Oligomerization of the Heptahelical G Protein Coupling Receptors: A Case for Association Using Transmembrane Helices

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Abstract: The heptahelical G protein coupling receptors oligomerize extensively *via* transmembrane domains, in association with heterotrimeric G proteins. This provides higher affinity for agonists, conformational stability necessary for signal transduction, and protection from intracellular proteinases. The oligomerization is relevant to organismic pathophysiology and could be targeted by natural or modified agonists.

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INTRODUCTION

G protein coupling receptors (GPCRs) may have started as light-driven ion transporters in prokaryotes, to develop into metazoan receivers of homeostatic messages (see e.g. [1]). Receptors for the various transmitters have developed forms of oligomeric association. Oligomerization can provide the anchoring and stability that help trapping of agonists and the regulation of ion passage, and that also can fortify and extend interactions with transducers, effectors, and the partners in adhesion or networking. Transmembrane dimerization is requisite for cell-connecting integrin receptors [2], as well as for membrane-stabilizing transmembrane proteins (see e.g. [3]).

Considerable evidence is available for both homomeric and heteromeric association of GPCRs (for recent reviews see [4-7]; for recommendations on receptor heteromultimer nomenclature see [8], and for a recent overview of heterotrimeric G proteins see [9]). Oligomerization that employs hydrophobic transmembrane segments of the receptors does not importantly depend on the level of receptor expression [10, 11]. Coupling through exocellular and even intracellular domains was however found for many receptors at high levels of expression. An overexpression of any protein can boost its interactivity. High physiological levels of GPCRs (> 1 pmol/mg membrane protein) are found in the pineal gland (e.g. the α1-adrenergic receptor [12]) and in the kidney cortex (e.g. the neuropeptide Y (NPY) Y2 receptor [13]), but the *in vivo* expression of many GPCRs is low [14]. A hormonally induced *in vivo* overexpression can modify surface organization of the receptor-expressing cells [15]. Demonstrations of specific mechanisms using the cell -line overexpressions of receptors and other components of signal transduction systems are valid if there are no large expression-induced changes in organization and function of the host cells.

At equal levels of expression, heterodimerization could even be preferred to homodimerization, e.g. for the co-expressed α2A and α2C-adrenergic receptors [16]. However, preference for homodimerization was also clearly established in several cases (the β2-adrenergic *vs.* the CCR5 and melatonin-1A receptors [17], the pancreatic polypeptide Y4 receptor *vs.* the Y1 and Y2 receptors [18], the α1A-adrenergic *vs*. the δ-opioid receptor [19], the histamine H1 *vs*. the H4 receptor [20]). It should also be recalled that many receptors are specifically expressed in particular tissues. A significant expression of thyroid hormone-stimulating (TSH) receptor in the ovary, or of the luteinizing hormone (LH) receptor in the thyroid gland, is not in evidence.

Across families of GPCRs [21], the adhesion receptors of family B (B-GPCRs) resemble cadherins in their long N-termini. N-terminal similarities with contemporary excitatory amino acid –gated channels are significant for all members of family C (C-GPCRs; see Table **S6**). The recently identified B-GPCRs with very large extracellular N-terminal domains [22, 23] may have multiple roles in oligomerization, ligand handling, and cell anchoring or translocation. However, among the family A GPCRs (A-GPCRs), long N-terminal exodomains up to the present have been found only for the leucine-repeat group (pituitary gonadotropin hormone and relaxin 1 and 2 receptors).

Currently there is no broad evidence for differentiation or specialization of protomers in families A and B of the G protein coupling receptors [24]. The γ-aminobutyric B (GABAB) family C receptor has functionally specialized non-identical, covalently linked subunits (see e.g. [25]). An unequal agonist affinity for two identical protomers is found for an A-GPCR, the NPY Y2 receptor, and that could be due to an allosteric influence of the occupation of the first site [26]. This ine-

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quality is of interest for the medicinal chemistry of GPCR agonists, and could also be involved in the allosteric binding to muscarinic [27], opioid ([28] and [29]) and other receptors.

1. ORGANIZATION OF GPCRS: OLIGOMER *AND* MONOMER RATHER THAN OLIGOMER *VERSUS* MONOMER, AND HOW THE PROTOMERS INTERACT

The GPCRs, including opsins, are known to function well as monomers [30, 31]. Some GPCRs are detected primarily as monomers in the brain environment, but as dimers in epithelial cell expressions [32]. However, a much stronger activation of the G protein α subunit by the dimer compared to the monomer was found for many GPCRs, including the leukotriene Blt-1 receptor [33], the D2 dopamine receptor [34], and the NPY Y2 receptor [32]. Dimerization also reduces the random mobility, helping productive collisions with agonists (including photons). Also, interactions with G protein transducers, or with effectors such as phospholipases, kinases and cyclases, are more stable in ordered, membrane - anchored aggregates. As arrayed in rafts, the receptor / G protein complexes represent a dynamic reserve that can be mobilized in response to agonist surges. Parts of the oligomeric assemblies would not necessarily diffuse out at the termination of a transductional event, and that could help a faster resetting. Organization of receptor dimers and other components in sheets and layers seems to be indispensable for the large-scale transduction in the visual process [35].

Connected to the above, GPCR dimers are expected to display a bilateral surface symmetry and an equivalency of the constituent protomers. The former expectation might be supported for all GPCRs, but the latter seems to be met only by the receptors that respond to small agonists (contrast e.g. the findings for an eicosanoid [33], or a metabotropic glutamate [36], with those for a peptide receptor [26]). In any case, only one protomer needs to bind agonist for activation of a G protein trimer [26, 37]. Activation of rhodopsin by a single photon [38] could reflect an inherent functional asymmetry which can also be expected from modeling with rhodopsin [39] and with the adenosine A3 receptor [40].

A transmembrane-domain asymmetry or quasi-symmetry was found for the 5-HT2c receptor [41]. Association by different transmembrane helices (e.g. tm4:tm5 [40], tm1:tm4 [42], or tm4:tm6 [43]) also may lead to a functional asymmetry. In an asymmetric / surface symmetric protomer pair (e.g. [40]), one member may have larger affinity for the G protein heterotrimer (G trimer). Also, if GPCR dimerization is completed by addition of Gα subunits to receptor dimer-Gβγ complex at the ER / Golgi stage [44, 45], attachment of Gα to Gβγ subunit that is associated with one of the protomers could allosterically alter conformation of the other protomer. Alternatively, only one G trimer would be associated with a receptor dimer. Indeed, several studies have detected heteropentamers ([33, 46, 47]; see the supplemental Fig. (**S2**) for examples with neuropeptide Y (NPY) receptors). However, the fully stoichiometric heterooctamers, predicted by modeling in the case of rhodopsin [48], were detected for the NPY Y2 receptor [26]. The above considerations are even more of interest with GPCR heterodimers, which could possess considerable functional asymmetry due to the lack of sequence identity of protomers.

2. NEW GPCRS OLIGOMERIZE USING TRANSMEMBRANE DOMAINS AT THE LEVEL OF THE ENDOPLASMIC RETICULUM

Opsins and opsin-like A-GPCRs and B-GPCRs with hormonal peptide agonists generally form dimers *via* hydrophobic, bilayer-passing (transmembrane) helices of the protomers. This was established for a number of receptors (Tables **2** and **S2**). Transmembrane dimers were also detected for the leucine-repeat A-GPCRs [49], and for metabotropic glutamate [50] and calcium-sensing [51] C-GPCRs. The latter also extensively dimerize *via* their large N-terminal extracellular domains [52, 53]. However, folding and processing of proteins in the endoplasmic reticulum (ER) and the Golgi area favor interactions between hydrophobic helices (see Chapter 12 in [54]). Transmembrane homodimers are also common for integrins (e.g. [55]) and other receptors, and for many other membrane proteins (e.g. [3]). The use of GPCR transmembrane dimers is schematized in Fig. (**S1**).

With many A-GPCRs, association through exodomains could interfere with agonist binding, and appears to be suppressed at the level of domain composition, as will be discussed later. Interactions of short cytoplasmic loops 1 and 2 (ic1 and ic2; see Table **S5A**) generally would face steric restraints. Association *via* transmembrane domains can generate transductionally active chain-like ensembles. There could be a preference for the tm1, tm4 and tm5 helices, which are somewhat apart from other domains within opsin-like molecules [56], are less stringently used in signal transduction, and also show fewer conserved features (Tables **1** and **S1**).

The fourth transmembrane domain also stands out in terms of both intrahelical and interhelical H-bonding triplets [57] (Fig. **1**), followed by the tm1 segment. The tm1 leads in hydrophobicity and the tm6 in bulky neutral residues (Fig. **1**). The above parameters support assemblies like tm1:tm4 for the α1B-adrenergic receptor [58], tm4:tm4 for the D2 dopamine receptor and bovine rhodopsin [34], tm4:tm5 for bovine rhodopsin [59] and the A3 adenosine receptor [40]. A helix bundling based on the tm6 could be present for the cholecystokinin-A (CCK-A) receptor [60]. The association using transmembrane helices could be largely responsible for the well-known grouping of opsins [61] and of neuronal GPCRs [62].

Dimers formed by transmembrane domains have been identified for a number of GPCRs (Tables **2** and **S2**). The initial assembly of dimers using the transmembrane helices should occur in the endoplasmic reticulum (ER) at the completion of protein synthesis, and should be aided by N-terminal receptor glycosylation (noting the abundant glycosylation motifs in the exocellular domains (Table **S4B**), as opposed to the transmembrane (Table **S3B**) and the intracellular domains (Table **S5B**)). The assembly involves a low-affinity 'packaging' with G proteins [45, 63, 64]. This should be supported by high levels of G proteins, which typically outnumber GPCRs by an order of magnitude or more (e.g. [32]), and could be the most readily available GPCR chaperones. Following final assembly in the Golgi area, these com-

Table 1. Residues and Motifs Shared with Bovine Rhodopsin in Transmembrane Segments of Human A-GPCRs

DOMAIN AND LENGTH	RESIDUES SHARED WITH BOVINE RHODOPSIN (% IDENTITY)	A-GPCR / *RHODOPSIN* MOTIF
TM1 (23)	N17 (100)	
TM2 (23)	L7 (96.7) A8 (83.6) A10 (71.9) D11 (98.1) L12 (70.9)	LAXA[S]D / *LAVAD*
TM3 (28)	L18 (86) R25 (98.4) Y26 (75.5)	D[E]RY[W] / *ERW*
TM4 (23)	W9 (97.9) P18 (65.6)	---- / *APPL* (17-20)
TM5 (23)	F11 (75.4) P14 (80.4) Y22 (76)	---- / *IPLI* (13-16)
TM6 (23)	F8 (87.6) C11 (75.1) W12 (79.4) P14 (80.4)	CWXP / *CWLP*
TM7 (25)	N19 (76.2) P20 (96.2) I22 (68.1) Y23 (92.7)	N[D]PXI[L]Y / *NPVLY*

The data are for 209 human A-GPCRs with known specific agonists and finalized sequences, compared with bovine rhodopsin. The positions within segments corresponds to those in bovine rhodopsin. Only residues shared by more than 65% of the examined A-GPCRs are shown. Residues in brackets are the variants found in more than 15% receptors at the position; x indicates a variety of residues at the position. The supplemental Table **S1** shows full alignments with the respective templates for the transmembrane segments of human family A (bovine rhodopsin template), family B (human glucagon receptor template) and family C (human metabotropic glutamate receptor-1 template).

plexes (possibly heterooctamers, with two G trimers per receptor dimer [26, 48]) get apposed to the plasma membrane to await activation by agonist binding, by 'constitutive' interactions with channels or effectors, or by osmotic and micromechanical events. In the absence of agonist influx, most of the receptor cycling is constitutive, but does depend on functional G protein α subunits (see e.g. [65]).

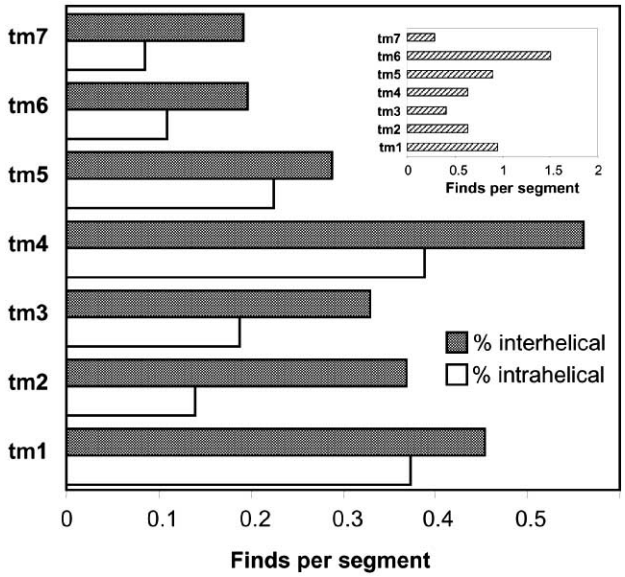

Fig. (1). The intrahelical and interhelical hydrogen bonding preferences for transmembrane helices of 209 human A-GPCRs. The triplets with intrahelical and interhelical H-bonding propensity [57] are, respectively, AGF, AGG, GLL, GFF, AGL, ALL, ALS, AGV, AAL, GLV, and AAA, AAG, AAI, AAL, AAM, AFS, AGF, AGG, AGL, AGV, AHS, AIM, AIP, ALF, ALS, GGF, GGL, GGV, GHT, GLF, GLL, HHV, IIL, LLS, LSV. The inset shows frequencies of 24 bulky hydrophobic triplets corresponding to permutations of Leu, Ileu, Val and Met residues.

The assembly using transmembrane segments could be non-covalent, e.g. by high-propensity interhelix H bonding triplets [57] and especially by H bonds of the aromatic π acceptors Trp and Tyr [66]. Hydrophobic 'zippers' [3, 67] could also be involved. Covalent assembly using intramembrane cysteine bridges was also reported [68].

Homodimerization predominates for clonal receptor expressions in cell lines, in the presence of usually low numbers of native GPCRs. In organismic systems, chances for heterodimerization are larger. However, in physiological conditions much of receptor mRNAs could be coordinately generated and exported to cytoplasm (e.g. [69]) in response to episodic homeostatic stimuli. This could favor a homodimeric assembly by higher expression of the receptor within a time frame. The transmembrane assembly need not be restricted to a single pair of domains. There is evidence for involvement of multiple transmembrane domains with the α1B-adrenergic receptor [70] and the CCK-A receptor [60]. This is further consonant with an important role of hydrophobic clinching in GPCR association *via* the tm domains [3, 67, 71].

The binding of agonist to a protomer enables a firmer association with the neighboring G protein αβγ trimer (G trimer), and could allosterically change the agonist binding affinity of the other protomer and/or the association of that protomer with G proteins. The initial activation stage can be detected as a ~180 kDa heteropentamer, demonstrated for rhodopsin and a number of A-GPCRs (also see Fig. **S2**). This could also result from agonist-promoted coupling to G trimer of a protomer in a G protein -free dimer that has re-formed from monomers at the plasma membrane. Reassociation with G proteins at the plasma membrane could be important in environments rich in G trimers (e.g. in neurons). In epithelial and endothelial cell lines, at least 50 clonal (as well as some native) GPCRs have been shown to cycle *via* agonist-induced internalization and return to the plasma membrane through a recycling endocytotic compartment, probably with reconstitution of the receptor dimer/G trimer complexes (Fig. **S1**). The monomerization followed by internalization was shown for several peptide receptors, including the CCK-A receptor [72], the pancreatic polypeptide Y4 receptor [18],

Table 2. Examples of GPCR Homodimers Connected by Transmembrane Domains

Receptor	Detection*	Comments	Reference
5-HT2c	ID , FRET	tm4:tm5; ER assembly	[41, 64, 110]
5-HT4	FRET	tm3-tm4 C-C	[68, 111, 112]
Adrenergic α1a, α1b	FRET	tm1:tm7, tm1:tm4	[19, 113-115]
Adrenergic α2a	FRET		[16, 46]
Adrenergic β1, β2	BRET, modeling	[tm5:tm6]	[17, 116, 117]
Dopamine D1, D2	ID , HA, FRET	tm4:tm4 or tm4:tm5	[118-122]
Histamine H1, H2, H4	BRET, FRET		[20, 123, 124]
Muscarinic m1-m4	FRET, yeast		[46, 125, 126]
Eicosanoid	CD, FRET	tm6 C:C ER	[33, 127]
C5a chemotactic	ID	tm1, tm2, tm4, ic2	[128]
CXCR1 (IL-8A)	FRET, BRET, ID	tm1:tm4	[115]
CXCR2 (IL-8B)	FRET	tm3:tm4 C:C or hydrophobic	[129, 130]
CXCR4	BRET, FRET, grad		[131]
CCR5	ID -HA, ID -FLAG	C in tm3, W in tm4-6	[42, 132, 133]
Adenosine A1, A2A, A3	FRET, BRET, modeling	tm4:tm5, tm5:tm5	[40, 46, 134-136]
Bradykinin B2	FRET		[137]
Bovine rod opsin 2 (rhodopsin)	microscopy, modeling, FRET	tm4 W / tm5 Y	[39, 48, 59, 61, 138]
Leucine-repeat	FRET, ID		[49, 139-141]
CCK-A	BRET, HA	tm6 or tm bundle	[60, 72]
Oxytocin, vasopressin V1a, V2			[44, 142]
Somatostatin sstr1, sstr2, sstr3, sstr5			[73, 143, 144]
NPY Y1, Y2, Y4, Y5	FRET, grad		[145], [18, 65]
Secretin, glucagon	BRET, modeling	[tm4:tm6]	[43, 146, 147]
CaSR human	BRET	tm7	[51]

*Abbreviations: ID = immunodetection / Western blotting; FRET = Förster resonance energy transfer; BRET = bioluminescence resonance energy transfer; grad = gradient centrifugation; HA = immunodetection of a N-terminal hemagglutinin sequence; FLAG = immunodetection of a N-terminal FLAG sequence; microscopy = atomic force microscopy; modeling = computer-assisted interface prediction; yeast = yeast two-hybrid system. For additional examples see the supplemental Table **S2**.

the somatostatin SSTR2 receptor [73], and the NPY Y1 [65] and Y2 [74] receptors.

3. FEATURES OF G PROTEIN -COUPLING RECEPTORS THAT COULD BE LINKED TO OLIGOMERIZATION

3.1. The Transmembrane Domains

The transmembrane (tm) helices serve to anchor and orient the receptor molecule, to provide the transductional switches operated by small ions and weak hydrogen bonding, and in many cases also to join the receptor monomers. There is a considerable sequence identity of these helical domains across the A-GPCRs, and at least six rhodopsin-aligning motifs or pivots are conserved in all members identified with agonists (the canonical receptors) in this family (Tables **1** and **S1A**). Many highly conserved residues are detected in the transmembrane segments of B-GPCRs, and also in C-GPCRs (Tables **S1A** and **S1B** and the legend of Table **S3A**). However, there are few studies on the mechanisms of transmembrane oligomerization for these receptors, and we shall focus here on A-GPCRs.

The first, fourth and fifth transmembrane helix (tm1, tm4 and tm5), frequently identified as contacts in the dimerization of A-GPCRs (see Tables **2** and **S2**), appear to have less direct roles in the signal transduction. The lack of conserved motifs in these segments (Tables **1** and **S1A**) may indicate adaptation for non-transductional tasks, including the coupling of transmembrane domains. It should be noted that the

tm4 has a low identity with the prototype receptor in all main groups of GPCRs (Table **S1**). For human canonical A-GPCRs, the positional correspondence with bovine rhodopsin is 36, 39, 36, 34, 40, 42 and 31% in tm1-tm7, respectively (Table **S1A**). From SSEARCH3 program [75], the average conservation in sequence overlaps relative to bovine rhodopsin for A-GPCRs is 22.6%, and over the total sequence length 17.4%. In family A, there is a high divergence of the transmembrane sequences both internally and relative to rod opsins (Table **S1A**). Families B and C show a high internal uniformity of transmembrane domains (Table **S1B** and **S1C**).

Abundances in the transmembrane segments of two potential connectors, cysteine and N-glycosylation motifs, are shown in supplementary Tables **S3A** and **S3B**, respectively. Cysteine is present in the tm6 of most A-GPCRs, and also is frequent in the tm4 and tm5, but not in transmembrane domains 1-3 (Table **S3A**). In B-GPCRs, peptide receptors show Cys in tm3 and tm7, anti-angiogenic receptors in all but the tm5, cadherin-like receptors only in the tm7, and Frizzled receptors in tm1, 2 and 6 (Table **S3A**). Among C-GPCRs, cysteine is poorly represented in the tm1, and strongly in tm2 and tm3 for all subgroups. N-glycosylation motifs are scantily represented in the transmembrane domains (Table **S3B**). Opsins and other A-GPCRs (excluding Leu-repeat receptors) have very few transmembrane N-glycosylation motifs (Table **S3B**).

Cysteines and aromatic residues in the transmembrane segments have been indicated as the dimer-coupling residues in many cases. Cysteine bridges between the transmembrane segments could result in a redox-sensitive, rather than phosphorylation / arrestin system –sensitive dimerization, and could reduce receptor cycling. Association *via* multiple hydrophobic or polar 'zippers' [67, 76, 77] is generally important in transmembrane dimerization [67, 71]. Motifs rich in hydroxy-amino acid residues could be important in both intrahelical and interhelical H bonding [57, 78]. Prolines in transmembrane segments 4 and 5 are found in most A-GPCRs that dimerize through transmembrane domains, possibly indicating importance of helix flexibility for the process. Dimers connected by the transmembrane segments are believed to be pulled apart by arrestins, dynamins or adaptins following multiple phosphorylations of intracellular domains (e.g. [79]). Tables **2** and **S2** present a number of reports of receptor dimerization *via* transmembrane domains across the GPCR families.

Attachment of oligosaccharides to transmembrane segments should be possible in ~40% of the residues that are not stably masked by the bilayer [56]. However, as seen in Table **S3B**, significant numbers of N-glycosylation sites are found only for the C-Taste 1, A-olfactory and B-cadherin-like receptors. The transmembrane segments of A-GPCRs and opsins are essentially devoid of N-glycosylation motifs (Table **S3B**).

3.2. The N-Terminal Exodomain

The N-terminal exodomain (ec1) contains the agonist-binding sites of B- and C-GPCRs, and of Leu-repeat A-GPCRs. However, most A-GPCRs with small agonists, and many A-GPCRs with peptide agonists, employ the transmembrane segments and extracellular loops to bind the cognate messengers.

The ec1 domains are typically short in opsins, as well as in A-GPCRs excepting the leucine-repeat group (average length about 40 residues; Table **S4A**). The leucine-repeat A-GPCRs (the receptors for glycoprotein hormones and relaxin) have about 380 residues in this domain. The peptide agonist -responding B-GPCRs have ~125 ec1 residues. However, the adhesion B-GPCRs have ~900 (the angiogenesis inhibitors), ~2500 (the cadherin-like) and even 5000 ec1 residues (the very long calx-rich [22] receptors). Many of the adhesion B-GPCRs could principally serve for broadly selective associations with neighboring cells, or with partners in the extracellular matrix (ECM). The Frizzled receptors, which have about 210 N-terminal outside residues, respond to Wingless-type (Wnt) proteins as agonists [80]. The metabotropic glutamate / calcium-sensing C-GPCRs have > 500 residues in this domain, about 2/3 of which are elaborately fashioned (using highly conserved cysteine bridges) into a ligand catching / binding "Venus flytrap" site [81]. In C-GPCRs this domain also shows a conserved alignment with ionotropic glutamate receptors outside of the agonist -binding site, pointing to an evolutionary link (Table **S6**). The Taste 1 sweet / umami (glutamate-sensing) receptors have the N-terminal exodomain of similar size, which aligns well with those of the canonical calcium-sensing and metabotropic glutamate C-GPCRs. It is tempting to speculate that the large N-termini of C-GPCRs could have arisen *via* exon shuffling from ionotropic precursors (see also the review [82]).

Among the ec1 constituents that could be critical for both intramolecular and intermolecular associations, cysteine is represented poorly in A-GPCRs (excepting the Leu-repeat group) compared especially to B-GPCRs, and also relative to C-GPCRs. The vertebrate opsins show no cysteine in the ec1 domain (Table **S4A**). Also, very few archaeal / fungal opsins (which are not sequence homologues of A-GPCRs, but could be the archetypal heptahelical light-driven transporters / transducers) have cysteine in the ec1 domain. The presence of cysteine in this domain could lead to significant internal covalent interactions (see [83]) e.g. at an ER stage, and it appears that N-terminal cysteine has been suppressed in the evolution of opsins. More than 40% of A-GPCRs also have no cysteine in the ec1 segment, a deficiency not encountered in any B- or C-GPCRs (Table **S4A**). Conserved multiple cysteines in the ec1 domain are known to be critical in the binding of peptide agonists to B-GPCRs (see [84] for the secretin receptor, [85] for the glucagon-like peptide-1 receptor). The leucine-repeat A-GPCRs have N-terminal extracellular domains almost tenfold larger than the average length found in other A-GPCRs (Table **S4A**). The conserved and relatively abundant cysteines in these segments stabilize and conform the binding sites of the large dimeric protein agonists [86]. That is also known for the highly conserved ec1 cysteines of C-GPCRs (e.g. [87]), in which the stabilization is critical for the capture and presentation of the small agonists.

The ec1 N-glycosylation motifs are well represented in A-GPCRs with short N-terminal segment, and in peptide B-

GPCRs (Table **S4B**). The average number of such motifs per segment is about 2 in short-ec1 A-GPCRs, about 5 in leucine-repeat A-GPCRs, close to 4 in the peptide B-GPCRs, and 6 or above in the C-GPCRs (Table **S4B**). This indicates a larger potential for surface stabilization *via* glycosylation in B- and C-GPCRs, and could also aid the possibly obligatory homodimerization *via* extracellular domains for the C-receptors (see also Table **S2**).

In summary of the features reviewed in this section, the A-GPCRs are not expected to widely dimerize *via* the N-terminal domain. The B-GPCRs with peptide agonists also may not use this domain extensively in oligomerization, as this could lead to silencing of their agonist -binding sites. However, a large utilization of this domain in oligomerization could be expected for the adhesion subgroups of B-GPCRs, and for all receptors of family C. As seen in Tables **2** and **S2**, the above expectations are essentially supported by the available evidence.

3.3. Other Extracellular Domains

The extracellular loops of A-GPCRs do not seem to serve frequently in receptor oligomerization. Use of these A-GPCR segments for stable intermolecular associations could interfere with agonist access and attachment. The three extracellular loops of A-GPCRs are on the average much shorter than the N-terminal segment. In 209 canonical human receptors, the length averages are 42 residues for the N-terminal segment, and 15, 25 and 12 residues, respectively, for loops 1, 2 and 3; nearly the same lengths are found for 996 non-human canonical A-GPCRs (Table **S4A**). With bacteriorhodopsins as possible fossils of the heptahelical GPCR design, one also finds short exoloops 1, 2 and 3 (extracellular domains 2, 3 and 4) of 19, 5 and 10 residues, respectively (in 30 receptors). The basic GPCR structure could have been set close to the emergence of the metazoa. The design obviously is conserved for the transmembrane helices from the cnidarian (e.g. sea anemone [88]) to the primate. This also forces restraints on the non-helical connecting parts. The exocellular N-termini have no such restraints, and did evolve an enormous variety of size, sequence, and structure. The first and third extracellular loops (extracellular segments 2 and 4, ec2 and ec4) are short in most receptors. The second exoloop (ec3) shows more diversity, but in most cases is quite short (Table **S4A**). Importantly, the exoloops show few free cysteines and glycosylation sites, and thus maintain the scarcity of oligomerization linkers that is found in all opsins. In mammalian rod opsins, there are two cysteines in the ec3 domain, possibly serving in a dynamic redox exchange [89, 90]. The extracellular loops of non-opsin GPCRs have few free cysteines (Table **S4A**), and most of these could be paired in internal disulfides. The above could help to explain the lack of reports on A-GPCR dimers connected by extracellular loops.

An intramolecular disulfide connecting the exoloops 1 and 2 could be present in most canonical A- and B-GPCRs. Use of these cysteines was reported for dimerization in a modified m3 muscarinic receptor [91]. Tables **S4A** and **S4B** summarize features of the three extracellular loops in heptahelical GPCRs. The third loop (ec4) is too short in any receptor to be a viable candidate for oligomerization, and also is lacking cysteine. Loops 1 (the ec2 domain) and 2 (the ec3 domain) were shown by peptide chemistry to be connected by a Cys-Cys bond in the m1 muscarinic [92] and in the GnRH receptor [93], and the same was indicated by site-directed mutagenesis for both these and more than twenty other A-GPCRs. Dynamic cysteine bridges between loops 1 and 2 could be important in the activation of A-GPCRs [89], and especially of rod opsins. The presence of this bond should strongly limit the potential for extracellular oligomerization in A-GPCRs.

In the C3a chemotactic receptor (family A), the large ec3 domain may serve in dimerization. However, this could interfere with attachment of the 76-residue C3a anaphylatoxin peptide. A similar consideration applies to many peptide receptors in families A and B. However, the binding of small agonists may not be affected by such interactions.

3.4. The Intracellular Segments

Homo- or heterodimerization of GPCRs *via* intracellular segments could be covalent and long -lived, as in the case of the γ-aminobutyric acid B (GABAB) receptor, but such complexes should rather be considered as single entities, and the GABAB receptor is the only currently known representative of the type across the heptahelical GPCRs.

The first intracellular loop is very short in most GPCRs (Table **S5A**), possibly connecting evolutionarily to the H-bond link of the tm1.17 Asn and the tm2.11 Asp (see Table **S1A** for the positions), and to H bonding of the tm1.17 Asn and a peptide bond carbonyl in the tm7 [56]. The second intracellular loop also is not long, which could evolutionarily reflect interactions of the tm2, tm3 and tm6 segments [56]. The third loop (located between unconnected transmembrane segments 5 and 6) and the intracellular 'tail' (the ic4 segment) have diversified greatly in size, sequence and structure. In many monoamine and in several peptide A-GPCRs, and in the canonical C-GPCRs, third intracellular loops and / or C-terminal segments are large (80 to 250 residues) and highly interactive. The confirmed interactants in addition to subunits of G proteins include the same receptor, other GPCRs, phospholipases, cyclases, sodium and calcium channels, protein 14-3-3, integrins, and filament proteins.

Cysteines in the intracellular tail could serve for membrane anchoring (e.g. the anchoring by palmitate [94]), rather than for dimerization. These cysteines are found in a majority of A-GPCRs (except the olfactory), and in most B- and C-GPCRs (Table **S5A**). The ic1 loop contains cysteine in the hormonal peptide B-GPCRs. The ic2 and ic3 loops have cysteine in many groups. Cysteine bridging could lead to impairment of palmitoylation and to poor retention in the plasma membrane for both GPCRs (e.g. [95]) and ionotropic receptors (e.g. [96]).

Glycosylation motifs are present in many ic1 segments of the canonical C-GPCRs, in ic2 segments of about 50% of Taste 2 GPCRs, and in a number of ic3 domains of the invertebrate, but not of the vertebrate, opsins (Table **S5B**). These motifs are found in many intracellular 'tails' of all GPCR groups, except opsins and olfactory receptors (Table **S5B**), and could help both homomeric and heteromeric associations.

4. PROSPECTS AND CHALLENGES

Study of the dimers and dimerization could allow a fresh look at the perennially baffling allostericity in the ligand binding to several receptors, including especially the muscarinic (e.g. [27]), opioid (e.g. [29, 97]) and dopamine [98] receptors. This of course needs to be preceded by a proper examination of the dimerization and of the G protein activation status of the receptors.

In G protein -rich tissues, such as the brain, accumulation of GPCR dimers could point to pathological changes. Treatments and conditions that affect the Golgi area, including decreases in cholesterol, could strongly reduce receptor dimers [99]. Oligosaccharide-processing enzymes are known to be carried by transmembrane-homodimerizing receptors [100], and the dimerization of these enzymes could be affected in storage diseases [101, 102]. This can also be connected to changes in oligomerization of the heptahelical GPCRs.

Downregulation of functional G proteins (in disease [103] or aging [104], by toxins [65, 74], or by siRNAs) could generally result in reduced levels of GPCR dimers. Accumulation of peptide receptor dimers could be expected in response to long-term reduction in levels of the cognate agonists. Overexpression of many GPCRs could also lead to high levels of dimers, and result in a heightened sensitivity to agonists. Heterodimerization may regulate activation of receptors, and for large agonists could mainly reduce the affinity and activity [105]. A forced co-expression of some receptor types might produce therapeutic rewards, by enhancing [106] or neutralizing [105] activity of the natively expressed receptors.

Mutation of transmembrane 'switches' in regions of A-GPCRs that are not important in oligomerization, e.g. replacement of the tm2.11 aspartate by a non-ionic sidechain (which results in attenuated signal transduction) could influence stability of the dimers. The change in free energy due to a single transmembrane mutation can critically influence the dimerization [107]. With all GPCRs, antagonists and partial agonists could concentration-dependently reduce dimer processing, and this could represent a target in the medicinal chemistry of GPCR ligands.

The levels and functional significance of GPCR dimers need to be examined in neuronal, endothelial and epithelial environments, especially in terms of regulation of the signal transduction based on alternative homo- and heterodimerization. For chemokine receptors, there could be heterodimerization at low and homodimerization at high agonist concentrations, resulting in switching between adhesion-promoting and chemotaxis-promoting signal transduction [108].

Dimerization by crosslinking agents at the cell surface could be a tool to explore the fraction of extruded receptors, as well as the rates of extrusion and internalization. This could have practical impact and in some cases was already explored [109].

There is need for comparisons with the obligatory physiological oligomers, including growth factor receptor and steroid receptor dimers, S100 Ca^{2+} - binding proteins, annexins, integrins and cadherins. This also connects to receptor compartmentalization (e.g. [47]), and to general involvement of GPCRs in cell motility, anchoring, adhesion and organization. Comparisons are also needed between monomeric and oligomeric GPCRs in interactions with ion channels and filament systems.

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ABBREVIATIONS

CCK	=	Cholecystokinin
GPCR	=	G protein-coupling receptor
ec1..4	=	Extracellular segments 1..4
ic1..4	=	Intracellular segments 1..4
tm1..7	=	Transmembrane segments 1..7
NPY	=	Neuropeptide Y
A-GPCR	=	Family A receptor
B-GPCR	=	Family B receptor
C-GPCR	=	Family C receptors
G trimer	=	G protein αβγ subunit heterotrimer

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