Handbook of Experimental Pharmacology 245

Alfredo Ulloa-Aguirre
Ya-Xiong Tao *Editors*

Targeting Trafficking in Drug Development

Springer

Handbook of Experimental Pharmacology

Volume 245

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ISSN 0171-2004 ISSN 1865-0325 (electronic)
Handbook of Experimental Pharmacology
ISBN 978-3-319-74163-5 ISBN 978-3-319-74164-2 (eBook)
DOI 10.1007/978-3-319-74164-2

Library of Congress Control Number: 2017964211

Printed on acid-free paper

This Springer imprint is published by the registered company Springer International Publishing AG part of Springer Nature.
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Preface

Orderly cellular function depends on many factors, including the absolute number and appropriate location of a diverse array of proteins. Proteins are synthesized in the endoplasmic reticulum at a very fast rate (~1–5 amino acids/second in eukaryotes), time which allows co-translational folding to occur. The endoplasmic reticulum has the daunting task of synthesizing nearly 100,000 proteins and providing the specialized environment necessary for folding, glycosylation, oxidation, signal peptide cleavage, and assembling of oligomeric proteins before their translocation to other cell domains, including the Golgi apparatus, where the proteins may be modified and prepared for secretion or transport to their final destination in the cell. Protein misfolding can arise spontaneously, or from mutations or alterations in the sequence of proteins, overexpression, changes in temperature, oxidative stress, and/or activation of signaling pathways associated with protein folding and quality control. Failure to achieve adequate folding, despite the effort of molecular chaperones to correct folding defects and prevent the accumulation and aggregation or proteasomal degradation of the misfolded protein, may lead to profound effects on the health of an organism. Understanding the molecular, cellular, and energetic mechanisms of protein folding and routing as well as those that govern the function of the quality control system of the cell may help to prevent or correct the structural defects linked to particular, protein misfolding-associated diseases.

This volume of the *Handbook of Experimental Pharmacology* compiles important information on misfolding and disordered intracellular traffic of different proteins associated with disease, including cancer, Golgi and neurodegenerative diseases, islet amyloidosis, cystic fibrosis, and some others, as well as on current therapeutic approaches based on pharmacological chaperones designed to correct particular protein folding defects and intracellular trafficking, the majority of which still are under intense investigation and development. To integrate this volume, the editors selected authors based on their research contributions in their corresponding fields and their ability to express their thoughts and ideas clearly. The editors would like to express their appreciation to the authors for their willingness to participate in this volume and for providing the excellent contributions in a timely fashion. We also thank the staff of Springer for helpful input.

The editors wish to dedicate this thematic volume of the *Handbook of Experimental Pharmacology* to the memory of one of its original editors, P. Michael Conn, Ph.D., who suddenly passed away before his time. Dr. Conn was an outstanding scientist, who dedicated his last 15 years of research activity to elucidate the structural basis and molecular physiopathogenesis of diseases caused by protein misfolding, particularly hypogonadotropic hypogonadism and nephrogenic diabetes insipidus due to mutation-caused misfolding of the gonadotropin-releasing hormone receptor and arginine-vasopressin receptor, respectively. He applied his clever mind and creative imagination to design and investigate on pharmacological chaperones potentially useful to treat these diseases, employing a variety of in vitro and in vivo experimental approaches and scenarios, which paved the way for research in this fascinating area. The scientific community will miss Michael Conn, an outstanding scientist and teacher, and an endearing friend.

Mexico City, Mexico — Alfredo Ulloa-Aguirre
Auburn, AL, USA — Ya-Xiong Tao

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Intracellular Trafficking of Gonadotropin Receptors in Health and Disease

Alfredo Ulloa-Aguirre, Teresa Zariñán, Rubén Gutiérrez-Sagal, and James A. Dias

Contents

Abstract

Gonadotropin receptors belong to the highly conserved subfamily of the G protein-coupled receptor (GPCR) superfamily, the so-called Rhodopsin-like family (class A), which is the largest class of GPCRs and currently a major drug target. Both the follicle-stimulating hormone receptor (FSHR) and the luteinizing hormone/chorionic gonadotropin hormone receptor (LHCGR) are

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A. Ulloa-Aguirre, Y.-X. Tao (eds.), *Targeting Trafficking in Drug Development*, Handbook of Experimental Pharmacology 245, DOI 10.1007/164_2017_49

mainly located in the gonads where they play key functions associated to essential reproductive functions. As any other protein, gonadotropin receptors must be properly folded into a mature tertiary conformation compatible with quaternary assembly and endoplasmic reticulum export to the cell surface plasma membrane. Several primary and secondary structural features, including presence of particular amino acid residues and short motifs and in addition, posttranslational modifications, regulate intracellular trafficking of gonadotropin receptors to the plasma membrane as well as internalization and recycling of the receptor back to the cell surface after activation by agonist. Inactivating mutations of gonadotropin receptors may derive from receptor misfolding and lead to absent or reduced plasma membrane expression of the altered receptor, thereby manifesting an array of phenotypical abnormalities mostly characterized by reproductive failure and/or abnormal or absence of development of secondary sex characteristics. In this chapter we review the structural requirements necessary for intracellular trafficking of the gonadotropin receptors, and describe how mutations in these receptors may lead to receptor misfolding and disease in humans.

Keywords
G protein-coupled receptors (GPCR) • Gonadotropin receptors • Gonadotropins • Intracellular traffic • Quality control system

1 Introduction

The pituitary gonadotropic hormones, follicle-stimulating hormone or follitropin (FSH) and luteinizing hormone or lutropin (LH), as well as placental chorionic gonadotropin (hCG), are glycoprotein hormones that play a pivotal role in reproduction. Their cognate receptors (FSHR and LHCGR -*the LH receptor binds both, LH and hCG*-) belong, together with the thyroid-stimulating hormone receptor (TSHR) expressed by thyroid follicular cells, to a highly conserved subfamily of the G protein-coupled receptor (GPCR) superfamily, the so-called Rhodopsin-like family (class A), which is the largest class of GPCRs and currently a major drug target. The FSHR and LHCGR are mainly expressed by specific cells in the gonads (Ascoli et al. 2002; Richards and Pangas 2010b; Simoni et al. 1997). The FSHR is expressed in ovarian granulosa cells and the testicular Sertoli cells of the seminiferous tubules. Here, the FSHR is essential for FSH-stimulated maturation of ovarian follicles and granulosa cell progesterone and estrogen production (Richards and Pangas 2010a), whereas in the testis, activation of the FSHR supports Sertoli cell growth and metabolism, promoting spermatogenesis (Huhtaniemi 2015). In males, LHCGR is expressed in the Leydig cells which comprise nests located between the seminiferous tubules, where LH stimulates androgen production, mainly testosterone which in addition to its effect on male secondary sex characteristics, is converted by Sertoli cells to estrogen (Haider 2004; Saez 1994). In females, the LHCGR is expressed in the ovarian theca cells lining the developing

follicle, where its cognate ligand induces production of aromatizable androgens, which are subsequently converted to estrogens in the granulosa cell layer (Richards and Pangas 2010b).

G protein-coupled receptors are membrane receptors that vary considerably in molecular size. Nevertheless, they share a common molecular topology consisting of a single polypeptide chain of variable length that traverses the lipid bilayer forming seven characteristic transmembrane hydrophobic α-helices [transmembrane domains (TMD)], connected by alternating extracellular and intracellular sequences or loops (EL and IL, respectively), with an extracellular NH_2-terminus and an intracellular carboxyl-terminal domain (Ctail) (Gershengorn and Osman 2001; Ulloa-Aguirre and Conn 1998). These receptors characteristically bind one or more heterotrimeric G proteins that become activated upon agonist binding, which in turn act as mediators of effector activation and intracellular signaling (Oldham and Hamm 2008). In particular, a large NH_2-terminal extracellular domain or ectodomain (ECD), where recognition and binding of their cognate ligands occurs, is characteristic of glycoprotein hormone receptors. This ECD is comprised of a central structural motif of imperfect leucine-rich repeats (LRR), a motif that is shared with a number of other membrane receptors involved in ligand selectivity and specific protein–protein interactions (Bogerd 2007). The carboxyl-terminal end of the large ECD displays the signal specificity subdomain (also called "hinge" region), which is an integral part of the ectodomain and that structurally links the leucine-rich ECD with the serpentine 7TMD of the receptor, where activation of the receptor occurs following conformational changes provoked by agonist interaction with the ECD (Chen et al. 2009; Krause et al. 2012; Majumdar and Dighe 2012). The hinge region has been structurally characterized for the human (h) FSHR (hFSHR) (Jiang et al. 2012) and evidence has linked this region to signaling functionality (Jiang et al. 2014).

The FSHR and the LHCGR exhibit a high degree of primary sequence homology. Whereas the ECD amino acid sequences of the gonadotropin receptors are approximately 46% identical, the 7TMD sequence portion of the receptors share nearly 72% homology (Dias and Van Roey 2001; Kleinau and Krause 2009). This high similarity between the 7TMD of the gonadotropin receptors might suggest similar mechanisms of receptor activation; however, it is noticeable that gain-of-function mutations in the 7TMD of the hFSHR are extremely rare when compared to the hLHCGR (Ulloa-Aguirre et al. 2014). A higher relative stability of the 7TMD of the hFSHR in the inactive state compared with that of the hLHCGR could explain this difference between gonadotropin receptors. Among the three domains, the intracellular regions have the lowest FSHR-LHCGR amino acid sequence homology (approximately 27% identity), with the exception of the NH_2-terminal end of the Ctail, which bear Cys residues for palmitoylation and a primary sequence motif ($F(x)_6LL$) that markedly influences trafficking from the endoplasmic reticulum (ER) to the cell surface plasma membrane (PM) (Duvernay et al. 2004, 2005; Timossi et al. 2004) (see Sects. 3.1.1 and 3.1.3).

Upon gonadotropin binding, the activated FSH and LHCG receptors trigger a number of intracellular signaling cascades. Although the classical $G\alpha_S$/cAMP/PKA

signaling pathway has been accepted as the main effector mechanism of gonadotropin biological action for a long time, it is currently clear that gonadotropin receptors (and the TSHR as well) may couple to other G protein subtypes and activate a number of distinct signaling pathways (Gloaguen et al. 2011; Ulloa-Aguirre et al. 2011), depending on the cell context and developmental stage of the host cells (Musnier et al. 2009).

It is well known that mutations resulting in changes in protein sequence may lead to misfolding, defined as a defect in protein folding due to a sufficient and persistent number of non-native interactions that significantly affect the overall architecture or conformation of the protein and/or its properties (Dobson 2003). Frequently, misfolding results in loss-of-function of the conformationally defective protein (Dobson 2004; Ulloa-Aguirre et al. 2004a) that may be transcribed and translated at normal levels, but is unable to reach its functional destination in the cell or to engage the secretory pathway (Dobson 2003, 2004). A number of mutations in several GPCRs associated with endocrine functions that lead to misfolding of the receptor protein and to partial or complete inability of the abnormal receptor to express at the PM level and interact with agonist have been described (Conn and Ulloa-Aguirre 2010). Several endocrine diseases caused by mutations in GPCRs that provoke protein misfolding and impaired traffic of the mutant to the PM include nephrogenic diabetes insipidus (which involves the vasopressin V2 Receptor; V2R) (Bichet 2006; Conn et al. 2007), familial hypocalciuric hypercalcemia (calcium-sensing receptor; CaSR) (Huang and Breitwieser 2007), congenital hypothyroidism (TSHR) (Calebiro et al. 2005), obesity (melanocortin-3 and -4 receptor; MC3R and MC4R, respectively) (Huang et al. 2017; Tao 2010; Tao and Conn 2014), and familial glucocorticoid deficiency (melanocortin-2 receptor; MC2R) (Clark et al. 2005). Mutation-provoked misfolding of GPCRs involved in the regulation of reproductive function may also occur and lead to distinct abnormalities, including hypogonadotropic hypogonadism [due to mutations in the gonadotropin-releasing hormone receptor or GnRHR (Ulloa-Aguirre et al. 2004b), neurokinin-3 receptor, prokineticin receptor-2, or kisspeptin receptor-1 (Francou et al. 2011; Monnier et al. 2009; Nimri et al. 2011)], male pseudohermaphroditism (hLHCGR), and ovarian failure (hFSHR) (Ulloa-Aguirre et al. 2014).

Before discussing the structural determinants involved in trafficking of the gonadotropin receptors and their pathogenic mutations, we will briefly review some of the general mechanisms dictating the intracellular trafficking of GPCRs, particularly of the rhodopsin-like receptors.

2 Endoplasmic Reticulum Quality Control System, Molecular Chaperones, and Regulation of Intracellular Trafficking

As any other protein produced by the cell, GPCRs begin their life cycle at the ER. Here, synthesis, folding, and assembly of proteins occurs (Fig. 1), and properly folded receptors that have reached a conformation compatible with ER export, are

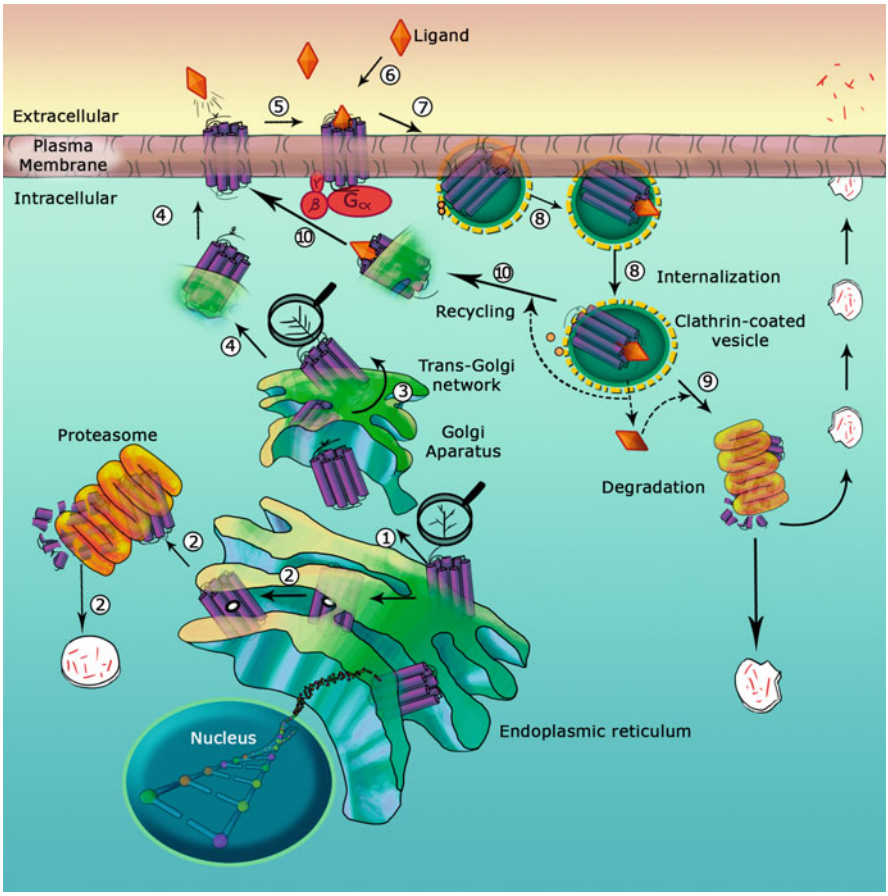

Fig. 1 Trafficking of rhodopsin-like G protein-coupled receptors. Newly synthesized proteins fold in the endoplasmic reticulum (step 1). Here, misfolded and misassambled products are retained and exposed to resident chaperones (oval structures) which attempt to correct folding and stabilize the protein in a conformation compatible with endoplasmic reticulum export. When correct folding fails, the misfolded protein is dislocated into the cytoplasm for proteosomal degradation (step 2). Correctly folded proteins are then translocated to the Golgi apparatus to complete processing such as glycosylation (magnifiers) (steps 1 and 3). Mature receptors are then exported to the plasma membrane (step 4) where they interact with cognate ligands (steps 5 and 6). Ligand activation of the receptor (step 6) is followed by phosphorylation (*orange circles*) of the receptor and recruitment of β-arrestins, which promote endocytosis (step 7) and internalization of the receptor-ligand complex (step 8). The receptor-ligand complex embedded in clathrin-coated vesicles may be either targeted to lysosomes for degradation, dissociate in the endosomal compartment with subsequent sorting of the ligand to lysosomal degradation (step 9) and the receptor to the recycling pathway, or recycled back to the plasma membrane (step 10) where agonist dissociates from the receptor to interact with agonist (Ascoli 1984; Sorkin and Von Zastrow 2002; Krishnamurthy et al. 2003; Melo-Nava et al. 2016)

then targeted to the ER-Golgi intermediate complex and thereafter to the Golgi apparatus and trans-Golgi network, where processing is completed and the receptor in transit is ready to continue their outward trafficking to the PM and become accessible to agonist (Broadley and Hartl 2009). Interaction between GPCRs and cognate agonists at the PM then stimulates downward trafficking which begins with internalization of the receptor through a series of distinct posttranslational modifications that include phosphorylation (which terminates G protein-mediated signaling) and β-arrestin recruitment, albeit in certain cell contexts GPCRs do not require β-arrestins for internalization (van Koppen and Jakobs 2004). β-arrestin recruitment by the phosphorylated receptor then allows interaction with clathrin and the clathrin adaptor AP2 to drive receptor internalization into endosomes, and either recycling of the receptor back to the PM or targeting to the lysosomes and/or proteasomes for degradation (Pavlos and Friedman 2017) (Fig. 1). Thus, a balance between synthesis and subsequent trafficking from the ER to the PM and the endocytosis-recycling/degradation pathway determine the net amount or density of functional receptor protein at the PM available to interact with agonist and provoke a biological response. Nonetheless, before reaching their final destination (e.g., the cell surface PM), newly synthesized GPCRs must be subjected to conformational screening by a strict quality control system (QCS) that monitors, and corrects if necessary, the folding of the nascent receptor into a three-dimensional structure compatible with ER export (Ulloa-Aguirre and Conn 2009). Monitoring the structural and conformational correctness of newly synthesized proteins by the QCS then determines the protein pools that must either be retained at the ER and eventually degraded in proteasomes or sorted to the Golgi apparatus and thereafter to the cell surface PM (Ulloa-Aguirre et al. 2004a). Thus, the QCS prevents accumulation of misfolded proteins that may aggregate and interfere with cell function. G protein-coupled receptor export from the ER to the Golgi is modulated by the interaction of the trafficking proteins with specialized folding factors, escort proteins, retention factors, enzymes, and members of the molecular chaperone families, which belong to the ER QCS and the so-called proteostasis network (Hartl et al. 2011; Hartl and Hayer-Hartl 2002; Hutt et al. 2009; Ron and Walter 2007). Specifically, molecular chaperones are key components of the ER QCS that screen native receptor conformation and promote delivery from the ER (Ellgaard et al. 2016; Ulloa-Aguirre et al. 2004a). Molecular chaperones not only recognize, but also retain and target misfolded, non-native protein conformers to degradation via the polyubiquitination/proteasome pathway (Chevet et al. 2001; Klausner and Sitia 1990; Schubert et al. 2000; Werner et al. 1996). Molecular chaperones may also disassemble protein aggregates and guard nascent polypeptides against unproductive and potentially toxic interactions that may occur during the various stages of folding (Duennwald et al. 2012). Surveillance of the QCS for correct folding and assembly of newly synthesized proteins relies more on some general structural features of the client protein (unpaired cysteines, exposure to hydrophobic shapes, immature glycans and specific sequence motifs), and thus possess the ability to recognize misfolded proteins when they expose hidden hydrophobic domains or particular sequences. For example, removal of the conserved ALAAALAAAAA