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Abstract:

The Renin-Angiotensin System (RAS) is important for the regulation of cardiovascular physiology, where it controls blood pressure, and salt- and water homeostasis. Dysregulation of RAS can lead to severe diseases including hypertension, diabetic nephropathy, and cardiac arrhythmia, and -failure. The importance of the RAS is clearly emphasised by the widespread use of drugs targeting this system in clinical practice. These include, renin inhibitors, angiotensin II receptor type I blockers, and inhibitors of the angiotensin converting enzyme. Some of the important effectors within the system are 7 transmembrane (7TM) receptors (or G-protein-coupled receptors) such as the angiotensin II Receptors type I and II (AT1R and AT2R) and the MAS-oncogene receptor. Several findings indicate that the 7TM receptors can form both homo- and heterodimers, or higher orders of oligomers. Furthermore, dimerization may be important for receptor function, and in the development of cardiovascular diseases. This is very significant, since "dimers" may provide pharmacologists with novel targets for improved drug therapy. However, we know that 7TM receptors can mediate signals as monomeric units, and so far it has been very difficult to establish if our observations reflect actual well defined dimerization or merely reflect close proximity between the receptors and/or various types of functional interaction. In this review, we will present and critically discuss the current data on 7TM receptor dimerization with a clear focus on the RAS, and delineate future challenges within the field.

1. Introduction:

The Renin-Angiotensin System (RAS) is a central component in the regulation of the cardiovascular system, where it controls blood pressure, and salt and water homeostasis. Therefore, dysregulation of RAS can lead to severe diseases, including hypertension, diabetic nephropathy, cardiac arrhythmia, and -failure. The essential role of the system is clearly emphasised by the widespread use of drugs targeting RAS in clinical practice (1-3). The complexity of the RAS is constantly being uncovered; today we know that the system comprises a variety of enzymes including renin, and Angiotensin Converting Enzymes 1 and 2 (ACE1 & 2). These enzymes are responsible for the conversion of angiotensinogen to biologically active hormones such as Angiotensin II (Ang II) and Ang (1-7). The RAS hormones primarily exert their biological function via receptors belonging to the family A of the 7TM receptors, including the Angiotensin IIs type I and II (AT1R and AT2R) and the MAS receptor (for a detailed review of the RAS components please see (4, 5)). Moreover, the RAS system, also closely interacts with other systems such as the kallikrein system (6).

Even though it has been demonstrated that GPCRs can signal as monomeric units (7, 8), there is mounting evidence that 7TM receptors may form both homo- and heterodimers, and that dimerization could be important for receptor function, with respect to ligand binding, receptor activation, desensitization, and trafficking (9-23). In this regard, the AT1R is particularly well studied (se figure 1). For example, it has been shown that the AT1R decreases Gαq coupling when the receptor interacts with either the MAS receptor or the AT2R (13, 17-19), while, its interaction with the Bradykinin B2 Receptor (B2R) enhances signalling (12, 14, 24). The AT1R can also form complexes with the beta 2 adrenergic receptors (β2AR) and it is possible to achieve dual receptor inhibition of AT1R and β2AR signalling using only a single receptor antagonist (16). The AT1R

also co-immunoprecipitates with the Dopamine D1, D3, and D5 and the Endothelin B receptors (20, 25-27). In addition to the AT1R dimers, the AT2R has been proposed to form homodimers (13, 28) and heterodimers with the B2R (29).

The notion that receptors can form dimeric interactions is especially interesting as it might be possible to generate receptor-subtype specific drugs to improve drug therapy. The biological significance of heterodimerization could also be profound, since it will add an additional layer of complexity to the understanding of how 7TM receptors are activated, and it provides yet another link for cross-talk between different receptor systems (30, 31). Even though much data present today suggests that 7TM receptor dimerization do occur and may have a functional purpose, the exact role of dimerization has not yet been defined. In addition, some of the data on the field are hard to reconcile. Furthermore, we have yet to identify the domains responsible for dimer formation. In reality, it is very difficult to establish whether the observations on dimerization are due to a well defined high-affinity interaction between the receptors or merely reflect close proximity between the receptors and/or various types of functional interaction. In this review we will present and critically discuss the current data on 7TM receptor dimerization in the RAS.

2. Angiotensin receptor homodimerization:

2.1 AT1R homodimerization and activation.

AT1 Receptor activation and signalling: Most of the known physiological effects of Ang II are mediated by AT1Rs. The AT1R is a key regulator of blood pressure and body fluid homeostasis and prolonged activation of the AT1R can lead to severe diseases including hypertension, diabetic nephropathy and cardiac arrhythmia (1, 2). Its importance in renal and cardiovascular pathophysiology is underscored by the widespread use of AT1R blockers (1, 2). The AT1Rs are

widely distributed in all organs, including liver, adrenals, brain, lung, kidney, heart, and vasculature. In humans there is a single AT1R gene, whereas rodents have genetic coding for two isoforms, dubbed AT1AR and AT1BR. In the rat the isoforms share 95% amino acid sequence identity (32). The AT1R activation mechanism has been studied in great detail and involves multiple signalling molecules, including heterotrimeric G-proteins, small G-proteins, kinases (such as G-protein-coupled Receptor Kinases (GRKs)) or second messenger kinases (such as Protein Kinase A or C (PKA, PKC)), and scaffold proteins (such as β-arrestins) (33-37).

Recent studies show, that it is possible to separate AT1R signalling into two principle components: the G-protein-dependent signalling and the G-protein-independent signalling (36, 37). These studies also show that selective G-protein-independent signalling can be obtained by pharmacological means. In particular, the Ang II analogue [SII] Ang II is a formidable example of this concept, as it is able to induce β-arrestin2-dependent signalling including receptor internalisation and ERK1/2 activation without generation of inositol phosphate second messengers by the AT1R (38-43). These observations have lead to concepts as "Functional selectivity" or "biased agonism", and forced a redefinition of fundamental pharmacological paradigms, to allow description of multiple discrete states of receptor activation and ligands with different 'collateral' degrees of efficacy for individual responses (44-47).

AT1R homodimerization: Today we know that 7TM receptors such as the β2AR (8) and rhodopsin (7) can signal as monomeric units. Since data indicate that family A 7TM receptors are activated through common mechanisms, it is reasonable to assume that this also applies for AT1R (34, 48). However, although data regarding AT1R dimerization are somewhat conflicting, a substantial

number of findings indicate AT1Rs can form dimers, and that dimerization may influence receptor activation mechanisms (10, 11, 13, 14, 26).

Early studies indicating AT1R dimerization were performed on AT1R-transfected cells or primary tissues, using Ang II affinity labelling, and/or chemical cross-linking experiments followed by gel filtration or SDS-PAGE analysis. These analyses showed that is was possible to identify AT1R receptor complexes with molecular weights corresponding to both a monomeric and a dimeric form of the receptor. However, these observations were not linked to any functional consequences, and it has been speculated, that the dimers could result from technical artefacts causing protein aggregation (9, 23, 49-53).

In 1996, Catherine Monnot from the group of Eric Clauser elegantly showed that it was possible to restore receptor binding capacity in intact cells by co-expression of the two Ang II binding deficient AT1Rs, K102A and K199A. Although co-expression could not restore AT1R signalling, this study shows, that receptors can in fact trans-complement each other and thereby posses the ability to functionally interact, possibly as dimers in living cells (54).

In our hands, AT1R also forms dimers or oligomers in living cells (10). Using $BRET^2$ analysis we showed that AT1Rs specifically form homodimeric or -oligomeric complexes in living cells that are unaffected by both receptor agonist and antagonist. Furthermore, these dimers are probably formed as early as the ER, which is in good agreement with the observations for most 7TM receptors (10, 22, 23). To make this conclusion, we took advantage of a system termed the regulated secretion/aggregation technology (RPD) developed by Rivera *et al.* (55). This technology enables ligand-gated release of receptors from the ER to the plasma membrane (se figure 2). The principle is

in brief that, the receptor of interest is N-terminally fused to a protein (dubbed F_m), which accumulates as aggregates in the endoplasmatic reticulum (ER) and Golgi. This aggregation is alleviated by incubation with a synthetic small-molecule drug (AP21998), thereby allowing the fusion receptors to escape the ER and Golgi and travel to the cell surface (55). With this assay we could demonstrate that the surface expression of an untagged AT1R-wt could be regulated by AP21998 incubation if co-expressed with a Fm-tagged, signalling-deficient, AT1R variant (mutated in the DRY-motif) (10). This demonstrates that the two types of AT1Rs interact in the ER. To asses the functional consequences of receptor dimerization, we over-expressed binding or signalling deficient mutants together with wild type receptors. Surprisingly, we observed that AT1R wild type G-protein coupling was diminished, whereas ERK activation and β-arrestin recruitment was unaffected by expression of the mutant receptors. Further, we observed that it was possible to "trans-activate" β-arrestin2 recruitment to a binding deficient AT1R mutant (K199A), by co-expression of the AT1R-K199A-Rluc, β-arrestin2-GFP[2] and an untagged version of the wild-type AT1R (10).

Using a very elegant experimental approach, Eszter Karip from the group of Laszlo Hunyady showed that AT1Rs can "cross-inhibit" each other with respect to G-protein signalling, which support the concept of ligand independent receptor oligomerization. To draw this conclusion Szidonya *et al.* developed an AT1R mutant (AT1R-S109Y) insensitive to binding of the AT1R blocker, candesartan, but with normal binding and signalling properties of Ang II (56). When this receptor was co-expressed with a AT1R-DRY mutant unable to activate the G-protein, they observed that candesartan could inhibit the Ang II induced G-protein activation, even though candesartan only was able to bind the signalling deficient DRY-mutant receptor (11). This suggests that one "inactive" protomer can negatively regulate signalling of the other subunit in a putative

dimer. Thus the AT1Rs might need two active subunits to activate G-protein signalling in a situation, where a dimer form is formed (11).

AbdAlla from the group of Ursula Quitterer have also shown that the AT1Rs can form homodimers (13-15). In 2001 they report H2O2 induced AT1R dimerization in HEK293 cells and platelets. The dimer formation is independent of Ang II and linked to receptor inactivation (14). When AT1Rs are expressed in PC-12 cells they form stable and ligand independent dimers that can be covalently linked using the cross-linker DST. The functional consequence of these dimers was not analyzed (13). In another study, they identify AT1R homodimers in isolated monocytes (15). These dimers are covalently cross-linked by factor XIIIA. This factor works as a transglutaminase mediating enzymatic cross-linking of membrane proteins by catalyzing the formation of stable amide bonds between proteins (57). This cross-linking is shown to depend both on ionophore activation of factor XIIIA, and the presence of Ang II (15). The site of cross-linking was located to amino acid Q315 in the helix8 of the AT1R, and mutating this residue to alanine abolished dimerization and factor XIIIA induced cross-linking. In contrast to the homodimers identified in platelets, this covalent dimer shows improved signalling in response to Ang II stimulation (15). In their quest for biological relevance of this dimer, they found that both Factor XIIIA activity and AT1R homodimer levels were increased in hypertensive patients (15). The glutamine Q315, which was responsible for the cross-linking is positioned in the predicted "helix 8" of the cytoplasmatic tail of the AT1R. This glutamine residue is present in 10% of the family A 7TM receptors, so it should be interesting to see if this mechanism for regulation applies to other receptors (58). AbdAlla *et al.* also report that levels of AT1R homodimers in hypertensive patients was reduced or normalized by chronic treatment with the ACE inhibitor captopril (15). Monocytes derived from hypercholesterolemic ApoE knockout mice also demonstrated increased levels of AT1 dimers that could be reduced by

captopril treatment or by expression of a factor XIIIA inhibitor (15). Captopril treatment and expression of the factor XIIIA inhibitor also reduced monocyte entry into the artery wall and inhibited development of atherosclerosis in hypercholesterolemic ApoE knockout mice (15). These findings point to a significant contribution of covalent dimerization of AT1Rs in pathogenic events that drive lesion formation (15). Recently Yamada et. al. reported that the vasoconstrictor effects mediated by aldosterone could be partly mediated by upregulation of intracellular transglutamisase induction and a following AT1R dimeriztion (59).

2.2 AT2R Homodimerization.

AT2R activation and signalling: AT2R is also a 7TM receptor activated by Ang II though it only shares 34% sequence identity to AT1R (60). AT2R is widely expressed during the foetal development (61, 62), whereas after birth the expression is decreased and confined to certain organs such as, the heart, kidney, vascular smooth muscle, lung, brain, adrenal cortex, uterus and ovarian follicles (63). Additionally, the AT2R is upregulated during certain pathological conditions such as, cardiac remodelling, skin healing, and inflammation to suggests that the receptor plays a role in differentiation, remodelling and healing (see (63) for review). The exact role of the AT2R in the cardiovascular system remains obscure (64, 65). AT2Rs exerts anti-proliferative and pro-apoptotic changes in cells, and in certain settings the AT2R is reported to counteract the effects of the AT1R (33, 63). However, this is not always the case. For example, AT2Rs can also promote ligand-independent constitutive hypertrophy in cardiomyocytes (65, 66). Even though the AT2R belongs to the family A of the 7TM receptors, and it can in fact signal through G-proteins of the Gαi class (67), much of AT2R signalling probably occur via G-protein independent mechanisms (33, 63, 67).

AT2R homodimerization: AT2R homodimerization has been assessed in two studies and in both cases they are reported to be constitutive dimers that do not depend on the presence of Ang II or other stimuli (13, 28). The functional importance of AT2 dimers were not analyzed in the study by AbdAlla (13), but Muira *et al.* suggested that dimer formation could be important both for the basal constitutive, and agonist induced AT2R signalling (28). It their study, they identify two cysteines, C35 (in the N-terminal) and C290 in ECL3, that are critical for dimer formation. Mutating these residues to alanines generates a receptor that neither signals nor dimerizes, though it still localizes to the plasma membrane and binds Ang II (28).

3. RAS receptor heterodimerization

3.1 AT1/AT2 heterodimerization:

In 2001 AbdAlla *et al.* shows that AT1R and AT2Rs form constitutive heterodimers in cell lines and human myometrial biopsies. They also show that AT2Rs antagonize AT1R mediated signal transduction (13). This is interesting since it provides a possible explanation for some of the functional crosstalk that has been reported in several studies (63, 65). In their study, simultaneous AT1R and AT2R expression leads to the formation of a dimeric complex, consisting of both receptors, which can be isolated by affinity purification (13). This dimerization could be of functional relevance since expression of the AT2R abolish both the AT1R catalyzed Gαq and Gαi protein activation (13). They also show that the AT2R does not need ligand binding abilities of functional coupling to inhibit the AT1R signalling, since both the AT2R-H273N mutant, which does not bind agonist and the AT2R-S243A, which does not activate intracellular protein phospatase are able to dampen AT1R signalling at a level identical to that of the wild type AT2R (13). To establish the *in vivo* relevance, they identify AT1/AT2R dimers in isolated foetal fibroblasts, and furthermore, AT2R antisense treatment of these cells increase AT1R signalling

(13). They also report that AT2R expression is decreased during pregnancy, with an accompanying increase in AT1R signalling in human female myometria (13).

3.2 AT1/Mas heterodimerization:

The Mas receptor was originally described as a proto-oncogene (68) but the receptor is now established as a part of RAS and it is known to be activated upon binding of the peptide hormone Ang (1–7), which is formed either from tissue specific endopeptidases or from direct ACE2 mediated digestion of Ang II (69-73). MAS receptor signalling has in several cases been shown to antagonize the effects of the AT1R signalling both *in vitro* and *in vivo* (5, 71, 74-76).

In 2005 Evi Kostenis reported, that the MAS receptor co-expressed together with the AT1R dampened the Ang II mediated AT1R-G-protein coupling, while at the same increasing AT1R expression (18). Very interestingly, they also observe that a MAS receptor knockout mouse has an increased vasopressor response to Ang II. This suggests that this functional interaction can be extrapolated to *in vivo* situations (18). To assess whether the functional interaction could result from dimerization, they perform a series of BRET experiments. Here, they co-express a MAS receptor C-terminally tagged with Renilla luciferase (MAS-Luc) and a C-terminally tagged AT1R with enhanced yellow fluorescent protein (AT1R-EYFP). In this analysis, they observe that the two receptors generate a BRET signal, which is unchanged by both agonist and antagonist incubation. To assess the specificity of the signal, they performed a BRET2 titration experiment and observe that the MAS-Luc receptor interacts more strongly and with higher apparent affinity with the AT1R-GFP2 in comparison to cytoplasmic GFP2 (18). This could indicate that the two receptors form dimers. However, since GFP2 is not the ideal molecule to use as a “control” for specificity when examining membrane protein interactions, it will be important to compare the BRET signal

generated from the AT1R-MAS interaction with the BRET signal generated from the interaction between the AT1R and an unrelated 7TM receptor (77).

The functional interaction between AT1R and MAS was confirmed in a very elegant study, performed by Meritxell Canals from the group of Graeme Milligan. In this study, the MAS receptor expression also results in a decreased AT1R G-protein coupling accompanied by a simultaneous increase in receptor surface expression (19). However, their data indicate that the receptor up-regulation and functional antagonism is not likely a result of dimerization, but is rather a result of constitutive activity of the MAS receptors, which then leads to PKC dependent AT1R phosphorylation, and accompanying receptor- desensitization (19). To make this conclusion they perform the following important observations. First, PKC inhibition abolished the MAS receptor-induced upregulation of AT1R expression levels. Furthermore, PMA induced PKC activation in itself resulted in increased AT1R expression. Secondly, they show that co-expression of a G-protein uncoupled MAS receptor mutant (MAS-I138D) does not enhance AT1R expression. And thirdly, removing the C-terminal tail of the AT1R, which contains the PKC phosphorylation sites, renders the receptor insensitive to MAS expression (19).

In a third study, Santos *et al.* also demonstrate that MAS receptor expression uncouples the wild type AT1R from the G-protein to a similar extent as previously reported (17). However, very interestingly, they also observe that MAS receptor expression can result in a gain-of-function of the mutant AT1R (K18A, K20A). This mutant receptor does not travel to the cell surface when it is expressed alone. However, surface expression, ligand binding and G-protein coupling is restored to AT1R wild-type levels upon MAS receptor expression (17). The authors also report that the two receptors co-localize and therefore might form dimers (17).

3.3 AT1/B2 receptor heterodimerization:

AT1R- and B2R signalling fuctionally interact on many levels *in vitro* and *in vivo* and they have important biological effects that are opposing each other (30, 31). In a series of reports Abdalla, Quitterer and co-workers have reported, that the AT1R forms heterodimers with the B2R (12, 14, 24). From these studies, they derive three important pharmacological conclusions: First; the AT1R and the B2R form heterodimers in all the cellular systems examined both including different cell lines (24), and primary cells such as vascular smooth muscle cells (24), neurons (12) and platelets (14). These data indicate that the system is "universal" and that AT1R/B2R heterodimerization occurs as a natural consequence of simultaneous expression of AT1R and B2R within the same cell (12, 14, 24). Second; when compared to the individually expressed receptors, the AT1R/B2R heterodimer signals with higher potency and efficacy upon Ang II stimulation, whereas Bradykinin signalling remains largely unaffected (24). Third; the B2R must be competent to engage its G-protein signalling pathway to produce the functional AT1R-potentiation (24).

The AT1R/B2R interaction is of great biological interest, since it could add to the complexity of the cross-talk between the RAS and the kallikrein-kinin system (30, 31). AT1R and B2R are naturally co-expressed in several cell types (78, 79) and both systems are intricately connected as show by several observations: 1) the AT1R functions as a vasoconstrictor while the B2R is a vasodepressor (30, 31); 2) ACE is responsible for both production of circulating Ang II and at the same time degradation of Bradykinin (80), and 3) AT1R activation leads to upregulation of the B2R (30, 31). It has also been shown that many biological effects of AT1R blockers such as Losartan are in fact mediated by the release of Bradykinin and, accordingly, can be blocked by the B2R inhibitor

HOE130 (81-83). In contrast to the paradigm described by AbdAlla *et al.*, the bradykinin B2R knockout mice have an enhanced susceptibility to Ang II induced hypertension (84).

From a medical perspective, the AT1R/B2R interaction would be of outstanding value, stressed by the finding by Abdalla *et al,* that B2 upregulation and AT1R potentiation can be linked to experimental hypertension (12), vascular smooth muscle cell contraction (24), and preeclampsia (14). The proposed disease relevance of the dimer implies that development of AT1R/B2R heterodimer specific antagonists could be beneficial for the treatment of cardiovascular diseases. This would be of particular value for preeclamptic patients, since they are characterized by an increased responsiveness to Ang II, but an unaltered concentration of circulating Ang II (85, 86). In their study AbdAlla and Quitterer suggest that B2R-expression levels, and also AT1R/B2R heterodimers, are upregulated in preeclamptic patients, and that this is the reason for their increased response to Ang II (14). If this is the case, a AT1R/B2R heterodimer specific antagonists could provide a new treatment paradigm, since the preeclamptic pregnant women cannot be treated with classic AT1R blockers as these cause fetal growth retardation, pulmonary hypoplasia, limb contractures, and calvarial hypoplasia in various combinations (87, 88). Hopefully, future studies will address the general significance of this potentially important finding as it remains to be reproduced by other research groups. It should be kept in mind that other mechanisms, such as the presence of agonistic auto-antibody to the AT1R, or the particularly high AT1R expression is decidua, also may explain the hyper-reactivity towards circulating Ang II in preeclamptic patients (89). For insightful reviews on this topic see (85, 86).

3.4 Other 7TM receptor-heterodimers in RAS:

AT1/β2AR: Barki-Harrington *et al.* show that AT1R can form complexes with the β2ARs, and that it is possible to achieve dual receptor inhibition of AT1R and β2AR signalling using only a single receptor antagonist (16). Interestingly, they provide evidence that the β-blocker propranolol, can inhibit the Ang II induced muscle contraction of mouse cardiomyocytes, and furthermore, that the AT1R blocker valsartan can inhibit the isoproterenol-mediated contractility. The mechanism for this trans-inhibitory effect of β-blockers and AT1R blockers is through receptor G-protein uncoupling and not due to changes in ligand binding; i.e., β-blockers interfere with AT1R-Gαq coupling, and valsartan interferes with β2AR-Gαs coupling, while none of the blockers affected the binding properties of the "trans-receptor". This interaction could be *in vivo* relevant, since, administration of the AT1R blocker valsartan to intact mice results in a significant reduction in the maximal response to catecholamine-induced elevation of heart rate (16). Furthermore, the RAS and adrenergic systems affect each other *in vivo* in a positive feed-back-loop, since RAS activity results in increased adrenal drive as well as adrenal drive results in renin release (90). β-blockers also interfere with Ang II signaling in cardiovascular diseases (1).

AT1/Dopamine D1 -D3 -D5 and Endothelin B receptors: In a number of papers Zeng and co-workers show that AT1Rs can interact with receptors from the doperminergic and endothelin families and that this might be important for the development of hypertension in spontaneously hypertensive rats (20, 25-27).

AT2R/B2: Recently, Abadir *et al.* showed that the AT2R and the B2R can form hetero-dimers. They detect the interaction between the two receptors with fluorescence resonance energy transfer (FRET) microscopy and co-immunoprecipitation (29). In their study, the rate of AT2R-B2R heterodimer formation is largely a function of the degree of AT2R-B2R expression. They

furthermore link hetero-dimerization to a diverse array of signalling events that may regulate NO production (29). This interaction could have a physiological relevance as functional interaction between AT2 and B2Rs have been implicated in left ventricular remodelling after post-myocardial infarction in mice (91).

4. How do we find out if dimerization is real?

As described above, several pieces of experimental data shows how 7TM receptors in RAS interact on a functional level affecting each others signalling properties with respect to ligand binding, signal transduction, desensitization and trafficking (9, 21, 22, 35). These findings can all be linked to the possibility that these receptors form dimers. However, today we know that 7TM receptors are able to signal to G-proteins and β-arrestin as monomeric units (7, 8). Thus, we are still far from knowing weather these family A receptors actually do form well defined dimers, and if so, what the functional purpose of the dimers is (21, 92). When examining the results in detail, it is difficult to assess if our observations are a result of dimerization or if they originate from various kinds of functional crosstalk and molecular crowding. In this section, we will provide an overview of how the functional crosstalk has been linked to dimer formation and briefly discuss the experimental challenges and problems with data interpretation with in this field. For comprehensive reviews on this topic please se (9, 21, 23, 92, 93).

4.1 Biochemical evidence for dimer formation and experimental challenges.

Several techniques have been developed to study dimerization. The most widely used are biochemical techniques such as co- immunoprecipitation, chemical cross-linking and the biophysical technologies relaying of resonance energy transfer between two excitable molecules in close proximity such as FRET and BRET (9, 21, 23, 94). However, protein complementation

technologies such as bimolecular fluorescence complementation (BiFC), (using either split-YFP (95) or split-GFP (96) molecules), or beta-galactosidase complementation assay (97, 98), also have been used. The different protein complementation technologies are based on similar concepts; the reporter protein (the fluorescent protein or beta-galactosidase) is split into two fragments, which are then fused to the proteins of interest. If the proteins of interest are in close proximity, the reporter protein moieties will re-associate to form a functional reporter unit capable of providing a detectable signal. Recently, BRET and BiFC has been used in combination to study the stoichiometry of larger protein complexes (99).

Although all these techniques are used in fine-tuned experimental settings, and all the proper control experiments are performed to ensure that the signal detected is a result of specific protein-protein interactions, it is never possible to completely ensure, that the detected signal is a result of specific protein-protein interactions rather than specific co-localization and accompanying random collision. The BRET technology has been refined tremendously during the last decade (23, 93). For example BRET50 values are measured to ensure that the protein-protein interaction has a “high affinity” compared to bystander BRET, which is defined as the BRET generated form random collision. For details see (23). It is also important to ensure that the BRET signal does not vary as a result of receptor expression levels. If it does, this could imply that the signal is caused by simple clustering. On the other hand, if it is stable over a broad range of expression levels, it could imply that the interaction is of high-affinity (23). Another important control for specificity is to over-express untagged receptors that must be able to cause a reduction of the BRET signal by intercalating with the tagged receptors, if the receptors interact specifically (23).

Nevertheless, even if all these control experiments seemingly provide us with the signs of specific protein-protein interaction, it is still not possible to exclude the possibility that the receptors are merely co-localized in the same cellular compartment. For example, a very high degree of co-localization of two proteins will generate a low BRET50 value compared to a BRET50 value for two proteins not co-localizing to a similar extent. Also, if a protein-protein interaction appears constant over a large range of protein-expression levels, the explanation could still be that they are located within the same microdomains, which are saturated at an even lower protein level. Just as well as, if the BRET ratio increases with expression levels, this does not necessarily exclude that some of the observed signal is in fact due to specific protein-protein interactions. Finally, if it is possible to reduce a BRET signal by over-expression of an untagged protein this does not exclude that the BRET signal results from unspecific protein assembly, since it is likely that the wild type protein will co-localize with the tagged receptors, and therefore interfere with unspecific signals as well. Comparable arguments can be made for the other techniques used to detect dimers.

4.2 A common dimer interface has not been identified and the crystal structures suggest that 7TM receptors are monomeric units.

If 7TM receptors are forming dimeric or oligomeric protein-protein interactions with high affinity it is puzzling why crystal structures of Rhodopsin (100-104) and the β2AR (105, 106) are not precipitated in a dimers or oligomeric form that makes biological sense. In certain cases the individual Rhodopsin monomers in the crystal were oriented asymmetrically with respect to "membrane-orientation" (101, 102). In other cases, Rhodopsin is crystallized as parallel dimers, but the is no consensus in the orientation, which suggests that these dimers are not physiologically relevant (100, 103, 104). When the β2AR is crystallized with a monoclonal antibody fab fragment that binds to the intra cellular loop 3 (IC3), it is precipitated as a monomer (105). However, when it

is crystallized with a T4 lysozyme situated in the IC3, it was precipitated as a dimer with the a primary contact point between K60 in the bottom of TM1 and E338 in helix 8 (106). Though it is possible that the dimer is disturbed in the packing of the crystal, the fact that these crystals precipitate without a common dimer interface could argue that Family A 7TM receptors prefer or only exists in a monomeric form, and that the dimeric interactions are of low-affinity. If proteins interact in a complex of high affinity, it is very often possible to precipitate these complexes into crystals that diffract well. For example, other membrane proteins such as the Voltage-dependent K+ (Kv) channels are crystallized in what is believed to be their functional unit consisting of four non-covalently linked T1 domains (107). It is convincingly established, that the Family C 7TM receptors form dimers trough the amino-terminal domain of the receptors (108-110). Accordingly, the amino-terminal domain of the mGluR1 forms dimers when this is crystallized (111). Also proteins that are believed to interact strongly, but transiently and reversibly, such as the Gβγ subunit and GKR2, has been crystallized in complex (112).

In general it has been difficult to establish the existence of a common interface although several studies have implicated TM4 as a mediator of homodimerization for the Dopanine D2 (113, 114), the C5a (115) and the serotonin 5HT2c receptor (116). Nevertheless, almost all seven helixes have been implicated in dimer formation (9, 21). The lack of a conserved dimer interface certainly points in the direction that dimerization is not directly involved in activation mechanisms of G-proteins and β-arrestin. Recently, it has also been shown in reconstituted systems that the β2AR (8) and rhodopsin (7) can signal as a monomer. Given the fact that family A 7TM receptors probably are activated through common mechanisms (34, 48), it is reasonable to assume that the dimeric interface should be conserved as well. Furthermore, most experimental data suggest that 7TM receptors interact with the G-protein and β-arrestin with a stoichiometry of 1:1, which further

argues that the monomeric receptor is indeed the functional unit. For a review of this topic please see (21, 92).

It should be kept in mind that "dimers" may still be used as a "pharmacological concept" even if it covers functional cross talk or receptor co-localization. In this scenario it may be possible to target specific subsets of receptors in various clusters with specific "heterodimeric" ligands, since many receptors show distinct ligand binding properties as a result of specific modification or the cellular milieu may be different.

4.3. Functional crosstalk - Indirect allosteric modulation, signal complementation or dimerization?

As depicted in figure 3, functional coupling between two receptors can be transmitted through many possible routes. Firstly, they can physically interact either through a specific dimer interface or through clustering. Secondly, each receptor can affect intracellular proteins that modify receptor function. Thirdly, each receptor can interact with particular proteins that are somehow limiting for receptor activity of the other receptor. Fourthly, each receptor can regulate the expression of the other. And lastly, the receptors can cause a paracrine ligand release of receptor agonist.

From the studies performed on the RAS receptors, it is difficult to establish exactly which mechanisms are responsible for the observations. For example, the cross-inhibition studies from our laboratory showing a dominant negative AT1R interfering with wild type signalling, (10) or the cross-inhibition of candesartan in the experiments from Karip (11) could easily be viewed as a demonstration that receptors can dimerize, and that this can have functional consequences. On the other hand, other more mundane explanations exist. First, allosteric interferences between the

mutant and wild type receptor due to simple clustering could produce similar outcomes. Second, the dominant negative effects could be caused by a titration of necessary factors by the co-transfected mutated receptor. Important control experiments therefore are to include over-expression of proteins from the designated signalling cascades, and to analysis for putative mutant-receptor effects on other 7TM receptors. However, given that the receptors of interest interact in close proximity or via "dimeric interactions" with the receptor mutant, these controls do not exclude that the dominant negative effect could be caused by a substrate limitation due to simple steric hindrance exerted by the mutant-receptor/substrate interaction. This type of hindrance would not limit activation of other receptors or be alleviated by over-expressing the signalling proteins. It should also be kept in mind that this work does not tell us whether two functional receptors are necessary or advantageous for receptor activation.

In the reverse example with apparent trans-activation, similar dilemmas occur. In this scenario the "active" receptor or the "heterodimer" could attract new proteins, or just recruit more proteins, such as G-proteins, kinases and scaffold proteins into the vicinity of the other receptor and this could result in a "gain-of-function" phenotype. In this scenario the receptors do not need to interact, they are just required to be within a range where their ancillary signal transduction molecules can interact. For example, in our studies of AT1R, we observe that β-arrestin was recruited to an Ang II binding defective receptor (AT1-K199A), by stimulation with Ang II only if this receptor was co-expressed with wild type AT1R (10). This apparent interaction does actually not have to be a result of direct receptor-receptor interaction, but could easily be explained by trans-phosphorylation of the mutated receptor.

The AT1/MAS receptor "heterodimer" is a formidable example of the complexity to this field. The receptors may interact physically, but on the other hand MAS is also constitutively active (17-19). Apart from the identified PKC activation that is responsible for the AT1R phosphorylation/uncoupling and accompanying increase of receptor expression, the MAS expression could also affect the entire cellular composition and in this way affect AT1R activation (19).

A very elegant example of how receptors functionally interact through limiting auxiliary proteins (or signalling cascades) has been demonstrated by Pernille Hansen and co-workers. They addressed the mechanisms underlying the functional potentiation between Ang II and adenosine mediated renovascular constriction (117, 118). In their studies they convincingly show that AT1R and Adenosin A1 receptor (A1R) stimulation mutually potentiate each others ability to mediate vasoconstriction. Furthermore, they show the enhanced vasoconstriction depends on the ability of the A1R to activate Gαi since Pertussis Toxin (PTX) incubation completely abolish the A1A mediated Ang II potentation (118). As an example of receptors that functionally interact through paracrine ligand release, Turu *et al.* elegantly demonstrate, that AT1R signalling can cause release of cell-derived endocannabinoid (eCB) mediators which then supports basal activity of cannabinoid CB1 receptors (CB1Rs) in non-neural cells and neurons (119).

5. Conclusion, Perspectives and Future challenges of dimerization.

Do 7TM receptors dimerize? - Where is the conserved interface? As described it has been clearly established that 7TM receptors in RAS interact on a functional level and that they may form dimers. Though these interactions clearly affect signalling properties, we do know that the minimal functional unit for receptor activation is a monomer (7, 8). However, this does not exclude that

dimers (or oligomers) can work as functional units in certain scenarios, but today we are still far from knowing weather these family A receptors actually do form well defined dimers and if so what their functional purposes are (21, 92). Currently, a conserved dimer interface has not yet been identified, tough it may very well exist, since the contradicting observations may reflect differences in the experimental conditions, and relatively few receptors have been thoroughly examined so far. Amongst 7TM receptors, the arrangement of the seven trans-membrane domains is conserved and the molecular structure of multiple interacting molecules including G-proteins, arrestins, receptor kinases, and scaffolding proteins are also conserved. This implies that the receptor activation mechanism is probably conserved as well. Accordingly, a conserved dimer interface is bound to exist if dimeric receptor units also activate these proteins. In the search for the ”dimer interface” it will be important to examine more receptors using several different techniques.

Dimers or functional crosstalk? – Perhaps it is not important for the pharmacological potential?

When looking very closely at the current data, it is difficult to establish if our observations are the results of dimerization or various kinds of functional crosstalk. Nevertheless, dimerization is one of the possible explanations for these observations, and in our opinion receptor “dimerization” is a very interesting concept with a great pharmacological potential. Even if these functional interactions are not caused by *de facto* high-affinity and well-defined dimerization domains, they still exist and may be biological relevant. Conceivably it is possible to generate drugs targeting a limited group of a given receptor that are functioning in a specific manner as a result of a particular functional interaction. It is well appreciated, that receptors change their ligand binding or signalling properties as a result of post-translational modifications or due to the presence of scaffold proteins (46). The clear pharmacological potential of the opioid 7TM receptor dimers has been elegantly demonstrated by Maria Waldhoer from the group of Jennifer Whistler (120). They demonstrate that

the opioid agonist ligand 6'-guanidinonaltrindole (6'-GNTI) has the unique property of selectively activating the delta opioid peptide and kappa opioid peptide receptors heterodimers, but not homodimers. Importantly, 6'-GNTI is an analgesic, thereby demonstrating that opioid receptor heterodimers are indeed functionally relevant *in vivo*. In addition to this, 6'-GNTI only induces analgesia when it is administered in the spinal cord, but not in the brain. This suggests that heterodimers are tissue-specific and therefore could provide an approach for designing analgesic drugs with reduced side effects (120).

Dimerization and Functional selectivity: So far many of the studies on of these dimers have been focussing strictly on the effects G-protein activation. However, many 7TM receptors including the AT1R and AT2R also signal through G-protein independent pathways both *in vivo* and *in vitro* (33, 35, 38-42). It will be interesting to determine how heterodimerization affects G-protein independent receptor activation as well.

How many 7TM receptors interact or dimerize? Though many functional interactions between 7TM receptors have been reported *in vitro* and *in vivo*, it is still difficult to assess the prevalence the phenomenon. Most studies only analyze the interaction of a particular "dimer of interest" and include a few control interactions to define specificity of the interaction. We still don't know how many 7TM receptors actually interact with each other in a given system. This question is of course very difficult if not impossible to answer comprehensively. Nevertheless, it would be very interesting to try to begin to study this. Even in very simple systems, it would be of great value to know how many receptor interact with each other physically or functionally – will it be one out of 100 receptors – or perhaps one out of ten?

Figure legends

Figure 1: AT1R dimerization. There is mounting evidence that 7TM receptors may form both homo- and heterodimers, and that dimerization could be important for the receptor function. As depicted schematically on the figure, the AT1R (show in green) forms both homodimers and heterodimers with several other 7TM receptors. When AT1R assembles with the B2R (shown in purple) it forms a heterodimeric receptor with enhanced signalling capabilities in response to Ang II (as illustrated by the thick arrow). On the other hand, when it assembles with other receptors, such as the MAS Receptor (shown in red), it forms a heterodimeric receptor with decreased signalling capabilities. This illustrated by the thin arrow. See text for details.

Figure 2: The regulated secretion/aggregation technology (RPD) – A means to detect dimerization. Many 7TM receptors probably dimerize as early as the ER. This can be indirectly detected using The Regulated secretion/aggregation Technology (RPD) (10, 55). As depicted in the figure, this technology enables ligand-gated release of receptors from the ER to the plasma membrane. The principle is in brief that, the receptor of interest is N-terminally fused to a protein (dubbed F_m – depicted in red), which accumulates as aggregates in the ER. This aggregation can be alleviated by incubation with a synthetic small-molecule drug (AP21998 – shown in black), thereby allowing the chimeric receptors to escape the ER and travel to the cell surface. In practice, this allows AP21998 mediated gating of Fm-tagged 7TM receptors. If a receptor (depicted in green) forms stable dimers with the Fm-tagged receptor (depicted in purple) in the ER, it will be possible to regulate the surface expression of this particular receptor using AP21998 even though this receptor does not contain a Fm-tag itself. On the other hand, a receptor (depicted in blue) that does not dimerize with the Fm-tagged receptor is not affected by AP21998 incubation, but can travel constitutively to the cell surface (10). See text for details.

Figure 3: Functional coupling between two receptors can be transmitted though many possible routes. The possible routes of functional interaction between two co-expressed receptors are shown as black arrows. This presentation is highly simplified as the interactions can be direct or include several other molecules, signalling systems, or even other cells. 1) The two receptors can physically interact either through a specific dimer interface or through clustering. 2) Each receptor can affect intracellular proteins that modify receptor function, such as kinases or scaffold proteins. 3) Each receptor can modulate the activity or expression of particular proteins that are somehow limiting for receptor activity of the other receptor. 4) Each receptor can regulate the expression of the other. 5) The receptors can cause a paracrine ligand release of receptor agonist (or antagonist). As discussed in the text, it is sometimes difficult to establish exactly with mechanisms are responsible for the observations we observe.

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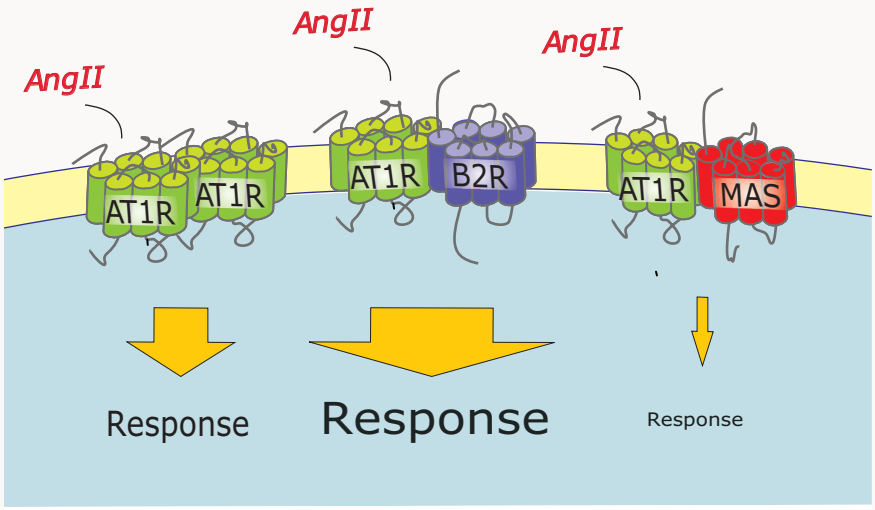

Figure 1

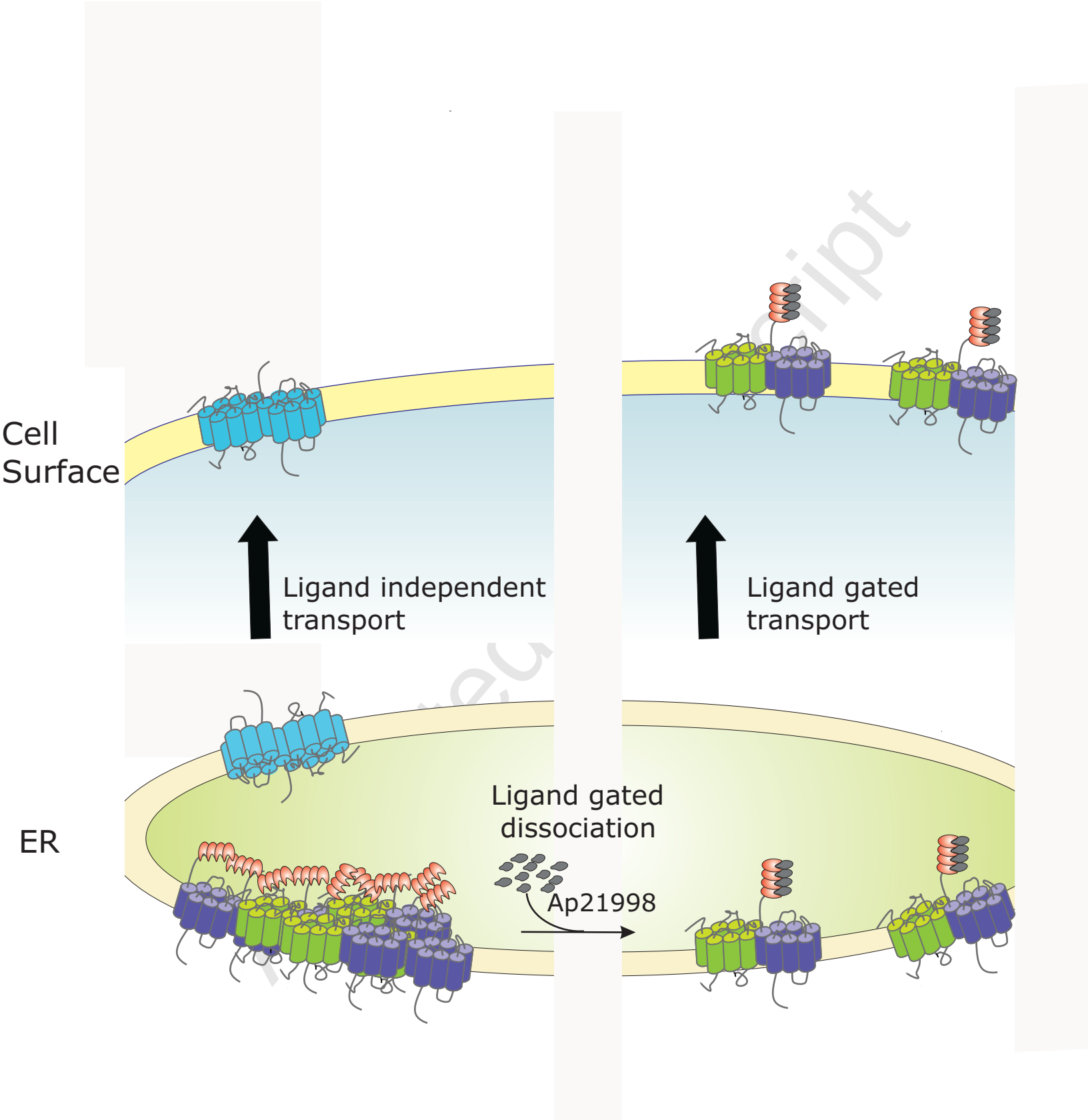

Figure 2

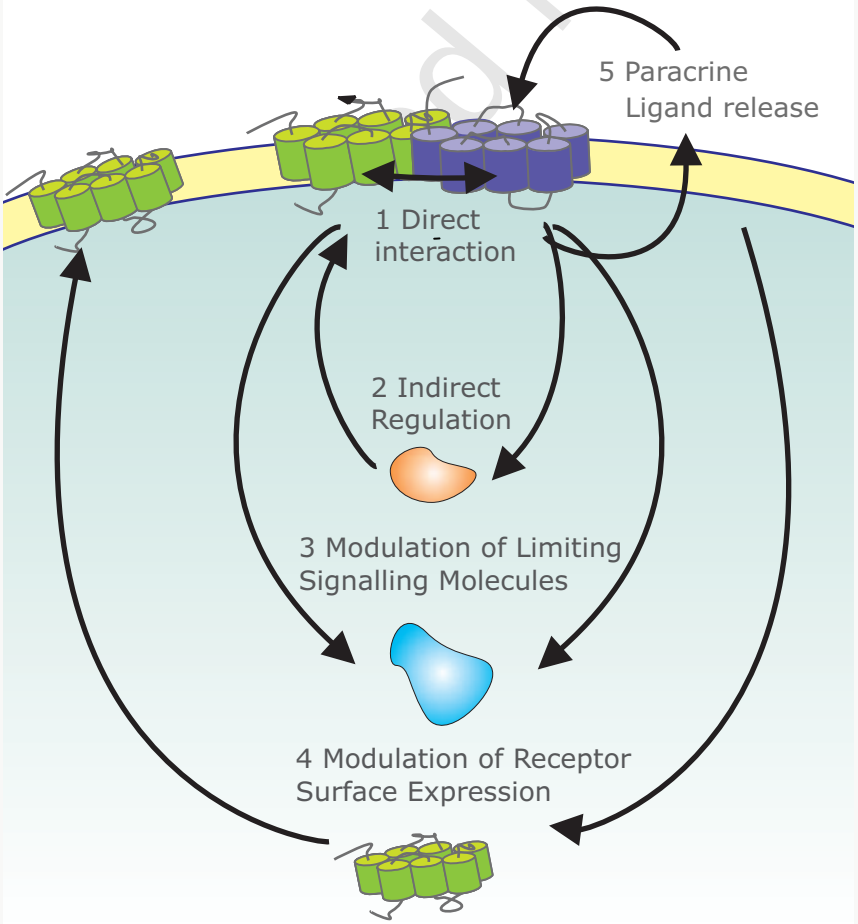

Figure 3