Summary

The G protein-coupled receptors (GPCRs) are considered as very diverse and also surprisingly successful structures during the whole evolutionary process, being capable of transducing the different forms of “information” within the cell and also between cells, such as different peptides, lipids, proteins, nucleotides, nucleosides, organic odorants and photons. Complex studies as well as two-dimensional crystallization of rhodopsin, their paradigm, led to the creation of a useful model having a common central core, consisting of seven transmembrane helical domains, which undergoes appropriate structural modification during activation and signal transduction.

After the complete delineation of the human genome, which is the apogee of human scientific civilization and culture, it was possible to identify more than 800 human GPCR sequences and in parallel analyze 342 unique functional nonolfactory human GPCR sequences with phylogenetic analyses. These results support, with high bootstrap values, the existence of five main families, named by the authors glutamate, rhodopsin, adhesion, frizzle/taste2, and secretin, forming the GRAFS classification system. Positions of the GPCRs in chromosomal paralogous regions indicate the importance of tetraploidizations or local gene duplication events during their creation. Some families of GPCRs show, however, very little or no similarity in the sequence of amino acid chains. They utilize an enormous number of different domains to bind ligands and to activate the appropriate G-proteins. The delicate tuning of their coupling to G proteins is further regulated by splicing, RNA editing and phosphorylation. A number of GPCRs may also form homodimers or heterodimers with structurally different GPCRs and also with membrane-bound proteins having one transmembrane domain. It should also be stressed that not all GPCRs are strictly faithful to G proteins because growing evidence indicates that they can interact directly, via their C-terminal domain, with proteins containing PDZ domains. These proteins organize the NMDA receptors and some K⁺ channels while their PDZ domains generally bind 3-4 amino-acid stretches of C-terminal sequences of target proteins. The -S/TXV motif was found in some PDZ target proteins. GPCRs can also interact with the Enabled/VASP homology (EVH)-like domain which interacts directly with group 1 mGluR receptors.

Every year brings new very important data in research of these proteins, which should be called "the most successful structures evolved during the whole of animal evolution".

Key words: receptor · GPCRs · signal transduction · evolution · genes · chromosome · GnRH · oxytocin · vasopressin · classification of GPCR receptors · rhodopsin · x-ray crystallography
INTRODUCTION

In the evolutionary process, in parallel with the formation of multicellular organisms, evolution formed the appropriate mechanisms of communication within the cell and between cells in order to allow cooperation in cell maintenance and among different functions.

About 25 years ago, it was discovered that membrane-bound receptors, dedicated to recognizing intercellular messenger molecules such as hormones, neurotransmitters and growth factors, and several sensory messages such as light and odors, belong to four or five protein families; the most common is the G protein-coupled receptor (GPCR) family. The diversity of structures and functions of GPCRs is immense. In vertebrates, this family contains 1000-2000 members, including more than 1000 coding for odorant and pheromone receptors [10].

GPCRs are involved in the recognition and transduction of messages as different as Ca²⁺, light, odorants, small molecules, including amino acid residues, nucleotides, peptides and proteins (Figure 1). They regulate the activity of enzymes, ion channels and transport of vesicles via the catalysis of the GDP-GTP exchange on heterotrimeric G proteins (Gα, Gβ, Gγ) (Figure 1) [10,11,30]. Comparison of sequences between the different GPCR proteins showed them to be different receptor families which have no sequence similarity. All these receptors, however, have in common a central core domain consisting of seven transmembrane helices (TM1-TM7), connected by three intracellular coils (IC1-IC3) and three extracellular C loops EC1-EC3 [5]. Two cysteine residues are present in EC1 and EC2, which are conserved in most GPCRs, forming disulfide bonds, which is highly important for the packing and stabilization of a restricted number of conformations among the seven TMs. Individual GPCRs have different length and function of their N-terminal EC domain, their C-terminal IC domain, and their intracellular loops, which provide among others specific properties to these different receptor proteins [10]. The seven TM (7TM) region constitutes the core domain of these receptors, and a change in configuration of this domain is certainly responsible and important for receptor activation. The switch from the inactive to the active conformation state is associated with a parallel change in relative orientation of TM-3 and TM-6, with a rotation of TM-6 and its separation from TM-3, which unmasks G protein-binding sites [10,12,21,38]. In the 1 GPCR family, one residue (Asp) in TM-2 and a tripeptide (DRY and ERW) at the interface of TM-3 and IC-2 are important for receptor activation [68,78].

The change in conformation of the core domain of a receptor generally affects the conformation of the 1-2 and 1-3 intracellular loops, which are directly linked to TM-3 and TM-6, respectively; that constitutes one of the key sites for G-protein recognition and activation [10,72,83,91].

It should be pointed out that a large variety of molecular mechanisms have been selected during evolution to allow the diverse, natural ligands to induce a change in conformation and activate the core domain of the receptor [10]. In the light-activated receptor, rhodopsin, the target of photons, retinal, is covalently linked in the cavity formed by TM-3 to TM-6, and its change in conformation induced by light activates the receptor. Other families of GPCRs are activated by short peptides which interact with the extracellular loops and the N-terminal domain. For the family of receptors which are activated by large peptides like glucagon or secretin, VIP or PACAP, the relatively long N-terminal domain also plays a role in the binding of the ligand [70].

The discovery of homo- or heterodimerization made a revolution in the current concepts of GPCR structures and functions. The functional analysis of chimeric and mutated receptors revealed that they can dimerize [53,62] possibly via a coiled-coil interaction of their sixth TM. Indeed, a peptide corresponding to the sixth TM of the β₂-adrenergic receptor inhibits both receptor dimerization and activation [31], suggesting that GPCR dimerization may be important for G-protein activation. Certain GPCRs need to form heterodimers with one TM domain protein, in order to be correctly folded, exported to the membrane, and in the case of CRLR (calcitonin receptor-like receptor) to obtain its final identity. It has been known for many years that nine A from Drosophila melanogaster and its vertebrate homologue, RanBP2, two cyclophilin-related proteins, bind opsins for folding and transport [4,22].

All these mentioned and many other data reveal a new level of complexity in the functioning of 7TM proteins, and may be of great help to elucidate the function of many GPCRs for which either the transduction pathway or the ligand (the so-called orphan receptor) is unknown [10]. Splicing with an insertion localized mainly at the
third intracellular loop or C-terminal domain has been selected during evolution to modify or regulate the specificity and intensity of GPCRs coupling to G proteins [39].

A very subtle regulation was discovered concerning GPCR coupling to G proteins. Transcripts encoding the 5-HT$_{2C}$ receptor, a PLC-coupled receptor, undergo RNA editing events in which the genomically encoded adenosine residues are converted to inosines by double-stranded RNA adenosine deaminases. Seven major 5-HT$_{2C}$ receptor isoforms are predicted, encoded by 11 distinct RNA species and differing in their second intracellular loops [14]. This post-transcriptional modification leads to a 10- to 15-fold reduction in efficacy of the coupling of 5-HT$_{2C}$ to the G protein. Generally, the post-transcriptional and post-translational modifications of GPCRs were selected during the evolutionary pathway to perform fine tuning of G proteins [10,81].

It was found that some GPCRs are unfaithful to G proteins and interact directly, via their C-terminal domain, with proteins containing PDZ and Enabled/VASP homology (EVH)-like domains. β$_2$-AR is unfaithful to G proteins in establishing a direct interaction between their C-terminal domain (DSSL) and a PDZ domain of the Na$^+$/H$^+$ exchanger regulatory factor (NHERF) [28]. mGluR1a and mGluR5 are unfaithful to G proteins in establishing a direct interaction between their Homer ligand sequence (PPXXP) and the EVH-like domain of Homer proteins [10,88,92].


**General and Methodological Information**

The superfamily of G-coupled receptors (GPCRs) is one of the largest families of proteins in the mammalian genome, which was evaluated after delineation of the complete structure of this genome [49,89]. The ligands for the GPCRs have great variation; the GPCR proteins are also highly variable. There are two main requirements for a protein to be classified as a GPCR. The first requirement relates to seven sequence stretches of about 25 to 35 consecutive residues that show a relatively high
degree of calculated hydrophobicity. These sequences are believed to represent seven α-helices that span the plasma membrane in a counter-clockwise manner, forming a receptor, or a recognition and connection unit, enabling an extracellular ligand to exert a specific effect on the cell. There second principal requirement is the ability of the receptor to react with a G-protein. There is great diversity in the functional coupling of the GPCRs; they have a number of alternative signaling pathways, interacting directly with a number of other proteins. Interaction with G-proteins has not been demonstrated for most GPCRs, in particular for those whose genes have just recently been sequenced. It may therefore be more technically correct to term this superfamily „seven transmembrane (TM) receptors”, but the GPCR terminology is more established [24]. G protein-coupled receptors (GPCRs) constitute a highly diverse and ubiquitous family of integral membrane proteins, transmitting signals inside the cells in response to an assortment of disparate extracellular stimuli. These receptors comprise the largest family of integral membrane proteins in human and animal genomes, having in humans about 800 GPCR family members identified [24].

The more than 800 identified human GPCR sequences together with 342 simultaneously analyzed unique functional nonolfactory human GPCR sequences underwent precise phylogenetic analyses. The obtained results reviewed five main families, named glutamate, rhodopsin, adhesion, frizzled/taste2, and secretin, forming the GRAFS classification system. The rhodopsin family is the largest and constitutes four main groups with 13 sub-branches. Positions of the GPCRs in chromosomal paralogous regions indicate the importance of tetraploidizations or local gene duplication events for their creation. New data showed several common structural features indicating that the human GPCRs in the GRAFS families share a common ancestor. It was possible to create a map of the GPCRs in a single mammalian genome. The new approach of precisely analyzing such large and diverse sequence sets may also be useful for the study of GPCRs in other genomes and divergent protein families [24]. These receptors communicate signals across the plasma membrane in response to a variety of extracellular stimuli, ranging from photons, ions and small molecules to peptides and proteins. The signals are amplified and transmitted to downstream effectors inside the cells primarily through coupling to heterotrimeric guanine nucleotide binding proteins (G proteins). Upon activation, receptors trigger complex cascades of reactions controlling crucial physiological and cellular processes [17]. GPCR family members exhibit common architecture of the 7 transmembrane α-helical bundle [9]. It is highly intriguing how such a relatively simple scaffold has evolved to selectively bind to thousands of diverse ligands transmitting signals to dozens of different effectors. The basic questions in the field of GPCR research include precise understanding of mechanisms of signal transduction and aspects of ligand selectivity and specificity. Many substantial questions can be answered only when biophysical and biochemical studies are combined with high-resolution delineation of structures of these proteins stabilized in particular functional states [17]. Development of integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors (GPCR) was essential for the progress of research in this fascinating field. The rapid pace of cloning and expression of G coupled receptors offers attractive opportunities to probe the structural basis of signal transduction mechanisms at the level of cell-surface receptors. Major insights have emerged from comparisons and classifications of the amino acid sequences of GPCRs into families defined by evolutionary developments and adapted to perform selective functions. Structural data on GPCRs, based on biochemical, immunological, and biophysical approaches, have validated consensus architecture of GPCRs with an extracellular N-terminus, a cytoplasmic C-terminus, and a transmembrane portion comprised of seven-transmembrane helical domains connected by loops. Developments in the molecular modeling and computational exploration of GPCR proteins indicate a tantalizing potential to alleviate some of these difficulties [6].

Agonist binding to G protein-coupled receptors (GPCRs) drives the receptor to assume a structure that can bind and activate the heteromeric G protein [9,10].

Rhodopsin, the visual pigment in rod photoreceptor cells, represents a paradigm for structure-function studies of GPCRs [33]. Detection of the photon is mediated by the 11-cis isomer of retinal, which in the dark acts as an inverse agonist on rhodopsin with an estimated half-life for thermal isomerization of 420 years [67,80]. In a very rapid, highly selective and effective reaction, light triggers conversion of the 11-cis double bond to trans, which initiates the visual cascade, leading to closure of ligand-gated calcium channels and excitation of the visual nerve [69]. At that time, three high-resolution structures of rhodopsin were published [66,69,79,86].

Okada et al. [67] described and discussed the new results of an X-ray structure study of rhodopsin. Substantial improvement of the resolution limit to 2.2 Å was achieved by new crystallization conditions, which also significantly reduced the probability of merohedral twinning in the crystals. The new structure completely resolved the polypeptide chain and provided further details of the chromophore binding site including the configuration about the C6-C7 single bond of the 11-cis-retinal Schiff base. Utilizing the results of earlier structure determinations and a new improved model of protein, the authors carried out a theoretical study of chromophore geometry using a combination of both quantum mechanics and force field molecular dynamics. They found significantly improved consistency between the experimental and calculated results for the 2.2 Å model, including the angle of the negatively twisted...
6-s-cis-bond. The new crystal structure refinement reveals significant negative pre-twist of the C11-C12 double bond and this is also supported by the theoretical calculation although the latter converges to a smaller value. Bond alteration along the unsaturated chain is significant, but weaker in the calculated structure than the one obtained from X-ray data [67].

Rapid progress in studying GPCRs, despite their high importance, was problematic due to difficulties in isolating large quantities of these membrane proteins in forms that retain ligand binding capabilities. Creating water-soluble variants of GPCRs by mutating the exterior, transmembrane residues provides a potential method to overcome these difficulties. Perez-Aguilar et al. [71] presented the first study involving the computational design, expression and characterization of a water-soluble variant of a human GPCR, the human mu-opioid receptor (MUR), which is involved in pain and addiction. An atomistic structure of the transmembrane domain was built using comparative (homology) modeling and known GPCR structures. This structure was highly similar to the subsequently determined structure of the murine receptor and was used to computationally design 53 mutations of exterior residues in the transmembrane region, yielding a variant intended to be soluble in aqueous media. The designed variant was expressed in high yield in Escherichia coli and was water soluble. This study exemplifies the computational approach to produce water-soluble variants of GPCRs amenable for more precise structural and functionally related characterization of them in aqueous solution with great hope to obtain new and exceptionally important results [71].

**Classification of GPCRs**

Several classification systems have been used to classify this superfamily. Some systems group the receptors by how their ligand binds, and others have used both physiological and structural aspects. One of the most frequently used systems uses clans (or classes) A, B, C, D, E, and F, and subclasses are assigned using roman number nomenclature [3,47]. This A-F system is designed to cover all GPCRs, in both vertebrates and invertebrates. Some families in the A-F system do not exist in humans. Certain species also exhibit a large difference in the numbers of receptor genes in different classes [24].

Fredriksson and co-workers [24] did enormous and greatly valuable research work, utilizing also the results of delineation of the complete human genome, collected a large set of GPCR sequences in the human genome and performed multiple phylogenetic analyses. They compiled a comprehensive data set with just a single copy of each gene. This approach allowed polymorphisms, pseudogenes, duplicates, and other related problems to be avoided. They identified more than 800 GPCRs in databases and simultaneously analyzed sequences of 342 unique functional nonolfactory human GPCRs and grouped them by phylogenetic analysis. The chromosomal localization and positioning in paralogous groups of the genes were studied to give an insight into the mechanism involved in creating the receptor genes. The different families were also studied, by the authors, for common sequence motifs, and finally they discussed the evidence for common descent of the families [24]. It should be underlined that they performed the first great phylogenetic study of the entire superfamily of GPCRs in a single mammalian genome. The detailed studies show, with high bootstrap support, that there are five main families of human GPCRs (Figure 2). Each of the receptors that are placed in one of the five families show appreciable bootstrap value in support of a phylogenetic relationship to the respective family. The results evidenced that the members within each family share a common evolutionary origin. The overall classification of the GPCRs has been hampered by large sequence differences between mammalian and invertebrate GPCRs. The GPCRs in Drosophila melanogaster show in several cases little resemblance to those in mammals [13]. Certain species also show a large difference in the numbers of receptor genes in different classes. Caenorhabditis elegans, a worm, has developed a remarkable number of chemosensory (olfactory) GPCRs related to the creature’s specific lifestyle. The chemosensory and olfactory receptors in Drosophila melanogaster have only slight resemblance to the olfactory receptors in humans [24].

Gene duplication occurs both by individual duplication, which often leaves the new gene near the parent gene, and by block duplications involving chromosomal regions or entire chromosomes. Large-scale duplications, including polyploidizations, are an important mechanism of vertebrate evolution. Two rounds of large-scale duplications, which presumably occurred in early vertebrate ancestry [32,52], resulting in up to four copies of each gene in mammals, originate from a common ancestor gene in a cephalochordate. It is now known as the “2R hypothesis” or “one-to-four model”. This led to the construction of maps that contain paralogous chromosomal regions, or paralogs [32,42,52,73], in vertebrates, which in combination with phylogenetic analysis can provide valuable information on gene relationships and origins. It is appropriate to mention here that sequenced genomes offer a new possibility to distinguish paralogues from orthologues, by comparing the location of the genes relative to neighboring genes on chromosomes. Genome databases can ideally provide a complete listing of the family members in each species. Many vertebrate gene families have expanded in two basal tetraploidizations (2R), and the teleost fish in a third tetraploidization (3R), after which some vertebrate lineages lost some of the duplicates [50].

Fredriksson and co-workers [24] assembled a primary data set of 802 unique GPCRs from the human genome completely delineated. They stated that the data taken for analysis contain most of the functional GPCRs in the
human genome. The obtained results indicate that the receptors cluster in five main families which they termed glutamate (G, with 15 members) rhodopsin (R, 701), adhesion (A, 24), frizzled/taste2 (F, 24), and secretin (S, 15), to which they applied the acronym GRAFS. Twenty-three protein sequences could not be assigned to any of the five families with appreciable bootstrap values (above 50%); these were placed by the authors separately as “other 7TM receptors.”

The rhodopsin family of the GPCR super-family of receptors (241 nonolfactory, total of 701) has the largest number of receptors. The rhodopsin family in this classification corresponds to what has previously been called either the rhodopsin-like receptors or class A in the A-F classification system. This family has several characteristics such as the NSxxNPxxY motif in TM-7, the DRY motif or D(E)-R-Y-(F) at the border between TM-3 and IL-2. Only a few receptors do not comply with these motifs, but these have other “fingerprint” elements that clearly link them to the rhodopsin family, apart from the phylogenetic analysis [24]. There are important exceptions, in particular for the glycoprotein binding receptors in this

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Fig. 2. Phylogenetic relationship between the GPCRs (TM1- TMVII) in the human genome. The tree was calculated using the maximum parsimony method on 1000 replicas of the data set terminally truncated GPCR, as was described under Materials and Methods in [24]. The position of the rhodopsin family was established by including twenty random receptors from the rhodopsin family. These branches were removed from this Figure for the better clarity of reading and replaced by an arrow toward the rhodopsin family receptors analysis, which was shortly described in the text of this article but precisely shown and discussed in the original authors article [24]. For details concerning the super-family of receptors tree, please see the paper of [24], modified.
The α-group of rhodopsin receptors (89). This group has five main branches: the protaglandin receptor cluster (15), the amine receptor cluster (40), the opsin receptor cluster (9), the melatonin receptor cluster (3) and the MECA receptor cluster (22). The bootstrap values that define these branches are very high.

The β-group of rhodopsin receptors (35). This group has no main branches and includes 36 receptors. All known ligands to these receptors are peptides: the group includes among others the thyrotropin releasing hormone receptor (TRHR), the ghrelin receptor, arginine vasopressin receptors (AVPRs) the gonadotropin-releasing hormone receptors (GnRHRs), the oxytocin receptor (OTR), and orphan receptors [24].

The γ-group of rhodopsin receptors (59). This group has three main branches: the SOG receptor cluster (15), MCH receptor cluster (2), and the chemokine receptor cluster (42). The bootstrap values that define these branches are high.

The δ-group of rhodopsin receptors (58, plus an estimated 460 olfactory). This group has four main branches: MAS-related receptor cluster (8), glycoprotein receptor cluster (8), purine receptor cluster (42), and the olfactory receptor cluster (estimated at 460). The glycoprotein receptor cluster contains the classic glycoprotein hormone receptors such as FSHR and TSHR [24].

Summarizing this part, Fredriksson et al. [24] in their outstanding and very valuable work generated the first map for one of the most studied GPCR superfamilies of proteins present in the human genome. They evidenced the existence of five distinct families of the GPCR superfamily and clearly showed the relationship of the genes within the subgroups of the great rhodopsin protein family. They also created a map which is very useful for further comparison of GPCRs in different species and will be very helpful in the better understanding of the path along which the structural and functional properties in this family evolved. The paralogon analysis brings strong evidence for common descent of the phylogenetic clusters and indicates how exon shuffling played a role in composition of some of the receptor genes. The diversity of structural elements found in this family of receptors may present excellent examples of evolutionary mechanisms that are formulated in this publication and certainly has general importance for analysis of many other protein families, typically those that share α-helical domains and TM regions that are combined with other functional elements.

The GnRH receptor and oxytocin/vasopressin receptors were selected for the illustration of the structure of individual receptors in this article, although I am aware of their relative similarity in structure, due to my previous research interest in these receptors; the reader may easily obtain more information on them, in two of my other publications [45,46].

Receptors of GnRH (GPCR superfamily)

β-group of rhodopsin receptors (according to the new classification of Fredriksson et al. [24])

Determination of the amino acid sequence in GnRH receptor type 1 was done for the first time in mouse after cloning this receptor from cell line aT3, and subsequently was validated by other publications [59,60]. After this achievement, it was isolated and DNA was identified in human [18,41], four mammals – rat [19], sheep [35], cow [41], pig [90] – and in wolffish [87]. The amino acid sequence of the GnRH receptor is highly conservative in these 6 species; identity is higher than 85%, while they are nearly identical within their TM domains. In human, cow and sheep GnRH receptor (GnRHR) molecules have 328 amino acid (AA) residues, but receptors in mice and rat have a 327 AA chain, because one residue is absent in the second extracellular domain (EC). The receptor of wolffish has 370 AA residues, because it also possesses 49 AA at the carboxyl end as an ending domain, which is absent in mammals [58]. The structure of the GnRH gene is shown in Figure 3. Cells of the pituitary aT3-1 cell line have in the GnRH gene cDNA a proximal 173-bp flanking region, which directs the expression of the GnRH gene in gonadotropic cells [63]. This regulatory region possesses two specific elements for gonadotropic cells having the sequence 5‘TGA/TCC-3’. Such elements ensure specific expression of a subunit of glycoprotein hormone in the particular cell [23] and LHβ [29] of genes in pituitary gonadotropes. Functional importance of steroidogenic factor-1(SF-1) in regulation of the GnRH gene was also discovered and confirmed by the results of mRNA expression type sense and anti-sense, when there was stimulation or repression of the native promoter, respectively [63]. Such gene expression, specific for a particular tissue, may be mediated in the specific tissue by differential utilization of the promoter in particular cell types [81].

Studies of homologous activation of the GnRH promoter of the mouse pituitary gonadotrope cell model aT3-1 cell line showed the important role of signalization path type consensus activating protein-1(AP-1), PKC and ERK1/2 [64]. This evident stimulatory influence of GnRH may be amplified through the pretreatment of activin A, which is inhibited by follistatin [65]. Homologous repression of the promoter of human GnRHR goes through active binding of c-Fos DNA in the place of AP-1. Such negative regulation of transcription mediated by GnRHR type I may serve as a mechanism of desensitization of pituitary
cells in the situation of prolonged stimulation. It is worth noting that in the situation when the same conditions induce a maximal stimulatory effect of GnRHR promoter of mouse and rat [64], substantial inhibition of the respective promoter in humans takes place [16]. These experiments support very well the hypothesis of the existence of specific mechanisms of transcriptional regulation of GnRH genes for individual species [15].

GnRH binds to protein G\textsubscript{q/11} in cells of the pituitary αT3-1 cell line. However, GnRHR also binds to protein G\textsubscript{s} in primary cells of the pituitary and in cells of the G-GH3 cell line, and when this protein activates adenyl cyclase, it leads in consequence to the synthesis of cAMP and to activation of protein kinase A (PKA) [51]. It was proven that GnRHR is also able to bind to all sub-families of G proteins – G\textsubscript{q/11}, G\textsubscript{s}, and G\textsubscript{1} – in the situation when it undergoes super-dispersion in cultures of pituitary cells and the G-G3 cell line. This fact is very important during every estimation of GnRHR binding to different G proteins and because of the possible danger of misinterpretation by making the extrapolation in studies of one cell type to another type [51].

Binding of GnRH to its receptor induces the heterotrimeric G proteins. This interaction initiates many intracellular reactions, among others the increase of metabolism of phosphoinositides, which in turn cause the elevation of intracellular diacylglycerol and calcium ions, but also to a certain degree the increase of cAMP concentration, which activates protein kinase C (PKC) and protein kinase A (PKA) [40].

In this way, activation of the transcription process of gonadotropin subunits begins. Many experiments also indicate that binding of GnRH to GnRHR leads to activation of phospholipase C and synthesis of 1,4,5-triphosphoinositol (IP\textsubscript{3}) and diacylglycerol, as it was mentioned before, which directly causes the increase of Ca\textsuperscript{2+} and activation of protein kinase C [51,59].

The signaling cascade sometimes needs the presence of direct and constant contact of a mediator with activated protein. Interruption of this interaction by introduction of one of the binding domains into the interior of the cell may block specific signaling paths. It was shown that interaction GPCR/G proteins causes the interruption, in vitro, of the activation process by peptides derived from the C end of G protein [84].

Receptor activation subsequently activates the signaling protein families ERK, N-terminal kinase c-Jun and p38 MAPK in the L\textsubscript{β}T2 cell line [51]. In these cells, there takes place expression of mRNA for GnRHR and subunits α\textsubscript{LH}, and β\textsubscript{LH}, and β\textsubscript{FSH}. For that reason, they are a relatively good model to study the signaling mechanisms and biosynthesis of pituitary gonadotropins [51]. Activation of ERK in the nucleus proceeds through via processes with the participation of PKC and MEK, and is dependent of them, while it is independent of calcium. There is also induction of c-Fos proteins and bLH. Induction of genes of both these proteins is independent from PKC in the cell line of L\textsubscript{β}T2 [51].

Activation of the signaling path after binding of GnRH to the receptor begins when protein G\textsubscript{qγ} is released from G\textsubscript{q}. Fig. 3. Two-dimensional structure of the human GnRH receptor. The 7-TM domains (boxed) are connected by 3 ECLs and 3 ICLs. Ligand binding residues (red) and residues thought to be important in receptor structure or binding pocket configuration (green) are shown. These include disulfide bond formation and glycosylation sites. Residues involved in receptor activation are shown in blue. Residues in squares are ones highly conserved throughout the rhodopsin family receptors (GPCRs). Residues involved in coupling to G proteins are shown in orange. Putative protein kinase C (PKC) and protein kinase A (PKA) phosphorylation sites are indicated. The intermolecular interactions between GnRH I residues and the receptor are indicated with red lines ([59,60], modified)
or $G_i$. Isoform $\beta_2$ of phospholipase C is activated by $G_{\beta\gamma}$ in a similar way as adenylate cyclase 2. Signaling may progress through the participation of $G_{\beta\gamma}$ in both signaling pathways, namely in $G_q$ and $G_i$, but there is a lack of inhibiting effect when $G_{\beta\gamma}$ is blocked, or after injection of GST-\betaARK, and the GnRH signaling proceeds mainly through the $\alpha$ subunit [51].

The best documented mechanism of GnRH action is the signaling pathway that is followed after binding to protein $G_q$; however, many results also indicate the physiological significance of cAMP in the pituitary as the mediator of GnRH action. The third intracellular coil (IC) of rat GnRHR binds both protein $G_q$ and $G_i$, which mediate the signaling paths of the G-GH3 cell line. cAMP signaling is dependent on the specific residues in the coil, which are not important for activation of the phosphoinositide signaling path [2]. Both GnRH and cAMP activate the promoter of GnRHR in mouse through an element reacting with cAMP in the G-GH3 cell line [55,56].

Studies utilizing pituitary cells of the fish tilapia showed that GnRH induces both genes of subunits $\alpha$ and $\beta$FSH and found good sensitivity of these cells to the inhibition of PKA. These data suggest the activation of cAMP signaling [27,51]. Simultaneously, induction of $\beta$LH was relatively resistant to inhibition of PKA but very sensitive to signalization of PKC and MEK. It was also found that resistance of pituitary cells to the action of GnRH may be induced through chronic signalization stimulating $G_q$ protein [51].

Receptors of oxytocin and vasopressin; GPCR superfamily, $\beta$-group of rhodopsin receptors (according to the new classification of [24])

**Receptor genes.** First identification of complete cDNA of the human oxytocin receptor (OTR) was done by Kimura et al. [43,44] utilizing the cloning strategy. After this discovery, the following sequences encoding OT from pig [26], rat [75], sheep [74], bovine [8], mouse [48], and rhesus monkey [76] were also isolated and identified [25].

The OTR is a typical member of the rhodopsin-type GPCR family. The human mRNA for the OT receptor (OTR) has two sizes, 3.6 kb in breast and 4.4 kb in ovary, endometrium and myometrium. The OTR gene is a single copy in the human genome and is localized as the gene locus 3p25–3p26.2 [36,57,82]. The gene spans 17 kb and contains 3 introns and 4 exons. Exons 1 and 2 cor-
respond to the 5'-prime noncoding region [25]. Exons 3 and 4 encode the amino acids in the OTR. Intron 3, the largest at 12 kb, separates the coding region immediately after the TM-6. Exon 4 contains the sequence encoding the TM-7, the COOH terminus, and the whole 3'-noncoding region, including the polyadenylation signals (Figure 4) [25].

The G-protein-coupled receptors (GPCR) normally do not possess introns, but the gene for the OT receptor has 3 introns; the gene for human V2 receptor contains one intron at the same location after transmembrane domain 6, similarly as the receptor family for GnRHs. The transcription start sites lie 618 and 621 bp upstream of the initiation codon, as demonstrated by primer extension analysis. Similarly, a TATA-like motif and potential SP-1 binding site are found in the human OT receptor gene [25]. The 5'-flanking region contains in its structure: inverted GATA-1 motifs, one c-Myb binding site, one AP-2 site, two AP-1 sites, and one, incomplete, ERE. Two half-palindromic 5’-GGTCA-3’ motifs and one half-palindromic 5’-TGACC-3’ motif of ERE have been found. Moreover, there are two nucleofactor interleukin-6 (NF IL-6) binding consensus sequences and two binding site sequences for an acute phase reactant-responsive element [25].

In the mouse OT receptor gene, the promoter region lacks an apparent TATA box but contains multiple interleukin-6-response elements, several half-palindromic motifs, and a classical ERE [48]. Rat OT receptor gene expression at parturition contains three transcripts that were identified and found to differ in the length of their 3'-untranslated regions [75]. The promoter region of the rat OT receptor gene also contains multiple putative interleukin-responsive elements, NF-6, and acute-phase response elements (APRE) [75].

Receptor proteins. The OT receptor is a polypeptide 389 amino acids long, with 7 transmembrane (TM) domains, and belongs to the G protein-coupled receptor (GPCR) superfamily. The active OT and VP receptor proteins are typical members of the rhodopsin-type I GPCR family (Figure 4). The seven transmembrane α-helices are highly conserved in the prevailing receptors of the GPCR family members. Generally, conserved sequence residues in GPCRs participate in a functional mechanism activating signal transduction to the G protein. In this type of receptor, the switch from the inactive to the active conformation is associated with a simultaneous change in the relative orientation of transmembrane domains 3 and 6, after which the G protein binding sites are unmasked.

Peripheral vasopressin and oxytocin receptors have been classified on the basis of both the second messenger system coupled to the receptors and the abundant series of vasopressin and oxytocin analogues with enhanced selectivity for a certain receptor type. These classification criteria have led to the distinction of V1a vasopressin (liver, smooth muscle cells from blood vessels, and most peripheral tissues expressing vasopressin receptors), V1b vasopressin (adenohypophysis), V2 vasopressin (kidney), and oxytocin (uterus, mammary gland) receptors [7,37].

Adenohypophysis V1b receptors also stimulate phospholipase C. Oxytocin receptors activate phospholipase C and induce an increase in the cytosolic Ca²⁺ concentration, which results in strong contractions of the uterus at term [1,54,64]. Single amino acids have been found to be important for the activation of this family of receptors. In the case of the rat V1a vasopressin receptor, the functional importance of the conserved Asp97 residue in TM II has been confirmed subsequently by mutational analysis; this was also found in many other GPCRs [77]. In human oxytocin receptors, it was found that arginine vasopressin (AVP) may act either as a complete agonist, as demonstrated by analysis of the electro-physiological response in Xenopus oocytes [43], or as a partial agonist, as demonstrated by measurement of the release of inositol phosphate in various cell lines. The latter property was utilized to identify amino acid residues that have a key role in regulating agonist-oxytocin receptor interactions. It was found that Tyr209 in the TM V and Phe284 in the TM VI of the human oxytocin receptor play such a role. Another highly conserved residue was shown to have a crucial role in the vasopressin/oxytocin receptor activation process. The conserved Pro322 situated on TM VII in the human V2 receptor is probably necessary to allow the relative movements within the helical bundle that are required for receptor activation [7].

Oxytocin receptors (OTR) are abundantly expressed in the ventromedial hypothalamus (VMH) in the rat brain. It is known that expression of these receptors is estrogen-dependent, but the OTRs present in the central nucleus of the amygdala do not appear to be so. The gene was isolated from a rat genomic library and its protomer region analyzed for response elements. Several regulatory elements of interest were identified, including cyclic adenosine monophosphate (cAMP) response element (CRE), a serum response element (SRE), and several activator protein-1 elements (AR-1), in addition to a palindromic estrogen response element (ERE). In transfection assays, protein kinase C (PKC) and protein kinase dependent on cAMP (PKA) activation by phorbol ester and forskolin treatment optimally enhanced the transcription of the OTR [20,85].

VP and OT strongly modulate autonomic fear responses, although the mechanisms of that are still not quite clear. Certainly, their action is through the network of their receptors abundantly present in the central and autonomic nervous systems. These neuropeptides excite distinct neuronal populations in the central amygdala, which provides the major output of the amygdaloid complex to the autonomic nervous system [34]. These authors identified two neuronal populations as part of an inhibitory network through which VP and OT modulate the integration of excitatory information from the
basolateral amygdala and cerebral cortex, in opposite manners. Through this network, the expression and endogenous activation of VP and OT receptors may regulate the autonomic expression of fear [34].

The possibility of research, in the near future, with high-resolution structures of vasopressin and oxytocin receptors will greatly facilitate establishing the precise three-dimensional models of these receptors and, subsequently, obtaining new knowledge on the receptor domains responsible for agonist or antagonist binding and for G protein activation [7]. Please also consult the author’s articles on GnRH and its evolution and on neurohormones and their evolution [45,46].

Concluding reflections

Professor Joel Bockaert (in the publication together with Dr J.P. Pin, 1999) reminds us of the famous idea of François Jacob: “Evolution is molecular tinkering”. This sentence possesses very deep biological meaning but also simultaneously very important philosophical and aesthetic meanings.

In the 2012 year publication, I mentioned that the knowledge of the fact that neurohormonal decapeptide GnRH II has a structure unchanged during evolution for 400 millions years, which is so perfect that it is not possible to improve its physiological value with any natural amino acid residue substitution, is very beautiful.

I would like to conclude that esthetic aspects of science have been important through the centuries both to scientists and to people in general.

The great scientist Maria Skłodowska-Curie said: “In my opinion, science is something very useful and also very beautiful.”

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