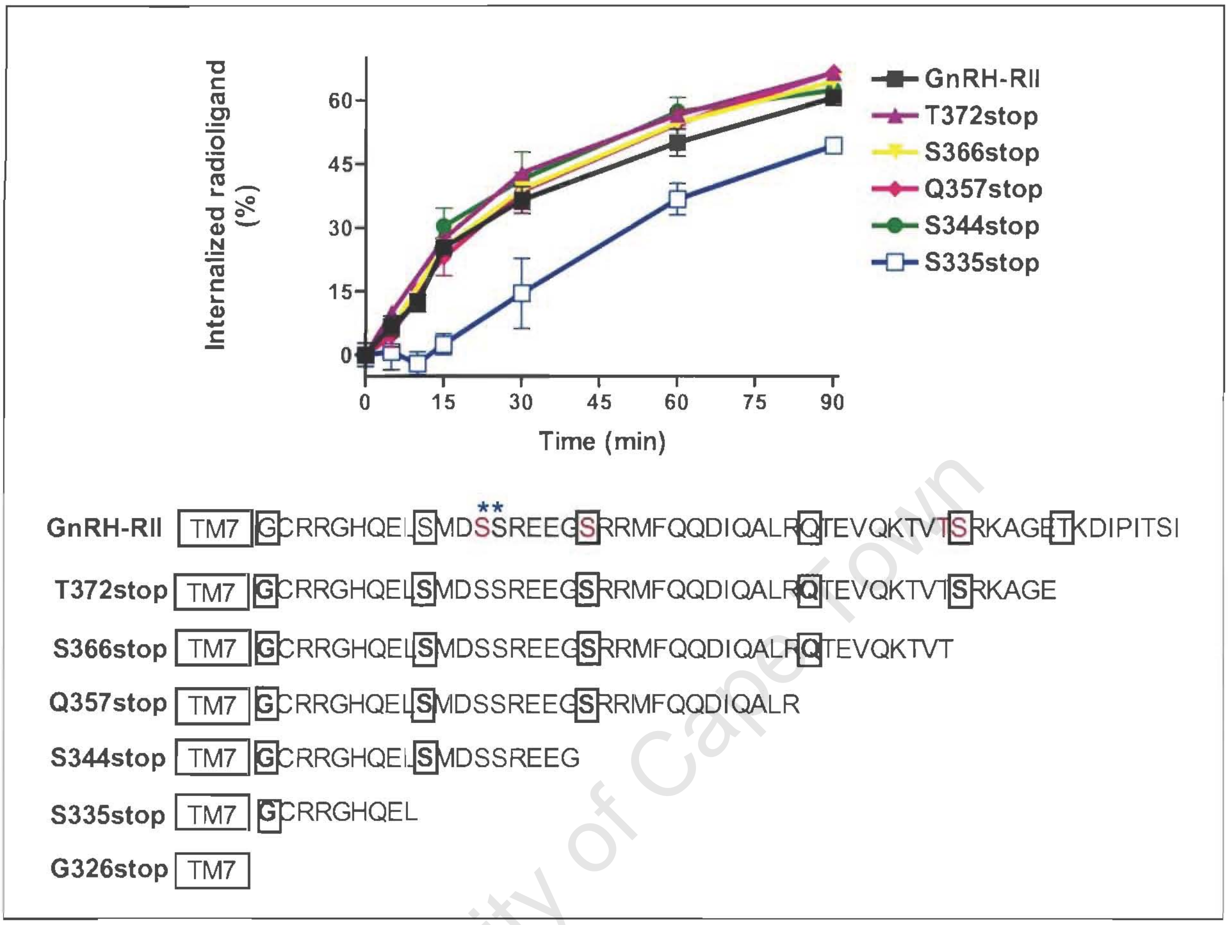

Fig. 4.2 Internalisation of the C-terminally truncated mutants of the type II GnRH receptor compared to the wild type GnRH-RII. Red amino acids indicate putative PKC phosphorylation sites within the C-terminal tail, blue asterics indicate putative CKII phosphorylation sites. Data shown are from a single representative experiment. Each experiment was performed at least three times in duplicate.

Internalisation kinetics of all mutants truncated by up to 37 amino acids (up to Ser 344) was identical with the wild type receptor (Fig. 4.2). However deletion of further nine amino acids (to Ser 335) resulted in a receptor, which showed a 75 % reduction of internalisation in the first thirty minutes after ligand stimulation compared to wild-type receptor. This receptor internalised fifteen minutes after agonist stimulation only 6.6 % of radioligand whereas the wild type receptor internalised 25.7 % of radioligand under the same conditions. Compared to the wild type receptor this is a reduction in internalisation of 75 % in the first fifteen minutes and

25 % at the sixty minutes time point, therefore this mutation affects particularly the early time points.

These results indicate that within the C-terminal tail of the type II GnRH receptor the region between Ser 335 and Ser 344 is critical for rapid receptor internalisation. The identified region contains a serine doublet (Ser 338 and Ser 339), which is part of consensus sequences for PKC and CKII. The same residues could be target for phosphorylation by GRKs. A consensus sequence for GRK phosphorylation sites has not been identified, however GRKs tend to phosphorylate serine and threonine residues, which have a pair of acidic residues on their N-terminal side (for review see (Pitcher *et al.* 1998)).

Deletion of the region containing a GRK phosphorylation site would abolish receptor phosphorylation and could therefore affect targeting of the receptor for endocytosis.

4.2.2 Ser 338 and 339 are the amino acids within the C-terminal tail critical for rapid internalisation

After identification of the region within the C-terminal tail that is critical for rapid receptor internalisation the next step was to find out which amino acid within the identified region are critical for rapid internalisation of the receptor. Since GRK is involved in the internalisation of the type II GnRH receptor it was investigated whether the putative serine phosphorylation sites Ser 338 and Ser 339 are the critical element within the region from Ser 335 to Gly 343. The serines were mutated to alanines using a PCR based site directed mutagenesis method as described in section 2.1. Internalisation of the S338,339A mutant was measured and compared to the internalisation of the S335stop mutant and the wild type receptor. The mutant lacking the putative phosphorylation sites showed an internalisation profile that was identical to the internalisation profile of the S335stop mutant (Fig. 4.3).

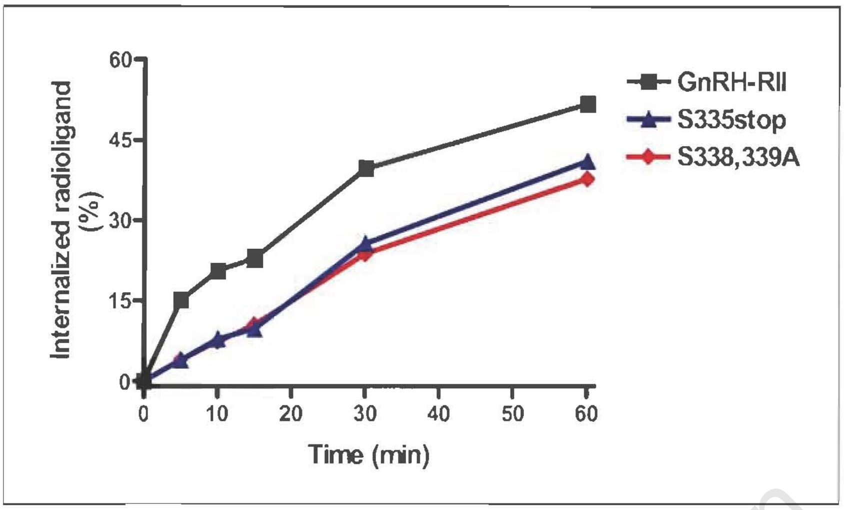

Fig. 4.3 Comparison of the internalisation of the type II GnRH receptor and the C-terminally truncated S335stop mutant with a mutant lacking the putative phosphorylation sites Ser 338 and Ser 339 (S338,339A). Data shown are from a single representative experiment. Each experiment was performed at least three times in duplicate.

These results show that Ser 338 and Ser 339 of the C-terminal tail are indeed the amino acids that are critical for rapid internalisation of the type II GnRH receptor of marmoset monkey. It is likely that these serine residues are phosphorylated by GRKs after agonist occupancy of the receptor and that the phosphorylation mediates rapid receptor internalisation and possibly also receptor desensitisation.

One indication that Ser 338 and Ser 339 are also involved in desensitisation of the type II GnRH receptor was obtained from IP assays. The EC_{50} values of the S338,339A were four fold lower compared to the wild-type (Table 4.1 and Fig. 4.5). The decreased EC_{50} value indicates that the mutant S338,339A cannot be desensitised as efficiently as the wild-type receptor and supports the hypothesis that these serine residues are phosphorylated by GRK after agonist activation of the receptor.

Although mutation of Ser 338 and 339 decreased receptor internalisation throughout the time course, particularly the early time points of internalisation were affected. This lead to the postulation that other putative phosphorylation sites located in ICL 3 might also be involved, since GRKs are known to phosphorylate GPCRs not only on the C-terminal tails, but also on the ICL 3s. Therefore the next step was to identify whether serine and threonine residues in ICL 3 also play a role in receptor endocytosis.

4.2.3 Ser 251 within ICL 3 plays a role in internalisation and signalling

The ICL 3 of the type II GnRH receptor contains four serine residues (Ser 231, Ser 232, Ser 239 and Ser 251) and one threonine residue (Thr 235) that could be target for GRK phosphorylation after agonist occupancy of the receptor (Fig. 4.1).

To investigate the role of these residues in ICL 3 the putative serine and threonine phosphorylation sites were mutated to alanines and the effect of the mutation was measured in internalisation assays. The mutant receptors S231,232A, T235A,S239A and S251A were created using a PCR based mutagenesis method described in 2.1. Abolishing the putative phosphorylation sites Ser 231, Ser 232, Thr 235 and Ser 239 had no effect on internalisation. The mutated receptors showed similar internalisation as the wild type receptor (Fig. 4.4). However mutation of Ser 251 to an alanine reduced the extent of internalisation particularly at later time points but had little influence on internalisation in the first 30 minutes of agonist stimulation. Internalisation of the S251A mutant is reduced by 30 % 60 minutes after agonist stimulation compared to wild type receptor.

These results show that within ICL 3 of the type II GnRH receptor of marmoset monkey Ser 251 plays a role in receptor internalisation. Like Ser 338 and 339 within the C-terminal tail of the receptor this residue might be a target of phosphorylation by GRK. Once mutated to an alanine Ser 251 cannot be phosphorylated and this could possibly lead to reduced internalisation of the S251A mutant compared to the wild type receptor.

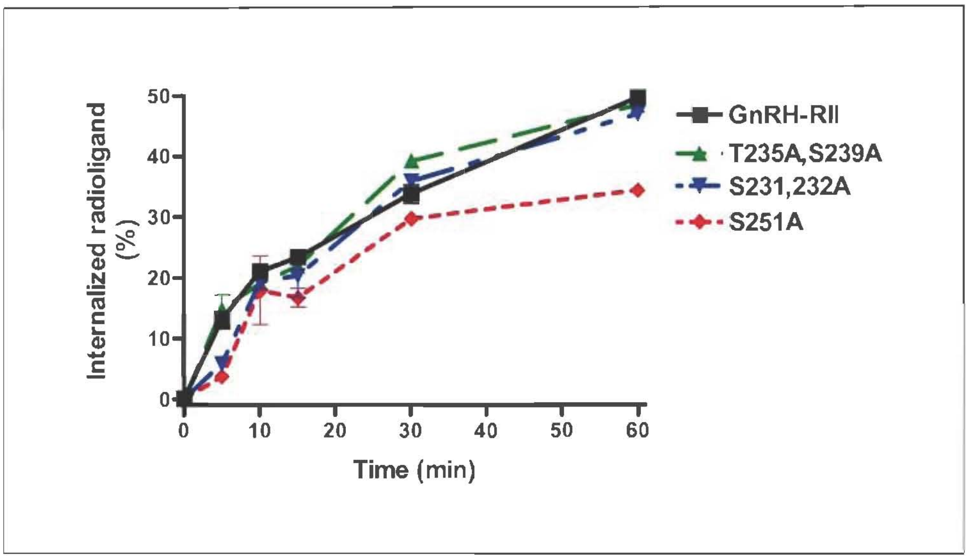

Fig. 4.4 Comparison of internalisation of three mutants lacking putative phosphorylation sites within ICL 3 with the wild type GnRH-RII. Data shown are from a single representative experiment. Each experiment was performed at least three times in duplicate.

IP assays revealed that mutation of Ser 251 to Ala causes a 10 fold increase in EC_{50} and a 67 % reduction in IPmax compared to the wild-type receptor (Table 4.1 and Fig. 4.5). These results indicate that the mutant S251A is partially uncoupled. Therefore Ser 251 does not only play a role in receptor internalisation, it also plays a role in cell signalling.

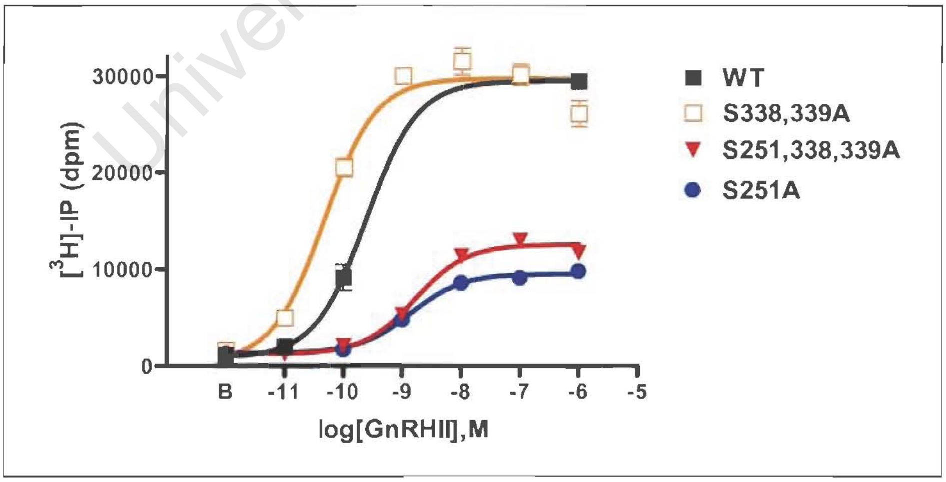

Fig. 4.5 GnRHII-stimulated IP production of the wild-type GnRH-RII and the receptor mutants, S251A, S338,339A and S251,338,339A. 48 hours after transfection cells were stimulated with the indicated concentrations of GnRHII for 1 hour before total IP accumulation was determined as described in *Materials and Methods*. IP production is expressed as dpm of 3[H]-IP. EC_{50} values are given in Table 4.1. Data shown are from a single representative experiment. The experiment was performed at least three times in duplicate.

4.2.4 Combined mutations of Ser 251 and Ser 338,339 to Ala causes additive inhibition of type II GnRH receptor internalisation

The previous results in section 4.2.2 and 4.2.3 showed that two serine residues within the C-terminal tail (Ser 338 and Ser 339) and one serine residue within ICL 3 (Ser 251) are important for internalisation of the type II GnRH receptor. As GRK is required for endocytosis of the type II GnRH receptor these serine residues are putative GRK phosphorylation sites.

Theoretically a combination of both mutations should cause greater reduction of internalisation than the individual mutations. Therefore all ICL 3 mutations discussed in section 4.2.3 were combined with the S338,339A mutation and the effect on internalisation was measured. The internalisation profile of the mutant receptors S231,232,338,339A and T235A,S239,338,339A was similar to that of a receptor that lacked the phosphorylation site within the C-terminal tail (Ser 338,339) (Fig. 4.6). This was consistent with the observation that Ser 231,232,239 and Thr 235 do not play a role in internalisation. However combination of the mutations S251A with S338,339A caused an additive inhibition of internalisation compared to the individual mutations. This receptor internalised 71 % less at 15 minutes and 67 % less at 60 minutes of agonist stimulation compared to the wild type receptor.

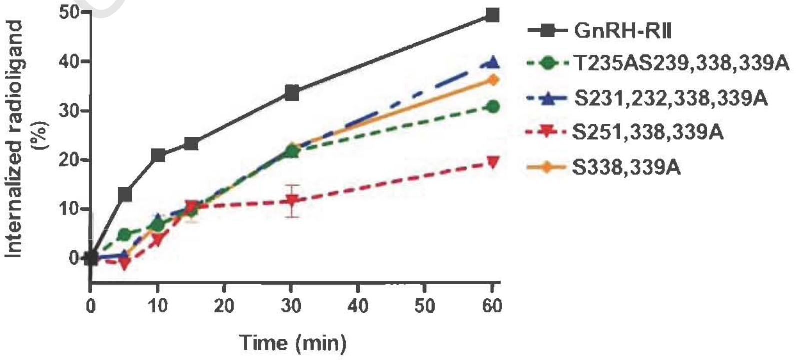

Fig. 4.6 Comparison of internalisation of mutants lacking putative phosphorylation sites within ICL 3 as well as the C-terminal tail (Ser 338,339) with the wild type GnRH-RII and the S338,339A mutant. Data shown are from a single representative experiment. Each experiment was performed at least three times in duplicate.

Chapter 5: Ser 251, 338 and 339 are putative phosphorylation sites, but are not required for β-arrestin dependent internalisation

5.1 Introduction

In the previous chapter Ser 251 in ICL 3 and Ser 338,339 were shown to play an important role in internalisation of the type II GnRH receptor. Mutation of these serine residues to alanines inhibited receptor internalisation by 71 % at 15 minutes and by 67 % at 60 minutes of agonist stimulation compared to the wild type receptor. Furthermore in section 3.2.2 the internalisation pathway of the type II GnRH receptor was shown to require GRK in COS-1 cells. These findings suggest that the serine residues in ICL 3 and the C-terminal tail undergo phosphorylation by GRK after agonist occupancy of the receptor.

To date the catfish type I GnRH receptor is the only GnRH receptor for which agonist stimulated phosphorylation of C-terminal tail residues could be shown (Willars *et al.* 1999). Receptor phosphorylation of other non-mammalian type I GnRH receptors or any type II GnRH receptor has never been tested. It has however been shown that the tail-less mammalian type I GnRH receptor does not undergo phosphorylation (Willars *et al.* 1999). Nevertheless when the C-terminal tail of the catfish receptor was added to the human receptor, the chimera displayed ligand induced receptor phosphorylation (Willars *et al.* 1999). Mutation of the phosphorylation sites of the catfish GnRH receptor resulted in reduced receptor internalisation (Blomenrohr *et al.* 1999). This observation is consistent with the finding that mutation of Ser 251, 338 and 339 to Ala inhibits endocytosis of the type II GnRH receptor.

Therefore it was examined whether the mammalian type II GnRH receptor undergoes agonist mediated receptor phosphorylation and whether the mutant lacking the putative phosphorylation sites cannot be phosphorylated. Furthermore we investigated whether these serine residues are important for β-arrestin dependent internalisation.

5.2 Results

5.2.1 Phosphorylation of the type II GnRH receptor cannot be detected by ^{32}P phosphorylation assays

To detect possible phosphorylation of the type II GnRH receptor Western Blots with an anti-phosphoserine antibody (Sigma-Aldrich, Steinheim, Germany) were performed. However due to lack in specificity this antibody did not detect the receptor (data not shown). Therefore an alternative method, the in vivo phosphorylation assay, was used.

^{32}P receptor phosphorylation assays were performed to investigate whether the serine residues that have been shown to be important for receptor internalisation are indeed phosphorylated after agonist stimulation. For receptor phosphorylation assays FLAG-tagged constructs were used. To make sure that a FLAG-tag does not interfere with the structure and charcteristics of the type II GnRH receptor an internalisation with both constructs, tagged and untagged, was performed (Fig. 5.1). Internalisation kinetics of the FLAG-tagged type II GnRH receptor are identical to the internalisation profile of the untagged GnRH receptor.

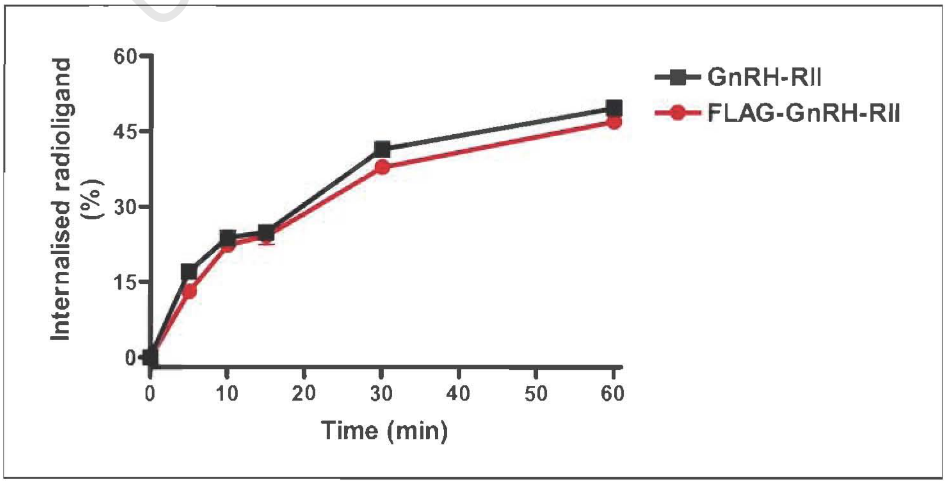

Fig. 5.1 Comparison of internalisation of FLAG-tagged and wild type GnRH-RII Data shown are from a single representative experiment. Each experiment was performed at least three times in duplicate.

For phosphorylation assays COS-1 cells were transfected with 2 µg of FLAG-tagged type II GnRH receptor as well as FLAG-tagged β_2-AR as a positive control using the lipofectamine method (see section 2.2). Receptor phosphorylation and immunoprecipitation was performed as described in Materials and Methods. The immunoprecipitated proteins of 10^6 cells were separated on an SDS-polyacryl amide gel per lane and the phosphorylated proteins were detected by autoradiography (exposure time five days). In parallel another SDS-gel with the same amount of proteins was blotted onto a PVDF membrane and the FLAG-tagged receptors were detected by Western Blot with an anti-FLAG M2 antibody from mouse and a anti-mouse HRP conjugated secondary antibody as described in Materials and Methods.

Fig. 5.2 (left panel) shows a Western Blot with the immunoprecipitated receptors. The arrows indicate low molecular weight β_2-AR bands and high molecular oligomers of the receptor (lane 1) as well as a single band and high molecular oligomers of FLAG-tagged GnRH receptor (lane 5 and 6). The right panel shows a autoradiograph of an SDS polyacrylamide gel with identical proteins as used for the Western Blot. Phosphorylation of epinephrine (0.1 mM) stimulated β_2-AR could be clearly detected as indicated by arrows (lane 1), however phosphorylation of GnRH II (1 µM) stimulated type II GnRH receptor could not be detected (lane 6). No difference between unstimulated GnRH receptor (lane 5) and ligand stimulated GnRH receptor (lane 5) could be seen. However in lane 5 and 6 there is a very faint band below the 45 kDa marker, which could be phosphorylated GnRH receptor, but there is no indication of phosphorylated high molecular receptor oligomers as it is the case with the β_2-AR. This band also appeared in the lane of the β_2-AR after longer exposure as well as with the mutant S251,338,339A (data not shown). Therefore it is most likely not a phosphorylated GnRH receptor band. Additionally this band was too faint to be quantified even by use of a phosphoimager and after several repetition of the phosphorylation assay under different conditions.

Western Blot analysis showed that the β_2-AR is higher expressed than the type II GnRH receptor. The differences in expression levels could be the reason why phosphorylation of the β_2-AR could be detected whereas phosphorylation of the type II GnRH receptor could not be shown.

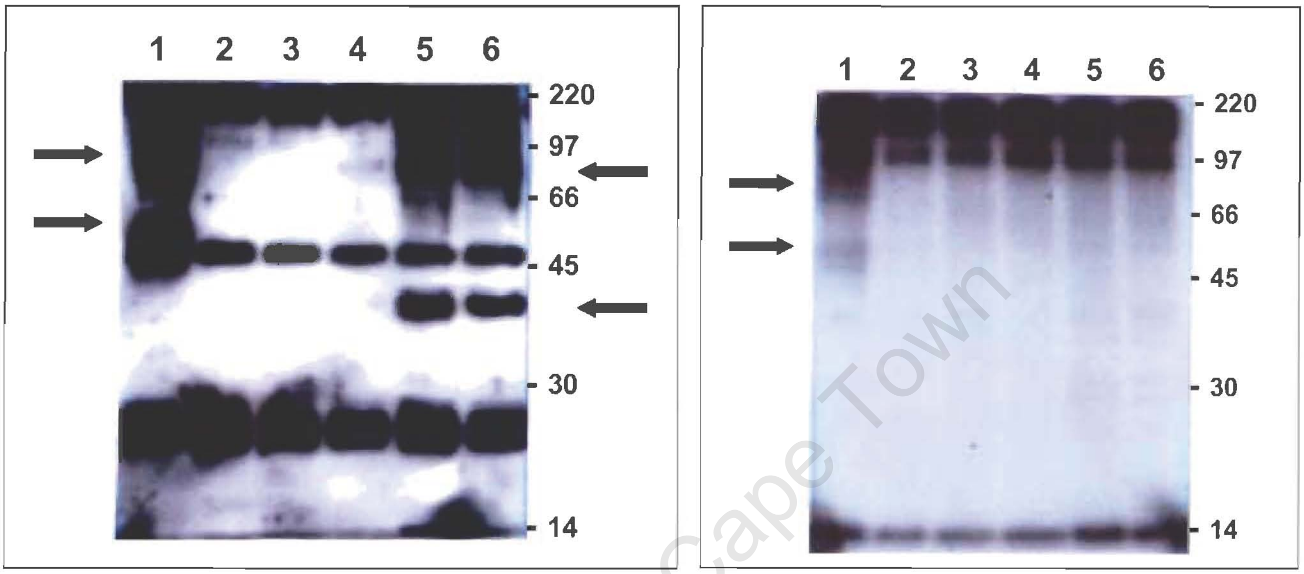

Fig. 5.2 Western Blot (left panel) and autoradiograph (right panel) of immunoprecipitated FLAG-tagged β_2-AR and type II GnRH receptors. As positive control epinephrine (0.1 mM) stimulated β_2-AR was used (lane 1). Lanes 2-4 are negative controls including immunoprecipitates from mock transfected cells (lane 2), unstimulated untagged type II GnRH receptor and GnRH II (1 μM) stimulated untagged type II GnRH receptor. Unstimulated FLAG-tagged type II GnRH receptor (lane 5), GnRH II (1 μM) stimulated FLAG-tagged type II GnRH receptor (lane 6). The arrows indicate a single β_2-AR band and high molecular oligomers of the receptor as well as a single band and high molecular oligomers of FLAG-tagged GnRH receptor. Data are shown from one single representative experiment. Each experiment was performed at least three times in duplicates.

5.2.2 Dominant negative GRK 2 cannot reduce internalisation of a mutant receptor lacking the putative phosphorylation sites

Since it was not possible to directly detect phosphorylation of the type II GnRH receptor neither by ^{32}P phosphorylation assays nor by Western Blots with an anti-phosphoserine specific antibody an alternative approach had to be used.

Knowing that internalisation of the type II GnRH receptor can be reduced by dominant negative GRK 2 (K220R), we hypothesised that if serines 251, 338 and 339 are GRK phosphorylation sites, then the dominant negative GRK 2 should not be able to inhibit internalisation of the mutant receptor lacking these putative phosphorylation sites. Therefore COS-1 cells were transfected with wild type GnRH receptor and the S251,338,339A mutant as well as the S338,339A mutant using the DEAE/Dextran method and an internalisation assay was performed as described in Materials and Methods in presence and absence of dominant negative GRK 2 (K220R).

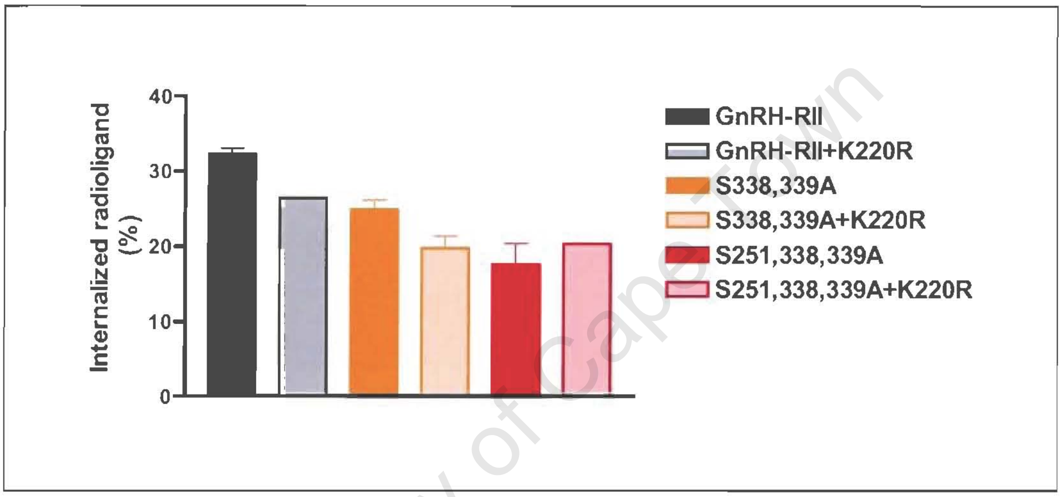

Fig. 5.3 Internalisation of the type II GnRH receptor and the mutants S338,339A and S251,338,339A in presence or absence of dominant negative GRK2 (K220R). Internalisation is expressed as % of internalised radioligand at 60 minutes after agonist stimulation. Data are shown from one single representative experiment. Each experiment was performed at least three times in duplicates.

As expected internalisation of the wild-type GnRH receptor could be reduced by dominant negative GRK, however internalisation of the mutant receptor lacking all serine residues, that have been shown to be critical for receptor internalisation, could not be inhibited by GRK 2 (K220R) (Fig. 5.3). Nevertheless this dominant negative mutant could reduce internalisation of the S338,339A mutant as well as of the S251A mutant (data not shown). These results suggest that Ser 251,338 and 339 are GRK phosphorylation sites and that one of the phosphorylation sites is enough for agonist induced GRK phosphorylation of the receptor.

5.2.3 Ser 251, 338 and 339 are not required for β-arrestin dependent internalisation

Having shown that Ser 251 within ICL3 and Ser 338 and 339 within the C-terminal tail are possibly GRK phosphorylation sites it had to be determined whether these sites are required for interaction of the type II GnRH receptor with β-arrestin.

The results from section 3.2.3 showed that the type II GnRH receptor does not internalise in a β-arrestin dependent manner in COS-1 cells. However co-expression of wild-type β-arrestin enhanced receptor internalisation by 45 % at fifteen minutes and by 22 % at sixty minutes indicating that the type II GnRH receptor is able to undergo β-arrestin dependent endocytosis in cell lines with higher endogenous levels of β-arrestin.

To find out whether Ser 251,338 and 339 are required for interaction with β-arrestin, β-arrestin induced augmentation of receptor internalisation was measured for the S251,338,339A mutant and compared to the wild-type receptor. β-arrestin could enhance internalisation of the mutant lacking the putative phosphorylations sites just to a similar extent than the wild-type receptor (Fig. 5.4).

These results suggest that even though Ser 251, 338 and 339 are required for β-arrestin independent receptor internalisation in COS-1 cells, they are not necessary for β-arrestin dependent internalisation of the type II GnRH receptor.

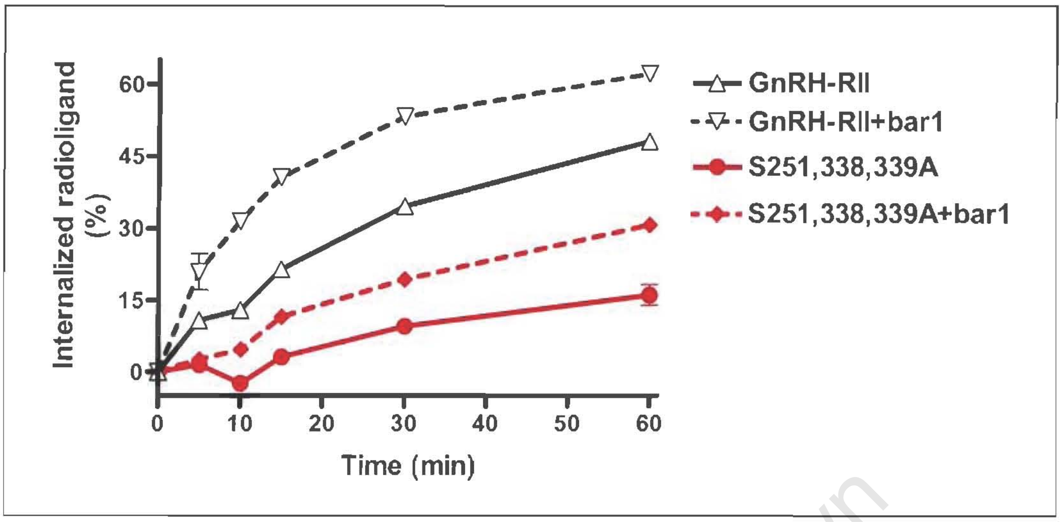

Fig. 5.4 Internalisation of the type II GnRH receptor and the S251,338,339A mutant in presence and absence of β-arrestin 1. Data are shown from one single representative experiment. Each experiment was performed at least three times in duplicates.

5.2.4 The C-terminal tail of the type II GnRH receptor is not required for β-arrestin dependent internalisation

Studies on the mammalian type I GnRH receptor, which does not have a C-terminal tail, have shown that internalisation of this receptor cannot be augmented by co-expression of β-arrestin (Heding *et al.* 2000). To verify this observation under our experimental conditions the internalisation of the type I GnRH receptor in the absence or presence of β-arrestin was compared it to the type II GnRH receptor. Indeed β-arrestin could not enhance internalisation of the type I GnRH receptor whereas it augmented internalisation of the type II GnRH recptor (Fig. 5.5). These results suggest that the determinants for β-arrestin mediated augmentation of internalisation might be located in the C-terminal tail.

After having shown that Ser 251, 338 and 339 are not necessary for β-arrestin dependent internalisation the question was raised whether any other area in the C-terminal tail of the type II GnRH receptor is important for β-arrestin mediated augmentation of internalisation. In the case that the C-terminal domain of the type II GnRH receptor plays an important role in β-arrestin dependent internalisation a mutant receptor lacking the C-terminal tail should not be enhanced by co-expression with β-arrestin. Therefore internalisation of the type II GnRH receptor and the C-terminally truncated mutant S335stop was measured in the presence and the absence of β-arrestin.

The results showed that co-expression of β-arrestin with the C-terminally truncated mutant resulted in a similar increase compared to the wild-type receptor (Fig. 5.6).

This result shows that in case of the type II GnRH receptor the C-terminal tail is not the region required for β-arrestin interaction and suggest that other regions of the receptor are important for β-arrestin dependent internalisation.

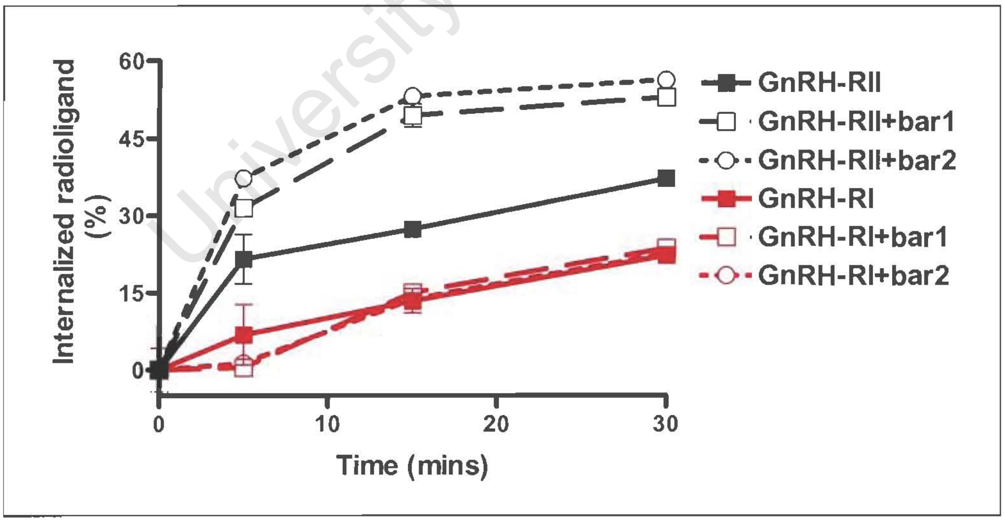

Fig. 5.5 Internalisation of the type II GnRH receptor and the type I GnRH receptor with and without β-arrestin 1 or 2. Data are shown from one single representative experiment. Each experiment was performed at least three times in duplicates.

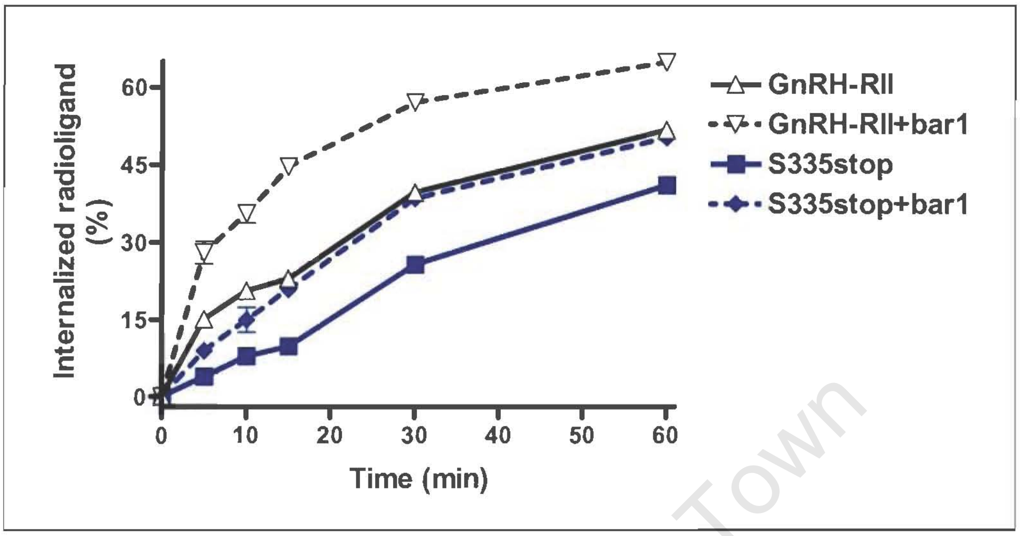

Fig. 5.6 Internalisation of the type II GnRH receptor and the C-terminally truncated mutant S335stop with and without β-arrestin 1. Data are shown from one single representative experiment. Each experiment was performed at least three times in duplicates.

Chapter 6: Discussion

6.1 The internalisation pathway of the mammalian type II GnRH receptor

The type II GnRH receptor was recently cloned from several monkey species (Millar *et al.* 2001; Neill *et al.* 2001). The internalisation of a mammalian type II GnRH receptor is not known yet. Therefore this thesis investigates the internalisation pathway of type II GnRH receptor of marmoset monkey and the role of the C-terminal tail as well as ICL 3 in endocytosis.

This study shows that in COS-1 cells the mammalian type II GnRH receptor internalises more rapidly than the mammalian type I GnRH receptor. The main difference between these two receptors is that the mammalian type I GnRH receptor lacks the cytoplasmic C-terminal domain. Therefore it is likely that the C-terminal tail is required for rapid internalisation, which will be discussed in section 6.2.

In COS-1 cells the type II GnRH receptor was shown to internalise in a β-arrestin independent, but GRK and dynamin dependent manner. It was found that co-expression of the dominant negative GRK 2 (K220R) reduced receptor internalisation by 44 % at fifteen minutes and by 28 % at sixty minutes of agonist stimulation. These data suggest that GRK mediated phosphorylation of the receptor is required for rapid internalisation. Furthermore co-expression of wild type GRK 1 and GRK 2 enhanced internalisation by 84 % at fifteen minutes indicating that higher levels of GRK can phosphorylate the receptor more efficiently, which in turn targets the receptor more efficiently for endocytosis and increases the percentage of internalised radioligand. According to the classical clathrin coated vesicle internalisation pathway GRK-mediated receptor phosphorylation is followed by β-arrestin binding to the activated and phosphorylated receptor (reviewed in (Ferguson 2001; Claing *et al.* 2002)). However internalisation of the type II GnRH receptor in COS-1 cells does not involve β-arrestin, since dominant negative mutants of both β-arrestin 1 (V53D, 319-418) and β-arrestin 2 (V54D) failed to reduce internalisation. COS cells have been reported to have low levels of β-arrestins (Menard *et al.* 1997), which could explain why dominant negative constructs of β-arrestin failed to reduce internalisation of the type II GnRH receptor. However for some GPCRs such as the TRH receptor, the PAFR and the AT_1 receptor a β-arrestin-dependent internalisation pathway has been demonstrated in this cell line, since dominant negative constructs of β-arrestin were able to reduce internalisation (Vrecl *et al.* 1998; Gaborik *et al.* 2001; Chen *et al.* 2002). Possibly the type II GnRH receptor has lower affinity for β-arrestin than the receptors mentioned and therefore at lower levels of β-arrestin the receptor could use an alternative β-arrestin independent pathway.

Over-expression of wild-type β-arrestin 1 and 2 enhanced internalisation by 87 % and 76 % respectively at fifteen minutes similarly to the effect of GRK over-expression. This indicates that the mammalian type II GnRH receptor is able to undergo β-arrestin dependent internalisation, if there is sufficient β-arrestin present in the cell. It is therefore likely that the type II GnRH receptor, which is expressed in a wide range of tissues (Millar *et al.* 2001), uses a β-arrestin-dependent internalisation pathway in other cell types.

The observation that the type II GnRH receptor internalises in a GRK dependent but β-arrestin independent manner in COS cells might seem contradictory, since the classical clathrin mediated endocytosis involves GRK mediated receptor phosphorylation as a prerequisite for β-arrestin binding. However some receptors such as the adenosine A3 receptor (Ferguson *et al.* 2000), the dopamine D2 receptor (Ito *et al.* 1999) and the M2 muscarinic receptor (Tsuga *et al.* 1994) become phosphorylated by GRKs after agonist occupancy, yet these GPCRs can be internalised in a β-arrestin-independent manner. Therefore GRK-mediated receptor phosphorylation does not always lead to β-arrestin binding. Furthermore studies on the luteinising hormone/choriogonadotropin and lutropin/choriogonadotropin receptors have shown that β-arrestin interaction with an activated GPCR can be phosphorylation independent (Min *et al.* 2002; Mukherjee *et al.* 2002).

The question arises whether a receptor like the type II GnRH receptor that internalises in a GRK- and dynamin-dependent, but a β-arrestin-independent manner, is still able to utilise the clathrin coated vesicle pathway. This endocytic pathway has been associated with the presence of β-arrestin, because β-arrestin is necessary for recruitment of clathrin and the AP2 complex (Goodman *et al.* 1996; Laporte *et al.* 1999). In recent years however it has been shown that internalisation via clathrin-coated vesicles does not necessarily depend on β-arrestin. It has been suggested that other molecules are able to take over the role of β-arrestin as an adapter between the activated receptor and the clathrin mediated internalisation machinery. One possibility could be that GRK 2 recruits clathrin. GRK 2 has been shown to possess a clathrin box (Shiina *et al.* 2001) similarly to β-arrestin and other clathrin interacting proteins. The ability of GRK 2 to directly interact with clathrin suggests that β-arrestin can be replaced as an adaptor molecule for clathrin mediated endocytosis especially for GPCRs that do not bind with β-arrestin. Therefore a receptor that requires GRK but not β-arrestin for its internalisation can still utilise the clathrin mediated internalisation pathway. One example for this β-arrestin independent clathrin mediated endocytosis is the PAR 1 receptor (Paing *et al.* 2002). The PAR 1 receptor requires β-arrestin for desensitisation, but not for internalisation. When expressed in mouse embryonic fibroblasts (MEF) derived from β-arrestin 1 and 2 knockout mice the phosphorylated PAR 1 receptor internalised in a clathrin dependent manner as efficiently as in wild-type MEF. Therefore the PAR 1 receptor utilises a distinct phosphorylation dependent, β-arrestin-independent pathway via clathrin-coated vesicles.

This leads to the question whether a GPCR, that internalises in a GRK and β-arrestin independent manner is still able to become internalised via clathrin coated vesicles. The mammalian type I GnRH receptor does not become phosphorylated by GRKs after agonist occupancy and cannot interact with β-arrestin possibly due to the lack of a C-terminal tail (Willars *et al.* 1999; Vrecl *et al.* 2000). Nevertheless a clathrin mediated pathway has been suggested for this receptor, based on the observation that this receptor colocalises with transferrin and that receptor internalisation could be abolished in the presence of sucrose (Vrecl *et al.* 1998) (Hislop *et al.* 2001). Even though the type II GnRH receptor could possibly internalise via clathrin coated vesicles, one cannot exclude that this receptor uses the caveolae pathway.

The GTPase, dynamin, plays an important role in vesicle fission in clathrin coated vesicle internalisation as well as in internalisation via caveolae. The involvement of dynamin in internalisation of the mammalian type I GnRH receptor is still controversial. While one group working on the rat GnRH receptor could see a 20 % reduction of internalisation in presence of dynamin (K44A) (Heding *et al.* 2000), another group working on the human GnRH receptor could not see an effect (Hislop *et al.* 2001). Co-expression of the dominant negative mutant of dynamin 1 (K44A) inhibited internalisation of the type II GnRH receptor by 38 % at 15 minutes of agonist stimulation similarly to the inhibition by GRK 2 (K220R), indicating that dynamin is required for internalisation of the receptor in COS cells. Nevertheless the dominant negative mutant dynamin 1 K44A could not completely abolish receptor internalisation. One possible explanation for this is that the major dynamin in COS cells is dynamin 2 (Gaborik *et al.* 2001), however dominant negative mutants of dynamin 1 and dynamin 2 have been shown to be equally efficient in inhibiting wild-type dynamin 1 (Gaborik *et al.* 2001). Therefore the possibility that a subpopulation of receptors uses a dynamin-independent pathway cannot be excluded. The same could be true for a GRK-independent pathway, since dominant negative GRK 2 K220R did not completely abolish internalisation of the type II GnRH receptor either. However low efficiency or low nexpression of the dominant negative constructs in competing with the endogenous wild-type proteins could also prevent complete inhibition.

Some GPCRs have been reported to be able to internalise in a β-arrestin-independent, dynamin-dependent pathway via caveolae. Examples are the vasoactive intestinal peptide type 1 (VIP1) receptor (Claing *et al.* 2000), the endothelin type B (ET_B) receptor (Claing *et al.* 2000), the endothelin type A (ET_A) receptor (Okamoto *et al.* 2000), the 5-hydroxytryptamine 2 A ($5\text{-}HT_{2A}$) receptor (Bhatnagar *et al.* 2001) or the prostacyclin receptor (Smyth *et al.* 2000). Furthermore it was observed that a receptor can require both caveolae and clathrin coated vesicles for internalisation (Lamb *et al.* 2001). It was suggested that caveolae and clathrin coated pits might be positioned in sequence in the same internalisation pathway. This pathway has been implicated in the internalisation of several growth hormone factor receptors, which first redistribute to caveolae and subsequently associate with clathrin-coated pits before being internalised (Li *et al.* 1995; Liu *et al.* 1996; Wu *et al.* 1997).

Internalisation of the type II GnRH receptor in presence of the caveolae inhibiting drug, filipin (Schnitzer *et al.* 1994), completely abolished receptor endocytosis. However hypertonic sucrose, an inhibitor of clathrin polymerisation and therefore of clathrin coated vesicle internalisation (Heuser and Anderson 1989), also abolished internalisation of the type II GnRH receptor. These results indicate that the type II GnRH receptor utilises both the caveolae and the clathrin internalisation pathways. Nevertheless it is possible that these two inhibitors have non-specific effects and could disrupt receptor internalisation indirectly. Filipin interferes with any cholesterol rich structures, not only caveolae but also lipid rafts, therefore one cannot exclude the possibility that the destruction of lipid rafts inhibits internalisation. As side effects of hypertonic sucrose reduction in ligand binding and accumulation of second messengers have been described (Lutz and Kumar 1993; Zhu *et al.* 1996). Reduction in ligand binding of the type II GnRH receptor was observed, however the internalisation assay used is designed to take differences in affinity and receptor expression into account, since the internalised ligand is calculated as a percentage of total cell associated ligand. Therefore reduction in ligand binding does not affect receptor internalisation.

This research tried to elucidate the internalisation pathway of a mammalian type II GnRH receptor. It was demonstrated that in COS cells the type II GnRH receptor requires GRK and dynamin for receptor endocytosis, but internalises in a β-arrestin-independent manner. However over-expression of β-arrestin enhanced internalisation kinetics. Therefore the type II GnRH receptor, which is widely expressed, and might use a β-arrestin-mediated internalisation pathway in a different cell type with higher levels of endogenous β-arrestin. The findings suggest that the mammalian type II GnRH receptor uses both the clathrin coated vesicle internalisation pathway as well as the caveolae pathway.

6.2 The role of the C-terminal tail and ICL 3 in receptor internalisation

The C-terminal tail of GPCRs plays a key role in receptor function from signalling, desensitisation and internalisation to receptor recycling and degradation (reviewed in (Ferguson 2001; Claing *et al.* 2002)). This thesis focuses on receptor internalisation. Therefore one aim was to elucidate the role of the C-terminal tail of the type II GnRH receptor in receptor internalisation.

One of the first findings of this research was that the mammalian type II GnRH receptor internalises more rapidly and to a greater extent than the mammalian type I GnRH receptor, which unlike other GPCRs lacks the C-terminal tail. This finding is consistent with other studies showing that GnRH receptors with a C-terminal tail undergo phosphorylation, rapid desensitisation and rapid internalisation kinetics (Heding *et al.* 1998; Willars *et al.* 1999).

Generally the C-terminal tail of GPCRs harbours phosphorylation sites for GRKs. However the importance of the C-terminal tail in receptor internalisation varies from receptor to receptor. The β_2-AR becomes phosphorylated within its C-terminal tail by GRKs (Fredericks *et al.* 1996) and truncation of the C-terminal tail or creation of a phosphorylation deficient mutant (Y326A) reduced receptor internalisation (Ferguson *et al.* 1995; Ferguson *et al.* 1996). Additionally mutation of GRK phosphorylation sites within the C-terminal tail of the β_2-AR reduced receptor internalisation (Seibold *et al.* 2000). Other examples of GPCRs that require the C-terminal tail for internalisation are the AT_{1A} receptor (Thomas *et al.* 1998), the D1 dopamine receptor (Lamey *et al.* 2002), the TRH receptor (Groarke *et al.* 2001), the thromboxane A2 receptor (Parent *et al.* 2001) or the thrombin receptor (Shapiro *et al.* 1996). However truncation of the C-terminal tail of the m1 muscarinic receptor does not affect receptor endocytosis (Lameh *et al.* 1992). Interestingly deletion of C-terminal amino acids from the FSH receptor even enhances internalisation (Kishi *et al.* 2002).

To investigate the role of the C-terminal tail of the mammalian type II GnRH receptor, C-terminally truncated receptor mutants were created. All truncated mutants of the type II GnRH receptor were functional in signalling. Even the receptor lacking the entire C-terminal tail (G326stop) was active in generating IP, although ligand binding was not measurable. This is in marked contrast to observations on other GnRH receptors with C-terminal domains. Complete truncation of the C-terminal tails of the catfish and the chicken GnRH receptors resulted in receptor mutants that were not active in cell signalling (Pawson *et al.* 1998; Blomenrohr *et al.* 1999). However studies on other GPCRs e.g. the thrombin receptor and the β-adrenergic receptor showed that receptor truncation did not disrupt signalling (Shapiro *et al.* 1996) (Bouvier *et al.* 1988).

Subsequently two serine residues (Ser 338 and Ser 339) within the C-terminal tail of the type II GnRH receptor were found to be critical for rapid receptor internalisation. Since the internalisation pathway of the type II GnRH receptor was shown to be GRK-dependent it is possible that these serine residues become phosphorylated after agonist occupancy of the receptor. In contrast to other kinases such as CKII, PKC or PKA a consensus sequence for GRK phosphorylation sites has not been identified. It has however been reported that GRK 2 prefers to phosphorylate serine and threonine residues that have acidic residues on their N-terminal side (Pitcher *et al.* 1998). The N-terminal adjacent amino acid of Ser 338 is Asp 337, therefore it is likely that this residue makes Ser 338/339 a target for GRK phosphorylation.

The only other serine or threonine residue that could be a GRK phosphorylation site due to an acidic amino acid neighbour is Ser 372, however deletion of the terminal amino acids including Ser 372 had no effect on receptor internalisation or signalling.

After GRK-mediated phosphorylation of the C-terminal tail many GPCRs bind β-arrestins. The role of the C-terminal tail of the mammalian type II GnRH receptor in β-arrestin-dependent internalisation was investigated. Augmentation of β-arrestin mediated internalisation of the C-terminally truncated mutant S335stop was measured and compared to the wild type receptor. Overexpression of β-arrestin enhanced internalisation of the truncated S335stop mutant as efficiently as of the wild type receptor. Therefore it can be concluded that the C-terminal tail of the type II GnRH receptor is not required for interaction of the receptor with β-arrestin.

Consistent with this result truncation of the C-terminal tail of the catfish GnRH receptor as well as truncation of the C-terminal tail of the β_2-AR could also not inhibit β-arrestin mediated augmentation of internalisation (Ferguson *et al.* 1996; Blomenrohr *et al.* 1999).

However the importance of the C-terminal tail in β-arrestin binding has been demonstrated with a number of GPCRs. Binding of β-arrestin to the TRH receptor can be abolished by truncation of the C-terminal tail (Groarke *et al.* 2001). A truncated receptor could in contrast to the wild-type receptor not translocate GFP-β-arrestin to the membrane after agonist stimulation. Furthermore, addition of the C-terminal tail of the TRH receptor to the rat GnRH receptor, which internalises in a β-arrestin independent manner, leads to a receptor chimera that uses a β-arrestin dependent pathway (Heding *et al.* 2000). These results indicate that a C-terminal tail may confer β-arrestin dependency. Nevertheless addition of the catfish C-terminal tail to the rat GnRH receptor did not confer β-arrestin dependency (Hanyaloglu *et al.* 2001), although the catfish GnRH receptor internalises in a β-arrestin dependent manner (Heding *et al.* 2000). Therefore the determinants for β-arrestin binding are more complex then simply the presence of a C-terminal tail and further research has to be done to investigate β-arrestin binding sites on GPCRs. A recent study on the PAR 2 receptor showed that basic residues in ICL 3 are important for β-arrestin dependent endocytosis and mutation of these residues reduces receptor internalisation (DeFea *et al.* 2000).

GRKs can not only phosphorylate serine and threonine residues located in the C-terminal tail, but also in ICL 3. One of the first reports demonstrating an important role of ICL 3 in receptor internalisation was a study on the m1 muscarinic receptor (Lameh *et al.* 1992). Mutation of serine and threonine within the C-terminal tail of this receptor as well as C-terminal truncation failed to affect internalisation. Therefore *Lameh et al.* created ICL 3 deletion mutants of the m1 muscarinic receptor. In the middle of ICL 3 a domain, which contained a cluster of four serine residues, was shown to be crucial for receptor internalisation. Subsequently agonist dependent phosphorylation sites were identified within ICL 3 of the related m2 muscarinic receptor (Nakata *et al.* 1994).

The function of putative phosphorylation sites in ICL 3 of the type II GnRH receptor was investigated by mutation of serine and threonine residues to alanines. Ser 251 was found to play a role in internalisation and signalling. Mutation of Ser 251 to an alanine reduced internalisation at sixty minutes of agonist stimulation, indicating that Ser 251 is a putative phosphorylation site. Additionally it was shown that Ser 251 also plays a role in signalling. IP assays reveiled that mutation of Ser 251 to Ala causes a reduction in IPmax as well as a 10 fold increase in EC_{50}. Therefore Ser 251 can either be a direct G protein interaction site or mutation of Ser 251 to Ala changes receptor structure to such an extent that the receptor cannot interact with the ligand or the G-protein efficiently. The fact that the significantly uncoupled S251A mutant can still internalise like wild-type within the first 40 minutes of agonist stimulation supports studies on other GPCRs like the β_2-AR that have shown that G protein coupling is not required for receptor internalisation (Mahan *et al.* 1985). However, if internalisation of the receptor is dependent on GRK recruitment a reduction in activation of G protein could recruit less GRK and explain weak internalisation.

This research revealed that Ser 338 and Ser 339 within the C-terminal tail of the mammalian type II GnRH receptor are critical for rapid receptor internalisation. Additionally a serine residue in ICL 3 (Ser 251) was identified to be important for G protein coupling as well as for internalisation. Generally GPCRs have phosphorylation sites either in their C-terminals tails or in their ICL3s. Interestingly for the type II GnRH receptor both regions contain putative phosphorylation sites that are important for receptor endocytosis.

6.3 The role of Ser 251, 338 and 339 in internalisation

Whereas the mutation S251A affected receptor internalisation at late time points, mutation S338,339A reduced internalisation at early time points after agonist stimulation. Interestingly mutation of both Ser 338,339 and Ser 251 combined the effect of the individual mutations and lead to a receptor that hardly internalised throughout a time course of 60 minutes. Similar observations have been made with the β_2-AR (Seibold *et al.* 2000). The β_2AR possesses a serine triplet in its C-terminal tail. Mutation of two serines to alanines causes a 21 % reduction in receptor internalisation. However mutation of all three serine residues causes an inhibition of 45 %.

The finding that these putative phosphorylation sites within the C-terminus and ICL 3 are crucial for internalisation of the type II GnRH receptor suggests that phosphorylation of these residues is necessary for receptor internalisation. This is consistent with many studies reporting the importance of receptor phosphorylation in desensitisation and internalisation (reviewed in (Ferguson 2001; Claing *et al.* 2002)). The importance of Ser 251, 338 and 339 is further underlined by the fact that these residues are conserved between all known mammalian type II GnRH receptors (Faurholm *et al.* 2001; Neill *et al.* 2001). Ser 251 within ICL 3 is also found in the non-mammalian type II GnRH receptor of bullfrog (Wang *et al.* 2001).

To investigate receptor phosphorylation ^{32}P whole cell phosphorylation assays were performed. However it was not possible to detect phosphorylation of the type II GnRH receptor even though the positive control the β_2-AR did show phosphorylation under the same experimental conditions. Receptor phosphorylation could also not be determined with an anti-phosphoserine specific antibody. The inability to detect phosphorylation of the type II GnRH receptor is probably due to the relatively low expression level of the type II GnRH receptor compared to the higher expressed β_2-AR as seen in Western Blot analysis.

Since receptor phosphorylation could not be shown directly, indirect approaches were used. One approach is to simulate phosphorylation by mutating a putatively phosphorylated serine or threonine residue to an acidic aspartate or glutamate residue, whose negative charge simulates the negativity of phospho-serine and phospho-threonine. Mutation of putatively phosphorylated residues in the AT_{1A} receptor to aspartate residues resulted in a receptor that internalised to the same extent as the wild type receptor whereas mutation of the same residues to alanine residues reduced internalisation (Thomas *et al.* 1998). Similar experiments were performed with the type II GnRH receptor (data not shown), however the findings were not as clear as in the case of the AT_{1A} receptor. In some instances mutation of a serine residue to an aspartate residue increased internalisation, whereas in other mutants it decreased internalisation. For example the mutant S251,338,339A internalised very weakly. Substitution of alanine 251 with an aspartate increased internalisation to the same level as the S338,339A mutant. This finding suggests that serine 251 does become phosphorylated. However a receptor with the single mutation S251D internalised less efficiently than the S251A mutant. It seems that a serine to aspartate mutation had a more disruptive effect than the serine to alanine mutation. These findings do not, therefore, clarify whether the type II GnRH receptor is phosphorylated or not.

As another approach to determine that Ser 251, 338 and 339 are indeed phosphorylation sites, the ability of dominant negative GRK (K220R) to reduce internalisation of the type II GnRH receptor mutant lacking all three putative phosphorylation sites (S251,338,339A) was tested. GRK 2 (K220R) could inhibit internalisation of the wild type receptor but not of the mutant lacking all phosphorylation sites indicating that these serines undergo phosphorylation, which is required for internalisation. Interestingly internalisation of a receptor mutant lacking only Ser 338, 339 or only Ser 251 could still be inhibited by dominant negative GRK. This shows that all three GRK phosphorylation sites have to be abolished in order to see an effect. The observation that mutating all residues causes an additive effect of the individual mutations and this GnRH receptor hardly internalises is similar to findings with the β_2AR (Seibold *et al.* 2000).

Another indication that Ser 338 and 339 are GRK phosphorylation sites was found in IP assays. Mutation of these serine residues to alanine caused a left shift of the EC_{50} indicating that the mutant S338,339A does not desensitise as efficiently as the wild-type receptor. Therefore it is likely that these serine residues are putative GRK phosphorylation sites and play a role in desensitisation as well as internalisation. However the shift in EC_{50} could also be explained by reduction of internalisation or by an increase in the number of receptors in the membrane.

Even though direct receptor phosphorylation could not be demonstrated, the experiments consistently support the conclusion that Ser 251, 338 and 339 are GRK phosphorylation sites.

Ser 338 and Ser 339 are not only putative GRK phosphorylation sites, but also part of a consensus sequence for CKII, while Ser 338 alone is located within a consensus sequence for PKC. Recently it has been demonstrated that CK II sites play a role in β-arrestin dependent internalisation of some GPCRs (Hanyaloglu *et al.* 2001). The TRH receptor has three CK II sites in its C-terminal tail and internalises in a β-arrestin dependent manner. Mutation of all CK II sites resulted in the loss of β-arrestin dependency. On the other hand the C-terminal tail of the catfish GnRH receptor does not contain any CK II sites and a chimera of a mammalian GnRH receptor with a catfish C-terminal tail does not require β-arrestin. However when CK II sites were added to the catfish tail the receptor chimera internalised in a β-arrestin dependent manner.

The role the CK II consensus sequence in the C-terminal tail of the type II GnRH receptor in β-arrestin binding was investigated. It could be shown that internalisation of a receptor mutant lacking the CK II sites (Ser

338 and 339) can still be enhanced by β-arrestin. In fact this mutant could internalise in a β-arrestin-dependent manner just as efficiently as the wild-type receptor. Therefore the CK II phosphorylation site within the C-terminal tail of the type II GnRH receptor is not required for β-arrestin dependent internalisation. Additionally we found that Ser 251 within intracellular loop is not critical for β-arrestin-mediated endocytosis. Studies on the β_2-AR receptor showed that internalisation of a phosphorylation deficient mutant, that internalises weakly, can be enhanced by over-expression of β-arrestin (Ferguson *et al.* 1996). Therefore β-arrestin does not absolutely require phosphorylated residues in order to interact with the receptor. In fact the LH receptor does not have to be phosphorylated in order to interact with β-arrestin (Mukherjee *et al.* 2002). Instead in this case an aspartate within the third intracellular loop is the site of β-arrestin interaction.

In summary this thesis elucidated the internalisation pathway of a mammalian type II GnRH receptor. In COS-cells the type II GnRH receptor internalises in a β-arrestin-independent, but GRK and dynamin-dependent manner. This receptor could internalise in a β-arrestin dependent manner in a cell type with higher levels of β-arrestin, since β-arrestin over-expression in COS-cells enhanced internalisation kinetics. Additionally it has been shown that sucrose as well as filipin can abolish internalisation indicating that both pathways, the clathrin coated vesicle pathway as well as the caveolae pathway, are involved in endocytosis of the type II GnRH receptor.

Two serine residues within the C-terminal tail (Ser 338 and 339) and one serine residue in ICL 3 (Ser 251) were shown to be critical for rapid internalisation. Based on the observation that dominant negative GRK 2 (K220R) could not reduce internalisation of a mutant receptor lacking these putative phosphorylation sites, but could reduce internalisation of the wild-type receptor, we suggest that Ser 251, 338 and/or 339 are phosphorylated after agonist occupancy of the receptor. However these phosphorylation sites as well as the C-terminal tail of the type II GnRH receptor are not important for β-arrestin mediated augmentation of receptor internalisation.

The finding that the mammalian type II GnRH receptor internalises more rapidly than the tail-less mammalian type I GnRH receptor is consistent with previous observations that the C-terminal tail mediates rapid desensitisation and internalisation (Heding *et al.* 1998; Blomenrohr *et al.* 1999; Willars *et al.* 1999). The lack of rapid desensitisation of the type I GnRH receptor has been thought to be the responsible for the prolonged LH surge in the late preovulatory phase (Pawson *et al.* 1998). Activation of the type II GnRH receptor leads mainly to FSH secretion (Millar *et al.* 2001; Millar 2002). FSH secretion is continuous throughout the reproductive cycle and does not display a prolonged surge like LH. This could be one explanation for the presence of a C-terminal tail, which mediates rapid desensitisation and internalisation in the type II GnRH receptor. The differential internalisation patterns of these two GPCRs could also be attributed to the different ligands. GnRH I is secreted in a pulsatile manner, which could lead to a slow internalisation profile. The way of secretion of GnRH II is not known, but could be responsible for rapid internalisation of the type II GnRH receptor. However it can only be speculated about the physiological role of the different internalisation patterns of the type I and type II GnRH receptors, particularly since the function of the type II GnRH receptor still has to be determined.

7. References

Acharjee, S., Maiti, K., Soh, J. M., Im, W. B., Seong, J. Y. and Kwon, H. B. (2002). "Differential desensitization and internalization of three different bullfrog gonadotropin-releasing hormone receptors." Mol Cells **14**(1): 101-107.

Ahn, S., Maudsley, S., Luttrell, L. M., Lefkowitz, R. J. and Daaka, Y. (1999). "Src-mediated tyrosine phosphorylation of dynamin is required for beta2- adrenergic receptor internalization and mitogen-activated protein kinase signaling." J Biol Chem **274**(3): 1185-1188.

Anborgh, P. H., Seachrist, J. L., Dale, L. B. and Ferguson, S. S. (2000). "Receptor/beta-arrestin complex formation and the differential trafficking and resensitization of beta2-adrenergic and angiotensin II type 1A receptors." Mol Endocrinol **14**(12): 2040-2053.

Arora, K. K., Krsmanovic, L. Z., Mores, N., O'Farrell, H. and Catt, K. J. (1998). "Mediation of cyclic AMP signaling by the first intracellular loop of the gonadotropin-releasing hormone receptor." J Biol Chem **273**(40): 25581-25586.

Arora, K. K., Sakai, A. and Catt, K. J. (1995). "Effects of second intracellular loop mutations on signal transduction and internalization of the gonadotropin-releasing hormone receptor." J Biol Chem **270**(39): 22820-22826.

Barak, L. S., Menard, L., Ferguson, S. S., Colapietro, A. M. and Caron, M. G. (1995). "The conserved seven-transmembrane sequence NP(X)2,3Y of the G-protein- coupled receptor superfamily regulates multiple properties of the beta 2-adrenergic receptor." Biochemistry **34**(47): 15407-15414.

Barak, L. S., Tiberi, M., Freedman, N. J., Kwatra, M. M., Lefkowitz, R. J. and Caron, M. G. (1994). "A highly conserved tyrosine residue in G protein-coupled receptors is required for agonist-mediated beta

2-adrenergic receptor sequestration." J Biol Chem **269**(4): 2790-2795.

Benovic, J. L., Kuhn, H., Weyand, I., Codina, J., Caron, M. G. and Lefkowitz, R. J. (1987). "Functional desensitization of the isolated beta-adrenergic receptor by the beta-adrenergic receptor kinase: potential role of an analog of the retinal protein arrestin (48-kDa protein)." Proc Natl Acad Sci U S A **84**(24): 8879-8882.

Bhatnagar, A., Willins, D. L., Gray, J. A., Woods, J., Benovic, J. L. and Roth, B. L. (2001). "The dynamin-dependent, arrestin-independent internalization of 5- hydroxytryptamine 2A (5-HT2A) serotonin receptors reveals differential sorting of arrestins and 5-HT2A receptors during endocytosis." J Biol Chem **276**(11): 8269-8277.

Block, M. R., Glick, B. S., Wilcox, C. A., Wieland, F. T. and Rothman, J. E. (1988). "Purification of an N-ethylmaleimide-sensitive protein catalyzing vesicular transport." Proc Natl Acad Sci U S A **85**(21): 7852-7856.

Blomenrohr, M., Heding, A., Sellar, R., Leurs, R., Bogerd, J., Eidne, K. A. and Willars, G. B. (1999). "Pivotal role for the cytoplasmic carboxyl-terminal tail of a nonmammalian gonadotropin-releasing hormone receptor in cell surface expression, ligand binding, and receptor phosphorylation and internalization." Mol Pharmacol **56**(6): 1229-1237.

Bockaert, J. and Pin, J. P. (1999). "Molecular tinkering of G protein-coupled receptors: an evolutionary success." Embo J **18**(7): 1723-1729.

Boekhoff, I., Inglese, J., Schleicher, S., Koch, W. J., Lefkowitz, R. J. and Breer, H. (1994). "Olfactory desensitization requires membrane targeting of receptor kinase mediated by beta gamma-subunits of heterotrimeric G proteins." J Biol Chem **269**(1): 37-40.

Bouvier, M., Hausdorff, W. P., De Blasi, A., O'Dowd, B. F., Kobilka, B. K., Caron, M. G. and Lefkowitz, R. J. (1988). "Removal of phosphorylation sites from the beta 2-adrenergic receptor delays onset of agonist-promoted desensitization." Nature **333**(6171): 370-373.

Bremnes, T., Paasche, J. D., Mehlum, A., Sandberg, C., Bremnes, B. and Attramadal, H. (2000). "Regulation and intracellular trafficking pathways of the endothelin receptors." J Biol Chem **275**(23): 17596-17604.

Brunger, A. T. (2000). "Structural insights into the molecular mechanism of Ca(2+)-dependent exocytosis." Curr Opin Neurobiol **10**(3): 293-302.

Budd, D. C., McDonald, J. E. and Tobin, A. B. (2000). "Phosphorylation and regulation of a Gq/11-coupled receptor by casein kinase 1alpha." J Biol Chem **275**(26): 19667-19675.

Bunemann, M. and Hosey, M. M. (1999). "G-protein coupled receptor kinases as modulators of G-protein signalling." J Physiol **517**(Pt 1): 5-23. =S0022375199092029.

Byrne, B., McGregor, A., Taylor, P. L., Sellar, R., Rodger, F. E., Fraser, H. M. and Eidne, K. A. (1999). "Isolation and characterisation of the marmoset gonadotrophin releasing hormone receptor: Ser(140) of the DRS motif is substituted by Phe." J Endocrinol **163**(3): 447-456.

Cao, T. T., Deacon, H. W., Reczek, D., Bretscher, A. and von Zastrow, M. (1999). "A kinase-regulated PDZ-domain interaction controls endocytic sorting of the beta2-adrenergic receptor." Nature **401**(6750): 286-290.

Cao, W., Luttrell, L. M., Medvedev, A. V., Pierce, K. L., Daniel, K. W., Dixon, T. M., Lefkowitz, R. J. and Collins, S. (2000). "Direct binding of activated c-Src to the beta 3-adrenergic receptor is required for MAP kinase activation." J Biol Chem **275**(49): 38131-38134.

Chen, A., Kaganovsky, E., Rahimipour, S., Ben-Aroya, N., Okon, E. and Koch, Y. (2002). "Two forms of gonadotropin-releasing hormone (GnRH) are expressed in human breast tissue and overexpressed in breast cancer: a putative mechanism for the antiproliferative effect of GnRH by down-regulation of acidic ribosomal phosphoproteins P1 and P2." Cancer Res **62**(4): 1036-1044.

Chen, H. J., Rojas-Soto, M., Oguni, A. and Kennedy, M. B. (1998). "A synaptic Ras-GTPase activating protein (p135 SynGAP) inhibited by CaM kinase II." Neuron **20**(5): 895-904.

Chen, Z., Dupre, D. J., Le Gouill, C., Rola-Pleszczynski, M. and Stankova, J. (2002). "Agonist-induced internalization of the platelet-activating factor receptor is dependent on arrestins but independent of G-protein activation. Role of the C terminus and the (D/N)PXXY motif." J Biol Chem **277**(9): 7356-7362.

Cheon, K. W., Lee, H. S., Parhar, I. S. and Kang, I. S. (2001). "Expression of the second isoform of gonadotrophin-releasing hormone (GnRH-II) in human endometrium throughout the menstrual cycle." Mol Hum Reprod **7**(5): 447-452.

Chi, L., Zhou, W., Prikhozhan, A., Flanagan, C., Davidson, J. S., Golembo, M., Illing, N., Millar, R. P. and Sealfon, S. C. (1993). "Cloning and characterization of the human GnRH receptor." Mol Cell Endocrinol **91**(1-2): R1-6.

Choi, K. C., Auersperg, N. and Leung, P. C. (2001). "Expression and antiproliferative effect of a second form of gonadotropin-releasing hormone in normal and neoplastic ovarian surface epithelial cells." J Clin Endocrinol Metab **86**(10): 5075-5078.

Claing, A., Chen, W., Miller, W. E., Vitale, N., Moss, J., Premont, R. T. and Lefkowitz, R. J. (2001). "beta-Arrestin-mediated ADP-ribosylation factor 6 activation and beta 2- adrenergic receptor endocytosis." J Biol Chem **276**(45): 42509-42513.

Claing, A., Laporte, S. A., Caron, M. G. and Lefkowitz, R. J. (2002). "Endocytosis of G protein-coupled receptors: roles of G protein-coupled receptor kinases and beta-arrestin proteins." Prog Neurobiol **66**(2): 61-79.

Claing, A., Perry, S. J., Achiriloaie, M., Walker, J. K., Albanesi, J. P., Lefkowitz, R. J. and Premont, R. T. (2000). "Multiple endocytic pathways of G protein-coupled receptors delineated by GIT1 sensitivity." Proc Natl Acad Sci U S A **97**(3): 1119-1124.

Cong, M., Perry, S. J., Hu, L. A., Hanson, P. I., Claing, A. and Lefkowitz, R. J. (2001). "Binding of the beta2 adrenergic receptor to N-ethylmaleimide-sensitive factor regulates receptor recycling." J Biol Chem **276**(48): 45145-45152.

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

Davidson, J. S., Assefa, D., Pawson, A., Davies, P., Hapgood, J., Becker, I., Flanagan, C., Roeske, R. and Millar, R. (1997). "Irreversible activation of the gonadotropin-releasing hormone receptor by photoaffinity cross-linking: localization of attachment site to Cys residue in N-terminal segment." Biochemistry **36**(42): 12881-12889.

Davidson, J. S., Flanagan, C. A., Zhou, W., Becker, II, Elario, R., Emeran, W., Sealfon, S. C. and Millar, R. P. (1995). "Identification of N-glycosylation sites in the gonadotropin-releasing hormone receptor: role in receptor expression but not ligand binding." Mol Cell Endocrinol **107**(2): 241-245.

Davidson, J. S., Wakefield, I. K. and Millar, R. P. (1994). "Absence of rapid desensitization of the mouse gonadotropin-releasing hormone receptor." Biochem J **300**(Pt 2): 299-302.

DeFea, K. A., Zalevsky, J., Thoma, M. S., Dery, O., Mullins, R. D. and Bunnett, N. W. (2000). "beta-arrestin-dependent endocytosis of proteinase-activated receptor 2 is required for intracellular targeting of activated ERK1/2." J Cell Biol **148**(6): 1267-1281.

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

Dohlman, H. G. and Thorner, J. (1997). "RGS proteins and signaling by heterotrimeric G proteins." J Biol Chem **272**(7): 3871-3874.

Dohlman, H. G., Thorner, J., Caron, M. G. and Lefkowitz, R. J. (1991). "Model systems for the study of seven-transmembrane-segment receptors." Annu Rev Biochem **60**: 653-688.

Eason, M. G., Moreira, S. P. and Liggett, S. B. (1995). "Four consecutive serines in the third intracellular loop are the sites for beta-adrenergic receptor kinase-mediated phosphorylation and desensitization of the alpha 2A-adrenergic receptor." J Biol Chem **270**(9): 4681-4688.

Edwardson, J. M. and Szekeres, P. G. (1999). "Endocytosis and recycling of muscarinic receptors." Life Sci **64**(6-7): 487-494.

Fan, G., Shumay, E., Malbon, C. C. and Wang, H. (2001). "c-Src tyrosine kinase binds the beta 2-adrenergic receptor via phospho- Tyr-350, phosphorylates G-protein-linked receptor kinase 2, and mediates agonist-induced receptor desensitization." J Biol Chem **276**(16): 13240-13247.

Faurholm, B., Millar, R. P. and Katz, A. A. (2001). "The genes encoding the type II gonadotropin-releasing hormone receptor and the ribonucleoprotein RBM8A in humans overlap in two genomic loci." Genomics **78**(1-2): 15-18.

Ferguson, G., Watterson, K. R. and Palmer, T. M. (2000). "Subtype-specific kinetics of inhibitory adenosine receptor internalization are determined by sensitivity to phosphorylation by G protein-coupled receptor kinases." Mol Pharmacol **57**(3): 546-552.

Ferguson, S. S. (2001). "Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling." Pharmacol Rev **53**(1): 1-24.

Ferguson, S. S., Downey, W. E., 3rd, Colapietro, A. M., Barak, L. S., Menard, L. and Caron, M. G. (1996). "Role of beta-arrestin in mediating agonist-promoted G protein-coupled receptor internalization." Science **271**(5247): 363-366.

Ferguson, S. S., Menard, L., Barak, L. S., Koch, W. J., Colapietro, A. M. and Caron, M. G. (1995). "Role of phosphorylation in agonist-promoted beta 2-adrenergic receptor sequestration. Rescue of a sequestration-defective mutant receptor by beta ARK1." J Biol Chem **270**(42): 24782-24789.

Flanagan, C. A., Fromme, B. J., Davidson, J. S. and Millar, R. P. (1998). "A high affinity gonadotropin-releasing hormone (GnRH) tracer, radioiodinated at position 6, facilitates analysis of mutant GnRH receptors." Endocrinology **139**(10): 4115-4119.

Flanagan, C. A., Rodic, V., Konvicka, K., Yuen, T., Chi, L., Rivier, J. E., Millar, R. P., Weinstein, H. and Sealfon, S. C. (2000). "Multiple interactions of the Asp(2.61(98)) side chain of the gonadotropin-releasing hormone receptor contribute differentially to ligand interaction." Biochemistry **39**(28): 8133-8141.

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

Foster-Barber, A. and Bishop, J. M. (1998). "Src interacts with dynamin and synapsin in neuronal cells." Proc Natl Acad Sci U S A **95**(8): 4673-4677.

Fredericks, Z. L., Pitcher, J. A. and Lefkowitz, R. J. (1996). "Identification of the G protein-coupled receptor kinase phosphorylation sites in the human beta2-adrenergic receptor." J Biol Chem **271**(23): 13796-13803.

Freedman, N. J., Ament, A. S., Oppermann, M., Stoffel, R. H., Exum, S. T. and Lefkowitz, R. J. (1997). "Phosphorylation and desensitization of human endothelin A and B receptors. Evidence for G protein-coupled receptor kinase specificity." J Biol Chem **272**(28): 17734-17743.

Fromme, B. J., Katz, A. A., Roeske, R. W., Millar, R. P. and Flanagan, C. A. (2001). "Role of aspartate7.32(302) of the human gonadotropin-releasing hormone receptor in stabilizing a high-affinity ligand conformation." Mol Pharmacol **60**(6): 1280-1287.

Gaborik, Z., Szaszak, M., Szidonya, L., Balla, B., Paku, S., Catt, K. J., Clark, A. J. and Hunyady, L. (2001). "Beta-arrestin- and dynamin-dependent endocytosis of the AT1 angiotensin receptor." Mol Pharmacol **59**(2): 239-247.

Gardner, B., Liu, Z. F., Jiang, D. and Sibley, D. R. (2001). "The role of phosphorylation/dephosphorylation in agonist-induced desensitization of D1 dopamine receptor function: evidence for a novel pathway for receptor dephosphorylation." Mol Pharmacol **59**(2): 310-321.

Garland, A. M., Grady, E. F., Lovett, M., Vigna, S. R., Frucht, M. M., Krause, J. E. and Bunnett, N. W. (1996). "Mechanisms of desensitization and resensitization of G protein-coupled neurokinin1 and neurokinin2 receptors." Mol Pharmacol **49**(3): 438-446.

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

Gilbert, T. L., Bennett, T. A., Maestas, D. C., Cimino, D. F. and Prossnitz, E. R. (2001). "Internalization of the human N-formyl peptide and C5a chemoattractant receptors occurs via clathrin-independent mechanisms." Biochemistry **40**(12): 3467-3475.

Goodman, O. B., Jr., Krupnick, J. G., Santini, F., Gurevich, V. V., Penn, R. B., Gagnon, A. W., Keen, J. H. and Benovic, J. L. (1996). "Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor." Nature **383**(6599): 447-450.

Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Hsuan, J., Booker, G. W. and et al. (1993). "The GTPase dynamin binds to and is activated by a subset of SH3 domains." Cell **75**(1): 25-36.

Gray, J. A. and Roth, B. L. (2002). "Cell biology. A last GASP for GPCRs?" Science **297**(5581): 529-531.

Groarke, D. A., Drmota, T., Bahia, D. S., Evans, N. A., Wilson, S. and Milligan, G. (2001). "Analysis of the C-terminal tail of the rat thyrotropin-releasing hormone receptor-1 in interactions and cointernalization with beta- arrestin 1-green fluorescent protein." Mol Pharmacol **59**(2): 375-385.

Hanyaloglu, A. C., Vrecl, M., Kroeger, K. M., Miles, L. E., Qian, H., Thomas, W. G. and Eidne, K. A. (2001). "Casein kinase II sites in the intracellular C-terminal domain of the thyrotropin-releasing hormone receptor and chimeric gonadotropin- releasing hormone receptors contribute to beta-arrestin-dependent internalization." J Biol Chem **276**(21): 18066-18074.

Hasbi, A., Allouche, S., Sichel, F., Stanasila, L., Massotte, D., Landemore, G., Polastron, J. and Jauzac, P. (2000). "Internalization and recycling of delta-opioid receptor are dependent on a phosphorylation-dephosphorylation mechanism." J Pharmacol Exp Ther **293**(1): 237-247.

Hauser, F., Sondergaard, L. and Grimmelikhuijzen, C. J. (1998). "Molecular cloning, genomic organization and developmental regulation of a novel receptor from Drosophila melanogaster structurally related to gonadotropin-releasing hormone receptors for vertebrates." Biochem Biophys Res Commun **249**(3): 822-828.

Heding, A., Vrecl, M., Bogerd, J., McGregor, A., Sellar, R., Taylor, P. L. and Eidne, K. A. (1998). "Gonadotropin-releasing hormone receptors with intracellular carboxyl- terminal tails undergo acute desensitization of total inositol phosphate production and exhibit accelerated internalization kinetics." J Biol Chem **273**(19): 11472-11477.

Heding, A., Vrecl, M., Hanyaloglu, A. C., Sellar, R., Taylor, P. L. and Eidne, K. A. (2000). "The rat gonadotropin-releasing hormone receptor internalizes via a beta- arrestin-independent, but dynamin-dependent, pathway: addition of a carboxyl-terminal tail confers beta-arrestin dependency." Endocrinology **141**(1): 299-306.

Henley, J. R., Krueger, E. W., Oswald, B. J. and McNiven, M. A. (1998). "Dynamin-mediated internalization of caveolae." J Cell Biol **141**(1): 85-99.

Hershko, A. and Ciechanover, A. (1998). "The ubiquitin system." Annu Rev Biochem **67**: 425-479.

Heuser, J. E. and Anderson, R. G. (1989). "Hypertonic media inhibit receptor-mediated endocytosis by blocking clathrin-coated pit formation." J Cell Biol **108**(2): 389-400.

Hislop, J. N., Everest, H. M., Flynn, A., Harding, T., Uney, J. B., Troskie, B. E., Millar, R. P. and McArdle, C. A. (2001). "Differential internalization of mammalian and non-mammalian gonadotropin-releasing hormone receptors. Uncoupling of dynamin-dependent internalization from mitogen-activated protein kinase signaling." J Biol Chem **276**(43): 39685-39694.

Hislop, J. N., Madziva, M. T., Everest, H. M., Harding, T., Uney, J. B., Willars, G. B., Millar, R. P., Troskie, B. E., Davidson, J. S. and McArdle, C. A. (2000). "Desensitization and internalization of human and xenopus gonadotropin- releasing hormone receptors expressed in alphaT4 pituitary cells using recombinant adenovirus." Endocrinology **141**(12): 4564-4575.

Illing, N., Jacobs, G. F., Becker, II, Flanagan, C. A., Davidson, J. S., Eales, A., Zhou, W., Sealfon, S. C. and Millar, R. P. (1993). "Comparative sequence analysis and functional characterization of the cloned sheep gonadotropin-releasing hormone receptor reveal differences in primary structure and ligand specificity among mammalian receptors." Biochem Biophys Res Commun **196**(2): 745-751.

Illing, N., Troskie, B. E., Nahorniak, C. S., Hapgood, J. P., Peter, R. E. and Millar, R. P. (1999). "Two gonadotropin-releasing hormone receptor subtypes with distinct ligand selectivity and differential distribution in brain and pituitary in the goldfish (Carassius auratus)." Proc Natl Acad Sci U S A **96**(5): 2526-2531.

Ito, K., Haga, T., Lameh, J. and Sadee, W. (1999). "Sequestration of dopamine D2 receptors depends on coexpression of G- protein-coupled receptor kinases 2 or 5." Eur J Biochem **260**(1): 112-119.

Kakar, S. S., Musgrove, L. C., Devor, D. C., Sellers, J. C. and Neill, J. D. (1992). "Cloning, sequencing, and expression of human gonadotropin releasing hormone (GnRH) receptor." Biochem Biophys Res Commun **189**(1): 289-295.

Karnik, S. S., Sakmar, T. P., Chen, H. B. and Khorana, H. G. (1988). "Cysteine residues 110 and 187 are essential for the formation of correct structure in bovine rhodopsin." Proc Natl Acad Sci U S A **85**(22): 8459-8463.

Kirchhausen, T. (2000). "Clathrin." Annu Rev Biochem **69**: 699-727.

Kirchhausen, T., Bonifacino, J. S. and Riezman, H. (1997). "Linking cargo to vesicle formation: receptor tail interactions with coat proteins." Curr Opin Cell Biol **9**(4): 488-495.

Kishi, H., Krishnamurthy, H., Galet, C., Bhaskaran, R. S. and Ascoli, M. (2002). "Identification of a short linear sequence present in the C-terminal tail of the rat follitropin receptor that modulates arrestin-3 binding in a phosphorylation-independent fashion." J Biol Chem **4**: 4.

Knox, C. J., Boyd, S. K. and Sower, S. A. (1994). "Characterization and localization of gonadotropin-releasing hormone receptors in the adult female sea lamprey, Petromyzon marinus." Endocrinology **134**(1): 492-498.

Kozasa, T. (2001). "Regulation of G protein-mediated signal transduction by RGS proteins." Life Sci **68**(19-20): 2309-2317.

Kraus, S., Naor, Z. and Seger, R. (2001). "Intracellular signaling pathways mediated by the gonadotropin-releasing hormone (GnRH) receptor." Arch Med Res **32**(6): 499-509.

Krupnick, J. G., Goodman, O. B., Jr., Keen, J. H. and Benovic, J. L. (1997). "Arrestin/clathrin interaction. Localization of the clathrin binding domain of nonvisual arrestins to the carboxy terminus." J Biol Chem **272**(23): 15011-15016.

Krupnick, J. G., Santini, F., Gagnon, A. W., Keen, J. H. and Benovic, J. L. (1997). "Modulation of the arrestin-clathrin interaction in cells. Characterization of beta-arrestin dominant-negative mutants." J Biol Chem **272**(51): 32507-32512.

Lamb, M. E., De Weerd, W. F. and Leeb-Lundberg, L. M. (2001). "Agonist-promoted trafficking of human bradykinin receptors: arrestin- and dynamin-independent sequestration of the B2 receptor and bradykinin in HEK293 cells." Biochem J **355**(Pt 3): 741-750.

Lameh, J., Philip, M., Sharma, Y. K., Moro, O., Ramachandran, J. and Sadee, W. (1992). "Hm1 muscarinic cholinergic receptor internalization requires a domain in the third cytoplasmic loop." J Biol Chem **267**(19): 13406-13412.

Lamey, M., Thompson, M., Varghese, G., Chi, H., Sawzdargo, M., George, S. R. and O'Dowd, B. F. (2002). "Distinct residues in the carboxyl tail mediate agonist-induced desensitization and internalization of the human dopamine D1 receptor." J Biol Chem **277**(11): 9415-9421.

Laporte, S. A., Oakley, R. H., Holt, J. A., Barak, L. S. and Caron, M. G. (2000). "The interaction of beta-arrestin with the AP-2 adaptor is required for the clustering of beta 2-adrenergic receptor into clathrin-coated pits." J Biol Chem **275**(30): 23120-23126.

Laporte, S. A., Oakley, R. H., Zhang, J., Holt, J. A., Ferguson, S. S., Caron, M. G. and Barak, L. S. (1999). "The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis." Proc Natl Acad Sci U S A **96**(7): 3712-3717.

Levi, N. L., Hanoch, T., Benard, O., Rozenblat, M., Harris, D., Reiss, N., Naor, Z. and Seger, R. (1998). "Stimulation of Jun N-terminal kinase (JNK) by gonadotropin-releasing hormone in pituitary alpha T3-1 cell line is mediated by protein kinase C, c-Src, and CDC42." Mol Endocrinol **12**(6): 815-824.

Li, S., Okamoto, T., Chun, M., Sargiacomo, M., Casanova, J. E., Hansen, S. H., Nishimoto, I. and Lisanti, M. P. (1995). "Evidence for a regulated interaction between heterotrimeric G proteins and caveolin." J Biol Chem **270**(26): 15693-15701.

Lin, H. C., Duncan, J. A., Kozasa, T. and Gilman, A. G. (1998). "Sequestration of the G protein beta gamma subunit complex inhibits receptor-mediated endocytosis." Proc Natl Acad Sci U S A **95**(9): 5057-5060.

Liu, P., Ying, Y., Ko, Y. G. and Anderson, R. G. (1996). "Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae." J Biol Chem **271**(17): 10299-10303.

Luttrell, L. M., Daaka, Y., Della Rocca, G. J. and Lefkowitz, R. J. (1997). "G protein-coupled receptors mediate two functionally distinct pathways of tyrosine phosphorylation in rat 1a fibroblasts. Shc phosphorylation and receptor endocytosis correlate with activation of Erk kinases." J Biol Chem **272**(50): 31648-31656.

Luttrell, L. M., Ferguson, S. S., Daaka, Y., Miller, W. E., Maudsley, S., Della Rocca, G. J., Lin, F., Kawakatsu, H., Owada, K., Luttrell, D. K., Caron, M. G. and Lefkowitz, R. J. (1999). "Beta-arrestin-dependent formation of beta2 adrenergic receptor-Src protein kinase complexes." Science **283**(5402): 655-661.

Lutz, W. and Kumar, R. (1993). "Hypertonic sucrose treatment enhances second messenger accumulation in vasopressin-sensitive cells." Am J Physiol **264**(2 Pt 2): F228-233.

Mahan, L. C., Koachman, A. M. and Insel, P. A. (1985). "Genetic analysis of beta-adrenergic receptor internalization and down- regulation." Proc Natl Acad Sci U S A **82**(1): 129-133.

Maney, D. L., Richardson, R. D. and Wingfield, J. C. (1997). "Central administration of chicken gonadotropin-releasing hormone-II enhances courtship behavior in a female sparrow." Horm Behav **32**(1): 11-18.

Marchese, A. and Benovic, J. L. (2001). "Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting." J Biol Chem **276**(49): 45509-45512.

Marinissen, M. J. and Gutkind, J. S. (2001). "G-protein-coupled receptors and signaling networks: emerging paradigms." Trends Pharmacol Sci **22**(7): 368-376.

McArdle, C. A., Franklin, J., Green, L. and Hislop, J. N. (2002). "Signalling, cycling and desensitisation of gonadotrophin-releasing hormone receptors." J Endocrinol **173**(1): 1-11.

McDonald, P. H., Chow, C. W., Miller, W. E., Laporte, S. A., Field, M. E., Lin, F. T., Davis, R. J. and Lefkowitz, R. J. (2000). "Beta-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3." Science **290**(5496): 1574-1577.

McDonald, P. H., Cote, N. L., Lin, F. T., Premont, R. T., Pitcher, J. A. and Lefkowitz, R. J. (1999). "Identification of NSF as a beta-arrestin1-binding protein. Implications for beta2-adrenergic receptor regulation." J Biol Chem **274**(16): 10677-10680.

McDowell, J. H., Nawrocki, J. P. and Hargrave, P. A. (1993). "Phosphorylation sites in bovine rhodopsin." Biochemistry **32**(18): 4968-4974.

Menard, L., Ferguson, S. S., Zhang, J., Lin, F. T., Lefkowitz, R. J., Caron, M. G. and Barak, L. S. (1997). "Synergistic regulation of beta2-adrenergic receptor sequestration: intracellular complement of beta-adrenergic receptor kinase and beta- arrestin determine kinetics of internalization." Mol Pharmacol **51**(5): 800-808.

Millar, R. P., Davidson, J., Flanagan, C.A. and Wakefield, I. (1995) "Ligand binding and second messenger assays for cloned Gq/G11-coupled neuropeptide receptors: the GnRH receptor", in Methods in Neurosciences, Receptor Molecular Biology (Ed Sealfon S.C.) pp 145-162, Academic Press, San Diego

Millar, R., Lowe, S., Conklin, D., Pawson, A., Maudsley, S., Troskie, B., Ott, T., Millar, M., Lincoln, G., Sellar, R., Faurholm, B., Scobie, G., Kuestner, R., Terasawa, E. and Katz, A. (2001). "A novel mammalian receptor for the evolutionarily conserved type II GnRH." Proc Natl Acad Sci U S A **98**(17): 9636-9641.

Millar, R. P. (2002). "GnRH II and type II GnRH receptors." Trends Endocrinol Metab **14**(1): 35-43.

Miller, W. E., Maudsley, S., Ahn, S., Khan, K. D., Luttrell, L. M. and Lefkowitz, R. J. (2000). "beta-arrestin1 interacts with the catalytic domain of the tyrosine kinase c-SRC. Role of beta-arrestin1-dependent targeting of c-SRC in receptor endocytosis." J Biol Chem **275**(15): 11312-11319.

Min, L., Galet, C. and Ascoli, M. (2002). "The association of arrestin-3 with the human lutropin/choriogonadotropin receptor depends mostly on receptor activation rather than on receptor phosphorylation." J Biol Chem **277**(1): 702-710.

Morgan, K., Conklin, D., Pawson, A. J., Sellar, R., Ott, T. R. and Millar, R. P. (2003). "A transcriptionally active human type II gonadotropin-releasing hormone receptor gene homolog overlaps two genes in the antisense orientation on chromosome 1q.12." Endocrinology **144**(2): 423-436.

Mukherjee, S., Gurevich, V. V., Preninger, A., Hamm, H. E., Bader, M. F., Fazleabas, A. T., Birnbaumer, L. and Hunzicker-Dunn, M. (2002). "Aspartic acid 564 in the third cytoplasmic loop of the luteinizing hormone/choriogonadotropin receptor is crucial for phosphorylation- independent interaction with arrestin2." J Biol Chem **277**(20): 17916-17927.

Myburgh, D. B., Millar, R. P. and Hapgood, J. P. (1998). "Alanine-261 in intracellular loop III of the human gonadotropin- releasing hormone receptor is crucial for G-protein coupling and receptor internalization." Biochem J **331**(Pt 3): 893-896.

Nakata, H., Kameyama, K., Haga, K. and Haga, T. (1994). "Location of agonist-dependent-phosphorylation sites in the third intracellular loop of muscarinic acetylcholine receptors (m2 subtype)." Eur J Biochem **220**(1): 29-36.

Naor, Z., Benard, O. and Seger, R. (2000). "Activation of MAPK cascades by G-protein-coupled receptors: the case of gonadotropin-releasing hormone receptor." Trends Endocrinol Metab **11**(3): 91-99. _00000232 _00000232.

Neill, J. D., Duck, L. W., Sellers, J. C. and Musgrove, L. C. (2001). "A gonadotropin-releasing hormone (GnRH) receptor specific for GnRH II in primates." Biochem Biophys Res Commun **282**(4): 1012-1018.

Oakley, R. H., Laporte, S. A., Holt, J. A., Barak, L. S. and Caron, M. G. (1999). "Association of beta-arrestin with G protein-coupled receptors during clathrin-mediated endocytosis dictates the profile of receptor resensitization." J Biol Chem **274**(45): 32248-32257.

Oakley, R. H., Laporte, S. A., Holt, J. A., Caron, M. G. and Barak, L. S. (2000). "Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors." J Biol Chem **275**(22): 17201-17210.

O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J. and Bouvier, M. (1989). "Palmitoylation of the human beta 2-adrenergic receptor. Mutation of Cys341 in the carboxyl tail leads to an uncoupled nonpalmitoylated form of the receptor." J Biol Chem **264**(13): 7564-7569.

Okamoto, P. M., Herskovits, J. S. and Vallee, R. B. (1997). "Role of the basic, proline-rich region of dynamin in Src homology 3 domain binding and endocytosis." J Biol Chem **272**(17): 11629-11635.

Okamoto, Y., Ninomiya, H., Miwa, S. and Masaki, T. (2000). "Cholesterol oxidation switches the internalization pathway of endothelin receptor type A from caveolae to clathrin-coated pits in Chinese hamster ovary cells." J Biol Chem **275**(9): 6439-6446.

Oppermann, M., Freedman, N. J., Alexander, R. W. and Lefkowitz, R. J. (1996). "Phosphorylation of the type 1A angiotensin II receptor by G protein- coupled receptor kinases and protein kinase C." J Biol Chem **271**(22): 13266-13272.

Paing, M. M., Stutts, A. B., Kohout, T. A., Lefkowitz, R. J. and Trejo, J. (2002). "beta -Arrestins regulate protease-activated receptor-1 desensitization but not internalization or Down-regulation." J Biol Chem **277**(2): 1292-1300.

Palczewski, K., Kumasaka, T., Hori, T., Behnke, C. A., Motoshima, H., Fox, B. A., Le Trong, I., Teller, D. C., Okada, T., Stenkamp, R. E., Yamamoto, M. and Miyano, M. (2000). "Crystal structure of rhodopsin: A G protein-coupled receptor." Science **289**(5480): 739-745.

Parent, J. L., Labrecque, P., Driss Rochdi, M. and Benovic, J. L. (2001). "Role of the differentially spliced carboxyl terminus in thromboxane A2 receptor trafficking: identification of a distinct motif for tonic internalization." J Biol Chem **276**(10): 7079-7085.

Pawson, A. J., Katz, A., Sun, Y. M., Lopes, J., Illing, N., Millar, R. P. and Davidson, J. S. (1998). "Contrasting internalization kinetics of human and chicken gonadotropin- releasing hormone receptors mediated by C-terminal tail." J Endocrinol **156**(3): R9-12.

Pfister, C., Chabre, M., Plouet, J., Tuyen, V. V., De Kozak, Y., Faure, J. P. and Kuhn, H. (1985). "Retinal S antigen identified as the 48K protein regulating light- dependent phosphodiesterase in rods." Science **228**(4701): 891-893.

Pierce, K. L., Luttrell, L. M. and Lefkowitz, R. J. (2001). "New mechanisms in heptahelical receptor signaling to mitogen activated protein kinase cascades." Oncogene **20**(13): 1532-1539.

Pippig, S., Andexinger, S. and Lohse, M. J. (1995). "Sequestration and recycling of beta 2-adrenergic receptors permit receptor resensitization." Mol Pharmacol **47**(4): 666-676.

Pitcher, J. A., Freedman, N. J. and Lefkowitz, R. J. (1998). "G protein-coupled receptor kinases." Annu Rev Biochem **67**: 653-692.

Pitcher, J. A., Payne, E. S., Csortos, C., DePaoli-Roach, A. A. and Lefkowitz, R. J. (1995). "The G-protein-coupled receptor phosphatase: a protein phosphatase type 2A with a distinct subcellular distribution and substrate specificity." Proc Natl Acad Sci U S A **92**(18): 8343-8347.

Premont, R. T., Claing, A., Vitale, N., Freeman, J. L., Pitcher, J. A., Patton, W. A., Moss, J., Vaughan, M. and Lefkowitz, R. J. (1998). "beta2-Adrenergic receptor regulation by GIT1, a G protein-coupled receptor kinase-associated ADP ribosylation factor GTPase-activating protein." Proc Natl Acad Sci U S A **95**(24): 14082-14087.

Prenzel, N., Zwick, E., Daub, H., Leserer, M., Abraham, R., Wallasch, C. and Ullrich, A. (1999). "EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF." Nature **402**(6764): 884-888.

Probst, W. C., Snyder, L. A., Schuster, D. I., Brosius, J. and Sealfon, S. C. (1992). "Sequence alignment of the G-protein coupled receptor superfamily." DNA Cell Biol **11**(1): 1-20.

Qanbar, R. and Bouvier, M. (2003). "Role of palmitoylation/depalmitoylation reactions in G-protein-coupled receptor function." Pharmacol Ther **97**(1): 1-33.

Ramjaun, A. R. and McPherson, P. S. (1998). "Multiple amphiphysin II splice variants display differential clathrin binding: identification of two distinct clathrin-binding sites." J Neurochem **70**(6): 2369-2376.

Rands, E., Candelore, M. R., Cheung, A. H., Hill, W. S., Strader, C. D. and Dixon, R. A. (1990). "Mutational analysis of beta-adrenergic receptor glycosylation." J Biol Chem **265**(18): 10759-10764.

Reinhart, J., Mertz, L. M. and Catt, K. J. (1992). "Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor." J Biol Chem **267**(30): 21281-21284.

Reiss, N., Llevi, L. N., Shacham, S., Harris, D., Seger, R. and Naor, Z. (1997). "Mechanism of mitogen-activated protein kinase activation by gonadotropin-releasing hormone in the pituitary of alphaT3-1 cell line: differential roles of calcium and protein kinase C." Endocrinology **138**(4): 1673-1682.

Rissman, E. F., Li, X., King, J. A. and Millar, R. P. (1997). "Behavioral regulation of gonadotropin-releasing hormone production." Brain Res Bull **44**(4): 459-464.

Roberson, M. S., Zhang, T., Li, H. L. and Mulvaney, J. M. (1999). "Activation of the p38 mitogen-activated protein kinase pathway by gonadotropin-releasing hormone." Endocrinology **140**(3): 1310-1318.

Robison, R. R., White, R. B., Illing, N., Troskie, B. E., Morley, M., Millar, R. P. and Fernald, R. D. (2001). "Gonadotropin-releasing hormone receptor in the teleost Haplochromis burtoni: structure, location, and function." Endocrinology **142**(5): 1737-1743.

Roseberry, A. G. and Hosey, M. M. (2001). "Internalization of the M2 muscarinic acetylcholine receptor proceeds through an atypical pathway in HEK293 cells that is independent of clathrin and caveolae." J Cell Sci **114**(Pt 4): 739-746.

Sallese, M., Mariggio, S., D'Urbano, E., Iacovelli, L. and De Blasi, A. (2000). "Selective regulation of Gq signaling by G protein-coupled receptor kinase 2: direct interaction of kinase N terminus with activated galphaq." Mol Pharmacol **57**(4): 826-831.

Schmid, S. L., McNiven, M. A. and De Camilli, P. (1998). "Dynamin and its partners: a progress report." Curr Opin Cell Biol **10**(4): 504-512.

Schnitzer, J. E., Oh, P., Pinney, E. and Allard, J. (1994). "Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules." J Cell Biol **127**(5): 1217-1232.

Sealfon, S. C. and Millar, R. P. (1995). "The gonadotrophin-releasing hormone receptor: structural determinants and regulatory control." Hum Reprod Update **1**(3): 216-230.

Sealfon, S. C., Weinstein, H. and Millar, R. P. (1997). "Molecular mechanisms of ligand interaction with the gonadotropin- releasing hormone receptor." Endocr Rev **18**(2): 180-205.

Seibold, A., Williams, B., Huang, Z. F., Friedman, J., Moore, R. H., Knoll, B. J. and Clark, R. B. (2000). "Localization of the sites mediating desensitization of the beta(2)- adrenergic receptor by the GRK pathway." Mol Pharmacol **58**(5): 1162-1173.

Shah, B. H., Alberto Olivares-Reyes, J., Yesilkaya, A. and Catt, K. J. (2002). "Independence of angiotensin II-induced MAP kinase activation from angiotensin type 1 receptor internalization in clone 9 hepatocytes." Mol Endocrinol **16**(3): 610-620.

Shapiro, M. J. and Coughlin, S. R. (1998). "Separate signals for agonist-independent and agonist-triggered trafficking of protease-activated receptor 1." J Biol Chem **273**(44): 29009-29014.

Shapiro, M. J., Trejo, J., Zeng, D. and Coughlin, S. R. (1996). "Role of the thrombin receptor's cytoplasmic tail in intracellular trafficking. Distinct determinants for agonist-triggered versus tonic internalization and intracellular localization." J Biol Chem **271**(51): 32874-32880.

Shenoy, S. K., McDonald, P. H., Kohout, T. A. and Lefkowitz, R. J. (2001). "Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin." Science **294**(5545): 1307-1313.

Sherwood, N. M., Lovejoy, D. A. and Coe, I. R. (1993). "Origin of mammalian gonadotropin-releasing hormones." Endocr Rev **14**(2): 241-254.

Shetzline, M. A., Walker, J. K., Valenzano, K. J. and Premont, R. T. (2002). "Vasoactive intestinal polypeptide type-1 receptor (VPAC1R) regulation: Desensitization, phosphorylation and sequestration." J Biol Chem **26**: 26.

Shiina, T., Arai, K., Tanabe, S., Yoshida, N., Haga, T., Nagao, T. and Kurose, H. (2001). "Clathrin box in G protein-coupled receptor kinase 2." J Biol Chem **276**(35): 33019-33026.

Sibley, D. R., Strasser, R. H., Benovic, J. L., Daniel, K. and Lefkowitz, R. J. (1986). "Phosphorylation/dephosphorylation of the beta-adrenergic receptor regulates its functional coupling to adenylate cyclase and subcellular distribution." Proc Natl Acad Sci U S A **83**(24): 9408-9412.

Slimp, J. C., Hart, B. L. and Goy, R. W. (1978). "Heterosexual, autosexual and social behavior of adult male rhesus monkeys with medial preoptic-anterior hypothalamic lesions." Brain Res **142**(1): 105-122.

Smyth, E. M., Austin, S. C., Reilly, M. P. and FitzGerald, G. A. (2000). "Internalization and sequestration of the human prostacyclin receptor." J Biol Chem **275**(41): 32037-32045.

Stoffel, R. H., 3rd, Pitcher, J. A. and Lefkowitz, R. J. (1997). "Targeting G protein-coupled receptor kinases to their receptor substrates." J Membr Biol **157**(1): 1-8.

Stojilkovic, S. S., Reinhart, J. and Catt, K. J. (1994). "Gonadotropin-releasing hormone receptors: structure and signal transduction pathways." Endocr Rev **15**(4): 462-499.

Stowell, M. H., Marks, B., Wigge, P. and McMahon, H. T. (1999). "Nucleotide-dependent conformational changes in dynamin: evidence for a mechanochemical molecular spring." Nat Cell Biol **1**(1): 27-32.

Strader, C. D., Fong, T. M., Graziano, M. P. and Tota, M. R. (1995). "The family of G-protein-coupled receptors." Faseb J **9**(9): 745-754.

Sun, Y. M., Flanagan, C. A., Illing, N., Ott, T. R., Sellar, R., Fromme, B. J., Hapgood, J., Sharp, P., Sealfon, S. C. and Millar, R. P. (2001). "A chicken gonadotropin-releasing hormone receptor that confers agonist activity to mammalian antagonists. Identification of D-Lys(6) in the ligand and extracellular loop two of the receptor as determinants." J Biol Chem **276**(11): 7754-7761.

Teixeira, A., Chaverot, N., Schroder, C., Strosberg, A. D., Couraud, P. O. and Cazaubon, S. (1999). "Requirement of caveolae microdomains in extracellular signal-regulated kinase and focal adhesion kinase activation induced by endothelin-1 in primary astrocytes." J Neurochem **72**(1): 120-128.

Thomas, W. G., Motel, T. J., Kule, C. E., Karoor, V. and Baker, K. M. (1998). "Phosphorylation of the angiotensin II (AT1A) receptor carboxyl terminus: a role in receptor endocytosis." Mol Endocrinol **12**(10): 1513-1524.

Thompson, H. M. and McNiven, M. A. (2001). "Dynamin: switch or pinchase?" Curr Biol **11**(21): R850.

Trejo, J. and Coughlin, S. R. (1999). "The cytoplasmic tails of protease-activated receptor-1 and substance P receptor specify sorting to lysosomes versus recycling." J Biol Chem **274**(4): 2216-2224.

Troskie, B., King, J. A., Millar, R. P., Peng, Y. Y., Kim, J., Figueras, H. and Illing, N. (1997). "Chicken GnRH II-like peptides and a GnRH receptor selective for chicken GnRH II in amphibian sympathetic ganglia." Neuroendocrinology **65**(6): 396-402.

Troskie, B. E., Hapgood, J. P., Millar, R. P. and Illing, N. (2000). "Complementary deoxyribonucleic acid cloning, gene expression, and ligand selectivity of a novel gonadotropin-releasing hormone receptor expressed in the pituitary and midbrain of Xenopus laevis." Endocrinology **141**(5): 1764-1771.

Tsuga, H., Kameyama, K., Haga, T., Honma, T., Lameh, J. and Sadee, W. (1998). "Internalization and down-regulation of human muscarinic acetylcholine receptor m2 subtypes. Role of third intracellular m2 loop and G protein- coupled receptor kinase 2." J Biol Chem **273**(9): 5323-5330.

Tsuga, H., Kameyama, K., Haga, T., Kurose, H. and Nagao, T. (1994). "Sequestration of muscarinic acetylcholine receptor m2 subtypes. Facilitation by G protein-coupled receptor kinase (GRK2) and attenuation by a dominant-negative mutant of GRK2." J Biol Chem **269**(51): 32522-32527.

Tsutsumi, M., Zhou, W., Millar, R. P., Mellon, P. L., Roberts, J. L., Flanagan, C. A., Dong, K., Gillo, B. and Sealfon, S. C. (1992). "Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor." Mol Endocrinol **6**(7): 1163-1169.

Vrecl, M., Anderson, L., Hanyaloglu, A., McGregor, A. M., Groarke, A. D., Milligan, G., Taylor, P. L. and Eidne, K. A. (1998). "Agonist-induced endocytosis and recycling of the gonadotropin-releasing hormone receptor: effect of beta-arrestin on internalization kinetics." Mol Endocrinol **12**(12): 1818-1829.

Vrecl, M., Heding, A., Hanyaloglu, A., Taylor, P. L. and Eidne, K. A. (2000). "Internalization kinetics of the gonadotropin-releasing hormone (GnRH) receptor." Pflugers Arch **439**(3): R19-20.

Walker, J. K., Premont, R. T., Barak, L. S., Caron, M. G. and Shetzline, M. A. (1999). "Properties of secretin receptor internalization differ from those of the beta(2)-adrenergic receptor." J Biol Chem **274**(44): 31515-31523.

Wang, L., Bogerd, J., Choi, H. S., Seong, J. Y., Soh, J. M., Chun, S. Y., Blomenrohr, M., Troskie, B. E., Millar, R. P., Yu, W. H., McCann, S. M. and Kwon, H. B. (2001). "Three distinct types of GnRH receptor characterized in the bullfrog." Proc Natl Acad Sci U S A **98**(1): 361-366.

Wang, L. H., Sudhof, T. C. and Anderson, R. G. (1995). "The appendage domain of alpha-adaptin is a high affinity binding site for dynamin." J Biol Chem **270**(17): 10079-10083.

Wess, J. (1998). "Molecular basis of receptor/G-protein-coupling selectivity." Pharmacol Ther **80**(3): 231-264.

White, R. B., Eisen, J. A., Kasten, T. L. and Fernald, R. D. (1998). "Second gene for gonadotropin-releasing hormone in humans." Proc Natl Acad Sci U S A **95**(1): 305-309.

Willars, G. B., Heding, A., Vrecl, M., Sellar, R., Blomenrohr, M., Nahorski, S. R. and Eidne, K. A. (1999). "Lack of a C-terminal tail in the mammalian gonadotropin-releasing hormone receptor confers resistance to agonist-dependent phosphorylation and rapid desensitization." J Biol Chem **274**(42): 30146-30153.

Wolf, R., Koch, T., Schulz, S., Klutzny, M., Schroder, H., Raulf, E., Buhling, F. and Hollt, V. (1999). "Replacement of threonine 394 by alanine facilitates internalization and resensitization of the rat mu opioid receptor." Mol Pharmacol **55**(2): 263-268.

Wu, C., Butz, S., Ying, Y. and Anderson, R. G. (1997). "Tyrosine kinase receptors concentrated in caveolae-like domains from neuronal plasma membrane." J Biol Chem **272**(6): 3554-3559.

Yamao, F. (1999). "Ubiquitin system: selectivity and timing of protein destruction." J Biochem (Tokyo) **125**(2): 223-229.

Yu, S. S., Lefkowitz, R. J. and Hausdorff, W. P. (1993). "Beta-adrenergic receptor sequestration. A potential mechanism of receptor resensitization." J Biol Chem **268**(1): 337-341.

Zhang, J., Ferguson, S. S., Barak, L. S., Menard, L. and Caron, M. G. (1996). "Dynamin and beta-arrestin reveal distinct mechanisms for G protein- coupled receptor internalization." J Biol Chem **271**(31): 18302-18305.

Zhou, W., Flanagan, C., Ballesteros, J. A., Konvicka, K., Davidson, J. S., Weinstein, H., Millar, R. P. and Sealfon, S. C. (1994). "A reciprocal mutation supports helix 2 and helix 7 proximity in the gonadotropin-releasing hormone receptor." Mol Pharmacol **45**(2): 165-170.

Zhu, S. J., Hatcher, L. I., Brown, J. C., 3rd, Whittle, S. M. and Toews, M. L. (1996). "Effects of hypertonic sucrose and potassium depletion on the binding properties of beta and alpha 1 adrenergic receptors measured on intact cells." Recept Signal Transduct **6**(3-4): 131-140.

Zhu, Y., Traub, L. M. and Kornfeld, S. (1999). "High-affinity binding of the AP-1 adaptor complex to trans-golgi network membranes devoid of mannose 6-phosphate receptors." Mol Biol Cell **10**(3): 537-549.

8. Publications and Presentations

Publication " Serine Residues in the C-terminal Tail and Intracellular Loop Three of the Type II GnRH Receptor are Critical for β-arrestin Independent Internalisation", Katharina Ronacher, Colleen A. Flanagan, Robert P. Millar and Arieh A. Katz, submitted to the Journal of Molecular Endocrinology, March 2003

First prize for best research presentation at the AstraZeneca Research Day at the Medical Research Council, Cape Town, South Africa

Poster presentation "Identification of Serine Residues within the C-terminal Tail of the Type II GnRH Receptor of Marmoset Monkey that are Critical for Rapid Internalisation", Ronacher K., Flanagan C.A., Millar R.P. and Katz A IUBMB, Cape Town, South Africa, November 2001

Oral presentation "Internalisation of the Type II GnRH Receptor of Marmoset Monkey", Ronacher K., Flanagan C.A., Millar R.P. and Katz A 5th International Congress in Neuroendocrinology, Bristol, UK, September 2002

Oral presentation "Identification of Serine Residues in ICL3 and the C-terminal Tail of the Type II GnRH Receptor that are Critical for Rapid Internalization" Ronacher K., Flanagan C.A., Millar R.P. and Katz A.
IUBMB Receptor-Ligand Interaction: Molecular, Physiological and Pharmacological Aspects, Bergen, Norway

Oral presentation "The Type II Gonadotropin-Releasing Hormone Receptor" Katz A., Faurholm B., Ronacher K., Flanagan,C.A. and Millar R.P., International Symposium on Signal Transduction in Health and Disease, Tel Aviv, Israel

9. Appendix

9.1 Primer Sequences

Vector specific primers for pcDNA 3.1+
T7 (sense) 5' TAA TAC GAC TCA CTA TAG GG 3'

BGH (antisense) 5' TAG AAG GCA CAG TCG AGG 3'

Gene specific primers for the type II GnRH receptor of marmoset monkey
The following antisense primers were used to create truncated receptor mutant in conjunction with the T7 sense primer. The nucleotides in boxes indicate the Xbal restriction site, that was used for cloning into pcDNA 3.1+.

Xbal
T372stop 5' CCG TCT AGA TCA TTC TCC TGC CTT TCT TGA TGT C 3'
S366stop 5' CCG TCT AGA TCA TGT CAC AGT TTT TTG TAC CTC CG 3'
Q357stop 5' CCG TCT AGA TCA TCT AAG GGC CTG AAT GTC CTG 3'
S344stop 5' CCG TCT AGA TCA CCC TTC TTC CCT AGA AGA GTC 3'
S335stop 5' CCG TCT AGA TCA AAG TTC TTG GTG CCC TC 3'
G326stop 5' CCG TCT AGA TCA AAG GGT GAA GGC CCC 3'

The following sense (s) and antisense (as) primers were used to create receptor mutants lacking putative phosphorylation sites. The nucleotides in open boxes indicate silent restriction sites to identify the mutants. The nucleotides in grey boxes indicate the serine or threonine to alanine mutations. These primers were used for bridge PCR in conjunction with the vecto specific T7 and BGH primers.

S338,339A_s Clal
5' GGG CAC CAA GAA CTA TCG ATG GAC GCT GCT AGG GAA GAA GGG 3'

S338,339A_as ClaI

5’ CCC TTC TTC CCT AGC AGC GTC CAT CGA TAG TTC TTG GTG CCC

S231,232A_s SacII

5’ GTG CTC GGT GTG GCC GCC CCG CGG ACA AGG 3’

S231,232_as SacII

5’ CCT TGT CCG CGG GGC GGC CAC ACC GAG CAC 3’

S251A_s StuI

5’ CTC CGT CGC GCC TTC GAC AAT AGG CCT CGT GTC CG 3’

S251A_as StuI

5’ CGG ACA CGA GGC CTA TTG TCG AAG GCG CGA CGG AG 3’

T235A,S239A_s ScII

5’ CCA GCC CTC GAG CAA GGA AGG GGG CCC ATG CCC C 3’

T235A,S239A_as ScII

5’ GGG GCA TGG GCC CCC TTC CTT GCT CGA GGG CTG G 3’