

THE ROLE OF THE GLU^{2.53(90)} RESIDUE IN GONADOTROPIN-RELEASING HORMONE RECEPTOR EXPRESSION AND FUNCTION

Ashmeetha Manilall

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Declaration

I, Ashmeetha Manilall, declare that this dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.



(Signature of candidate)

15th day of MAY 20 17 in JHB

Abstract

Gonadotropin-releasing hormone (GnRH) binds to GnRH receptors (GnRHR) in the pituitary and stimulates release of gonadotropins, which control reproduction. It has been proposed that the congenital Glu^{2.53(90)}Lys GnRHR mutation causes infertility by disrupting a salt-bridge important for GnRHR protein expression. To investigate its role in GnRHR function, Glu^{2.53(90)} was mutated to residues that mimic or remove its side-chain properties. Mutant receptors were assessed for inositol phosphate signaling and radioligand binding. Receptors with small or negatively-charged substitutions for Glu^{2.53(90)} exhibited no measurable function. Stabilizing receptor expression by appending a carboxy-terminal tail recovered function of the Glu^{2.53(90)}Lys and Glu^{2.53(90)}Ala GnRHRs, but not the conservative Glu^{2.53(90)}Asp mutant. Receptors with uncharged (Gln) or hydrophobic (Leu, Phe) substitutions that cannot form salt-bridges with Lys^{3.32(121)} were fully functional. Although the positively-charged Arg substitution decreased binding affinity, it preserved GnRHR function, confirming that interaction with the positively-charged Lys^{3.32(121)} is not required. Comparing the GnRHR with structurally-related G protein-coupled receptors revealed that the equivalent residue of rhodopsin, Met^{2.53(86)}, interacts with Trp^{6.48(265)}. Mutating Trp^{6.48(280)} of the GnRHR to Ala and Arg disrupted GnRH-stimulated function, confirming a role in expression. The Trp^{6.48(280)}Arg GnRHR with an appended carboxy-terminal tail had decreased GnRH binding affinity. The preserved function of mutant receptors with large hydrophobic or positively-charged amino acid substitutions suggests that the size of the Glu^{2.53(90)} is important for stabilizing GnRHR structure. Decreased affinity of mutant receptors with larger (Arg) substitutions for Glu^{2.53(90)} and Trp^{6.48(280)} suggest that both residues make conserved intramolecular interactions that stabilize receptor protein expression and configure the extracellular GnRHR structure.

(250 words)

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Presentations arising from this research project

Poster presentation WITS Research Day, September 2014

The role of the Glu^{2.53(90)} residue in gonadotropin-releasing hormone receptor expression and function

Poster presentation WITS Research Day, September 2016

Gonadotropin-releasing hormone receptors with mutations in transmembrane helices 2 or 6 have similar phenotypes, supporting disruption of a conserved water-mediated hydrogen bond network

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List of abbreviations and symbols

μ	Micro
Å	Angstrom
Ala, A	Alanine
Arg, R	Arginine
Asn, N	Asparagine
Asp, D	Aspartic acid
B0	Total binding
BSA	Bovine serum albumin
C	Carbon
CaCl ₂	Calcium chloride
CONH ₂	Amide
COOH	Carboxy-/Carboxylic
CT	Carboxy-terminal tail
Cys, C	Cysteine
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagles Medium
DOR	Delta-opioid receptor
EC ₅₀	Effective concentration for 50% of maximal response
ECL	Extracellular loop
EDTA	Ethylenediaminetetraacetic acid
E _{max}	Maximal response
ER	Endoplasmic reticulum
FBS	Foetal bovine serum
FGFR1	Fibroblast growth factor receptor 1 gene
FSH	Follicle-stimulating hormone

G protein	Guanosine triphosphate-binding protein
GDP	Guanosine diphosphate
Gln, Q	Glutamine
Glu, E	Glutamic acid
Gly, G	Glycine
GnRH	Gonadotropin-releasing hormone
GnRHR	Gonadotropin-releasing hormone receptor
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
hCG	Human chorionic gonadotropin
HCl	Hydrochloric acid
HH	Hypogonadotropic hypogonadism
His, H	Histamine
IC ₅₀	Inhibitory concentration for 50% of maximal response
ICL	Intracellular loop
Ile, I	Isoleucine
IP ₃	Inositol-1,4,5-triphosphate
KAL-1	Kallmann syndrome 1 gene
KCl	Potassium chloride
KOR	Kappa-opioid receptor
KS	Kallmann's syndrome
Leu, L	Leucine
LH	Luteinizing hormone
LiCl	Lithium chloride
Lys, K	Lysine
MAPK	Mitogen activated protein kinase

MD	Molecular dynamics
Met	Methionine
MgCl ₂	Magnesium chloride
mM	Millimolar
NaCl	Sodium chloride
NaH ₂ PO ₄	Sodium hydrogen phosphate
NH ₂	Amidogen
NH ₄ HCO ₃	Ammonium hydrogen carbonate
NMR	Nuclear magnetic resonance
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
Pen	Penicillin
Phe, F	Phenylalanine
PIP2	Phosphatidylinositol biphosphate
PKC	Protein kinase C
PLC β	Phospholipase C β
Pro, P	Proline
QCS	Quality control system
R	Inactive receptor conformation
R*	Active receptor conformation
SCAM	Substituted cysteine accessibility method
SDS	Sodium dodecyl sulphate
Ser, S	Serine
Strep	Streptomycin
TM	Transmembrane
Trp, W	Tryptophan
Tyr, Y	Tyrosine
Val, V	Valine
α	Alpha
β	Beta

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1. Chapter 1: Introduction and literature review

1.1. Development of human reproductive function

Human reproductive function depends on coordinated communication amongst the hypothalamus, pituitary and gonads (ovaries in females and testes in males). The hypothalamus synthesizes and releases gonadotropin-releasing hormone (GnRH), which is transported in hypothalamic-pituitary portal vessels to the anterior pituitary gland where it binds to GnRH receptors (GnRHR) on gonadotrope cells. This GnRH-GnRHR interaction stimulates the production of second messenger, inositol-1,4,5-triphosphate (IP3), leading to synthesis and release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which act on the gonads to initiate gametogenesis. Primary sexual characteristics are established during gestation according to the genotype of an individual. A 46XX genotype results in a female phenotype, while a 46XY genotype results in a male phenotype. The 'Y' chromosome carries essential genes for male anatomical development, which are absent in females. Male and female gonadal function is regulated hormonally in a sex-specific manner throughout an individual's lifetime, commencing during gestation (Levy, Berne, Koeppen, 2005).

During embryonic development, GnRH neurons originate with olfactory neurons in the olfactory placode (6 weeks gestation). GnRH neurons then migrate to- and mature in the hypothalamus (González-Martínez *et al.*, 2004). Failure of this neuronal migration can arise from mutations of the Kallmann syndrome 1 (KAL-1) and fibroblast growth factor receptor 1 (FGFR1) genes, which encode proteins that direct migration of GnRH neurons to the hypothalamus and olfactory neurons to the nasal epithelia (González-Martínez *et al.*, 2004). Incorrect localization of the olfactory cells causes anosmia, while failure of the GnRH neurons to reach the hypothalamus causes infertility by disrupting GnRH secretion.

Individuals that possess both of these abnormal phenotypes are diagnosed with the Kallmann's syndrome form of hypogonadotropic hypogonadism (HH) (González-Martínez *et al.*, 2004).

GnRH production varies during the different stages of human life. Since peripheral GnRH concentrations are very low (too low to measure), circulating gonadotropin concentrations are considered to reflect GnRH production levels and GnRH concentrations in the pituitary. LH and FSH concentrations are low in infancy and suppressed during childhood, which are the non-reproductive years of human life. Gonadotropin concentrations increase at puberty and are maintained through the reproductive years (Levy, Berne & Koeppen, 2005).

Follicles of the female ovary and seminiferous tubules of the male testis are sites for the development of gametes. The gonads contain endocrine cells (granulosa and theca cells in ovaries and Sertoli and Leydig cells in testes) that secrete sex steroid hormones (androgens and oestrogens), which promote gametogenesis. The sex steroids also stimulate development of secondary sexual characteristics at puberty. Puberty is the start of the reproductive stage of life initiated by an increase in GnRH production. This GnRH increase is believed to be due to disinhibition of an upstream regulator of GnRH secretion. Upstream regulators of GnRH include neuropeptide ligands, such as kisspeptin and neurokinin B, which activate receptors on the GnRH-producing neurons (Silveira *et al.*, 2010; Ohkura *et al.*, 2009). Kisspeptin has been identified as a potent stimulator of gonadotropin secretion, especially LH, by regulating its stimulation of GnRH release. Neurokinin B has been identified as having an important role in the reproductive axis at the level of GnRH secretion (Silveira *et al.*, 2010; Navarro, 2013). The pubertal GnRH surge is responsible for the accompanying increase in gonadotropin levels. The gonadotropin increase promotes a testosterone increase in males causing the testes to enlarge and begin producing sperm for the first time. In females, GnRH stimulates cyclical release of LH and FSH, which results in active ovarian cycles that begin at puberty and

continue until menopause. Menopause occurs when the ovarian follicles are depleted and the ovaries no longer produce gonadal steroids. Lack of gonadal steroids causes reproductive function to cease in menopausal females. LH primarily stimulates production of androgens by theca and Leydig cells. FSH stimulates production of estrogens, mainly estradiol, by granulosa cells in females. In males, FSH acts on Sertoli cells in the seminiferous tubules to stimulate spermatogenesis. Gonadal steroids are responsible for the secondary sexual characteristics that develop at puberty. Distinguishing secondary sexual characteristics in males include deepening of the voice, broadening of shoulders and male pattern hair growth pattern on the face, chest and body. Female secondary sexual characteristics include breast development, widening of the hips and absence of hair growth on the face and chest (Levy, Berne, Koeppen, 2005).

An initial increase in oestrogen concentrations secreted by maturing ovarian follicles during the first 2 weeks of the female menstrual cycle causes thickening of the endometrium. Oestrogen from the follicles also causes an increase in GnRH and GnRH pulse frequency which stimulates a surge in LH concentrations. The LH surge causes a mature follicle to rupture and release an ovum into the fallopian tube. This ovum is then ready to be fertilized by male sperm to form an embryo. The ruptured ovarian follicle develops into a corpus luteum, which produces oestrogen and progesterone. If fertilization does not occur, the corpus luteum degenerates causing a decrease in oestrogen and progesterone levels, which results in breakdown of the endometrial lining and menstrual bleeding. However, should fertilization and implantation occur, the chorionic membrane between the mother and her developing foetus produces human chorionic gonadotropin (hCG). hCG is a heterodimeric protein with an α subunit identical to those of LH and FSH and a β subunit that is 80% identical to that of LH (Stenman & Alfthan, 2013). Owing to this structural similarity, LH and hCG interact with the same receptor, the LH/CG receptor (Choi & Smitz, 2014b). hCG prevents degeneration of

the corpus luteum during the first trimester of pregnancy and stimulates production of estrogen and progesterone. hCG production increases until the placenta is fully developed and can support growth of the foetus during the remaining term of pregnancy. The homology of LH and hCG allows for a functional substitution during the different life stages. In addition to stimulating ovarian steroid production, hCG also regulates foetal development. In the male foetus, placental hCG acts on the foetal testes to stimulate production of testosterone in the environment of low maternal and foetal LH. This early hCG-stimulated testosterone exposure stimulates development of male anatomy in the foetus (Choi & Smitz, 2014a). In contrast, LH and FSH are required for sexual development at puberty and a deficiency results in decreased production of gonadal steroids and decreased gonadal growth that results in delayed or absent puberty. Individuals that have delayed pubertal development and infertility caused by low levels of gonadotropins are diagnosed with HH (Choi & Smitz, 2014a).

1.2. Etiology of hypogonadotropic hypogonadism

As mentioned earlier, failed migration of GnRH neurons to the hypothalamus causes infertility by disrupting GnRH production. Reduced or absent GnRH secretion results in decreased levels of LH and FSH that causes the Kallmann's syndrome form of HH (Fraietta *et al.*, 2013). HH can also occur without anosmia and is known as normosmic HH, which has a variety of etiologies that manifest variable clinical features, ranging from partial to complete forms of the hypogonadism (Fraietta *et al.*, 2013).

HH can arise from mutations of the GnRH gene that result in aberrant peptide that cannot activate its receptor, disrupting stimulation of LH and FSH release (Maione *et al.*, 2013; Bouligand *et al.*, 2009). Disruption of gonadotropin secretion results from mutations of the genes for GnRH regulators, such as kisspeptin and neurokinin B, or their receptors (Navarro, 2013). However, population studies have shown that mutations of the GnRHR gene are the most common cause of HH (Francou *et al.*, 2016). These mutations partially or

completely disrupt the ability of GnRH to stimulate gonadotropin production (Kottler *et al.*, 1999).

1.3. GnRHR mutations that cause HH

Mutations of the GnRHR potentially impact receptor function via reduced binding affinity for GnRH, decreased signalling in response to GnRH binding or decreased expression of the receptor protein on the gonadotrope cell surface (Kottler *et al.*, 1999; Tello *et al.*, 2012). Owing to the recessive inheritance of HH, causal mutations tend to go undetected in asymptomatic carriers and only manifest a diseased state in homozygous individuals and in compound heterozygotes (Francou *et al.*, 2016). A number of HH cases are sporadic forms, arising from *de novo* mutations of the GnRHR gene (Fraietta *et al.*, 2013).

The first description of a HH-causing mutation of the human GnRHR was the Glu^{2.53(90)}Lys (for explanation of numbering scheme, please see section 1.6) missense mutation. This involves substitution of the glutamic acid residue at position 2.53(90) (refer to page 11 for residue numbering scheme) of the GnRHR protein with lysine. The mutation causes a complete HH phenotype in homozygous carriers (Söderlund *et al.*, 2001). To date, 26 naturally-occurring mutations that cause HH have been identified in the human GnRHR gene (Table 1.1) (Seminara *et al.*, 1998; Tello *et al.*, 2012). *In vitro* analysis has shown that most of these mutations disrupt expression of the GnRHR protein and some disrupt receptor function (Table 1.1).

In vitro studies, in which the Glu^{2.53(90)}Lys mutant GnRHRs expressed in COS7 cells, showed no measurable binding of GnRH and no measurable GnRH-stimulated inositol phosphate (IP) production (Leanos-Miranda, 2002; Tello *et al.*, 2012). Small membrane-permeable GnRH antagonist molecules, referred to as pharmacological chaperones, enhance the cell surface expression of misfolded and poorly expressed mutant receptor proteins by acting as scaffolds to stabilize and correct their folding (Ulloa-Aguirre *et al.*, 2014).

Depending on the GnRHR mutation (see table 1.1), this resultant enhanced expression sometimes leads to restoration of mutant receptor expression, with function that is similar to wild type, as is the case for the Glu^{2.53(90)}Lys mutant GnRHR (Ulloa-Aguirre *et al.*, 2014).

Confocal microscopy of fluorescently labelled GnRHR showed that prior to pharmacological chaperone treatment, the Glu^{2.53(90)}Lys mutant protein was retained in the endoplasmic reticulum (ER) and was not expressed on the plasma membrane. In contrast, the wild type GnRHR was localized both on the plasma membrane and intracellularly (Brothers *et al.*, 2004). This suggests that the Glu^{2.53(90)}Lys mutation disrupts GnRHR trafficking to the cell membrane. Thus, poor expression of GnRHR protein *in vivo* is the likely cause of HH in patients who are homozygous for the Glu^{2.53(90)}Lys GnRHR mutation. It has not been established how the Glu^{2.53(90)}Lys mutation disrupts the structure of the GnRHR at the molecular level that results in this decreased expression. Therefore, the aim of this study is to determine the role of the Glu^{2.53(90)} residue in the expression and function of the GnRHR.

1.4. Protein biosynthesis and the quality control system

Biosynthesis of membrane proteins occurs in the ER. This involves targeting to- and insertion into- the ER membrane, which stabilizes correct folding of the membrane-spanning segments (Schlinkmann *et al.*, 2012). To obtain its functional state, a protein must transition from unfolded to folded by crossing a free-energy barrier to reach its most energetically stable free-energy conformation (Chung *et al.*, 2015; Herczenik & Gebbink 2008). Owing to the complexity of this process, protein translation and assembly is error-prone (Hebert & Molinari 2007; Broadley & Hartl 2009). Therefore, the ER has a quality control system (QCS), comprised of endogenous chaperone proteins that recognize misfolded nascent proteins and correct their folding. This enhances membrane protein assembly and improves transport of the correctly folded protein to the plasma membrane. A combination of defects in protein structure, including exposed, rather than buried, hydrophobic amino acid side-chains,

incorrect salt-bridge formation or incomplete disulfide or hydrogen bonds, contributes to the protein being recognized as misfolded by the QCS (Araki & Nagata, 2011; Chung et al, 2015). Variations in amino acid composition that result from mutations of the gene encoding a protein may destabilize protein folding (Herczenik & Gebbink 2008). If the QCS chaperones cannot correct folding of the mutant protein, the misfolded protein is not delivered to the plasma membrane and it is usually ubiquitin-tagged and targeted to proteasome complexes for degradation (Tan *et al.*, 2004; Alfredo Ulloa-Aguirre *et al.*, 2004; Conn & Janovick, 2009).

Degradation of misfolded and unfolded proteins prevents continuous unsuccessful attempts of the chaperones to correctly fold ‘un-rescuable’ proteins and is essential to maintain protein homeostasis within the cell (Broadley & Hartl, 2009; Araki & Nagata, 2011). The intracellular accumulation of misfolded and/or unfolded proteins has been recognized as the cause of diseases including Alzheimer’s, Parkinson’s and Huntington’s diseases, type 2 diabetes and retinitis pigmentosa (Hartong *et al.*, 2006; Herczenik & Gebbink, 2008; Reynaud, 2010). The aggregates formed by misfolded proteins that are responsible for these and other diseases, disrupt normal functioning of the cell within which they accumulate. Disruption of cellular function results from toxicity induced by increases in oxidative stress, ER stress and malfunction, increased membrane permeability, altered calcium concentrations, malfunctioning mitochondria and/or eventual stimulation of apoptosis leading to untimely cell death (Herczenik & Gebbink, 2008; Reynaud, 2010).

1.5. Rescue of misfolded mutant GnRHRs

Of the 26 known HH-causing mutant GnRHRs that were transfected into cells, 20 showed little to no GnRH-stimulated IP production or binding of GnRH (table 1.1). Treatment of these cells with membrane-permeable small molecule GnRHR ligands resulted in measurable GnRHR expression and function *in vitro*. The small molecule GnRHR

antagonists act as artificial targeted protein folding chaperones, which are thought to stabilize intramolecular interactions within the mutant protein that may allow correct folding. The correctly folded proteins are then trafficked to- and expressed on the cell surface. This suggests that 20 of the 26 characterized HH-causing mutations of the GnRHR disrupt protein folding.

Table 1.1: Effect of HH-associated GnRHR gene mutations. The effects of GnRHR gene mutations on disease severity and on *in vitro* GnRHR expression and function are summarised.

Mutation	HH severity	Expression ^a	Ligand Binding	Rescue? ^b	Signalling	Reference
Ala ¹²⁹ Asp in TM3	Complete	NR ^d	No binding	NR	None	(Caron et al. 1999)
Asn ¹⁰ Lys,Gln ¹¹ Lys in amino terminal	Partial	Normal	↓ ^c affinity	NR	↓	(Costa et al. 2001; Beneduzzi et al. 2012)
Thr ³² Ile in TM1	Complete	↓	↓affinity	Yes	↓	(Bédécarrats et al. 2003)
Glu ⁹⁰ Lys in TM2	Complete	↓	No binding	Yes	None	(Söderlund et al. 2001; Tello et al. 2012)
Thr ¹⁰⁴ Ile in ECL1	Complete	↓	↓affinity	Yes	↓	(Maya-Núñez et al. 2011)
Gln ¹⁰⁶ Arg in ECL1	Partial	Normal	↓	NR	↓	(Costa et al. 2001)
Tyr ¹⁰⁸ Cys in ECL1	Complete	↓	No binding	Yes	None	(Maya-Núñez et al. 2011)
Arg ¹³⁹ His in TM3 and ICL2	Complete	Normal	No binding	Yes	None	(Ballesteros et al. 1998)
Arg ¹³⁹ Cys in TM3 and ICL2	Complete	↓	Unchanged affinity	Yes	↓	(Topaloglu et al. 2006)
Pro ¹⁴⁶ Ser in ICL2	Partial	NR	NR	NR	↓	(Vagenakis et al. 2005)
Ser ¹⁶⁸ Arg in TM4	Complete	Normal	No binding	NR	None	(Pralong et al. 1999)
Ala ¹⁷¹ Thr in TM4	Complete	Normal	No binding	NR	None	(Beate Karges et al. 2003)
Cys ²⁰⁰ Tyr in ECL2	Partial	↓	↓affinity	Yes	↓	(Bédécarrats et al. 2003)
Ser ²¹⁷ Arg in TM5	Variable	NR	↓affinity	NR	↓	(de Roux et al. 1999)
Arg ²⁶² Gln in ICL3	Partial	↓	Unchanged affinity	NR	↓	(de Roux et al. 1997)
Leu ²⁶⁶ Arg in ECL3	Complete	NR	No binding	Yes	None	(Bédécarrats et al. 2003)
Cys ²⁷⁹ Tyr in TM6	Complete	NR	No binding	Yes	None	(Bédécarrats et al. 2003)
Pro ²⁸² Arg in TM6	Complete	↓	No binding	No	None	(Tello et al. 2012)
Tyr ²⁸³ His in TM6	Complete	NR	NR	NR	NR	(Beneduzzi et al. 2012)
Tyr ²⁸⁴ Cys in TM6	Complete	NR	↓affinity	Yes	↓	(Layman et al. 1998)
Leu ³¹⁴ X in TM7	Complete	NR	No binding	No	None	(Kottler et al. 1999)
Pro ³²⁰ Leu in TM7	Complete	Normal	No binding	NR	None	(Meysing et al. 2004)
Tyr ³²³ Cys in TM7	Partial	↓	Unchanged affinity	Yes	↓	(Tello et al. 2012)
Exon 2 deletion	Complete	None	No binding	No	None	(Silveira et al. 2002)

^a Expression addresses detection of mutant GnRHR on the cell surface

^b Rescue refers to increased expression of poorly expressed mutants following treatment with pharmacological agents *in vitro*

^c Decreased compared with wildtype

^d NR, Not reported

When cells transfected with the Glu^{2.53(90)}Lys mutant GnRHR were treated with the small molecule antagonists IN3 or NBI-42902, the mutant receptor subsequently showed GnRH binding and second messenger responses to GnRH stimulation similar to the wild type receptor (Brothers *et al.*, 2004; Leanos-Miranda, 2002; Tello *et al.*, 2012). The rescued function of the Glu^{2.53(90)}Lys mutant GnRHR expression suggests that this mutation causes misfolding of the GnRHR, but has no other effect on the molecular function.

Other forms of *in vitro* ‘rescue’ for the poorly expressed mutant GnRHRs involve genetic modification, including the addition of a carboxy-terminal tail. It has been shown that expression of the human GnRHR is enhanced by addition of the carboxy-terminal tail of a catfish GnRHR (Lin *et al.*, 1998; Janovick *et al.*, 2003). The Glu^{2.53(90)}Lys mutant GnRHR with the catfish carboxy-terminal tail showed enhanced expression, which was accompanied by GnRH-stimulated IP production similar to wild type levels, indicative of rescued function (Janovick *et al.*, 2003). Appending the carboxy-terminal tail of the human type II GnRHR to the human GnRHR enhanced expression of other poorly expressed mutant GnRHRs (Flanagan *et al.*, 2000). Expression of the wild type GnRHR showed a similar increase when a carboxy-terminal tail was appended. This suggests that these receptors are not maximally expressed and may be partially misfolded even in the absence of mutations (Janovick *et al.*, 2003).

1.6. G protein-coupled receptors: Homology

The GnRHR belongs to the large family of G protein-coupled receptors (GPCRs) (Millar *et al.*, 2004). GPCRs are integral membrane proteins that transmit extracellular signals, conveyed by diverse ligands, across a cellular membrane to activate cytosolic G proteins (Naor, 2009). These extracellular signals encompass a vast array of stimuli, including light, ions, odours, hormones, neurotransmitters, drugs and cytokines (Gether & Kobilka, 1998; Pardo *et al.*, 2007). GPCRs are integral to many physiological systems and are

therefore involved in the pathophysiology of many diseases, making them important targets for therapeutic intervention (Tehan *et al.*, 2014). The tertiary structure of the GnRHR has not been elucidated and it has been possible to obtain crystal structures of only a small, but growing, number of related GPCRs (Standfuss *et al.*, 2011; Rasmussen *et al.*, 2011; Cherezov *et al.*, 2007; Deupi *et al.*, 2012). Therefore, much of what is known about the GnRHR structure has been inferred from molecular models based on available GPCR structures (Söderhäll *et al.*, 2005; Mayevu *et al.*, 2015). The GnRHR exhibits characteristic features of GPCRs, including seven membrane-spanning segments, which form a bundle of α -helices relatively perpendicular to the plane of the membrane. The membrane-spanning segments are connected by three intracellular loops (ICL) and three extracellular loops (ECL). The membrane-spanning segments are amphipathic with amino acid residues that face the membrane lipids being hydrophobic, whereas the side chains of residues oriented towards the interior of the helical bundle are largely hydrophilic. The extracellular region, which is variable among different GPCRs, is primarily responsible for ligand recognition. The transmembrane helix bundle forms the structural core, which conveys the ligand binding message to the cytosolic surface of the receptor that interacts with cytosolic proteins, including G proteins (Forfar & Lu, 2011). The mammalian GnRHR also has a unique feature, when compared with most other GPCRs, in that it lacks an intracellular carboxy-terminal tail (Thompson & Kaiser, 2014), although the tail is present in non-mammalian and type II GnRHRs (Lin *et al.*, 1998). A second form of GnRH, GnRH II, is present in a number of species ranging from amphibians to humans. The type II GnRHRs are the cognate receptors for GnRH II, and have carboxyl-terminal tails (Millar *et al.*, 2001). In humans, the type II GnRHR has a stop codon within its coding sequence, which results in a truncated receptor protein. If this truncated receptor is expressed, its function has not been defined (Millar, 2003).

Sequence alignment of GPCRs led to the observation of highly conserved residues and amino acid motifs within the seven transmembrane (TM) segments, from which different GPCR classes were identified (Baldwin, 1993). GPCRs are divided into five different classes, A, B, C, F and O, based on sequence similarities. The GnRHR has the conserved sequences and motifs that are characteristic of class A, which includes the most widely studied GPCRs, rhodopsin and the β_2 -adrenergic receptor (Isberg *et al.*, 2015). Class B includes secretin and adhesion receptors, class C includes glutamate and taste I receptors, while classes F and O include Frizzled receptors and taste II receptors respectively (Isberg *et al.*, 2015). Since the different class A GPCRs vary in the lengths of the extracellular and intracellular loops, Ballesteros and Weinstein proposed a universal numbering system for amino acids of different class A GPCRs. The most conserved residue in each TM is assigned the arbitrary reference number 50, preceded by the TM number. Residues in each TM are numbered relative to the most conserved residue, with the sequence number of the residue in the receptor in parenthesis (Ballesteros & Weinstein, 1995). Thus, the Glu⁹⁰ residue of the GnRHR is designated Glu^{2.53(90)} because it is located three residues past the location of the most conserved residue of TMD2, Asn^{2.50(87)}. Similarly, the Trp²⁸⁰ residue of the GnRHR is designated Trp^{6.48(280)} because it is located two residues before the most conserved residue of TM6, Pro^{6.50(282)}. This consensus numbering scheme allows the comparison of equivalent TM amino acids in different rhodopsin-like GPCRs, facilitating extrapolation of the role of a particular residue from one GPCR to another (Van Rhee *et al.*, 2011). Figure 1 depicts a schematic of the GnRHR sequence showing the positions of the most conserved residues in GPCRs and the positions of the Glu^{2.53(90)} and Trp^{6.48(280)} residues.

The sequences of class A GPCRs show highly conserved amino acid motifs, conserved residues that are found grouped together, within TM domains. Three key motifs of class A GPCRs are the Asp/Glu^{3.49}-Arg^{3.50}-Tyr^{3.51} ((D/E)RY) motif at the cytosolic end of

TM3, the Cys^{6.47}-Trp^{6.48}-x^{6.49}-Pro^{6.50}-Tyr^{6.51} (CWxPY) motif in TM6 and the Asn^{7.49}-Pro^{7.50}-x^{7.51}-x^{7.52}-Tyr^{7.53} (NPxxY) motif in TM7 where 'x' is any amino acid (Palczewski, 2006; Smith, 2010; Vohra *et al.*, 2013). It has been proposed that the highly conserved amino acids of class A GPCRs are the key to mediating the conserved GPCR function by facilitating similar changes in protein conformation that constitute receptor activation in response to agonist binding (Dalton *et al.*, 2015). Comparison of the crystal structures of inactive and active GPCRs suggests that the residues of conserved motifs have conserved roles in the rearrangements of TMs 3, 5, 6 and 7 that occur during GPCR activation (Angel *et al.*, 2009; Miura *et al.*, 2003; Tehan *et al.*, 2014).

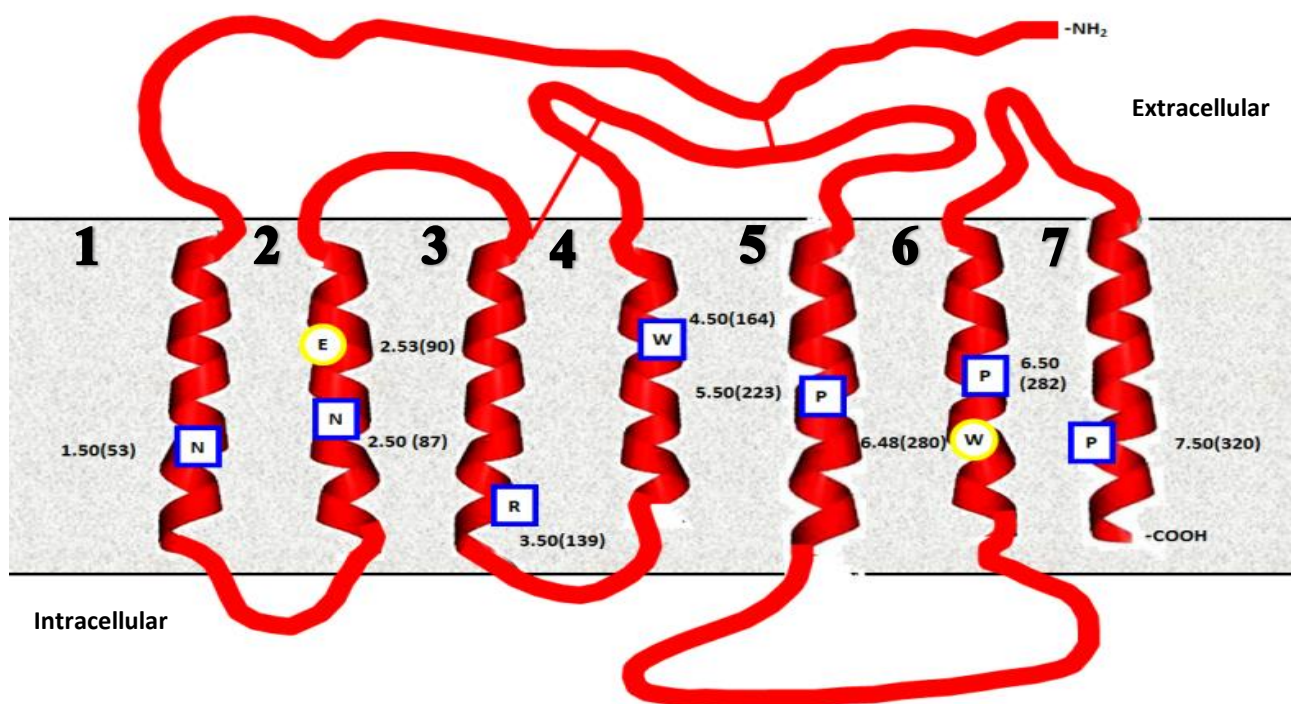


Figure 1: Schematic two-dimensional representation of the human GnRH receptor structure. The seven TM segments are connected by three intracellular and three extracellular loops (red). The amino terminus is shown on the extracellular side of the membrane but the GnRHR lacks a carboxy-terminal tail at its intracellular end (red). Squares in blue represent the most highly conserved residues of each TM helix, bold numbers 1-7 indicate the helices/TM segments. The circles in yellow indicate the locations of Glu^{2.53(90)} and Trp^{6.48(280)} residues.

1.7. G protein-coupled receptors: Theoretical models of receptor activation

A GPCR that is bound by an agonist changes conformation within the cell membrane and subsequently activates membrane-associated cytosolic proteins, particularly G proteins,

which induce intracellular signalling. An agonist binds to a receptor and initiates a physiologic response by stabilizing the receptor in an active conformation. An antagonist will prevent the physiologic response by binding to a receptor and preventing an agonist from activating the receptor. Models of receptor activation evolved from the simple notion of a lock and key mechanism to more complex concepts with added mathematical variables (Kenakin, 2009). Pharmacological studies of drug and receptor interactions led to the two-state or binary receptor model, which proposes that a GPCR exists in equilibrium between one of two functional states or conformations; active (R^*) or inactive (R) (Gether & Kobilka, 1998; Khilnani & Khilnani, 2011). A GPCR is activated by binding of an agonist that induces a conformational change of the receptor stabilizing the R^* or active conformation, which binds to G protein. In contrast, a GPCR is inactivated by binding of an antagonist, which prevents agonist binding, receptor activation and coupling to G protein. This model evolved into the extended ternary complex model to accommodate the discovery that some GPCRs, including adrenergic and opioid receptors, may induce a signalling response in the absence of an agonist. This spontaneous mode of GPCR activation and coupling to G protein is known as constitutive activity. Ligands that are able to bind to, and cause a GPCR to transition from active to inactive, are known as inverse agonists. Inverse agonists decrease constitutive activity of a receptor by stabilizing it in the inactive conformation despite the GPCR being coupled to a G protein (Kenakin, 2009; Khilnani & Khilnani, 2011). Evidence that certain agonists and inverse agonists can partially activate or partially inactivate a GPCR led to the idea that receptors exist in multiple states, whereby a single GPCR can have a collection of conformations. Each of these conformations interacts with a ligand in a different way, giving rise to different possibilities of signalling responses for a single GPCR (Kenakin, 2009). Mutations may stabilize inactive receptor conformations, preventing the R to R^* transition thereby maintaining the inactive state. Some receptor mutations stabilize the R^* conformation

causing constitutive activity. Most mutations of the GnRHR do not result in constitutive activity, but there has been a single report of constitutive activity in a highly modified form of the Glu^{2.53(90)}Lys mutant GnRHR in ECL2 (Janovick *et al.*, 2011).

1.8. G protein-coupled receptors: Function

The physiological function of all GPCRs involves transmission of extracellular signals, particularly agonist binding, across a cellular membrane to activate various intracellular signalling pathways (Naor, 2009). Models of GPCR activation imply that agonist binding is associated with a conformational change of the transmembrane helical bundle that stabilizes the active state(s), which activates cytosolic G proteins. Structural rearrangements of the membrane-spanning segments arise from rearrangement of intramolecular interactions within individual TM segments and between different TM segments (Gether & Kobilka, 1998). It is assumed that because the GnRHR has the highly conserved residues characteristic of class A GPCRs, it undergoes movements that are similar to those of other class A GPCRs (Mayevu *et al.*, 2015).

G proteins are heterotrimeric membrane-associated proteins, consisting of $G\alpha$, β and γ subunits. This family of evolutionarily conserved proteins controls the onset of many of the intracellular signalling pathways that mediate GPCR functions. G proteins exist in an inactive state, which is characterised by the $G\alpha$ subunit of the heterotrimer being bound to guanosine diphosphate (GDP). G protein activation is initiated by release of GDP and binding of guanosine triphosphate (GTP) to the $G\alpha$ subunit. Following binding of GTP to $G\alpha$, the $G\beta\gamma$ dimer dissociates and the separated subunits interact with effector proteins (Milligan & Kostenis, 2009; Johnston & Siderovski, 2007). The $G\alpha$ -GTP subunit and the $G\beta\gamma$ dimer each modulate downstream effectors, including nucleotide cyclases, ion channels and phospholipases. This regulates production of second messengers, such as cyclic AMP (cAMP), IP3 and diacylglycerol (DAG), which initiate cellular signalling pathways that

translate the external stimulus to a physiological response (Naor, 2012). G proteins have been classified into four subfamilies; $G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q/11}$ and $G_{\alpha 12/13}$, based on sequence homology of α subunits that activate distinct signalling pathways. $G_{\alpha s}$ activates adenylyl cyclase, which catalyses cAMP production; $G_{\alpha i/o}$ inhibits adenylyl cyclase activity and $G_{\alpha 12/13}$ activates small GTPases involved in cell migration. The GnRHR couples to the $G_{\alpha q/11}$ family of G proteins, which activates phospholipase C β (PLC β). PLC β catalyses the production of second messengers, IP3 and DAG, from the membrane phospholipid, phosphatidylinositol bisphosphate (PIP2). IP3 stimulates mobilization of Ca²⁺ ions from the ER. DAG and cytosolic Ca²⁺ activate protein kinase C (PKC). PKC then activates mitogen-activated protein kinase (MAPK) (Naor & Huhtaniemi, 2012; Naor, 2009; Grosse *et al.*, 2000), which, in turn, regulates gonadotropin production and secretion.

1.9. G protein-coupled receptors: Crystal structures

Obtaining tertiary structures of GPCR proteins is difficult. As GPCRs are integral membrane proteins, they become unstable when removed from their natural membrane environment to be crystallized. GPCRs are often poorly expressed, with their low abundance making it difficult to obtain enough protein to generate crystals. Receptor proteins are naturally flexible and exist in multiple conformations. Therefore, another challenge is to obtain a single conformation from the numerous conformations that individual protein molecules assume in order to successfully generate a single static crystal structure (Carpenter *et al.*, 2008; Ghosh *et al.* 2015). Because of these difficulties, crystal structures of only a small number of different GPCRs have been determined. Rhodopsin and the β -adrenergic receptors are the most widely studied and understood of the class A GPCRs. This detailed knowledge has allowed crystallization of these receptors in both inactive and active states (Standfuss *et al.*, 2011; Rasmussen *et al.*, 2011; Cherezov *et al.*, 2007; Deupi *et al.*, 2012). More recently, advances in crystallographic technology and strategies to overcome the technical challenges

have led to an increase in the number of different GPCRs that have been crystalized (Carpenter *et al.*, 2008; Ghosh *et al.*, 2015). Since there is no available crystal structure of the GnRHR, I provide a detailed review of crystal structures of other class A GPCRs to assess the functions of residues in position 2.53 in order to better understand how the Glu^{2.53(90)}Lys mutation disrupts GnRHR function. In particular, the earlier rhodopsin and β_2 -adrenergic receptor crystals and some of the more recent structures such as the neurotensin receptor (NT1R) have been used as homology models for GnRHR structure (Mayevu *et al.*, 2015). The information gained from probing GPCR crystal structures has given insight into areas of GPCR research that were previously lacking, including the nature of receptor interactions with ligands and G proteins at the structural level, whereas comparison of inactive and active receptor structures has improved the understanding of receptor activation mechanisms (Deupi, 2014; Rasmussen *et al.*, 2011).

1.10. Crystal structures of inactive rhodopsin

1.10.1. Overall fold and proline kinks

The first GPCR crystal structure to be determined was a low resolution structure of the inactive conformation, or dark-state, of rhodopsin, consisting of the apoprotein, opsin, covalently bound to 11-*cis*-retinal, which acts as an inverse agonist (Palczewski *et al.*, 2000). This crystal structure confirmed predictions from sequence analyses that GPCRs would have seven membrane-spanning α -helical segments with an extracellular NH₂-terminus and an intracellular COOH-terminal tail. Positions of the highly conserved residues of each membrane-spanning segment, as well as the highly conserved structural motifs within these TMs were also defined. The proline residues, Pro^{5.50}, Pro^{6.50} and Pro^{7.50}, which are highly conserved in TMs 5, 6 and 7 of class A GPCRs were found to cause structural distortions in the α -helices in the form of bends or kinks, but these are not classical proline kinks. For example, Pro^{6.50(267)} of the CWxPY motif in TM6, was shown to have a more exaggerated

bend angle than a standard proline-kink and it was hypothesized that a change in this bend angle would be involved in receptor activation (Palczewski *et al.*, 2000; Trzaskowski *et al.*, 2012). To achieve this bend angle, Pro^{6.50(267)} is stabilized by an interaction with a water molecule, which makes hydrogen bonds to Cys^{6.47(264)}, Tyr^{6.51(268)} of the CWxPY motif and Pro^{7.38(291)} (Standfuss *et al.*, 2011). The overall GPCR fold is stabilized by interactions involving non-covalent intrahelical and interhelical interactions (Venkatakrisnan *et al.*, 2013). Some of the hydrogen bond interactions are mediated by water molecules, which form a network of hydrophilic interactions (Pardo *et al.*, 2007; Angel *et al.*, 2009).

1.10.2. Water-mediated hydrogen bond networks and the CWxPY motif

The most highly conserved residues within inactive rhodopsin crystals are connected by hydrogen bonds with nearby water molecules. It is believed that these water molecules mediate long distance hydrogen bond interactions between residues of different helices that are too far apart to make direct interactions. For example the Trp^{6.48(265)} residue of the CWxPY motif in TM6 is connected to Asp^{2.50(83)}, the most conserved residue of TM2, as well as to Asn^{7.49(302)} of the NPxxY motif and Ser^{7.45(298)} in TM7 (Li *et al.*, 2004; Standfuss *et al.*, 2011). An interaction between the TM2 Asp^{2.50} and TM7 Asn^{7.49} residue had been predicted, based on the reciprocal interchange of Asp^{2.50} and Asn^{7.49} to Asn^{2.50} and Asp^{7.49} in the GnRHR, when compared with other class A GPCRs (Zhou *et al.*, 1994; Flanagan *et al.*, 1999). The rhodopsin crystal structures showed that this interaction could not be direct owing to the distance between the Asp^{2.50} and Asn^{7.49} residues. However, an indirect interaction via hydrogen bonding to water molecules was noted (Palczewski *et al.*, 2000). The inactive rhodopsin crystals show a hydrophobic barrier located on the cytosolic side Asp^{2.50(83)} that comprises six residues of TMs 2, 3 and 6 including, Leu^{2.43(76)}, Leu^{2.46(79)}, Leu^{3.43(128)}, Leu^{3.46(131)}, Met^{6.36(253)} and Met^{6.40(257)}. The barrier separates the ligand binding pocket from the cytoplasmic G protein binding site, which is occluded and inaccessible in the inactive state

(Standfuss *et al.*, 2011). Met^{2.53(86)} was proposed to interact with Trp^{6.48(265)} of rhodopsin to separate the water networks at the extracellular and intracellular sides (Deupi *et al.*, 2012).

1.10.3. Ligand binding pocket

All seven TM segments and a region of the extracellular part of rhodopsin contribute to binding of *cis*-retinal in the inactive state (Menon *et al.*, 2001). Retinal is covalently linked to the positively charged Lys^{7.43(296)}, which is stabilized by the negatively charged counterion Glu^{3.28(113)} via a salt-bridge interaction. The binding pocket itself places retinal between the TMs and means that it is buried by side-chains of surrounding TMs (Palczewski *et al.*, 2000; Teller *et al.*, 2001). Overall, about 22 residues are located close enough to interact with retinal and form the ligand binding pocket of inactive rhodopsin (Li *et al.*, 2004). Of these residues, Trp^{6.48(265)} of the CWxPY motif was found to make hydrophobic contact with carbon 18 (C18) of the β -ionone ring of 11-*cis*-retinal (Li *et al.*, 2004). Several other residues make hydrophobic contacts with the ligand including Met^{5.42(207)}, Phe^{4.47(212)} and Tyr^{6.51(268)}. However, far fewer residues contact the ligand by polar interactions (Li *et al.*, 2004). There was no mention of a direct interaction of retinal with the Met^{2.53(86)} residue of inactive rhodopsin. (Li *et al.*, 2004; Venkatakrishnan *et al.*, 2013).

1.10.4. Ionic-lock

The inactive rhodopsin crystal showed that the Arg^{3.50(135)} of the conserved (D/E)RY motif at the cytosolic end of TM3 formed a salt-bridge with the adjacent Glu^{3.49(134)} of the (D/E)RY motif and formed an interhelical salt-bridge with Glu^{6.30(247)} at the cytosolic end of TM6 (Palczewski *et al.*, 2000). This interaction linking TMs 3 and 6 is known as the ionic-lock, which had been predicted to stabilize inactive GPCR conformations (Vogel *et al.*, 2008).

1.10.5. Location of residues 5.58 and 7.53

Tyr^{7.53(306)} of the NPxxY motif is constrained in the inactive rhodopsin crystal by an aromatic interaction with the Phe^{7.60(313)} residue of TM8, placing it away from the TM core bundle and toward TM1 (Goncalves *et al.*, 2010; Deupi *et al.*, 2012). It is believed that this constraining interaction is one that stabilizes the inactive state of rhodopsin (Trzaskowski *et al.*, 2012; Goncalves *et al.*, 2010). Like Tyr^{7.53(306)}, another highly conserved residue in TM5, Tyr^{5.58(223)} is also positioned facing away from the TM core bundle in the inactive rhodopsin crystal (Goncalves *et al.*, 2010).

1.11. Crystal structures of rhodopsin-like GPCRs in inactive conformations

The first crystal structures of class A GPCRs other than rhodopsin, referred to here as ‘rhodopsin-like’ GPCRs, were crystallized long after the initial rhodopsin crystal owing to technical difficulties that required multiple methodological innovations in crystallography (Ghosh *et al.*, 2015). The main difficulties are the flexibility and instability of receptor proteins. There are three types of receptor flexibility, the first arising simply from ‘loose ends’ such as amino and carboxy termini and long ECLs within the 7TM structure. Technical innovations to overcome some of the problems associated with protein flexibility involved subjecting the receptor itself to recombinant technologies such as the truncation of ‘loose ends or loops’ and/or incorporation of stabilizing proteins, including the T4 lysozyme in ICL3 to form a fusion protein. The T4 lysozyme is a stable and water-soluble protein. Inserted proteins allow for hydrophilic contacts that aid in crystallography (Zhou *et al.*, 2012). A second type of flexibility is the conformational flexibility of the TM bundle itself that requires the introduction of thermostabilizing mutations to achieve the stability needed for crystallography. Many thermostabilizing mutations are known to affect receptor function and may thus cause artefacts in crystal structures (Ghosh *et al.*, 2015).

A third type of flexibility arises from spontaneous conformational interchanges of the protein. Since unliganded GPCR proteins exist in equilibria of active and inactive conformations, co-crystallization of receptors with ligands was needed to stabilize either active or inactive receptor conformations. Such ligands may dissociate from the receptor and allow changes in conformation that again induce flexibility that hinders crystal formation. Development of covalently bound or slowly diffusing, high affinity full inverse agonists was needed to overcome this problem (Ghosh *et al.*, 2015). Nevertheless many of these ligands do not fully stabilize the receptor in a fully inactive state and crystal structures may represent a conformation that is not fully inactive, but rather partially active (Dror *et al.*, 2009).

Despite the fact that a crystal structure might not be resolved without incorporation of recombinant receptor modifications, these modifications may introduce structural bias or artefacts that compromise the reliability of these structures (Deupi, 2014; Dror *et al.*, 2009). It has been suggested that since the rhodopsin crystal structures do not contain artefacts from engineered stabilizing mutations they may be more reliable as models for comparison of inactive, partially active and active GPCRs (Deupi, 2014).

The early rhodopsin-like receptor crystals included GPCR proteins co-crystallized with inverse agonists, which stabilize the inactive receptor state. These structures included the β_1 and β_2 -adrenergic receptors, A_{2A}-adenosine, histamine H1 and dopamine D3 receptors (Warne *et al.*, 2008; Doré *et al.*, 2011; Shimamura *et al.*, 2011; Chien *et al.*, 2011). This was followed by crystal structures of receptors that bind larger peptide ligands including the opioid receptors and the CCR5 and CXCR4 chemokine receptors (Huang *et al.*, 2015; Tan *et al.*, 2013; Wu *et al.*, 2010).

1.11.1. Overall fold

The inactive rhodopsin-like GPCR crystal structures showed a similar structural fold to the inactive rhodopsin crystal. The fold is supported by conserved interactions of the highly

conserved amino acids and many interactions believed to be important in maintaining the inactive conformation of rhodopsin were confirmed in the rhodopsin-like receptor crystals (Chien *et al.*, 2011; Doré *et al.*, 2011; Shimamura *et al.*, 2011; Wu *et al.*, 2010; Dror *et al.*, 2009; Wu *et al.*, 2012).

1.11.2. Ionic-lock

The ionic-lock was present in some of the inactive rhodopsin-like receptor crystals, including the D3 dopamine receptor and the A_{2A} adenosine receptor, confirming that these receptors were in the inactive state (Chien *et al.*, 2011; Doré *et al.*, 2011; Huang *et al.*, 2015). Several other rhodopsin-like receptor crystals, including the M2 muscarinic receptor, histamine H1 receptor and the β_2 -adrenergic receptor, did not display an ionic-lock (Dror *et al.*, 2009; Huang *et al.*, 2015; Shimamura *et al.*, 2011; Haga *et al.*, 2012). It has been suggested that the broken ionic-lock may be explained by stabilization of a conformation that is not fully inactive, by ligands that may be partial inverse agonists. For example, it has been suggested that the β_2 -adrenergic receptor in complex with dissociable ligands was not a fully inactive receptor crystal, but rather a crystal of an intermediate conformation, which does not contain an ionic-lock (Dror *et al.*, 2009). Alternatively, the broken ionic-lock of inactive crystal structures incorporating the T4 lysozyme protein may result from presence and location of the T4 lysozyme in ICL3, which may hinder interactions of Arg^{3.50} with the Glu^{6.30} at the ICL3 end of TM6 (Dror *et al.*, 2009). The inactive CCR5 chemokine receptor crystal displayed a modified ionic-lock due to the introduction of the thermostabilizing mutation, Ala^{6.33(233)}Asp. The charged Asp forms a salt-bridge with Arg^{3.50}, which stabilizes the receptor in an inactive state (Tan *et al.*, 2013). Like 80% of rhodopsin-like GPCRs (Mirzadegan *et al.*, 2003), the μ -opioid receptor lacks an acidic Glu amino acid at position 6.30 of TM6 that can form a salt-bridge with the conserved Arg^{3.50(163)} of TM3. However, Arg^{3.50(163)} forms a hydrogen bond with a nearby Thr^{6.34(279)} instead (Huang *et al.*, 2015). The GnRHR has an Arg

in position 6.30 but, there is a Thr residue at position 6.33. Therefore, Thr^{6.33(278)} could mimic the role of Thr^{6.34(279)} of the μ -opioid receptor and form a similar alternative ionic-lock interaction in the GnRHR.

1.11.3. Ligand binding pocket

Binding pockets of GPCRs with small ligands, like the rhodopsin binding pocket, are largely within the TM domain. This allows for direct interactions with the highly conserved residues (Deupi *et al.*, 2012). Comparisons of the ligand binding pockets across the different inactive GPCR crystals has highlighted a group of amino acid loci, which, regardless of the amino acid at the locus, contribute to a conserved ligand binding cradle. This ligand binding cradle includes the highly conserved Trp^{6.48} sidechain (Venkatakrishnan *et al.*, 2013). For example, inverse agonists complexed with the inactive CCR5 chemokine receptor, A_{2A} adenosine receptor and histamine H1 receptors make direct contacts with the highly conserved Trp^{6.48} residue of the CWxPY motif to stabilize the inactive conformation (Shimamura *et al.*, 2011; Tan *et al.*, 2013). However, Trp^{6.48} does not make direct contact with inverse agonists of the β_2 -adrenergic receptor (Deupi & Standfuss, 2011). It has been proposed that the inverse agonists that interact with Trp^{6.48} inhibit movement or rotation of TM6 (Foucaud *et al.*, 2008; Trzaskowski *et al.*, 2012). The binding pockets of rhodopsin-like GPCRs that bind large peptide-ligands are more extracellularly located when compared to rhodopsin or small ligands that bind at the same GPCR. For example, the peptide antagonist, CVX15, binds more to the extracellular domain of the CXCR4 chemokine receptor and makes fewer direct interactions with the highly conserved residues in the TM bundle (Trzaskowski *et al.*, 2012; Wu *et al.*, 2010).

1.11.4. Water-mediated hydrogen bond networks

Like rhodopsin, the rhodopsin-like GPCR crystal structures show cavities or channels within the TM core, which are thought to be supported by water molecules that stabilize

receptor folding (Hulme, 2013). The highly conserved residues of rhodopsin and rhodopsin-like GPCRs hydrogen bond to water molecules forming a hydrogen bond network that is conserved among class A GPCRs. The conservation suggests that the network is important for GPCR structure (Huang *et al.*, 2015; Pardo *et al.*, 2007; Blankenship *et al.*, 2015). In addition to the water molecules, high resolution crystal structures of the inactive β_1 -adrenergic, δ -opioid, A_{2A} adenosine and PAR1 protease-activated receptors show a sodium ion that coordinates with the highly conserved, acidic, Asp^{2.50} side-chain via a salt bridge and with a number of water molecules, which interact with other highly conserved amino acid residues (Katritch *et al.*, 2014; Liu *et al.*, 2012). The sodium ion with its coordinated water molecules creates a link between the conserved Asp^{2.50} in TM2 and the conserved Trp^{6.48} and Asn^{7.49} residues in TMs 6 and 7 (Fenalti *et al.*, 2014; Katritch *et al.*, 2014; Nygaard *et al.*, 2010; Liu *et al.*, 2012). Since the sodium ion is known to be an allosteric inhibitor of GPCR signalling and is present only in the inactive structures, it is likely that the sodium ion stabilizes the hydrogen bonding network that maintains the inactive receptor state (Katritch *et al.*, 2014; Nygaard *et al.*, 2010). This stabilization may not be necessary for rhodopsin, which does not have the sodium ion, because it has a covalently bound inverse agonist ligand. Binding of agonist is proposed to displace the sodium ion towards the cytoplasm (Liu *et al.* 2012). Mutagenesis studies support a role for Asp^{2.50} in GPCR activation (Katritch *et al.*, 2014).

Sodium ions inhibit binding of GnRH agonists to the GnRHR at physiological concentrations (10mM), but have less effect on GnRH antagonist binding (Wormald *et al.*, 1985; Marian & Conn, 1980; Hazum, 1987; Hazum, 1981; Heitman *et al.*, 2008), suggesting that sodium ions also allosterically inhibit the GnRHR. The GnRHR has Asn^{2.50(87)} instead of an Asp at position 2.50, but has an Asp^{7.49(319)} instead of Asn at position 7.49. Mutagenesis studies suggest that the function of Asp^{2.50} of other GPCRs may be transferred to Asp^{7.49(319)} in the GnRHR. The Asn^{2.50(87)} is needed for GnRHR expression, but Asp^{7.49(319)} is required for

activation of G protein-dependent signalling (Flanagan *et al.*, 1999). It is possible that the Asp at position 7.49 is part of the sodium ion/water network of the GnRHR. In summary, compared with rhodopsin, rhodopsin-like GPCRs have sodium ions and a larger cytoplasmic water-mediated polar network, which act together to stabilize the inactive receptor conformation in the absence of a covalently bound inverse agonist (Huang *et al.*, 2015; Standfuss *et al.*, 2011).

1.12. Crystal structures of active rhodopsin

When photons of light interact with the retinal that is covalently bound to rhodopsin in photoreceptor cells, retinal isomerizes, changing from “bent” 11-*cis*-retinal to “straight” all-*trans*-retinal (Shichida & Morizumi, 2007). The longer “straight” all-*trans*-retinal cannot be accommodated within the binding pocket defined by the inactive rhodopsin structure. The opsin apoprotein undergoes conformational transitions through several intermediate states, each corresponding to a specific spectroscopic absorption profile. These include photorhodopsin, bathorhodopsin, lumirhodopsin, and metarhodopsin I before the active metarhodopsin II conformation is formed (Zhou *et al.*, 2012). Metarhodopsin II is unstable with all-*trans*-retinal bound and consequently splits into active opsin apoprotein and free all-*trans*-retinal. The active opsin binds and activates the G protein, transducin, which then activates downstream signalling pathways (Zhou *et al.*, 2012).

As was the case for the inactive receptor structures, rhodopsin was also the first GPCR to be crystalized in the active state. Rhodopsin was initially crystalized in spectroscopically-defined, partially active states, including metarhodopsin I, which provided insight into the process of activation, but did not define the fully active conformation (Ruprecht *et al.*, 2004; Deupi *et al.*, 2012; Deupi, 2014). A fully active, ligand-free rhodopsin structure was then crystallized in complex with a synthetic peptide derived from the carboxy terminus of the α subunit of transducin (G α CT) to stabilize the active conformation (Scheerer *et al.*, 2008). A

limitation of the ligand-free opsin structure is that it does not reveal how agonist binding initiates the transition of the receptor from inactive to active conformation. Therefore, two constitutively active rhodopsin mutants were co-crystallized with all-*trans*-retinal and a GαCT peptide to give insight into the ligand binding pocket of active rhodopsin (Deupi *et al.*, 2012; Standfuss *et al.*, 2011). These crystals are examples of two types of constitutively active mutant rhodopsin crystal structures. The first type contains a mutation (Glu^{3.28(113)}Gln) of the binding pocket, that has unchanged amino acid sequence around the G protein binding site, allowing investigation of G protein binding (Standfuss *et al.*, 2011). The second constitutively active rhodopsin had a mutation, Met^{6.40(257)}Tyr, near the G protein binding site, but an unmutated retinal binding pocket that was compared with other rhodopsin structures to identify the ligand-interacting residues of the active receptor conformation (Deupi *et al.*, 2012). The constitutively active mutants were confirmed as being fully active Metarhodopsin II-like structures by spectroscopic characterization (Deupi *et al.*, 2012; Standfuss *et al.*, 2011).

1.12.1. Ligand binding pocket

When compared with inactive rhodopsin, active rhodopsin crystals show a larger binding pocket, which accommodates the longer “straight” all-*trans*-retinal configuration (Deupi & Standfuss, 2011). The larger binding pocket results from outward movement of the extracellular end of TM6, together with repositioning of bulky side-chains during receptor activation (Deupi *et al.*, 2012). During retinal isomerization, the β-ionone ring of all-*trans*-retinal moves 4.3Å into a space between TMs 5 and 6 forcing the outward rotation of TM6. The Trp^{6.48(265)} side-chain, which interacts with the methyl group on C18 of the β-ionone ring in the inactive rhodopsin crystal, shows a co-ordinated movement of 3.6Å that maintains its interaction with the retinal during transition to the active conformation (Standfuss *et al.*, 2011).

Metarhodopsin II crystals were also prepared by incubating the wild type apoprotein with all-*trans*-retinal, which became covalently bound. One of these active crystals included a GaCT peptide, whereas the other was prepared without the G protein mimetic. The ligand binding pockets of these structures were similar to those of the constitutively active mutant rhodopsins, except for the presence of a hydrophobic interaction of C15 of all-*trans*-retinal with Met^{2.53(86)} (Choe *et al.*, 2011). Compared with inactive rhodopsin, active rhodopsin showed a structural rearrangement of the back-bone of TM2 around a Gly^{2.56(89)}-Gly^{2.57(90)} motif that causes movement of the side-chain of Met^{2.53(86)}. The movement of the Met^{2.53(86)} and Trp^{6.48(265)} side-chains away from their central location within the TM core of rhodopsin, is reported to allow water molecules to penetrate further into the central core of the TM bundle of the active receptor (Deupi *et al.*, 2012; Choe *et al.*, 2011).

1.12.2. Unchanged Pro^{6.50} kink and rotation via Trp^{6.48}

Specific residues within the conserved GPCR motifs, such as Trp^{6.48}, are believed to have a conserved role in GPCR activation (van Rhee *et al.*, 2011; Mayevu *et al.* 2015; Wu *et al.*, 2012; Kobilka, 2007; Hulme 2013). Biophysical studies showed an outward motion of the cytosolic end of TM6 during rhodopsin activation, which was associated with the conserved Pro^{6.50} of the CWxPY motif (Hubbell *et al.* 2003). The Pro^{6.50} was predicted to serve as a hinge that moved TM6 by changing the angle of the bend in the helix (Trzaskowski *et al.*, 2012; Tehan *et al.*, 2014; Altenbach *et al.*, 2008). However, the crystal structures of activated rhodopsin show that TM6 undergoes a whole body movement that is rotational, rather than hinged, and has an unchanged bend angle at the proline-kink in the CWxPY motif. This rotational outward movement of the cytosolic end of TM6 is associated with movement of Trp^{6.48} of the CWxPY motif, which changes its position in response to retinal isomerization and movement of the β -ionone ring (Standfuss *et al.*, 2011; Mayevu *et al.*, 2015). The constitutively active Glu^{3.28(113)}Gln rhodopsin mutant structure showed that the movement of

the cytosolic end of TM6 is amplified by the presence of the exaggerated bend angle of Pro^{6.50(267)} (Standfuss *et al.*, 2011). The crystal structures of constitutively active rhodopsin mutants were similar at the cytoplasmic side, showing that the agonist ligand is enough to stabilize a fully active conformation of rhodopsin in the absence of a G protein peptide (Choe *et al.*, 2011).

1.12.3. Open ionic-lock

Another difference observed in the active- compared with the inactive- rhodopsin crystal structures was the open ionic-lock between residues in TMs 3 and 6, which results from the outward rotation of the cytosolic end of TM6 in response to retinal isomerization (Standfuss *et al.*, 2011). The opsin apoprotein crystals show that the side-chain of Arg^{3.50(135)}, (which is freed when the ionic lock connecting it to Glu^{3.49(134)} and Glu^{6.30(247)} is broken by movement of TM6,) is positioned near the side-chains of the conserved residues Tyr^{5.58(223)} of TM5 and Tyr^{7.53(306)} (of the NPxxY^{7.53} motif in TM7). Compared with inactive rhodopsin, the Tyr^{5.58(223)} and Tyr^{7.53(306)} side-chains are rotated inward towards Arg^{3.50(135)} (Goncalves *et al.*, 2010; Hoffmann *et al.*, 2000). More recently, a high resolution structure of activated opsin, which shows a more complete water network, revealed direct interaction of Arg^{3.50(135)} with Tyr^{5.58(223)}, which then makes a water-mediated hydrogen bond with Tyr^{7.53(306)} (Blankenship *et al.*, 2015). The close proximity of Tyr^{5.58(223)}, Tyr^{7.53(306)} and Arg^{3.50(135)} in the active structure suggests that these interactions may contribute to stabilizing the active state of rhodopsin (Goncalves *et al.*, 2010). The functional importance was supported by the constitutively active Met^{6.40(257)}Tyr rhodopsin structure in which the hydroxyl group of the mutant Tyr^{6.40(257)} side-chain mimics the hydroxyl of a water molecule that is part of this interaction (Blankenship *et al.*, 2015). The mutant Tyr^{6.40(257)} residue also destabilizes the inactive receptor state by disrupting the hydrophobic barrier that is present in the inactive receptor and absent from the active receptor structure (Deupi *et al.*, 2012). The absence of the

hydrophobic barrier allows formation of a continuous hydrophilic channel that extends from the ligand binding pocket to the G protein binding site in the active rhodopsin structure (Tehan *et al.*, 2014; Deupi 2014).

1.12.4. Water-mediated hydrogen bond network

Like the inactive structures, active rhodopsin structures showed water molecules that form hydrogen bonding networks. Molecular modelling that involved omission of structural water molecules from active rhodopsin showed a disordered structure, whereas the structure with the waters present was more energetically favourable and stable, supporting structural importance of the water (Standfuss *et al.*, 2011). Two active rhodopsin crystal structures with enlarged retinal binding pockets showed water-mediated hydrogen bond interactions that differ from the network in the inactive rhodopsin crystals. This suggests that each set of hydrogen bond networks specifically stabilizes either the active or the inactive receptor conformation (Choe *et al.*, 2011). This observation in rhodopsin led to a proposal that the water molecules may be conserved structural elements of GPCRs that participate in the activation process and facilitate coupling to G proteins (Standfuss *et al.*, 2011; Pardo *et al.*, 2007).

A high resolution structure of activated opsin revealed that water molecules can be divided into three categories, based on their apparent roles (Blankenship *et al.*, 2015). One set of water molecules occupied similar positions in both the inactive and the active conformations, suggesting that they have a structural, rather than a signalling, role. A second set of water molecules was present in both the active and inactive rhodopsin structures and maintained hydrogen bonding with the same residue in both conformations, although the amino acid side-chains moved. It is likely that these water molecules facilitate movement and repositioning of the interacting residues. The final set of water molecules were observed to be

in different positions in the inactive and active rhodopsin structures, suggesting a role in stabilizing a single receptor conformation (Blankenship *et al.* 2015).

The water network of the active rhodopsin structure creates a continuous hydrophilic channel from retinal in the binding pocket to the G protein-binding site when the hydrophobic barrier of the inactive receptor conformation is broken (Standfuss *et al.*, 2011; Blankenship *et al.*, 2015) and the Met^{2.53(86)} and Trp^{6.48(265)} side-chains move away from the centre of the TM bundle (Choe *et al.*, 2011; Deupi *et al.*, 2012). The altered arrangement of the water network of the active rhodopsin crystals arises from a rearrangement of water-mediated hydrogen bonds between highly conserved residues, including Trp^{6.48(265)} of TM6. A subsequent review of the rhodopsin structures proposed a conserved water-mediated interaction of the less obviously conserved residue in position 2.53 with the conserved Trp^{6.48(265)} in class A GPCRs (Deupi, 2014). This suggested to us that the side-chain of the Glu^{(2.53)90} residue of the GnRHR may interact with Trp^{(6.48)280}. Thus, the HH-associated Glu^{(2.53)90}Lys mutation may disrupt GnRHR expression by disrupting this interaction.

1.13. Active rhodopsin-like GPCR crystal structures

Based on theoretical models of GPCR activation, it is expected that GPCRs must be co-crystallized with agonist ligands to obtain structures of active GPCR conformations. Unlike the case for rhodopsin, it was found that simple agonist binding was not sufficient to obtain fully active structures. Rather, agonist-bound GPCRs had to be co-crystallized with a G protein or G protein mimetic to stabilize the high agonist affinity GPCR conformation that defines the fully active receptor state (Dror *et al.*, 2012; Nygaard *et al.*, 2013; Sounier *et al.*, 2015). Therefore, several agonist-bound GPCR crystals, such as the agonist-bound A_{2A} adenosine and rat neurotensin (NTSR1) receptors, which did not include G protein mimetics, were only partially active structures (Lebon *et al.*, 2011; Xu *et al.*, 2011; White *et al.*, 2012). Based on comparisons with partially and fully active rhodopsin crystals (see section 1.12),

these structures did not exhibit the full extent of movement of TMs 6 and 7 (Xu *et al.*, 2011). An exception is the highly constitutively active viral GPCR, US28, in complex with the CX3CL1 chemokine ligand, which showed very similar structures when crystallized with- or without- the G protein mimetic, Nb7 (Burg *et al.*, 2015).

Since co-crystallization of G proteins is difficult to achieve, G protein mimetics have been incorporated into GPCR crystallography. Commonly used G protein mimetics include antibody Fab fragments (Steyaert & Kobilka, 2011) or nanobodies (very small antibodies produced by llamas), which were selected for their ability to stabilize the high agonist binding affinity GPCR conformation (Rasmussen *et al.*, 2011a). Whereas these antibodies may mimic the effect of the G protein on receptor conformation, they are likely to make slightly different binding contacts with receptors (Rasmussen *et al.*, 2011a). This review will focus primarily on the fully active rhodopsin-like GPCR structures.

1.13.1. Ligand binding pocket

Although ligand binding pockets of active GPCR crystals must differ to accommodate the binding of their different ligands, it has been observed that many ligands contact the highly conserved residues, including Trp^{6.48}. This suggests a globally conserved role for such residues in the activation of class A GPCRs and Trp^{6.48} has even been proposed to be part of a conserved binding pocket (Venkatakrisnan *et al.*, 2013). Not all agonist ligands make direct contact with Trp^{6.48}. The crystal structures of the active β_2 -adrenergic receptor showed no direct contact of agonist with Trp^{6.48(286)}, although it forms part of the ligand binding pocket (Rasmussen *et al.*, 2011). Similarly, the recent description of chemokine GPCR crystal structures make no mention of agonist interaction with the Trp^{6.48} residue (Burg *et al.*, 2015; Qin *et al.*, 2015). In contrast, Trp^{6.48(264)} was shown to contact the agonist ligand in the human A_{2A} adenosine crystal (Xu *et al.*, 2011).

Although some peptide ligands partially enter the TM core of receptors (Wu *et al.*, 2010; Standfuss, 2015), because of their larger size, it has long been expected that peptides would bind more extracellularly making contact with the extracellular loops and the upper part of the TM bundle (Shonberg *et al.*, 2014). It has also been predicted that some peptide and protein ligands bind exclusively to the extracellular domains of GPCRs (Krumm & Grisshammer, 2015). This mode of binding would limit the contact of such ligands with conserved residues that are located within the TM bundle (Berthold & Bartfai, 1997; Shonberg *et al.*, 2014). Supporting this, crystal structures of the peptide agonist-bound NTSR1 neurotensin receptor, showed an open ligand binding pocket exclusively on the extracellular surface of the receptor (White *et al.*, 2012; Egloff *et al.*, 2014; Krumm *et al.*, 2015). The carboxy terminus of the peptide ligand interacts with two Arg residues near the extracellular surface. The Arg side-chains make hydrogen bonds with Tyr^{6.51} of the CWxPY motif. The Tyr^{6.51} forms hydrophobic stacking interactions with Phe^{7.42}, which mediates planar interactions with Trp^{6.48}. The Trp^{6.48} then makes hydrophobic stacking interactions with the conserved Phe^{6.44}, which is part of the hydrophobic core activation switch (Krumm *et al.*, 2015; Tehan *et al.*, 2014). This suggests a role for Trp^{6.48} in coupling extracellular agonist binding to the conserved activation switches of the NTSR1 receptor.

We propose that, like neurotensin, GnRH interacts largely with the extracellular domain of the GnRHR. This is supported by site-directed mutagenesis studies that have identified only residues that are extracellular to the conserved network as ligand contacts (Millar *et al.*, 2004; Mayevu *et al.*, 2015). Earlier reports proposed an interaction of Trp^{6.48} of the GnRHR with Trp³ of the GnRH ligand (Chauvin *et al.*, 2000; Davidson *et al.* 1994; Karges *et al.*, 2003). However, systematic mutagenesis of Trp^{6.48(280)} to Ala, His, Ser, Gln and Met affected only expression of mutant GnRHR proteins and had no effect on GnRH binding affinity or GnRH signalling (Coetsee *et al.*, 2006). A separate study showed that mutation of

Trp^{6.48(280)} to Phe had small effects on GnRH binding affinity, but had a larger effect on affinity of a small molecule antagonist that was predicted to bind more deeply within the TM bundle (Betz *et al.*, 2006). This supports the idea that ligands that bind more deeply within the TM bundle may contact the Trp^{6.48}, whereas the GnRH peptide probably does not (Betz *et al.*, 2006).

The residue at position 2.53 has not generally been considered to be a ligand contacting residue (Madabushi *et al.*, 2004; Rodriguez *et al.*, 2010). Of all the GPCRs crystallized up to 2013, only the human κ -opioid receptor showed direct contact of its ligand, an antagonist, with the residue in position 2.53 (Venkatakrishnan *et al.*, 2013; Wu *et al.*, 2012), which is also supported by site-directed mutagenesis studies (Vortherms *et al.*, 2007).

Mutagenesis studies suggest no direct contact of Glu^{2.53(90)} of the GnRHR with the GnRH peptide. Although, mutation of Glu^{2.53(90)} to Lys abolishes GnRHR function, when expression of the mutant receptor is rescued, its GnRH binding affinity is the same as in the wild type GnRHR (Tello *et al.*, 2012; Conn & Janovick, 2009; Chevrier *et al.*, 2011), as was the case for mutations of Trp^{6.48(280)} (Coetsee *et al.*, 2006). The unchanged GnRH binding affinities of GnRHRs with mutations of Glu^{2.53(90)} and Trp^{6.48(280)} suggest that neither of these residues directly contacts the ligand. Therefore it is likely that Glu^{2.53(90)} and Trp^{6.48(280)} have structural roles in the GnRHR that contribute to receptor expression, but they do not contact peptide ligands.

1.13.2. Overall fold and movement of TMs

The overall fold of a nanobody-stabilized β_2 -adrenergic receptor structure was nearly identical to the β_2 -adrenergic receptor in complex with the G_s protein, especially around the most highly conserved residues. This confirmed that the nanobody, Nb80, stabilized a fully active conformation of the β_2 -adrenergic receptor by effectively mimicking the structural

effects of G protein binding (Rasmussen *et al.*, 2011a; Rasmussen *et al.*, 2011b; Huang *et al.*, 2015).

Both the G_s-coupled and nanobody-stabilized active structures of the β_2 -adrenergic receptor showed outward movement of the cytosolic end of TM6 and repositioning of Tyr^{7.53(326)} of the NPxxY motif near Arg^{3.50(131)}, as was seen for rhodopsin (Rasmussen *et al.*, 2011a; Rasmussen *et al.*, 2011b; Ring *et al.*, 2013). The β_2 -adrenergic receptor was subsequently crystallized in complex with a different nanobody, Nb6B9, and three different agonists including the natural ligand, adrenaline (Ring *et al.*, 2013). Although the agonists in the Nb6B9-stabilized β_2 -adrenergic receptor structures were chemically diverse, the receptor structures showed similar overall folds. A notable similarity between the G_s- and nanobody-stabilized active β_2 -adrenergic receptor structures is that, as was the case for rhodopsin, there is no switch of the Trp^{6.48} side-chain rotamer (Shonberg *et al.*, 2014). In rhodopsin, the Trp^{6.48} shifts position because retinal pushes it aside during isomerization, whereas in the active β_2 -adrenergic receptor structures, Trp^{6.48} is shifted because the surrounding TMs “push” TM6 (Rasmussen *et al.*, 2011a; Rasmussen *et al.*, 2011b; Ring *et al.*, 2013). Although the G_s- and nanobody-stabilized β_2 -adrenergic receptor structures differ very slightly overall, the discrepancies noted are mostly at the cytosolic ends of TMs 5 and 6, because the receptors interact with different proteins, namely the G protein or the nanobody. Nevertheless, the structures are nearly identical around the highly conserved motifs, such as DRY and NPxxY at the cytosolic ends of TMs 3 and 7 respectively (Rasmussen *et al.*, 2011b).

Comparison of the inactive and active structures of the M₂ muscarinic receptor supports outward movement of TM6 and inward movement of TM7 that is similar to structures of rhodopsin and the β_2 -adrenergic receptor (Shonberg *et al.*, 2014). The high-resolution active μ -opioid receptor structure showed a similar outward movement of TM6 that was stabilized by a clearly defined water-mediated hydrogen bonding network (Huang *et al.*,

2015). Combined with NMR spectral studies, the increasing number of crystal structures suggests that the movements of TMs 5, 6 and 7 are conserved features of the activation process of rhodopsin-like GPCRs (Nygaard *et al.*, 2013; Shonberg *et al.*, 2014). Thus, although the agonist ligand binding pockets differ among class A GPCRs, the structures at the intracellular side of the receptors, which interact with the G proteins, are largely similar (Huang *et al.*, 2015; Sounier *et al.*, 2015).

1.13.3. Open ionic-lock

The active rhodopsin-like GPCRs typically display an open ionic-lock, as movement of TMs 3 and 6 away from each other breaks the salt-bridge between Arg^{3.50} and Glu^{6.30} that restrains the receptor in the inactive state (Rasmussen *et al.*, 2011a). It has been proposed that in addition to the ionic lock, a water-mediated hydrogen bonding network at the intracellular side of GPCRs stabilizes an inactive conformation and that binding of the G protein destabilizes this network to allow receptor activation (Huang *et al.*, 2015).

Like the active rhodopsin structures (Blankenship *et al.*, 2015), the high-resolution μ -opioid receptor structure shows that Arg^{3.50} interacts with Tyr^{5.58}, which hydrogen bonds via water to Tyr^{7.53} (Huang *et al.*, 2015). However, in the Nb80-stabilized β_2 -adrenergic receptor structure, this interaction is prevented by interaction of Arg^{3.50(131)} with the nanobody (Rasmussen *et al.*, 2011a). Subsequent nanobody-stabilized high resolution structures of the β_2 -adrenergic receptor, allowed clear mapping of many water molecules (Ring *et al.*, 2013). These active structures showed a water-mediated hydrogen bond between Tyr^{5.58(219)} and Tyr^{7.53(326)} as was seen in active rhodopsin (Shonberg *et al.*, 2014; Blankenship *et al.*, 2015). The G_s-stabilized β_2 -adrenergic receptor showed that Arg^{3.50(131)} forms parts of the open ionic-lock and also interacts with G_s (Rasmussen *et al.*, 2011b). However, the lower resolution of this structure did not allow mapping of many water molecules and the water-mediated hydrogen bond between Tyr^{5.58(219)} and Tyr^{7.53(326)} was not noted (Rasmussen *et al.*, 2011b).

Despite some variations, the active structures of rhodopsin-like GPCRs show interactions between highly conserved residues that are repositioned close enough to interact directly, or indirectly via water, because of the opening of the ionic lock (Blankenship *et al.*, 2015; Huang *et al.*, 2015; Romo *et al.*, 2010).

1.13.4. Water-mediated hydrogen bonding networks

As with rhodopsin, rhodopsin-like GPCRs have a network of polar interactions extending from the ligand binding pocket to the G protein-binding surface (Huang *et al.*, 2015). In contrast to the inactive structures, the active structures do not contain sodium ions. The movement of TMs 6 and 7 during activation changes the size of the sodium binding pocket such that the sodium ion is unable to bind within the confined space. The TM movement is thought to expel the sodium ion to the cytoplasmic side (Liu *et al.*, 2012; Katritch *et al.*, 2014). The agonist-bound NTSR1 neurotensin receptor structures show that Trp^{6.48(321)} forms water-mediated hydrogen bonds with other residues of the sodium ion binding pocket that occlude access of the sodium ion (Krumm *et al.*, 2015).

Like rhodopsin, the nanobody-stabilized active β_2 -adrenergic receptor showed a continuous water channel. It was proposed that the structural rearrangements of TMs during receptor activation are associated with changes in this water network that stabilize inactive and active states (Rasmussen *et al.*, 2011a). A detailed analysis of the water molecules in the high resolution structure of the active μ -opioid receptor showed that the water molecules interact predominantly with the highly conserved residues in rhodopsin-like GPCRs, connecting them via a hydrogen-bonding network. Low resolution structures of active GPCRs, in which water molecules could not be resolved, showed highly conserved residues in the same positions as they are in the high resolution active μ opioid receptor structure. Therefore, it is suggested that water molecules may be present and form a similar hydrogen-bonding network in all active GPCRs (Huang *et al.*, 2015). Notably, as in rhodopsin, the

highly conserved Trp^{6.48(293)} makes water-mediated contacts with other structurally important residues (Asp^{2.50(114)}, Asn^{3.35(150)}, Ser^{3.39(154)}) that contribute to activation of the μ -opioid receptor (Huang *et al.*, 2015). Based on this, the Trp^{6.48(280)} residue of the GnRHR may make water-mediated interactions with the equivalent residues Asn^{2.50(87)}, Ser^{3.35(124)} and Tyr^{3.39(126)}.

1.14. The relationship between the residue at the 2.53 locus and the conserved Trp^{6.48}

1.14.1. Class A GPCRs

90% of Class A GPCRs have an aromatic amino acid at position 6.48 and of these, 90% are Trp (Mirzadegan *et al.*, 2003). It is well established that the highly conserved Trp^{6.48} residue is important for GPCR function (Ahuja & Smith, 2009; Malherbe *et al.*, 2014; Trzaskowski *et al.*, 2012; Yuan *et al.*, 2014). Although, the amino acid at position 2.53 has not usually been considered a conserved residue, a high proportion of rhodopsin-like GPCRs (69%) have large and hydrophobic residues (Ile, Met and Val) at position 2.53, whereas acidic residues are rare (2%) (Mirzadegan *et al.*, 2003). Because the side-chain of the residue at position 2.53 is usually hydrophobic, it was not considered in analyses of conserved hydrophilic interactions (Venkatakrishnan *et al.*, 2013) or the conserved water-mediated hydrogen bond network (Huang *et al.*, 2015). However, evolutionary trace analysis identified Met^{2.53(86)} of rhodopsin among the top 20% of conserved residues in class A GPCRs. The analysis showed that Met^{2.53(86)} is not involved in any retinal-specific functions of rhodopsin, but it is likely to be important in all class A GPCRs (Madabushi *et al.*, 2004). Substituted cysteine accessibility method (SCAM) analysis of the D2 dopamine receptor showed that the Val^{2.53(83)} side-chain is oriented towards the water-accessible core of the TM bundle (Javitch *et al.*, 1999), where it may form intra-molecular interactions. Nuclear magnetic resonance (NMR) analysis of the β 2-adrenergic receptor showed that the environment of the Met^{2.53(86)} side-chain was different between active and inactive receptor conformations. It was suggested

that change in the environment of the Met^{2.53(86)} resulted from a change in its distance from Trp^{6.48(286)} (Kofuku *et al.*, 2012; Nygaard *et al.*, 2013). It is possible that an interaction between these residues that is present in the inactive, but absent from the active conformation of the β 2-adrenergic receptor causes repositioning of the side-chains. The changed environment may also reflect protonation of the nearby Asp^{2.50}, which is the counter ion of the sodium ion that stabilizes the inactive structure (Dror *et al.*, 2009; Ranganathan *et al.*, 2014; Liu *et al.*, 2012). It was proposed that Trp^{6.48} and Met^{2.53} are part of a conserved “ligand sensing” region of GPCRs where residues that are not part of the ligand binding pocket are allosterically coupled to ligand binding (Madabushi *et al.*, 2004). The observation by Deupi *et al.* that movement of Met^{2.53(86)} and Trp^{6.48(293)} in rhodopsin in response to retinal isomerization contributes to opening of the hydrophilic channel is consistent with this (Deupi *et al.*, 2012; Deupi, 2014). A follow-up evolutionary trace study found that mutation of Val^{2.53(83)} of the D2 dopamine receptor decreases dopamine binding affinity. It was concluded that Val^{2.53(83)} is “part of a set of less conserved residues outside of the ligand binding pocket that couple agonist binding to the highly conserved switch residues”. Specifically, Val^{2.53(83)} was proposed to communicate with Trp^{6.48} and/or Tyr^{6.51} of the CWxPY motif, via intramolecular water molecules (Rodriguez *et al.*, 2010). A recent analysis of conserved direct inter-helical interactions, including hydrophobic interactions in all GPCR crystals, showed that the residue at the 2.53 locus, regardless of the amino acid present, interacts directly with the residue at the 3.35 locus in TM3. It was also found that Trp^{6.48} makes conserved direct interactions with residues at the adjacent locus 3.36 and the 7.42 locus (Cvacek *et al.*, 2016). The conserved interactions of the residues at 2.53 and 6.48 respectively, with the neighbouring loci, 3.35 and 3.36, in TM3 of all GPCR crystals imply that the side-chains of the 2.53 and 6.48 residues must be positioned close to each other. The interactions with TM3 are present in both active and inactive structures, showing that changes in these interactions probably do not constitute

part of the conserved GPCR activation mechanism. However, these interactions are likely to have structural roles that affect receptor folding and the configuration of the ligand binding pocket (Cvicek *et al.* 2016; Madabushi *et al.* 2004). Thus, mutations of these residues may disrupt receptor expression, but may also distort the ligand binding surface, affecting affinity, or disrupt coupling to the conserved activation switches, affecting signalling. We reviewed the effects of mutagenesis of the residues in the 2.53 locus of class A GPCRs and the results are summarized in table 1.2. Mutagenesis of the locus 2.53 residue had varying effects in different receptors, including disrupting receptor expression, decreased agonist and/or antagonist binding affinity and constitutive activity in the case of the thyroid-stimulating hormone receptor. This indicates that the residue in the 2.53 locus of class A GPCRs has diverse roles in receptor folding and configuration of the ligand binding pocket, but is mostly not directly involved in ligand binding.

1.14.2. GnRHRs

Trp^{6.48} is 100% conserved in GnRHRs (Millar *et al.*, 2004) and was proposed to be a ligand-contacting residue that is directly involved in binding of peptide ligands (Söderhäll *et al.*, 2005; Chauvin *et al.*, 2001). However, systematic mutagenesis of the Trp^{6.48(280)} residue disrupted GnRHR expression, but had no effect on signalling or binding affinity (Betz *et al.* 2006; Coetsee *et al.*, 2006). This suggests that Trp^{6.48(280)} has a role in maintaining the structural stability of the GnRHR, but does not directly contact the ligand.

The Glu side-chain is a carboxylic (COOH) acid, which can be either negatively charged (COO⁻) or uncharged (COOH), depending on the pH of its microenvironment within the protein (Betts & Russell, 2007). It has been proposed that the Glu^{2.53(90)} residue of the GnRHR forms an intramolecular salt-bridge with the basic side-chain of Lys^{3.32(121)} in TM3 (Janovick *et al.*, 2011; Janovick *et al.*, 2009; Stewart *et al.*, 2012; Hoffmann *et al.*, 2000). It was suggested that the presence of the basic Lys side-chain in position 2.53(90) of the HH-

associated Glu^{2.53(90)}Lys GnRHR mutant disrupts receptor folding by disrupting the proposed intramolecular salt-bridge (Janovick *et al.*, 2011; Blumenröhr *et al.*, 2001; Stewart *et al.*, 2012; Janovick *et al.*, 2009; Tello *et al.*, 2012).

Although Glu^{2.53(90)} is conserved in mammalian type I GnRHRs, non-mammalian- and type II- GnRHRs have large hydrophobic residues (Val, Met or Leu) in position 2.53 (Millar *et al.*, 2004). Combined with presence of hydrophobic residues at position 2.53 in other rhodopsin-like GPCRs, this suggests that the carboxyl group of Glu^{2.53} may not be important in the function of GnRHRs. This is supported by a mutation of the Glu^{2.53(90)} residue in the mouse GnRHR to uncharged, Gln, which resulted in a mutant receptor that was indistinguishable from the wild type GnRHR (Flanagan *et al.*, 1994). Since the Glu^{2.53(90)}Gln GnRHR raises questions about the proposed salt-bridge interaction of the Glu^{2.53(90)}, it is unclear why the Glu^{2.53(90)}Lys mutation disrupts GnRHR folding.

This project has investigated the chemical properties of the Glu^{2.53(90)} side-chain that might explain the effects of the Glu^{2.53(90)}Lys and Glu^{2.53(90)}Gln mutations, using systematic site-directed mutagenesis of Glu^{2.53(90)}. Since crystal structures of class A GPCRs show conserved orientation of the side-chains at position 2.53 toward the hydrophilic centre of the TM bundle (Deupi, 2014; Deupi & Standfuss, 2011; Cvicek *et al.*, 2016), it is likely that the Glu^{2.53(90)} side-chain is oriented towards the core of the TM bundle of the inactive GnRHR. Proposals of a water-mediated interaction between the residue at the 2.53 locus and the conserved Trp^{6.48} in class A GPCRs (Deupi, 2014; Deupi & Standfuss, 2011), suggest that the side-chains of these residues may be oriented toward each other within the TM bundle of the GnRHR. This suggests that mutating these residues may disrupt GnRHR expression by disrupting the conserved water network. To test for a possible interaction of Glu^{2.53(90)} with Trp^{6.48(280)}, we also mutated the Trp^{6.48(280)} residue of the GnRHR. The more recent report that the residues at the 2.53 and 6.48 loci make conserved direct interactions (Cvicek *et al.*, 2016)

suggests that Glu^{2.53(90)} and Trp^{6.48(280)} may interact respectively with the Ser^{3.35(124)} and Met^{3.36(125)} residues of the GnRHR. This would also support roles for Glu^{2.53(90)} and Trp^{6.48(280)} in GnRHR structure and function. Mutagenesis of Ser^{3.35(124)} to Ala preserved wild type GnRHR function, whereas, mutation to Asp abolished receptor function (Betz *et al.*, 2006), suggesting that introduction of Asp instead of Ala in close apposition to Glu^{2.53(90)} may disrupt GnRHR expression. This proposal also places the side-chains of Glu^{2.53(90)} and Trp^{6.48(280)} within a shared environment via interaction with adjacent residues in TM3 of the GnRHR. This study has used additional site-directed mutagenesis of Trp^{6.48(280)} to assess its role in configuration of the ligand binding pocket of the GnRHR in addition to its structural role.

Thus, the aim of this study was to determine the role of the chemical properties of the Glu^{2.53(90)} side-chain in GnRHR structure and function, using systematic site-directed mutagenesis of Glu^{2.53(90)}. Trp^{6.48(280)} was also mutated to investigate a possible relationship with Glu^{2.53(90)} in the GnRHR. Mutant and wild type GnRHR constructs were transiently transfected into COS7 cells. Mutant receptors were assessed for inositol phosphate signaling to test functional capacity and radioligand binding assays were performed as a means of testing for receptor expression relative to wild type GnRHRs.

As summarized in table 3.1, we show that small amino acid substitutions for Glu^{2.53(90)} (Asn, Ser) abolish GnRH-stimulated IP production and ligand binding, suggesting disruption of GnRHR expression. Addition of a carboxy-terminal tail, which enhances Glu^{2.53(90)}Lys receptor expression, did not yield measurable IP production or ligand binding in these mutant GnRHRs. The Glu^{2.53(90)}Ala mutation, which removes the carboxyl side-chain of Glu^{2.53(90)} and introduces only a methyl group, abolished GnRHR function, which was only partially recovered by addition of the carboxy-terminal. A conservative substitution with Asp, which, like Glu, has a COOH side-chain, also completely abolished IP production and ligand binding, both in the absence and presence of a carboxy-terminal tail. This indicates that

presence of a COOH group does not stabilize GnRHR expression, showing that the conservative acidic Asp is less well tolerated than the basic Lys side-chain. In contrast, substitution of Glu^{2.53(90)} with large, uncharged (Phe, Leu, Gln) amino acids results in robust IP production and ligand binding with wild type-like GnRH affinity. The unchanged affinities show that Glu^{2.53(90)} is not part of the GnRHR ligand binding pocket. To confirm that Glu^{2.53(90)} does not form a salt-bridge with a positively-charged residue, we substituted Glu^{2.53(90)} with Arg. Surprisingly, the Arg substitution preserves GnRH-stimulated IP production, even without a carboxy-terminal tail. This shows that the GnRHR is well-expressed with a basic residue at the 2.53 locus. However, GnRH had lower potency and affinity suggesting that the Arg mutation distorts the ligand binding pocket. Based on proposals that the residue at the 2.53 locus of other GPCRs may interact with the conserved Trp^{6.48} via the water-mediated hydrogen bond network (Deupi, 2014), we mutated Trp^{6.48(280)} of the GnRHR. Substitution of Trp^{6.48(280)} with Arg also decreased GnRH potency and binding affinity, suggesting that this Arg side-chain also distorts the ligand binding pocket. Our results suggest that Glu^{2.53(90)} might not directly interact with Trp^{6.48(280)}, but that these two residues have a similar role in maintaining the overall GnRHR fold that contributes to stable cell surface expression of the receptor.

Table 1.2: Summary of the role of the residue at locus 2.53 in class A GPCRs

GPCR	Residue		Reference
β_2 -adrenergic	Met(82)	Not directly involved in ligand binding, NMR spectroscopy shows environment of 2.53 side-chain changes upon receptor activation	(Kofuku <i>et al.</i> , 2012) (Nygaard <i>et al.</i> , 2013)
Serotonin (5-hydroxytryptamine) 5HT _{2B}	Val(87)	Predicted to face the ligand binding pocket, Val ^{2.53} makes van der Waals interactions with two methyl groups of the subtype specific analogue, S-(+)-Norfenfluramine (SNF) Val ^{2.53} Ala mutation decreases SNF binding affinity 190-fold Val ^{2.53} Leu decreases SNF binding affinity 17-fold – MD simulations show a loss of one van der Waals interaction between 2.53 and an SNF methyl group owing to the loss of the bulky Val side-chain Val ^{2.53} Ile conservative mutation showed unchanged SNF binding affinity as the bulky side-chain maintains interactions with both SNF methyl groups as it does in the wild-type receptor	(Setola <i>et al.</i> , 2005)
Rat urotensin-II	Tyr100	SCAM mutation Tyr100Cys had no effect on receptor function, may be part of the ligand binding pocket, proposed interaction with 2 antagonists linking Tyr100 to orthosteric binding	(Sainsily <i>et al.</i> , 2013)
Tachykinin NK3	Met(134)	Ala mutant showed 53-fold decrease in binding affinity for antagonist, RO5328673 and abolished effect of another antagonist	(Malherbe <i>et al.</i> , 2014) (Malherbe <i>et al.</i> , 2008)
Rhodopsin	Met(86)	Side-chain movement resulting from a helix bend in TMD2 which is necessary for receptor activation, possible water-mediated interaction with Trp ^{6.48(265)}	(Deupi <i>et al.</i> , 2012) (Deupi, 2014)
Kappa-opioid (KOR)	Val(100)	Water accessible by SCAM analysis, may modulate orientation of residues within the ligand binding pocket to confer subtype selectivity of Salvinorin A for KOR over MOR and DOR Contacts the selective antagonist, JDTC	(Vortherms <i>et al.</i> , 2007) (Wu <i>et al.</i> , 2012)
Delta-opioid (DOR)	Ala(98)	Water inaccessible by SCAM analysis, may be obscured by large side-chain of a neighbouring residue	(Vortherms <i>et al.</i> , 2007)
Thyroid-stimulating hormone (TSHR)	Met(463)	Met ^{2.53} Val is a naturally-occurring mutant that may cause constitutive activity, mutant receptor expression was significantly lower than wildtype, phenotype of non-autoimmune hyperthyroidism due to gain-of-function mutation	(Fuhrer <i>et al.</i> , 2000)
M1 Muscarinic	Ile(74)	Ile ^{2.53} Ala showed reduced signalling capacity, nearly no effect on receptor expression and a small decrease in acetylcholine binding affinity, Ile ^{2.53} has a role in G protein coupling	(Bee & Hulme, 2007)

Dopamine (D2R)	Val(83)	Val ^{2.53} Leu showed impaired ligand binding, indicating that valine ^{2.53} contributes to recognition of Dopamine. This residue may also communicate with Trp ^{6.48} via water molecules Val ^{2.53} is water-accessible by SCAM analysis, placing it within a hydrophilic environment and may contact a subtype selective antagonist	(Rodriguez <i>et al.</i> , 2010) (Javitch <i>et al.</i> , 1999)
Neurokinin-1 (NK1R)	Met(81)	Met ^{2.53} thought to be part of the binding pocket, mutation to Ala showed no change in binding affinity for agonist (Substance P) or antagonist (CP-96,345). Not likely to be a ligand contacting residue	(Fong <i>et al.</i> , 1994)
Endothelin A	Tyr(129)	Mutations of Tyr ^{2.53} to Ala, His, Lys, Ser, Gln, Asn and Phe showed changes in affinity for some ligands but not others and partially decreased signalling, suggests that Tyr ^{2.53} confers subtype selective ligand binding	(Lee <i>et al.</i> , 1994) (Webb <i>et al.</i> , 1996) (Krystek <i>et al.</i> , 1994)
Mouse GnRHR	Glu(90)	Mutation of Glu ^{2.53} to the isosteric amide, Gln, showed no change in GnRH-stimulated signalling. Suggests that Gln can substitute for Glu ^{2.53} in the mouse GnRHR	(Flanagan <i>et al.</i> , 1994)
Human GnRHR	Glu(90)	Mutation of Glu ^{2.53} to Ala abolished ligand binding and signalling. Was proposed that Ala caused a distortion in the ligand binding pocket or in the overall receptor fold that decreased GnRHR expression	(Hoffmann <i>et al.</i> , 2000)

2. Chapter 2: Materials and methods

2.1. Site-directed mutagenesis

PCR-based whole plasmid site-directed mutagenesis was used to generate mutant GnRHRs. Mutagenic primers were designed to substitute Glu^{2.53(90)} with Ala, Arg, Asn, Asp, Phe, Gln, Leu, Lys and Ser (table 2.1). Mutating Glu to the small amino acid, Ala, which has only a methyl group, effectively removes the side-chain. Mutation to Asp preserves the carboxyl group of Glu^{2.53(90)}. Asn and Ser have shorter side-chains than Glu and are uncharged but can form hydrogen bonds. Gln is isosteric with Glu^{2.53(90)}, therefore the side-chain length is maintained but Gln has uncharged hydrogen-bonding groups instead of a carboxyl group. Whereas, Phe and Leu are similar in size to Glu, they have hydrophobic side-chains that do make hydrogen bonds and favor being buried within the protein core. The substitution of Glu with Lys replicates the naturally-occurring Glu^{2.53(90)}Lys mutant and the introduction of positively-charged Lys would be expected to cause a charge repulsion if Glu^{2.53(90)} interacts with Lys^{3.32(121)} in the wild type GnRHR. Substitution with Arg introduces a larger positively-charged side-chain than Lys that would be expected to have a similar effect to that of the Lys substitution. All mutagenic primers incorporated unique silent restriction enzyme sites for each mutant that allowed for identification of mutant DNA by restriction digest analysis (table 2.1).

Wild type human GnRHR cDNA cloned into pcDNA3 (10 ng; Invitrogen, Carlsbad, California) was used as template and mixed with mutagenic primers (0.3 μ M), dimethylsulfoxide (5%), deoxynucleotide triphosphate (dNTP) mix (0.3 mM each dNTP, KAPA Biosystems, Cape Town, South Africa) and high fidelity DNA polymerase enzyme (0.5 U, KAPA Biosystems) in a final reaction volume of 50 μ l of high fidelity PCR buffer (KAPA Biosystems).

Table 2.1: Primer sequences used to generate mutant GnRH receptors. Wild type GnRH receptor sense and antisense cDNA sequences are shown with the Glu^{2.53(90)} codon underlined. Sequences of mutagenic primers are shown with amino acid codons underlined and the silent restriction enzyme sites shaded.

GnRH receptor construct	Primer Sequences	Silent Restriction Enzyme	Digest ^a
Wild type	5' CCTTAGCCAACCTGTTGGAGACTCTGATTGTCATGCC3' 3' GGAATCGGTTGGACAACCTCTGAGACTAACAGTACGG5'	-	-
Glu ^{2.53(90)} Ala	5' CCTTAGCCAACCTGTTGGCCACTCTGATTGTCATGCC3' 5' GGCATGACAATCAGAGTGGCCAACAGGTTGGCTAAGG3'	<i>MscI</i> 5'TGG↓CC3'	4363, 2283
Glu ^{2.53(90)} Asn	5' CCTTAGCCAACCTGCTGAACACTCTGATTGTCATGCC3' 5' GGCATGACAATCAGAGTGTTTCAGCAGGTTGGCTAAGG3'	<i>BveI</i> 5'ACCTGC(N)4 ↓3'	4893, 922, 450, 381
Glu ^{2.53(90)} Asp	5' CCTTAGCCAACCTTCTAGACACTCTGATTGTCATGCC3' 5' GGCATGACAATCAGAGTGTCTAGAAGGTTGGCTAAGG3'	<i>XbaI</i> 5'T↓CTAGA3'	5726, 920
Glu ^{2.53(90)} Phe	5' CCTTAGCCAACCTGCTGTTCACTCTGATTGTCATGCC3' 5' GGCATGACAATCAGAGTGAACAGCAGGTTGGCTAAGG3'	<i>BveI</i> 5'ACCTGC(N)4 ↓3'	4893, 922, 450, 381
Glu ^{2.53(90)} Gln	5' CCTTAGCCAACCTGCTGCAGACTCTGATTGTCATGCC3' 5' GGCATGACAATCAGAGTCTGCAGCAGGTTGGCTAAGG3'	<i>PstI</i> 5'CTGCA↓G3'	4123, 2240, 283
Glu ^{2.53(90)} Leu	5' CCTTAGCCAACCTGCTGTTAACTCTGATTGTCATGCC3' 5' GGCATGACAATCAGAGTTAACAGCAGGTTGGCTAAGG3'	<i>HpaI</i> 5'GTT↓AAC3'	6646
Glu ^{2.53(90)} Lys	5' CCTTAGCCAACCTGCTTAAGACTCTGATTGTCATGCC3' 5' GGCATGACAATCAGAGTCTTAAGCAGGTTGGCTAAGG3'	<i>AflIII</i> 5'C↓TTAAG3'	3567, 2077, 1002
Glu ^{2.53(90)} Arg	5' CCTTAGCCAACCTGTTGCGCACTCTGATTGTCATGCC3' 5' GGCATGACAATCAGAGTGCAGCAACAGGTTGGCTAAGG3'	<i>NsbI</i> 5'TGC↓GCA3'	2361, 2303, 1982
Glu ^{2.53(90)} Ser	5' CCTTAGCCAACCTGCTGTCGACTCTGATTGTCATGCC3' 5' GGCATGACAATCAGAGTCGACAGCAGGTTGGCTAAGG3'	<i>SalI</i> 5'G↓TCGAC3'	3193, 2188, 1198, 67
Trp ^{6.48(280)} Ala	5' TCATTTACTGTCTGCGCAACTCCCTACTATGTC3' 5' GACATAGTAGGGAGTTGCGCAGACAGTAAATGA3'	<i>NsbI</i> 5'TGC↓GCA3'	2537, 2361, 1748
Trp ^{6.48(280)} Arg	5' TCATTTACTGTCTGCAGAACTCCCTACTATGTC3' 5' GACATAGTAGGGAGTTCTGCAGACAGTAAATGA3'	<i>PstI</i> 5'CTGCA↓G3'	2523, 1670, 853

^aDigest, DNA fragment lengths generated by digestion with specific silent restriction enzymes in the presence of the mutation. Except for *HpaI*, all restriction enzymes digest either the GnRHR gene or the vector in addition to the restriction site introduced by the mutation

After an initial denaturation step (5 min, 95°C), DNA was amplified for 16 cycles (98°C for 20 seconds, 63°C for 15 seconds and 72°C for 7 min) followed by a final extension step (72°C for 5 min) (T100 Thermal Cycler, Biorad, Hercules, California). *DpnI* restriction endonuclease (2 U, Thermo Scientific, Waltham, Massachusetts) was added directly to the PCR reaction and incubated (37°C, overnight). *DpnI* digests only methylated template DNA, while unmethylated mutant PCR products remain intact.

DpnI-digested PCR products (3 µl) were transformed into competent *JM109 E. coli* cells (100 µl) and cultured on Luria agar plates with ampicillin (1 µg/ml, Sigma Aldrich, Saint Louis, Missouri). Colonies were cultured in Luria broth with ampicillin (37°C, 16 hours) for small scale plasmid DNA isolation. Cultures were centrifuged to pellet cells (1500 g, 10 min) and dispersed into resuspension buffer (50 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid (EDTA) pH8, 10 mg/ml RNase A), before mixing with lysis buffer (100 mM NaOH, 10% sodium dodecyl sulphate (SDS)) to release the DNA. Cell lysates were neutralized (3 M potassium acetate, pH 5.5), incubated on ice (5 min) and centrifuged (13500 g, 10 min). Supernatants were mixed with silica (5% suspension in water, Sigma Aldrich, Saint Louis, Missouri), incubated (5 minutes on ice) and centrifuged (13500 g, 1 min). Supernatants were discarded and silica pellets were resuspended in wash buffer (10 mM Tris-HCl pH 8, 100 mM NaCl, 2.5 mM EDTA, 55% ethanol) and centrifuged (13500 g, 1 min). DNA was eluted from the silica in Tris-EDTA buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) at 55°C. Restriction digest analysis was performed to identify mutant DNA. Mutant constructs were sequenced (Inqaba Biotechnologies, Johannesburg, South Africa) to confirm the presence of intended mutations and the absence of unintended PCR errors.

Wild type and mutant receptors were subcloned into the *EcoRI* and *XhoI* sites of the improved pcDNA3.1+ expression vector (Invitrogen, Carlsbad, California). Subcloning served to exclude any PCR errors in the parent vector that might compromise the

experimental data. It has previously been found that appending a catfish carboxy-terminal tail enhances expression of both wild type and mutant human GnRHRs (Janovick *et al.*, 2003). Appending a human type II GnRHR carboxy-terminal has been shown to enhance expression of wild type human GnRHRs (Flanagan *et al.*, 2000). Therefore to enhance expression of poorly-expressed Glu^{2.53(90)} mutant GnRHRs, these GnRHRs were subcloned into the *EcoRI* and *EcoNI* sites of a GnRHR construct with a human type II GnRHR carboxy-terminal domain (Flanagan *et al.*, 2000) as a means of appending a carboxy-terminal tail.

The Trp^{6.48(280)} mutant GnRHRs were generated using overlap extension PCR because the annealing temperatures of the mutagenic primer pairs for the Trp^{6.48(280)} mutations were different, making it nearly impossible to amplify the complementary primers in a single PCR reaction. The overlap extension PCR involved three separate rounds of cycling. For each Trp^{6.48(280)} GnRHR mutant, two separate PCR reactions were performed with a vector primer and the corresponding mutagenic primer (T7 primer and the reverse mutagenic primer = PCR1, BGH primer and the forward mutagenic primer = PCR2). Plasmids containing wild type human GnRHR cDNA or the wild type GnRHR with the human type II GnRHR carboxy-terminal domain were used as templates. Template DNA (10ng) was mixed with mutagenic primers (0.3 μ M), vector primers (T7 or BGH, 0.3 μ M), dimethylsulfoxide (5%), dNTP mix (0.3 mM each dNTP) and high fidelity DNA polymerase enzyme (0.5 U) in a final reaction volume of 50 μ l of high fidelity PCR buffer. After an initial denaturation step (5 min, 95°C), DNA was amplified for 30 cycles (98°C for 20 seconds, 55°C for PCR1 or 62°C for PCR2 for 15 seconds and 72°C for 2 min) followed by a final extension step (72°C for 5 min). PCR products were subjected to electrophoresis (1% agarose gel at 65V for 1 hour) and extracted from the gel using a gel preparation kit (Favorgen, Biocom Biotech) and used as templates for the subsequent overlap PCR reaction.

PCR1 extract (3µl) was combined with PCR2 extract (2µl) and mixed with dNTP mix (0.3 mM each dNTP) and high fidelity DNA polymerase enzyme (0.5 U), but no primers, in a final reaction volume of 20 µl of high fidelity PCR buffer and nuclease-free water. After an initial denaturation step (5 min, 94°C), PCR products were extended for 5 cycles (94°C for 30 seconds, 55°C for 20 min and 72°C for 2.5 min) and held at 4°C. Vector primers (T7 and BGH, 0.3 µM), dimethylsulfoxide (5%), dNTP mix (0.3 mM each dNTP), high fidelity PCR buffer and high fidelity DNA polymerase enzyme (0.5 U) were added to each reaction to give a final reaction volume of 25 µl. After an initial denaturation step (30 seconds, 95°C), DNA was amplified for 35 cycles (95°C for 30 seconds, 50°C for 30 seconds, 72°C for 5 min) followed by a final extension step (72°C for 10 min). These PCR products were electrophoresed (1% agarose gel at 65V for 1 hour), excised and extracted from the gel to be ligated and cloned into the *EcoRI* and *XhoI* sites of the pcDNA3.1+vector. This resulted in two sets of Trp^{6.48(280)} mutant GnRHRs, with and without a carboxy-terminal tail, depending on the template DNA used. All subcloned wild type and mutant receptor DNA constructs were then prepared for functional assays by large scale plasmid isolation (PureYield™ plasmid maxiprep system, Promega, Madison, Wisconsin).

2.2. Cell culture and transfection

COS7 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Biowest, France) supplemented with 10 % foetal bovine serum (FBS, Thermo Scientific, Waltham, Massachusetts) and Pen-Strep (0.5mg/ml penicillin, 0.1mg/ml streptomycin) at 37°C in 10 % CO₂. Wild type and mutant GnRH receptor constructs were transiently transfected into COS7 cells, which do not naturally express GnRH receptors. A transfection complex was prepared by mixing 5 µg of DNA, 5 µl of Plus Reagent™ and 20 µl of Lipofectamine® LTX (Invitrogen, Carlsbad, California) in 500 µl of HEPES (20 mM)-buffered DMEM (HEPES-DMEM, pH7.4). The transfection complex was incubated at room

temperature for 30 minutes and added to COS7 cells in a 100 mm dish containing 4 ml of culture medium. Cells were incubated at 37°C in 10 % CO₂ for 24 hours. Transfected cells were detached from transfection dishes with PBS-EDTA (10 mM EDTA, 1 x PBS), pelleted by centrifugation (500 g, 3 minutes), plated into 12-well plates and cultured overnight (37°C in 10 % CO₂).

2.3. IP accumulation assays

Production of IP was used to assess the ability of wild type and mutant GnRH receptors to mediate GnRH-stimulated signaling essentially as previously described (Millar *et al*, 1995). Transfected cells were radiolabelled with [³H]-myoinositol (1 µCi/ml, 1ml/well) (Perkin Elmer, Boston, Massachusetts) in DMEM supplemented with FBS (2 %) overnight. Labeling medium was aspirated and cells were pre-incubated with pre-warmed buffer I (140 mM NaCl, 4 mM KCl, 8.6 mM glucose, 1 mM CaCl₂, 1 mM MgCl₂, 0.1 % bovine serum albumin (BSA), 10 mM LiCl, pH 7.4; 1 ml/well, 37°C, 15 minutes). Cells were stimulated by incubation in the absence or presence of various concentrations (10⁻¹¹ M, 10⁻¹⁰ M, 10⁻⁹ M, 10⁻⁸ M, 10⁻⁷ M, 10⁻⁶ M) of GnRH (37°C, 60 min) in buffer I. The incubation was terminated by replacing the medium with formic acid (10 mM, 1 ml, 4°C) and incubation (4°C, 30 min) to lyse the cells. Hypotonic lysis releases the cytoplasmic cellular contents including the radiolabeled [³H]-IP. Cell extracts were applied to Dowex-1 resin (1 ml; Sigma Aldrich, Saint Louis, Missouri) in 10 ml chromatography columns (Biorad, Hercules, California) prewashed with 3 M ammonium formate/0.1 M formic acid (3 ml) and distilled water (10 ml). After washing with water (10 ml) and 0.1 M formic acid (5 ml), bound [³H]-IP was eluted with 1 M ammonium formate/0.1M formic acid (3 ml). Eluates were collected in scintillation fluid (3 ml, Optiphase Hisafe III, Perkin Elmer, Boston, Massachusetts) and counted by liquid scintillation spectroscopy (Tri-Cab Liquid Scintillation Analyzer, United Technologies Packard, Paolo Alto, California).

2.4. Radioligand competition binding assays

2.4.1. Radio-iodination of [His⁵,D-Tyr⁶]-GnRH

The high affinity GnRH analogue, [His⁵,D-Tyr⁶]-GnRH, was radio-labelled and used as a tracer in ligand binding assays. The chloramine T iodination method (Flanagan *et al*, 1998) was adapted. [His⁵,D-Tyr⁶]-GnRH peptide (5 µg) was incubated in NaH₂PO₄ (0.5 M, 25 µl, pH 7.4) with NaI¹²⁵ (1 mCi; Perkin Elmer) and chloramine T (10 µl, 85 nM in 0.5 M NaH₂PO₄) for 10 seconds. The reaction was stopped by addition of sodium metabisulfite (50 µl, 85 nM in 0.5 M NaH₂PO₄). The iodinated peptide was separated from free I¹²⁵ by QAE Sephadex purification. QAE Sephadex A25 (Sigma Aldrich, Saint Louis, Missouri) and Sephadex G25 (Sigma Aldrich, Saint Louis, Missouri) beads were presoaked in water overnight. The top of a 10 ml serological plastic pipette was cut off and the tip was plugged with glass wool to act as a disposable chromatography column. The column was prepared by pouring and packing ~10 ml of QAE Sephadex A25 suspension followed by ~2 ml of G25 Sephadex applied on top and was equilibrated with 0.1 M NH₄HCO₃ to pH 9.2. The iodination mixture was applied to the column and eluted with 0.1 M NH₄HCO₃ (pH 9.2). 1 ml fractions of eluate were collected into glass tubes containing 10 µl 10% BSA and 400 µl 0.1 M acetic acid. 10 µl of these fractions were transferred to 1ml of 0.1 M NH₄HCO₃ (pH 9.2) and 100 µl of these mixtures were counted using a gamma counter (Perkin Elmer, Wizard 2.1 sample detector). Fractions with highest radioactivity were aliquoted, frozen (-70°C) and tested for specific binding. Fractions with the highest specific binding were used in competition binding assays.

2.4.2. Competition binding assay

Transfected cells were washed with 1 ml cold binding buffer (HEPES-DMEM with 0.1 % BSA; pH 7.4) and incubated (4°C, 5 hours) with ¹²⁵I-[His⁵,D-Tyr⁶]-GnRH (~100 000 cpm) in the absence or presence of various concentrations (10⁻¹⁰ M, 10⁻⁹ M, 10⁻⁸ M, 10⁻⁷ M,

10^{-6} M) of unlabelled GnRH or unlabelled [His⁵,D-Tyr⁶]-GnRH for homologous competition in a final volume of 500µl binding buffer. The incubation was terminated by washing 3 times with cold PBS to remove unbound radioligand and bound radioligand was collected from plates by solubilization of cells with 0.1 M NaOH (1 ml/ well). The radioactivity was counted in a gamma counter.

2.5. Data analysis

IP production and radioligand binding assays were performed at least 3 times in duplicate for each GnRHR mutant and the wild type GnRHR was included in every assay. For the IP assays, maximal response (E_{max}) and effective concentrations for 50% of maximal response (EC_{50}) were determined from sigmoidal dose-response curves fitted to experimental data sets using non-linear regression (GraphPad Prism software, version 5, La Jolla, California). Similarly, total binding in the absence of unlabelled ligand (B_0) and 50% inhibitory concentrations (IC_{50}) for radioligand binding assays were determined using non-linear regression. pEC_{50} and pIC_{50} values were calculated as negative logarithms of EC_{50} and IC_{50} values respectively. This transformed the mean $\pm$ SD data to a normal distribution that could be used for group comparison statistical analysis (Flanagan, 2016). A one-way analysis of variance with post-hoc Tukey's test was used to determine whether pEC_{50} and pIC_{50} values for each mutant GnRHR differed from the values of the wild type GnRHRs.

3. Chapter 3: Results

3.1. Confirmation that the naturally-occurring Glu^{2.53(90)}Lys mutant GnRHR is non-functional

COS7 cells transfected with wild type human GnRHR showed robust GnRH-stimulated IP production with an EC₅₀ value of 0.26 ± 0.31 nM, whereas vector-transfected cells showed no IP response to GnRH stimulation (figure 3.1, table 3.1). This result confirms expression of the wild type human GnRHR in COS7 cells and coupling of the receptor to IP signalling. In contrast, COS7 cells transfected with the Glu^{2.53(90)}Lys mutant GnRHR showed no detectable GnRH-stimulated IP production (figure 3.1, table 3.1). Consistent with the signalling results, the wild type GnRHR displayed high total ligand binding (figure 3.1, table 3.1), and the Glu^{2.53(90)}Lys mutant GnRHR showed no detectable binding. The lack of detectable binding indicates minimal cell surface expression of the Glu^{2.53(90)}Lys mutant GnRHR.

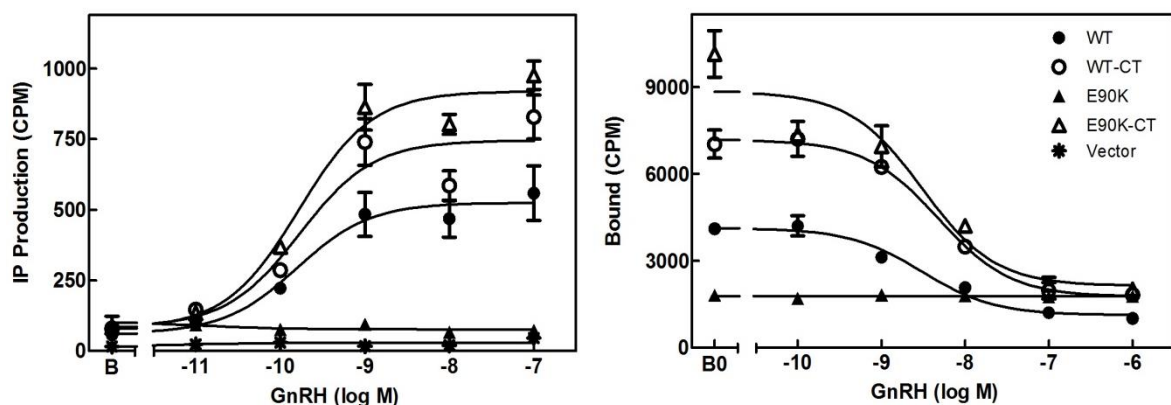


Figure 3.1: GnRH-stimulated IP accumulation and competition binding in COS7 cells expressing wild type and Glu^{2.53(90)}Lys GnRHRs. COS7 cells transfected with wild type (WT, ●), Glu^{2.53(90)}Lys (E90K, ▲), tailed wild type (WT-CT, ○) or tailed Glu^{2.53(90)}Lys (E90K-CT, △) GnRHRs were treated with increasing concentrations of GnRH before IP extraction (Left panel) or cells were incubated with ¹²⁵I-[His⁵,D-Tyr⁶]-GnRH in the presence of increasing concentrations of GnRH and bound radioactivity was counted (Right panel). 'B' represents basal IP production in the absence of ligand. 'B0' represents total binding in the absence of unlabelled or competing ligand. Data are from a single experiment, representative of experiments that were performed at least three times in duplicate.

3.2. Rescue of Glu^{2.53(90)}Lys mutant GnRHR expression and function by appending the Type II GnRHR carboxy-terminal tail

Appending the human Type II GnRHR carboxy-terminal tail to the wild type GnRHR (see methods) increased the maximum GnRH-stimulated IP production in most experiments, but

had no significant effect on GnRH potency (EC_{50} , 0.21 ± 0.25 nM vs 0.26 ± 0.31 nM for the untailed wild type GnRHR, figure 3.1, table 3.1). Appending the carboxy-terminal tail to the wild type GnRHR increased total binding of I^{125} -[His⁵,D-Tyr⁶]-GnRH (figure 3.1) without changing affinity for GnRH (IC_{50} , 4.76 ± 1.35 nM vs 3.33 ± 1.26 nM for the untailed wild type GnRHR, figure 3.1, table 3.1). This is consistent with enhanced receptor expression as previously described (Flanagan *et al.*, 2000). Similar findings were reported when the carboxy-terminal tail of a catfish GnRHR was appended to wild type and several mutant GnRHRs (Janovick *et al.*, 2003). COS7 cells expressing the Glu^{2.53(90)}Lys mutant GnRHR with the carboxy-tail showed detectable binding of I^{125} -[His⁵,D-Tyr⁶]-GnRH and GnRH-stimulated IP production similar to levels of the untailed wild type GnRHR (figure 3.1, table 3.1), which is consistent with enhanced expression and functional rescue of this mutant receptor as previously described (Flanagan *et al.*, 2000; Janovick *et al.*, 2009; Maya-Nuñez *et al.*, 2002). The affinity of the rescued Glu^{2.53(90)}Lys mutant GnRHR with the carboxy-tail was unchanged from wild type (IC_{50} , 3.2 ± 0.05 nM vs 3.3 ± 1.26 nM for the untailed wild type GnRHR, figure 3.1, table 3.1), as previously described (Tello *et al.*, 2012; Söderlund *et al.*, 2001).

3.3. IP signalling and ligand binding of mutant GnRHRs with small amino acid substitutions for Glu^{2.53(90)}

COS7 cells expressing mutant GnRHRs in which Glu^{2.53(90)} was substituted with Ala, Ser and Asn showed no detectable GnRH-stimulated IP production (Figure 3.2, table 3.1) and no detectable I^{125} -[His⁵,D-Tyr⁶]-GnRH binding (figure 3.2, table 3.1). The mutant receptor with the conservative, acidic, Asp substitution for Glu^{2.53(90)} also showed no detectable GnRH-stimulated IP production (figure 3.2, table 3.1) and no detectable I^{125} -[His⁵,D-Tyr⁶]-GnRH binding (figure 3.2, table 3.1). This indicates that the negatively-charged carboxyl group of the Asp sidechain is not sufficient to substitute for the native Glu^{2.53(90)} in expression of the GnRHR. Appending the carboxy-tail to the Glu^{2.53(90)}Ala mutant GnRHR resulted in

measurable IP production and binding ($E_{\max}$, 35 ± 15 % and B_0 , 38 ± 7 % of untailed wildtype GnRHR, figure 3.2, table 3.1). Potency was not different from the untailed wildtype GnRHR (EC_{50} , 0.10 ± 0.09 nM vs 0.26 ± 0.31 nM for the untailed wild type GnRHR, figure 3.2, table 3.1), but affinity of the tailed Glu^{2.53(90)}Ala mutant GnRHR was reduced (IC_{50} , 19.95 ± 0.01 nM vs 3.33 ± 1.26 nM for the untailed wild type GnRHR, figure 3.2, table 3.1). In contrast, the Glu^{2.53(90)}Ser, Glu^{2.53(90)}Asn and Glu^{2.53(90)}Asp mutant GnRHRs with carboxy-tails showed no measurable IP production or total ¹²⁵I-[His⁵,D-Tyr⁶]-GnRH binding (figure 3.2, table 3.1). The addition of the carboxy-terminal tail rescued expression of the Glu^{2.53(90)}Ala mutant GnRHR but not the expression of mutant GnRHRs with small and polar side-chains, suggesting that these mutations may introduce destabilizing interactions that cannot be overcome by the carboxy-terminal tail.

Table 3.1: GnRH-stimulated IP production and competition binding of COS7 cells transfected with wild type and mutant GnRHRs. Data are means $\pm$ SD of the indicated numbers of experiments performed in duplicate. All experiments included the wild type GnRHR and tailed wild type GnRHR.

GnRH Receptor	IP Production			Competition Binding		
	EC ₅₀ (nM)	pEC ₅₀	E _{max} (% WT)	IC ₅₀ (nM)	pIC ₅₀	B0 (% WT)
Wild type	0.26 $\pm$ 0.31	9.58 $\pm$ 0.64 (n=19)	100	3.33 $\pm$ 1.26	8.59 $\pm$ 0.26 (n=13)	100
Wild type-CT ^a	0.21 $\pm$ 0.25	9.89 $\pm$ 0.41 (n=17)	146 $\pm$ 43	4.76 $\pm$ 1.35	8.34 $\pm$ 0.14 (n=13)	200 $\pm$ 61
Glu ^{2.53(90)} Lys	-	- (n=3)	nms ^b	-	- (n=3)	nmb ^c
Glu ^{2.53(90)} Lys-CT	0.14 $\pm$ 0.14	9.97 $\pm$ 0.15 (n=3)	132 $\pm$ 48	3.2 $\pm$ 0.05	8.50 $\pm$ 0.01 (n=3)	185 $\pm$ 35
Glu ^{2.53(90)} Ala	-	- (n=4)	nms	-	- (n=4)	nmb
Glu ^{2.53(90)} Ala-CT	0.10 $\pm$ 0.09	9.80 $\pm$ 0.20 (n=3)	35 $\pm$ 15	19.95 $\pm$ 0.01	7.70 $\pm$ 0.3* (n=3)	38 $\pm$ 7
Glu ^{2.53(90)} Asp	-	- (n=4)	nms	-	- (n=4)	nmb
Glu ^{2.53(90)} Asp-CT	-	- (n=4)	nms	-	- (n=4)	nmb
Glu ^{2.53(90)} Ser	-	- (n=3)	nms	-	- (n=3)	nmb
Glu ^{2.53(90)} Ser-CT	-	- (n=3)	nms	-	- (n=3)	nmb
Glu ^{2.53(90)} Asn	-	- (n=3)	nms	-	- (n=3)	nmb
Glu ^{2.53(90)} Asn-CT	-	- (n=3)	nms	-	- (n=5)	nmb
Glu ^{2.53(90)} Gln	0.23 $\pm$ 0.06	9.70 $\pm$ 0.1 (n=3)	84 $\pm$ 18	5.04 $\pm$ 2.19	8.33 $\pm$ 0.28 (n=3)	58 $\pm$ 21
Glu ^{2.53(90)} Gln-CT	0.27 $\pm$ 0.31	9.80 $\pm$ 0.56 (n=3)	148 $\pm$ 106	3.34 $\pm$ 1.44	8.5 $\pm$ 0.21 (n=3)	107 $\pm$ 10
Glu ^{2.53(90)} Phe	0.27 $\pm$ 0.12	9.51 $\pm$ 0.15 (n=3)	110 $\pm$ 51	2.97 $\pm$ 0.40	8.53 $\pm$ 0.10 (n=3)	76 $\pm$ 4
Glu ^{2.53(90)} Phe-CT	0.30 $\pm$ 0.20	9.63 $\pm$ 0.35 (n=3)	160 $\pm$ 34	2.99 $\pm$ 0.51	8.50 $\pm$ 0.15 (n=3)	252 $\pm$ 9
Glu ^{2.53(90)} Leu	0.19 $\pm$ 0.21	9.70 $\pm$ 0.35 (n=6)	100 $\pm$ 34	4.39 $\pm$ 1.07	8.37 $\pm$ 0.11 (n=6)	108 $\pm$ 85
Glu ^{2.53(90)} Leu-CT	0.23 $\pm$ 0.24	10.0 $\pm$ 0.61 (n=3)	119 $\pm$ 7	2.83 $\pm$ 1.03	8.50 $\pm$ 0.10 (n=3)	119 $\pm$ 20
Glu ^{2.53(90)} Arg	80.3 $\pm$ 7.60	7.13 $\pm$ 0.60* (n=6)	64 $\pm$ 26	82.1 $\pm$ 0.09	7.08 $\pm$ 0.17* (n=6)	25 $\pm$ 8
Glu ^{2.53(90)} Arg-CT	7.47 $\pm$ 7.39	8.27 $\pm$ 0.40* (n=7)	121 $\pm$ 70	79.4 $\pm$ 0.02	7.10 $\pm$ 0.02* (n=7)	58 $\pm$ 4
Trp ^{6.48(280)} Ala	-	- (n=8)	nms	-	- (n=8)	nmb
Trp ^{6.48(280)} Ala-CT	0.21 $\pm$ 0.14	9.82 $\pm$ 0.33 (n=5)	80 $\pm$ 18	4.39 $\pm$ 1.07	8.43 $\pm$ 0.12 (n=5)	96 $\pm$ 13
Trp ^{6.48(280)} Arg	-	- (n=6)	nms	-	- (n=6)	nmb
Trp ^{6.48(280)} Arg-CT	53.3 $\pm$ 46.9	7.40 $\pm$ 0.37* (n=7)	64 $\pm$ 15	27.73 $\pm$ 6.74	7.57 $\pm$ 0.11* (n=7)	97 $\pm$ 30

*Significantly different from wild type GnRHR

p<0.05; ^a-CT, Carboxy terminal-tail appended

^bnms, no measurable stimulation

^cnmb, no measurable binding

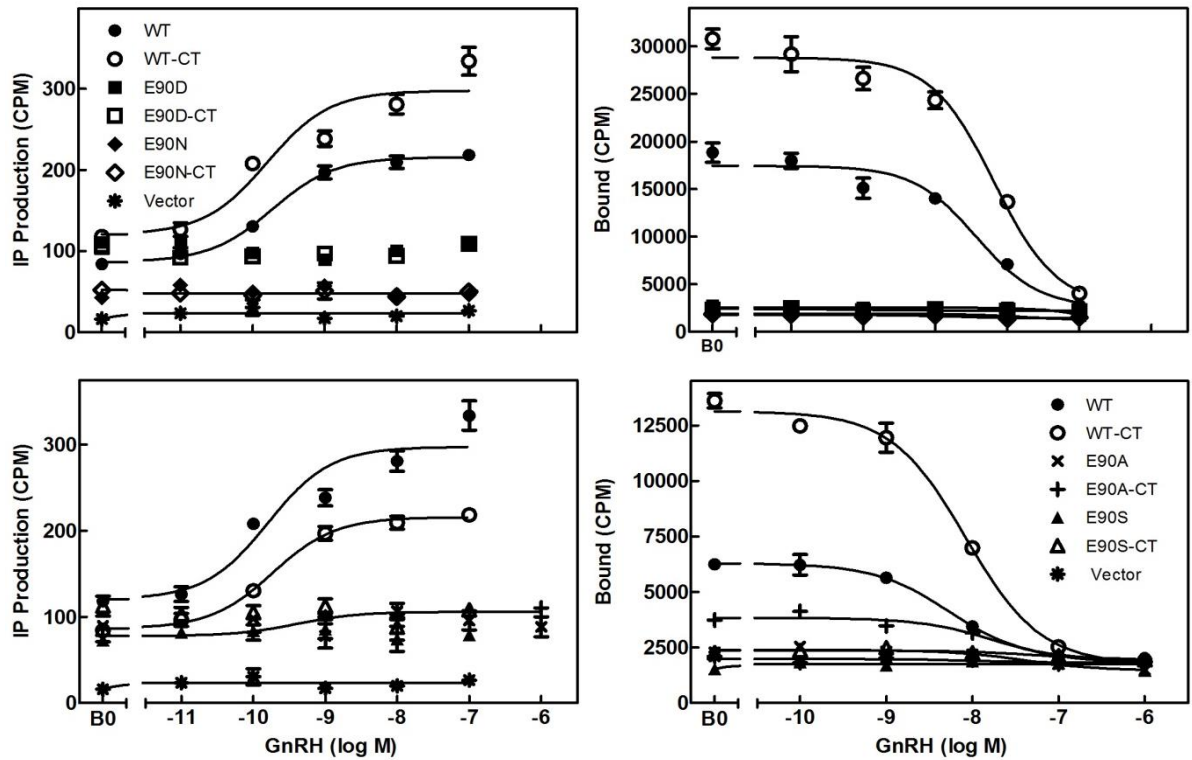


Figure 3.2: GnRH-stimulated IP accumulation and competition binding in COS7 cells expressing mutant GnRHRs with small amino acid substitutions for Glu^{2.53(90)}. COS7 cells transfected with wild type (WT, ●), tailed wild type (WT-CT, ○), Glu^{2.53(90)}Ala (E90A, x), tailed Glu^{2.53(90)}Ala (E90A-CT, +), Glu^{2.53(90)}Asp (E90D, ■), tailed Glu^{2.53(90)}Asp (E90D-CT, □), Glu^{2.53(90)}Asn (E90N, ◆), tailed Glu^{2.53(90)}Asn (E90N-CT, ◇), Glu^{2.53(90)}Ser (E90S, ▲) or tailed Glu^{2.53(90)}Ser (E90S-CT, △) GnRHRs were treated with increasing concentrations of GnRH before IP extraction (Left panel) or cells were incubated with ¹²⁵I-[His⁵,D-Tyr⁶]-GnRH in the presence of increasing concentrations of GnRH and bound radioactivity was counted (Right panel). Data are from a single experiment, representative of experiments that were performed at least three times in duplicate.

3.4. IP signalling and ligand binding of mutant GnRHRs with large, uncharged amino acid substitutions for Glu^{2.53(90)}

COS7 cells expressing mutant GnRHR in which Glu^{2.53(90)} was substituted with the isosteric Gln residue showed GnRH-stimulated IP production with similar potency to that of the wild type GnRHR (EC_{50} , 0.23 ± 0.06 nM vs 0.26 ± 0.31 nM for the wild type GnRHR, figure 3.3, table 3.1) and bound ¹²⁵I-[His⁵,D-Tyr⁶]-GnRH without any change in affinity (IC_{50} , 5.04 ± 2.19 nM vs 3.33 ± 1.26 nM for the wild type GnRHR, figure 3.3, table 3.1). This is consistent with previously reported function of the Glu^{2.53(90)}Gln mouse GnRHR (Flanagan et al. 1994). The isosteric Gln differs from Glu only in having an amide (CONH₂) rather than a carboxyl group (COO⁻) in its sidechain. The unchanged function of the Glu^{2.53(90)}Gln mutant GnRHR suggests that the negative charge of the Glu^{2.53(90)} is not necessary for GnRHR expression or function. COS7 cells expressing the Glu^{2.53(90)}Gln mutant GnRHR with the carboxy-terminal

tail showed minimal change to maximum IP production and total ^{125}I -[His⁵,D-Tyr⁶]-GnRH binding compared with the untailed mutant GnRHR (figure 3.3, table 3.1).

COS7 cells expressing mutant GnRHRs in which Glu^{2.53(90)} was substituted with the large hydrophobic Phe or Leu residues showed GnRH-stimulated IP production with E_{max} values similar to wildtype (figure 3.4, table 3.1). GnRH potency and receptor affinity for GnRH remained unchanged (figure 3.4, table 3.1). This suggests that the hydrophilic properties of the native Glu^{2.53(90)} sidechain might not be necessary for GnRHR expression or function. The Glu^{2.53(90)}Phe and Glu^{2.53(90)}Leu GnRHRs with the carboxy-terminal tail showed increased maximum GnRH-stimulated IP production (E_{max} , $160 \pm 34 \%$ and $119 \pm 7 \%$ of the untailed wild type GnRHR, figure 3.4, table 3.1). The Glu^{2.53(90)}Phe GnRHR with the carboxy-terminal tail showed increased total binding of ^{125}I -[His⁵,D-Tyr⁶]-GnRH (B0, $252 \pm 29 \%$ of the untailed wild type GnRHR, figure 3.4, table 3.1). Both of these mutant GnRHRs showed unchanged affinities for GnRH.

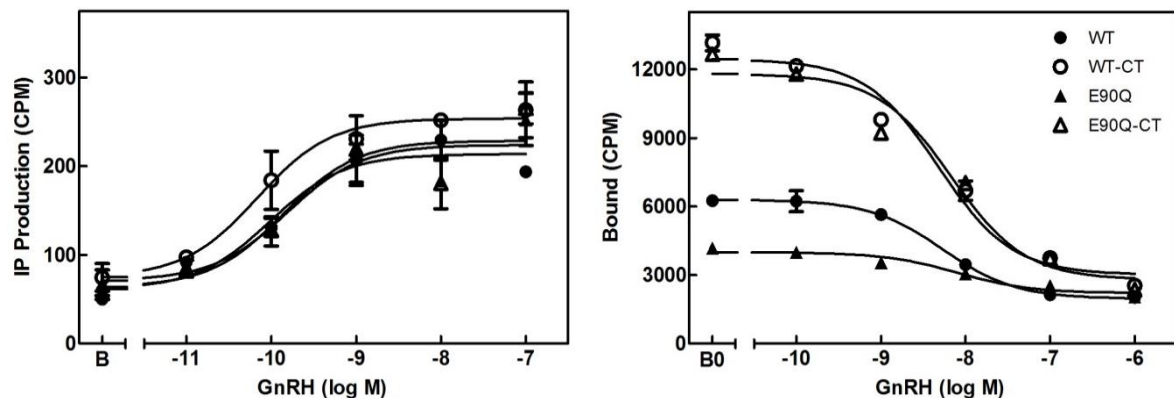


Figure 3.3: GnRH-stimulated IP accumulation and competition binding in COS7 cells expressing mutant GnRHRs with Gln substituted for Glu^{2.53(90)}. COS7 cells transfected with wild type (WT, ●), tailed wild type (WT-CT, ○), Glu^{2.53(90)}Gln (E90Q, ▲) or tailed Glu^{2.53(90)}Gln (E90Q-CT, △) GnRHRs were (Left panel) treated with increasing concentrations of GnRH before IP extraction or (Right panel) were incubated with ^{125}I -[His⁵,D-Tyr⁶]-GnRH in the presence of increasing concentrations of GnRH and bound radioactivity was counted. Data are from a single experiment, representative of experiments that were performed at least three times in duplicate.

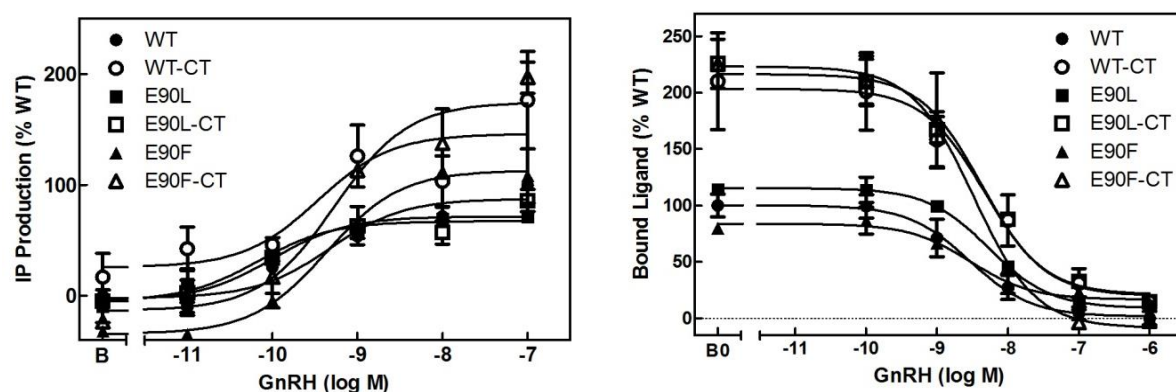


Figure 3.4: GnRH-stimulated IP accumulation and competition binding in COS7 cells expressing mutant GnRHRs with large and hydrophobic amino acid substitutions for Glu^{2.53(90)}. COS7 cells transfected with wild type (WT, ●), tailed wild type (WT-CT, ○), Glu^{2.53(90)}Leu (E90L, ■) or tailed Glu^{2.53(90)}Leu (E90L-CT, □), Glu^{2.53(90)}Phe (E90F, ▲) or tailed Glu^{2.53(90)}Phe (E90F-CT, △) GnRHRs were (Left panel) treated with increasing concentrations of GnRH before IP extraction or (Right panel) were incubated with ¹²⁵I-[His⁵,D-Tyr⁶]-GnRH in the presence of increasing concentrations of GnRH and bound radioactivity was counted. Data are from a single experiment, representative of experiments that were performed at least three times.

3.5. IP signalling and ligand binding of mutant GnRHRs with a positively charged amino acid substitution for Glu^{2.53(90)}

The sidechain of Arg is basic, like that of Lys of the HH-associated and non-functional Glu^{2.53(90)}Lys mutant GnRHR. Several authors have modelled an interhelical salt-bridge between the native, acidic, Glu^{2.53(90)} and Lys^{3.32(121)} of TM3 (Stewart *et al.*, 2012; Janovick *et al.*, 2009). The Arg sidechain is longer than Lys and contributes a positive charge owing to a guanidinium group on its sidechain (Hoffmann *et al.*, 2000). If a salt-bridge were important for GnRHR folding, it would be expected that an Arg substitution for Glu^{2.53(90)} would disrupt expression giving rise to a receptor that behaves similarly to the non-functional Glu^{2.53(90)}Lys mutant GnRHR. COS7 cells transfected with the Glu^{2.53(90)}Arg mutant GnRHR showed GnRH-stimulated IP production with a maximum that was similar to the untailed wild type GnRHR (E_{max} , 64 ± 24 % of the untailed wild type GnRHR, figure 3.5, table 3.1). However, GnRH potency was significantly decreased (EC_{50} , 80.3 ± 7.6 nM, figure 3.5, table 3.1) compared with the untailed wild type GnRHR (0.26 ± 0.31 nM, figure 3.5, table 3.1). The Glu^{2.53(90)}Arg mutant GnRHR with the carboxy-terminal tail showed measurable IP production with decreased potency like its untailed counterpart (EC_{50} , 7.47 ± 7.39 nM, figure

3.5, table 3.1). The decreased potency of the Glu^{2.53(90)}Arg mutant receptors compared with wild type may be a result of partial uncoupling from the IP signalling pathway or decreased binding affinity for GnRH. The Glu^{2.53(90)}Arg mutant GnRHR showed decreased total binding of ¹²⁵I-[His⁵,D-Tyr⁶]-GnRH and a decreased affinity for GnRH (IC₅₀, 82.1 ± 0.09 nM) compared with the wild type receptor (3.33 ± 1.26 nM, figure 3.5, table 3.1). The Glu^{2.53(90)}Arg mutant GnRHR with the carboxy-terminal tail also showed decreased total binding of ¹²⁵I-[His⁵,D-Tyr⁶]-GnRH and a decreased affinity for GnRH (IC₅₀, 79.4 ± 0.02 nM, figure 3.5, table 3.1) compared with the wild type receptor. The Glu^{2.53(90)}Arg mutant GnRHRs showed no change in affinity for the high affinity analogue [His⁵,D-Tyr⁶]-GnRH (IC₅₀, 3.21 ± 1.34 nM and 4.96 ± 1.15 nM) compared with the untailed wild type GnRHR (2.43 ± 0.95 nM, figure 3.5, table 3.1). This unchanged affinity for the constrained GnRH analogue explains why the mutant receptor showed measurable binding of ¹²⁵I-[His⁵,D-Tyr⁶]-GnRH in spite of decreased affinity for GnRH.

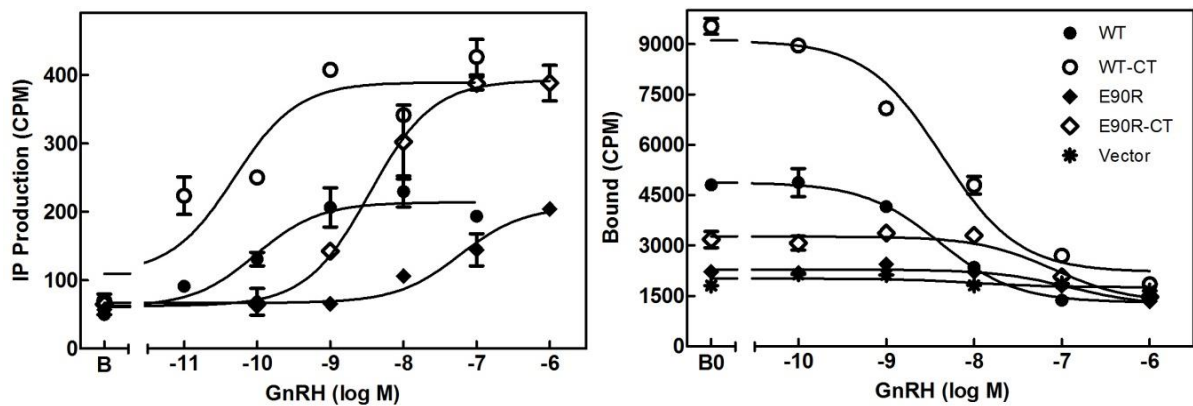


Figure 3.5: GnRH-stimulated IP accumulation and competition binding of wild type and Glu^{2.53(90)}Arg GnRHRs. COS7 cells transfected with wild type (WT, ●), tailed wild type (WT-CT, ○), pcDNA3.1+ vector (vector, *), Glu^{2.53(90)}Arg (E90R, ◆) or tailed Glu^{2.53(90)}Arg (E90R-CT, ◇).GnRHRs GnRHRs were (Left panel) treated with increasing concentrations of GnRH before IP extraction or (Right panel) were incubated with ¹²⁵I-[His⁵,D-Tyr⁶]-GnRH in the presence of increasing concentrations of GnRH and bound radioactivity was counted. Data are from a single experiment, representative of experiments that were performed at least three times in duplicate.

3.6. IP signalling and ligand binding of mutant GnRHRs with Ala and Arg substitutions for Trp^{(6.48)280}

Trp^{6.48} is a highly conserved residue among GPCRs forming part of the CWxPY motif in TM6. As discussed in the introduction, it has been suggested that an interaction between Trp^{6.48} and the residue at position 2.53 may regulate receptor activation in rhodopsin and rhodopsin-like GPCRs (Deupi, 2014; Deupi *et al.*, 2012). To investigate a possible interaction in the GnRHR, we mutated the Trp^{(6.48)280} residue to Ala and Arg.

COS7 cells transfected with the Trp^{6.48(280)}Ala mutant GnRHR showed no detectable GnRH-stimulated IP production and no detectable binding of ¹²⁵I-[His⁵,D-Tyr⁶]-GnRH (Figure 3.6, table 3.1). The Trp^{6.48(280)}Ala mutant with the carboxy-terminal tail showed IP production similar to wild type levels ($E_{\max}$, 80 ± 18 % of untailed wild type GnRHR, figure 3.6, table 3.1) and GnRH potency was similar (figure 3.6, table 3.1). This is consistent with a previous report of unchanged pharmacology of this mutant receptor when rescued by pre-treatment with small molecule antagonist, IN3 (Coetsee *et al.*, 2006). The tailed Trp^{6.48(280)}Ala mutant showed binding of ¹²⁵I-[His⁵,D-Tyr⁶]-GnRH similar to that of the untailed wild type GnRHR. This mutant GnRHR behaved similarly to the Glu^{2.53(90)}Lys mutant GnRHR in that appending the carboxy-terminal tail rescued receptor function from non-functional to wild type levels, with unchanged GnRH potency and unchanged affinity for GnRH. We hypothesized that if the Glu^{2.53(90)} and the Trp^{6.48(280)} side-chains are oriented towards each other within the TM bundle, substitution of Trp^{6.48(280)} with Arg might result in similar distortion of GnRHR function as found for the Glu^{2.53(90)}Arg mutant GnRHR. The Trp^{6.48(280)}Arg mutant GnRHR showed no detectable IP production or binding of ¹²⁵I-[His⁵,D-Tyr⁶]-GnRH (figure 3.6, table 3.1). However, similar to the Glu^{2.53(90)}Arg mutant, the Trp^{6.48(280)}Arg mutant GnRHR with the carboxy-terminal tail showed GnRH-stimulated IP production with decreased GnRH potency (EC_{50} , $53.3 \pm 46.9^*$ nM) compared with the wild type GnRHR (0.26 ± 0.31 nM, figure 3.6, table 3.1). The tailed Trp^{6.48(280)}Arg mutant GnRHR showed binding of ¹²⁵I-[His⁵,D-Tyr⁶]-

GnRH that was similar to levels of the untailed wild type GnRHR (B0, 97 ± 30 % of untailed wild type GnRHR, figure 3.6, table 3.1). Also, similar to the Glu^{2.53(90)}Arg mutant, the tailed Trp^{6.48(280)}Arg mutant GnRHR showed decreased affinity for GnRH (IC_{50} , 27.7 ± 6.74 nM) compared with the wild type GnRHR (3.33 ± 1.26 nM, figure 3.6, table 3.1). Thus, the decreased potency may be explained by the lower affinity of GnRH for this tailed Trp^{6.48(280)}Arg mutant GnRHR. The tailed Trp^{6.48(280)} mutant GnRHRs showed no change in affinity for the high affinity analogue [His⁵,D-Tyr⁶]-GnRH (IC_{50} , 3.37 ± 0.32 nM for the tailed Trp^{6.48(280)}Ala mutant GnRHR and 1.76 ± 1.74 nM for the tailed Trp^{6.48(280)}Arg mutant GnRHR vs 2.43 ± 0.95 nM for the untailed wild type GnRHR). This unchanged affinity explains why the mutant receptor showed measurable binding of ¹²⁵I-[His⁵,D-Tyr⁶]-GnRH.

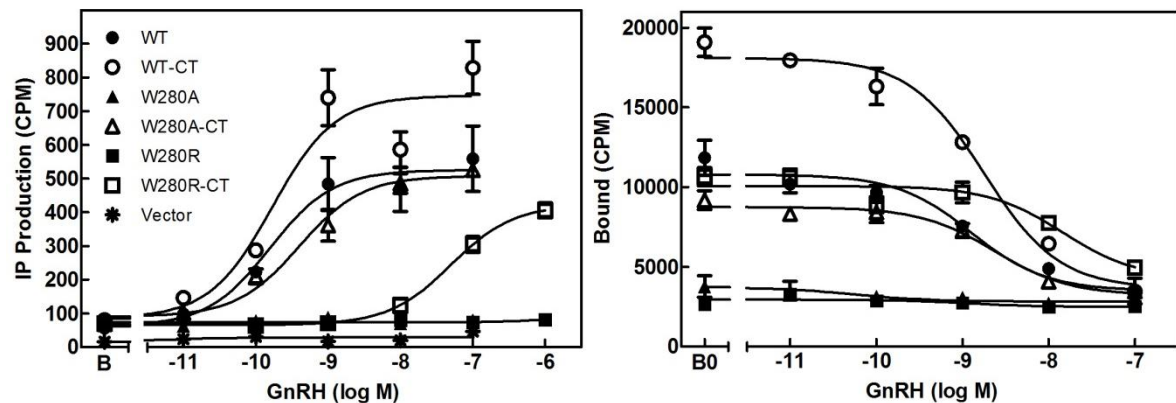


Figure 3.6: GnRH-stimulated IP accumulation and competition binding of wild type, Trp^{(6.48)280}Ala and Trp^{(6.48)280}Arg mutant GnRHRs. COS7 cells transfected with wild type (WT, ●), tailed wild type (WT-CT, ○), Trp^{6.48(280)}Ala (W280A, ▲), tailed Trp^{6.48(280)}Ala (W280A-CT, △), Trp^{6.48(280)}Arg (W280R, ■) or tailed Trp^{6.48(280)}Arg (W280R-CT, □) GnRHRs were (Left panel) treated with increasing concentrations of GnRH before IP extraction or (Right panel) were incubated with ¹²⁵I-[His⁵,D-Tyr⁶]-GnRH in the presence of increasing concentrations of GnRH and bound radioactivity was counted. Data are from a single experiment, representative of experiments that were performed at least three times in duplicate.

3.7. Summary of results

We have confirmed that the Glu^{2.53(90)}Lys mutation disrupts GnRHR signalling and ligand binding and shown that its function can be recovered to wild type levels by appending the Type II GnRHR carboxy-terminal tail. Mutation of Glu^{2.53(90)} to smaller amino acids, including the conservative Glu-to-Asp mutant, also disrupted GnRHR function. However, addition of the carboxy-terminal tail to these mutant receptor constructs could only recover

function of the Glu^{2.53(90)}Ala mutant. In contrast, mutant GnRHRs with large uncharged and hydrophobic substitutions for Glu^{2.53(90)} retained full wild type-like GnRHR function. The Glu^{2.53(90)}Arg mutant GnRHR showed robust IP production and binding of radiolabelled GnRH agonist, but GnRH affinity and potency were decreased. We have shown that in contrast to the Trp^{6.48(280)}Ala mutant GnRHR, the Trp^{6.48(280)}Arg GnRHR that was recovered by appending a carboxy-terminal tail showed decreased affinity for GnRH, similar to that of the Glu^{2.53(90)}Arg mutant GnRHR. Our results show that the GnRHR is fully functional with residues at the 2.53(90) locus that cannot form a salt-bridge with Lys^{3.32(121)} and suggest that the size of the Glu^{2.53(90)} side-chain may be important for stabilizing the structure of the GnRHR.

4. Chapter 4: Discussion

We have investigated the role of the Glu^{2.53(90)} residue in GnRHR signalling and ligand binding. We have confirmed that the Glu^{2.53(90)}Lys mutant GnRHR is non-functional, but show that function can be recovered by addition of the carboxy-terminal tail of the human type II GnRHR. As expected, the recovered Glu^{2.53(90)}Lys mutant GnRHR exhibited GnRH-stimulated IP production and GnRH binding affinity that was indistinguishable from that of the wild type GnRHR. The Glu^{2.53(90)}Ala mutation, which removes the carboxyl side-chain of Glu^{2.53(90)} and introduces only a methyl group, abolished GnRHR function, which was only partially recovered by addition of the carboxy-terminal (maximal IP 35% of wild type), showing that the Ala substitution is more disruptive than the large, positively-charged Lys substitution. Substitution of Glu^{2.53(90)} with small hydrophilic amino acids (Asn, Ser) or with the conservative carboxylic Asp resulted in no measurable GnRHR function in the presence or absence of a carboxy-terminal tail, showing that these substitutions are more disruptive to the GnRHR than either the Ala or the Lys substitutions. The unrecoverable function of the Glu^{2.53(90)}Asp GnRHR shows that a carboxyl group at the 2.53 locus is not sufficient for GnRHR function. A mutant GnRHR with isosteric Gln substituted for Glu^{2.53(90)} showed wild type-like activity even in the absence of a carboxy-terminal tail, showing that a negative charge is not needed at the 2.53 locus for GnRHR function. This was further supported by the wild type-like activities of mutant GnRHRs with large hydrophobic (Phe, Leu) substitutions for Glu^{2.53(90)}. The unchanged GnRH binding affinities of these mutants and of the recovered Glu^{2.53(90)}Lys GnRHR show that the residue at the 2.53 locus is not directly involved in ligand binding. The unchanged expression (as assessed by total binding in the absence of an unlabelled competitor, B0) of GnRHRs with hydrophobic substitutions for Glu^{2.53(90)} indicates that an interaction of the Glu^{2.53(90)} side-chain with a basic side-chain is not important for GnRHR expression. To test this, we substituted Glu^{2.53(90)} with Arg. High maximal IP signalling of the Glu^{2.53(90)}Arg mutant GnRHR shows that the mutant was well-expressed. The

good expression shows that the Arg at the 2.53 locus is unlikely to be closely apposed to the Lys^{3.32(121)} and suggests that the Glu^{2.53(90)} of the wild type GnRHR probably also is not in close proximity to the Lys^{3.32(121)} and thus does not form a salt-bridge interaction. Nevertheless, the decreased GnRH binding affinity at the Glu^{2.53(90)}Arg GnRHR suggests that the Glu^{2.53(90)} side-chain makes a non-salt-bridge interaction that configures the ligand binding pocket and thus contributes to GnRHR structure. Based on proposals that the residue at the 2.53 locus of other GPCRs may interact with the conserved Trp^{6.48} via the conserved water-mediated hydrogen bond network, we mutated Trp^{6.48(280)} of the GnRHR. The wild type-like potency and affinity of GnRH at the Trp^{6.48(280)}Ala GnRHR with an appended carboxy-terminal tail shows that Trp^{6.48(280)} is not directly involved in ligand binding, but may have a role in GnRHR folding and expression. The decreased GnRH potency and affinity at the recovered Trp^{6.48(280)}Arg mutant GnRHR suggests that the introduction of the Arg disrupts the configuration of the ligand binding pocket and that Trp^{6.48(280)} makes intramolecular interactions that affect the structure of the ligand binding pocket. Thus, both Glu^{2.53(90)} and Trp^{6.48(280)} have roles in stable folding and expression of the GnRHR and both contribute to the structural configuration of the GnRH binding pocket. Based on comparisons with other GPCRs, it is likely that Glu^{2.53(90)} and Trp^{6.48(280)} configure the structure of the GnRH binding pocket via interactions with the adjacent residues Ser^{3.35(124)} and Met^{3.36(125)} in TM3 of the GnRHR.

4.1. The COOH group of the Glu^{2.53(90)} is not sufficient for GnRHR expression and function

We show that appending the human type II GnRHR carboxy-terminal tail recovers function of the Glu^{2.53(90)}Lys mutant GnRHR, as did appending the catfish carboxy-terminal tail or incubation with small molecule antagonists or pharmacoperones in other studies (Lin *et al.*, 1998; Conn & Janovick 2009; Tan *et al.*, 2004, Conn & Janovick 2010). The pharmacoperones are said to bind to misfolded proteins, acting as folding templates to

stabilize the receptor for trafficking and cell surface expression as opposed to being targeted for degradation (Janovick *et al.*, 2009). Therefore, it is likely that the appended carboxy-terminal tail stabilizes GnRHR expression by enhancing trafficking to the cell surface and anchoring the receptor at the cell membrane by similar mechanisms as appending a catfish carboxy-terminal tail (Maya-Núñez *et al.*, 2011). The wild type-like GnRH affinity of the recovered Glu^{2.53(90)}Lys GnRHR indicates that Glu^{2.53(90)} does not directly interact with the ligand, but has a structural role in GnRHR folding.

Substitution of Glu^{2.53(90)} with the small amino acid, Ala, abolished both GnRHR signalling and ligand binding. The null mutation shows that some aspect of the Glu^{2.53(90)} side-chain is necessary for GnRHR expression or function. Our result is consistent with a previous report (Hoffmann *et al.*, 2000), which went on to propose that the Glu^{2.53(90)}Ala GnRHR was non-functional because the Ala substitution disrupted a proposed salt-bridge between Glu^{2.53(90)} and Lys^{3.32(121)} residue in TM3. Adding a carboxy-terminal tail to the Glu^{2.53(90)}Ala mutant GnRHR only partially recovered IP signalling, showing that the stabilization provided by the carboxy-terminal tail only partially compensates for the disruptive effect of the Ala substitution. Decreased GnRH binding affinity suggests that the Ala substitution causes a change in the GnRHR structure that distorts its extracellular surface where the ligand binds.

Since Asp has a side-chain that is very similar to that of Glu^{2.53(90)}, except that it is shorter by one CH₂ moiety, it is expected that the Glu^{2.53(90)}Asp GnRHR would be functional owing to the preserved carboxylic group if Glu^{2.53(90)} does make a salt-bridge with Lys^{3.32(121)}. In contrast, substitution of Glu^{2.53(90)} with Asp also abolished GnRHR signalling and ligand binding. Unlike the Glu^{2.53(90)}Lys and Glu^{2.53(90)}Ala GnRHRs, function of the Glu^{2.53(90)}Asp GnRHR could not be recovered by addition of a carboxy-terminal tail. This shows that the carboxylic group is not sufficient to preserve GnRHR function, but also shows that mis-positioning of the carboxylic group disrupts GnRHR structure more than Ala, which cannot make hydrogen bonds.

Mutant GnRHRs with uncharged, small hydrophilic (Asn, Ser) substitutions for Glu^{2.53(90)} behaved similarly to the Glu^{2.53(90)}Asp GnRHR, in that these mutant GnRHRs exhibited no measurable IP signalling or ligand binding and could not be recovered by addition of a carboxy-terminal tail. This suggests unfavourable hydrogen bond interactions that favour misfolding of the GnRHR and direct mutant receptors to degradation, rather than allowing cell surface expression. Since both IP signalling and ligand binding assays depend on binding of GnRH agonist to the GnRHR, it is possible that these mutant GnRHRs are expressed on the cell surface but are undetectable due to lack of GnRH binding.

4.2. Large, uncharged and hydrophobic amino acid substitutions stabilize GnRHR function

The substitution of isosteric uncharged Gln for Glu^{2.53(90)} preserves GnRHR expression and function, with ligand binding and signalling that is indistinguishable from the wild type GnRHR. This shows that charge is unnecessary and suggests to us that Glu^{2.53(90)} may be protonated and thus uncharged within the GnRHR TM bundle. Hoffmann *et al.* (2000) proposed that the preservation of wild type-like behaviour in the Glu^{2.53(90)}Gln mouse GnRHR (Flanagan *et al.*, 1994) may have been a result of Gln making hydrogen bonds with Lys^{3.32(121)} of TM3 that could substitute for the proposed salt-bridge interaction. However, our mutations of Glu^{2.53(90)} to large and hydrophobic amino acids (Phe, Leu), which cannot make hydrogen bonds, also preserve GnRHR expression and function, with ligand binding and signalling that is indistinguishable from the wild type GnRHR. This shows that the hydrophilic properties of the Glu^{2.53(90)} side-chain are not necessary for GnRHR expression and function. If it is not the carboxylic group or the hydrogen bonding capacity of the Glu^{2.53(90)} that is needed for GnRHR expression and function, then perhaps the size or side-chain length of Glu^{2.53(90)} is important. The presence of amino acids that are approximately the same size as the native Glu^{2.53(90)} (Phe, Leu, Gln) may support an appropriate spacing of the TM helices that results in good expression of GnRHRs with unchanged affinity. This suggests a possible spacer role of the

Glu^{2.53(90)} that contributes to the overall fold of the GnRHR that is necessary for cell surface expression. If this is true, the small amino acid substitutions (Ala, Asn, Asp, Ser) for Glu^{2.53(90)} may have caused the TM helices to collapse towards one another, as small amino acids may not sufficiently fill the space of the Glu^{2.53(90)}, resulting in a misfolded GnRHR that is targeted for degradation, instead of being trafficked to the plasma membrane to be expressed as a functional receptor protein.

4.3. It is unlikely that Glu^{2.53(90)} forms a salt-bridge with Lys^{121(3.32)}, but it is important for GnRHR expression

Our results showing that mutant GnRHRs containing hydrophobic substitutions for Glu^{2.53(90)} are well expressed show that a salt-bridge interaction of the residue at the 2.53 locus is not necessary for robust GnRHR expression and suggest that Glu^{2.53(90)} may not form a salt-bridge. However, it has been proposed that the decreased expression of the HH-associated Glu^{2.53(90)}Lys mutant GnRHR results from the positive charge of the Lys at the 2.53 locus that disrupts an interaction with Lys^{3.32(121)} by causing an insurmountable charge repulsion within the TM helices (Blomenröhr *et al.*, 2001; Conn & Ulloa-aguirre 2010; Conn & Ulloa-Aguirre 2009; Janovick *et al.*, 2011). If this is true, it would be expected that introduction of an Arg at the 2.53 locus would have a similar effect on GnRHR expression, as the guanidium groups of Arg side-chains within proteins are always positively charged (Betts & Russell 2007; Mayevu *et al.*, 2015; Palczewski *et al.*, 2000; Armstrong *et al.*, 2016). The Glu^{2.53(90)}Arg mutation preserved cell surface expression, even in the absence of a carboxy-terminal tail, as evidenced by the wild type-like maximal IP signalling. The preservation suggests that the Arg side-chain at the 2.53 locus is not in close apposition to Lys^{3.32(121)} and suggests that Glu^{2.53(90)} is not close enough to Lys^{3.32(121)} to form a salt-bridge in the wild type GnRHR. It also suggests that Arg makes interactions that stabilize GnRHR folding, but the decreased affinity suggests that Arg interactions distort the ligand binding pocket. Since other mutations show that the Glu^{2.53(90)} side-chain is not directly involved in ligand binding, the effect of the Arg

substitution at the 2.53 locus is likely to be indirect. The Arg side-chain is longer than Glu^{2.53(90)} and all other substitutions (Leu, Phe, Gln) that preserved GnRHR expression, suggesting that the presence of Arg may increase the distance between the TM helices, “pushing” the helices apart. This may cause a distortion in the configuration of the ligand binding pocket that could account for the decreased ligand binding affinity. A longer side-chain being necessary at the 2.53 locus is also supported by the decreased affinity of the Glu^{2.53(90)}Ala mutant GnRHR, which may result in a distortion to the ligand binding pocket, possibly caused by the TM helices collapsing towards one another.

4.4. Trp^{6.48(280)} may also be important for GnRHR structure

Since our results for the Glu^{2.53(90)}Arg mutant GnRHR exclude an interaction of Glu^{2.53(90)} with Lys^{3.32(121)}, we investigated other potential intramolecular interactions that could account for the role of Glu^{2.53(90)} in GnRHR expression. Based on a proposed conserved water-mediated interaction of the residue at the 2.53 locus of class A GPCRs with the highly conserved Trp^{6.48} (Deupi, 2014; Deupi & Standfuss, 2011), we mutated the Trp^{6.48(280)} residue of the GnRHR. We confirmed lack of function of the Trp^{6.48(280)}Ala mutant GnRHR (Coetsee *et al.*, 2006) and showed recovery to wild type-like function by addition of a carboxy-terminal tail. Recovery of the function of this mutant suggests that, like Glu^{2.53(90)}, the Trp^{6.48(280)} residue may be important for expression of the GnRHR, but is not a ligand-contacting residue. Coetsee *et al.* (2006) showed that a range of substitutions for Trp^{6.48(280)}, including Ala, His, Gln and Met, resulted in severely decreased ligand binding, but all mutant GnRHRs showed binding affinity similar to wild type once recovered using the pharmacoperone, IN3. This suggests that Trp^{6.48(280)} does not directly interact with GnRH. We show that substitution of Trp^{6.48(280)} with Arg did not preserve GnRHR function in the absence of a carboxy-terminal tail. Thus, Arg disrupts folding, but when folding is recovered, there is decreased GnRH binding affinity suggesting that the receptor fold is distorted in the recovered mutant GnRHR.

4.5. The similar phenotypes of both Trp^{6.48(280)}Arg and Glu^{2.53(90)}Arg mutant GnRHRs suggest a similar disruption of the GnRHR

Decreased GnRH binding affinity of the Trp^{6.48(280)}Arg GnRHR with appended carboxy-terminal tail suggests that substituting Trp^{6.48(280)} with Arg affects the relative positioning of TM helices, causing a disruption of the configuration of the ligand binding pocket. The most recent analysis of intramolecular interactions in Class A GPCR crystal structures reported that the residues at the 2.53 and 6.48 loci have conserved interactions with adjacent residues, 3.35 and 3.36, in TM3 (Cvick et al., 2016). Considering these conserved interactions, it is likely that Glu^{2.53(90)} directly contacts Ser^{3.35(124)} in TM3 of the GnRHR. This is supported by a mutagenesis study in which a Ser^{3.35(124)}Ala mutant GnRHR had wild-type-like function, whereas, mutation of Ser^{3.35(124)} to Asp abolished receptor function (Betz et al., 2006), suggesting that a repulsive interaction of the Asp with Glu^{2.53(90)} disrupts GnRHR folding. The conserved interactions suggest that Trp^{6.48(280)} of the GnRHR would make a direct contact with Met^{3.36(125)}, placing the side-chains of Glu^{2.53(90)} and Trp^{6.48(280)} within a shared environment. The Ser^{3.35(124)} and Met^{3.36(125)} residues are only three and four loci, i.e. one helical turn, away from the Lys^{3.32(121)}, previously proposed to form a salt-bridge interaction with Glu^{2.53(90)}. These predictions are largely consistent with a homology model of the inactive human GnRHR from GPCRdb (figure 4.1, http://www.gpcrdb.org/structure/homology_models), which shows that Glu^{2.53(90)} is oriented toward Ser^{3.35(124)} in TM3, whereas Lys^{3.32(121)} is oriented away from Glu^{2.53(90)} but is positioned closer to Asp^{2.61(98)}. This model also shows that Trp^{6.48(280)} of the inactive GnRHR is positioned in close proximity to Met^{3.36(125)} in TM3, and that Glu^{2.53(90)} and Trp^{6.48(280)} are in fact within a shared environment (figure 4.1). It is possible that modelling a negatively-charged glutamate side-chain, rather than an uncharged glutamic acid side-chain may have biased the modelling process to create a salt-bridge interaction with the positively-charged Lys^{3.32(121)} instead of the highly conserved interaction of the 2.53 locus with the 3.35 locus.

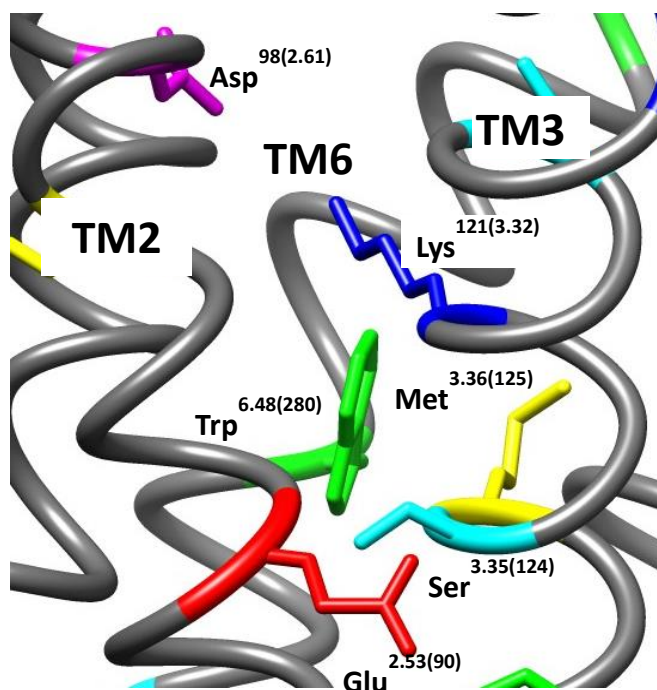


Figure 4.1: Homology model of the inactive human GnRHR showing relative positions of residues Glu^{2.53(90)}, Ser^{3.35(124)}, Lys^{3.32(121)}, Asp^{2.61(98)}, Trp^{6.48(280)} and Met^{3.36(125)}. This model was downloaded from the GPCRdb website (www.gpcrdb.org/structure/homology_models) and viewed using USCF Chimera software package, version 1.11.2, San Francisco to show the spatial positioning of Glu^{2.53(90)} (red) and Trp^{6.48(280)} (green) relative to the neighbouring residues; Ser^{3.35(124)} (light blue), Lys^{3.32(121)} (dark blue), Asp^{2.61(98)} (magenta), and Met^{3.36(125)} (yellow).

Our results and the conserved intramolecular interactions with adjacent residues of TM3 are consistent with the proposal that Trp^{6.48(280)} and Glu^{2.53(90)} are part of a conserved “ligand sensing” region of class A GPCRs, where residues that are not a part of the ligand binding pocket are allosterically coupled to ligand binding (Madabushi *et al.*, 2004). In conclusion, we have confirmed the importance of the Glu^{2.53(90)} in the function of the GnRHR and shown that this may be because it has a role as a space-filling residue in GnRHR folding. A negative charge of the side-chain is not necessary for GnRHR function and Glu^{2.53(90)} may be uncharged within the protein. Our results suggest that the Glu^{2.53(90)} probably does not form a salt-bridge interaction with Lys^{3.32(121)}, but may occupy a space within the TM bundle of the GnRHR that is shared with Trp^{6.48(280)}. Our results suggest that Glu^{2.53(90)} might not directly interact with Trp^{6.48(280)}, but that these two residues have a similar role in maintaining the overall GnRHR fold that contributes to stable cell surface expression of the receptor and configuration of the ligand binding pocket. We are currently involved in a collaboration that will hopefully yield molecular models to further integrate our mutagenesis data.

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6. Appendices

6.1. Appendix A: Ethics waiver

Human Research Ethics Committee (Medical)

Research Office Secretariat: Senate House Room SH 10005, 10th floor. Tel +27 (0)11-717-1252
Medical School Secretariat: Medical School Room 10M07, 10th Floor. Tel +27 (0)11-717-2700
Private Bag 3, Wits 2050, www.wits.ac.za. Fax +27 (0)11-717-1265



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05/02/2014

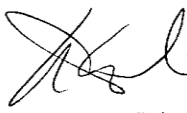
TO WHOM IT MAY CONCERN:

Waiver: This certifies that the following research does not require clearance from the Human Research Ethics Committee (Medical).

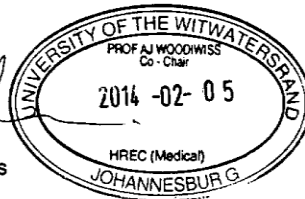
Investigator: Ashmeetha Manilall (0504496W)

Project title: The role of the Glu2.53(90) residue in gonadotropin-releasing hormone receptor expression and function.

Reason: This research for an MSc dissertation is an in vitro study using commercially available cell lines and recombinant DNA only. No humans or patient records are involved. An application for biosafety clearance has been submitted to the WITS biosafety committee.


Professor Angela Woodiwiss

Co-Chair: Human Research Ethics Committee (Medical)



Copy: Anisa Keshav, Research Office, Senate House, Wits

6.2. Appendix B: Turnitin report results

turnitinashnov2016.pdf

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