Glu$^{2.53(90)}$ of the GnRH receptor is part of the conserved G protein-coupled receptor structure and does not form a salt-bridge with Lys$^{3.32(121)}$

Ashmeetha Manilall[a], B. Andre Stander[b], Michael T. Madziva[a], Robert P. Millar[c], Colleen A. Flanagan[a,*]

[a] School of Physiology, University of the Witwatersrand Faculty of Health Sciences, Parktown, Johannesburg, South Africa

[b] Department of Physiology, Faculty of Health Sciences, University of Pretoria, Pretoria, South Africa

[c] Centre for Neuroendocrinology, University of Pretoria, Pretoria, South Africa

*Corresponding author. School of Physiology, University of the Witwatersrand Faculty of Health Sciences, Private Bag 3, Wits, 2050, South Africa.
Colleen.Flanagan@wits.ac.za

Highlights

- Mutation of Glu^{90} of the GnRH receptor to Lys or Asp causes congenital infertility.
- Glu90 interactions form a network that is important in G protein-coupled receptors.
- Glu^{90} mutations to Lys and Asp disrupt conserved intramolecular interactions.
- Glu^{90} mutation to Arg preserves receptor function, but decreases its GnRH affinity.
- Disruption of conserved G protein-coupled receptor networks causes infertility.

Abstract

GnRH receptor mutations, Glu$^{2.53(90)}$Lys and Glu2$^{.53(90)}$Asp, cause congenital hypogonadotropic hypogonadism. The Glu$^{2.53(90)}$ side-chain has been proposed to form an intramolecular salt-bridge with Lys$^{3.32(121)}$, but conserved intramolecular interaction networks in G protein-coupled receptor crystal structures predict that it interacts with Ser$^{3.35(124)}$ and Trp$^{6.48(280)}$. We investigated interhelical interactions of Glu$^{2.53(90)}$ that stabilise GnRH receptor folding using functional analyses and computational modelling of mutant receptors. The Glu$^{2.53(90)}$Asp mutant was non-functional, but mutants with hydrophobic amino acids or Arg substituted for Glu$^{2.53(90)}$ were functional, excluding a salt-bridge interaction. The Glu$^{2.53(90)}$Arg and Trp$^{6.48(280)}$Arg mutants had decreased affinity for GnRH. Models showed that congenital Glu$^{2.53(90)}$Lys and Glu$^{2.53(90)}$Asp mutations disrupt interactions with Ser$^{3.35(124)}$ and Trp$^{6.48(280)}$ respectively, whereas the Glu$^{2.53(90)}$Arg and Trp$^{6.48(280)}$Arg mutations preserve intramolecular contacts, but increase distance between the transmembrane helices. Our results show that disruption of interhelical contacts that are conserved in G protein-coupled receptors accounts for the effects of some disease-associated GnRH receptor mutations.

Keywords: GnRH receptor; G protein-coupled receptor; Interhelical contact; Receptor structure

1. Introduction

Gonadotropin-releasing hormone (GnRH) is a key regulator of reproduction. Mutations of the GnRH receptor disrupt the GnRH-dependent gonadotropin production that initiates pubertal development, resulting in the congenital hypogonadotropic hypogonadism (cHH) form of infertility, which is transmitted in an autosomal recessive pattern (Balasubramanian and Crowley, 2007 [Updated, 2017 Mar 2]; Chevrier et al., 2011; Gianetti et al., 2012).

The GnRH receptor is a membrane protein belonging to the large family of G protein-coupled receptors (GPCRs), which regulate diverse physiological systems. GPCRs share a conserved mechanism of action based on a conserved structure, consisting of an extracellular amino terminus, seven membrane-spanning α-helices connected by intracellular and extracellular loops and a cytoplasmic carboxy-terminal tail. The human GnRH receptor has the conserved amino acids characteristic of class A GPCRs, which include well-characterised receptors such as rhodopsin and the β-adrenergic receptors, but it lacks the cytoplasmic carboxy-terminal tail (Chi et al., 1993; Flanagan and Manilall, 2017; R. P. Millar et al., 2004). Appending carboxy-terminal tails from type II GnRH receptors stabilises the protein folding during biogenesis and enhances cell surface expression of the tailless mammalian GnRH receptor proteins (Flanagan et al., 1999, 2000; Lin et al., 1998; Maya-Nunez et al., 2000, 2002). The three-dimensional structure of the GnRH receptor has not been directly determined, but a growing number of other GPCR crystal structures show that the GPCR proteins share a highly conserved structural fold that is stabilised by non-covalent intramolecular interactions between different transmembrane (TM) helices (Bortolato et al., 2014; Cvicek et al., 2016; Mahoney and Sunahara, 2016; Venkatakrishnan et al., 2013). Forty interhelical contacts are conserved in class A GPCR structures, in both active and inactive conformations. These conformation-independent interhelical contacts connect amino acids in topologically equivalent positions of the GPCR proteins to form a conserved structural network, but the amino acids making the contacts are not all conserved (Bortolato et al., 2014; Cvicek et al., 2016; Venkatakrishnan et al., 2013). The conserved

Abbreviations: cHH, congenital hypogonadotropic hypogonadism; ECL, extracellular loop; GnRH, gonadotropin-releasing hormone; GPCR(s), G protein-coupled receptor(s); ICL, intracellular loop; IP, inositol phosphate; TM, transmembrane

interhelical contacts are likely to be present in the GnRH receptor and to contribute to stable folding and cell surface expression of the receptor protein (Flanagan and Manilall, 2017). In addition to the direct interhelical contacts, recent high-resolution GPCR crystal structures show that intramolecular water molecules form a conserved hydrogen bond network that indirectly connects highly conserved polar amino acids in class A GPCRs (Angel et al., 2009; Blankenship et al., 2015; Deupi et al., 2012; Flanagan and Manilall, 2017; Huang et al., 2015; Standfuss et al., 2011; Trzaskowski et al., 2012; Yuan et al., 2014).

Homozygous mutation of the $Glu^{2.53(90)}$ residue of the GnRH receptor to Lys caused cHH that was completely refractory to intravenous administration of GnRH (see materials and methods for an explanation of the residue numbering system) (Soderlund et al., 2001). *In vitro* analysis showed that the $Glu^{2.53(90)}$Lys mutant receptor was not expressed in transfected cells, but expression and function were rescued by deletion of a destabilizing residue of the receptor (Maya-Nunez et al., 2002) or by pre-treatment of transfected cells with membrane-permeable small molecule GnRH antagonists (Janovick et al., 2002; Leanos-Miranda et al., 2002; Tello et al., 2012). It was subsequently found that the $Glu^{2.53(90)}$Lys- and many other-mutant GnRH receptors are misfolded during biosynthesis, due to failure to form correct intramolecular contacts (Conn and Janovick, 2009; Tao and Conn, 2014). The misfolded nascent membrane proteins are identified by the endoplasmic reticulum-associated degradation system and exported to the proteosomal compartment, where they are degraded, whereas correctly folded receptor proteins are transported to the cell membrane (Tao and Conn, 2014). It has been proposed that the $Glu^{2.53(90)}$Lys mutation destabilises mutant receptor protein folding by breaking an interhelical salt-bridge with the basic $Lys^{3.32(121)}$ residue in TM3 and introducing charge repulsion (Janovick et al., 2009, 2011; Tao and Conn, 2014). However, it has recently been reported that the conservative $Glu^{2.53(90)}$Asp mutation, which would be expected to preserve the salt-bridge, also causes cHH (Marcos et al., 2014).

$Glu^{2.53(90)}$ is conserved in the tailless type I GnRH receptors (R. P. Millar et al., 2004; Sefideh et al., 2014), but the related type II GnRH receptors and most class A GPCRs have large hydrophobic residues in position 2.53 (R. P. Millar et al., 2004; Mirzadegan et al., 2003; Sefideh et al., 2014). The lack of conservation suggests that the negatively-charged carboxylate side-chain of $Glu^{2.35(90)}$ is not essential for GnRH receptor folding. Supporting this, substitution of $Glu^{2.53(90)}$ with uncharged Gln in the mouse GnRH receptor resulted in a fully functional mutant receptor (Flanagan et al., 1994), although the null mutation, to Ala, completely disrupted receptor function (Hoffmann et al., 2000). This suggests that the $Glu^{2.53(90)}$ side-chain makes one- or more-interhelical contacts that are important for folding of the GnRH receptor during biogenesis, but these contacts probably do not involve a salt-bridge.

It has been proposed that residues in position 2.53 of class A GPCRs have a conserved water-mediated interaction with the conserved $Trp^{6.48}$ residue, of the CWxPY motif ($Cys^{6.47}$-$Trp^{6.48}$-any amino acid-$Pro^{6.50}$-$Tyr^{6.51}$) in TM6 (Deupi, 2014). Thus, the $Glu^{2.53(90)}$ side-chain of the GnRH receptor may interact with $Trp^{6.48(280)}$ via the conserved water-mediated hydrogen bond network to stabilise expression and the cHH-associated $Glu^{2.53(90)}$ mutations may disrupt this interaction. A more recent analysis of non-covalent direct interhelical contacts in GPCR crystal structures found a conserved hydrophobic interhelical contact between the residues in positions 2.53 and 3.35 of all GPCRs (Cvicek et al., 2016). This suggests that direct contact of $Glu^{2.53(90)}$ with the side-chain of $Ser^{3.35(124)}$ in TM3 may be needed for stable folding and expression of the GnRH receptor. The $Glu^{2.53(90)}$ side-chain potentially forms additional intramolecular contacts that are not conserved in other GPCRs, but may also contribute to correct folding of the GnRH receptor.

We have used systematic site-directed mutagenesis of $Glu^{2.53(90)}$ and $Trp^{6.48(280)}$, functional assays that report cell surface expression and computational modelling to understand the interhelical interactions of the $Glu^{2.53(90)}$ side-chain that stabilise GnRH receptor folding during biogenesis. Our results show that mutant GnRH receptors with amino acids smaller than Glu, including the negatively-charged Asp, all disrupted GnRH receptor function, whereas substitution of $Glu^{2.539(90)}$ with larger uncharged or hydrophobic amino acids, or the positively-charged Arg, all preserved receptor function, excluding a salt-bridge interaction of $Glu^{2.53(90)}$ with $Lys^{3.32(121)}$. Our models show that the cHH-associated mutations disrupt interhelical interactions with $Ser^{3.35(124)}$ or with $Trp^{6.48(280)}$, whereas the $Glu^{2.53(90)}$Arg mutation preserves both of these interactions. Our results suggest that disruption of interhelical contacts that are conserved in GPCRs should be considered as a mechanism for the effects of some cHH-causing mutations.

2. Materials and methods

2.1. Amino acid numbering system

The Ballesteros and Weinstein numbering system is used to facilitate comparison of amino acids of the GnRH receptor with equivalent residues in other class A GPCRs. Residues in each TM are numbered relative to the most conserved residue, which is designated .50, with the sequence number of the residue in the receptor in parenthesis (JA Ballesteros and Weinstein, 1995; van Rhee and Jacobson, 1996). Thus, the Glu^{90} residue of the GnRH receptor is designated $Glu^{2.53(90)}$ because it is located three residues past the location of the most conserved residue of TM2, $Asn^{2.50(87)}$.

2.2. Site-directed mutagenesis

PCR-based whole plasmid site-directed mutagenesis (high fidelity PCR readymix, KAPA Biosystems, Cape Town, South Africa) was used to generate mutant GnRH receptors using wild-type human GnRH receptor cDNA cloned into pcDNA3 (Invitrogen, Carlsbad, California) as a DNA template. Mutagenic primers with unique silent restriction enzyme sites were designed to substitute $Glu^{2.53(90)}$ with Ala, Arg, Asn, Asp, Phe, Gln, Leu, Lys and Ser. Similarly, $Trp^{6.48(280)}$ was mutated to Ala and Arg. *DpnI*-digested PCR products (3 µl) were transformed into competent *JM109 E. coli* cells (100 µl) and cultured on Luria-Bertani agar plates with ampicillin (1 µg/ml, Sigma Aldrich, Saint Louis, Missouri). Colonies were cultured in Luria broth with ampicillin (37 °C, 16 h) for small-scale plasmid DNA isolation. 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 pcDNA3.1 + expression vector (Invitrogen, Carlsbad, California). To enhance expression of poorly-expressed $Glu^{2.53(90)}$ mutant GnRH receptors, mutant constructs were subcloned into the *EcoRI* and *EcoNI* sites of a GnRH receptor construct with a human type II GnRH receptor carboxy-terminal domain (Flanagan et al., 2000).

2.3. Cell culture and transfection

COS7 cells (ATCC CRL-1651) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Biowest, France) supplemented with 10% foetal bovine serum (FBS, Thermo Scientific, Waltham, Massachusetts) at 37 °C in 10% CO_2. Wild-type and mutant GnRH receptor constructs were transiently transfected into COS7 cells using Lipofectamine LTX (Invitrogen, Carlsbad, California), according to the manufacturer's instructions. Transfected cells were incubated in DMEM with FBS and Pen-Strep (0.5 mg/ml penicillin, 0.1 mg/ml streptomycin) at 37 °C in 10% CO_2 for 24 h, detached from transfection dishes with PBS-EDTA (10 mM EDTA, 1 x PBS), pelleted by centrifugation (500 g, 3 min), plated into 12-well plates and cultured overnight.

Table 1
GnRH-stimulated IP production and competition binding of COS7 cells transfected with wild-type and mutant GnRH receptors. Data are means ± SD of the indicated numbers of experiments performed in duplicate. All experiments included the wild-type GnRH receptor and tailed wild-type GnRH receptor.

GnRH Receptor	IP Production			Competition Binding			Coupling Coefficient
	EC_{50} (nM)	pEC_{50}	Emax (%WT)[b]	IC_{50} (nM)	pIC_{50}	B0 (%WT)[b]	
Wild-type	0.26 ± 0.31	9.58 ± 0.64 (n = 19)	100	3.33 ± 1.26	8.59 ± 0.26 (n = 13)	100	6.90
Wild-type-CT[a]	0.21 ± 0.25	9.89 ± 0.41 (n = 17)	146 ± 43	4.76 ± 1.35	8.34 ± 0.14 (n = 13)	200 ± 61	8.64
$Glu^{2.53(90)}$Lys	–	- (n = 3)	nms[b]	–	- (n = 3)	nmb[c]	–
$Glu^{2.53(90)}$Lys-CT	0.14 ± 0.14	9.97 ± 0.15 (n = 3)	132 ± 48	3.2 ± 0.05	8.50 ± 0.01 (n = 3)	185 ± 35	8.51
$Glu^{2.53(90)}$Ala	–	- (n = 4)	nms	–	- (n = 4)	nmb	–
$Glu^{2.53(90)}$Ala-CT	0.55 ± 0.51	9.48 ± 0.55 (n = 4)	30 ± 19	19.95 ± 0.01	7.70 ± 0.3* (n = 3)	38 ± 7	14.7
$Glu^{2.53(90)}$Asp	–	- (n = 4)	Nms[c]	–	- (n = 4)	nmb[d]	–
$Glu^{2.53(90)}$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 ± 0.06	9.70 ± 0.1 (n = 3)	84 ± 18	5.04 ± 2.19	8.33 ± 0.28 (n = 3)	58 ± 21	16.6
$Glu^{2.53(90)}$Gln-CT	0.27 ± 0.31	9.80 ± 0.56 (n = 3)	148 ± 106	3.34 ± 1.44	8.5 ± 0.21 (n = 3)	107 ± 10	9.25
$Glu^{2.53(90)}$Phe	0.27 ± 0.12	9.51 ± 0.15 (n = 3)	110 ± 51	2.97 ± 0.40	8.53 ± 0.10 (n = 3)	76 ± 4	8.68
$Glu^{2.53(90)}$Phe-CT	0.30 ± 0.20	9.63 ± 0.35 (n = 3)	160 ± 34	2.99 ± 0.51	8.50 ± 0.15 (n = 3)	252 ± 29	3.48
$Glu^{2.53(90)}$Leu	0.19 ± 0.21	9.70 ± 0.35 (n = 6)	100 ± 34	4.39 ± 1.07	8.37 ± 0.11 (n = 6)	108 ± 85	11.2
$Glu^{2.53(90)}$Leu-CT	0.23 ± 0.24	10.0 ± 0.61 (n = 3)	119 ± 7	2.83 ± 1.03	8.50 ± 0.10 (n = 3)	119 ± 20	6.65
$Glu^{2.53(90)}$Arg	80.3 ± 7.60	7.13 ± 0.60* (n = 6)	64 ± 26	82.1 ± 0.09	7.08 ± 0.17* (n = 6)	25 ± 8	2.59
$Glu^{2.53(90)}$Arg-CT	7.47 ± 7.39	8.27 ± 0.40* (n = 7)	121 ± 70	79.4 ± 0.02	7.10 ± 0.02* (n = 7)	58 ± 4	12.1
$Trp^{6.48(280)}$Ala	–	- (n = 8)	nms	–	- (n = 8)	nmb	–
$Trp^{6.48(280)}$Ala-CT	0.21 ± 0.14	9.82 ± 0.33 (n = 5)	80 ± 18	4.39 ± 1.07	8.43 ± 0.12 (n = 5)	96 ± 13	9.13
$Trp^{6.48(280)}$Arg	–	- (n = 6)	nms	–	- (n = 6)	nmb	–
$Trp^{6.48(280)}$Arg-CT	53.3 ± 46.9	7.40 ± 0.37* (n = 7)	64 ± 15	27.73 ± 6.74	7.57 ± 0.11* (n = 7)	97 ± 30	0.50

*Significantly different from wild-type GnRH receptor $p < 0.05$.
[a] -CT, Carboxy terminal-tail appended.
[b] Emax and B0 values are presented as % of values obtained for cells transfected with the wild-type GnRH receptor in the same assay to accommodate for variation in radiolabelling efficiency.
[c] nms, no measurable stimulation.
[d] nmb, no measurable binding.

2.4. IP accumulation assays

Transfected cells were radiolabelled overnight with [^{3}H]-*myo*inositol (1 μCi/ml, 1ml/well) (Perkin Elmer, Boston, Massachusetts) in DMEM supplemented with FBS (2%). Cells were pre-incubated with pre-warmed buffer I (140 mM NaCl, 4 mM KCl, 8.6 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% bovine serum albumin (BSA), 10 mM LiCl, pH 7.4; 1 ml/well, 37 °C, 15 min) and incubated (37 °C, 60 min) in the absence or presence of various concentrations of GnRH (10^{-11} to 10^{-6} M) 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. Cell lysates were extracted on Dowex-1 resin (1 ml; Sigma Aldrich, Saint Louis, Missouri) anion exchange chromatography, as previously described (R.P. Millar et al., 1995) and counted by liquid beta scintillation spectroscopy.

2.5. Radioligand competition binding assays

The high affinity GnRH analog, [His^5,D-Tyr^6]-GnRH, was radioiodinated using chloramine T (Flanagan et al., 1998) and purified on a QAE sephadex column. Fractions with highest radioactivity were aliquoted and frozen (−70 °C). Transfected cells were washed with 1 ml cold binding buffer (HEPES-DMEM with 0.1% BSA; pH 7.4) and incubated (4 °C, 5 h) with ^{125}I-[His^5,D-Tyr^6]-GnRH (~100 000 cpm) in the absence or presence of various concentrations (10^{-11} - 10^{-6} M) of unlabelled GnRH or unlabelled [His^5,D-Tyr^6]-GnRH in binding buffer (final volume, 500 μl). The incubation was terminated by washing 3 times with cold PBS to remove unbound radioligand and bound radioligand was collected from plates by solubilizing cells with 0.1 M NaOH (1 ml/well). The radioactivity was counted in a gamma counter.

2.6. Computational modelling

A homology model of the wild-type human GnRH receptor was generated as described in Supplementary Material. Homology models of mutant receptors were generated by substituting Lys, Asp or Arg for $Glu^{2.53(90)}$ and Arg for $Trp^{6.48(280)}$. Molecular dynamics simulations were performed on wild-type and mutant GnRH receptor homology models as described in Supplementary Material. Interhelical contacts between amino acids in the resulting models are defined as present when the distance between any pair of atoms (side-chain and/or main-chain atoms) is within the Euclidean distance (the sum of the van der Waal's radii of the atoms plus 0.6 Å) as previously described (Venkatakrishnan et al., 2013). Wild-type and mutant receptor models were then subjected to 100 ns molecular dynamics production runs, as described in Supplementary Material, to assess the stability of interhelical interactions formed by side-chains of the residues in position 2.53(90).

2.7. Data analysis

IP production and radioligand binding assays were performed at least 3 times in duplicate for each GnRH receptor mutant and the wild-type GnRH receptor 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, version 5, La Jolla, California). Similarly, total binding in the absence of unlabelled ligand (B0) 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 transforms the mean ± SD data to a normal distribution that can be used for

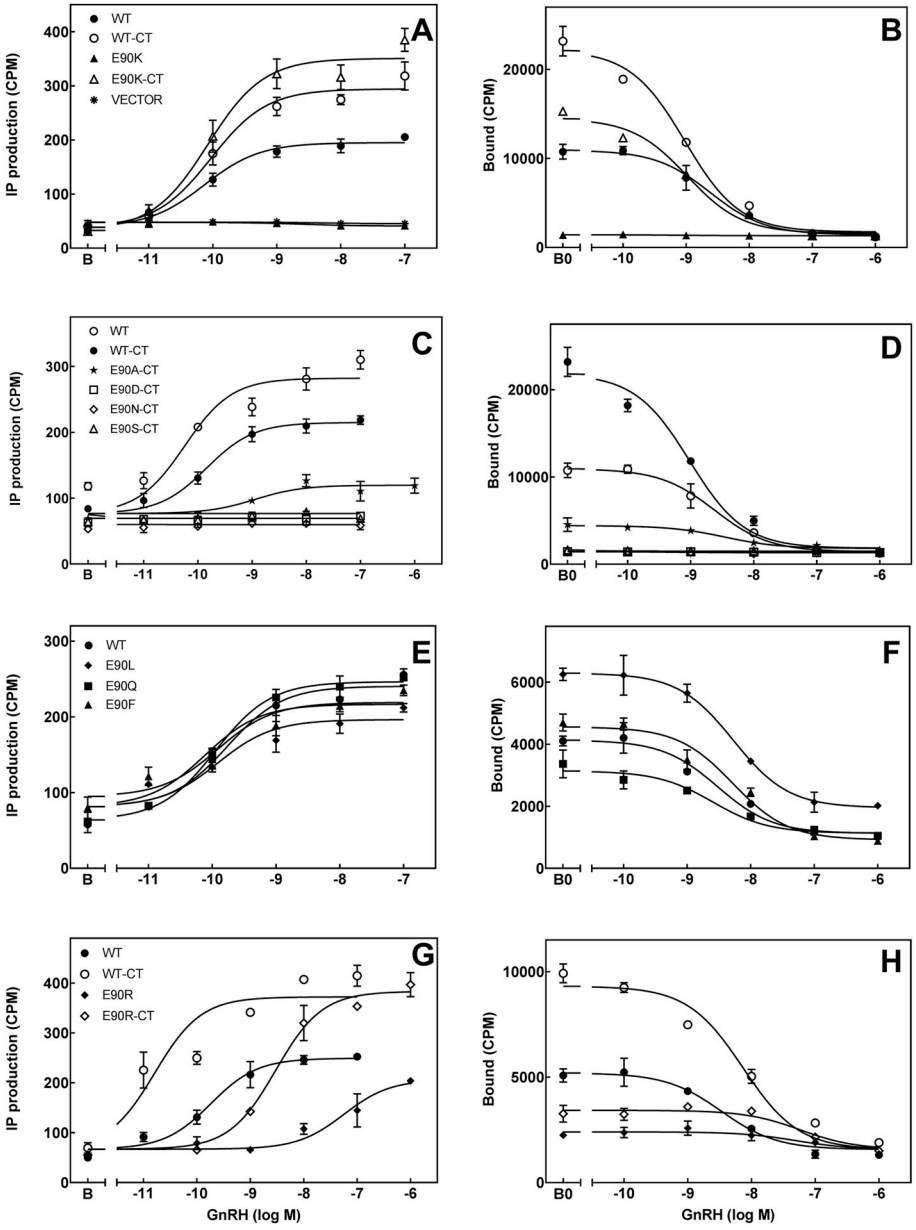

Fig. 1. Effects of mutations of the Glu$^{2.53(90)}$ residue of the GnRH receptor on GnRH-stimulated IP production and ligand binding. COS7 cells transfected with wild-type or mutant GnRH receptors, without or with an appended carboxy-terminal tail (CT), were labelled with [^{3}H]-*myo*inositol and incubated with varying concentrations of GnRH (left panels) or incubated with ^{125}I-[His^5,D-Tyr^6]-GnRH and varying concentrations of GnRH (right panels). Data are means ± range from representative experiments performed at least 3 times in duplicate.

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 GnRH receptor differed from the values of the wild-type GnRH receptors. Coupling coefficients were calculated using the formula $Q = 0.5\ [(IC_{50} + EC_{50})/EC_{50}](E_{max}/B_{max})$ using the data in Table 1, as previously described (J Ballesteros et al., 1998; Mayevu et al., 2015). Two-tailed T tests were used to compare interatomic distances in models of the wild-type and mutant GnRH receptors.

3. Results

3.1. Confirmation that the Glu$^{2.53(90)}$Lys mutant GnRH receptor is non-functional and rescue by appending the human type II GnRH receptor carboxy-terminal tail

COS7 cells transfected with the wild-type human GnRH receptor showed robust GnRH-stimulated IP production and high total ligand binding (Fig. 1, Table 1). In contrast, COS7 cells transfected with the Glu$^{2.53(90)}$Lys mutant GnRH receptor showed no IP production and no detectable binding (Fig. 1, Table 1). Appending the human type II GnRH receptor carboxy-terminal tail to the wild-type GnRH receptor increased the maximum GnRH-stimulated IP production in most experiments and increased total binding of I^{125}-[His^5,D-Tyr^6]-GnRH, without changing affinity for GnRH (Fig. 1, Table 1), consistent with increased expression of functional GnRH receptor protein (Flanagan et al., 2000). The Glu$^{2.53(90)}$Lys mutant receptor with the appended carboxy-terminal tail bound I^{125}-[His^5,D-Tyr^6]-GnRH, had the same affinity for GnRH as the wild-type receptor (Fig. 1B, Table 1) and mediated GnRH-stimulated IP production with EC_{50} and E_{max} values similar to those for the untailed wild-type GnRH receptor (Fig. 1A, Table 1). Since it has previously been shown that the lack of measurable function of the untailed Glu$^{2.53(90)}$Lys mutant receptor is due to lack of

receptor protein expression (Maya-Nunez et al., 2002), the binding and IP production show that appending the human type II GnRH receptor carboxy-terminal tail stabilises expression of the mutant receptor, which has ligand binding and ligand-stimulated signaling properties that are indistinguishable from those of the wild-type GnRH receptor. We therefore appended the carboxy-terminal tail to other mutant receptors in order to increase their cell surface expression without changing their GnRH binding and signaling properties.

3.2. IP signaling and ligand binding of mutant GnRH receptors with small amino acid substitutions for $Glu^{2.53(90)}$

COS7 cells expressing mutant GnRH receptors in which $Glu^{2.53(90)}$ was substituted with Ala, Ser, Asn and Asp showed no detectable GnRH-stimulated IP production and no detectable ^{125}I-[His^5,D-Tyr^6]-GnRH binding (Table 1). This indicates that the negatively-charged carboxyl group of the Asp side-chain is not sufficient to substitute for the native $Glu^{2.53(90)}$ in expression of the GnRH receptor. Appending the carboxy-terminal tail to the $Glu^{2.53(90)}$Ala mutant GnRH receptor resulted in some measurable IP production and binding (Fig. 1C, Table 1). GnRH potency was the same as at the untailed wild-type GnRH receptor (Fig. 1C, Table 1), but GnRH binding affinity was reduced six-fold (Fig. 1D, Table 1). The $Glu^{2.53(90)}$Ser, $Glu^{2.53(90)}$Asn and conservative $Glu^{2.53(90)}$Asp mutant GnRH receptors with appended carboxy-tails showed no measurable IP production or ^{125}I-[His^5,D-Tyr^6]-GnRH binding (Fig. 1, Table 1).

3.3. IP signaling and ligand binding of mutant GnRH receptors with large, uncharged amino acid substitutions for $Glu^{2.53(90)}$

COS7 cells expressing the mutant GnRH receptor with isosteric, uncharged Gln substituted for $Glu^{2.53(90)}$ showed GnRH-stimulated IP production similar to that of the wild-type GnRH receptor and had unchanged GnRH binding affinity (Fig. 1, Table 1). GnRH receptors with the large hydrophobic residues, Phe and Leu, substituted for $Glu^{2.53(90)}$ mediated GnRH-stimulated IP production with E_{max} values similar to wild-type (Fig. 1E, Table 1). GnRH potency and affinity were also unchanged (Fig. 1, Table 1).

3.4. IP signaling and ligand binding of mutant GnRH receptors with a positively charged amino acid substitution for $Glu^{2.53(90)}$

The guanidinium side-chain of Arg is basic and positively charged, but longer than the Lys side-chain. The $Glu^{2.53(90)}$Arg GnRH receptor mediated significant GnRH-stimulated IP production (Fig. 1G, Table 1), although GnRH potency was decreased (Fig. 1G, Table 1). The $Glu^{2.53(90)}$Arg mutant GnRH receptor with a carboxy-terminal tail showed IP production with decreased GnRH potency like its untailed counterpart (Fig. 1G, Table 1). The $Glu^{2.53(90)}$Arg mutant receptors showed decreased total binding of ^{125}I-[His^5,D-Tyr^6]-GnRH and decreased affinity for GnRH (Fig. 1H, Table 1). The $Glu^{2.53(90)}$Arg mutant GnRH receptors showed no change in affinity for the high affinity analog [His^5,D-Tyr^6]-GnRH (IC_{50}, 3.21 ± 1.34 nM and 4.96 ± 1.15 nM, wild-type GnRH receptor 2.43 ± 0.95 nM). This unchanged affinity for the constrained GnRH analog explains why the mutant receptor showed measurable binding of ^{125}I-[His^5,D-Tyr^6]-GnRH in spite of decreased affinity for GnRH.

3.5. IP signaling and ligand binding of mutant GnRH receptors with Ala and Arg substitutions for $Trp^{(6.48)280}$

COS7 cells transfected with the $Trp^{6.48(280)}$Ala mutant GnRH receptor showed no detectable GnRH-stimulated IP production and no detectable binding of ^{125}I-[His^5,D-Tyr^6]-GnRH (Fig. 2, Table 1). The $Trp^{6.48(280)}$Ala mutant with the carboxy-terminal tail showed IP production and binding similar to that of the wild-type GnRH receptor (Fig. 2, Table 1). 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 distortion of GnRH receptor function similar to what we found for the $Glu^{2.53(90)}$Arg mutant GnRH receptor. The $Trp^{6.48(280)}$Arg mutant GnRH receptor showed no detectable IP production or binding. The $Trp^{6.48(280)}$Arg mutant GnRH receptor with the appended carboxy-terminal tail showed decreased GnRH potency, decreased affinity for GnRH and decreased coupling efficiency (Fig. 2, Table 1). The tailed $Trp^{6.48(280)}$ mutant GnRH receptors showed no change in affinity for the [His^5,D-Tyr^6]-GnRH analog used as the tracer in the ligand binding assays.

3.6. Homology modelling and molecular dynamics of wild-type and mutant GnRH receptors

The wild-type GnRH receptor model showed that the $Glu^{2.53(90)}$ side-chain makes direct contact with the side-chains of $Ser^{3.35(124)}$ in TM3 and $Trp^{6.48(280)}$ in TM6 as well as with the $Phe^{7.43(313)}$ and $Pro^{7.45(316)}$ residues in TM7 (Fig. 3A, Table 2). These contacts were preserved during molecular dynamics (Supplementary Figs. S7 and S8), indicating that they are stable. $Glu^{2.53(90)}$ did not stably contact $Lys^{3.32(121)}$ (Fig. 3A, Supplementary Fig. S9).

The starting model of the $Glu^{2.53(90)}$Lys mutant GnRH receptor showed a van der Waals interaction of the introduced $Lys^{2.53(90)}$ side-chain with $Ser^{3.35(124)}$ (Fig. 3B), similar to that in the wild-type GnRH receptor model, but it was broken during the molecular dynamics simulation (Table 2, Supplementary Fig. S7), suggesting that it is unstable. The introduced $Lys^{2.53(90)}$ preserved the interaction with $Trp^{6.48(280)}$ (Table 2, Supplementary Fig. S8). The starting $Glu^{2.53(90)}$Asp mutant GnRH receptor model showed a preserved contact of $Asp^{2.53(90)}$ with $Ser^{3.35(124)}$, but there was no interaction with $Trp^{6.48(280)}$ (Fig. 3C, Table 2, Supplementary Figs. S7 and S8).

The model of the $Glu^{2.53(90)}$Arg mutant GnRH receptor showed that the introduced $Arg^{2.53(90)}$ side-chain made stable interhelical contacts with both $Ser^{3.35(124)}$ and $Trp^{6.48(280)}$ (Fig. 3D, Table 2, Supplementary Figs. S7 and S8). The $Trp^{6.48(280)}$Arg mutant GnRH receptor showed stable interhelical contacts of $Glu^{2.53(90)}$ with $Ser^{3.35(124)}$ and $Arg^{6.48(280)}$ (Table 2, Supplementary Figs. S7 and S8). To better understand the decreased binding affinities of mutant receptors with Arg in positions 2.53(90) or 6.48(280), we measured the distances between the backbone carbons of the amino acids in these loci (Fig. 4). Models of the $Glu^{2.53(90)}$Arg and $Trp^{6.48(280)}$Arg mutant receptors showed increased average interhelical distances (14.33 ± 0.018 Å and 13.11 ± 0.008 compared with 12.34 ± 0.017 Å in the wild type receptor) (Fig. 4). More detailed molecular modelling results are available in Supplementary Material.

In summary, we have shown that mutation of $Glu^{2.53(90)}$ to smaller amino acids, including the conservative Asp substitution, disrupted GnRH receptor function and addition of the carboxy-terminal tail to these mutant receptor constructs could recover function of only the $Glu^{2.53(90)}$Ala mutant. In contrast, mutant GnRH receptors with large uncharged and hydrophobic substitutions for $Glu^{2.53(90)}$ retained full wild-type-like GnRH receptor function. Furthermore, the $Glu^{2.53(90)}$Arg mutant GnRH receptor showed robust IP production and ligand binding, but GnRH affinity and potency were decreased. The $Trp^{6.48(280)}$Arg mutant receptor with a carboxy-terminal tail had decreased affinity for GnRH, similar to that of the $Glu^{2.53(90)}$Arg mutant GnRH receptor. Computational modelling of the wild-type GnRH receptor showed that the $Glu^{2.53(90)}$ side-chain makes a conserved interhelical contact with $Ser^{3.35(124)}$ in TM3 and a direct contact with $Trp^{6.48(280)}$ in TM6, but no stable contact with $Lys^{3.32(121)}$. The model of the cHH-associated $Glu^{2.53(90)}$Lys mutant showed an unstable $Lys^{2.53(90)}$-$Ser^{3.35(124)}$ interhelical contact, while the $Glu^{2.53(90)}$Asp mutant showed no interaction of $Asp^{2.53(90)}$ with $Trp^{6.48(280)}$. Models of the $Glu^{2.53(90)}$Arg and $Trp^{6.48(280)}$Arg mutant receptors showed stable contacts of the amino acids in position 2.53(90) with $Ser^{3.35(124)}$ and

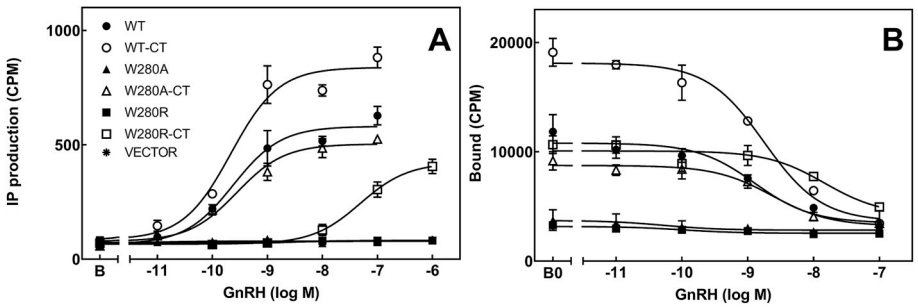

Fig. 2. Effects of mutations of the Trp$^{6.48(280)}$ residue of the GnRH receptor on GnRH-stimulated IP production and ligand binding. COS7 cells transfected with wild-type or mutant GnRH receptors, without or with an appended carboxy-terminal tail (CT), were labelled with [^{3}H]-*myo*-inositol and incubated with varying concentrations of GnRH (A) or incubated with ^{125}I-[His^{5},D-Tyr^{6}]-GnRH and varying concentrations of GnRH (B). Data are means ± range from representative experiments performed at least 3 times in duplicate.

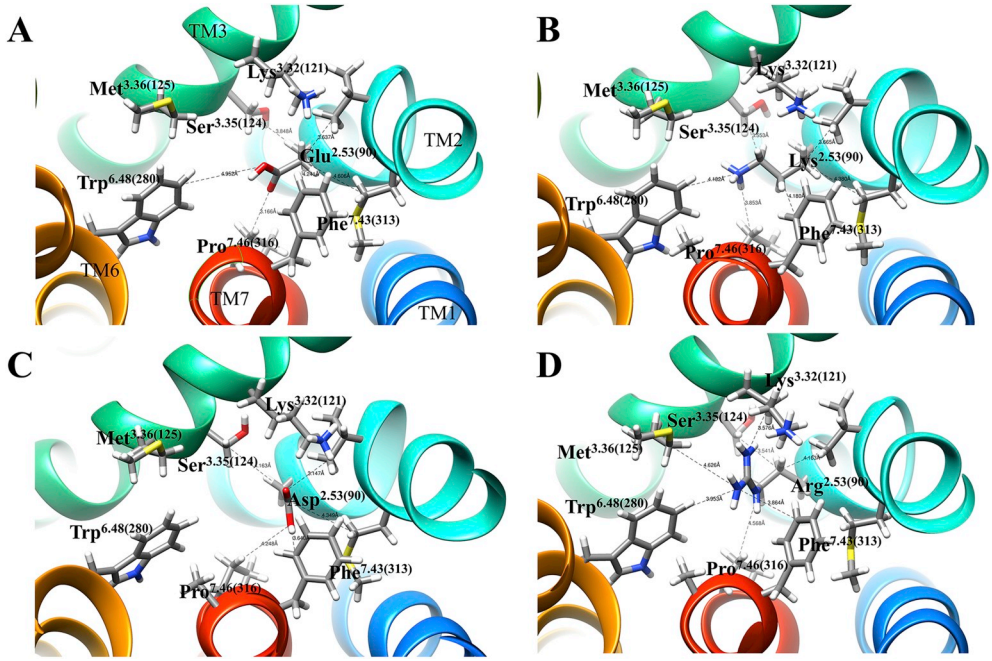

Fig. 3. Models of the wild-type and mutant GnRH receptors showing interactions of residues in position 2.53(90). Homology models of wild-type (A) and Glu$^{2.53(90)}$Lys (B), Glu$^{2.53(90)}$Asp (C) and Glu$^{2.53(90)}$Arg (D) mutant receptors were generated using the NTSR1 rat neurotensin receptor crystal structure as a template for the TM domain and subjected to energy minimisation and molecular dynamics as described under materials and methods. Residues within the Euclidian distance of the residues in position 2.53(90) are shown.

Table 2

Lengths of interhelical contacts of amino acids in position 2.53(90) of wild type and mutant GnRH receptors. Average distances between the indicated atoms of the amino acid in position 2.53(90) and the indicated atoms of residues in TM helices other than TM2 that were within the Euclidian distance of the Glu$^{2.53(90)}$ side-chain in the starting wild type GnRH receptor model, were calculated during the 100 ns molecular dynamics simulations of the wild type and mutant GnRH receptor models. Time courses of changes in interatomic distance are presented in Supplementary Fig. 7, for interaction with Ser$^{3.35(124)}$, and in Supplementary Fig. 8 for other interactions. Data are mean ± SEM of the interatomic distances in the 2001 snapshots before and during the 100ns molecular dynamics production runs. *, significantly different from wild type, $p < 0.05$.

Interhelical contact residue, atom	Residue in position 2.53(90)				
	Glu$^{2.53(90)}$	Lys$^{2.53(90)}$	Asp$^{2.53(90)}$	Arg$^{2.53(90)}$	Glu$^{2.53(90)}$
	(interacting atom, Å)				
Ser$^{3.35(124)}$, CB	CB	CB	CB	CB	CB
	4.55 ± 0.01	4.70 ± 0.02*	4.03 ± 0.01*	4.06 ± 0.01*	4.73 ± 0.01*
Trp$^{6.48(280)}$, CH2	OE1	NZ	OD2	NH1	–
	4.25 ± 0.01	4.28 ± 0.01*	6.35 ± 0.02*	5.39 ± 0.04*	
Arg$^{6.48(280)}$, CZ	–	–	–	–	OE1
					3.73 ± 0.01*
Phe$^{7.43(313)}$, CE2	OE2	CE	OD1	NH2	OE2
	4.52 ± 0.01	6.01 ± 0.03*	5.80 ± 0.03*	6.89 ± 0.03*	6.48 ± 0.02*
Pro$^{7.46(316))}$, CG	OE2	CE	OD2	NH2	OE2
	5.10 ± 0.03	5.77 ± 0.02*	4.80 ± 0.03*	7.90 ± 0.03*	5.19 ± 0.01*

between the residues at positions 2.53(90) and 6.48(280), with increased TM2-TM6 interhelical distances.

4. Discussion

We have investigated the role of interhelical interactions of the Glu$^{2.53(90)}$ side-chain in GnRH receptor biogenesis using systematic site-directed mutagenesis of Glu$^{2.53(90)}$ and Trp$^{6.48(280)}$, functional assays that report cell surface expression and computational modelling. We found that all mutations of Glu$^{2.53(90)}$ to amino acids with side-chains shorter than the side-chain of Glu (Ala, Ser, Asn and the acidic Asp) resulted in no measurable function of the mutant receptors. Appending

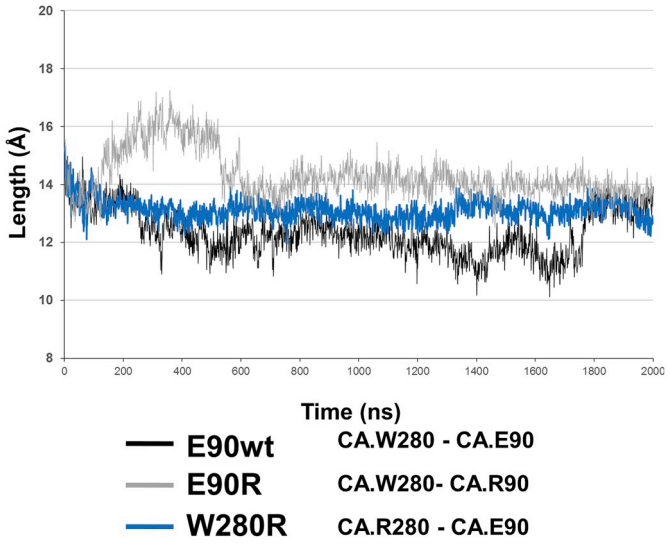

Fig. 4. Distances between TM2 and TM6 in wild-type and mutant GnRH receptor models. The distances between the backbone (CA) carbons of residues in position 2.53(90) and 6.48(280) for wild-type, $Glu^{2.53(90)}$Arg and $Trp^{6.48(280)}$Arg mutant GnRH receptors during the 100 ns molecular dynamics production runs.

a carboxy-terminal tail partially recovered function of the $Glu^{2.53(90)}$Ala mutant receptor, but not others, suggesting that small amino acid substitutions severely disrupt GnRH receptor biogenesis. In contrast, all of our mutations to amino acids with side-chains the same length as- or longer than that of Glu (Gln, Leu, Phe and Arg) preserved receptor function, showing that these mutations do not disrupt receptor biogenesis. This suggested to us that mutations to short side-chain amino acids disrupt one or more intramolecular interactions that are needed for stable GnRH receptor folding and that these interactions are preserved or mimicked in mutants with long side-chain substituents for $Glu^{2.53(90)}$ (except for the $Glu^{2.53(90)}$Lys mutant). We then used molecular models to identify interhelical contacts of the $Glu^{2.53(90)}$ side-chain in the wild-type GnRH receptor and to assess which of these contacts are present, absent or unstable when $Glu^{2.53(90)}$ is substituted. The models show that $Glu^{2.53(90)}$ makes direct interhelical contacts with $Ser^{3.35(124)}$ and $Trp^{6.48(280)}$ in the wild-type receptor, whereas the cHH-associated $Glu^{2.53(90)}$Lys and $Glu^{2.53(90)}$Asp mutant receptors show disruption of the interhelical contacts with $Ser^{3.35(124)}$ and $Trp^{6.48(280)}$ respectively. Both contacts are intact in the model of the well-expressed $Glu^{2.53(90)}$Arg mutant, in spite of its positively-charged Arg side-chain.

The only functional feature that differed among the well-expressed mutant receptors was the decreased GnRH affinity and potency at the $Glu^{2.53(90)}$Arg mutant. The $Trp^{6.8(280)}$Arg mutation (but not other $Trp^{6.48(280)}$ mutations) also decreased GnRH affinity and potency. Therefore, we inspected our models to interpret this result and found that both mutations increase the distance between TM2 and TM6. Since residues at the extracellular ends of both TM2 and TM6 of the GnRH receptor directly contact GnRH, it is likely that the increased interhelical distances increase the distance between ligand-contacting residues of the binding pocket and this distortion decreases tightness (i.e. affinity) of GnRH binding.

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

It is well-established that the $Glu^{2.53(90)}$Lys mutation disrupts formation of intramolecular contacts necessary for correct folding of the GnRH receptor protein during biogenesis, leading to intracellular degradation of the nascent protein, instead of transport to the plasma membrane (Maya-Nunez et al., 2002; Tan et al., 2004; Tao and Conn, 2014). We show that appending the human type II GnRH receptor carboxy-terminal tail recovers function, and thus cell surface expression of the $Glu^{2.53(90)}$Lys mutant GnRH receptor. Based on this, we assume that all of our mutant receptors that show no measureable IP production and ligand binding are poorly expressed at the cell surface due to misfolding and the recovered function on appending the carboxy-terminal tail reflects recovery of cell surface expression of the mutant protein.

It has previously been proposed that the misfolding of the $Glu^{2.53(90)}$Lys mutant GnRH receptor during biogenesis results from charge repulsion that disrupts a putative interhelical salt-bridge interaction of $Glu^{2.35(90)}$ with $Lys^{3.32(121)}$ in the wild-type receptor (Janovick et al., 2009, 2011; Tao and Conn, 2014). Our molecular model of the wild-type GnRH receptor shows no interaction of $Glu^{2.53(90)}$ with $Lys^{3.32(121)}$. Instead it shows the interhelical contact with $Ser^{3.35(124)}$ that is conserved in all GPCR crystal structures (Cvicek et al., 2016; Flanagan and Manilall, 2017) and direct contact with $Trp^{6.48(280)}$, instead of the expected water-mediated contact (Deupi, 2014; Flanagan and Manilall, 2017). Our model of the $Glu^{2.53(90)}$Lys mutant receptor shows that the introduced Lys side-chain destabilises the conserved interaction with $Ser^{3.35(124)}$, but preserves the interhelical contact with $Trp^{6.48(280)}$. This modest destabilisation of a single interhelical contact may explain why the low cell surface expression of $Glu^{2.53(90)}$Lys mutant protein can be recovered by the stabilising effects of the appended type II GnRH receptor tail.

The disruptive effect of the null mutation of $Glu^{2.53(90)}$ to the small amino acid, Ala, shows that the effect of the $Glu^{2.53(90)}$Lys mutation does not arise solely from the presence of a positive charge. The partial recovery of receptor function on adding a carboxy-terminal tail suggests that the null mutation is more disruptive than the $Glu^{2.53(90)}$Lys mutation. The $Glu^{2.53(90)}$Asp GnRH receptor mutation, which has recently been associated with cHH (Marcos et al., 2014), preserves the carboxylic group of Glu, but is shorter by one CH_2 moiety and so might be expected to substitute for Glu in forming an intramolecular salt-bridge. We found that the lack of function of the $Glu^{2.53(90)}$Asp GnRH receptor was not recovered by addition of a carboxy-terminal tail, showing that the Asp side-chain is more disruptive than both the Ala and Lys substitutions and cannot substitute for $Glu^{2.53(90)}$. Mutant GnRH receptors with small uncharged hydrophilic (Asn, Ser) substitutions for $Glu^{2.53(90)}$ were similar to the $Glu^{2.53(90)}$Asp GnRH receptor, showing no measurable IP signaling or ligand binding with- or without a carboxy-terminal tail. This suggests severe destabilisation of mutant receptor biogenesis, although it is possible that the mutant proteins were expressed at the cell surface, but undetectable due to lack of GnRH binding. Our molecular models indicate that the $Glu^{2.53(90)}$Asp mutation disrupts GnRH receptor structure by failing to make the TM2-TM6 interhelical contact with $Trp^{6.48(280)}$. These results suggest that a side-chain that is long enough to contact $Trp^{6.48(280)}$ is necessary for stable cell surface expression of the GnRH receptor, but the disruptive effect of the $Glu^{2.53(90)}$Lys mutation shows that a long side-chain is not sufficient, as a stable interhelical contact with $Ser^{3.35(124)}$ is also required.

4.2. Large, uncharged, hydrophobic and basic amino acid substitutions preserve GnRH receptor function

The wild-type-like function of mutant GnRH receptors with uncharged (Gln) or hydrophobic (Phe and Leu) substituted for $Glu^{2.53(90)}$ shows that mutant receptors are well-expressed at the cell surface and neither the charge nor hydrophilic interactions of the $Glu^{2.53(90)}$ side-chain are necessary for stable GnRH receptor folding during biogenesis. Non-mammalian and type II GnRH receptors (R. P. Millar et al., 2004; Sefideh et al., 2014) and most class A GPCRs have large hydrophobic side-chains in the 2.53 locus (Isberg et al., 2016; Mirzadegan et al., 2003), which form a conserved hydrophobic interhelical contact with the residue in the 3.35 locus (Cvicek et al., 2016). The preserved function of mutant receptors with hydrophobic substitutions for

Glu$^{2.53(90)}$ is consistent with the interhelical contacts of Glu$^{2.53(90)}$ with Ser$^{3.35(124)}$ in TM3 and Trp$^{6.48(280)}$ in TM6 of our molecular model and does not support a charge-based interaction with Lys$^{3.32(121)}$.

To test our conclusion that the Glu$^{2.53(90)}$ side-chain does not form a salt-bridge, we substituted Glu$^{2.53(90)}$ with Arg, which has a guanidinium side-chain that is more basic than the Lys in the cHH-associated mutant (Armstrong et al., 2016; Betts and Russel, 2007). The preserved function of the Glu$^{2.53(90)}$Arg mutant receptor shows that the Arg side-chain fully substitutes for Glu$^{2.53(90)}$ in stabilising GnRH receptor folding during biogenesis. It shows that the Arg$^{2.53(90)}$ side-chain is not close to Lys$^{3.32(121)}$ and indicates that the wild-type Glu$^{2.53(90)}$ side-chain does not form a salt-bridge with Lys$^{3.32(121)}$. The model of the Glu$^{2.53(90)}$Arg mutant receptor shows that the Arg$^{2.53(90)}$ side-chain forms stable interhelical contacts with both Ser$^{3.35(124)}$ and Trp$^{6.48(280)}$, consistent with preserved receptor expression.

The Glu$^{2.53(90)}$Arg mutant receptor differed from the other mutants in having decreased affinity for GnRH. The unchanged affinity of most other mutant receptors shows that the Glu$^{2.53(90)}$ side-chain does not directly contact GnRH. This suggests that the Arg substitution affects GnRH binding affinity indirectly, by distorting the ligand contact surface. Since the Arg side-chain is longer than Glu$^{2.53(90)}$ and all other substitutions (Leu, Phe, Gln) that had wild-type-like GnRH binding affinity, Arg may distort the ligand binding pocket by increasing the distance between the TM helices, forcing the helices apart. This is supported by our molecular models which show that the distance between the backbone carbons of Arg$^{2.53(90)}$ and Trp$^{6.48(280)}$ is on average 2.1 Å longer than between the Glu$^{2.53(90)}$ and Trp$^{6.48(280)}$ in the wild-type receptor. This increased distance is likely to result in increased distance between residues at the extracellular ends of TM2 (Asp$^{2.61(98)}$, Asn$^{2.65(102)}$) and TM6 (Tyr$^{6.58(290)}$), which directly contact the GnRH peptide (Coetsee et al., 2008; Davidson et al., 1996; Flanagan and Manilall, 2017; Flanagan et al., 2000). Such a change in the relative positions of ligand-contacting residues may account for the decreased GnRH binding affinity.

4.3. Similar phenotypes of Trp$^{6.48(280)}$Arg and Glu$^{2.53(90)}$Arg mutant GnRH receptors suggest a similar disruption of the GnRH receptor

Since the robust expression of the Glu$^{2.53(90)}$Arg mutant GnRH receptor makes an interaction of Glu$^{2.53(90)}$ with Lys$^{3.32(121)}$ unlikely, we investigated other potential intramolecular interactions that could account for the role of Glu$^{2.53(90)}$ in GnRH receptor 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$^{6.48}$ in TM6 (Deupi, 2014; Deupi and Standfuss, 2011), we mutated the Trp$^{6.48(280)}$ residue of the GnRH receptor. The lack of function of the Trp$^{6.48(280)}$Ala mutant GnRH receptor and the recovery to wild-type-like function with addition of a carboxy-terminal tail, along with a previous report that multiple substitutions for Trp$^{6.48(280)}$ severely decreased GnRH receptor function, which was recovered using a pharmacological chaperone (Coetsee et al., 2006), shows that the Trp$^{6.48(280)}$ residue is important for expression of the GnRH receptor, but does not contact the ligand. GPCR crystal structures show that the Trp$^{6.48}$ side-chain makes conserved conformation-independent interactions with the residues in positions 3.36 and 7.42 (Cvicek et al., 2016; Venkatakrishnan et al., 2013). Our molecular models show that Trp$^{6.48(280)}$ makes interhelical contacts with Met$^{3.36(124)}$ in TM3 and Ala$^{7.42(312)}$ in TM7 as well as with Glu$^{2.53(90)}$, all of which are preserved in the Trp$^{6.48(280)}$Arg mutant. Disruption of any of these interactions may account for decreased expression of GnRH receptors with mutations of Trp$^{6.48(280)}$. The decreased GnRH binding affinity of the "tailed" Trp$^{6.48(280)}$Arg mutant GnRH receptor, but not other Trp$^{6.48(280)}$-substituted GnRH receptors, suggests that the Arg distorts the GnRH binding surface of the receptor. This distortion may arise from the increased TM2-TM6 interhelical distance in the Trp$^{6.48(280)}$Arg mutant, but also may reflect altered distances between TM6 and TM3 or TM7. The decreased coupling efficiency of the Trp$^{6.48(280)}$Arg mutant supports reports that the Trp$^{6.48}$ residue is part of a conserved network of intramolecular contacts that configure GPCR ligand binding pockets and couple them to the conserved G protein-activating mechanism (Deupi, 2014; Venkatakrishnan et al., 2013).

4.4. cHH-causing GnRH receptor mutations commonly affect conserved interhelical contacts

Many cHH-associated mutations disrupt GnRH receptor folding during biogenesis, which can be rescued by pharmacological chaperones *in vitro* (Flanagan and Manilall, 2017; Tao and Conn, 2014). In addition to the contacts in this study, other conserved interhelical contacts of the GnRH receptor are affected by cHH-associated mutations (see Supplementary Table S1) (Flanagan and Manilall, 2017), suggesting that disruption of conserved interhelical contacts provides a mechanism for understanding some cHH-associated misfolding mutations. The recent approval of the orally-active GnRH receptor antagonist, Elagolix, for treatment of endometriosis provides potential for pharmacological chaperone treatment of cHH patients with mutations that disrupt GnRH receptor folding during biogenesis.

In conclusion, our *in vitro* functional analyses and computational models show that the Glu$^{2.53(90)}$ side-chain of the GnRH receptor does not form an interhelical salt-bridge with Lys$^{3.32(121)}$, but suggest that it forms a conserved contact with Ser$^{3.35(124)}$ in TM3 and a direct contact with Trp$^{6.48(280)}$ in TM6 that likely stabilise correct folding of the protein during biosynthesis. Confirmation of these contacts will depend on direct determination of the GnRH receptor protein structure. The cHH-associated Glu$^{2.53(90)}$Lys mutation appears to disrupt the interhelical contact with Ser$^{3.35(124)}$ and the previously-uncharacterised cHH-associated, conservative, Glu$^{2.53(90)}$Asp mutation, which has a more disruptive effect on receptor function, is associated with inability of the Asp side-chain to form an interhelical contact with Trp$^{6.48(280)}$. Our mutation of the Glu$^{2.53(90)}$ and Trp$^{6.48(280)}$ residues to Arg has revealed a previously unanticipated role of these residues in configuring the structure of the GnRH binding pocket that is consistent with their roles as part of the conserved amino acid network that senses agonist binding to GPCRs and triggers receptor activation.

Funding

This work was supported by grants from the South African National Research Foundation [grant numbers 93464 and 81184] to CAF, from the South African National Research Foundation [grant number 105824] to RPM and an award from the Harry Oppenheimer Trust to RPM.

References

Angel, T.E., Chance, M.R., Palczewski, K., 2009. Conserved waters mediate structural and functional activation of family A (rhodopsin-like) G protein-coupled receptors. Proc. Natl. Acad. Sci. U. S. A. 106, 8555–8560.

Armstrong, C.T., Mason, P.E., Anderson, J.L., Dempsey, C.E., 2016. Arginine side chain interactions and the role of arginine as a gating charge Carrier in voltage sensitive ion channels. Sci. Rep. 6, 21759.

Balasubramanian, R., Crowley, W.F.J., 2007. [Updated 2017 mar 2]. Isolated gonadotropin-releasing hormone (GnRH) deficiency. In: Adam, M.P., Ardinger, H.H., Pagon, R.A., Wallace, S.E., Bean, L.J.H., Mefford, H.C., K, S., Amemiya, A., Ledbetter, N. (Eds.), GeneReviews® [Internet]. University of Washington, Seattle (WA.

Ballesteros, J., Kitanovic, S., Guarnieri, F., Davies, P., Fromme, B.J., Konvicka, K., Chi, L., Millar, R.P., Davidson, J.S., Weinstein, H., Sealfon, S.C., 1998. Functional microdomains in G-protein-coupled receptors. The conserved arginine-cage motif in the gonadotropin-releasing hormone receptor. J. Biol. Chem. 273, 10445–10453.

Ballesteros, J.A., Weinstein, W., 1995. Integrated methods for the construction of three-

dimensional models and computational probing of structure-function relations in G protein-coupled receptors. Methods Neurosci. 25, 366–428.
Betts, M.J., Russel, R.B., 2007. Amino-acid properties and consequences of substitutions. In: R, B.M. (Ed.), Bioinformatics for Geneticists. John Wiley & Sons, Ltd, Chichester, UK, pp. 311–342.
Blankenship, E., Vahedi-Faridi, A., Lodowski, D.T., 2015. The high-resolution structure of activated opsin reveals a conserved solvent network in the transmembrane region essential for activation. Structure 23, 2358–2364.
Bortolato, A., Dore, A.S., Hollenstein, K., Tehan, B.G., Mason, J.S., Marshall, F.H., 2014. Structure of Class B GPCRs: new horizons for drug discovery. Br. J. Pharmacol. 171, 3132–3145.
Chevrier, L., Guimiot, F., de Roux, N., 2011. GnRH receptor mutations in isolated gonadotropic deficiency. Mol. Cell. Endocrinol. 346, 21–28.
Chi, L., Zhou, W., Prikhozhan, A., Flanagan, C., Davidson, J.S., Golembo, M., Illing, N., Millar, R.P., Sealfon, S.C., 1993. Cloning and characterization of the human GnRH receptor. Mol. Cell. Endocrinol. 91, R1–R6.
Coetsee, M., Gallagher, R., Millar, R., Flanagan, C., Lu, Z.L., 2006. Role of Trp280(6.48) in the Gonadotropin-releasing Hormone (GnRH) Receptor, vol. 2006 Abstrct, BioScience, Glasgow UK.
Coetsee, M., Millar, R.P., Flanagan, C.A., Lu, Z.L., 2008. Identification of Tyr(290(6.58)) of the human gonadotropin-releasing hormone (GnRH) receptor as a contact residue for both GnRH I and GnRH II: importance for high-affinity binding and receptor activation. Biochemistry 47, 10305–10313.
Conn, P.M., Janovick, J.A., 2009. Drug development and the cellular quality control system. Trends Pharmacol. Sci. 30, 228–233.
Cvicek, V., Goddard 3rd, W.A., Abrol, R., 2016. Structure-based sequence alignment of the transmembrane domains of all human GPCRs: phylogenetic, structural and functional implications. PLoS Comput. Biol. 12, e1004805.
Davidson, J.S., McArdle, C.A., Davies, P., Elario, R., Flanagan, C.A., Millar, R.P., 1996. Asn102 of the gonadotropin-releasing hormone receptor is a critical determinant of potency for agonists containing C-terminal glycinamide. J. Biol. Chem. 271, 15510–15514.
Deupi, X., 2014. Relevance of rhodopsin studies for GPCR activation. Biochim. Biophys. Acta 1837, 674–682.
Deupi, X., Standfuss, J., 2011. Structural insights into agonist-induced activation of G-protein-coupled receptors. Curr. Opin. Struct. Biol. 21, 541–551.
Deupi, X., Standfuss, J., Schertler, G., 2012. Conserved activation pathways in G-protein-coupled receptors. Biochem. Soc. Trans. 40, 383–388.
Flanagan, C.A., 2016. GPCR-radioligand binding assays. Methods Cell Biol. 132, 191–215.
Flanagan, C.A., Becker, I.I., Davidson, J.S., Wakefield, I.K., Zhou, W., Sealfon, S.C., Millar, R.P., 1994. Glutamate 301 of the mouse gonadotropin-releasing hormone receptor confers specificity for arginine 8 of mammalian gonadotropin-releasing hormone. J. Biol. Chem. 269, 22636–22641.
Flanagan, C.A., Fromme, B.J., Davidson, J.S., Millar, R.P., 1998. A high affinity gonadotropin-releasing hormone (GnRH) tracer, radioiodinated at position 6, facilitates analysis of mutant GnRH receptors. Endocrinology 139, 4115–4119.
Flanagan, C.A., Manilall, A., 2017. Gonadotropin-releasing hormone (GnRH) receptor structure and GnRH binding. Front. Endocrinol. 8, 274.
Flanagan, C.A., Rodic, R., Konvicka, K., Yuen, T., Chi, L., Rivier, J.E., Millar, R.P., Weinstein, H., Sealfon, S.C., 2000. Specific interactions of the Asp2.61(98) side chain of the gonadotropin-releasing hormone receptor contribute differentially to ligand interaction and receptor expression. Biochemistry 39, 8133–8141.
Flanagan, C.A., Zhou, W., Chi, L., Yuen, T., Rodic, V., Robertson, D., Johnson, M., Holland, P., Millar, R.P., Weinstein, H., Mitchell, R., Sealfon, S.C., 1999. The functional microdomain in transmembrane helices 2 and 7 regulates expression, activation, and coupling pathways of the gonadotropin- releasing hormone receptor. J. Biol. Chem. 274, 28880–28886.
Gianetti, E., Hall, J.E., Au, M.G., Kaiser, U.B., Quinton, R., Stewart, J.A., Metzger, D.L., Pitteloud, N., Mericq, V., Merino, P.M., Levitsky, L.L., Izatt, L., Lang-Muritano, M., Fujimoto, V.Y., Dluhy, R.G., Chase, M.L., Crowley Jr., W.F., Plummer, L., Seminara, S.B., 2012. When genetic load does not correlate with phenotypic spectrum: lessons from the GnRH receptor (GNRHR). J. Clin. Endocrinol. Metab. 97, E1798–E1807.
Hoffmann, S.H., ter Laak, T., Kuhne, R., Reilander, H., Beckers, T., 2000. Residues within transmembrane helices 2 and 5 of the human gonadotropin- releasing hormone receptor contribute to agonist and antagonist binding. Mol. Endocrinol. 14, 1099–1115.
Huang, W., Manglik, A., Venkatakrishnan, A.J., Laeremans, T., Feinberg, E.N., Sanborn, A.L., Kato, H.E., Livingston, K.E., Thorsen, T.S., Kling, R.C., Granier, S., Gmeiner, P., Husbands, S.M., Traynor, J.R., Weis, W.I., Steyaert, J., Dror, R.O., Kobilka, B.K., 2015. Structural insights into μ-opioid receptor activation. Nature 524, 315–321.
Isberg, V., Mordalski, S., Munk, C., Rataj, K., Harpsoe, K., Hauser, A.S., Vroling, B., Bojarski, A.J., Vriend, G., Gloriam, D.E., 2016. GPCRdb: an information system for G protein-coupled receptors. Nucleic Acids Res. 44, D356–D364.
Janovick, J.A., Maya-Nunez, G., Conn, P.M., 2002. Rescue of hypogonadotropic hypogonadism-causing and manufactured GnRH receptor mutants by a specific protein-folding template: misrouted proteins as a novel disease etiology and ther-apeutic target. J. Clin. Endocrinol. Metab. 87, 3255–3262.
Janovick, J.A., Patny, A., Mosley, R., Goulet, M.T., Altman, M.D., Rush 3rd, T.S., Cornea, A., Conn, P.M., 2009. Molecular mechanism of action of pharmacoperone rescue of misrouted GPCR mutants: the GnRH receptor. Mol. Endocrinol. 23, 157–168.
Janovick, J.A., Pogozheva, I.D., Mosberg, H.I., Conn, P.M., 2011. Salt bridges overlapping the gonadotropin-releasing hormone receptor agonist binding site reveal a coin-cidence detector for G protein-coupled receptor activation. J. Pharmacol. Exp. Therapeut. 338, 430–442.
Leanos-Miranda, A., Janovick, J.A., Conn, P.M., 2002. Receptor-misrouting: an unexpectedly prevalent and rescuable etiology in gonadotropin-releasing hormone re-ceptor-mediated hypogonadotropic hypogonadism. J. Clin. Endocrinol. Metab. 87, 4825–4828.
Lin, X., Janovick, J.A., Brothers, S., Blomenrohr, M., Bogerd, J., Conn, P.M., 1998. Addition of catfish gonadotropin-releasing hormone (GnRH) receptor intracellular carboxyl-terminal tail to rat GnRH receptor alters receptor expression and regulation. Mol. Endocrinol. 12, 161–171.
Mahoney, J.P., Sunahara, R.K., 2016. Mechanistic insights into GPCR-G protein interactions. Curr. Opin. Struct. Biol. 41, 247–254.
Marcos, S., Sarfati, J., Leroy, C., Fouveaut, C., Parent, P., Metz, C., Wolczynski, S., Gerard, M., Bieth, E., Kurtz, F., Verier-Mine, O., Perrin, L., Archambeaud, F., Cabrol, S., Rodien, P., Hove, H., Prescott, T., Lacombe, D., Christin-Maitre, S., Touraine, P., Hieronimus, S., Dewailly, D., Young, J., Pugeat, M., Hardelin, J.P., Dode, C., 2014. The prevalence of CHD7 missense versus truncating mutations is higher in patients with Kallmann syndrome than in typical CHARGE patients. J. Clin. Endocrinol. Metab. 99, E2138–E2143.
Maya-Nunez, G., Janovick, J.A., Conn, P.M., 2000. Combined modification of intracellular and extracellular loci on human gonadotropin-releasing hormone receptor provides a mechanism for enhanced expression. Endocrine 13, 401–407.
Maya-Nunez, G., Janovick, J.A., Ulloa-Aguirre, A., Soderlund, D., Conn, P.M., Mendez, J.P., 2002. Molecular basis of hypogonadotropic hypogonadism: restoration of mutant (E(90)K) GnRH receptor function by a deletion at a distant site. J. Clin. Endocrinol. Metab. 87, 2144–2149.
Mayevu, N.M., Choe, H., Abagyan, R., Seong, J.Y., Millar, R.P., Katz, A.A., Flanagan, C.A., 2015. Histidine in the conserved peptide receptor activation domain of the gonadotropin releasing hormone receptor couples peptide binding and receptor activation. Mol. Cell. Endocrinol. 402, 95–106.
Millar, R.P., Davidson, J., Flanagan, C., Wakefield, I., 1995. Ligand binding and second-messenger assays for cloned Gq/G11-coupled neuropeptide receptors: the GnRH receptor. Methods Neurosci. 25, 145–162.
Millar, R.P., Lu, Z.L., Pawson, A.J., Flanagan, C.A., Morgan, K., Maudsley, S.R., 2004. Gonadotropin-releasing hormone receptors. Endocr. Rev. 25, 235–275.
Mirzadegan, T., Benko, G., Filipek, S., Palczewski, K., 2003. Sequence analyses of G-protein-coupled receptors: similarities to rhodopsin. Biochemistry 42, 2759–2767.
Sefideh, F.A., Moon, M.J., Yun, S., Hong, S.I., Hwang, J.I., Seong, J.Y., 2014. Local duplication of gonadotropin-releasing hormone (GnRH) receptor before two rounds of whole genome duplication and origin of the mammalian GnRH receptor. PloS One 9, e87901.
Soderlund, D., Canto, P., de la Chesnaye, E., Ulloa-Aguirre, A., Mendez, J.P., 2001. A novel homozygous mutation in the second transmembrane domain of the gonadotrophin releasing hormone receptor gene. Clin. Endocrinol. 54, 493–498.
Standfuss, J., Edwards, P.C., D'Antona, A., Fransen, M., Xie, G., Oprian, D.D., Schertler, G.F., 2011. The structural basis of agonist-induced activation in constitutively active rhodopsin. Nature 471, 656–660.
Tan, C.M., Brady, A.E., Nickols, H.H., Wang, Q., Limbird, L.E., 2004. Membrane trafficking of G protein-coupled receptors. Annu. Rev. Pharmacol. Toxicol. 44, 559–609.
Tao, Y.X., Conn, P.M., 2014. Chaperoning G protein-coupled receptors: from cell biology to therapeutics. Endocr. Rev. 35, 602–647.
Tello, J.A., Newton, C.L., Bouligand, J., Guiochon-Mantel, A., Millar, R.P., Young, J., 2012. Congenital hypogonadotropic hypogonadism due to GnRH receptor mutations in three brothers reveal sites affecting conformation and coupling. PloS One 7, e38456.
Trzaskowski, B., Latek, D., Yuan, S., Ghoshdastider, U., Debinski, A., Filipek, S., 2012. Action of molecular switches in GPCRs-theoretical and experimental studies. Curr. Med. Chem. 19, 1090–1109.
van Rhee, A.M., Jacobson, K.A., 1996. Molecular architecture of G protein-coupled receptors. Drug Dev. Res. 37, 1–38.
Venkatakrishnan, A.J., Deupi, X., Lebon, G., Tate, C.G., Schertler, G.F., Babu, M.M., 2013. Molecular signatures of G-protein-coupled receptors. Nature 494, 185–194.
Yuan, S., Filipek, S., Palczewski, K., Vogel, H., 2014. Activation of G-protein-coupled receptors correlates with the formation of a continuous internal water pathway. Nat. Commun. 5, 4733.