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Structural and functional plasticity of the luteinizing hormone/choriogonadotrophin receptor

Britta Troppmann[1], Gunnar Kleinau[2], Gerd Krause[3], and Jörg Gromoll[1,*]

[1]Centre of Reproductive Medicine and Andrology, Institute of Reproductive and Regenerative Biology, University Hospital Muenster, Albert-Schweitzer-Campus 1, D11, Muenster 48149, Germany [2]Institute of Experimental Pediatric Endocrinology, Charité-Universitätsmedizin Berlin, Augustenburger Platz 1, Berlin 13353, Germany [3]Leibniz Institut für Molekulare Pharmakologie, Robert-Rössle-Strasse 10, Berlin 13125, Germany

Correspondence address. Tel: +49-251-83-56447; Fax: +49-251-83-56093; E-mail: joerg.gromoll@ukmuenster.de

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BACKGROUND: In recent years it became evident that several types of the luteinizing hormone/choriogonadotrophin receptor (LHCGR) exist. In addition to the classical receptor type known in rodents, an *LHCGR* type containing an additional exon is present in primates and humans. This specific exon 6A introduces a hitherto unknown regulatory pathway of the *LHCGR* at the transcriptional level which can lead to the expression of an alternative protein covering the extracellular part only. Furthermore, an *LHCGR* type lacking exon 10 at the mRNA and protein levels has been described in the New World primate lineage, giving rise to an additional receptor type in which amino acids of the extracellular hinge region connecting the leucine-rich repeat domain and transmembrane domain are missing.

METHODS: Topic-related information was retrieved by systematic searches using Medline/PubMed. Structural homology models were retrieved from a glycoprotein hormone receptors web application and from recent publications.

RESULTS: In a novel approach, we combine functional aspects with three-dimensional properties of the LHCGR and the different receptor types to deduce causative relationships between these two parameters. On this basis, the physiological impact and patho-physiological consequences of the different LHCGR types are inferred.

CONCLUSIONS: The complex system of different LHCGR types and two corresponding hormones (LH and CG) represents a major challenge for future studies on selective hormone binding, signal transduction and receptor regulation. The presence of these naturally occurring LHCGR types requires re-examining of our present view on receptor function, experimental set-ups and data interpretation, but also offers new clinical approaches to interfere with LH/CG action in humans.

Key words: gonadotrophins / luteinizing hormone receptor / choriogonadotrophin / evolution / genomic organization

Introduction

The luteinizing hormone/choriogonadotrophin receptor (LHCGR) is a member of the superfamily of guanine nucleotide-binding protein coupled receptors (GPCRs) and belongs to the glycoprotein hormone receptors (GPHRs), a subfamily of the family A GPCRs (Ascoli *et al*., 2002; Fredriksson *et al*., 2003). This subfamily also contains the follicle-stimulating hormone receptor (FSHR; Simoni *et al*., 1997) and the thyroid-stimulating hormone receptor (TSHR; Vassart and Dumont, 1992). The LHCGR mediates the action of two closely related hormones, luteinizing hormone (LH) produced by the pituitary and chorionic gonadotrophin (CG) secreted by the placenta, which are heterodimeric glycoproteins consisting of a common alpha-subunit non-covalently associated with a hormone-specific beta-subunit defining their individual biological properties (Pierce and Parsons, 1981). The CG beta-subunit (CGB) derives from duplications of the LH beta-subunit gene and is characterized by 24 additional amino acids at the C-terminus and a higher degree of glycosylation, resulting in an extended circulatory half-life (Talmadge *et al*., 1984; Henke and Gromoll, 2008).

The crucial role of the LHCGR and the corresponding hormones in physiological aspects of gonadal maturation and reproductive processes is reflected by the predominant expression of the receptor in ovarian theca, granulosa and luteal cells and in the Leydig cells of the testes (reviewed in Ascoli *et al*., 2002). At female puberty, LH stimulates the conversion of androgen from the theca cells to estradiol and thereby regulates the development of female secondary sex characteristics. Postpubertally, LH promotes the production of androgen precursors in the theca cells for aromatization to estradiol by granulosa cells during the follicular phase of the menstrual cycle (reviewed in Young and McNeilly, 2010). While the theca cells constitutively express the LHCGR, the granulosa cells express the LHCGR only in the later stages of follicular development. During its mid-cycle surge, LH induces follicular maturation and ovulation (reviewed in Conti *et al*., 2012). In the luteal phase, LH regulates the formation of the corpus luteum and stimulates progesterone secretion (Benyo and Zeleznik, 1997; Niswender *et al*., 2000). Following fertilization, luteal function induced by LH is maintained by the action of placental CG. During male fetal development, the LHCGR plays a pivotal role by mediating increased androgen synthesis of fetal testicular Leydig cells upon stimulation by placental CG (Diez d'Aux and Pearson Murphy, 1974; Huhtaniemi *et al*., 1977; Sharpe, 2006). Post-natally, LH stimulates testicular testosterone production, triggering pubertal development and is essential for the development of male secondary sex characteristics and spermatogenesis (reviewed in Grumbach, 2002). The importance of the LHCGR function in reproduction is demonstrated by a plethora of naturally occurring mutations and single nucleotide polymorphisms of the LHCGR linked to reproductive disorders and disorders of sex development, including male-limited gonadotrophin-independent precocious puberty, Leydig cell hypoplasia and anovulation/amenorrhoea (reviewed in Themmen and Huhtaniemi, 2000; Huhtaniemi and Themmen, 2005; Piersma *et al*., 2007a; Arnhold *et al*., 2009; Segaloff, 2009; Salvi and Pralong, 2010; Coviello *et al*., 2012; Latronico and Arnhold, 2012; Wang *et al*., 2012).

As one of the objectives of this review is to reconsider the receptor nomenclature currently used, it might be helpful to have a short view on the historical evolution which led to the actual official name, i.e. LH/CG receptor, as found today in databases (http://www.iuphar-db.org/; LHCGR). Over decades, it was assumed that due to nearly indistinguishable biological effects of LH and CG and the lack of a specific CG receptor (Lee and Ryan, 1971; Huhtaniemi and Catt, 1981, Casarini *et al*, 2012), the term 'LHCGR' would nicely describe this type of receptor. However, the fact that CGB genes are not found in rodents and most other mammals was continuously neglected (Wurzel *et al*., 1983; Jameson *et al*, 1984; Tepper and Roberts, 1984; Maston and Ruvolo, 2002). The majority of functional receptor studies were performed in rodents and CG was more easily available and better to handle (e.g. radioiodation for binding studies) and even today studies are predominantly done for CG and not for LH. In view of current knowledge on different receptor types and the LH and CG hormone system, we believe that a revisit of the nomenclature is timely and warranted.

This review focuses on the recent discovery and characterization of different types of the LHCGR in primates and humans, reflecting the unexpected evolutionary plasticity of this receptor. We will propose a new nomenclature of the different receptor types to facilitate more precise communication in this area. In a novel approach, we will combine functional aspects with three-dimensional properties of the LHCGR and the different receptor types to propose important causative relationships between these two parameters and thereby update current concepts of the structure and function of this receptor system. We discuss subsequent possible physiological consequences and resulting challenges, which must be considered when studying LHCGR function in different model systems and under clinical aspects. The general LHCGR topology and signalling mechanisms have been extensively reviewed by others (Ascoli *et al*., 2002; Puett *et al*., 2005, 2007; Menon and Menon, 2012) and are not the focus of this review. Thus, we will only briefly summarize the basics and latest insights into different LHCGR types, according to their significance for functional, physiological and pharmacological aspects.

Methods

Searches were performed in Medline/PubMed to identify relevant studies using key words. Journal articles were included based on high quality and relevance. Our journal database searches were not restricted by species; however, we focused our review on human and primates. Structural homology models from previously published studies (Kleinau and Krause, 2009; Heitman *et al*., 2012; Kossack *et al*., 2013) or a GPHR web application (Kreuchwig *et al*., 2011) were used and modified for visualization of particular aspects described here.

LHCGR gene and protein structures

The *LHCGR* gene

The *LHCGR* gene is a single-copy gene consisting of either 11 or 12 exons and 10 or 11 introns and this principle genomic organization is highly conserved between species. The human *LHCGR* is located on the short arm of chromosome 2 (2p21) and consists of 11 constitutive exons which is complemented by the primate-specific exon 6A, whereas the *Lhr* of the mouse located on chromosome 17 only contains 11 exons (Fig. 1A, Table I; Minegishi *et al*., 1990; Rousseau-Merck *et al*., 1990; Atger *et al*., 1995; Kossack *et al*., 2008). Exon 1 of the *LHCGR* encodes the signal peptide and the *N*-terminal cysteine-rich region (Fig. 1A, 1B;

Atger *et al*., 1995). The leucine-rich repeat domain (LRRD) and the *N*-terminus of the hinge region, connecting the LRRD and transmembrane helix (TMH) 1, arise from splicing of exons 1–10. Exon 11 encodes the C-terminal segment of the hinge region and the entire serpentine-like membrane-spanning domain constituted by the TMHs and connecting loops, as well as the C-terminal intracellular part (reviewed in Dufau, 1998; Ascoli *et al*., 2002; Puett *et al*., 2005). The major transcriptional start sites of the *LHCGR* are located within 176 bp upstream of the ATG start codon and promoter activation is modulated by Sp1-binding sites and an upstream inhibitory motif that binds nuclear orphan receptors (Geng *et al*., 1999; Zhang and Dufau, 2003; reviewed in Dufau *et al*., 2010). Furthermore, *LHCGR* gene expression is regulated via epigenetic modulations, whereby local chromatin changes at the promoter resulting from histone acetylation and methylation are critical (Zhang and Dufau, 2002; Zhang *et al*., 2005).

LHCGR protein structure and mechanisms of signal transduction

Hormone binding and signalling at the extracellular region

The mature human LHCGR consists of 699 amino acids (Fig. 1B). Due to massive glycosylation, the molecular mass of the mature LHCGR present in the cell membrane is higher (85–95 kDa) than the predicted molecular protein mass of ~75 kDa (reviewed in Ascoli *et al*., 2002).

Although the crystal structure of CG has been known for nearly two decades (Lapthorn *et al*., 1994; Wu *et al*., 1994; Lustbader *et al*., 1995), structures of the hormone bound to the extracellular region of the LHCGR are not yet available. Models of the particular tertiary structure of proteins including details of amino acid arrangements on the level of atoms can be determined via X-ray diffraction of well-ordered crystallized molecules of interest and would allow deeper insights into the LHCGR complexed with its ligand. However, for the homologous FSHR, a crystal structure of FSH bound to a fragment of the extracellular LRRD has been available since 2005 (Fan and Hendrickson, 2007). A recent extended crystal structure shows a nearly complete FSHR extracellular region in complex with FSH and reveals substantial new information for the homologous GPHRs (Jiang *et al*., 2012). General characteristics visible with this extended structure may also be assumed for the LHCGR. The crystallized LRRD is formed by around 260 amino acids and starts with cysteine-box 1 (Cb-1) and ends at cysteine-box 2 (Cb-2) (Fig. 2). This LRRD comprises 11 repeats in contrast to the 9 repeats observed in the previous, more fragmentary FSHR structure (Fan and Hendrickson, 2007). Each of the 11 repeats exhibits a β-strand forming a concave β-sheet, where hormone binding occurs (Fig. 2). Apart from a common general binding mode at this β-sheet for the GPHs (Smits *et al*., 2003; Bogerd, 2007), specific characteristics have also been reported in the interaction between the GPHs and their corresponding receptors (Caltabiano *et al*., 2008; Angelova *et al*., 2010). It has been shown by site-directed mutagenesis that repeats two to eight of the LRRD are key players for high-affinity binding of LH and CG (Braun *et al*., 1991; Bhowmick *et al*., 1996, 1999; Vischer *et al*., 2003; Angelova *et al*., 2010).

One feature of the new structural extension concerns repeat 11, that is, apart from the β-strand, which is also specifically characterized by a short helical structure (positions 272–280). It contains two consecutive cysteines (FSHR: Cys275, Cys276; LHCGR: Cys279, Cys280) that are bridged to the last two extracellular cysteines of Cb-3 close to TMH 1. One of these cysteines is located at an additional short β-strand element (FSHR: Cys346, LHCGR: Cys343) that is assembled parallel to the concave LRRD β-strand 11 and the second cysteine is adjacent to TMH 1 (FSHR: Cys356, LHCGR: Cys353). Thus, Cb-2 (LRRD) and Cb-3 (close to TMH 1) are in tight spatial proximity and these disulfide bridges have been shown to be important for the global LHCGR structure and full signalling capacity (Zhang *et al*., 1996).

Moreover, a second hormone-binding site is constituted by Asp330 and Tyr331, which are located at the C-terminal part of the LHCGR hinge region (Bonomi *et al*., 2006; Bruysters *et al*., 2008b). The interaction between the sulphated tyrosine (as exhibited in all GPHRs (FSHR: Tyr335, LHCGR: Tyr331, TSHR: Tyr385)) at the C-terminus of the hinge region and the hormone is now visible as a further important feature of the new FSHR crystal structure (Jiang *et al*., 2012). The hinge region of the LHCGR and other GPHRs is generally known to be involved in determinating endogenous ligand binding (Costagliola *et al*., 2002; Bonomi *et al*., 2006; Bruysters *et al*., 2008b; Kleinau *et al*., 2011) and selectivity (Bernard *et al*., 1998), signalling pathway regulation (Nurwakagari *et al*., 2007) and signalling features (Moyle *et al*., 2004; Urizar *et al*., 2005). In conclusion, the new FSHR crystal structure and several experimental studies support, also for the LHCGR, two separate binding sites of the hormones at the extracellular region of the receptor: one at the LRRD and the other at the hinge region around Tyr331 (LHCGR number). This interplay conveys the signal to the serpentine domain by a yet unknown mechanism. It might be assumed that structural modifications induced by hormone binding at both the LRRD and the hinge region finally lead to changes at the disulphide-bridged unit between the LRRD C-terminus and Cb-3 located close to the serpentine domain. Especially amino acids Pro276 and Ser277 at the C-terminal LRRD in repeat 11 and transition to the hinge region should play a fundamental role for signalling regulation as mutations at these amino acids constitutively activate the LHCGR (Nakabayashi *et al*., 2000, 2003; Zeng *et al*., 2001; Sangkuhl *et al*., 2002).

Of specific note, the new FSHR/FSH crystal structure (Jiang *et al*., 2012) is a trimeric receptor/ligand complex and indeed, GPHRs are known to form higher order complexes (Urizar *et al*., 2005). However, the previous FSHR/FSH crystal structure (Fan and Hendrickson, 2007) has a dimeric arrangement which does not overlap with the recently published structure with interactions between the receptor monomers. Moreover, experimental studies at the previously observed LRRD dimer contacts failed to support the significance of these interactions (Guan *et al*., 2010). Therefore, the relevance of the previously dimeric and recently observed trimeric-extracellular arrangement must be very carefully considered because of lacking supporting experimental evidence (also for the LHCGR); it might be that this observed constellation is forced by the crystallization procedure and not due to naturally occurring interaction patterns.

Signal transduction at the serpentine domain

During receptor activation, structural re-arrangements between the extracellular loops and the hinge region likely also directly affect the intramolecular network of side-chain interactions at the serpentine domain. Several amino acids are known either to be important to keep the receptor in the inactive state (characterized by occurrence of constitutively activating mutations), or they are involved in switching the LHCGR into the active-state conformation (as mutations at these positions lead to receptor inactivation, collected in the GPHR information database available at

Figure 1 (**A**) Genomic organization of the human *LHCGR type 1* (Gene ID: 3973) and the mouse *Lhr* gene (Gene ID: 16867). The size of the exons (in base pairs) is given above and the intronic size (in kilo base pairs) is given below the genomic elements. (**B**) Alignment of the receptor types described in this review. The amino acid sequence comparison (alignment) between the different receptor types reveals similarities and differences in amino acid composition and sequence length. Additionally, structural features related to specific sequence regions are annotated (LRRD, leucine-rich repeat domain; SD, serpentine domain; Ctt, C-terminal tail; Cb-1, 2, 3, cysteine-boxes 1, 2, 3) and the exon 10- or exon 6A-encoded regions are boxed. Colour code of amino acids: green/cyan, hydrophobic; orange, hydrophilic; red, negatively charged; blue, positively charged; brown, cysteines; black, prolines.

http://www.ssfa-gphr.de, Kleinau *et al.*, 2007; Kreuchwig *et al.*, 2011). Pathogenic LHCGR mutations (Table II, Fig. 2) assign important spatial regions for activation-related receptor components. As presented in Fig. 2, mapping of activating mutations on the LHCGR model highlights TMH 6 as most crucial for signal transduction at the transmembrane region. Inactivation of the receptor by mutation can be found over the

Table I Summary and nomenclature of the different LHCGR types.

Gene name	Gene structure	Full-length receptor encoded by exons	Previous nomenclature	Proposed new nomenclature	Presence	Hormones	Comment
Lhcgr: *Mus musculus*; Gene ID: 16867	11 exons	1–11	*Lhcgr*	*Lhr*	Rodents, probably all mammals (except primates)	LH	These species do not possess CG (except horse).
LHCGR: *Homo sapiens*; Gene ID: 3973	12 exons, 1–11 and 6a	1–11	*LHCGR/LHR*	*LHCGR type 1*	Primates (except New World monkeys)	LH and CG	For the clear assignment of counting in exon 6A one should outline bp 1–300 and the deduced amino acids 1–30 of exon 6A as ins6A.
LHCGR: *Callithrix jacchus*	12 exons, 1–11 and 6a	1–9 and 11	*LHCGR type 2*	*LHCGR type 2*	New World monkey lineage	CG	The hinge region is 26 amino acids shorter compared with LHCGR type 1.

entire receptor structure, also at amino acids in the peripheral regions. Mutations at these amino acid sites interfere with processes such as hormone-ligand or G-protein binding or they diminish the receptor cell-surface expression level.

Furthermore, several amino acids at each helix are key players for receptor functions. This fact is already reflected by their high degree of evolutionary conservation among family A GPCRs. These amino acids have been studied by several groups for different purposes such as investigation of basal activity regulation, constitutive activation or signal transformation between the helices (Fernandez and Puett, 1996; Kosugi *et al*., 1998; Min *et al*., 1998; Alvarez *et al*., 1999; Angelova *et al*., 2002; Fanelli *et al*., 2004). A remarkable recent study was performed to describe information pathways (for activation) with conserved amino acids participating in structure networks deputed to intramolecular communication (Angelova *et al*., 2011).

Although detailed evidence based on the combined panel of experimental and structural implications of specific amino acids is available, the particular steps leading to LHCGR activation upon hormone binding are not yet understood in full detail. Diverse models suggest receptor activation via interactions between the ligand bound to the extracellular region and the transmembrane helices (Vassart *et al*., 2004; Puett *et al*., 2005; Menon and Menon, 2012). For the TSHR and the FSHR, functional studies proposed an inhibitory effect of the extracellular region, keeping the receptor in an inactive state unless ligand binding occurs (Vlaeminck-Guillem *et al*., 2002). While this was not found for the LHCGR (Sangkuhl *et al*., 2002; Nurwakagari *et al*., 2007), an extracellularly localized intramolecular agonistic unit that becomes activated by hormone binding is commonly assumed (reviewed in Kleinau and Krause, 2009), which likely triggers the serpentine domain (termed also heptahelical or transmembrane region).

In the serpentine domain spatial re-arrangements between the helices 3, 5, 7 (rotation or/and lateral movement relative to each other) and to the greatest extent at helix 6 are significant for the transition between inactive and active conformations (reviewed in Lebon *et al*., 2012). In brief, the GPCR ligand induces, by binding, modifications of intramolecular interactions shifting the equilibrium between the inactive and active conformation towards the signalling-active state of the receptor. This restructuring enables specific interactions with the G-protein. The TMH 6 seems to be a key for this mechanism and moves 4–14 Å towards the membrane. This principle mechanism for family A GPCRs was previously suggested based on results from biophysical studies (reviewed in Schwartz *et al*., 2006) and have been confirmed by the recent crystallization of active-state conformations like for opsin (Scheerer *et al*., 2008) and a complex between ADRB2 and Gs (Rasmussen *et al*., 2011). In addition, the G-protein supports stabilization of the active state and simultaneously increases the ligand-affinity (Lebon *et al*., 2012). Finally, the GPCR triggers intracellular activation of the G-protein (and other effectors) by specific interactions.

One interesting evolutionary aspect of the LHCGR is linked with the capability to bind low-molecular-weight (LMW) ligands (Moore *et al*., 2006; Arey, 2008; Heitman and Ijzerman, 2008). GPCRs of family A evolved 570–700 million years ago (reviewed in Strotmann *et al*., 2011). They bind diverse ligands such as amines, purines, lipids or peptides between the transmembrane helical bundle, with the exception of GPHR ligand-hormone binding that occurs endogenously only at the extracellular region (Fig. 3A). Since the bulky hormones bind to the extracellular receptor region (orthosteric site) of the GPHRs, a recent study of the LHCGR suggests that drug-like LMW ligands bind alternatively to an allosteric site located in the transmembrane helical bundle (Fig. 3A; Heitman *et al*., 2012). These drug-like ligands act as allosteric agonists (activators) or signalling modulators (increasing or decreasing effects on signalling of further ligands). In addition, it is hypothesized that multiple allosteric-binding pockets in the serpentine domain of the human LHCGR do exist, which reveals new opportunities to achieve selectivity of drug action. The importance of this subject is reflected by the finding that a second allosteric LHCGR-binding site closely resembles the orthosteric-binding site of the adenosine A3 receptor and both receptors are able to bind identical molecules (Heitman *et al*., 2012). Of note, LHCGR and the A3 receptor are expressed in reproductive

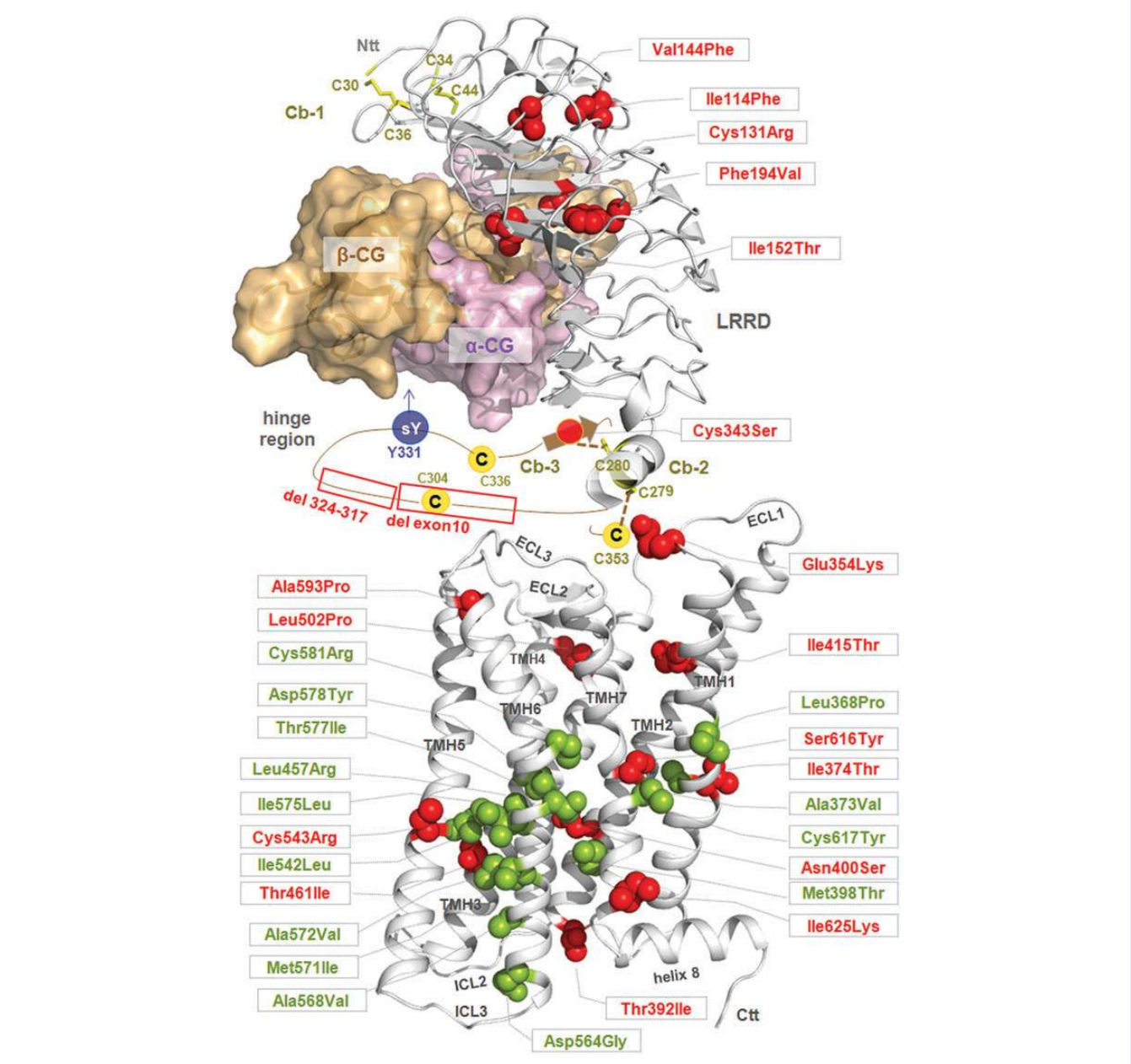


Figure 2 Fragmentary structural homology model of the LHCGR type 1 combined with functional information. The LHCGR type 1 (model-backbone white) binds the hormone (CG subunits light-brown/violet, surface and backbone) at the extracellular side between the LRRD and the hinge region. In principle, the LRRD, the hinge region and the serpentine domain are arranged sandwich like, however, the precise spatial orientation to each other is unknown (an exemplary plot is shown). Cysteine-bridges (yellow) between the LRRD and the hinge region attach these fragments tightly together in spatial terms. The exon 10-encoded region (red box) is located at the hinge region. (Pathogenic deletions are known for both exon 10 and an adjacent fragment, positions 317–324; Table II). A sulphated tyrosine (blue) at the hinge region interacts with the hormone CG. The transmembrane helices (TMHs) 1–7 spanning the membrane are connected by intra- (ICLs) and extracellular loops (ECLs). In this model, positions of naturally occurring inactivating and activating single-point mutations are mapped (Table II). Activating mutations (green spheres) either disrupt the inactive state or stabilize the active-state conformation. For the LHCGR type 1, constitutively activating mutations are cumulatively localized in the centre of the TMH core. In contrast, inactivating mutations (red spheres) occur throughout the entire receptor, including the LRRD. These mutations probably either (i) modify the capability of the receptor to interact with the hormone (mutations located at the LRRD or hinge region) or (ii) they might prevent signal transduction through the transmembrane region or (iii) they may interfere with activation of the G-protein (located intracellularly).

tissues (Rivkees, 1994; Zhang *et al*., 2001) and in the adrenal cortex (Pabon *et al*., 1996; Atkinson *et al*., 1997). Finally, the capability of the LHCGR to bind molecules allosterically at the transmembrane region is an example of conservation of a ligand-binding sensitive region which is not occupied by the endogenous ligand. However, activation of the LHCGR by the hormone or by small molecules is defined as a

Table II Naturally occurring mutations reported for the LHCGR.

Location	Mutation	Amino acid change	Effect	Reference
Signal peptide (exon 1)	ins c.54CTGCTGAAGCTGCTGC TGCTGCTGCAG(CTGCAG)	ins19LLKLLLLLQ(LQ)	Inactivating	Wu *et al.* (1998); Richter-Unruh *et al.* (2002a); Sinha *et al.* (2011)
Signal peptide (exon 1)	c.59A>C	Gly20Pro	Inactivating	Bentov *et al.* (2012)
LRRD hinge (exons 1–10)	Δstarting in exon 1/ending within exon 10	Δexon1–10	Inactivating	Richard *et al.* (2011)
LRRD (exon 4)	c.340A>T	Ile114Phe	Inactivating	Leung *et al.* (2006)
LRRD (exon 5)	c.391T>C	Cys131Arg	Inactivating	Misrahi *et al.* (1997); Richard *et al.* (2011)
LRRD (exon 5)	c.430G>T	Val144Phe	Inactivating	Richter-Unruh *et al.* (2004)
LRRD (exon 5)	c.455T>C	Ile152Thr	Inactivating	Qiao *et al.* (2009)
LRRD (intron 6/ exon 7)	c.537–3C>A	Δexon 7	Inactivating	Qiao *et al.* (2009); Han *et al.* (2012)
LRRD (exon 6A)	ins6A c.557A>C	Change in splicing pattern	Inactivating	Kossack *et al.* (2008)
LRRD (exon 6A)	ins6A c.558G>C	Change in splicing pattern	Inactivating	Kossack *et al.* (2008)
LRRD (exon 6A)	ins6A c.580A>G	Change in splicing pattern	Inactivating	Kossack *et al.* (2013)
LRRD (exon 7)	c.580 T>G	Phe194Val	Inactivating	Gromoll *et al.* (2002)
LRRD (exon 8)	Δundefined	Δexon 8	Inactivating	Laue *et al.* (1996a)
Hinge (intron 9/ exon 10)	Δstarting in intron 9/ending within intron 10	Δexon 10	Inactivating	Gromoll *et al.* (2000)
Hinge (intron 10/ exon 11)	c.955-1G>A	ΔTyr317Ser324	Inactivating	Bruysters *et al.* (2008a)
Hinge (exon 11)	c.1027T>A	Cys343Ser	Inactivating	Martens *et al.* (2002)
Hinge (exon 11)	c.1060G>A	Glu354Lys	Inactivating	Stavrou *et al.* (1998)
TMH 1 (exon 11)	c.1103T>C	Leu368Pro	Activating	Latronico *et al.* (2000)
TMH 1 (exon 11)	c.1118C>T	Ala373Val	activating	Gromoll *et al.* (1998)
TMH 1 (exon 11)	c.1121T>C	Ile374Thr	Inactivating	Pals-Rylaarsdam *et al.* (2005)
TMH 2 (exon 11)	c.1175C>T	Thr392Ile	Inactivating	Pals-Rylaarsdam *et al.* (2005)
TMH 2 (exon 11)	c.1193T>C	Met398Thr	Activating	Evans *et al.* (1996); Ignacak *et al.* (2000), (2002); Mao *et al.* (2010)
TMH 2 (exon 11)	c.1199A>G	Asn400Ser	Inactivating	Yariz *et al.* (2011)
TMH 2 (exon 11)	c.1244T>C	Ile415Thr	Inactivating	Kossack *et al.* (2013)
TMH 3 (exon 11)	c.1370T>G	Leu457Arg	Activating	Latronico *et al.* (1998a)
TMH 3 (exon 11)	c.1382C>T	Thr461Ile	Inactivating	Kossack *et al.* (2008)
TMH 4 (exon 11)	c.1473G>A	Trp491*Stop*	Inactivating	Richter-Unruh *et al.* (2002a)
TMH 4 (exon 11)	c.1505T>C	Leu502Pro	Inactivating	Leung *et al.* (2004)
TMH 5 (exon 11)	c.1624A>C	Ile542Leu	Activating	Laue *et al.* (1995a)
TMH 5 (exon 11)	c.1627T>C	Cys543Arg	Inactivating	Martens *et al.* (2002)
TMH 5 (exon 11)	c.1635C>A	Cys545*Stop*	Inactivating	Laue *et al.* (1995b); Wu *et al.* (1998)
ICL 3 (exon 11)	c.1660C>T	Arg554*Stop*	Inactivating	Latronico *et al.* (1996)
ICL 3 (exon 11)	c.1691A>G	Asp564Gly	Activating	Laue *et al.* (1995a)
ICL 3 (exon 11)	c.1703C>T	Ala568Val	Activating	Latronico *et al.* (1995)
TMH 6 (exon 11)	c.1713G>A	Met571Ile	Activating	Kremer *et al.* (1993); Kosugi *et al.* (1995)

Continued

Table II *Continued*

Location	Mutation	Amino acid change	Effect	Reference
TMH 6 (exon 11)	c.1715C>T	Ala572Val	Activating	Yano *et al.* (1995)
TMH 6 (exon 11)	c.1723A>C	Ile575Leu	Activating	Laue *et al.* (1996b); Kremer *et al.* (1999)
TMH 6 (exon 11)	c.1730C>T	Thr577Ile	Activating	Kawate *et al.* (1995); Kosugi *et al.* (1995); Cocco *et al.* (1996)
TMH 6 (exon 11)	c.1732G>T/C	Asp578Tyr/His	Activating	Muller *et al.* (1998); Liu *et al.* (1999); Richter-Unruh *et al.* (2002b)
TMH 6 (exon 11)	c.1733A>G	Asp578Gly	Activating	Shenker *et al.* (1993); Yano *et al.* (1994)
TMH 6 (exon 11)	c.1734T>A	Asp578Glu	Activating	Wu *et al.* (1999)
TMH 6 (exon 11)	c.1741T>C	Cys581Arg	Activating	Laue *et al.* (1995a)
TMH 6 (exon 11)	ins c.1765T	Phe588fs (CHLSCLQSTSYHSNQL*Stop)*	Inactivating	Richter-Unruh *et al.* (2005)
TMH 6 (exon 11)	c.1777G>C	Ala593Pro	Inactivating	Kremer *et al.* (1995); Toledo *et al.* (1996)
TMH 7 (exon 11)	Δc.1822–1827	ΔLeu608Val609	Inactivating	Latronico *et al.* (1998b)
TMH 7 (exon 11)	c.1847C>A	Ser616Tyr	Inactivating	Laue *et al.* (1996a); Latronico *et al.* (1996)
TMH 7 (exon 11)	c.1850G>A	Cys617Tyr	Activating	Nagasaki *et al.* (2010)
TMH 7 (exon 11)	c.1874 T>A	Ile625Lys	Inactivating	Martens *et al.* (1998)

LRRD, leucine-rich repeat domain; TMH, transmembrane helices; ICL, intracellular loop.

timely and spatially ordered sequence of structural shifts between different receptor components, finally to enable activation of G-proteins.

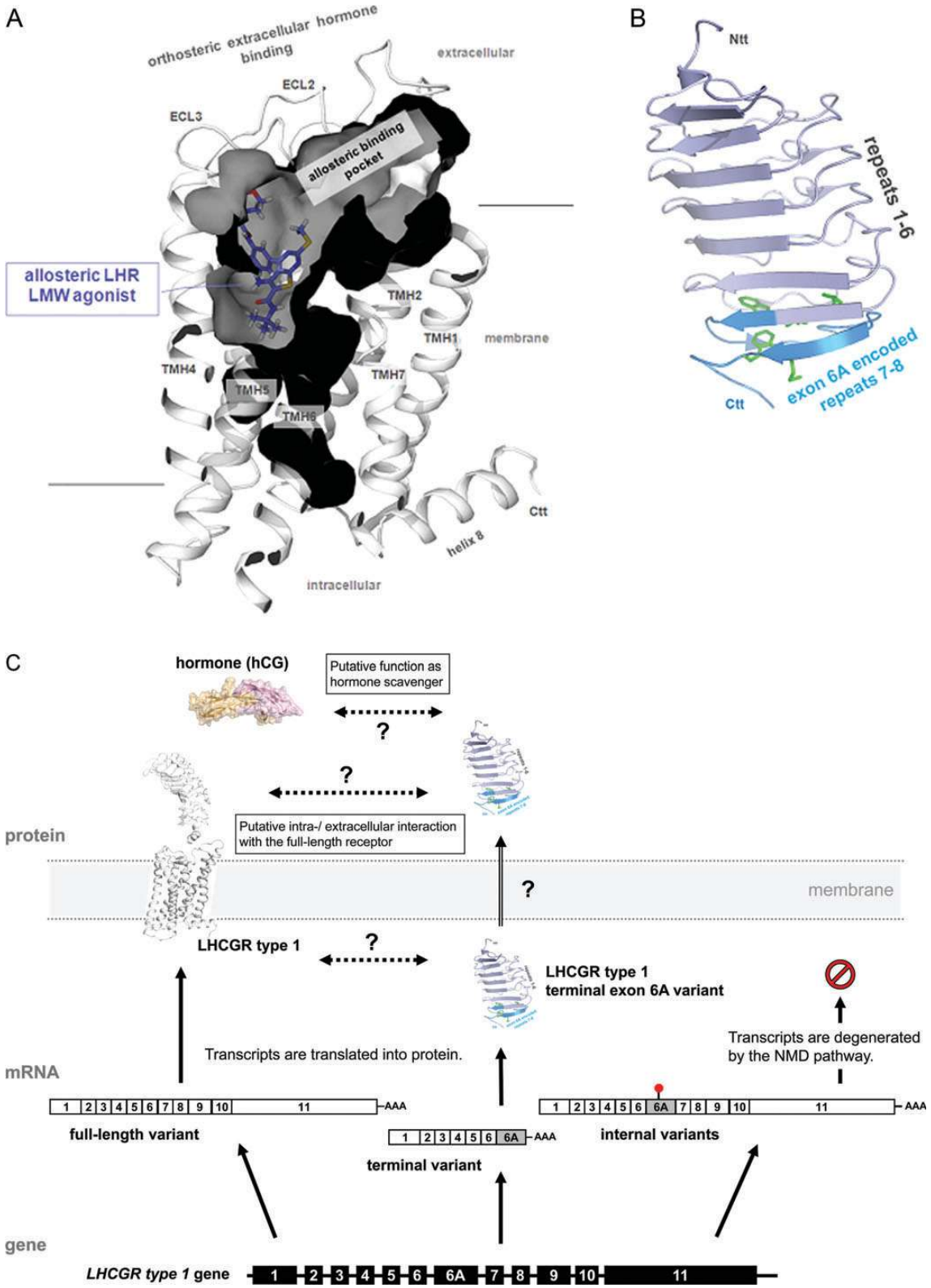
The majority of LHCGR-dependent effects are mediated by the activation of the canonical Gαs/cAMP/PKA signalling pathway. However, the LHCGR belongs to a group of GPCRs prone to promiscuous coupling to multiple classes of G-proteins including inhibitory G-proteins (Gudermann *et al.*, 1992; Gilchrist *et al.*, 1996; Herrlich *et al.*, 1996; Kühn and Gudermann, 1999; Ascoli *et al.*, 2002; Ulloa-Aguirre *et al.*, 2011). The specific recruitment of G-proteins as well as additional LHCGR-dependent signalling pathways (e.g. ERK1/2-pathway) is cell-type specific and differs upon stimulation with LH or CG (Ascoli *et al.*, 2002; Casarini *et al.*, 2012).

Amino acids at the intracellular site and spatial properties between the helical ends and the intracellular loops (ICLs) decipher complementary properties for G-protein recognition and activation. For the LHCGR, those mechanisms are only partially investigated, e.g. the contribution of specific amino acids of ICL2 and ICL3 in Gs protein coupling (Angelova *et al.*, 2008), or coupling of the extreme C-terminal region of Gαs (DeMars *et al.*, 2011). Strikingly, it was found that the transition between the intracellular end of TMH 3 and ICL2 is important for Gs coupling. This is in accordance with data showing that this region acts as a regulatory element of activity states in interplay with residues of TMH 6 (Feng *et al.*, 2008). These general implications are complementary to insights from the TSHR/G-protein interaction process (Kleinau *et al.*, 2010) and are in accordance with latest insights from newly solved complex crystal structures (Chung *et al.*, 2011).

Dimeric receptor organization

A crucial aspect of LHCGR function is its capacity to form higher order complexes, namely homo-dimers (LHCGR protomer—LHCGR protomer) or homo-oligomers. For reasons of simplification here, we will use the term 'dimers' or 'dimerization', although it is possible that the receptor complexes contain more than two receptors which cannot yet clearly be distinguished experimentally. In 1997, it was shown that co-expression of binding-deficient (but with full signalling capacity) and signalling-inhibited (but with diminished binding capacity) receptor fragments partially restores ligand-induced signal generation (Osuga *et al.*, 1997). This was the first hint of a close spatial proximity and functional interrelation between LHCGR monomers. This study guided further approaches leading in 2002 to the description and dissection between cis- (receptor is activated by the bound hormone) and trans- (binding of hormone at the first receptor protomer activates a second receptor) activation mechanisms (Jeoung *et al.*, 2007) for the LHCGR (Ji *et al.*, 2002). For the FSHR, it was additionally found that trans-activation probably induces biased signalling in terms of generation of only one of two hormone signals but not both simultaneously (Ji *et al.*, 2004).

In 2004, constitutive and agonist-dependent self-association of the LHCGR was shown (Tao *et al.*, 2004; Fanelli, 2007) as well as negative effects of inactive LHCGR mutants on wild-type receptor signalling in dimeric constellations (Zhang *et al.*, 2009). The main interface contact between the protomers is suggested to be located between the transmembrane helices, but the extracellular portion might modulate dimeric interrelations (Urizar *et al.*, 2005).



In 2010, an *in vivo* study was published that used a mouse Lhr as a model GPCR to demonstrate that transgenic mice co-expressing formerly described binding-deficient and signalling-deficient forms of the Lhr can re-establish normal LH actions through intermolecular functional complementation of the mutant receptors (Rivero-Muller *et al*., 2010). This study was one of the first to provide *in vivo* evidence for cooperation between two receptor protomers. However, this topic is still under lively discussion as reflected by a recently published study substantively questioning the concept of functional reconstitution between Lhr mutants *in vivo* (Zhang *et al*., 2012).

Different LHCGR subtypes

Beyond the conserved structural and functional similarities, a presumably evolutionary drive resulted in the development of differing receptor characteristics and multiple receptor types. Despite the high degree of conservation of the general genomic organization of the *LHCGR* gene between species, a DNA insertion of a 2.7 kbp long genomic region between exons 6 and 7 resulted in the generation of an additional exon present in primates and humans only (Fig. 1A). A further regulatory genetic event, the consecutive skipping of exon 10 in the New World monkey lineage, led to an additional primate-specific receptor type, where exon 10 became a pseudo-exon and the receptor lacking exon 10 represents the wild-type form. For a comprehensive assignment of the different types of receptor, we suggest a new nomenclature designating the classical receptor known in rodents as *Lhr*, whereas the receptor type containing exon 6A and present in species owning primordially LH and CG as endogenous hormones should be designated as *LHCGR* (Table I). The *LHCGR* is further split into *LHCGR type 1* and *type 2*, based on the constitutive presence or skipping of exon 10, respectively.

The proposed new nomenclature scheme for the receptor indicated in Table I should facilitate more precise, fact-driven communication in the area of LHCGR research and takes into account the following considerations: (i) the 'classical' receptor consisting of 11 exons and present in model organisms such as mouse and rat is referred to as *Lhr*, since in these species LH is the only endogenous ligand for this receptor known so far; (ii) due to the evolutionary development of the dual system of LH and CG mediating their effect via one receptor in humans and primates, this receptor type is designated as *LHCGR*; (iii) this receptor type is characterized by an additional primate-specific exon, which represents a new genetic element for the regulation of receptor expression and (iv) a further differentiation of this receptor type into *LHCGR type 1* and *type 2* is required due to the fact that in the New World monkey lineage, exon 10 became a pseudo-exon.

In the two following sections, the LHCGR type 1 and type 2 present in primates and humans only are characterized in more detail.

LHCGR type 1-containing exon 6A

LHCGR type 1 was first identified in 2008 by Kossack *et al*., who analysed unusual *LHCGR* mRNA variants consisting of exons 1–6 and additional unknown sequences either terminated by a poly(A) tail or continuing with exons 7–11. The unknown sequences revealed a perfect match with the intronic region between exons 6 and 7 of the human *LHCGR type 1* gene. Comparable sequences are only present in other primates and are completely lacking in all other investigated species so far, leading to the speculation that the appearance of exon 6A in the primate *LHCGR* gene is related to the evolutionary appearance of CG in primates. Furthermore, the high level of conservation of exon 6A in primates indicates strong functional constraints of this element, thereby forming the framework of critical biological functions across species. A 3′ splice acceptor site (AG) and two internal 5′ splice sites were detected, giving rise to a novel internal exon of 159 bp (short) or 207 bp (long). Additionally, a 3′ polyadenylation signal (AATAAA) was identified and, in cooperation with the 3′ splice acceptor site, yields a terminal exon. The newly identified internal or terminal exon within intron 6 was designated 'exon 6A' and established the new type of the LHCGR present in the human (Kossack *et al*., 2008). The presence of exon 6A gives rise to a number of different *LHCGR* mRNAs, which are present in varying ratios under normal physiological or patho-physiological circumstances. More precisely, the primary transcript of the *LHCGR type 1* gene can give rise to the full-length *LHCGR type 1* mRNA containing exons 1–11. Additionally, exon 6A can be spliced into the mature transcript as a terminal or internal exon (Fig. 3C). The three variants, *LHCGR type 1* exon 6a short, long and terminal, are present in human tissues such as adult and fetal testes, granulosa cells and adrenal gland (Kossack *et al*., 2008; Fowler *et al*., 2009). The internal variants are both expressed at relative low levels, due to the presence of premature stop codons which designate these variants as putative targets for nonsense-mediated mRNA decay (NMD), while the terminal variant is highly abundant

Figure 3 The allosteric ligand-binding pocket and the LHCGR type 1 terminal exon 6A variant. (**A**) A remarkable aspect of the LHCGR is an allosteric-binding region, which is different from the extracellular binding site of the endogenous hormone ligand (Fig. 2). It has been shown that LMW ligands (here the LMW agonist Org43553) can occupy this transmembrane pocket, which is the preferred binding region in family A GPCRs (clipped side view, pocket as inner surface). This binding-sensitive region is located between the transmembrane helices towards the extracellular side, including the ECL2. This binding site is conserved among the family A GPCRs, although not always used by the endogenous ligand as in case of the LHCGR. (**B**) The structural homology model of the LHCGR type 1 exon 6A terminal variant (white-blue backbone ribbon) is based on a modified FSHR LRRD crystal structure. Repeats one to six are identical to the full-length receptor. The amino acid sequence alignment (Fig. 1B) predicts that exon 6A might encode structural repeats and β-strands 7 and 8 (blue). The resulting LRRD of LHCGR type 1 exon 6A is formed by repeats 1–8 instead of 11, as in the full-length LHCGR type 1 (Fig. 2). Of note, amino acids (numbers 180 and 182) in the putative β-strand 7 which are oriented to the concave LRRD site (hormone-binding site) are different in their biophysical properties compared with the full-length receptor (Fig. 1B). (**C**) Hypothetical model of the transcriptional network of the *LHCGR type 1* gene (modified according to Kossack *et al*., 2008). The primary transcript of the *LHCGR type 1* gene can, in the mature mRNA, give rise to the full-length *LHCGR type 1* containing 11 exons. Additionally, exon 6A can be spliced into the terminal or internal *LHCGR type 1* exon 6A variants. The terminal variant is translated into a truncated LHCGR protein consisting of seven exons, which could hypothetically be secreted and interact either with the full-length LHCGR type 1 protein or serve as a hormone scavenger via binding to LH and/or CG. The premature stop codon (red circle) in exon 6A designates the internal *LHCGR type 1* exon 6A variants as putative targets for the NMD pathway since on the first round of translation any premature in-frame stop codon found more than 50 nucleotides upstream of the splice junction triggers NMD.

expressed in amounts comparable to the full-length transcript or even higher (Fig. 3C; Kossack *et al*., 2008; Fowler *et al*., 2009).

The incorporation of exon 6A as a terminal exon inserts a stop codon and a polyadenylation site, thereby resulting in a truncated LHCGR type 1 protein of 209 amino acids, wherein exon 6A only encodes 30 amino acids (Figs 1B and 3B). The LHCGR type 1 exon 6A terminal variant, therefore, encodes a sequence of exons 1–6 which is identical to the full-length LHCGR type 1 and forms structural repeats one to six of the LRRD. The amino acids encoded by exon 6A also contain typical residue motifs constituting β-strands in the hormone-binding site, which suggests a continued typical fold of this short LRRD variant. Therefore, the LHCGR type 1 exon 6A terminal variant can be hypothesized to encode 8 repeats, instead of 10, as postulated for the full-length LHCGR LRRD (Kossack *et al*., 2013). Moreover, residues at β-strands 2–8 are experimentally evidenced as most relevant for hormone binding at LRRDs of the GPHRs (Caltabiano *et al*., 2008; Angelova *et al*., 2010), which might be a hint that the LHCGR type 1 exon 6A terminal variant should be capable of hormone binding to an unknown extent and specificity, but without any signalling activity. This scenario would support the concept of a hormone scavenger which modulates the active serum hormone levels by binding.

The physiological importance of exon 6A is highlighted by naturally occurring mutations within this exon leading to severe disorders of sexual differentiation (Kossack *et al*., 2008, 2013). In patients with the clinical phenotype of Leydig cell hypoplasia, where no causative mutations have been identified in routine clinical screening of the *LHCGR type 1*, mutations were identified in exon 6A, indicating the necessity of including the sequencing of the entire *LHCGR*-coding region, also covering exon 6A with its splicing sites, in the screening. The mutations identified in exon 6A led to a dramatically increased inclusion rate of exon 6A into the primary transcript and thereby to a decreased number of transcripts contributing to generation of the full-length receptor protein. Thus, these mutations highlight the important role of exon 6A as an additional regulatory element of the *LHCGR type 1* gene and of mutations not causing alterations at the protein level, but at the transcriptional level. The mutations located within exon 6A which thereby alter the ratios of the *LHCGR type 1* variant transcripts with and without exon 6A additionally demonstrated that a distinct ratio of these variants is required for proper LHCGR type 1 function. Further evidence that these ratios between the different exon 6A variants and the full-length receptor are of physiological relevance has been provided by a study investigating developmental changes in fetal expression of testicular *LHCGR type 1* and the effects of maternal cigarette smoking (Fowler *et al*., 2009). In testes of morphologically normal human male fetuses of women undergoing termination, they measured hormone levels and testicular *LHCGR type 1* expression. Firstly, they demonstrated that the proportion of *LHCGR type 1* transcript variants encoding functional LHCGR type 1 is different between fetal human testis and adult human testis, with fetal testes containing higher proportions of functional variants (Kossack *et al*., 2008; Fowler *et al*., 2009). Furthermore, maternal smoking resulted in a reduced amount of fetal CG and revealed slight effects on the ratio of different *LHCGR type 1* variants. Functional *LHCGR type 1* transcripts were increased in mothers who smoked, which might indicate an adaptive mechanism maintaining the vital output of testosterone in these fetuses (Fowler *et al*., 2009).

The putative biological relevance of exon 6A as a regulatory element is additionally underscored by high amounts of transcripts containing this exon in native tissues (Kossack *et al*., 2008; Fowler *et al*., 2009). Future studies are necessary to unravel the putative role of the LHCGR type 1 terminal exon 6A variant as a hormone modulator. The LHCGR type 1 terminal exon 6A variant might be secreted by cells expressing the LHCGR type 1 and thereby play a role by interacting with circulating hormone molecules (Fig. 3C). The concept that the extracellular part of the LHCGR type 1 could function as a hormone modulator by interacting with the hormone is supported by the observed massive alternative splicing of the *LHCGR* gene, giving rise to variants bearing only the extracellular hormone-binding domain (Zhang *et al*., 1994; Kossack *et al*., 2008). Furthermore, experimental evidence that a soluble form of the Lhr as well as the LHCGR type 1 exists has accumulated over recent decades. First, the existence of Lhr outside of cells was described in CG-affinity purified follicular fluid from porcine (Kolena and Sebokova, 1986; Kolena *et al*., 1986). Later, an LH-Lhr complex from Leydig cell culture media and a soluble protein acting as an LHCGR type 1 antagonist in serum from uraemic boys suffering from hypogonadism were described (West and Cooke, 1991; Dunkel *et al*., 1997). Recently, the release of soluble LHCGR type 1 from transfected cells into the culture medium and from placental explants into the bloodstream of pregnant women was demonstrated (Chambers *et al*., 2011a). This soluble LHCGR type 1 also exists in a complex with LH and can be measured under both conditions in human serum and follicular fluid (Chambers *et al*., 2011b). The specific shape and structure of this cell-free receptor as well as the species-specificity, physiological function and the clinical significance remains elusive. However, it was reported that the separated extracellular region of GPHRs is capable of binding their corresponding hormones (Osuga *et al*., 1997).

In addition, ancient GPH subunits have been found to be distributed in the central nervous system, stomach, pancreas, testis and other reproductive tissues of different animals (Hsu *et al*., 2002; Li *et al*., 2004; Sun *et al*., 2010) and might also be considered (as single subunits or as heterodimeric hormone) (Dos Santos *et al*., 2009) as a potential hormone-ligand, beside LH and CG, for those *N*-terminal LHCGR variants with unknown ligand specificity. Another putative function of truncated receptor variants has been demonstrated for common splice variants of the rat Lhr as well as the human LHCGR type 1, which regulate cell-surface expression and/or receptor functionality of the full-length receptor in heterologous expression systems via dimerization with the full-length receptor (Nakamura *et al*., 2004; Apaja *et al*., 2006; Dickinson *et al*., 2009). Thus, regulation of the LHCGR by its corresponding splice variants may be a more common mechanism than anticipated so far, indicating the possibility that the LHCGR type 1 exon 6A terminal variant could have a functional role in LH/CG target cells of humans and primates by regulating the number of functional receptors via intra- or extracellular di-/oligomerization (Fig. 3C).

LHCGR type 2-lacking exon 10 at the mRNA and protein levels

Evolutionary divergent developments resulted in different types of the LHCGR and diversified functions of LH and/or CG in primates. The LHCGR type 1, as described above, is present in ancestral primate suborders as well as in evolutionarily advanced primate species. Evolutionary events altering the splicing mechanism regarding exon 10 resulted in the emergence of an additional receptor type, LHCGR type 2, which is present in the New World monkey lineage. In the New World monkey lineage, the *LHCGR* mRNA lacking exon 10 represents the wild-type form and exon 10 acts as a pseudo-exon (Gromoll *et al*., 2003). In

the *LHCGR type 1*, exon 10 encodes 27 amino acids in the putative hinge region of the extracellular region (Fig. 1B). First, constitutive skipping of exon 10 was reported in the *LHCGR* of the common marmoset *Callithrix jacchus* (Zhang *et al*., 1997; Gromoll *et al*., 2003). In splicing studies, 11 different *LHCGR type 2* isoforms were identified in testis and ovary tissues of marmoset monkeys; exon 10 was absent in all variants, demonstrating that exon 10 has the characteristics of a pseudo-exon in this species (Michel *et al*., 2007). The constitutive skipping of exon 10 was confirmed in four different monkey species from *Platyrrhini* and was proposed for the complete New World monkey lineage (Zhang *et al*., 1997; Gromoll *et al*., 2003). Despite extensive attempts, no transcript variant of the *LHCGR type 1* with exon 10 skipped was detected in the human (Madhra *et al*., 2004), indicating a crucial impact of the presence of this exon for the biological functionality of the receptor. However, variants of the *Lhr* lacking exon 10 have been described in other species such as bovine (Kawate and Okuda, 1998; Robert *et al*., 2003) and sheep (Bacich *et al*., 1994; Abdennebi *et al*., 2002). These species- and/or lineage-specific events of alternative or constitutive splicing regarding exon 10 illustrate a putative path of diversified receptor activity regulation in species with differing hormonal reproductive mechanisms of the hormones LH and CG.

Since exon 10 is present in the genomic sequences of the *LHCGR type 2* in the New World monkey lineage, the general lack of exon 10 at the mRNA and protein levels is due to aberrant transcriptional events in which exon 10 splicing is prevented. The genomic organization of exon 10 in the marmoset is characterized by expansion of the circumjacent intronic regions mainly due to the presence of LINE-1 elements while the sequence of exon 10 is highly conserved and the splice sites are intact (Gromoll *et al*., 2007). However, the LINE-1 elements found in the intronic regions of the marmoset *LHCGR type 2* gene do not obviously influence exon inclusion or exclusion. The molecular mechanism underlying constitutive exon 10 inclusion in the human and exon 10 skipping in the marmoset *LHCGR* was attributed to nucleotides at the intron 9/exon 10 boundary. By sequence comparison of primate sequences of this genomic region, the nucleotides at positions −10, −19 and +26 were shown to differ in the New World monkey lineage and to navigate exon 10 skipping or splicing of the *LHCGR* by alterations in the secondary RNA structure (Gromoll *et al*., 2007). Changes within this secondary structure context prevent the identification of selected sequences by splicing factors and result in general skipping of exon 10, which could be experimentally reversed by interchanging the responsible nucleotides present in the New World monkey lineage via mutagenesis into the human sequence (Gromoll *et al*., 2007).

The species-specific constitutive skipping of exon 10 in the New World monkey lineage leads to functional novelty of the LHCGR type 2 protein in the affected species and causes fundamental functional differences in comparison with orthologous receptors, reflected by an additional distinctive feature of the New World monkey lineage, the functional replacement of LHB by CGB, which is therefore the only gonadotrophin with luteinizing function in this species (Müller *et al*., 2004; reviewed in Henke and Gromoll, 2008).

Functional and clinical characteristics of exon 10 in terms of specific ligand binding and signalling become evident by analysing genetic alterations present in this region. It has been shown that the 291Asn/Ser SNP (rs12470652) in exon 10 of the human *LHCGR type 1* results in increased receptor sensitivity and affects the response of the receptor to CG as well as LH, probably due to the presence or absence of glycan side chains at this position (Piersma *et al*., 2007b). A further SNP in exon 10, 312Ser/Asn (rs2293275), was identified as a risk allele for breast cancer, probably acting in linkage with a functional polymorphism, since no effect of this SNP on any receptor function could be demonstrated (Piersma *et al*., 2007b). Important clinical and functional insights illuminating the role of exon 10 in the differential action of LH and CG were derived from a patient with a homozygous deletion of ∼5 kb encompassing exon 10 of the *LHCGR type 1* gene (Gromoll *et al*., 2000). The patient displayed a normal male phenotype associated with delayed pubertal development and hypogonadism. Normal testosterone production and complete spermatogenesis was induced by CG administration. The LHCGR type 1 lacking exon 10 (LHCGR type 1 Δ10) showed normal CG binding and CG-induced cAMP and inositol trisphosphate signal transduction *in vitro* (Müller *et al*., 2003). In addition, displacement experiments demonstrated comparable binding of LH to the wild-type LHCGR type 1 or the LHCGR type 1 Δ10 (Müller *et al*., 2003); while in the LHCGR type 1 Δ10 cAMP production was impaired significantly when stimulated by LH, although CG stimulation was not affected. Therefore, exon 10 of the LHCGR type 1 does not seem to be important for binding of CG and LH, but plays a pivotal role in differing responsiveness of the receptor to LH and CG (Müller *et al*., 2003). This conclusion was confirmed by a study on the functional role of the hinge region of the LHCGR type 1 (Bruysters *et al*., 2008a). In the LHCGR type 1 lacking exon 10, the EC50 value for LH was 15-fold higher, while the EC50 for CG was only slightly increased. The function of exon 10 encoded amino acids for LH and CG induced signalling seems rather to be attributed to the overall length of the entire hinge region and is not dependent on the biophysical properties of the particular residues within exon 10. This conclusion is supported by chimeric and LHCGR type 1 deletion-mutant studies (Bruysters *et al*., 2008a).

Based on the crystal structure of the FSHR (Jiang *et al*., 2012) a homology model for the LHCGR hinge region can be generated, especially concerning the repeat 11 helix that contains the two cysteines (LHCGR: Cys279 and Cys280) linking the LRRD towards the transmembrane domain and also the sulphated tyrosin 331 (hinge region) that forms a second binding site to the hormones. However, the middle of the hinge region is not provided in the FSHR crystal structure. Unfortunately, the sequence of exon 10 in LHCGR comprises this missing part and therefore the molecular details of this portion prevent a structural description without further experimental input. Nevertheless, it is conceivable that deletion of exon 10 in the LHCGR shortens and modifies the hinge region of the LHCGR considerably due to the missing amino acid sequence itself (Fig. 1B). Furthermore, this is supported by the homologous model, although the structure of the hinge region is only presented fragmentarily (Fig. 2). As a consequence, the residues upstream are joined to the downstream residues of exon 10. Such a radical reduction very likely leads to a spatial displacement of the downstream part containing the sulphated tyrosin 331, the second hormone binding site. This seems to be sensitive for LH signalling but can obviously be tolerated by CG as it might interact with the hinge region differently from LH.

Cumulating evidence suggests that LH and CG, although binding and activating the same receptor, can cause distinct responses in their target cells, supposedly due to different binding sites for CG and LH, which might result in the activation of different target proteins (Gudermann *et al*., 1992; Gilchrist *et al*., 1996; Galet and Ascoli, 2005; Gupta *et al*., 2012). Recently, the first report dealing directly with quantitative and qualitative differences of LH and CG action on LHCGR type 1

signalling revealed unequal activity of both hormones at the receptor and the activation of different signal transduction pathways (Casarini *et al*., 2012). It was reported that CG acts more potently on cAMP production than LH, while LH is more potent for ERK and AKT activation, and that the expression of LH and CG target genes partly involves the activation of different pathways, depending on the ligand. These results point to an induced biased agonism signalling cascade related to the particular hormone subtype. Of note, biased agonism at the LHCGR was also reported for a small molecule that only induces activation of Gs-related pathways (van Koppen *et al*., 2008), which further supports differential regulation of signalling at the LHCGR.

Moreover, the non-equivalence of the two hormones in terms of receptor function requires further detailed analyses starting at the level of molecular events with differing binding and activation processes and including *in vivo* studies unravelling the complete physiological impact of signalling differences. The human LHCGR type 1, where a genomic deletion led to the loss of exon 10, where the functional differences between LH and CG became first apparent, turned out to be a suitable tool for further investigation of this topic. The detailed analysis of the molecular and mechanical reasons for differences between LH and CG binding and signalling for LHCGR type 1 Δ10 may reveal which parts of exon 10 are involved in hormone selectivity of LH and CG, and whether the C-terminal part of the extracellular domain of the receptor can be used in the design and development of hormone-specific analogues.

Evolution and plasticity of the LHCGR

A comprehensive understanding in terms of the differing receptor characteristics seen today also requires insight into the evolutionary dynamics of the ancestral receptor towards the Lhr and LHCGR as well as into the evolution of the corresponding hormones. Specific GPCR signatures can be found in all eukaryotic species, demonstrating the ancient origin of GPCRs (Schoneberg *et al*., 2007). Studies regarding the molecular evolution of leucine-rich repeat-containing GPCRs (LGRs) encoding genes hypothesized that the common ancestor of these genes emerged early during evolution, before the radiation of metazoan phyla, since different types of LGRs can already be identified in molluscs, nematodes, cnidaria and insects, representing ancient homologs of mammalian GPHRs (Kudo *et al*., 2000; Hsu *et al*., 2002; Park *et al*., 2005; Van Loy *et al*., 2008). Evolutionary events of sequential duplications led from an ancestral LGR that regulated physiological functions in the common ancestral metazoan to the three GPHRs (Oba *et al*., 2001; Park *et al*., 2005). New insights into the molecular structure and function of GPHRs of lampreys, one of the most ancient lineages of vertebrates, demonstrated that the genetic structure as well as functionality of the GPHRs was highly conserved during the long period of divergent evolution (Freamat and Sower, 2008, 2010). The appearance of LHCGR and FSHR is hypothesized to be the result of duplication processes before the emergence of *Gnathostomata* (Oba *et al*., 2001). As proposed for the cognate receptors, the duplication events leading to the presence of different GPH beta-subunits took place prior to radiation of the *Gnathostomata* (Dos Santos *et al*., 2011). The duality of the LH/FSH signalling system is conserved throughout vertebrates, and it is assumed that duplication of an ancestral gene in *Agnatha* gave rise to LH and FSH in the *Gnathostomata* (Kawauchi and Sower, 2006). Parallel expansion of the GPH alpha- and beta-subunits and their corresponding receptors through gene duplication of common molecular ancestors enabled a process of divergence leading to the development of new and sub-functions of the ligand–receptor pairs in the endocrine systems in vertebrates.

The diverse plasticity of this hormone-receptor complex is thereby expanded by evolutionary divergent developments concerning the function of LH and/or CG in primates, based on different types of the LHCGR. This two hormone/one receptor system is exclusive to the human and certain primates, indicating a very recent evolutionary event resulting in the formation of these two reproductive hormones (Hallast *et al*., 2008). Sequence comparisons of the human *LHB* and *CGB* genes displayed high levels of homology and founded the concept of a common evolutionary origin by duplication of *CGB* from *LHB* ~55–30 million years ago, after splitting of the *Strepsirrhini* from the anthropoid line but before splitting of *Platyrrhini* and *Catarrhini* (Talmadge *et al*., 1984; reviewed in Henke and Gromoll, 2008; Nagirnaja *et al*., 2010). During evolution, new and sub-functionalization of duplicated proteins enabled divergent expression of duplicated paralogous GPHs and their receptors in selected tissues. Further gene duplications of the *LHB* and *CGB* genes occurred in the Old World monkey and great ape lineages, which have resulted in variable numbers of gene copies and diversification of this genomic region among species (Maston and Ruvolo, 2002; reviewed in Henke and Gromoll, 2008). The New World monkey lineage originally must have possessed both genes, *LHB* and *CGB*, but *LHB* became pseudogenized and the CGB adopted the functions of LHB (Müller *et al*., 2004; Scammell *et al*., 2008). As a functional consequence, CGB is highly expressed in the pituitary of the common marmoset to display and coordinate functions beyond pregnancy formerly performed by LH. Therefore, in the common marmoset a system evolved for the tissue-specific expression of the single-copy *CGB* gene (Henke *et al*., 2007; Adams *et al*., 2011).

The primate LHCGR type 1 is present, on the one hand, in very ancestral primate suborders such as the *Strepsirrhini*, and, on the other hand, in evolutionarily advanced primate species (Fig. 4). Possibly as a consequence of the hormone inactivation of LHB in the New World monkey lineage, the LHCGR type 2 lacking exon 10 on the mRNA level developed as a new subclass of LHCGR, revealing distinct hormone selectivity (Henke and Gromoll, 2008). In the *LHCGR type 2* of the New World monkey lineage, exon 10 is a pseudo-exon resulting in an mRNA variant lacking exon 10 as the wild-type form of the receptor (Gromoll *et al*., 2003). Since *LHCGR* mRNA including exon 10 exists in *Strepsirrhini* species, the molecular event resulting in the phenomenon of splicing out exon 10 took place after splitting of the *Platyrrhini* but before splitting of the suborders within the New World monkeys, approximately between 40 and 35 million years ago (Gromoll *et al*., 2003).

In conclusion, the *Lhr* consisting of 11 exons on the genomic level is present in evolutionary lower species, while at the onset of primate evolution, the event of a DNA insertion containing exon 6A led to the formation of the modified receptor type *LHCGR*, which then contains 12 exons and is present in all primate lineages. In the New World monkey lineage, an *LHCGR* subtype evolved by changes in the splicing mechanism regarding exon 10, resulting in the exclusion of exon 10 on the mRNA and therefore the protein level. It is believed that the evolutionary development of this LH/CG/LHCGR system is dynamically ongoing.

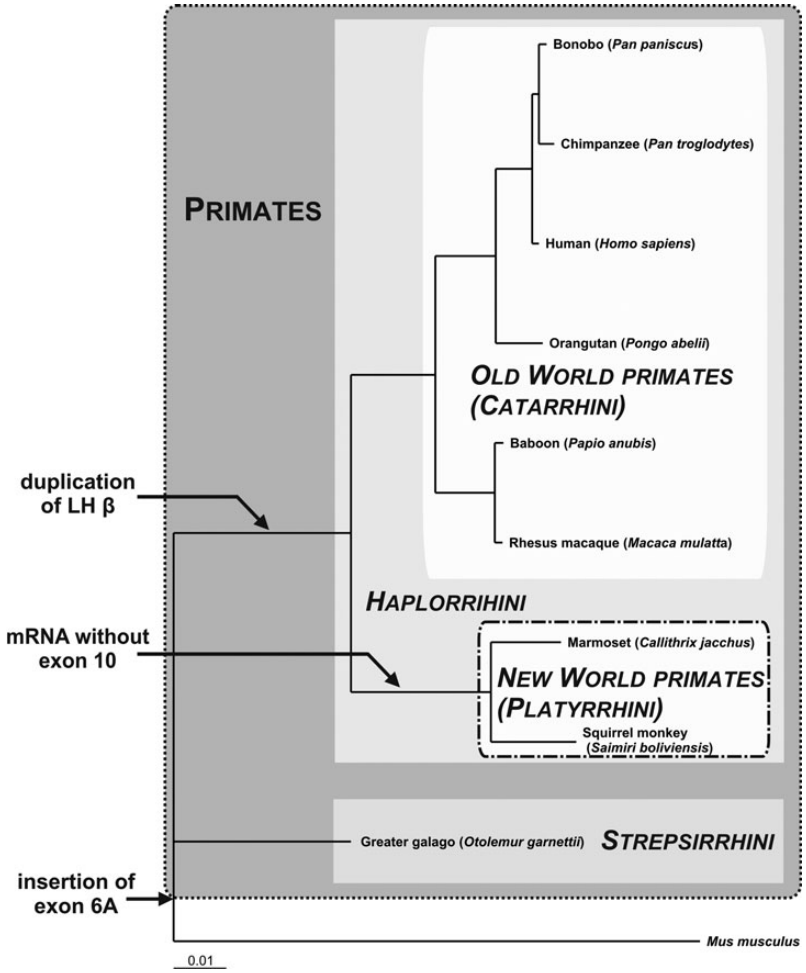


Figure 4 Phylogenetic tree of the LHCGR of primates. The deduced LHCGR amino acid sequences of nine primate species were analysed using the Clustal method (Larkin *et al*., 2007) and displayed with TreeView (Page, 1996) using the LHCGR of *Mus musculus* (NP_038610.1) as an outgroup. LHCGR used: *Pan paniscus* (XP_003822738.1), *Pan troglodytes* (XP_003309053.1), *Homo sapiens* (NP_000224), *Pongo abelii* (XP_002812087.1), *Papio anubis* (XP_003908693.1), *Macaca mulatta* (XP_001114090.1), *Callithrix jacchus* (AAB53698.1), *Saimiri boliviensis* (XP_003922921.1), *Otolemur garnettii* (XP_003787958.1). Dots, LHCGR containing exon 6A; broken lines, LHCGR lacking exon 10 on the protein level.

Challenges of studying LHCGR functions

Today, we are facing the complex situation that three different receptor types (Lhr, LHCGR type 1 and 2) and two corresponding hormones (LH and CG) exist. While fundamental insights unravelling central questions on receptor activation and signalling due to high structural conservation and overall similarities could be transferable between the different receptor types, investigators must keep in mind the existing differences in structure and in endocrine functions (Malassine *et al*., 2003). The multifaceted setting of receptor types and function therefore represents a major challenge for future studies on selective hormone binding, signal transduction and receptor regulation. Beyond this challenge, the existence of these three receptor types might also have hitherto unknown major physiological implications. Awareness of differences between the endocrine setting in animal models and humans is of paramount importance. For example, studies on the human LHCGR type 1 should bear in mind the existence of exon 6A and studies in New World monkeys should consider that only CG is endogenously present and not LH. Consequently, limitations of model systems need to be considered. The extrapolation of results conducted in rodent studies to humans or vice versa might be limited as well. The design and development of efficient gonadotropic drugs and optimal clinical protocols for fertility treatment can only be achieved by the use of appropriate model systems or at least using homologous cell line systems, e.g. human granulosa cells for studies on the human LHCGR.

A further challenge is the difference in signalling mechanisms between the two hormones LH and CG at the same receptor type (Casarini *et al*., 2012). The molecular details of these differing activation mechanisms have to be thoroughly clarified before drawing any conclusions regarding the physiological and patho-physiological consequences with respect to envisaged novel therapeutical strategies.

Although LH and not CG is the physiological hormone in men and in non-pregnant women, the vast majority of our current knowledge of *in*

vitro actions mediated by the LHCGR on Leydig and ovarian cells was obtained using CG due to its facile availability by urinary extraction and its comparable longer half-life. Moreover, these properties also led to the use of CG as medication of choice in clinical applications including induction of ovulation in assisted reproductive technologies as well as induction of spermatogenesis in men with hypogonadotrophic hypogonadism. With the advent of recombinant gonadotrophins, the development from native hormones to recombinant medications was introduced and now opens the possibility of using recombinant LH instead of urinary or recombinant CG. Some studies have indicated that recombinant LH, due to its shorter half-life, might be safer and equally as effective compared with urinary CG for ovulation induction reducing the incidence of ovarian hyperstimulation syndrome (European Recombinant LH Study Group, 2001; Ludwig *et al*., 2003), while others have claimed that there is no evidence of differences in clinical outcome and therefore recommend the continuation of the use of urinary CG because of availability and cost-effectiveness (Al-Inany *et al*., 2005; Youssef *et al*., 2011). The treatment of male infertility in cases of hypogonadotropic hypogonadism attempts to mimic the normal physiological secretion of gonadotrophins by intramuscular injections of CG, as a replacement for LH, and human menopausal gonadotrophin for FSH. To our knowledge, no studies on recombinant LH as surrogate for CG in the clinical treatment of hypogonadotropic hypogonadism are available yet. A study in healthy men demonstrated differences in both bioavailability and time courses of action of recombinant LH and CG; nevertheless the response of testicular Leydig cells to recombinant LH is comparable to CG (Cailleux-Bounacer *et al*., 2008). Although so far no clear superior action of LH over CG in terms of clinical parameters has been demonstrated, recent data on quantitative as well as qualitative differences in signalling properties of both hormones should lead to a reassessment of both hormones in clinical regimens.

The potential interaction between receptors, their variants and the functional consequences of homo- and hetero di/oligomerization as well as trans- or cis-activation (Osuga *et al*., 1997; Jeoung *et al*., 2007) is another emerging and challenging field for future receptor studies. This complex three-facet relationship between structural (oligomeric receptor), functional (signalling pathway(s)), and mechanical (activation by different hormones in cis- and/or trans-modes) properties, however, also opens new avenues for system regulation and therefore also pharmaceutical interventions.

The fact that the expression of different receptor variants is not only developmentally specific but also tissue specific might be another aspect of extra-gonadal Lhr and LHCGR function and physiology. Numerous studies have revealed that the receptors are also present in non-gonadal tissues and both gonadal and extra-gonadal tumours, indicating a putative extra-gonadal and tumorigenic role for LH and CG (reviewed in Ziecik *et al*., 1992; Fields and Shemesh, 2004; Huhtaniemi, 2010). This growing body of evidence has led to a number of suggestions and concepts for the physiological functions of extra-gonadal expressed Lhr and LHCGR (Toth *et al*., 2001; Ziecik *et al*., 2007; Banerjee and Fazleabas, 2011), however, functional data and implied physiological actions are not fully elucidated and remain controversial (reviewed in Ziecik *et al*., 1992; Pakarainen *et al*., 2007).

Authors' roles

All authors contributed substantially to the concept and design of this review and were involved in approval of the final version. B.T. and G.Kl. contributed to the literature search, analysis and interpretation of data and writing of the manuscript. G.Kl. designed and visualized all structural information. G.Kr. contributed to the interpretation of data and writing of the manuscript. J.G. initiated the study and contributed to the interpretation of data and writing of the manuscript.

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Conflict of interest

All authors have nothing to declare.

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