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Review Article

SEVEN TRANSMEMBRANE RECEPTORS (7TM) IN THE VIEW OF DIMERIZATION AND EXPERIMENTAL METHODS TO STUDY THEIR DIMERIZATION AND CROSS-TALK

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Summary: Seven transmembrane (7TM) receptors constitute the largest and the most diverse superfamily of proteins encoded in the human genome comprising at least 850 putative members. These receptors are widely expressed in the body and play a fundamental role in physiology and pathophysiology. Not only do they regulate many physiological processes, but drugs that target these receptors and either activate or inactivate them, account for the most prosperous drugs sold worldwide. Of approximately 500 currently marketed drugs, more then 30 % are modulators of 7TM receptor function. In the last two decades, impressive progress in the understanding of 7TM receptor function has been achieved, though dimerization or oligomerization of 7TM receptors is still a novel and controversial concept. Although a large quantity of data, obtained by different biophysical, biochemical, structural and functional approaches e.g. coimunoprecipitation, Western blot, bioluminescence and fluorescence resonance energy transfer (BRET and FRET, respectively) argue for dimerization or oligomerization of these receptors, several publications criticized the applied methods and challenged the concept. In this paper the main and the most important techniques are presented and complemented with our ideas.

Key words: 7TM receptors, structure, dimerization, experimental methods

Introduction

With its 865 members, according to recent bioinformatics analysis of human genome (1) the group of seven transmembrane (7TM) receptors, also known as G protein-coupled receptors constitute the largest superfamily of proteins in humans, as well as in animals. As the first name implies, these integral membrane molecules share a common tertiary structure characterized by seven membrane-spanning α- helices connected by alternating intra- and extracellular loops. As the second name implies, the receptors are able to signal by activating heterotrimeric intracellular guanine-nucleotide binding proteins (G-proteins) (2, 3).

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The 7TM receptors are targets for an extraordinary repertoire of extracellular endogenous ligands, which show a vast variation in structure, size and chemical nature. This includes large proteins, peptides, amino acids, lipids, biogenic amines, nucleotides and even single ions. This diverse group of ligands illustrates the involvement of these receptors in a great variety of physiological processes. What these diverse ligands have in common is their ability to functionally activate 7TM receptors and thereby propagate signals to the intracellular environment and in such way to serve as molecular switches for cells, which is crucial for cellular survival and cellular adaptation. As a result, receptors exert a wide range of physiological functions, for instance they mediate neurotransmission, hormone response, inflammation, regulate heart rate and blood pressure, modulate skin pigmentation, direct the site-specific

trafficking of leukocytes, control neuronal activity in specific areas of the brain, contribute to the progression of infectious diseases, or are involved in recognizing light-sensing molecules in the eye, several hundred of distinct odorants in the olfactory system and a large number of taste molecules in taste buds (2, 4, 5, 6, 7).

Defects in receptor signalling systems and their regulation are responsible for many diseases. These include defects in heart rhythm and motor skills, metabolic diseases like obesity and type II diabetes, mental disorders, as well as some viral infections, like those observed with influenza, herpesvirus infections and some forms of cancer. Given this functional diversity, it is not surprising that approximately a third of the commercially available drugs modulate 7TM receptor activity and 7TM receptors remain highly interesting, both from a biological perspective and as future drug targets (7). 7TM receptor agonists and antagonists have therapeutic benefit across a broad spectrum of diseases including pain (opioid receptor agonists), asthma (β_2-adrenergic receptors agonists), peptic ulcers (histamine receptors antagonists) and hypertension (angiotensin receptor antagonists) (6, 8). They have also been proven as relatively easy drug targets to modulate cellular responses, since these drugs can act without entering the cell. However, around 100 7TM receptors, according to the list maintained by the International Union of Basic and Clinical Pharmacology (IUPHAR), have not yet been paired with their endogenous ligands and are known as "orphan" receptors (9).

7TM receptors have no overall sequence homology (10); though, they share a basic common architecture of seven hydrophobic TM domains consisting of 20-25 amino acids, while the total length of 7TM receptors is generally 400-480 residues. The TMs form α helices connected by alternating three intracellular loops (IC-loops) and three extracellular loops (EC-loops) and are flanked by an extracellular amino-terminal tail and intracellular carboxyl-terminal tail. Apart from the overall common architecture, most 7TM receptors have structural similarities also designated as "fingerprint residues", which are distributed as clusters of conserved residues throughout the receptor. Based on conserved key residues and structural motifs, the 7TM receptor superfamily has been subdivided into three major families denoted A, B and C. In addition, three minor families exist namely the frizzled/smoothened, taste TR2 and pheromone VR1 receptor families (6). The A family receptors are by far the most studied. Rhodopsin (light receptor of the eye) and β_2-adrenergic receptors (receptors that target cateholamines) are prototypical members and their structures have been determined at the molecular level. Rhodopsin was the first 7TM receptor crystallized (11) and several years later two groups have successfully crystallized β_2-adrenergic (12) and β_1-adrenergic receptors (13). Other important members of the A family are chemokine receptors and receptors regulated by viruses. The B family of receptors are characterised by a large (~ 100 amino acids) N-terminal domain to which peptide hormones bind and activate this group. Metabotropic glutamate receptors (mGlu) are the prototypical receptors that make up the C family. Family C members have a very large N-terminal domain (> 350 residues), which is responsible for binding their endogenous ligands.

The 7TM receptors primarily signal through the family of G-proteins. These heterotrimeric compounds consist of the three subunits, designated α, β and γ, of approximately 40, 35 and 7 kDa, respectively. Agonist activation of a 7TM receptor induces conformational change within the receptor resulting in increased affinity for the G-protein and exchange of GDP and GTP on the Gα subunit. Subsequently, both the Gα-GTP and Gβγ are activated and able to modulate signalling molecules. The intrinsic activity of the Gα subunits hydrolyzes the GTP back to GDP, which promotes reassociation with the Gβγ and both signalling molecules are returned to their inactive states. Diversity of G-protein signalling leads to a wide range of possible biological responses including cell proliferation, differentiation, development, survival, angiogenesis, hypertrophy or oncogenesis. This is accomplished by a plethora of Gα and Gβγ subunits, which stimulate a variety of intracellular signalling pathways and other downstream targets (14, 15). Traditionally, G-proteins have been classified into four families based on sequence homologies among the α-subunits, namely $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12}$ (16). A total of 16 genes encode Gα proteins and further diversity is generated through transcriptional splice variation (17). The Gβ and Gγ subunits are tightly linked and can be considered as a single entity. There are five Gβ genes and twelve Gγ genes and also many splice variants have been described. Multiple pairings between Gβ and Gγ subunits are possible.

Members of the $G\alpha_s$ family primarily induce the activity of adenylyl cyclase (AC), leading to cAMP formation. On the contrary, most members of the $G\alpha_i$ family inhibit the activity of AC. The $G\alpha_q$ subunits

primarily stimulate different subtypes of phospholipase C (PLC) β, which results in inositol 1,4,5 triphosphate (IP3) and diacylglycerol (DAG) accumulation, while the $G\alpha_{12}$ subunits regulate the ras homology guanine exchange factor activity. As opposed to the Gα families, the action of the βγ-subunits does not primarily act upon one type of effector. The βγ-subunits are capable of regulating a multitude of effector molecules including all the abovementioned as well as phophoinositide 3-kinases, G-protein coupled receptor kinases (GRKs), tyrosin kinases and ion channels among others (11). In addition to signalling through G-proteins, 7TM receptors can also transduce signals through G-protein independent mechanisms. This was first reported in 1988, when acetylcholine was found to induce a potassium current through muscarinic receptors in the absence of G-protein (18). However, this remained highly controversial until recently as the tools available to rule out a G-protein mediated response have made G-protein-independent signalling unequivocal. The existence of G protein-independent, GRK/β-arrestin-mediated signal transduction has been established for several 7TM receptors (19) and may represents a potentially important mechanism by which 7TM receptors modulate physiologic processes as recently suggested in the cardiovascular system (20).

Idea about 7TM receptors dimerization and their cross-talk

7TM receptors are traditionally thought to act as monomers that engage their cognate G-proteins with a stochiometry of 1:1 (6). The traditional view of 7TM receptors signalling (described above), a cell-surface receptor is activated by the binding of a single agonist ligand, which further activates one G-protein (21, 22). Rhodopsin, the first 7TM receptor which was purified, was characterized as a single polypeptide that functions as an isolated monomer 30 years ago. Initially it was assumed that it binds like other 7TM receptor members to one heterotrimeric G-protein, thus a single receptor can activate sequentially a large number of G-proteins. However, this idea has been challenged during the 1970s and 1980s, with the usage of radiation inactivation as well as accumulating pharmacological and biochemical data regarding the association of many 7TM receptors types into higher-ordered structures (23, 24). On the other hand, crystalographic analyses of two new crystal forms of rhodopsin (25) revealed an interaction surface which may be involved in the formation of functional dimers or oligomers and implicates TM-I, TM-II, and helix 8 as the main contact surface stabilizing the dimers; this supports a stochiometry of 2:1 (2 receptors, 1 G-protein). Most evidence suggests that 7TM receptors dimerize, although formations of higher 7TM receptor complexes are also probable, as receptor dimers may represent the "building blocks" of higher order oligomers. This idea that receptors might dimerize was first proposed in 1982 (26) although the idea only gained some acceptance after more than a decade. 7TM receptors can be either connected to identical partner(s), which results in formation of homodimers (or homo-oligomers), or to structurally different receptor(s), which results in formation of heterodimers (hetero-oligomers) (27).

7TM receptor dimerization was proposed to play a potential role in i) receptor maturation and correct transport to the plasma membrane, ii) ligand-promoted regulation, iii) pharmacological diversity, iv) signal transduction, and v) receptor internalization and desensitization (28, 29).

Dimerization interface and domain swapping

Many different interfaces of dimerization are reported in literature, involving the transmembrane domains and ectodomains, such as C-termini. A considerable amount of evidence supports a TM-V and TM-VI dimerization interface (30), however on the other hand, functional site was identified also in helices TM-II and TM-III. Nevertheless, the interface may depend on additional stabilizing factors such as the coiled-coil interactions reported in the $GABA_B$ receptor and the disulfide bridge interactions in the muscarinic and the other class C receptors. $GABA_B$ receptors are an example of an interaction involving their C-termini (31). The evidence from several laboratories (reviewed in 30) support the TM-V and TM-VI dimerization interface, known to play a role in activation. Helices TM-V and TM-VI can form two dimer types: TM-V, TM-VI-contact dimers, and TM-V, TM-VI-domain-swapped, dimers. Simple TM-V, TM-VI contact dimer would use exactly the same V-VI interface and would differ only in the relative orientation of IC loop3. Domain swapping was proposed by Gouldson et al. (32) as an explanation for the functional rescue observed on co-expression of adrenergic-muscarinic chimeric receptors (33). The ability of helices TM-I and TM-V and TM-VI and TM-VII to function as separate A and B domains has been shown for the rhodopsin, adrenergic, muscarinic,

vasopressin, and GnRH receptors. Such evidence for a dynamic structure reinforces the idea that these receptors could domain-swap (30). The functional interaction site identified in helices TM-II and TM-III is not as well-defined as the site on helices TM-V and TM-VI and there are not many experiments that clearly implicate the external face of helices TM-II and TM-III in dimerization activity (34).

Methods applied for studying 7TM receptor dimerization and cross-talk

Different methods have been employed in order to resolve how 7TM receptors work, showing that they might not act as single monomeric units. Despite many different techniques applied to resolve this issue, this new concept is still debated (35). One concern has been that 7TM receptors homo-/heterodimerization might be an artefact of receptor over-expression and/or a result of the techniques used to study receptor associations. Some of these methods are discussed below.

Sucrose density gradient fractionation and immunoblot analyses

Initially, analysis of the receptor species for neurotransmitters, such as dopamine, opioids, and other peptides present on plasma membranes obtained by sucrose density gradient fractionation and visualized by immunoblot, revealed receptor monomers, dimers, and higher molecular weight forms. Tetramers of 7TM receptors have also been reported in immunoblot analyses of membranes from cells expressing the receptors and in native brain tissue (36). This method never gained general acceptance, partly due to the fact that there were observations of some oligomers with odd an number of receptors (e.g. trimers, pentamers) (23).

Co-immunoprecipitation and Western blot

One of the most frequently used approaches was co-immunoprecipitation of differentially-tagged receptors, which has also been used to track other protein partners. Immunoprecipitation is a useful method for isolating proteins of interest from cellular extracts using specific antibodies. Following immunoprecipitation of a protein of interest, it can be determined via Western blot whether any other proteins have co-immunoprecipitated. 7TM receptors can be tagged with different epitopes (e.g. HA-, c-myc-) and the same 7TM receptor can be co-expressed in two different foms in a heterologous expression system. Initially this technique was used to co-express c-myc- and HA-tagged forms of β_2-AR in insect Sf9 cells and demonstrated interaction between the two isoforms of the receptor as both were present in imunoprecipitates generated using either anti-HA or anti-c-myc antibodies (37). These studies also noted the resistance to monomerization during SDS/PAGE resolution of simple membrane preparations, while higher-ordered structures were observed, under denaturating conditions (in the presence of sodium dodecyl sulfate; SDS), which were interpreted as potential artefacts. Because of hydrophobic nature of the receptor TM domains there were opinions that co-immunoprecipitation might reflect nonspecific aggregations due to detergent extraction of membranes or cells. However, Salim et al. (38) showed interactions between several 7TM receptors. The first widely accepted demonstration of 7TM receptor heterodimerization came from the $GABA_B$ (GB_BR) receptors, which only responded to GABA when co-expressed (39).

Atomic force microscopy (AFM)

Rhodopsin is the only 7TM receptor for which the presumed higher-order oligomeric state has been demonstrated in the native disk membrane. Recent atomic force microscopy (AFM) studies revealed an oligomeric arrangement of rhodopsin and opsin in the form of large paracrystalline arrays, which shows receptors organized into rows of dimers (40, 41).

AFM is a very high-resolution scanning probe microscope, with demonstrated resolution of fractions of nm, more than 1000 times better than the optical diffraction limit. The AFM can be used to image and manipulate atoms and structures on a variety of surfaces. The atom at the apex of the tip "senses" individual atoms on the underlying surface when it forms initial chemical bonds with each atom. Because these chemical interactions gently alter the tip's vibration frequency, they can be detected and mapped. AFM has also been used to measure the forces required to unfold single rodopsin molecules, thereby revealing which residues are responsible for its stability. Recent functional analyses of fractions from solubilized disk membranes revealed that higher-order rhodopsin oligomers are the most active species. Based on these data and the X-ray structure, an atomic mod-

el of rhodopsin dimers has been proposed, a model that is currently analyzed and utilized in various ways (42).

Fluorescence resonance energy transfer (FRET) technique

Fluorescence resonance energy transfer (FRET) is a biophysical method that enables proximity assessment of appropriately tagged receptor proteins in live cells. Initially it was used to demonstrate conformational changes in 7TM receptor monomers upon agonist binding and was later used to detect 7TM receptor dimers. Förster resonance energy transfer (RET) is based on non-radiative transfer of energy between suitable donor and acceptor that are less than 100 Å apart and properly orientated (43, 44). The basic principle (described in 45) of this method of detection uses two fluorescently labeled proteins, the green fluorescent protein (GFP) and a mutation of this protein with altered spectral characteristics, e.g. cyan fluorescence protein (CFP), yellow fluorescence protein (YFP) or others variants. The transfer of energy is a consequence of the spectral overlap between the donor emission and acceptor excitation profiles. Although, FRET enables the visualization of protein interactions in living cells, the problems associated with FRET (e.g. high fluorescent background, autofluorescence, photobleaching) make BRET, which uses bioluminescence donor instead of a fluorescent one, the technology of choice for several applications. Also a distinct time-resolved FRET-based approach to monitor interactions between 7TM receptors at the cell surface is based on using anti-epitope tag antibodies labeled with suitable donors and acceptors, which are modifications of earth elements lanthanides. N-terminally epitope-tagged forms of 7TM receptors should only be accessible to such antibodies in intact cells if they have been delivered successfully and inserted in plasma membrane (46). Advanced version is a combination of time-resolved FRET and SNAP-tag technologies, which enables quantitative analysis of protein-protein interactions. SNAP-tag technology enables direct covalent labeling of the proteins of interest, eliminating the need for antibodies and reducing the assay complexity. It consists of a protein tag that reacts covalently with a labeled substrate, forming a stably labeled fusion protein. Substrates are derivatives of O_6-benzylguanine (BG) that can carry a wide range of different labels (46, 47).

Bioluminescence resonance energy transfer (BRET)

BRET is principal method by which scientists currently investigate 7TM receptors oligomerization (28). BRET was first described in a study on dimerization of the bacterial Kai B clock protein (43). Subsequently, BRET was introduced in the 7TM receptor and tyrosine kinase receptor field demonstrating β_2-AR dimerization (48) and ligand-induced conformational changes in the insulin receptor (49). Further on it has been suggested as a suitable tool for characterization and detection of 7TM receptors homo/heterodimerization (50). Since then, existence of several homo- and heterodimers has been reported in living cells. Renilla luciferase (RLuc) and green fluorescent protein (GFP) used as energy donor an acceptor, respectively are fused to the proteins of interest and co-expressed in the same cells (described in 45). In the presence of the substrate (e.g. coelenterazine, DeepBlueC), RLuc emits light at a specific wavelength and after interaction of labeled proteins RET occurs, when energy is transferred from RLuc to GFP and light is emitted at different wavelength. Following BRET, an advantageous version, termed BRET^2 was developed with improvements in the substrate (instead of coelenterazine h, a coelenterazine analog DeepBlueC was employed) and Stokes shift (GFP was replaced by GFP mutant GFP^2), which gives better separation of RLuc and GFP light and hence higher sensitivity (51). However, quantification of the fraction of 7TM receptors existing as momoner vs. dimer or higher-order oligomer remains a challenging task. With a modified form of the Veatch and Stryer model (52) and using BRET, we proposed a theoretical model to distinguish between these possibilities (50).

Controlled dimerization system

This system was developed by Patricia Hinkle group (53) in order to study the functional importance and consequences of homodimerization of thyrotropin-releasing hormone receptor (TRH). In this system, which allows artificially induced homodimerization of TRH receptor, the C-terminal tail of receptor was linked to HA-tagged human FK506 binding protein (FKBPv). Furthermore, a dimeric ligand was created by linking together to monomeric ligands, which each bind to a single FKBPv and used to induce dimerization of FKBPv-TRH receptor fusion protein.

Chimeric approach - employment of quality control system of GB_BR

The most direct and convincing evidence for receptor dimerization came from studies on the GABA (GB) receptors, the first 7TM receptors shown to be functionally combined of two distinct subunits, $GABA_{B1}$ (GB_{1a}) and $GABA_{B2}$ (GB_2), as an obligatory constitutive heterodimer (39, 54, 55, 56), thus representing a good model for studying the functional relevance of 7TM receptor dimerization. The GB_{1a} found to be largely nonfunctional in the terms of ligand binding when expressed alone and GB_2 nonfunctional in signalling properties. Co-expression of the two subunits is required for proper insertion of the heterodimer to plasma membrane. The GB_{1a} subunit contains an endoplasmic reticulum (ER) retention RSRR signal in its C-terminal tail, preventing it from reaching the cell surface as a monomer. Only when associated with GB_2, GB_{1a} retention signal is masked and the GB_{1a} subunit can reach the cell surface as a functional receptor (56). Although no covalent linkage between the subunits has been observed, these dimers are likely to be very stable due to α coiled-coil interaction. To determine whether the GB_{1a} subunit is required for all GB_BR pharmacology, transgenic mice were generated with a GB_{1a} receptor deletion. In these mice, pre- and postsynaptic GB_BR function was absent, which suggests that GB_{1a} is essential for all GB_BR pharmacology. In addition, the epileptic phenotype of heterozygote animals indicated that both GB_BR agonist and antagonists might have therapeutic benefit in treatment of neurological and psychiatric disorders (57). In a study on mGlu5 receptor (58), a member of C family of 7TM receptors, chimeric mGlu5 receptor was engineered by exchange of the mGlu5 receptor tail with GB_{1a} and GB_2 tails, developing mGlu5-GB_{1a} and mGlu5-GB_2 chimeras (receptors that have combined attributes from different sources). The study demonstrated that the quality control system of GB_BR could be transferred to other family C members. In our study this principle was transferred to a member of the A family of 7TM receptor (59).

Concatameric approach

For studying dimerization, a new approach of forced obligatory dimers, also termed concatamers can be used. The term concatamer or concatameric receptors is used when two or more linear molecular units are covalently linked in tandem. Previously this system was used to study the stoichometry of acetylcholine ion gated channel - a heteromer consisting of five subunits (60) and was later transferred to the 7TM receptor field by Terpager (61). In the latter study two 7TM receptors were forced together by linkage with an artificial transmembrane region, since 7TM receptors have an uneven number of transmembrane segments, which places the N-terminal and C-terminal parts on the opposite side of membrane, we can not fuse them together in a classical way. Concatamers were engineered by linking the C-terminal end of "receptor A" to the N-terminal end of "receptor B" through a spacer construct comprising of an artificial transmembrane helix. As model systems, two unrelated 7TM receptors e.g. NK_1-R and β_2-AR were chosen, which, at present, are not expected to form heterodimers by themselves, but homodimers of the β_2-AR were (37, 48) and were also connected together in a fusion protein. The same system was used to study homodimerization of the ghrelin receptor (unpublished).

Dimerization observed in different families of 7TM receptors

Heterodimerization in the C family of receptors has been most extensively studied and for some experts in the field of 7TM receptors the only one demonstrated to form real dimers. In this family of 7TM receptors heterodimerization is important for either receptor function, proper expression on the cell surface or enhancing receptor activity. In the A family of receptors, dimerization was extensively studied, however it remains extensively controversial. On the other hand, this view my change due to recent compelling evidence for the dimerization in the family A 7TM receptors that was elegantly demonstrated for the first time *in vivo* by Huhtaniemi's group who was able to rescue the LH receptor knockout phenotype by complementation i.e. co-expressing two nonfunctional receptor mutants in the knockout mice (62). Review of examples is presented in Table 1.

Clinical relevance of 7TM receptor homo- and heterodimerization

Importance of 7TM receptor dimerization is already obvious in cross-talk between different classes of drugs. Beta blockers can, in some cases, block signalling by both β_2-AR and angiotensin AT_1R. Furthermore, the interaction between σ-OPR and μ-OPR

Table 1: Overview of dimerizing 7TM receptors

7TM receptors FAMILY C	Type/ Mechanism	Effect	Method	Reference
GABA receptors $GB_{1a}R$, GB_2R	heterodimerization via C-terminal tail coil-coiled interaction	$GB_{1a}R$ binds the ligand, GB_2R is required for cell-surface expression, together are functional	yeast two-hybrid screening; co-immuno- precipitation	(54, 63, 64)
glutamate receptor mGluR1	*homodimerization* via a disulfide bridge in the N-terminal domain	each bind a ligand, might be required for proper expression on the cell surface	different crystal structures of the extracellular ligand-binding region of mGluR1	(65)
Taste receptors (T_1R_2), (T_1R_3)	heterodimerization	sweet response	transgenic rescue experiments	(66)
Taste receptors T_1R_1 and T_1R_3	heterodimerization	umami taste responses effect on pharmacology of receptors	transgenic rescue experiments	(66)
FAMILY A				
adrenergic receptors (AR) β_2-AR	*homodimerization*	targeting of receptor to the cell surface	coimmunoprecipitation; rescue of a constitutively desensitized form of the receptors; BRET; mutation of a putative dimerization motif	(37, 48, 50, 67)
- β_2-AR - β_1-AR or β_3-AR	heterodimerization	reduction in the rate of agonist-induced internalization of β_2-AR; reduced ability of the receptor to stimulate ERK phosphorylation; **proper regulation of cardiac contractility**	co-immunoprecipitation; BRET; knock out mice; co-immunoprecipitation	(68, 69, 70)
β_1-AR/ β_2-AR α_2-AR	heterodimerization	cross-internalization of the receptors following agonist stimulation	BRET	(71)
β_2-AR opioid receptors	heterodimerization	significant cross-internalization of the receptor complex by adrenergic and opioid ligands	BRET	(72)
dopamine receptors D_1R D_2R	heterodimerization	heterodimer exhibits different function than the individual momomer receptors	transgenic mice experiments	(73)
opioid peptide receptor (OPR) family	*homodimerization*	interconversion between the dimeric and monomeric forms plays a role in opioid receptor internalization	BRET; agonist-induced internalization with differentially (Flag and c-Myc) epitope-tagged receptors; biochemical and pharmacological methods	(72, 74, 75)
opioid peptide receptor (OPR) family δ-OPR κ-OPR	heterodimerization	change in pharmacological properties of the receptors	biochemical and pharmacological methods	(75)

δ -OPR μ-OPR	heterodimerization	influences receptor pharmacology and results in synergistic signalling, delta opioid receptor ligands enhance morphine's potency	co-immunoprecipitation; *in vivo* experiment on mice	(76)
δ-OPR/κ-OPR β_2-AR	heterodimerization	influences receptor internalization	BRET;	(72)
μ-OR somatostatin receptor $SSTR_2A$	heterodimerization	significant co-internalization and cross-desensitization	co-immunoprecipitation;	(77)
μ-OR NK_1-R	heterodimerization	significant co-internalization and cros-desensitization	co-immunoprecipitation; BRET	(78)
somatostatin receptor family $SSTR_5$, $SSTR_1$ D_2R	heterodimerization	significant co-internalization and cross-desensitization	biochemical and pharmacological methods	(79)
purinergic receptor subtypes A_1R, $P2Y_1R$	heterodimerization	exhibits a distinct pharmacological profile, which may contribute to the diversity of purinergic receptor binding sites	co-immunoprecipitation; confocal laser microscopy	(80)
A_1R D_1 dopamine receptors	heterodimerization	exhibits a distinct pharmacological profile	co-immunoprecipitation; confocal laser microscopy	(81)
A_1R mGluR1	heterodimerization	new molecular and functional interaction between two functionally unrelated types of G protein-coupled receptors and receptors cross-talk	co-immunoprecipitation	(82)
$A_{2A}R$ mGluR5	heterodimerization	synergistic signaling between adenosine receptor and glutamate receptor agonists	co-immunoprecipitation; confocal laser microscopy; *in vivo* experiment on mice	(83)
olfactory receptors (ORs) M71 β_2-AR	heterodimerization	a striking enhancement in the surface trafficking of M71 in heterologous cells	co-immunoprecipitation; co-internalization	(84)
vasopressin oxytocin receptor (OTR) subtypes $V_{1a}R/V_2R$, $V_{1a}R$/OTR, V_2R/OTR $V_{1a}R$//OTR	heterodimerization	two receptors can be endocytosed as stable heterodimers	co-internalization	(85)
vasopressin bradykinin B_2 receptor	heterodimerization	effect on the cell surface expression	co-immunoprecipitation; BRET	(86)
muscarinic acetylcholine M_2 M_3	heterodimerization	functial interaction between receptors, cross-interaction between receptor subtypes	chimeric receptors	(87)
M_3 α_2C	heterodimerization	functial interaction between receptors, cross-interaction between receptor subtypes	chimeric receptors	(87)

thyrotropin-releasing hormone receptors TRHR1 TRHR2	heterodimerization	interactions of TRHRs with beta-arrestin may be altered by hetero-oligomer formation	BRET; confocal microscopy	(88)
Angiotensin AT_1 bradykinin B_2	heterodimerization	alteration in the hetero-dimerization of 7TM receptors was **linked to preeclampsia, first known disorder caused by heterodimerization of 7TM receptors**	pharmacological methods; *in vivo* experiment on mice	(89)
angiotensin AT_1 and AT_2	heterodimerization	AT_1 antagonises AT_2 receptor	pharmacological methods; *in vivo* experiment on mice	(90)
cholecystokinin type A and B	heterodimerization	heterodimeriaztion enhances signalling and promotes cell growth	pharmacological methods; co-internalization	(91)
endothelin type A and B receptors	heterodimerization	influence on ligand induced internalization	co-immunoprecipitation; co-internalization	(92)
thyrotropin (TSHr) and lutropin (LH/CGr) receptors	*homodimerization* and heterodimerization	link between homodimerization and negative cooperativity	BRET; TR-FRET: functional and biochemical approaches	(93)
relaxin family peptide receptor 1 and 2	*homodimerization* and heterodimerization	link between homodimerization and negative cooperativity	BRET	(94, 95)
FAMILY B				
gastric inhibitory polypeptide receptor (GIP-R)	*homodimerization*	new insight into the ability of family B 7TM receptors to dimerize	BRET	(50)
(GIP-R with β_2-AR	heterodimerization	heterodimerization between members of different families of 7TM receptors	BRET	(50)
vasoactive intestinal polypeptide receptors (VPAC1 VAPC2)	*homodimerization* and heterodimerization	new insight into the ability of family B 7TM receptors to dimerize	BRET	(96)
(VPAC1 and VAPC2) with secretin receptor	heterodimerization	new insight into the ability of family B 7TM receptors to dimerize	BRET	(96)
Calcitonin-like receptor protein (CLRL9, receptor-activating-modifying protein (RAMP) family	dimerize with non-7TM receptor proteins	expressed alone, it seems to be non-functional, but when co-expressed with a member of the receptor-activating-modifying protein (RAMP) family, becomes functional receptor	co-immunoprecipitation	(97)

(**bold** – known relevance in organism, *italic* - homodimerization)

has been postulated to account for the well-known effects of σ-OPR on μ-OPR mediated-analgesia. Synergistic and antagonistic interactions between drugs are extremely important to consider in a clinical setting and heterodimerization between receptors represents a specific mechanism that may potentially underlie certain drug-drug interactions at the molecular level. In the field of chemokine receptors, two subtypes of chemokine receptor, CCR5 and CXCR4 are known to act as co-receptors for HIV entry into cells. Evidence exists that dimerization or cross-talk between 7TM receptors in host cell and viral 7TM receptor can enable the activity of viruses.

Further evidence for the clinical significance of 7TM receptor heterodimerization has also come from recent studies on Wnt receptors. Wnts are

secreted glycoproteins that play diverse roles in regulating cell fate and proliferation via activation of 7TM receptors belonging to the frizzled (Fz) family. Mutations to the Fz4 subtype have been found to underlie an autosomal-dominant form of a disease known as familial exudative vitreoretinopathy (FEVR), which is characterized by impaired growth of retinal capillaries leading to eventual retinal degeneration. The mutant Fz4 receptor in FEVR can form homodimers with wild-type Fz4 leading to ER retention of these receptors (98). Preeclampsia was the first disorder associated with altered 7TM receptor heterodimerization of AT_1/B_2 receptors (89). AT_1 is a receptor for the vasopressor angiotensin II and the B_2-receptor for the vasodepressor bradykinin 2. Upon AT_1–B_2-receptor heterodimerization, angiotensin- II–stimulated signaling is strongly enhanced. AT_1–B_2-receptor heterodimerization in preeclampsia is mediated by a 4–5-fold increase in B_2-receptor protein levels. A significant induction of B_2 receptors can be observed with the major clinical signs of preeclampsia.

7TM receptor homo- and heterodimerization could therefore represent a novel aspect of 7TM receptor biology that has exciting potential in drug discovery.

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RECEPTORJI S SEDMIMI TRANSMEMBRANSKIMI OBMOČJI (7TM) V LUČI DIMERIZACIJE TER EKSPERIMENTALNI PRISTOPI ZA PROUČEVANJE NJIHOVE DIMERIZACIJE IN MEDSEBOJNIH VPLIVOV

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Povzetek: Receptorji s sedmimi transmembranskimi območji (receptorji 7TM) oblikujejo največjo in najbolj raznoliko superdružino proteinov, kodiranih v človeškem genomu, ki šteje vsaj 850 domnevnih predstavnikov. Receptorji 7TM so v organizmu splošno izraženi in igrajo temeljno vlogo v fiziologiji ter patofiziologiji. Ker uravnavajo mnogo fizioloških procesov, so pomembna tarča za zdravila, ki jih bodisi aktivirajo ali inaktivirajo in so posledično najbolj uspešno prodajana zdravila. Od približno 500 trenutno prodajanih zdravil jih več kot 30 odstotkov vpliva na delovanje receptorjev 7TM. V zadnjih dveh desetletjih je znanje na področju delovanja receptorjev 7TM močno napredovalo, kljub temu pa je koncept njihovega združevanja v bodisi dimere ali oligomere še vedno sporna ter neobičajna tematika. Kljub temu da obstaja mnogo podatkov o njihovi dimerizaciji, pridobljenih z različnimi biofizikalnimi, biokemijskimi, strukturnimi in funkcionalnimi pristopi, kot so na primer tehnike koobarjanja, prenos po metodi western ter metodi bioluminiscenčnega in fluoroscenčnega prenosa resonančne energije (BRET, FRET), so številne objave kritizirale obstoječe metode in izzvale omenjeno zamisel. V članku predstavljamo osrednje in najpomembnejše metode za proučevanje dimerizacije, ki so dopolnjene še z našimi pristopi.

Ključne besede: 7TM receptorji; struktura; dimerizacija; eksperimentalni pristopi