

# LUTEINIZING HORMONE RECEPTOR

Expression and post-translational regulation of the rat receptor  
and its ectodomain splice variant

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Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in Auditorium 5 of the Institute of Dentistry, on November 25th, 2005, at 12 noon

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## *Abstract*

The luteinizing hormone receptor (LHR) is a G protein-coupled receptor (GPCR) that has a large N-terminal ligand binding ectodomain. The LHR ectodomain splice variant, expressed concomitantly with the full-length LHR in tissues, has an unknown biological function. GPCRs are a major pharmacological target, however, very little is known about the intracellular regulation of these receptors. In the present work, expression and maturation of the rat LHR and its variant were elucidated using both tissues and heterologous expression systems. A special effort was made to identify the role of developmental stage and tissue type on the LHR maturation and to find out about the molecular role of the ectodomain splice variant.

We found two sites of localization for the receptor, namely the sensory system and urogenital tissues. This was demonstrated at mRNA and protein level and by rat LHR promoter-driven  $\beta$ -galactosidase ( $\beta$ -Gal) expression in the mice. In neurons, the  $\beta$ -Gal co-localized with the cytochrome P450 side chain cleavage enzyme, which may indicate a novel role in the neurosteroid synthesis.

The neuronal LHR was expressed in the mature and immature protein forms in both developing and adult tissues, being able to bind hormone with similar high-affinity as gonadal receptors. In contrast, only immature receptors were detected in the fetal rat urogenital structures. A significant novel finding was substantial upregulation of the LHR in pregnant female rat adrenal glands and kidneys at a time that coincides with the differentiation of the fetal urogenital tissues.

The mice overexpressing the ectodomain splice variant showed interference in pituitary-gonadal functions and morphological changes in the urogenital tissues. The studies showed that the variant was an endoplasmic reticulum (ER)-retained soluble protein. It accumulated in juxtannuclear regions of the ER together with ER folding chaperones and was a substrate for ER associated degradation (ERAD). The co-expression of the variant with the full-length receptor decreased the amount of receptors and misrouted them to the juxtannuclear ER subcompartment.

Taken together, we suggest that the maturation of the LHR protein is developmentally and physiologically regulated at the post-translational level in tissues. The LHR ectodomain splice variant possibly modulates post-translationally the number of full-length receptors through physiological signals. Our observation of the chaperone and protein accumulation into a specific ER subcompartment may represent a protein quality control holding compartment for inefficiently/misfolded ERAD substrates.

*Keywords:* endoplasmic reticulum, G protein-coupled receptor, luteinizing hormone receptor, membrane proteins, splice variants



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Oulu, October 2005

Pirjo Apaja

## Abbreviations

AKAPs	A-kinase anchoring proteins
ATF	Activating transcription factor
ATP	Adenosine triphosphate
$\beta$ -Gal	$\beta$ -galactosidase
BiP	Binding protein
Ca <sup>2+</sup>	Calcium
C-terminal	Carboxy-terminal
cAMP	Adenosine-3'-5'-cyclic monophosphate
CCD	C-terminal cysteine-rich domain
cDNA	Complementary deoxyribonucleic acid
CFTR	Cystic fibrosis transmembrane conductance regulator
CHO	Chinese hamster ovary
CNS	Coat protein nine signalosome
COS	African green monkey kidney
DAG	Diacylglycerol
3D	Three dimensional
dpc	Days post coitum
EDEM	ER-degradation enhancing 1,2-mannosidase-like protein
EM	Electron microscopy
Endo H	Endoglycosidase H
EOR	Endoplasmic reticulum overload response
EPACs	Guanine nucleotide-exchange proteins activated by cAMP
ER	Endoplasmic reticulum
ERAC	ER-associated compartment
ERAD	ER-associated degradation
ERGIC	ER-to-Golgi intermediate compartment
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
FITC	Fluorescein 5-isothiocyanate
FSH	Follicle-stimulating hormone
G protein	Heterotrimeric guanine nucleotide-binding protein

GABA	$\gamma$ -amino-butyrlic acid
GFP	Green fluorescent protein
GlcNac	N-acetylglucosamine
Gln	Glucose
GnRH	Gonadotropin releasing hormone
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
HA	Hemagglutinin
hCG	Human chorionic gonadotropin
HEK	Human embryonic kidney
Hsp	Heat shock protein
IgG	Immunoglobulin G
IP	Inositol phosphate
IRE	Inositol requiring kinase
kDa	Kilodalton
LacZ	$\beta$ -galactosidase gene
LGR	Leucine-rich repeat-containing GPCR
LH	Luteinizing hormone
LRR	Leucine-rich repeat
mAb	Monoclonal antibody
Man	Mannose
MAPK	Mitogen-activated protein kinase
mMT-1	Mouse metallothionein 1
mRNA	Messenger ribonucleic acid
MT	Microtubule
MTOC	MT organizing center
NCD	N-terminal cysteine-rich domain
N-terminal	Amino-terminal
p450 <sup>scc</sup>	Cytochrome p450 side chain cleavage
pAb	Polyclonal antibody
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PE	Phycoerythrin
PERK	Protein kinase-like ER-kinase
PK	Protein kinase
PL	Phospholipase
PNGase F	N-glycosidase F
R	Receptor
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase-PCR
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacryl amide gel electrophoresis
StAR	Steroidogenic acute regulatory protein
TSH	Thyroid-stimulating hormone
UDP-GT	UDP:glucose-glycoprotein glycosyltransferase
UPR	Unfolded protein response

## **List of original articles**

- I Apaja P, Harju K, Aatsinki J, Petäjä-Repo U & Rajaniemi H (2004) Identification and structural characterization of the neuronal luteinizing hormone receptor associated with sensory systems. *J Biol Chem* 279: 1899-906
- II Apaja P, Aatsinki J, Rajaniemi H & Petäjä-Repo U (2005) Expression of the mature luteinizing hormone receptor in rodent urogenital and adrenal tissues is developmentally regulated at a post-translational level. *Endocrinology* 146:3224-32
- III Apaja P, Poutanen M, Aatsinki J, Petäjä-Repo U & Rajaniemi H (2005) Overexpression of the luteinizing hormone receptor ectodomain splice variant in transgenic mice. (Submitted)
- IV Apaja P, Tuusa J, Pietilä M, Rajaniemi H & Petäjä-Repo U (2005) Luteinizing hormone receptor ectodomain splice variant misroutes the full-length receptor into a subcompartment of the endoplasmic reticulum. (Submitted)

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Additionally, some unpublished results are presented



# Contents

Abstract	
Acknowledgements	
Abbreviations	
List of original articles	
Contents	
1 Introduction .....	13
2 Review of the literature .....	14
2.1 General features of GPCRs .....	14
2.1.1 Function .....	15
2.1.2 Classification .....	17
2.1.3 Protein structure .....	18
2.1.4 Post-translational modifications .....	20
2.1.5 Influence of oligomerization, splice variants and mutants on GPCR trafficking .....	21
2.2 LHR .....	23
2.2.1 Structure and domains .....	23
2.2.2 Ligands and expression in tissues .....	25
2.2.3 Function .....	26
2.2.4 Post-translational modifications and maturation .....	29
2.2.5 Splice variants .....	31
2.3 Transport through the ER .....	34
2.3.1 Molecular chaperones and quality control in the ER .....	34
2.3.2 ER-associated degradation .....	36
2.3.3 Intracellular protein accumulation .....	37
2.3.3.1 Unfolded protein response .....	37
2.3.3.2 Cytosolic protein accumulation .....	38
2.4 Efficiency of protein maturation .....	39
3 Aims of the present work .....	41
4 Materials and methods .....	42
4.1 Summary of methods .....	42
4.2 DNA constructs used in cell lines .....	43

4.3 Tissues and transgenic animals .....	44
4.4 Cell culture .....	45
4.5 Drug and hormone treatments .....	46
4.6 Antibodies .....	47
4.7 Light microscopy .....	48
4.8 Conventional confocal microscopy .....	48
4.9 Transmission and immunoelectron microscopy.....	48
4.10 Fluorescence-activated cell sorting.....	48
4.11 Statistical analysis.....	48
4.12 Effect of hCG on the LHR promoter-driven transgene.....	49
5 Results .....	50
5.1 Identification of the rat LHR tissue distribution (I, II) .....	50
5.1.1 Expression of the LHR in the sensory nervous tissue (I).....	50
5.1.2 Activity of the LHR promoter-driven transgene co-localizes with the P450 $\alpha$ 17 enzyme immunoreactivity in neurons (I).....	51
5.1.3 Expression of the LHR in urogenital and adrenal tissues (II).....	52
5.1.4 Expression of the LHR is hormonally regulated (II) .....	52
5.2 LHR protein in tissues (I, II).....	54
5.2.1 Mature LHR is expressed in the nervous tissue (I).....	55
5.2.2 Mature LHR is developmentally and hormonally regulated at the post-translational level in the rat urogenital and adrenal tissues (II) .....	55
5.3 Overexpression of the LHR ectodomain splice variant in transgenic mice (III) .....	56
5.3.1 LHR ectodomain splice variant interferes with the pituitary- gonadal functions .....	57
5.3.2 LHR ectodomain splice variant causes morphological changes in the adrenal glands, kidney and urinary bladder .....	58
5.4 Intracellular trafficking of the LHR ectodomain splice variant in association with the full-length LHR (IV).....	59
5.4.1 Identification of the rat LHR ectodomain splice variant protein .....	59
5.4.2 LHR ectodomain splice variant is a misfolded ER protein and substrate to ERAD .....	60
5.4.3 LHR ectodomain splice variant colocalizes with ER chaperones and causes their redistribution .....	61
5.4.4 LHR ectodomain splice variant accumulates in the ER.....	61
5.4.5 LHR ectodomain splice variant misroutes the full-length LHR into a subcompartment of the ER .....	62
6 Discussion .....	64
6.1 Maturation of the LHR in the sensory nervous system.....	64
6.2 Maturation of the LHR in the urogenital tissues.....	65
6.3 LHR ectodomain splice variant and the ER subcompartment .....	67
6.4 Post-translational regulation of the mature LHR.....	68
7 Conclusions .....	70
References	
Original articles	

# 1 Introduction

G protein-coupled receptors (GPCRs) represent the largest class of transmembrane receptors in the body. They traverse the membrane bilayer seven times. The leucine-rich repeat-containing GPCRs (LGRs) have the same basic assembly, but they diverge structurally in having a large N-terminal domain that is involved in ligand binding (Jiang *et al.* 1995, Kajava 1998). The luteinizing hormone receptor belongs to this GPCR subfamily of the glycoprotein hormone receptors, as do also the follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH) receptors, and several novel LGRs (Hsu *et al.* 1998, McDonald *et al.* 1998, Hermey *et al.* 1999, Hsu *et al.* 2000, Hsu *et al.* 2002). The LHR is essential in the gonads (Pierce & Parsons 1981, Ascoli *et al.* 2002), but it is also found in several other tissues, in which its functional significance is still under debate.

More than 50% of all medicines available today act on GPCRs, making them a major target for the development of new medicines. GPCRs are mainly involved in signaling from outside the cell to inside the cell. Stimuli for GPCRs are as diverse as light, odorants,  $\text{Ca}^{2+}$ , hormones, and peptides. The physiological processes regulated by these proteins are likewise varied (Bockaert & Pin 1999). While the cell surface action of GPCRs has been studied extensively, little is known about their intracellular processing. Increasing evidence shows that during their biosynthesis some GPCRs have a tendency to accumulate in the ER, suggesting that they may mature inefficiently. Additionally, some GPCRs exist in divergent protein forms that are created through alternative splicing of the gene transcript. GPCRs have traditionally been regarded as monomeric proteins, until relatively recently when the studies have shown that both the cell surface and intracellular receptors can exist oligomers. All these intracellular aspects of GPCRs might provide a mean to regulate their expression profile in the cell.

In the following chapters the terms *maturation* and *processing* are used to define the post-translational modifications and aspects in protein folding that are needed to produce cell surface receptor forms. This work was conducted to find out more about the expression and processing of the rat LHR in natural tissues and heterologous expression systems to elucidate the GPCR maturation process. Special attention was focused on the effect of developmental stage, tissue type and the LHR ectodomain splice variant on the cellular maturation of the LHR.

## 2 Review of the literature

### 2.1 General features of GPCRs

GPCRs are transmembrane proteins that respond to a wide range of relatively small and structurally diverse chemicals such as biogenic amines, peptides, hormones, neurotransmitters, odorants,  $\text{Ca}^{2+}$  and even light with global changes in receptor conformation that leads to larger scale protein-protein interactions. They mediate the majority of transmembrane signal transduction in living cells. The ubiquitous tissue distribution of GPCRs and their capacity to regulate virtually all known physiological processes has made this family of receptors the most important target for drug research. (Bockaert & Pin 1999, Christopoulos & Kenakin 2002).

It is estimated that approximately 1% of the mammalian genome codes for GPCRs, predicting the existence of thousands of these types of receptors. The human genome has been approximated to have 950 genes encoding proteins in the GPCR superfamily (Takeda *et al.* 2002). Of these, ~ 300 full open reading frames can be identified from the public databases and only ~ 200 are classified as known receptors. Ligand binding data is available for more than 80 receptors and 300 ligands. However, substantial portion of GPCRs are described as orphan receptors and, it is assumed that the human genome contains 200-500 orphan GPCRs yet to be identified. (Howard *et al.* 2001, Horn *et al.* 2003).

GPCRs are found in a diverse range of species. The amino acid sequence identity is generally less than 45%, being strongest in the transmembrane region. GPCRs are usually targeted to the plasma membrane. However, this view has been challenged relatively recently by the finding of intracellularly localized GPCRs from the nucleus and melanosomes (Bhattacharya *et al.* 1998, Bhattacharya *et al.* 1999, Schiaffino *et al.* 1999, Lee *et al.* 2004). Integral membrane proteins constitute in many cells about 1% of total proteins. According to the expressed sequence tag databases, the match for GPCRs is typically only 0.01 to 0.001% (Fredriksson & Schioth 2005). Low expression level of GPCRs in native tissues has hindered the mapping of expression sites and made a closer analysis and identification a challenging task.

### 2.1.1 Function

GPCRs transduce the information provided by the stimuli into intracellular second messengers, which amplify the signal and are then interpreted as meaningful signs by the cell. In contrast to the large number of GPCRs, the number of identified effectors is considerably smaller. Because cells can have multiple types of GPCRs, cross-regulation occurs in the signaling pathways leading to diverse physiological responses. Since GPCRs modulate also other proteins' signaling pathways, the outcome is complicated and sometimes even unpredictable.

In principle, a GPCR can exist in an active or inactive form. Binding of an agonist favors the active form and several GPCRs seem to exhibit constitutive activity in the absence of ligand also in normal circumstances (Fong 1996, Kobilka *et al.* 1998). In the classical model of GPCR signaling, the activation of the GPCR leads to the activation of heterotrimeric G (guanine nucleotide binding) proteins located at the cytosolic side of the plasmamembrane, which dissociate into  $\alpha$ - and  $\beta\gamma$ -subunits. These subunits activate effector molecules, which include second messenger generating systems (see Figure 4). The guanine-nucleotide binding site and GTPase activity is in the  $\alpha$ -subunit, where as the  $\beta\gamma$ -subunit forms a tightly bound dimer. The multiplicity of subunits (20  $\alpha$ -, 5 $\beta$ - and 12 $\gamma$ -subunits) allows diversity in signaling. The GPCR promotes the release of GDP from the inactive  $\alpha$ -subunit and binding of GTP, which leads to the free  $\alpha$ -GTP and  $\beta\gamma$ -subunit. These subunits interact with a diverse array of effectors, including adenylyl cyclase, phospholipase C (PLC), G-protein gated potassium channels, voltage-sensitive calcium channels and molecules in the mitogen-activated protein kinase (MAPK) pathway (Belcheva & Coscia 2002, Hur & Kim 2002, Neves *et al.* 2002). G proteins and their splice variants have also been detected on intracellular membranes, such as the Golgi apparatus (Montmayeur & Borrelli 1994, Maier *et al.* 1995, Ugur & Jones 2000) and the endoplasmic reticulum (ER) (Audigier *et al.* 1988). In the brain G proteins have been localized also to the mitochondria and nucleus (Khan & Gutierrez 2004). It is suggested that these intracellular pools perform different functions from the G proteins at the plasma membrane. There is some evidence showing they may modulate protein trafficking (Bomsel & Mostov 1992, Helms 1995) and can influence endocytotic pathways (Melancon *et al.* 1987).

The G proteins are classified into four classes,  $G_s$ ,  $G_{i/o}$ ,  $G_{q/11}$  and  $G_{12/13}$  (Conklin & Bourne 1993, Rens-Domiano & Hamm 1995, Neer & Smith 1996). The stimulatory  $G_s$  subfamily activates adenylyl cyclase resulting in the activation of the cyclic adenosine monophosphate (cAMP) and protein kinase A (PKA) pathway as well as the MAPK pathway through Src-family tyrosine kinases. Several other proteins are also activated by cAMP, like cyclic-nucleotide-gated channels, phosphodiesterases and guanine nucleotide-exchange proteins activated by cAMP (EPACs) (Wong & Scott 2004). The inhibitory  $G_i$  inhibits adenylyl cyclase and activates the MAPK pathway through Src-family tyrosine kinases, tyrosine kinase Raf, and small GTPase Ras. The  $G_q$  subfamily activates PLC resulting in release of intracellular  $Ca^{2+}$  by the protein kinase C (PKC) and inositol triphosphate receptor ( $IP_3$ ) and also the MAPK pathway activation through tyrosine kinase Raf. The  $G_{12}$  subfamily is suggested to regulate small GTP binding proteins (Belcheva & Coscia 2002, Hur & Kim 2002). It seems that most GPCRs are

preferentially coupled to a certain subfamily of G proteins, although they are capable of activating other G proteins with reduced efficiency (Gudermann *et al.* 1996, Gudermann *et al.* 1997).

The finding of new signaling pathways has shown that the classical model of GPCR signaling is only a part of the story. GPCRs associate with a variety of molecules other than heterotrimeric G proteins, although very little is known about the physiological relevance of these associations. For example, some GPCRs like the M<sub>3</sub> muscarinic acetylcholine receptor and H<sub>1</sub> histamine receptor stimulate phospholipase D in a small G protein (Arf and RhoA) -dependent manner without heterotrimeric G proteins (Mitchell *et al.* 1998). The  $\beta_2$ -Adrenergic receptor has been found to associate with the Na<sup>+</sup>/H<sup>+</sup> exchanger regulatory factor in an agonist-dependent manner. This association enhances the exchanger activity, when typically increases in the cAMP would inhibit it (Hall *et al.* 1998, Hall & Lefkowitz 2002). Also some protein enriched in postsynaptic sites (Homer proteins and Shank) have been proposed to function as adaptors for coupling some GPCRs to IP<sub>3</sub> receptor and in this way to regulate intracellular Ca<sup>2+</sup> stores (Hur & Kim 2002). The list of GPCR-interacting proteins is growing rapidly bringing new impressing complexity to the GPCR action.

The classical signaling model is based on the free diffusion of proteins at the plasma membrane. The specificity is dependent on the three dimensional (3D) recognition of protein surfaces. However, this view has been challenged. The signaling in intact cells is greater, quicker, and more specific than in build-up systems, suggesting that receptors, G proteins and effectors are not randomly mobile, but organized to specific microdomains (Neubig 1994). Specialized microdomains such as caveolae and lipid rafts contain a variety GPCRs and signaling molecules as also many other proteins (Shaul & Anderson 1998, Moffett *et al.* 2000). Especially the  $\beta$ -arrestin has been described to serve as a scaffold molecule in the formation of signal transduction complexes (Perry & Lefkowitz 2002). CAMP, PKA and some other signalling molecules are attached to the A-kinase anchoring proteins (AKAPs) (Wong & Scott 2004). The interaction in microdomains and AKAPs could compartmentalize and integrate the signaling events to activate or terminate the relay and to disseminate the receptor signal from other signals.

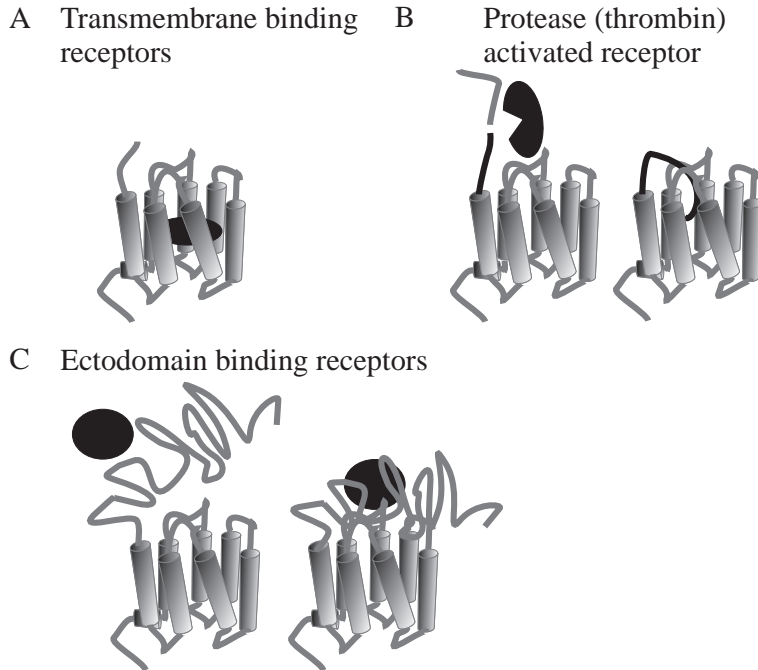
The agonist dependent activation of GPCRs often results in rapid decrease of receptor responsiveness termed as desensitization. Firstly, desensitization includes uncoupling the receptor from G proteins in response to receptor phosphorylation by second messenger-dependent protein kinases and GPCR kinases. Alternatively, G proteins (G<sub>i</sub> and G<sub>q</sub>) signaling is dampened via proteins called regulators of G protein signaling. Secondly, phosphorylation promotes  $\beta$ -arrestin-mediated uncoupling from G proteins and also targets many GPCRs for internalization in clathrin-coated vesicles. Thirdly, the signal transduction pathways down-regulate the receptor number at the cell surface through reducing the transcription and protein synthesis together with the lysosomal and plasma membrane degradation of the corresponding GPCR. The time frame of these processes range from seconds (phosphorylation) to minutes (internalization) and hours (down-regulation). The desensitization provides a mean to terminate the signaling completely or attenuate the agonist potency and responsiveness. However, prolonged desensitization would leave a cell unable to response to the appropriate stimuli. As the desensitization protects the cell from overstimulation, resensitization protects cells from a prolonged lack of receptors. This is achieved through dephosphorylating the internalized GPCRs and

recycling receptors back to the cell surface. This process is slow and inefficient and does not compensate the lost receptors. Thus, many GPCR agonists have limited long-term therapeutic usefulness because of the development of tolerance (Bohn *et al.* 2000, Borgland 2001, Ferguson 2001).

### 2.1.2 Classification

GPCR proteins are highly variable. There are two main requirements for a protein to be classified as a GPCR: seven hydrophobic membrane spanning  $\alpha$ -helices and the ability of the receptor to interact with a G protein. However, there are a number of other proteins coupled to GPCRs and the interaction with G proteins has not been demonstrated for most receptors. Therefore, a more correct definition would be seven transmembrane receptors, but the term GPCR is more established.

GPCRs can be classified in several ways. The most widely used classification system is based on sequence homology, ligand structure and receptor function, defining families A-E and Frizzled/Smoothed, in both vertebrate and invertebrate GPCRs. The family A is for rhodopsin-like, B for secretin-like, C for metabotropic glutamate/pheromone, D for fungal pheromone and E for cAMP receptors. For mammals, also the seven subfamily (A, B, large N-terminal B-7 transmembrane helix, C, Frizzled/Smoothed, taste 2, vomeronasal 1 receptors) classification is used. The rhodopsin family has by far the largest number of receptors, and is sometimes divided into four main subgroups ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ) in phylogenetic trees (Figure 3). According to the ligand binding site, GPCRs can also be divided as those having ligand binding site within of the seven transmembrane helices (e.g. peptide, amine, eucosanoid and nucleotide receptors), extracellular N-terminal domain (glycoprotein hormone and many neurotransmitter e.g. glutamate and  $\text{Ca}^{2+}$  receptors) and protease (thrombin) activated GPCRs (Figure 1) (Kolakowski 1994, Ji *et al.* 1998, Morris & Malbon 1999, Fredriksson *et al.* 2003).



**Fig. 1. Generalized presentation of ligand binding sites in GPCRs. In most GPCRs, the ligand binding site lies within the transmembrane helices (A), some can be activated by protease (thrombin) (B), or ligand can bind to the extracellular domain of the receptor (C) (Kolakowski 1994, Ji *et al.* 1998, Morris & Malbon 1999).**

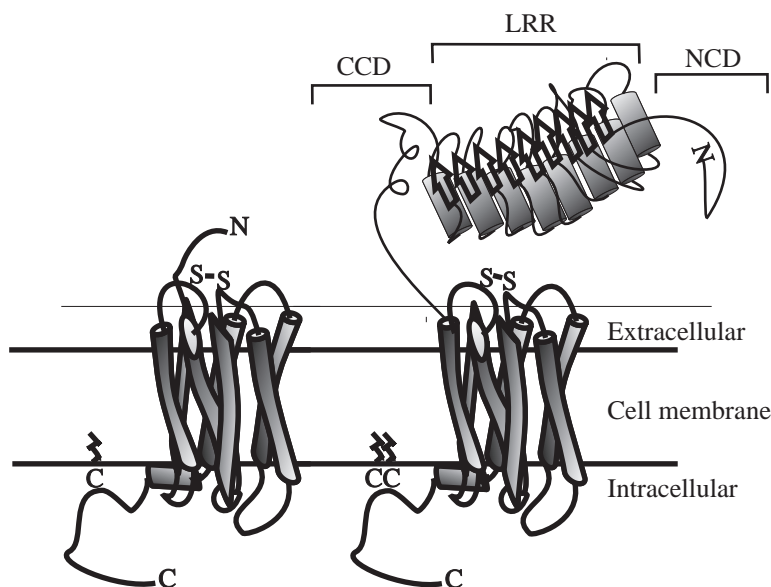
### 2.1.3 Protein structure

All GPCRs have a common central transmembrane core domain connected by three intracellular and three extracellular loops. The  $\alpha$ -helices made up of 22-28 hydrophobic amino acids traverse the lipid bilayer in the counter-clockwise manner. The N-terminus is located on the extracellular surface and the C-terminus on the cytoplasmic surface. In most GPCRs two highly conserved cysteine residues in the extracellular loop one and two that are thought to form a disulfide link. It is likely to be important for the packing and stabilization of a restricted number of conformations at the transmembrane helices, especially because GPCR activation needs conformational flexibility that may lead to instability in the transmembrane structure. Structurally, GPCRs differ in the length and function of their N-terminal extracellular domain, their C-terminal intracellular domain and intracellular loops. Each of these domains provide specific properties to the receptor protein (Figure 2) (Baldwin 1993, Morris & Malbon 1999). Cell surface receptors often contain a cleavable N-terminal hydrophobic signal sequence, which directs the nascent receptor to the ER membrane. However, according to the GPCR protein sequence entries, only a few family A receptors include this kind of signal sequence, whereas nearly all

receptors have it in families B, large N-terminal B-7 transmembrane helix, C, and Frizzled/Smoothed. The first or second transmembrane helix may also be able to function as a signal sequence (Kolakowski 1994, Wallin & von Heijne 1995).

For years, there were no 3D structural data available for GPCRs, and bacteriorhodopsin was used as a reference. The X-ray structure of bovine rhodopsin was solved recently and it is the only crystal structure available today for any GPCR (Palczewski *et al.* 2000). The bovine rhodopsin structure revealed several kinks in transmembrane helices, and a short hinge region after the seventh transmembrane helix immediately followed by an eighth  $\alpha$ -helix running parallel to the cytoplasmic surface (Figure 2). Although some of the proline and glycine residues causing the characteristic kinks in transmembrane helices are conserved in GPCRs, there are differences in amino acid composition that may cause flexibility differences to the bundle structure. However, the overall packing arrangement of the transmembrane region can be assumed to be similar in all GPCRs (Palczewski *et al.* 2000). Thus, 3D models of GPCRs should now allow the use of productive structure-based approaches for drug discovery.

According to the several molecular modeling and mutagenesis studies, transmembrane helices one, two and seven form an inner cavity containing a cluster of polar residues. The side-chains in these residues are conserved in family A receptors and interact with the parallel  $\alpha$ -helix eight possibly transducing the rearrangement of the transmembrane helices to the helix eight. Experiments suggest that the short hinge region connecting helices seven and eight functions as a switch between inactive and active conformations and that the cavity of polar residues with the third intracellular loop is involved in receptor activation and G protein coupling. Based on crystal structure of rhodopsin, activation of GPCRs probably involves separation of transmembrane helices three and six and a twist in helix seven, which pulls the third intracellular loop toward the cytosol resulting in GPCR-G protein interaction (Palczewski *et al.* 2000, Shapiro *et al.* 2002).



**Fig. 2.** The topography of GPCRs. GPCRs have seven transmembrane helices. The N-terminus is extracellular and the C-terminus cytosolic. Generally, most GPCRs have rather short N-terminus and the ligand binding site lies within the transmembrane helices (left model). In the LHR the ligand binds to the large ectodomain. The ectodomain comprises leucine-rich repeats (LRR) flanked by N-terminal (NCD) and C-terminal cysteine-rich domain (CCD) (right model). In most GPCRs, two highly conserved cysteine residues in extracellular loop one and two are thought to form a disulfide link (S-S). One or more cysteine residues conserved in the C-terminal end of the eighth  $\alpha$ -helix are suggested to be palmitoylated anchoring that part of the tail into the plasma membrane (Baldwin 1993, Palczewski *et al.* 2000, Vassart *et al.* 2004).

### 2.1.4 Post-translational modifications

The most common form of post-translational modifications for GPCRs are N-linked glycosylation, disulfide bridges between cysteine residues, signal sequence cleavage, phosphorylation by protein kinases and palmitoylation.

The N-terminal extracellular domain has one or more consensus sequences for N-linked glycosylation (Asn-X (not Pro or Asp)-Ser/Thr) in most known GPCRs. Only very few exceptions in GPCRs are known not to go through N-glycosylation, exhibiting some differences between species. Several chemokine and  $\alpha_{1D}$ -adrenergic receptors belong to this group (Schoneberg *et al.* 2002). In addition, glycosylation in the extracellular loop two occurs in a few family A receptors (Garcia Rodriguez *et al.* 1995). It seems that N-glycosylation is needed for proper folding of the receptor in the ER and trafficking to the cell surface, but has no effect on ligand binding or functional activity in those receptors expressed at the plasma membrane. Some of the N-glycosylation sites are more critical to

the receptor folding and in many cases only a few of the potential sites are used (Schoneberg *et al.* 2002).

The O-linked glycosylation has been documented recently for the V<sub>2</sub> vasopressin (Sadeghi & Birnbaumer 1999), human  $\delta$  opioid (Petäjä-Repo *et al.* 2000) and for some large N-terminal B-7 transmembrane helix receptors having ~20% serine and threonine in their N-terminal domain (Stacey *et al.* 2000). O-linked glycans are added on serine and threonine residues, but there does not appear to be any conserved motif. Generally, it is hypothesized that large O-linked glycans may be needed for maintaining the proper 3D structure, for intermolecular interactions, and for altering the functional state of proteins (Van den Steen *et al.* 1998). Hence, their functional significance for GPCRs needs further investigation.

The disulfide bridges between cysteine residues may have a general role in the 3D conformational stabilization. A majority of GPCRs have conserved cysteine residues in the extracellular loop one and two. In addition, several cysteine residues in the N-terminal domain are conserved in GPCRs having specific cysteine-rich domains associated with leucine-rich repeats (LRR) (Zhang *et al.* 1996), hormone binding (Bazarsuren *et al.* 2002) and frizzled cysteine-rich domains (Dann *et al.* 2001). These domains are important for proper membrane insertion, ligand binding and as in the case of frizzled domain, for a dimer formation interface that may be needed in signaling. In some GPCRs a highly conserved disulfide bridge between cysteines in the third transmembrane helix and the second extracellular loop is important for the maintenance of a proper ligand binding pocket (Horn *et al.* 2003).

Many GPCRs have one or more cysteine residue conserved in the C-terminal end of the eighth  $\alpha$ -helix that was found in the crystal structure of rhodopsin (Palczewski *et al.* 2000). The model of palmitoylating these cysteines and anchoring that part of the C-terminal tail into the plasma membrane has been generalized to almost all GPCRs, even though not directly proven in most cases. However, palmitoylation may not be limited only to this location, since mutating the two conserved C-tail cysteines in e.g. rat  $\mu$  opioid receptor failed to prevent palmitate incorporation (Chen *et al.* 1998). It is suggested that palmitoylated cysteines may be important for normal processing of GPCRs e.g. targeting the receptor to microdomains and especially in activation, desensitisation and internalization processes through affecting the receptor phosphorylation rate (Qanbar & Bouvier 2003).

### ***2.1.5 Influence of oligomerization, splice variants and mutants on GPCR trafficking***

GPCRs have been traditionally regarded as monomeric proteins until relatively recently when the oligomeric nature of GPCRs has been demonstrated in several studies. GPCRs may exist as homo- or heterodimers/oligomers. The ligand-promoted dimerization at the cell surface has been proposed for several GPCRs, but the oligomeric assembly in intracellular compartments appears to be possible. Dimerization/oligomerization at the cell surface might provide a means to aggregate, facilitate and alternate the signaling events depending on the nature of the formed GPCR complex. Alternatively, or perhaps

additionally, formation of oligomeric complexes in the ER may affect the intracellular trafficking and destination of the corresponding receptor complex (Angers *et al.* 2002, Park *et al.* 2004). It is noteworthy that bioluminescence resonance energy transfer studies done with different GPCRs demonstrated homo- and hetero-oligomer formation to prefer closely related sequences rather than more distantly related sequences (Ramsay *et al.* 2002).

Several plasma membrane glycoproteins form oligomeric complexes in the ER (Hurtley & Helenius 1989). The existence of complexes formed soon after biosynthesis raises a possible novel role for GPCR expression profile in the cell. Association of GPCRs in the ER could lead to intracellular retention or enhancement of the cell surface expression. Similarly, splice variants, mutant or even polymorphic forms of the GPCRs could modulate the cell surface expression of their corresponding wild-type receptors. Perhaps the best example demonstrating the functional importance of dominant positive oligomerization is given by the  $\gamma$ -aminobutyric acid (GABA<sub>B</sub>) receptor, which exists in two R1 and R2 splice forms. When expressed alone, the GABA<sub>B1</sub> retained in the ER as an immature form, whereas the GABA<sub>B2</sub>, incapable of binding ligand, is targeted to the plasma membrane. When expressed together, the GABA<sub>B2</sub> masks the ER retention signal in the C-terminal coiled-coil domain of the GABA<sub>B1</sub> allowing the receptor complex to reach the plasma membrane and bind ligand (Jones *et al.* 1998, Kaupmann *et al.* 1998, White *et al.* 1998). Interestingly, a similar example of obligate heterodimerization has been reported for the T1R2 and T1R3 which, upon co-assembly, form a sweet taste GPCR (Nelson *et al.* 2001). In contrast, homodimerization, mediated at least in part by formation of N-terminal disulfide bonds, is reported for GPCRs like the metabotropic glutamate receptors 1 and 5 (Ray & Hauschild 2000, Tsuji *et al.* 2000, Romano *et al.* 2001), and extracellular Ca<sup>2+</sup>-sensing receptor (Zhang *et al.* 2001c). A dominant negative effect causing misrouting in wild-type GPCR trafficking is described even more often to mutants and splice variants. For example, the mutant frizzled 4 receptor associated with vitreoretinopathy traps the wild-type receptor in the ER by dimerization (Kaykas *et al.* 2004). Similar event is described for the gonadotropin-releasing hormone (GnRH) receptor causing hypogonadotropic hypogonadism (Leanos-Miranda *et al.* 2003, Brothers *et al.* 2004) and the chemokine CCR5 Caucasian receptor allele (Samson *et al.* 1996) leading to a delayed onset of acquired immunodeficiency syndrome. Splice variants causing misrouting of the wild-type receptor are described at least for the dopamine D3 (Grosse *et al.* 1997), GnRH (Karpa *et al.* 2000), vasopressin V2 receptor (Sarmiento *et al.* 2004), 5-HT<sub>4</sub> receptors (Pindon *et al.* 2002), and recently also for the LHR (Nakamura *et al.* 2004).

Several GPCRs have been described in alternatively spliced forms, which usually are a result of modifications in the C-terminal part of the receptor. GPCR domains involved in protein interactions are often sites for C-terminal splicing and also alterations in the third intracellular loop. In contrast, the N-terminal splice variants have been proposed to have more influence on ligand binding specificity (Minneman 2001). Changes in physiological stimuli or synaptic activity could regulate the alternative splicing pattern of GPCRs. This could control the forward traffic and amount of cell surface receptors as well as the quality of interacting proteins. Thus, controlling of GPCR export and splicing may provide a cellular mechanism for determining the homeostasis of the cell.

## 2.2 LHR

### 2.2.1 Structure and domains

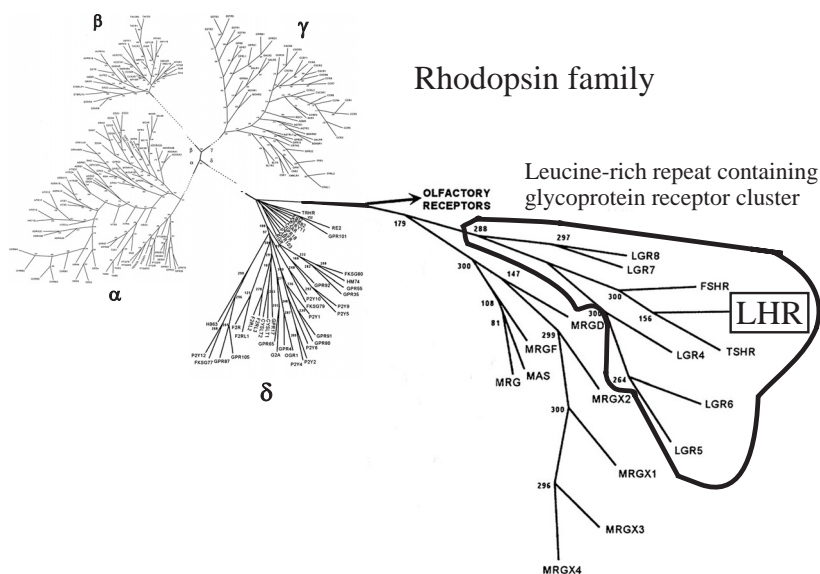
The LHR belongs to the subfamily of LGRs that have the same basic structure with seven transmembrane domains as other GPCRs but diverge in having a large extracellular N-terminal ectodomain of less than 350 and up to 500 residues. The N-terminal domain is involved in selective and high-affinity ligand binding (Jiang *et al.* 1995, Kajava 1998). The LRR domain at the LHR contains nine LRR motifs comprised of ~25 imperfectly repeated amino acids. The motif is flanked by N- and C-terminal cysteine-rich domains (NCD and CCD, respectively) that are thought to protect hydrophobic core of the LRR from the solvent. The CCD domain is sometimes referred to as a hinge region in the LGRs. Multiple LRR motifs have been identified from many proteins which appear to be involved in protein-protein recognition. Direct crystal structure information for the LRR motif is available for two proteins, the pig ribonuclease inhibitor and spliceosomal U2A'. Each LRR repeat is based on a short  $\beta$ -strand and an  $\alpha$ -helix almost parallel to each other. All strands and helices are parallel to a common axis, resulting in a horseshoe-like curved molecule, in which all  $\beta$ -strands are lining the inner and  $\alpha$ -helices the outer part of the shoe (Kobe & Kajava 2001, Vassart *et al.* 2004) (Figure 2).

LGRs belong to the family A, rhodopsin-like GPCR family. In mammals the LGR subfamily contains receptors for LH/human chorionic gonadotropin (hCG), FSH and TSH as well as several recently identified novel receptors, LGR4-8 (Table I) (Hsu *et al.* 1998, McDonald *et al.* 1998, Hermey *et al.* 1999, Hsu *et al.* 2000, Hsu *et al.* 2002). In the phylogenetic tree, the LHR can be found from the subgroup  $\delta$  in the glycoprotein receptor cluster (8 members) together with the MAS-related (8 members), purin (42 members) and olfactory receptor (~460 members, unidentified) clusters (Figure 3) (Fredriksson *et al.* 2003).

Table I. Human leucine-rich repeat (LRR)-containing G protein-coupled receptors (LGRs)

Human LGRs	Number of LRRs	Ligand	Reference
LHR	9	LH	(Minegishi <i>et al.</i> 1990)
FSHR	9	FSH	(Minegishi <i>et al.</i> 1991)
TSHR	9	TSH	(Frazier <i>et al.</i> 1990)
LGR4 (GPR48)	15	Orphan receptor	(Loh <i>et al.</i> 2001)
LGR5 (HG38, GPR49, GPR67)	17	Orphan receptor	(Hsu <i>et al.</i> 1998)
LGR6	11	Orphan receptor	(Hsu <i>et al.</i> 2000)
LGR7 (Relaxin receptor1)	10 + one low-density lipoprotein receptor domain	Relaxin	(Hsu <i>et al.</i> 2000)
LGR8 (Relaxin receptor2, GREAT)	10 + one low-density lipoprotein receptor domain	Relaxin, Leydig insulin-like peptide	(Hsu <i>et al.</i> 2002)

Contrary to other family A GPCRs, the hormone binds to the N-terminal ectodomain of the LHR instead of the transmembrane domains (Jiang *et al.* 1995, Kajava 1998). Several independent studies have established that endogenous ligand interacts with the N-terminal ectodomain and extracellular loops one and two. The interaction between the ectodomain and the loop two has been shown to keep the LHR in inactive state, whereas the ligand binding releases it for receptor activation (Nishi 2002). The cysteine-rich domains may participate in binding by blocking the hormone to a firm hold thus increasing the binding affinity. Thus, the hormone-ectodomain complex would thereby interact with the transmembrane domains causing the rearrangement of helices and initiation of signal transduction (Zeng *et al.* 2001). The LHR has an acidic groove in the center of the LRR7  $\beta$ -strand, which has been identified to contain the critical amino acids (two glutamic acids and lysine) for specific hCG binding (Smits *et al.* 2003). In contrast, the LRR2 has a single residue (isoleucine in human and serine in rat) that is important for affinity of human LH binding (Galet & Ascoli 2005). Studies done with the truncated rat LHRs have defined that the CCD at the extracellular part and the transmembrane domain do not participate in hormone binding (Xie *et al.* 1990, Braun *et al.* 1991, Hong *et al.* 1998, Zeng *et al.* 2001). It could, however, be possible that sulfation on tyrosines (Tyr-Asp-Tyr) in the CCD may determine some of the high-affinity binding (Costagliola *et al.* 2002). However, the truncated rat LHR containing only the ectodomain (amino acids 1-338) was found to be retained intracellularly. The hormone binding could be detected only by solubilizing cells with the non-ionic detergent and glycerol (Xie *et al.* 1990). In spite of the intracellular retention, the truncated LHR was found to bind hormone with identical affinity to the full-length receptor (Tsai-Morris *et al.* 1990, Xie *et al.* 1990). Thereby, it seems that the CCD and transmembrane domains are needed for receptor activation and/or trafficking to the cell surface, but not for high-affinity hormone binding. Co-expression of the separate ectodomain and transmembrane domains has resulted in both receptors incapable of stimulating cAMP production in human embryonic kidney (HEK) 293 cells (rat LHR)(Osuga *et al.* 1997) and in forming functional receptors in African green monkey fibroblasts (COS) cells (porcine LHR)(Remy *et al.* 1993). The reason for this discrepancy is unclear. However, it seems that at least one transmembrane sequence is needed to have some cell surface expression, if only at a reduced level when compared to the full-length receptor (Osuga *et al.* 1997, Sangkuhl *et al.* 2002).



**Fig. 3.** The phylogenetic tree of rhodopsin-like GPCRs. The most frequently used classification system for GPCRs defines family A for rhodopsin-like receptors. According to this phylogenetic analysis, the rhodopsin family is divided into four main subgroups ( $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\delta$ ). The LHR can be found in the subgroup  $\delta$  in the glycoprotein receptor cluster (8 members) (modified from Fredriksson *et al.* 2003).

### 2.2.2 Ligands and expression in tissues

It has been estimated that 80% of hormones and neurotransmitters act on GPCRs (Birnbauer 1990). The LHR is recognized by two cognate ligands; glycoprotein hormones LH secreted by the pituitary gland and its functional homolog, placentally secreted CG in humans. These hormones are dimeric proteins with a molecular weight of ~30 kDa composed of two non-covalently linked  $\alpha$ - and  $\beta$ -subunits. Gonadotropins, LH, hCG, FSH, and also TSH share a common  $\alpha$ -subunit, but the  $\beta$ -subunit provides hormone specificity. The LH and hCG are the major regulators of reproductive-related endocrine functions and steroidogenesis (Pierce & Parsons 1981).

The LHR was first found to be expressed in testicular and ovarian cells probably because it has an unusually high expression level for a GPCR in these organs (Pierce & Parsons 1981, Ascoli *et al.* 2002). The LH promotes ovulation and steroidogenesis in ovarian theca, granulosa and luteal cells stimulating the production of steroid hormones estrogens and progesterone. During pregnancy, hCG stimulates progesterone production in the corpus luteum to maintain endometrial environment favorable for the fertilized ovum until the placenta is developed. In the testis the LH activates steroid and especially testosterone synthesis in the Leydig cells (Pierce & Parsons 1981, Dufau 1998). This has

created the classical description for the LHR as being a receptor controlling gonadal reproductive processes. Accumulative data from different sources has, however, confirmed that the LHR expression pattern is more scattered. The LHR expression has been found in the reproductive tract (Reshef *et al.* 1990, Derecka *et al.* 1999, Zhang *et al.* 2001b), nervous system (Lei *et al.* 1993, al Hader *et al.* 1997, al-Hader *et al.* 1997), prostate (Reiter *et al.* 1995, Tao *et al.* 1995, Dirnhofer *et al.* 1998), adrenal gland (Pabon *et al.* 1996, Kero *et al.* 2000), uterus (Reshef *et al.* 1990, Derecka *et al.* 1999, Stepien *et al.* 2000, Zhang *et al.* 2001b) and even in sperm (Eblen *et al.* 2001) and ovum (Patsoula *et al.* 2001, Patsoula *et al.* 2003). Rao and co-workers have also reported the presence of the LHR in an extremely wide variety of tissues (Rao 2001). In addition, the LHR expression has been monitored in some pathological conditions. In many cases, the expression is based on the LHR mRNA detection. The biological role of the LHR in these tissues is still under investigation.

In addition to the gonadal LHR, another example describing some possible functional LHRs is found in the adrenal glands associated with elevated serum hCG/LH levels (Pabon *et al.* 1996, Rilianawati *et al.* 1998, Kero *et al.* 2000, Lacroix *et al.* 2001, Hämäläinen *et al.* 2002, Piltonen *et al.* 2002, Beuschlein *et al.* 2003). In addition, several clinical findings may be explained by the expression of the LHR in the adrenal cortex (Lacroix *et al.* 2001). The gain-of-function and loss-of-function mutations of the LHR gene (Latronico & Segaloff 1999, Themmen & Huhtaniemi 2000, Lei *et al.* 2001, Zhang *et al.* 2001a) created major functional changes into the gonads, resulting in infertility and abnormal steroid hormone balance. This has made it difficult to evaluate the potential role of the LHR in the other tissues such as the adrenal glands. However, it appears that steroid hormone replacement therapy in males or ovary transplantation in females can partially restore fertility in the LHR knock-out mice (Pakarainen *et al.* 2005a, Pakarainen *et al.* 2005b). Another approach to study the physiological significance of the LHR has been the overexpression of cognate ligands in transgenic mice (Risma *et al.* 1995, Nilson *et al.* 2000, Matzuk *et al.* 2003, Huhtaniemi *et al.* 2005). The phenotypes have been substantially more pronounced in female mice, which have had alterations and tumorigenesis in ovaries, pituitary gland, kidneys and adrenal glands. The male mice seem to be more resistant to elevated hCG levels and tumorigenic effect (Huhtaniemi *et al.* 2005).

The third acknowledgeable potential role of the LHR has arisen recently in the pathogenesis of Alzheimer's disease. Elevated LH expression has been found to co-localize with neurons vulnerable in the Alzheimer's disease, and individuals with the disease have a two-fold elevation in the serum concentrations of the gonadotropins (Bowen *et al.* 2002, Bowen *et al.* 2004).

### **2.2.3 Function**

The well-established signal transduction pathways for the LHR are adenylate cyclase/cAMP/PKA and PLC/IP/diacylglycerol (DAG) routes through heterotrimeric G proteins (Figure 4) (Dufau *et al.* 1977, Gudermann *et al.* 1992a, Gudermann *et al.* 1992b). Even though it is evident that the LHR activates G<sub>s</sub>, it is suggested that the LHR

can function also through  $G_i$ ,  $G_q$  and  $G_{13}$  at a slower rate (Herrlich *et al.* 1996, Rajagopalan-Gupta *et al.* 1997). It seems that the cAMP/PKA pathways functions as a mediator of steroidogenesis, but the role of the PLC pathway is still controversial. It is proposed that this pathway is activated only in females during pregnancy and the LH surge, since the activation requires high concentrations of LH/hCG hormones (Gudermann *et al.* 1992b).  $IP_3$  mobilizes intracellular ER  $Ca^{2+}$  stores and possibly also stimulates  $Ca^{2+}$  entry into the cell through channels triggering calcium responsive protein actions, while DAG activates the PKC mediated protein phosphorylation in a similar manner as PKA (Nishizuka *et al.* 1984, Hur & Kim 2002). In addition to the classical pathways, the Src tyrosine kinase is thought to mediate LHR activation through the MAPK pathway (Taylor *et al.* 1996, Hirakawa & Ascoli 2003). The MAPKs are a group of protein serine/threonine kinases that mediate extracellular signals to the nucleus through the protein phosphorylation cascade. This cascade regulates cell growth, division and differentiation. The extracellular signal-regulated kinases (ERKs) belong to this group (Belcheva & Coscia 2002). It is suggested that LH/hCG inhibits steroidogenesis through ERKs regulating the steroidogenic acute regulatory protein (StAR) expression (Seger *et al.* 2001). This would mean that the LHR activation not only stimulates, but may also limit the stimulation of steroidogenesis.

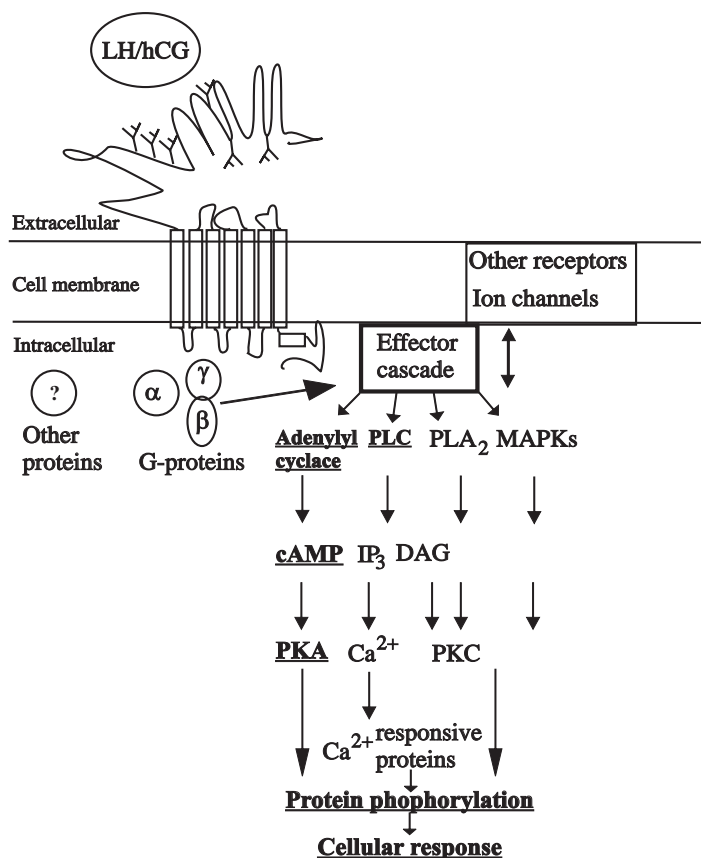


Fig. 4. Schematic presentation of the main signal transduction pathways and the putative topography of the LHR. The LHR has six potential N-glycosylation sites in the ectodomain. The hormones (LH/hCG) bind to the extracellular ectodomain of the LHR creating conformational change that activates G proteins and possibly also some other, yet unidentified proteins, consequently leading to the effector cascade regulation. The LHR was one of the first GPCRs shown to activate adenylyl cyclase and PLC pathways (bold and underline (Gudermann *et al.* 1992a). The activation of preferred routes may have cell type specific differences, and cross-talk between pathways and other receptors and ion channels may occur. (G proteins=heterotrimeric guanine-nucleotide binding proteins, cAMP=cyclic adenosine 3', 5'-monophosphate, PKA=protein kinase A, PLC=phospholipase C, DAG=diacylglycerol, IP<sub>3</sub>=inositol triphosphate, PKC=protein kinase C, PLA<sub>2</sub>=phospholipase A<sub>2</sub>, MAPK=mitogen-activated protein kinase (modified from Richards 1994, Ascoli *et al.* 2002).

As for other GPCRs, high concentrations of agonist cause LHR desensitization. The LHR forms a complex with  $\beta$ -arrestin mediating uncoupling from the G-protein (Mukherjee *et al.* 2000). The cytoplasmic serine residues appear to be important for phosphorylation mediated uncoupling and internalization (Wang *et al.* 1997). Conflicting data exists, however, whether phosphorylation of the LHR is needed for  $\beta$ -arrestin binding. In other GPCRs, the formation of the GPCR/ $\beta$ -arrestin complex depends mostly on receptor

phosphorylation rather than on receptor activation (Perry & Lefkowitz 2002). In contrast, the human LHR association with  $\beta$ -arrestin is suggested to be dependent on receptor activation (Min *et al.* 2002). However, the  $\beta$ -arrestin association at the human FSH was dependent on phosphorylation. It is thereby suggested that the LHR might form an exception among GPCRs concerning  $\beta$ -arrestin binding (Krishnamurthy *et al.* 2003). Upon desensitization the LHR seems to form self-associated aggregates in the cell surface while exhibiting more diffuse distribution during active signaling (Hunzicker-Dunn *et al.* 2003). The long-term effect in agonist exposure is the down-regulation of the LHR mRNA. However, there may be tissue-specific and even developmental specific-differences. A high concentration of LH causes down-regulation of the LHR in the gonads (Catt *et al.* 1980, Metsikkö & Rajaniemi 1984, Pakarinen *et al.* 1990), but induces LHR expression and steroidogenesis in the adrenal glands (Rilianawati *et al.* 1998, Kero *et al.* 2000, Beuschlein *et al.* 2003). In fetal testis, hCG upregulates the LHR (Huhtaniemi 1994) but down-regulates it in the adult testis (Hsueh *et al.* 1977, Pakarinen *et al.* 1990). Menon *et al.* have suggested that the down-regulation is not due to decreased transcription of the gene, but increased degradation of the mRNA. They identified a protein termed as the LHR binding protein 1 from the ovary and later characterized it as the mevalonate kinase. Mevalonate kinase is an enzyme involved in cholesterol biosynthesis. The LHR activation increases mevalonate kinase and lipoprotein receptor levels, increasing also the binding of the mevalonate kinase to the LHR mRNA, resulting in destabilization. This process suppresses the LHR cell surface levels and increases the capability of the cells to replenish cellular cholesterol pools (Kash & Menon 1998, Nair *et al.* 2002, Wang & Menon 2005).

#### ***2.2.4 Post-translational modifications and maturation***

The polypeptide chain of the human LHR is composed of 674 and the rat LHR of 675 amino acids residues in addition to a signal sequence of 25-26 residues. The NCD domain involves 28, LRR domain 240 and C-terminal cystein-rich domain 88 residues, and in total it can be considered that there are ~363 residues forming the N-terminal ectodomain. The transmembrane helices and intervening loops are formed of ~268 and the C-terminal cytosolic tail of 70 residues. The amino acid identity between rat and human is ~88% in the ectodomain, ~92% in the transmembrane domain and ~69% in the C-terminal tail (Ascoli *et al.* 2002).

The cell surface mature form of the LHR has been reported to have a molecular mass of 85-95 kDa, which arises from the ER localized immature form of 68-75 kDa (Keinänen *et al.* 1987, Roche & Ryan 1989, Zhang & Menon 1989, Hipkin *et al.* 1992, Pietilä *et al.* 2005). In addition, LHR species with a molecular mass 165-200 kDa have been described. These species are assumed to be oligomeric forms of the immature LHR based on their susceptibility to endoglycosidase H (Endo H), a glycosidase that removes the ER associated high-mannose type carbohydrates, and the fact that the time course of the oligomer formation is identical to that of the immature form (Davis *et al.* 1997, Kawate *et al.* 1997, Fabritz *et al.* 1998). However, the identity of the oligomer has not been directly characterized, leaving options for alternative associations. Studies done in

HEK 293 cells have shown that the immature rat LHR forms oligomeric complexes with a 120 kDa protein in the ER (Pietilä *et al.* 2005). The FSHR ectodomain occupied with its ligand FSH is capable of forming dimers through tyrosine in position 110 with the interface in the LRR2-4 (Fan & Hendrickson 2005). Because tyrosine 110 is completely conserved, a homologous receptor, such as the LHR, may form dimers in a similar manner.

The glycoprotein nature of the LHR has been confirmed with tissue purified and heterologously expressed receptor. The LHR has six consensus sequences for N-glycosylation in the N-terminal ectodomain, three of them laying in the LRR domain and three in the CCD. These sites are conserved in the mammalian LHRs. According to the mass spectrometric analysis, at least five of these sites contain carbohydrates (Vu-Hai *et al.* 2000). Studies both with the human and rat LHR have shown that N-linked glycosylation has no effect on ligand binding or functional activity of the receptor (Petäjä-Repo *et al.* 1993, Davis *et al.* 1997), which is in accordance with similar studies done with other GPCRs (Schoneberg *et al.* 1999). Thus, glycosylation appears to facilitate the LHR folding, since the lack of glycosylation decreased LHR processing and targeted the receptors for degradation thereby leading to decreased cell surface expression (Clouser & Menon 2005). The ER lectin binding chaperones calnexin and calreticulin associate with immature forms of the human and rat LHR (Rozell *et al.* 1998, Mizrahi & Segaloff 2004), possibly preventing their too early entrance to the cell surface or degradation pathway.

For the rat LHR, site-directed mutagenesis experiments have suggested that cysteines in the CCD and cysteines in the extracellular loops one and two are important for membrane insertion, whereas cysteines in the NCD and the cysteine bridge in LRR region are important for hormone binding (Zhang *et al.* 1996). The cysteines in the C-terminal cytoplasmic tail are palmitoylated and needed for normal maturation of the LHR, since the mutation of the sites resulted in intracellular retention (Zhu *et al.* 1995). Furthermore, it has been shown that palmitoylation determines the recycling efficiency of the internalized human LHR recycling (Munshi *et al.* 2005).

The rat LHR maturation process has been studied in the HEK 293 cells (Hipkin *et al.* 1992, Zhu *et al.* 1995, Li *et al.* 2000, Pietilä *et al.* 2005). These studies have shown that the conversion of the immature form into the mature one is slow and inefficient. In contrast, it seems that the human LHR maturation is more efficient, resulting in over eight-fold more mature receptors at the cell surface than for the rat LHR (Ascoli *et al.* 2002). A large portion of the rat LHR immature form is possibly degraded and never converted to the mature form. Both the LHR and glycosylation mutants of the LHR undergo degradation that appear to be dependent on ubiquitination (Clouser & Menon 2005, Pietilä *et al.* 2005). The degradation of the immature rat LHR is enhanced by a cytosolic protein p38<sup>JAB1</sup>, which associates with the C-terminal tail of the LHR (Li *et al.* 2000). The p38<sup>JAB1</sup> is a component of the coat protein (COP) 9 signalosome (CNS) (Naumann *et al.* 1999) and eight of the CNS subunits share significant sequence similarity with the eight subunits of the 26S proteasome lid (Seeger *et al.* 1998) and with the translation initiation complex eIF3 (Glickman *et al.* 1998). The CNS is thought to cooperate with the ubiquitin/26S proteasome system in the regulation of protein stability by mediating the phosphorylation of the target protein. The CNS also interacts with certain ubiquitin ligases regulating their activity state (Lyapina *et al.* 2001, Wee *et al.* 2005).

There is an increasing body of evidence that the CNS is involved in regulating ubiquitination. However, the LHR degradation pathway is rather unknown and has not been fully studied with regard to phosphorylation and ubiquitination.

### 2.2.5 *Splice variants*

The coding region of the LHR gene consists of 11 exons and 10 introns (Figure 5) (Koo *et al.* 1991). It has been speculated that the gene has originally arisen from assimilation of two different genes. Hence, the exon 11 greatly resembles most of the GPCR genes by lacking introns (Dufau 1998). The basal promoter region at the mouse and rat LHR gene lies within the first 180 bp, and most of the identified main regulatory elements are located within the 1000 bp upstream of the translation initiation codon (Tsai-Morris *et al.* 1995, El Hefnawy *et al.* 1996, Chen *et al.* 1999). The exons 1-10 encode the signal peptide and the extracellular part of the receptor excluded by half of the CCD, which is encoded by the beginning of the exon 11 (141 nucleotides). All the introns lay within this region. The rest of the exon 11 encodes the GPCR part of the protein, namely the transmembrane domain, intervening loops and cytosolic C-terminal tail (Tsai-Morris *et al.* 1991).

An increasing number of GPCRs has alternatively spliced variants. The LHR have several splice variants that to some extent exhibit species specificity. The splicing seems to occur mostly in the exon 11, but also skipping in the exons encoding the LRR has been described. Potentially, alternative splicing toward the LRR encoding region may modulate the hormone binding properties or create receptor with differential activity state conformations, leading to, for example, the usage of the alternative signal transduction pathways. Splicing toward the exon 11, on the other hand, affects more the transmembrane domain, possibly creating soluble products. The exon 11 contains two out-of-frame alternative splice sites, the usage of which would encode the complete hormone binding domain without the G protein-coupled domain (Tsai-Morris *et al.* 1991). Different splice variants have been described at least for pig (Loosfelt *et al.* 1989), rat (Segaloff *et al.* 1990, Tsai-Morris *et al.* 1990, Aatsinki *et al.* 1992), sheep (Bacich *et al.* 1994) and human (Minegishi *et al.* 1997, Reinholz *et al.* 2000). One of the most repeatedly cloned splice variant in different species differs from the full-length LHR mRNA by having a deletion of 266 bp, resulting from the usage of a first alternative splice site in exon 11 (=ectodomain splice variant). This deletion causes a frame shift in the reading frame and creates a truncated translation product lacking half of the CCD in the ectodomain, transmembrane domain and C-terminal tail (amino acids 295-674 in the rat), and concomitantly creating a unique C-terminus. This variant was first cloned from pig and rat (Loosfelt *et al.* 1989, Tsai-Morris *et al.* 1990). As the variant also contains a signal sequence, it may be secreted. In the gonads, this variant transcript is not downregulated by hCG as are other LHR transcripts (LaPolt *et al.* 1991, Wang *et al.* 1991). It is noteworthy that this variant does not contain the C-terminal tail binding site characterized for mevalonate kinase mediated LHR mRNA degradation in the downregulation event (Nair *et al.* 2002). In pigs, 40% of the transcripts are estimated to correspond to variants lacking the transmembrane domain (VuHai-LuuThi *et al.* 1992). A

transcript corresponding to the extracellular domain of the LHR can be detected as early as 13.5 dpc (days post coitum) in the rat ovary and testis (Sokka *et al.* 1996).

The biological role of the LHR splice variants has remained largely unrevealed. There is, however, accumulating evidence suggesting that alternative splicing of the LHR gene transcript could regulate functional receptor levels in various physiological conditions. For example, changes in alternative splicing appear to be associated with cycle-dependent regulation of LHR mRNA levels in human endometrium (Licht *et al.* 2003) and receptor downregulation in rat corpora lutea (Lakkakorpi *et al.* 1993). Furthermore, various rodent tissues have been shown to display changes in the LHR splicing pattern during development and cellular differentiation (Sokka *et al.* 1992a, Sokka *et al.* 1992b, Vihko *et al.* 1992, Tena-Sempere *et al.* 1994, Zhang *et al.* 1994). Variations in the splicing pattern have also been shown to take place in pathophysiological conditions, such as endometrial carcinomas that contain elevated levels of spliced LHR transcripts (Lin *et al.* 1994) or in human ovarian (Reinholz *et al.* 2000, Steinmeyer *et al.* 2003) and breast epithelial cell (Jiang *et al.* 2002) tumors, in which the alternative splicing pattern seems to be altered or ceased when compared to normal cells. In the Leydig tumor cells, the transcript corresponding to the extracellular domain is the predominant mRNA (Wang *et al.* 1992).

There is very little information available on possible protein products of the alternatively spliced transcripts. Some studies have been carried out with artificially created transcripts encoding the extracellular ectodomain of the LHR (Xie *et al.* 1990, Braun *et al.* 1991, Hong *et al.* 1998, Zeng *et al.* 2001), which do not fully correspond to the naturally occurring splice variants. However, they do give some information on the ways the ectodomain behaves without the transmembrane domain. It is evident that the ectodomain binds hormone with high affinity (Tsai-Morris *et al.* 1990, Xie *et al.* 1990, Braun *et al.* 1991, Osuga *et al.* 1997, Sangkuhl *et al.* 2002), but information on its potential secretion capability is contradictory (Table 1). It was found that this truncated receptor is not secreted into the culture media but remains trapped within the HEK 293, COS cells, and *Escherichia coli* (Xie *et al.* 1990, Braun *et al.* 1991, Chen & Bahl 1993). However, it was unexpectedly found to be secreted in varying extents in a study done in Sf9 insect cells (Bozon *et al.* 1995). Also, the porcine LHR ectodomain coexpressed in COS cells with hCG was secreted as a complex with the hormone (Remy *et al.* 2001). One possible explanation for this discrepancy is the used cells or the amino acids included to determine the intersection of the ectodomain. The true, endogenously expressed rat LHR ectodomain splice variant was found to be secreted (Tsai-Morris *et al.* 1990) whereas the porcine variant was both secreted and to be intracellularly retained in COS cells (VuHai-LuuThi *et al.* 1992) and secreted in the mammary epithelial HC11 cells (Pajot-Augy *et al.* 1997). Importantly, recent studies have provided some direct evidence on the functional significance of the LHR splice variants, as a human ovarian variant with a deletion of 63 amino acids of exon 9 was shown to be expressed in transfected COS cells. This variant misrouted the immature full-length receptor in the dominant negative manner causing a decrease in the cell surface LHR expression (Minegishi *et al.* 1997, Nakamura *et al.* 2004). Hence, it should be pointed out that there is no data available on tissues demonstrating protein products arising from these alternatively spliced transcripts.

Table 2. Secretion capability of the LHR ectodomain

Ectodomain	Amino acids	Domains	Number of the N-glycosylation sites	Cell type	Secretion	Reference
Rat ectodomain, constructed	1-338	NCD, LRR, CCD	6	HEK 293	no	(Xie <i>et al.</i> 1990)
Human ectodomain, constructed	1-244	NCD, LRR 1-8	3	COS	no	(Braun <i>et al.</i> 1991)
Rat ectodomain, constructed	1-294	NCD, LRR, 49 amino acids of CCD	6	Escherichia coli	no	(Chen & Bahl 1993)
Porcine ectodomain, constructed	1-297	NCD, LRR, 30 amino acids of CCD	5	Sf9	yes	(Bozon <i>et al.</i> 1995)
Porcine ectodomain, constructed	1-277	NCD, LRR, 10 amino acids of CCD	3	COS secreting hCG	yes	(Remy <i>et al.</i> 2001)
Rat ectodomain splice variant	1-294+22	NCD, LRR, 49 amino acids of CCD+22 novel	6	COS	yes	(Tsai-Morris <i>et al.</i> 1990)
Porcine ectodomain splice variant	1-316+13	NCD, LRR, 44 amino acids of CCD+13 novel	6	COS	yes	(VuHai-LuuThi <i>et al.</i> 1992)
Porcine ectodomain splice variant	1-316+13	NCD, LRR, 44 amino acids of CCD+13 novel	6	HC11	yes	(Pajot-Augy <i>et al.</i> 1997)

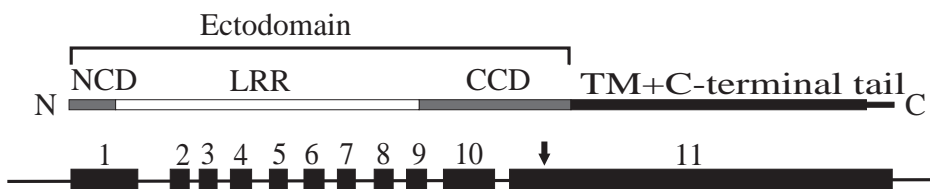


Fig. 5. Schematic structure of the rat LHR gene and protein domains. The ectodomain comprises leucine-rich repeats (LRR) flanked by the N-terminal (NCD) and C-terminal cysteine-rich domain (CCD) encoded by exons 1-10 and the beginning of the exon 11. Exon 11 encodes mostly the transmembrane domain and cytosolic C-terminal tail of the receptor. The arrow indicates the out-of-frame alternative splice site that is used to create the LHR ectodomain splice variant. TM, transmembrane (based on publications Koo *et al.* 1991, Vassart *et al.* 2004).

## 2.3 Transport through the ER

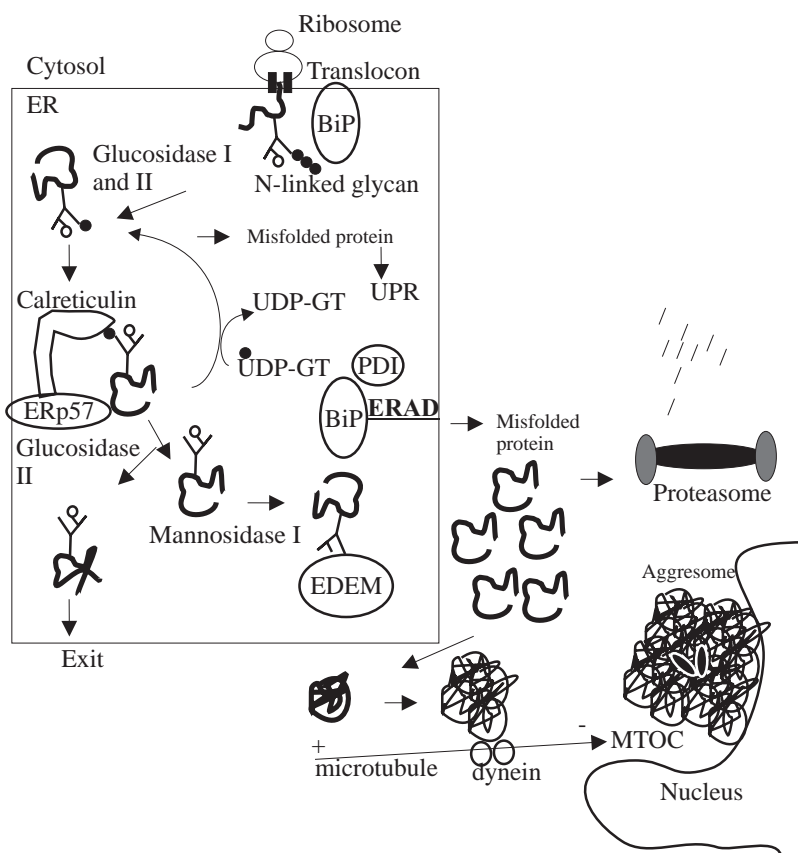
### 2.3.1 Molecular chaperones and quality control in the ER

The proper conformational maturation of nascent proteins is both aided and monitored by a number of proteins in a complex process termed the ER quality control that prevents the premature progression of misfolded proteins through the secretory pathway. This process is controlled by molecular chaperones that recognize unfolded proteins and promote protein folding by preventing irreversible aggregation (Hendrick & Hartl 1993, Hammond & Helenius 1994). The ER contains chaperones that belong to the classical chaperone families present also in the cytosol. The main chaperone families in the ER are heat shock protein 70s (Hsp), from which the binding protein (BiP) is the most common one, taking part in many quality control processes, e.g. the folding of the newly synthesized proteins, ER-associated degradation (ERAD) and the UPR. Hsp40s, Hsp90s (GRP94) and peptidyl-prolyl isomerases are also found in the ER. The ER also contains chaperones that are unique, such as calnexin and calreticulin and the family of thiol-disulfide oxidoreductases, such as protein disulfide isomerase (PDI) and ERp57 (Ellgaard & Helenius 2003).

The components and mechanisms of action of the two major chaperone systems are the most widely studied. The first system is dependent on the presence of both monoglucosylated N-linked glycans and unfolded regions on nascent glycoproteins. The second major ER chaperone system is only dependent on the presence of unfolded regions on proteins containing hydrophobic residues, which are recognized by the ER chaperone BiP (Flynn *et al.* 1991, Blond-Elguindi *et al.* 1993). The main feature of the glycan-dependent system is the calnexin-calreticulin cycle (Figure 6) (Parodi 2000). Calnexin and calreticulin are two homologous lectins, of which calnexin is a type 1 membrane protein whereas calreticulin is a luminal ER protein. The N-linked glycosylation involves transfer of a triglucosylated oligosaccharide ( $\text{Gln}_3\text{Man}_9\text{GlcNAc}_2$ ) to a consensus sequence in the newly synthesized polypeptide. The ER enzymes glucosidase I and II trim the oligosaccharide to the monoglucosylated form ( $\text{Gln}_1\text{Man}_9\text{GlcNAc}_2$ ), which interacts with calnexin and calreticulin (Hammond *et al.* 1994). Interaction with these two lectins is accompanied by a thiol oxidoreductase ERp57, which is likely to be involved in disulfide bond formation of the nascent polypeptide (Oliver *et al.* 1997). The last glucose is removed by glucosidase II, which abrogates the lectin binding site (Trombetta & Parodi 1992, Sousa & Parodi 1995). If the glycoprotein has reached the native conformation, it is successfully transported to the Golgi complex. However, if the protein is partially folded, the folding sensor UDP:glucose-glycoprotein glycosyltransferase (UDP:GT) recognizes the hydrophobic unfolded regions and adds a single glucose back to the oligosaccharide (Trombetta & Parodi 1992, Sousa & Parodi 1995, Ritter *et al.* 2005), and the calnexin-calreticulin cycle is repeated.

The second major ER chaperone system is dependent on the presence of unfolded regions on proteins containing hydrophobic residues. Some calnexin/calreticulin substrates can bind to BiP instead if N-linked glycosylation is blocked (Balow *et al.* 1995,

Zhang *et al.* 1997). Also, the initial choice of the chaperone seems to be dependent on the location of N-linked glycans in the growing nascent chain. Direct interaction with calnexin and calreticulin without prior interaction with BiP was found to occur if glycans were present within about 50 residues from the amino-terminus in the Semliki forest virus and influenza hemagglutinin glycoprotein (Molinari & Helenius 2000). BiP and all Hsp70 proteins are believed to undergo ATP dependent cycles of binding and release from unfolded proteins (Gamer *et al.* 1996, Bukau & Horwich 1998). A number of chaperones and cofactors both can positively and negatively regulate the ATPase cycle of Hsp70 proteins in yeast. However, the mammalian ER homologues of most of these proteins are poorly known. The role of most of the proteins in the ER quality control and their relationship with the two major chaperone systems has not been clearly elucidated.



**Fig. 6. Schematic model of glycoprotein transport through the ER.** Translocated glycoproteins are core-glycosylated and then trimmed by glucosidases I and II. The monoglucosylated protein forms bind to calreticulin/calnexin that are associated with the thiol oxidoreductase ERp57. Glucosyl transferase (UDP-GT) adds the last glucose (●) back to the unfolded glycoproteins to repeat the folding cycle. The accumulation of folding intermediates in the ER can trigger the stress cascade reaction, UPR. Folded proteins exit from the ER to the Golgi, and unfolded (mannose o) mannosidase I trimmed proteins are recognized by the ER-degradation enhancing 1,2-mannosidase-like protein (EDEM). These misfolded proteins are targeted to ERAD. ERAD substrates are retrotranslocated from the ER to the cytosol. Aggregation in the cytosol happens if the misfolded proteins are not degraded by the proteasome. Formed aggregates are transported via microtubules (MT) to the MT organizing center (MTOC) near the nucleus that requires the dynein/dynactin motor complex (modified from Garcia-Mata *et al.* 2002, Ellgaard & Helenius 2003).

### 2.3.2 ER-associated degradation

To travel along the secretory pathway and eventually reach their appropriate cellular destinations, newly synthesized proteins must fold and assemble correctly. Proteins that

are not folded correctly are bound to be degraded. A glycoprotein can exit the calnexin-calreticulin cycle when the UDP-GT fails to add a single glucose back to the oligosaccharide (Figure 6). For the degradation of glycoproteins, the ER mannosidase I removes a single mannose from the middle of the oligosaccharide leading to the form ( $\text{Man}_8\text{GlcNAc}_2$ ) that is bound by ER-degradation enhancing 1,2-mannosidase-like protein (EDEM) (Hosokawa *et al.* 2001, Jakob *et al.* 2001, Molinari *et al.* 2003). The EDEM targets glycoproteins for ERAD and maintains the glycoprotein folding capacity and secretion efficiency (Eriksson *et al.* 2004). Studies on yeast have shown that in principle, a single context-specific N-linked glycan can determine the targeting of misfolded glycoprotein for ERAD (Kostova & Wolf 2005, Spear & Ng 2005). In mammalian cells, the degradation of a misfolded glycoprotein usually starts after a lag period of 30-90 min. The calnexin-calreticulin cycle seems to protect glycoproteins against degradation creating the lag phase. However, if the glycoprotein does not enter the calnexin-calreticulin cycle, the degradation can start without a lag (Mancini *et al.* 2003, Molinari *et al.* 2003). The ERAD substrates are retrotranslocated (dislocated) across the ER membrane possibly through the Sec61 translocon channel and assisted by an associated protein complex into the cytosol (Plemper *et al.* 1999). Derlin-1 has also been identified to move misfolded proteins into the cytosol (Lilley & Ploegh 2004, Ye *et al.* 2004). BiP may have a role in retrotranslocation and in keeping the protein unfolded after the calnexin-calreticulin cycle. Microscopic studies with different ERAD substrate proteins have shown accumulation into a novel preGolgi compartment that was adjacent to the centrosome, the Golgi complex, and the ER-to-Golgi intermediate compartment (ERGIC). The ER chaperones calreticulin and calnexin, but not BiP or PDI, concentrated in this subcellular region. This compartment is microtubule dependent and possibly a subcompartment of the ER. It is suggested that it might be the site for concentration and retrotranslocation of misfolded proteins (Kamhi-Nesher *et al.* 2001, Frenkel *et al.* 2004). In the cytosol, proteins are ubiquitinated by ubiquitin enzymes that covalently attach a 76-residue ubiquitin protein to lysine residues on substrate proteins. Ubiquitinated proteins are degraded through the 26S proteasome complex (Biederer *et al.* 1997, Plemper & Wolf 1999, Gilon *et al.* 2000, Wilhovsky *et al.* 2000, Ellgaard & Helenius 2003, Helenius & Aebi 2004). Some other alternative pathways besides ERAD have also been suggested, but not yet identified (Mancini *et al.* 2003).

### **2.3.3 Intracellular protein accumulation**

#### *2.3.3.1 Unfolded protein response*

Inefficient maturation or mutations can easily cause accumulation of misfolded proteins into the ER. In addition to the misfolded proteins, the ER protein aggregates often contain molecular chaperones and thiol-disulfide oxidoreductases (Gibson *et al.* 1979, Molinari *et al.* 2002). The ER controls the entire endomembrane and endocytic system and is the major  $\text{Ca}^{2+}$  storage organelle. The ER homeostasis is thereby vital for the cell and the ER transmits signals in response to environmental cues by releasing  $\text{Ca}^{2+}$ . The incapability of

the ER to process misfolded proteins and their accumulation into the ER causes stress termed as UPR (Figure 6). However, experiments have revealed that not all unfolded proteins are able to activate the response. It is suggested that proteins that bind BiP activate the UPR, whereas those that bind for instance calnexin do not (Ma & Hendershot 2004). The UPR involves a signaling network in mammalian cells. The ER transmembrane proteins inositol requiring kinase 1 (IRE1), protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6) mediate the regulation of both transcriptional and translational levels during ER stress (Kaufman 1999, Harding *et al.* 2000). In unstressed cells, BiP keeps these proteins in an inactive state (Bertolotti *et al.* 2000). PERK mediates the attenuation of the protein translocation into the ER and prevents the overloading. IRE1 and ATF6 mediate activation of genes that encode components that increase protein folding, export and degradation (Kaufman 1999, Harding *et al.* 2000). Cells that have irreversible ER stress undergo programmed cell death, apoptosis. The ER elicits a proapoptotic signal that results in transmission of  $\text{Ca}^{2+}$  to the mitochondria. Increasing  $\text{Ca}^{2+}$  causes mitochondrial fragmentation and eventually the release of cytochrome c into the cytosol and rapid activation of the apoptotic signal transduction pathways (Breckenridge *et al.* 2003, Orrenius *et al.* 2003). Another pathway that may initiate in the ER is called the ER overload response (EOR). The EOR is a result of accumulation of proteins in the ER membrane, which may increase its  $\text{Ca}^{2+}$  permeability possibly because of impaired  $\text{Ca}^{2+}$ -ATPase function or increased protein-to-lipid ratio in the ER membrane (Pahl 1999).

Conformational diseases arise when a protein is incapable of folding correctly and is prone to aggregate. The protein aggregates can locate into the ER, nucleus, or cytosol. The UPR may play a role in the pathogenesis of a number of diseases, including in ischemia, neurodegeneration, heart disease and diabetes. Viral infection, tissue hypoxia and hypoglycemia may cause a protein misfolding and ER stress. Interestingly, autosomal dominant diabetes in mice results from the accumulation of misfolded proinsulin 2 in the ER and impairment in insulin secretion, and  $\beta$ -cell death. ER stress is also found in liver and adipose tissue of obese mice and mice with unhealthy diets (Ron 2002, Xu *et al.* 2005).

### 2.3.3.2 Cytosolic protein accumulation

Misfolded proteins are retrotranslocated from the ER into the cytosol and are delivered to the proteasome for degradation. Protein aggregation in the cytosol occurs when the accumulation rate of misfolded or aggregation prone proteins in cytosol exceeds the capacity of chaperones to unfold substrates into a state, in which they can be presented to the proteasome system, rather than malfunction in the proteasome activity (Muchowski *et al.* 2000, Dul *et al.* 2001). Aggregated particles are transported toward the microtubule (MT) organizing center (MTOC), in which they form a perinuclear, centrosome-associated aggregate (Figure 6). Johnston *et al.* named this MT-dependent aggregate in the mammalian cells as an aggresome in order to distinguish it from inclusion bodies (Johnston *et al.* 1998). In cell culture conditions, the aggresome formation appears to be a natural consequence of experimental usage of proteasome inhibitors and an indication of

the target protein degradation by the proteasome (Kopito & Ron 2000). Aggresomes are not static, but recruit a number of cytosolic chaperones like Hsp70, Hsc70 and Hsp40 family members, ubiquitination enzymes and proteasome components. Aggresomes are likely to be formed around the MTOC to prevent aggregated proteins from interfering with normal cellular functions. It is also possible that the centralization of proteins facilitates autophagic disposal. The formation of multilamellar autophagosomes that mature into lysosomes is the major process by which many cytoplasmic components are degraded (Johnston *et al.* 1998, Muchowski *et al.* 2000, Dul *et al.* 2001, Garcia-Mata *et al.* 2002). Aggresome formation also leads to the disruption of the Golgi complex despite the vesicular transport being unaltered (Garcia-Mata *et al.* 1999).

Cytoplasmic aggregates are features in many diseases, especially in neurodegenerative ones. The aggresomes are nearly always associated with abnormally phosphorylated intermediate filaments like neurofilaments and vimentin. The MT-associated transport of aggregated proteins may be a cytoprotective mechanism toward the toxicity of aggregates until the pathway is overwhelmed. There are several diseases described to be caused by aggregation-prone proteins in the cytosol, such as Alzheimer's, Huntington's, Parkinson's and Prion diseases (Tran & Miller 1999, Garcia-Mata *et al.* 2002).

## 2.4 Efficiency of protein maturation

Proteins have distinctive differences in their folding efficiency. Some polypeptides are trapped into the misfolded conformation even with the assistance of chaperones. Between 30 to 75% of newly synthesized polypeptides are rapidly degraded within 20 minutes (Lin *et al.* 1998, Schubert *et al.* 2000, Yewdell *et al.* 2001), while some are degraded already as the growing nascent chain (Turner & Varshavsky 2000). Inefficient maturation has been found to be characteristic of some GPCRs (Fishburn *et al.* 1995, Petäjä-Repo *et al.* 2000, Imai *et al.* 2001, Lu *et al.* 2003, Wüller *et al.* 2004), such as human  $\delta$ -opioid receptor (Petäjä-Repo *et al.* 2000, Petäjä-Repo *et al.* 2001) and also of other proteins, such as the cystic fibrosis transmembrane conductance regulator (CFTR) (Kopito 1999). Nearly 70% of the CFTR and 50% of the newly synthesized human  $\delta$ -opioid receptor was targeted to degradation. The same inefficient maturation appears to apply also for the rat LHR (Pietilä *et al.* 2005). A poor maturation rate is often observed also for proteins that need hetero-oligomerization for correct folding (Merlie & Lindström 1983). Yewdell *et al.* suggest that inefficient maturation serves an important immunological function via introducing degraded protein peptides, both the cells' own and viral, on the cell surface for T-cell detection (Yewdell *et al.* 2001). Also, the immunoglobulin light chain is always excessively expressed when compared to the heavy chain to guarantee the heavy chain oligomerization and prevent it from forming possibly toxic free forms (Sitia & Cattaneo 1995). In some cases, the availability of ligand determines the maturation efficiency, such as the availability of high affinity peptide for the major histocompatibility complex class I molecules (Cresswell 2000). This suggests that the ligand may stabilize of the partially folded or misfolded proteins, possibly through conformational changes within the hydrophobic core of the protein. An elegant approach using pharmacologically selective membrane permeable compounds, termed

pharmacological chaperones, has been used to stabilize the inefficiently matured GPCRs, facilitating their export from the ER to the cell surface. This rescue of misfolded GPCRs has been successfully demonstrated for both wild-type (Petäjä-Repo *et al.* 2002, Van Craenenbroeck *et al.* 2005) and mutant receptors (Morello *et al.* 2000, Janovick *et al.* 2002, Noorwez *et al.* 2003). The protein maturation efficiency can also be a regulated event and directly linked to the physiological signals. This is demonstrated by the observation that the absence of synaptic activity improved the maturation efficiency and export of the glutamate-gated ion channels, the N-methyl-D-aspartate receptors, from the ER by changing an alternative C-terminal tail of the receptors through alternative splicing of the mRNA (Mu *et al.* 2003). Controlling of protein maturation through physiological signals may thereby be in close connection with both the oligomerization event in the ER and the protein variants created through alternative splicing of the mRNA transcript.

### **3 Aims of the present work**

There is very little information available on molecular mechanisms regulating the processing and maturation of GPCRs, how these proteins are transported through the secretory pathway, and on factors affecting the folding in the ER.

The overall aim of this work was to assess LHR expression and maturation in natural tissues and heterologous expression systems and to elucidate the molecular significance of the LHR ectodomain splice variant. The specific aims were:

1. To identify the rat LHR tissue distribution at gene activation, mRNA and protein levels during development and adulthood
2. To determine the role of developmental stage and tissue type on the LHR maturation
3. To study the LHR structural counterpart's, the ectodomain splice variant's cellular maturation, destination and biological role in association with the full-length LHR.

## 4 Materials and methods

The materials and methods are summarized below in tables and chapters and described in detail in the original publications (I-IV).

### 4.1 Summary of methods

*Table 3. Summary of methods used in original publications I-IV*

Method	Original publication
Biochemical techniques	
Autoradiography	I, IV
Centrifugation techniques	I, II, III, IV
Deglycosylation	I, II, IV
Immunoprecipitation	I, II, IV
Lectin affinity chromatography	IV
Ligand affinity chromatography	I, II, IV
Ligand binding assays	I
Metabolic labelling	IV
Protein degradation and synthesis rate measurements	IV
Protein denaturation/precipitation	IV
Radioiodination	I
Scintillation counting	IV
Sodium dodecyl-sulfate polyacryl-amide gel electrophoresis (SDS-PAGE)	I, II, IV
Subcellular fractionation	IV
Unfolded protein response test	IV
Western blot analysis	I, II, IV
Molecular biological techniques	
DNA construct cloning	I, II, III, IV
Genomic library cloning	I
Polymerase chain reaction (PCR)	I, II, III

*Table 3. continued.*

Method	Original publication
Quantitative PCR	IV
RNA purification	I, II, III, IV
Reverse transcriptase (RT)-PCR	I, II, III, IV
Cell Biological techniques	
Cell culture	III, IV
Establishing cell lines (stable and transient)	IV
Histological methods (sectioning, staining)	I, II, III, IV
Immunohistochemistry	I, III, IV
Indirect immunofluorescence	IV
Transgenic animals (microinjection, maintaining, analysis)	I, II, III
Microscopy and cytometry techniques	
Confocal laser scanning microscopy	IV
Electron microscopy	IV
Fluorescence activated cell sorting (FACS)	IV
Light microscopy	I, II, III
Miscellaneous	
Hormone measurements	III
Densitometric scanning	IV
Statistical analysis	I, III, IV
Ligand binding data analysis	I

## 4.2 DNA constructs used in cell lines

The DNA cassettes containing the Kozak sequence (gccacc) followed by a codon for the starting methionin, the hemagglutinin (HA) signal sequence (MKTIIALS<sup>Y</sup>IFCLVFA), the N-terminal epitope sequence Myc (EQKLISEEDL) or HA (YPYDVPDYA), the polylinker site and the C-terminal epitope sequence FLAG (DYKDDDDK) followed by a stop-codon were constructed to create expression vectors for epitope-tagged protein production. The cassettes were subcloned to plasmids pcDNA5/FRT/TO and pcDNA3. The appropriate cDNAs were subcloned to a polylinker site between the tag sequences without the proteins' own signal sequence. The rat LHR with an endogenous signal sequence was used to create the construct with a C-terminal green fluorescent protein (GFP) tag. To integrate the cDNA of interest into a specific site in the cells' genome by Flp recombinase-mediated integration, pOG44 (encoding Flp-In recombinase) was used (Table 4).

Table 4. DNA constructs and vectors used to generate stably or transiently transfected cells

cDNAs, DNA cassettes and tags	Vector	Source of the vector	Construct name	Original publication
Flp-In recombinase enzyme	pOG44	Invitrogen	pOG44	IV
Kozak-HA signal sequence- <i>Myc</i> -polylinker- <i>Flag</i> -stop codon	pcDNA5/FRT/TO	Invitrogen	<i>Myc-Flag</i>	IV
Kozak-HA signal sequence- <i>HA</i> -polylinker- <i>Flag</i> -stop codon	pcDNA5/FRT/TO	Invitrogen	<i>HA-Flag</i>	IV
Kozak-HA signal sequence- <i>HA</i> -polylinker- <i>Flag</i> -stop codon	pcDNA3	Invitrogen	<i>pcDNA3-HA-Flag</i>	IV
Rat LHR without stop codon	<i>Myc-Flag</i>	Homemade	<i>Myc-rLHR-Flag</i>	IV
Rat LHR with stop codon	<i>pcDNA3-HA-Flag</i>	Homemade	<i>HA-rLHR</i>	IV
Rat LHR	pEGFP-E3	Invitrogen	<i>rLHR-GFP</i>	IV
Rat LHR ectodomain splice variant	<i>Myc-Flag</i>	Homemade	<i>Myc-rLHRvariant</i>	IV
Rat LHR ectodomain splice variant	<i>HA-Flag</i>	Homemade	<i>HA-rLHRvariant</i>	IV
Human $\delta$ OR	<i>HA-Flag</i>	Homemade	<i>HA-h<math>\delta</math>OR-Flag</i>	IV
Human $\mu$ OR	<i>HA-Flag</i>	Homemade	<i>HA-h<math>\mu</math>OR-Flag</i>	IV

### 4.3 Tissues and transgenic animals

Two different size 5' flanking regions (-2060 or -3970 bp from translation initiation site) of the rat LHR gene in front of the reporter gene in the *LacZ*-vector pNASS $\beta$  were used to produce transgenic mice for the rat LHR expression studies (I, II) (Table 5). The constructs were microinjected into pronuclei of (C57BL/6 X DBA)F2 mouse zygotes, after which they were transferred into the oviduct of pseudopregnant NMRI mice.

The rat LHR ectodomain splice variant cDNA was cloned into the expression vector pNeoNUT containing the mouse metallothionein I promoter (mMT-1) (table 5). The construct was microinjected into pronuclei of inbred FVB/N mouse zygotes and procreated transgenic mice were used to identify the effect of the rat LHR ectodomain splice variant production on mouse phenotype (III). Founder animals were identified from tail or ear DNA by PCR analysis using either *LacZ*-specific primers (I, II) or rat LHR-specific primers (III).

Table 5. DNA constructs and vectors used in transgenic animals

DNA inserts	Vector	Source of the vector	Construct name	Original publication
Rat LHR promoter ~2 and 4 kb inserts	pNASS $\beta$	Clontech	2 or 4 LHRpromoter- NASS	I, II
Rat LHR ectodomain splice variant	pNeoNut with mMT-1 promoter	I Huhtaniemi	mMT-LHRvariant	III

Transgenic rat LHR promoter and wild-type mouse fetuses or adult mouse tissues were used to detect specific  $\beta$ -galactosidase activity indicating the LHR expression sites (I, II). Transgenic rat LHR ectodomain splice variant and wild-type adult mice were used in morphological studies (III). Rat tissues and fetuses were used for RT-PCR, receptor purification, and ligand binding assays (I, II) (Table 6).

*Table 6. Tissues and fetuses used in original publications (I-III)*

Tissues	Origin	Original publication
Fetuses 11.5, 12.5, 13.5, 14.5, 15.5, 16.5 17.5 and 19.5 dpc, and one-day-old neonates	Transgenic rat LHR promoter and C57BL/6 mice	I, II
Adult brain, trigeminal ganglia, kidneys, adrenal glands, gonads, uterus, oviduct	Transgenic rat LHR promoter and C57BL/6 mice	I, II
Adult kidneys, adrenal glands, gonads, pituitary, urethra, urinary bladder, seminal vesicles, prostate gland, liver, uterus, oviduct	Transgenic rat LHR ectodomain splice variant and FVB/N mice	III
Fetuses 15.5, 16.5, 17.5, 18.5 and 19.5 dpc, and one-day-old neonates	Sprague-Dawley rat	I, II
Adult brain, trigeminal ganglia, kidneys, adrenal glands, gonads, liver and muscle	Sprague-Dawley rat	I, II
Pregnant female 15.5- and 19.5-dpc kidney, adrenal glands and gonads	Sprague-Dawley rat	II
Pseudopregnant ovary	Sprague-Dawley rat	I, II

The use of animals was approved by the University of Oulu committee for the care of experimental animals.

#### 4.4 Cell culture

Flp-In HEK 293 or Chinese hamster ovary (CHO) cells were used to generate stable, constitutive mammalian expression cell lines (HEK293<sub>c</sub> or CHO<sub>c</sub>) (Table 7). Flp-In HEK 293 inducible expression cell line (HEK293<sub>i</sub>) was generated with T-Rex Core Kit according to the instructions of the manufacturer (Invitrogen). Cells were grown routinely in Dulbecco's modified Earl's (HEK-293) or Ham's F-12 (CHO) medium containing 10% (v/v) fetal calf serum, 100 units of penicillin/ml, and 100  $\mu$ g streptomycin/ml with appropriate selection antibiotics in a humidified atmosphere of 5% CO<sub>2</sub> at 37 C.

For generating stable expression cell lines to integrate the cDNA of interest into a specific site in the genome by Flp recombinase-mediated integration, Flp-In HEK293<sub>c</sub> or i or CHO<sub>c</sub> cells were transfected with pOG44 (which encodes the Flp-In recombinase) and appropriate DNA construct. LipofectAMINE 2000 (Invitrogen) was used as a transfection reagent. Hygromycin was used as a selection antibiotic for HEK293<sub>c</sub> (100  $\mu$ g/ml) and CHO<sub>c</sub> (500  $\mu$ g/ml) derived cell lines and blasticidin S (4  $\mu$ g/ml) was used in addition for HEK293<sub>i</sub> derived cell lines (Invitrogen). Selected cell colonies were tested for the correct

integration site for their zeocin sensitivity (100  $\mu\text{g/ml}$ ) and the lack of  $\beta\text{-Gal}$  activity. To generate stable, double expression cell lines the *HA-rLHR* or *rLHR-GFP* constructs were transfected to a random integration site in the inducible HEK293<sub>i</sub>-*Myc-rLHRvariant* cells. The cells were selected for G-418 (400  $\mu\text{g/ml}$ ) resistance. For transient transfections the cells were cultured on glass coverslips or in 6-well plates and transfected with the appropriate DNA construct with LipofectAMINE 2000 or Fugene-6 reagent (Roche). The recombinant protein production in inducible cell lines was activated with a medium containing tetracycline.

*Table 7. Stable cell lines used in original publication (IV).*

Cell lines	Source/reference	Original publication
Flp-In HEK 293 (HEK293 <sub>c</sub> )	Invitrogen	IV
Flp-In CHO (CHO <sub>c</sub> )	Invitrogen	IV
CHO <sub>c</sub> - <i>Myc-LHRvariant</i>	Homemade	IV
CHO <sub>c</sub> - <i>Myc-LHR-Flag</i>	Homemade	IV
HEK293 <sub>i</sub>	Homemade	IV
HEK293 <sub>i</sub> - <i>Myc-LHR-Flag</i>	Homemade	IV
HEK293 <sub>i</sub> - <i>Myc-LHRvariant</i>	Homemade	IV
HEK293 <sub>i</sub> - <i>Myc-LHRvariant+HA-LHR</i>	Homemade	IV
HEK293 <sub>i</sub> - <i>Myc-LHRvariant+LHR-GFP</i>	Homemade	IV

## 4.5 Drug and hormone treatments

Hormones were used in ligand binding assays, hCG in the transgenic LHR promoter mice, and hCG and drugs in cell culture experiments.

*Table 8. Drug and hormone treatments used in original publications (I-IV).*

Drug or hormone	Source	Publication
Brefeldine A (5 $\mu\text{g/ml}$ )	Alexis	IV
hCG (50-100 U/animal)	Sigma	I, II
hCG (9 fM-70 nM in binding assays)	Sigma	I
hFSH (36 nM in binding assays)	Sigma	I
[ <sup>125</sup> I]hCG (1.8 nM in binding assays)	Sigma	I
hLH (9 fM-75 nM in binding assays)	Sigma	I
Lactacystin (10 $\mu\text{M}$ )	Alexis	IV
Tetracycline (0.5 $\mu\text{g/ml}$ )	Invitrogen	IV
Tunicamycin (5 $\mu\text{g/ml}$ )	Sigma	IV
ZnSO <sub>4</sub> x 7H <sub>2</sub> O (15 mg/kg)	Merck	III

## 4.6 Antibodies

Antibodies were used in immunoblottings or in immunohistochemistry, immunofluorescence and immunoelectron microscopy.

*Table 9. Antibodies and peptides used in original publications (I-IV).*

Antibody	Source	Original publication
Primary antibody		
$\beta$ -actin mAb	Sigma	IV
BiP mouse mAb	Sigma	IV
Calnexin N-terminal and C-terminal pAb	Stressgen.	IV
Calreticulin pAb	Stressgen	IV
LHR C-terminal and N-terminal pAb	Homemade	I, II, III, IV
cMyc mAb	Stressgen or Dept. of Biochemistry, University of Montréal, Canada	IV
cMyc pAb	SantaCruz	IV
GM130 mAb	Becton Dickinson Transduction	IV
HA mAb	Sigma	IV
Hsp70/Hsc70 mAb	Stressgen	IV
Mouse IgG	Sigma	I, III
NeuN mAb	Chemicon	I
P450scc pAb	Chemicon	I,III
PDI pAb	Stressgen	IV
S100 pAb	DakoCytomation	I
Secondary antibody		
AlexaFluor488, goat anti-mouse IgG	Molecular Probes	IV
AlexaFluor488, goat anti-rabbit IgG	Molecular Probes	IV
AlexaFluor568, goat anti-mouse IgG	Molecular Probes	IV
AlexaFluor568, goat anti-rabbit IgG	Molecular Probes	IV
Biotin-streptavidin-conjugated goat anti-rabbit IgG	DakoCytomation	I, III
Biotin-streptavidin-conjugated goat anti-mouse IgG	DakoCytomation	I, III
Horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG	Jackson ImmunoResearch	I, II, IV
Phycoerythrin (PE)-conjugated goat anti-mouse IgG	Becton Dickinson	IV
Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG	Becton Dickinson	IV
FITC-conjugated goat anti-rabbit IgG	Becton Dickinson	IV
Peptides		
Myc	Sigma	IV
FLAG	Dept. of Biochemistry, University of Oulu, Finland	IV

## **4.7 Light microscopy**

Tissue sections stained for hematoxylin and eosin (III), safranin (I, II),  $\beta$ -Gal activity (I, II), and specific antibodies (I, III) were examined with a Nikon Eclipse E600 microscope. The microdissections (II) and  $\beta$ -Gal activity studies (I, II) were done as whole-mounts under a stereomicroscope.

## **4.8 Conventional confocal microscopy**

Direct or indirect immunofluorescence stained cells (IV) were examined with a Zeiss LSM510 or Olympus Fluoview FV1000 confocal laser scanning microscope. Images were processed with Adobe Photoshop<sup>®</sup>.

## **4.9 Transmission and immunoelectron microscopy**

Thin sections of embedded cells were cut with a Reichert Ultracut microtome. Thin cryosections were cut with a Leica Ultracut UCT microtome and immunolabeled with the anti-cMyc monoclonal antibody and protein A-gold complex. Sections were examined using a Philips CM100 transmission electron microscope.

## **4.10 Fluorescence-activated cell sorting**

Direct or indirect immunofluorescence stained cells (IV) were collected using a Becton Dickinson FACSCalibur and quantified using CellQuest Pro<sup>™</sup> software. The data were analyzed with the GraphPad Prism version 4 by using geomean values.

## **4.11 Statistical analysis**

GraphPad Prism software 4 was used for the ligand binding data analysis (I), for the one-way ANOVA to compare the hormone concentrations in serum and intratesticular testosterone samples and for the two-way ANOVA to determine the effect of age on the same samples (III). Statistical *t* tests were used in cell culture experiments (IV). Significance was set at  $p < 0.05$ . The values are presented as mean  $\pm$  SEM.

#### **4.12 Effect of hCG on the LHR promoter-driven transgene**

Female mice carrying the LHR promoter transgene were housed in for 11.5, 12.5, 13.5, 14.5 and 15.5 dpc in their pregnancy and injected intraperitoneally with a single dose of either vehicle (0.1 % bovine serum albumin in phosphate-buffered saline) or hormone (hCG) 24 hours or 48 hours before collecting the fetuses. Each timepoint contained a group of five to six transgenic female mice, half of which received the the hormone, the other half the vehicle, serving as controls. The hCG dosage used was either 50 U or 100 U. The same vehicle and hormone treatment was performed with eight transgenic non-pregnant females. The fetuses and adult female tissue samples were stained for  $\beta$ -Gal activity as described earlier (I) and observed as whole mounts under a stereoscope.

## 5 Results

### 5.1 Identification of the rat LHR tissue distribution (I, II)

The overall spatial and temporal tissue expression pattern of the LHR mRNA was evaluated by using RT-PCR analysis of total RNA from adult and fetal rat tissues (I, Figure 1 and II, Figures 4, 5 and 6). The primers used in RT-PCR (I, Figure 1) flanking the entire coding region of the LHR to amplify cDNAs with full-length open reading frames. On the second amplification round, the final products were slightly smaller than the original cDNA due to the used nested primers. The regional and cell-type specific expression was determined by using transgenic mice expressing the *LacZ* gene under control of the rat LHR promoter fragments of 2060 bp and 3970 bp in length (I, Figure 4). The 2060-bp and the 3970-bp LHR promoter fragments were able to directly express the *LacZ* gene in three and eight transgenic mouse lines, respectively. A representative mouse line expressing the *LacZ* gene under the 3970-bp LHR promoter was used in the analyses.

#### 5.1.1 Expression of the LHR in the sensory nervous tissue (I)

The LHR was found to be expressed at the mRNA level in the developing and adult sensory nervous system. The 1.9-kb and 1.7-kb species that corresponded to the full-length LHR and the most common receptor splice variant (Tsai-Morris *et al.* 1990, Aatsinki *et al.* 1992, Bacich *et al.* 1994) were obtained from the adult testis, ovary, placenta, and nervous tissue (e.g. from trigeminal ganglion, thalamus, olfactory bulb and pituitary), and from the heads of the 14.5-dpc and the 19.5-dpc fetuses. Muscle, liver, and spinal cord showed no amplification of LHR mRNA. The amount of the LHR mRNA transcripts in the nervous system was very low, because the transcripts were detected only after the second amplification round. Interestingly, several smaller mRNA transcripts were also observed, possibly representing developmental stage or tissue specific variants due to alternative splicing of the primary transcript (Tsai-Morris *et al.* 1990, Wang *et al.* 1991, Aatsinki *et al.* 1992, Hipkin *et al.* 1992, Sokka *et al.* 1992a, Zhang *et al.* 1994).

This was especially evident in the pituitary, which exhibited unique-sized mRNAs compared to the other brain areas.

Whole-mount staining of the developing mouse fetuses (I, Figure 4) revealed that the transgene expression is clearly dependent on the developmental stage of the fetus. No differences among female and male fetuses were observed. The most distinct expression of  $\beta$ -Gal appeared in the ganglia of the sensory and autonomic systems and the olfactory bulb. The transgene was first expressed in the sensory ganglia (11.5-dpc fetuses), after which the expression increased in cranial and spinal ganglia, peripherally and centrally outsprouting nerve fibers, olfactory bulb and the thalamic and brain stem areas (14.5-dpc fetuses). In the 16.5-dpc fetuses, the expression was decreased in the sensory ganglia, but was still clearly detectable in the peripherally and centrally outgrowing nerves. This was particularly clear in the one-day-old neonates.

Isolated whole brains of fetal and adult mice (I, Figure 5) indicated that transgene expression in the brain continues at varying levels until adulthood and is then restricted to specific brain areas involved in olfaction and some other sensory functions. The  $\beta$ -Gal expression was apparent in the thalamic and brain stem structures of the 14.5-dpc fetuses, but seemed to have decreased by 16.5 dpc, except for in the olfactory bulb. In the adult, the most distinct staining was found in cortical cell layers of the cerebrum and cerebellum, thalamic region and brain stem areas. Examination of sagittal slices of the adult brain (I, Figure 6) further revealed that the  $\beta$ -Gal expression in the cortical cell layers of the cerebrum was particularly localized to the olfactory (piriform and entorhinal cortex), auditory, visual, and somatosensory cortices. The expression appeared to follow the direct connections between the olfactory bulb and the entorhinal cortex. In addition, the expression was apparent in the posterior hippocampus. The spinal cord was negative. The trigeminal nerves, however, showed strong expression.

### ***5.1.2 Activity of the LHR promoter-driven transgene co-localizes with the P450ssc enzyme immunoreactivity in neurons (I)***

The  $\beta$ -Gal positive staining was solely confined to nerve cells when the LHR promoter-driven  $\beta$ -Gal expression was analyzed microscopically from the thin brains sections (I, Figure 7). The  $\beta$ -Gal activity was restricted to the trigeminal and spinal ganglia and peripherally and centrally outgrowing nerves. Glial-specific S-100 immunoreactivity was not detected in cells that showed  $\beta$ -Gal activity, whereas neuron-specific NeuN immunoreactivity and  $\beta$ -Gal activity co-localized in the same cells.

An important result was that the  $\beta$ -Gal activity was found to co-localize in the same neurons with the p450ssc enzyme that is involved in steroid biosynthesis (I, Figure 8). This coordinate expression in the nervous system suggests that the LHR may be involved in the regulation of neurosteroid synthesis. LH and hCG regulate steroidogenesis in the ovaries and testes by regulating the expression of steroidogenic enzymes (Saez 1994). The P450ssc is an enzyme that catalyzes the initial reaction in the steroidogenic pathway and has been shown to localize in steroidogenic peripheral cells (Voutilainen *et al.* 1986, Voutilainen & Miller 1988) and in steroidogenic neurons and glial cells of the central nervous tissue (Le Goascogne *et al.* 1987, Compagnone *et al.* 1995).

### ***5.1.3 Expression of the LHR in urogenital and adrenal tissues (II)***

As with the nervous system, the developing urogenital structures were found to express the full-length LHR mRNA early on during differentiation. With the exception of the adrenal glands, the amount of transcripts appeared to be higher in males, although there seemed to be no differences in the onset of mRNA expression in males and females. As expected, two amplified products of 2.1 kb and 1.8 kb were detected after the first amplification round in the adult testes and ovaries (II, Figure 4), corresponding to the full-length LHR and the most common receptor variant. The full-length transcripts were present in the gonads of the 17.5-dpc fetuses, as well as in the one-day old neonates. The two major mRNA products were also found in the fetal and neonatal adrenal glands and kidneys (II, Figure 5) as well as in the genital tubercles (data not shown). In the developing testes, the transcripts were apparent already after the first amplification round, but in fetal and neonatal ovaries, the LHR mRNA transcripts were detected only after the second amplification round. When RNA from adult female adrenal glands was used as a template, the transcripts were detectable already after the first amplification round, whereas the male samples revealed transcripts only after the second round. The adult kidneys yielded no amplification products (II, Figure 5).

During organogenesis the LHR promoter-driven transgene was first activated in the developing genital tubercles and in the paired genital swellings representing the developing external genitalia at around 11.5 dpc and from the 14.5 dpc onward, also in the urogenital fold. In the one-day-old neonates, the  $\beta$ -Gal activity was clearly apparent in the external genitalia in both females and males and no distinct sex-dependent differences were observed (II, Figure 3).

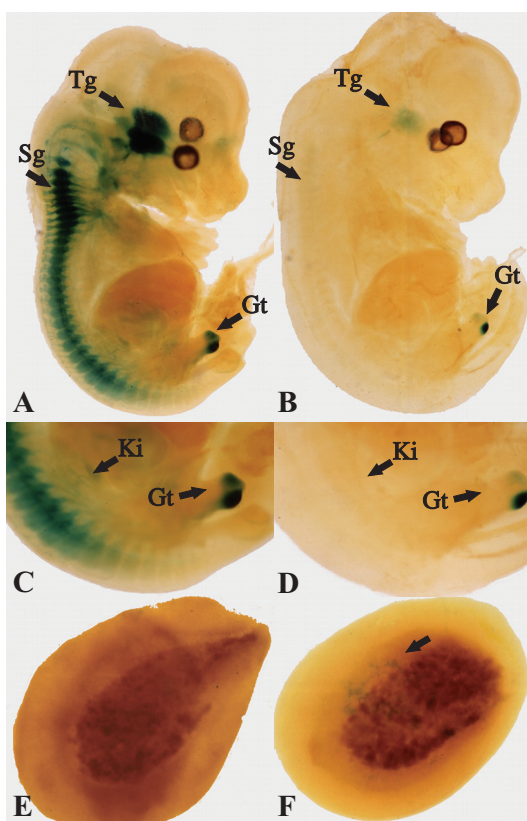
The  $\beta$ -Gal activity was first detectable at around 14.5 dpc in the developing gonads and surprisingly, in the adrenal glands and kidneys. Similar staining was also detected in the 16.5-dpc female and male fetuses. In the one-day-old neonates, distinct staining was found in the medulla and cortex of the transgenic but not in the non-transgenic adrenal glands (II, Figures 1 and 2). In the developing ovaries, the activity remained at a low level until in the nearly fertile 30-day old mice, in which distinct staining in the developing follicles was detected. In the adult females, transgene activity was clearly apparent in the maturing ovarian follicles and corpora lutea and was located in granulosa, thecal and luteal cells and in a varying degree to the ova and interstitial glandular cells. In the developing testes, specific  $\beta$ -Gal expression was detected in the interstitial tissue but not in the seminiferous tubules, and was clearly restricted to the Leydig cells in the adult testes. The endogenous  $\beta$ -Gal activity in the epididymis and ductus deferens was so strong that specific activity could not be determined.

### ***5.1.4 Expression of the LHR is hormonally regulated (II)***

In contrast to male and non-pregnant female adrenal glands and kidneys, the mRNA expression was substantially higher in the same tissues of pregnant female rats of 15.5 dpc and 19.5 dpc. As expected, the ovaries of the pregnant females displayed higher expression compared to non-pregnant ones (II, Figure 6). Importantly, the transcripts

were clearly detectable at 15.5 dpc after the first amplification round, which was in clear contrast to the findings obtained for the non-pregnant females (II, Figure 4). However, unlike in the ovaries, the expression decreased at 19.5 dpc, (II, Figure 6) and was detected in the kidneys only after the second amplification round (data not shown).

The 3970-bp fragment of the LHR promoter has been shown to contain elements that mediate regulation of LHR levels in gonadal tissues (Hammond *et al.* 1994, Nelson *et al.* 1994, Tsai-Morris *et al.* 1994). Thus, the functional activity of the promoter was tested by injecting the pregnant female mice (12.5-15.5 dpc) with 50 or 100 U of hCG. We reasoned that if the reporter activity would change, this should indicate crosstalk between maternal and fetal tissues, taken that hCG is capable of crossing the mother-placenta barrier. Alternatively, and perhaps also more likely, taken the molecular size of hCG (~30kDa), change of the promoter activity could happen through secondary pathways activated in females by hCG. When the transgenic fetuses were analysed 24 h later, the transgene activity was clearly decreased in the fetuses (Figure 7B and D), whereas vehicle administration had no effect (Figure 7A and C). The gonadotropin hCG has been shown to increase the LHR content in the female mice adrenal gland (Kero *et al.* 2000, Lacroix *et al.* 2001). Therefore, we activated the LHR-promoter-driven transgene in a similar manner by injecting 100 U of hCG to fertile female mice. The whole mount staining of the adrenal gland after 24 h of the injection showed patch-like  $\beta$ -Gal activity in the zona reticularis and faint striated staining in some parts of the zona glomerulosa (Figure 7F compare to vehicle 7E). These results suggest that the LHR promoter was fully functional and, furthermore, confirm that the hCG is able to affect the promoter activity through mother. In addition, the activation of the transgene with a high dose of hCG in the female adrenal gland suggests that the situation may mimic the elevated LHR expression observed in the day 15.5-pc pregnant female rat adrenal gland.



**Fig. 7. HCG-mediated down- and up-regulation of LHR promoter-driven transgene expression.** Human CG (100 U) (B, D, F) or vehicle (A, C, E) were injected into pregnant female mice at 12.5 dpc (A-D) or into transgenic nonpregnant female mice (E, F). The mice were sacrificed 24 h later and the transgenic fetuses (A-D) and adrenal glands (E, F) were dissected and whole-mount  $\beta$ -galactosidase staining was performed. The arrow in F indicates the innermost cortical layer of the adrenal gland. Ki, kidney; Gt, genital tubercle; Sg, spinal ganglion; Tg, trigeminal ganglion.

## 5.2 LHR protein in tissues (I, II)

Triton X-100-solubilized membranes from rat tissue samples were subjected to immunoprecipitation using a LHR-specific polyclonal antibody directed against the C-terminal domain of the protein and to hCG affinity chromatography. The purified samples were analyzed by Western blotting using the LHR-specific polyclonal antibody directed against the C-terminal or N-terminal domains of the protein.

### ***5.2.1 Mature LHR is expressed in the nervous tissue (I)***

The  $M_r$  90,000 and  $M_r$  73,000 LHR protein forms were purified from the adult rat thalamus and the developing nervous systems of the 14.5- and 19.5-dpc fetal heads (I, Figure 2). The  $M_r$  90,000 and  $M_r$  73,000 forms were also purified from the pseudopregnant rat ovary indicating that the receptor protein is processed in a similar manner in the ovaries and neuronal tissues. In contrast, the two forms were not purified from muscle, which is in accordance with the absence of LHR mRNA in this tissue (I, Figure 1).

The  $M_r$  90,000 and  $M_r$  73,000 receptor forms contained fully or partially processed oligosaccharides, respectively, representing immature and mature forms of the receptor. Glycosidase treatments revealed that two forms differ in their post-translational carbohydrate modifications (I, Figure 2). Endo H reduced the molecular mass of the  $M_r$  73,000 LHR form to about  $M_r$  62,000, demonstrating that it represents a full-length receptor bearing high mannose-type oligosaccharides (Maley *et al.* 1989). The resistance of the  $M_r$  90,000 receptor species to Endo H treatment suggests that this species contains complex-type N-linked oligosaccharides. In contrast, PNGase F, an enzyme that can remove all types of N-linked oligosaccharides from glycoproteins (Maley *et al.* 1989), was able to digest the  $M_r$  90,000 receptor.

The neuronal LHR has an ability to bind ligand, since the two receptor species were also purified by hCG affinity chromatography (I, Figure 2). The ligand binding capacity of the neuronal LHR was tested directly in competition binding experiments by incubating crude membrane particles from the 19.5-dpc rat fetus heads with [ $^{125}$ I]hCG in the presence of an increasing amount of unlabeled human CG, LH or FSH (I, Figure 3). hCG and hLH inhibited the binding of [ $^{125}$ I]hCG in a dose-dependent manner with half-maximal inhibition of binding obtained at concentrations of  $0.10 \pm 0.05$  (mean  $\pm$  SEM) nM and  $2.63 \pm 0.46$  nM, respectively. HFSH, on the other hand, was not able to displace [ $^{125}$ I]hCG binding, confirming the hormone-binding specificity of the neuronal LHR. The inhibitory dissociation constant ( $K_i$ ) obtained for hCG ( $0.08 \pm 0.04$  nM) was in good agreement with that reported previously for rat gonadal LHRs (Segaloff & Ascoli 1993, Ascoli *et al.* 2002). The maximal binding capacity ( $B_{max}$ ) for hCG, determined from the homologous competition experiments, was  $31.5 \pm 1.1$  fmol/mg of membrane protein in the fetal head membranes as compared to  $3.68 \pm 0.22$  pmol/mg in the pseudopregnant rat ovaries.

### ***5.2.2 Mature LHR is developmentally and hormonally regulated at the post-translational level in the rat urogenital and adrenal tissues (II)***

The ovary and testis of the adult rat carried a mature LHR species with fully processed N-linked oligosaccharides as did to some extent the adult female adrenal glands. Importantly, only immature receptor forms were expressed in fetal and neonatal rat urogenital structures. This apparent tissue- and age-dependent difference in the ability of

cells to process the LHR protein to the mature form may indicate a developmental and physiological regulation mechanism.

Two receptor species with  $M_r$  90,000 and 73,000 were detected from the adult rat ovary. Two receptor species were also immunoprecipitated from the adult testes but the larger species migrated more slowly ( $M_r$  100,000), most likely due to tissue-specific differences in the glycan moieties. Importantly, both the mature and immature receptor species were purified by ligand (hCG) affinity chromatography showing that they were able to bind hormone (II, Figure 4).

Surprisingly, none of the developing or adult non-gonadal urogenital tissues were found to express the mature LHR, except for the adult female adrenal glands, in which the mature receptor was just barely detectable (II, Figure 5). Only the  $M_r$  73,000 receptor species was immunoprecipitated from the fetal and one-day-old female gonads (II, Figure 4), adrenal glands (II, Figure, 5) and kidneys (data not shown). Two receptor species of  $M_r$  73,000 and  $M_r$  70,000 were purified from the corresponding one-day-old male tissues (II, Figures 4 and 5). These two receptor species were also purified from the female and male fetal and neonatal genital tubercles (data not shown).

The immature LHRs were the predominant receptor species in the adult female rat adrenal glands. However, there was a clear shift to an increased level of mature receptors during pregnancy both in the adrenal glands and kidneys. This finding suggests that LHR expression is hormonally regulated in these non-gonadal tissues, observation that was further supported by the fact that the hCG was able to affect on the LHR promoter activity in the adrenal glands and fetuses (Figure 7). The high amount of mRNA transcripts correlated to expression of mature receptors in these tissues. Two receptor species of  $M_r$  90,000 and  $M_r$  73,000 were found from the ovaries, adrenal glands and kidneys of the 15.5-dpc pregnant females. Both of these receptor species were purified by ligand affinity chromatography, indicating that they are capable of hormone binding. Near delivery, at 19.5 dpc, the expression decreased approximately to the same level as in the non-pregnant females, and the adrenal glands and kidneys again appeared to contain mostly the immature LHR form (data not shown). Only the adrenal gland displayed a small amount of receptor corresponding to the mature form (II, Figure 6).

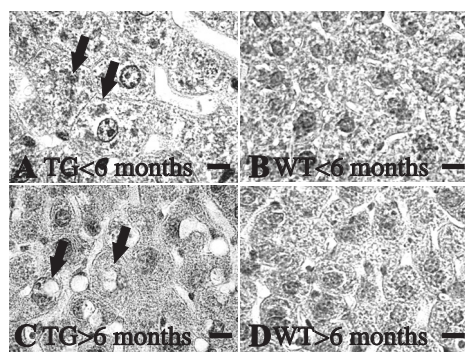
### **5.3 Overexpression of the LHR ectodomain splice variant in transgenic mice (III)**

The role of the LHR ectodomain splice variant was studied by using transgenic mice overexpressing this variant under the control of the mMT-1 promoter (III, Figure 1). The offspring of four transgenic mouse lines (average litter size 6-8) expressed the transgene in a similar manner, were viable, and developed into adulthood.

The RT-PCR analysis of total RNA from adult mouse tissues was used to study the expression of the rat LHR ectodomain variant in transgenic and non-transgenic mice. The LHR specific primers used allowed the identification of endogenous expression of the LHR ectodomain variant in the gonads. The transgene mRNA was expressed at a high level in both sexes, especially in the gonads, but also in the kidney and liver. Also the

adrenal and pituitary glands of both sexes showed increased transgene mRNA expression (III, Figure 1).

The full-length LHR and none of its splice variants are found to be expressed endogenously in the liver. Thereby the LHR ectodomain splice variant expression in the transgenic liver must be considered as ectopic caused by the used mMT-I promoter. The liver cells showed interesting morphology (Figure 8). The young transgenic mice (2-6 months) liver cells contained dense inclusion bodies (Figure 8A compare to wild-type 8B), whereas the aged transgenic mice (>6 months) had vacuolar formations close to the nucleus (Figure 8C compare to wild-type 8D).



**Fig. 8. Liver morphology of transgenic LHR ectodomain splice variant and wild-type mice. A, C transgenic and B, D wild-type mouse liver. The paraffin-embedded liver was sectioned (5  $\mu\text{m}$ ) and stained with hematoxylin and eosin. The arrows in A show dense inclusion bodies and in C vacuolar formations close to the nucleus. TG, transgenic; WT, wild type. Scale bars 10  $\mu\text{m}$ .**

We were unable to identify specific LHR ectodomain splice variant expression in tissues or serum at a protein level due to the lack of proper antibody for immunoprecipitation. In addition, the hCG affinity purification did not reveal any specific ectodomain protein, giving the impression that the variant is not capable of ligand binding (data not shown). Heterologous expression systems with the epitope-tagged variant were used in subsequent studies to characterize the variant protein and possible molecular mechanisms that may have created the observed phenotype in the transgenic mice (IV and 5.4).

### ***5.3.1 LHR ectodomain splice variant interferes with the pituitary-gonadal functions***

The gonadal morphology was altered in the transgenic mice overexpressing the LHR ectodomain splice variant. The effects were consistently more prominent in the males than in the females. The testes showed a progressive degeneration of spermatogonia already in the young transgenic males (2-6 months) that was dependent on the developmental stage of the seminiferous epithelium. This progressed later into a state

where the structure of seminiferous epithelium was disrupted. Interestingly, sufficient amounts of mature sperm were apparently produced in the transgenic mice as they were fertile, but after the age of five months, the litter number declined severely (III, Figure 2).

In contrast to males, the overall changes found in the gonads of transgenic female mice were minor. Nevertheless, corpora lutea and degenerating small follicles were more numerous in the ovaries of young transgenic mice. In the aged transgenic animals, large foamy cells appeared in the ovarian stroma, possibly originating from stromal glandular cells, and the corpora lutea were less abundant than in the younger animals. Thecal cells showed some vacuolarization, as did the uterine and oviductal epithelial cells (data not shown) but no other major changes were observed in these tissues (III, Figure 3).

The testicular and ovarian phenotypes of the transgenic mice point to impairment in the LH production and/or gonadal steroidogenesis. The measurement of the serum LH, FSH, testosterone and testicular testosterone levels indicated that the most distinct changes were in the serum LH levels both in transgenic males and females as compared to controls. The LH levels were significantly decreased in both young and aged transgenic males, but only in the aged females (III, Figure 4). The transgenic male mice had also significantly decreased testicular testosterone levels in both age groups (III, Figure 4). Serum FSH levels were mostly unaltered, and only slightly increased in the young transgenic females (data not shown).

The decreased testicular testosterone level in the transgenic males indicates impairment in testicular androgen synthesis, and the decreased p450<sub>scc</sub> enzyme immunoreactivity in the Leydig cells confirmed this (III, Figure 5). The non-transgenic testes showed clear immunoreactivity in the interstitial Leydig cells (III, Figure 5).

### ***5.3.2 LHR ectodomain splice variant causes morphological changes in the adrenal glands, kidney and urinary bladder***

Previous studies have shown that the LHR is expressed in the rodent and human adrenal glands (Pabon *et al.* 1996, Rilianawati *et al.* 1998, Kero *et al.* 2000, Hämäläinen *et al.* 2002) and that the receptor expression in the adrenal glands may be under LH regulation (Warren *et al.* 1984, Kero *et al.* 2000). In the present work (II), we confirmed the expression in the rat adrenal glands and in addition demonstrated expression in the kidneys. Both of these tissues showed pronounced alterations in transgenic mice overexpressing the ectodomain variant, especially in males. The adrenal cortex of the transgenic males displayed a hypertrophic fasciculate layer, which contained large adenomatous foci of cells with varying size. The foci were surrounded by dense and shrunken cells, possibly undergoing degeneration. In the female transgenic mice, the most distinct changes were observed in the X-zone. This zone develops after birth and is unique to the mouse. In males it regresses at puberty (Howard-Miller 1928), whereas in females it continues to thicken until the first pregnancy and regresses thereafter (Holmes & Dickson 1971). In the young transgenic females, the regression of the X-zone with extensive cellular vacuolization occurred much earlier than in the non-transgenic females. The cells in this zone formed nests of foamy cells in the older animals, which resembled

morphologically foamy cells found in the ovarian stroma. Unlike in the transgenic males, the other cortical layers were unaltered (III, Figure 6).

The transgenic males displayed also a unique phenotype in the urinary organs (III, Figure 7 and 8). In the younger animals the majority of the glomeruli and tubules were unaltered. The changes in the kidneys were progressive, and the aged transgenic mice displayed large fluidic cysts, glomeruli with dilated Bowman's space, and degenerative lesions with infiltrated inflammatory cells. Interestingly, these males also had a dilated urinary bladder and problems with penile function, which may have been a secondary cause for the changes in the kidneys. However, histological examination of the lower urinary tract of the transgenic male mice revealed that the accessory sex glands and urethra were virtually normal and devoid of obstructions. The smooth muscle layer of the urinary bladder was markedly thinner and the mucous membrane was devoid of normal folding. Transgenic females showed only occasionally cortical fluidic cysts and degenerative corpuscles in the kidney, but no progress of these changes was found in aging animals (data not shown).

## **5.4 Intracellular trafficking of the LHR ectodomain splice variant in association with the full-length LHR (IV)**

Stable, tetracycline inducible Flp-In HEK 293 (HEK293<sub>i</sub>) and constitutive Flp-In CHO (CHO<sub>c</sub>) cell lines were used for recombinant protein production. This allowed the integration of the appropriate cDNA into a definite site in the genome by Flp recombinase and created a system, in which the different receptor proteins were expressed in an identical background. A Myc tag was used for the rat LHR ectodomain splice variant (*Myc-rLHRvariant*) and the Myc-Flag tag as used for the rat LHR (*Myc-rLHR-Flag*) to facilitate immunological detection and purification of the receptor protein. Both the full-length LHR and ectodomain variant were stably transfected to HEK293<sub>i</sub> cells (HEK293<sub>i</sub>-*Myc-rLHR-Flag* and HEK293<sub>i</sub>-*Myc-rLHRvariant*, respectively) (Table 7). For co-expressing the variant and full-length LHR, two differentially tagged LHR receptor constructs (*HA-rLHR* or *rLHR-GFP*) were stably transfected to HEK293<sub>i</sub>-*Myc-rLHRvariant* cells (HEK293<sub>i</sub>-*Myc-rLHRvariant+HA-rLHR* and HEK293<sub>i</sub>-*Myc-rLHRvariant+rLHR-GFP*, respectively) into a random integration site. These cell lines expressed the LHR constitutively and the variant under induction.

### **5.4.1 Identification of the rat LHR ectodomain splice variant protein**

The rat LHR ectodomain splice variant contains a cleavable signal sequence and six potential N-glycosylation sites. The potential glycoprotein nature of the variant and secretion into the medium were tested. The immunoprecipitation, lectin (concanavalin A) chromatography (data not shown), and direct lysate preparation (IV, Figure 2) with subsequent immunoblotting with a cMyc-tag specific antibody recognized M<sub>r</sub> 52,000 and 54,000 species for the ectodomain variant. The two variant species migrated faster under

non-reducing conditions, suggesting that they contain disulfide bonds (IV, Figure 2). The medium was also subjected to lectin and hCG affinity chromatography and immunoprecipitation, but no secretion was detected (data not shown). An alternative integration site-specific Flp-In CHOc cell line expressing stably and constitutively the rat LHR and the variant, produced the same molecular size proteins as the HEK293i cells. No secreted variant was found from the medium, confirming that the receptor is similarly processed in different cell lines (data not shown). Furthermore, the variant protein was not tightly bound peripheral or an integral membrane protein, which could have explained the inability to detect the secreted forms. This was tested with a sequential high-salt extraction that disrupts weak electrostatic interactions, and alkaline extraction that disrupts hydrophobic interactions which associate peripheral membrane proteins with lipid bilayers. Alkaline treatment also converses closed vesicles into open membrane sheets (Fujiki *et al.* 1982). The full-length LHR was resistant to both treatments (data not shown), but the variant behaved as the soluble ER protein BiP and dissociated from the membranes after high salt treatment (IV, Figure 2). A subpopulation of the variant might be more tightly attached, since some protein could be detected in the last fraction. It might represent proteins on the way of translocation into or out of the ER (unpublished data). Subcellular fractionation of frozen and freshly harvested cells confirmed further that the variant is a soluble ER protein (data not shown).

The identities of variant species were studied further. The glycosidase treatment with Endo H and peptide-N-glycosidase (PNGase) F gave the same result for the *Myc*-LHR-*Flag* as the rat tissue LHR (see 5.2) and also showed characteristic inefficient processing typical for the rat LHR (Pietilä *et al.* 2005). In contrast, the rat LHR ectodomain splice variant was harboring only high mannose-type oligosaccharides since both Endo H and PNGase F treatments resulted in a  $M_r$  40,000 form (IV, Figure 2).

#### ***5.4.2 LHR ectodomain splice variant is a misfolded ER protein and substrate to ERAD***

The rat LHR ectodomain splice variant contains the critical amino acids described for hormone binding (Smits *et al.* 2003, Galet & Ascoli 2005). The hCG affinity chromatography purification indicated that a very small fraction of the variant is able to bind hormone, suggesting that it tends to fail in finding the correct conformation (IV, Figure 3). As expected, both the  $M_r$  76,000 immature and 94,000 mature receptor species were purified from the LHR expressing cells and as the mature receptor form was clearly more intense than the immature one the former is likely to contain more receptors capable of hormone binding compared to the immature form. The LHR variant was not detected with the same protein amounts, but after a longer exposure time the  $M_r$  52,000-54,000 species could be seen, indicating that only a small fraction of the protein was able to bind hormone.

The inhibition of proteosomal activity led to a  $1.6 \pm 0.4$  –fold increase in the amount of the variant. This indicated that the variant is a substrate for ERAD and is directed to proteasomal degradation. However, no degradation intermediates or high molecular

weight aggregates were detected (IV, Figure 3). Similarly, no n-dodecyl- $\beta$ -maltodise-insoluble, SDS-soluble variant forms were observed (data not shown).

### ***5.4.3 LHR ectodomain splice variant colocalizes with ER chaperones and causes their redistribution***

The intracellular location of the soluble LHR ectodomain splice variant was studied visually by using double-label immunofluorescence with ER and Golgi markers. The perinuclear staining of the LHR variant in induced cells co-localized with the ER resident proteins, calreticulin and calnexin, showing an intriguing redistribution of the staining pattern of these ER chaperones as compared to non-induced cells. The Golgi protein GM130 did not reside in the same cellular compartment with the variant. The data showed that the variant expression is accompanied with a redistribution of the ER proteins, calnexin and calreticulin, to a more compartmentalized location (IV, Figure 4). This is likely to be part of the ER quality control process and reflects the fact that the variant is a substrate for ERAD. A similar kind of effect could be observed not only for ER chaperones, but also for the cytosolic intermediate filament vimentin. This also happened when proteasomes were inhibited with lactacystin and the variant formed cytosolic nocodazole-sensitive aggresomes (unpublished results).

### ***5.4.4 LHR ectodomain splice variant accumulates in the ER***

The ER quality control is a process, in which the misfolded proteins are retained in the ER and targeted for degradation. We noticed that the LHR ectodomain variant gathered into a perinuclear area and caused a curious redistribution of ER resident proteins calnexin and calreticulin. A few misfolded membrane-bound and secreted proteins have been shown to accumulate in pericentriolar aggresomes, especially if their proteasomal degradation is compromised (Johnston *et al.* 1998, Illing *et al.* 2002, Junn *et al.* 2002, Saliba *et al.* 2002). The above-mentioned phenomena together with the morphological appearance of the immunofluorescence stainings and the fact that the variant was not found in a deglycosylated form from the cell cytosol in fractionation experiments, alerted us to further explore its exact location. This question was approached by antibody transfections (IV, Figure 5), FACS (data not shown), and immunoelectron microscopy (IV, Figure 6). Intact cells internalize certain amount of antibodies that reach the epitopes available in the cytosol but not inside the ER. The LHR and calnexin are type I membrane proteins and are inserted into the ER membrane with the N-terminus being inside and the C-terminus outside the ER. We used this positioning for marking cytosolic staining by using the calnexin C-terminal antibody and the LHR C-terminal Flag antibody. The N-terminal antibody for calnexin and N-terminal cMyc antibody for the LHR and variant was used to reveal the luminal portion of the ER. The LHR was studied following blockade of receptor transport in the ER with brefelding A (BFA) (Lippincott-Schwartz *et al.* 1989). Both antibody transfections and the FACS analysis showed that the

C-terminal Flag and calnexin C-terminal antibodies recognized the cytosolic part of the proteins. The calnexin N-terminal and the anti-cMyc antibodies did not find the epitope, indicating that the variant is inside the ER. This was confirmed by staining permeabilized cells. The specificity of the calnexin C-terminal antibody internalization in FACS was controlled by blocking the cell surface with the sodium-azide (data not shown). When the cells expressing the LHR or the variant were treated with lactacystin to block proteasomal degradation, the N-terminal part of the proteins was delivered into the cytosol and the Myc-epitope became available (IV, Figure 5). This confirmed that both the LHR and the variant are substrates for ERAD. Ultrastructural analysis of non-induced and induced HEK293<sub>r</sub>-Myc-rLHRvariant cells revealed that the accumulation was indeed in the ER (IV, Figure 6). The labeled anti-cMyc antibody localized close to the luminal ER membrane. However, proteasomal blockade with lactacystin led to appearance of gold particles also in aggregates outside the ER lumen (IV, Figure 6). The appearances of aggresomes in lactacystin treated cells were accompanied by an increase in the number of lysosomes (data not shown). Transmission electron microscopy analysis revealed prominent juxtannuclear tubulo-vesicular structures with electron dense material (IV, Figure 6). This data confirmed the results (IV, Figure 2), which indicated that the LHR variant is in the core-glycosylated, ER-retained form and is attached in to some extent to the ER membrane. This data allowed us to conclude that the variant was retained in the ER in a specialized subcompartment, and although targeted for proteasomal degradation, it accumulated in the cytosol only if proteasomal degradation was compromised.

#### ***5.4.5 LHR ectodomain splice variant misroutes the full-length LHR into a subcompartment of the ER***

Since the LHR ectodomain splice variant was not able to fold correctly and was stopped by the ER quality control, we investigated what effect the variant expression might have on the full-length LHR, a situation that is biologically equivalent to natural tissues. The HA-rLHR and rLHR-GFP were used to constitutively express the LHR in cells that expressed the Myc-tagged variant under induction. We used immunofluorescence anti-cMyc staining to perform a qualitative comparison of the plasmamembrane staining of cells with a fluorescence microscope and a quantitative one with FACS. Both methods showed that the LHR ectodomain variant cannot be found at the cell surface even in the presence of the full-length LHR (data not shown). The same protein purifications for the cell lysate and medium were performed as for the variant expressing cells, but also in this case the variant was found as a core-glycosylated, ER resident M<sub>r</sub> 52,000-54,000 species and was not found to be secreted into the medium (data not shown).

The above findings suggest that the LHR and variant potentially interact in the ER. The LHR variant production had an impact on the LHR immature and mature forms when measured by FACS or immunoprecipitation methods (IV, Figure 7). The results revealed that the non-induced cells converted the LHR precursor to mature one extremely inefficiently and only 25-30% of the LHR matured to cell surface form. The LHR ectodomain splice variant was found to decrease the LHR amount (IV, Figure 7) also in metabolic labeling experiments (data not shown). The decrease in cell surface form was

in average about 32-42%. We also observed that some of the intracellular GFP, representing unfolded LHR, emit fluorescence signal in more red-shifted wavelengths, and the variant induction promoted this signal. Furthermore, hCG affinity chromatography showed less purification for the LHR immature form under induction, indicating that there may be a less correctly folded receptor capable at hormone binding (data not shown).

By performing several investigations to rule out possible secondary effects that the induction might have created, we unambiguously demonstrated that the LHR ectodomain splice variant caused the observed reduction in the LHR. We tested the overall protein synthesis rate by using metabolic labeling and scintillation counting, but no decrease was observed. The possible activation of UPR was measured by Western analysis (BiP, PDI) and real time quantitative PCR (BiP). A positive UPR response was clear in induced tunicamycin treated cells, but no response was observed in the 5h-48h induced cells (data not shown).

The full-length LHR was found to be misrouted to the ER subcompartment shortly after synthesis in the variant expressing cells. This possibly caused the observed decrease in the number of LHRs. The full-length LHR was observed as a diffuse network with clear nuclear envelope staining in permeabilized, non-induced cells under the fluorescence microscope (IV, Figure 8). The variant production made the LHR to partially lose its web-like structure and be redistributed to a more compact location, colocalizing to the perinuclear area with the variant. The GFP tagged LHR gave plasmamembrane and strong intracellular fluorescence a signal confirming that most of the LHR is converted inefficiently to the mature plasmamembrane form. The intracellular GFP that emitted fluorescence signal in more red-shifted wavelengths was directed to perinuclear structures. This change in the fluorescence signal is consistent with FACS results from the same cell line (data not shown). The perinuclear staining of the LHR as well as the variant in induced cells colocalized with the ER resident proteins calreticulin and calnexin (data not shown). The results suggested that the immature LHR and variant reside in the same ER subcompartment. The same redistribution of the LHR was observed when the HA-tagged variant was transiently transfected to HEK293<sub>i</sub>-*Myc-rLHR-Flag* cells (IV, Figure 8). In addition, transiently transfected cells showed that the human  $\mu$  and  $\delta$  opioid receptor were transported to cell surface (data not shown) and the intracellular location did not show any alterations under the variant induction (IV, Figure 8). The ER staining of the variant did not colocalize significantly with the receptors, suggesting that their intracellular trafficking route may be different.

## 6 Discussion

### 6.1 Maturation of the LHR in the sensory nervous system

The LHR has generally been regarded as a protein expressed mainly in the gonads. We demonstrate in this work that the LHR is not expressed solely in the reproductive organs but is also present as a functional protein in the nervous system. The expression appears to commence prenatally in both the peripheral and central nervous system and continues through adulthood, especially in specific restricted areas of the brain involved in sensory functions. The LHR thus resembles other LGRs (LGR4-8) which display a wide tissue distribution (Hsu *et al.* 1998, McDonald *et al.* 1998, Hermey *et al.* 1999, Hsu *et al.* 2000, Hsu *et al.* 2002), suggesting that this property may be conserved within the LGR subfamily of GPCRs. Furthermore, the presented results demonstrate that the LHR expression results in multiple receptor mRNA transcripts and that a concomitant synthesis of receptor protein exhibits specific high affinity ligand binding. The similar size mature ( $M_r$  90,000) and immature ( $M_r$  73,000) LHR receptor forms were also present in the ovaries, which is in agreement with previous reports (Roche & Ryan 1989, Zhang & Menon 1989, Hipkin *et al.* 1992), showing that the receptor is processed in these tissues in a similar manner. The immature  $M_r$  73,000 forms carried high-mannose type *N*-linked glycans (Keinänen 1988, Kusuda & Dufau 1988, Hipkin *et al.* 1992, VuHai-LuuThi *et al.* 1992, Beau *et al.* 1997, Fabritz *et al.* 1998), which are typical of glycoproteins residing in the ER. Rao *et al.* have previously identified a LHR form of  $M_r$  80,000 in the rat and human nervous tissue (Lei *et al.* 1993, al Hader *et al.* 1997, al-Hader *et al.* 1997). This apparent discrepancy to our results might be a reflection of the different methods used (Western blotting versus immunoprecipitation). Because of the very low level of LHRs in the nervous tissue, we were not able to detect any specific bands for the neuronal LHRs with direct Western blotting of the solubilized membranes (data not shown). This kind of low expression is typical of GPCRs. The high amount of the immature forms most likely reflects inefficient receptor maturation (Pietilä *et al.* 2005), as has been previously reported for the human  $\delta$ -opioid receptor (Petäjä-Repo *et al.* 2000) and for the ovarian FSHRs (Vannier *et al.* 1996). The inefficient processing of the LHR may provide a means to control receptor numbers at post-translational level.

Immunoprecipitation, hCG affinity chromatography, and competition binding experiments showed that the neuronal LHR is a functional protein and capable of similar hormone-binding specificity as gonadal LHRs (Segaloff & Ascoli 1993, Ascoli *et al.* 2002). Our data suggests that functional cell surface neuronal LHRs precedes those expressed in the gonads. The early neuronal LHR expression suggests a role in the regulation of neuronal growth and differentiation. This may be possible, since the administration of hCG has been found to result in neurite outgrowth of cultured rat neuronal cells (al Hader *et al.* 1997). The LHR gene was also active in distinct areas of the adult brain, especially areas involved in olfaction, but also in behaviour and memory. The sensory functions are more qualitative in nature, which may explain the undetectable changes in the LHR knock-out mice nervous system (Lei *et al.* 2001, Zhang *et al.* 2001a) and also the fact that in most cases neuronal defects are compensated by alternative pathways. The neuronal LHR does not seem to have importance directly in reproduction, since the ovary transplantation was sufficient to restore the fertility of knock-out mice (Pakarainen *et al.* 2005b). Our results also indicated that hCG administration to mother can alter the LHR gene activity in fetuses and that gene activity can be downregulated with high hCG levels, suggesting that maternal hormones could regulate the fetal LHR gene. In the gonads, the LHR regulates StAR and other steroidogenic enzymes (Saez 1994). An interesting possibility is that the LHR may take part in the regulation of neurosteroid production in a similar manner. The neurosteroids are known to act as signaling factors that coordinate environmental cues to reproductive behavior and regulate neuronal growth and differentiation (Mensah-Nyagan *et al.* 1999, Mellon *et al.* 2001). The LHR promoter-driven  $\beta$ -Gal activity was found to co-localize with in the cytochrome P450<sub>scc</sub> in the same neurons. In addition, the expression pattern of the transgene was very similar to enzymes involved in neurosteroid synthesis, such as the cytochrome P450<sub>c17</sub> (Le Goascogne *et al.* 1987, Compagnone *et al.* 1995) and StAR (King *et al.* 2002). It has been suggested that the marked increase in serum LH following menopause/andropause could be a pathophysiologically relevant signal that could promote amyloid-beta secretion and deposition in the aging brain and in the pathogenesis of Alzheimer's disease. Our observations of the neuronal LHR, neurosteroids and the LHR gene downregulation in the nervous system by hCG may provide an interesting issue to be addressed in future studies.

## 6.2 Maturation of the LHR in the urogenital tissues

We demonstrated that, in addition to the nervous system and gonads, the LHR is expressed also in other urogenital structures, such as kidneys, genital tubercles, and adrenal glands. The receptor gene activity appears to start early during fetal development, since the genital tubercles showed  $\beta$ -Gal activity at 11.5 dpc. Our results also indicated that hCG administration to mother can alter the LHR gene activity in fetuses through an unknown mechanism. Importantly, no apparent sex differences in the onset of mRNA expression were observed. In contrast to these findings, mouse LHR promoter fragments showed no detectable transgene expression in prenatal transgenic mice and were found to show sex- and tissue-dependent differences in adult mice tissues (Hämäläinen *et al.*

2002). Also, we detected full-length mRNA transcripts in the gonads at 17.5 dpc. Previous findings reported rat ovaries to express only truncated mRNA species before birth (Sokka *et al.* 1992a). The promoter results may be due to differences in species and in the case of mRNA detection, the low amount and different techniques used to detect the full-length receptor transcripts. We detected the transcripts in the fetal ovaries by RT-PCR only after the second amplification round, whereas those in the testes were easily detectable after the first round. This sex-dependent difference was not unexpected, because the same difference was clearly apparent in the adult gonads as well. Whether this has some functional significance remains to be determined in the future.

Expression of LHRs in the developing urogenital tissues suggests that the receptor may have a functional role in the differentiation of these tissues. Nevertheless, it is unlikely that the intracellular immature receptors detected in the developing fetal tissues are fully functional. They are, therefore, unlikely to respond to circulating hormones. It is notable, however, that the immature receptors were found to be capable of hormone binding. This suggests that the hormone binding sites detected previously in developing testes represent immature non-functional receptors (Warren *et al.* 1984). The LHR might have a negligible role in intrauterine sex differentiation. For example, studies on LHR knockout mice have shown that the newborns are born phenotypically normal and only the postnatal sexual development is impaired (Lei *et al.* 2001, Zhang *et al.* 2001a). Fertility can be later restored with steroid hormones (Pakarainen *et al.* 2005a, Pakarainen *et al.* 2005b). In addition, prenatal testosterone levels are normal in hypogonadal (hpg) mice lacking circulating gonadotropins (O'Shaughnessy *et al.* 1998). Although it is possible that some mature LHRs may be expressed in the developing urogenital tissues, our data support the notion that the LHR may have a negligible role in the development of the gonadal and non-gonadal urogenital tissues. Instead, the cognate hormones may have a more important functional role in the fetal nervous system where the mature LHR was expressed during development.

Previously published studies have demonstrated LHR expression in rodent and human adrenal glands (Pabon *et al.* 1996, Rilianawati *et al.* 1998, Kero *et al.* 2000, Hämäläinen *et al.* 2002). Our results support and extend these findings. The receptor expression was low in adult females but increased substantially during pregnancy. Importantly, the pregnant adrenal glands expressed mostly the mature receptor form, suggesting that these receptors are very likely to be fully functional. Both direct and indirect evidence in the literature suggests that this may indeed be the case. Human fetal adrenal glands have been shown to respond to hCG administration by increasing dehydroepiandrosterone sulfate secretion (Serón-Ferré *et al.* 1978), and adrenal glands of transgenic female mice expressing a chimeric protein (the  $\alpha$ -subunit of bovine LH and a fragment of the  $\beta$ -subunit of hCG) were found to express the LHR and respond to hCG with significantly increased cAMP, progesterone, and corticosterone production (Kero *et al.* 2000). There are descriptions on cases of Cushing's syndrome in which the adrenocortical hyperfunction was found to be LH/hCG dependent (Lacroix *et al.* 2001). We also showed that the administration of hCG to female mice can activate the rat LHR gene promoter in the adrenal glands. Whether LH and hCG are able to regulate adrenal gland steroid production during pregnancy remains to be tested in the future. In addition, the factor(s) that regulate LHR expression in the adrenal glands and cause upregulation during pregnancy remain to be identified. An interesting candidate hormone is prolactin, which

is known to upregulate LHRs in the corpus luteum during pregnancy (Gåfvels *et al.* 1992, Bjurulf *et al.* 1994), and numerous placentally-produced lactogens are secreted during mid and late pregnancy (Linzer & Fisher 1999, Lacroix *et al.* 2002).

### **6.3 LHR ectodomain splice variant and the ER subcompartment**

Studies done with transgenic mice overexpressing the rat LHR ectodomain splice variant provided direct evidence that the variant can modify the physiological effects of LH/hCG in target tissues and manifest pathophysiological functions. The variant had the most profound effects on the pituitary-gonadal functions. The phenotype was more severe in the transgenic male mice, suggesting that either the variant or decreased serum LH has more functional impact on males, possibly because of the LHR's role on testosterone production in the Leydig cells. This may also hint that decreased serum LH levels are more critical to males and, as has been observed in previous studies, increased levels to females (Kero *et al.* 2000, Beuschlein *et al.* 2003). The adrenocortical changes in transgenic mice are likely to result from decreased circulating LH levels and/or changes in receptor number in the adrenal glands. These hypotheses are reasonable, because results presented in this work and by others have shown that the human and rodent adrenal glands express functional LHRs (Pabon *et al.* 1996, Rilianawati *et al.* 1998, Lacroix *et al.* 2001, Hämäläinen *et al.* 2002, Piltonen *et al.* 2002) and the receptor number is upregulated by elevated LH levels upon gonadectomy in adrenal tumors (Kero *et al.* 2000, Beuschlein *et al.* 2003) and during pregnancy. Moreover, maintenance of the X-zone in mice adrenal glands appears to be dependent on LH action (Deacon *et al.* 1986). The observed decrease in testicular testosterone may also be caused by increased androgen production in the adrenal tumors of the transgenic male mice (Lacroix *et al.* 2001). The changes in the urinary organs occurred under conditions in which serum LH levels and testicular testosterone production were chronically decreased. This, together with the earlier finding that the rat testicular steroidogenesis is disrupted in the nephritic syndrome (Menjívar *et al.* 2003), suggests that the observed renal changes in transgenic males may be linked to impaired gonadal steroidogenesis. According to our results on the LHR expression in the sensory ganglia, we can also hypothesize that local overexpression of the receptor variant might indirectly lead to weakening of the miction reflex and subsequently lead to urine retention and dilation of the bladder.

The mechanism of how the LHR ectodomain variant overexpression leads to morphological changes in tissues, especially in males, and impairment in steroidogenesis remains to be tested in the future. However, the most likely possibility is that it impairs LH production and/or action on target cells. Recent reports in the literature provide two alternatives. The variant might alter the secretion of the hormone in the LH-producing gonadotrophs. The porcine LHR ectodomain coexpressed in cells with hCG was secreted as a complex with the hormone (Remy *et al.* 2001). Alternatively, the variant expressed in the target cells may locally impair LH action by modulating the number and/or function of the full-length LHR. The human LHR splice variant lacking exon 9 has been shown to have an effect on the full-length receptor expression level in a heterologous expression system (Nakamura *et al.* 2004). According to our own results in HEK 293 cells, the

explanation for this phenotype is likely to be misrouting of the full-length LHR within the cell by the variant into the ER subcompartment. Our data showed that in HEK 293 cells, the variant is a soluble, core-glycosylated ER-retained protein that has a tendency to fold incorrectly. The transgenic mice liver cells showed vacuolization and aggregated material in the absence of the full-length LHR, which might be a direct consequence of the incorrectly folded variant. Nevertheless, it indicated an interesting cellular phenomenon possibly pointing toward the splice variant's natural hydrophobic protein nature and trafficking inside the cell. Only a very small portion of the variant protein was able to bind hormone in HEK 293 cells. This could be true also in tissues, since no purification of the variant protein was observed in hCG affinity chromatography. Furthermore, the variant accumulated and co-localized with ER folding chaperones to a specific ER subcompartment and also redistributed the full-length LHR into this same location in HEK 293 cells. This could be the same compartment of the ER located adjacent to the centrosome and ERGIC described for some ERAD substrate proteins in mammalian cells when proteasomal degradation is inhibited (Kamhi-Nesher *et al.* 2001, Frenkel *et al.* 2004), and equivalent for the yeast ER-associated compartment (ERAC) (Huyer *et al.* 2004). Our observation of the variant and immature LHR accumulation in the specific ER subcompartment instead of cytosol is a rather novel idea to represent protein holding site. It might be more advantageous for the cell to maintain aggregation-prone misfolded proteins in a soluble form in the ER by chaperones, where they still could have the possibility to fold correctly instead of trafficking them to the cytosol where they might aggregate and interfere with cellular functions. The variant was a substrate for ERAD, since the inhibition of proteasomal activity led to an increase in the amount of the variant and its appearance in the cytosol. Furthermore, the inhibition redistributed chaperones and intermediate filaments and the variant formed nocodazole sensitive aggresomes. The misrouting of the LHR would provide a mean to regulate the number of newly synthesized receptors that would mature and reach the cell surface and possibly hasten the ERAD process of inefficiently maturing receptor proteins. This may be indicated by the appearance of a specific ER subcompartment and redistribution, but the issue remains to be further examined in detail. The co-expression of the variant with the LHR hints toward the possibility of the degradation of the immature form of the LHR being controlled through the expression of its ectodomain splice variant. The modulation of full-length receptor expression levels is the most interesting from a physiological point of view, as the variant is highly expressed in the LH target tissues. Most importantly, the variant expression has been shown to change in these tissues depending on the physiological stimuli, cellular differentiation, and tumorigenic cell growth (Sokka *et al.* 1992a, Sokka *et al.* 1992b, Vihko *et al.* 1992, Lakkakorpi *et al.* 1993, Tena-Sempere *et al.* 1994, Zhang *et al.* 1994, Jiang *et al.* 2002, Licht *et al.* 2003).

## 6.4 Post-translational regulation of the mature LHR

The developing rat urogenital structures and adrenal glands showed that the receptor protein is expressed only at an immature level. Mature receptors were detected only in adult gonads and, surprisingly, in pregnant female adrenal glands and kidneys. Our

results implicate that the expression of mature and functional LHRs is tightly regulated in both gonadal and urogenital tissues. The detected smaller species may be an intact receptor precursor with a fewer number of N-linked glycans than the  $M_r$  73,000 form. Alternatively, the  $M_r$  70,000 receptor form may result from alternative splicing of the primary transcript and represent a receptor variant lacking some of the exon(s) coding for the extracellular domain of the receptor (Tsai-Morris *et al.* 1990, Aatsinki *et al.* 1992, Bacich *et al.* 1994).

It has been suggested that LHR expression in the developing gonads is regulated at a post-transcriptional level, as the onset of expression of receptors capable of hormone binding in the developing testes and ovaries appears to coincide with the change in the alternative splicing pattern of the LHR mRNA (Sokka *et al.* 1992a, Zhang *et al.* 1994). Our results extend these findings and argue that the expression of fully functional mature receptors is regulated at post-translational level, both in the gonads and urogenital tissues. This notion was supported by the fact that there was a clear age-, tissue- and sex-dependent variation in the relative amount of mature receptors to immature ones. The expression of mature receptors was clearly upregulated in the adrenal glands and kidneys of pregnant female rats, whereas the corresponding tissues in non-pregnant ones expressed mainly the immature receptor. Thus, although the gonadal and urogenital tissues appear to express the protein constitutively, the level of mature receptors seems to be developmentally and physiologically regulated.

It remains a mystery how mammalian cells maintain a constant balance between protein synthesis and degradation, although there is evidence that tight coupling between protein synthesis and degradation can depend on extracellular signals (Franklin & Johnson 1998). It can be hypothesized that the number of fully mature LHRs at the cell surface may be upregulated due to enhanced maturation and cell surface targeting of immature receptors retained in the ER or, alternatively, lengthening the half-life of the mature receptors at the cell surface. The former possibility is more likely, since in HEK 293 cells newly synthesized LHRs were prone to premature degradation and inhibition of degradation leads to enhanced maturation of the ER-retained immature receptors (Pietilä *et al.* 2005). Our results presented here showed that the inefficient LHR maturation is a natural condition in rat tissues. One possibility is that the degradation of the immature LHR is regulated by extracellular signals through a phosphorylation dependent kinase cascade. This is supported by the fact that the CNS component, protein p38<sup>JAB1</sup>, binds to the C-terminal tail of the rat LHR and enhances receptor degradation (Li *et al.* 2000). An interesting candidate for the direct post-translational regulator is the LHR ectodomain splice variant. Our results obtained from HEK 293 cells expressing the variant with the full-length LHR support this view. The ectodomain splice variant may function as a closed circuit degradation assistant and provide a cellular mechanism to control the LHR export. The connection between extracellular signals, LHR splice variants, oligomerization, and CNS to the full-length LHR need to be determined further.

## 7 Conclusions

We have identified and characterized the rat LHR expression and maturation in the nervous system and urogenital structures. The LHR has been previously considered to be a protein expressed solely in the reproductive organs. However, our study showed that the LHR resembles other LGRs, which display wide tissue distribution. The neuronal LHR existed in a mature plasma membrane form and exhibited specific high affinity ligand binding. The neuronal LHR may be involved in neurosteroid production and functions involved with sensory systems. The developing rat urogenital structures and adrenal glands expressed only the immature LHR. Mature receptors were detected only in adult gonads and female adrenal glands and, surprisingly strongly in pregnant female adrenal glands and kidneys at a time that coincides with the differentiation of fetal urogenital structures. We propose that the mature LHR expression is tightly regulated at post-translational level in gonadal and non-gonadal tissues. Furthermore, the pregnancy-induced upregulation of mature LHR expression in the female adrenal glands and kidneys predicts novel roles for LH and hCG.

The rat LHR ectodomain splice variant may have a role in post-translational regulation of the LHR. The variant could decrease the amount of the full-length LHR by a dominant negative manner possibly through routing the immature LHR to the degradation pathway. The variant may be especially important during downregulation event in preventing the ER exit of the LHR to the cell surface. The described new ER quality control subcompartment may be a novel holding site for ERAD substrates and connected with inefficient maturation. The mechanisms causing the ER chaperone and intermediate filaments redistribution and accumulation during ER quality control and ERAD need further investigations. The variant overexpressed in the transgenic mice showed that it can modify gonadal, urogenital, and adrenal functions either at the pituitary and/or gonadal target cell level. The results suggest a novel and yet unidentified complex regulatory role for the GPCR splice variants as factors that may determine the homeostasis of the cell.

The studies presented here provide essential information on molecular mechanisms underlying the maturation of GPCRs by connecting physiological signals to the splicing, inefficient maturation, oligomerization, and ER quality control/ERAD.

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