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Ligand Regulated Oligomerization of ß$_2$-Adrenoceptors in a Model Lipid Bilayer

Juan Jose Fung, Xavier Deupi, Leonardo Pardo, Xiao Jie Yao, Gisselle Velez-Ruiz, Brian DeVree, Roger Sunahara

Corresponding author: Brian K. Kobilka, Stanford University

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 18 May 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, both referees 1 and 2 show significant interest in your work, and are supportive of publication, contingent upon a number of issues being addressed. Referee 3, on the other hand, is more negative, and does not recommend publication. However, given the positive recommendations of the majority of reviewers, we are prepared to consider a revised version of your manuscript - provided you are able to answer the criticisms of the reviewers adequately. In particular, referee 3 finds further validation would be required to substantiate your proposal that inverse agonist binding affects oligomerisation of the receptor. In addition, referee 2 highlights concerns as to the GTPγS binding assays, and as to the effects of G protein binding on oligomerisation status. It will also be important to address the comments of the referees regarding discussion of how your data fits with already published findings on ß$_2$AR and other GPCR oligomerisation.

I would therefore like to invite you to submit a revised version of the manuscript, addressing all the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final version of the manuscript.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

This manuscript describes a series of FRET-based studies examining receptor-receptor interactions between highly-purified beta-2-adrenergic receptors ($ß_2$AR) reconstituted into artificial lipid bilayers. The authors report evidence that the receptors can associate into oligomers, most likely tetramers, and that the interactions between the receptors are altered by treatment with inverse agonists. The authors describe many rigorous control experiments they performed to convince themselves that the observed receptor-receptor interactions were not simply a result of random collisions or artifacts of absurdly high packing of receptors into the lipid bilayers. Overall, the data shown here are extremely convincing and the paper is very clearly written.

These studies are important because of the intense current interest in G protein-coupled receptor (GPCR) oligomerization and the controversial nature of some of the past findings in this area. Almost all previous studies of GPCR oligomerization have been performed using intact cells, of course, with FRET-based techniques or co-immunoprecipitation approaches being employed to assess the formation and regulation of receptor-receptor complexes. A major issue with many of these previous studies is that it has been impossible to rule out scenarios in which the observed receptor oligomers might just be held together by scaffold proteins, such that the receptors are close to each other (and tethered together by joint interactions with other proteins) but not engaging in authentic receptor-receptor interactions per se. A major advantage of the current study is the highly-purified nature of the preparation, which means that there are no other proteins present to hold the receptors together. This leads the authors to the convincing conclusion that the receptors do in fact have some native ability to associate with each other to form oligomers.

My only criticism of this elegant work is that I think there should be more discussion comparing the ligand regulation data in the current study with the published findings for ligand regulation of $ß_2$AR homo-oligomerization in cells. Many previous papers, mostly from the Bouvier group, have reported an increase in $ß_2$AR homo-oligomerization in cells following agonist stimulation. In the current paper, using the highly purified $ß_2$AR preparation, agonist stimulation has little effect on the extent of the receptor-receptor interactions, whereas inverse agonist stimulation has a bigger effect. I believe that the authors should be more explicit in addressing what seems to be a discrepancy between the current work and past findings. Might the lack of G proteins (or other cellular factors) in the present studies contribute to the difference between the present work in the purified preparation and the earlier-published studies in cells? Do the receptors in this highly-purified preparation have an unusually high level of constitutive activity, such that agonist stimulation is bound to have little effect on conformation, whereas inverse agonists are more likely to induce conformational changes? It would enhance the manuscript if these issues could be addressed in the Discussion as clearly and directly as possible.

Referee #2 (Remarks to the Author):

Oligomerization of Family A GPCRs and its biological role is a hotly debated issue. Here the authors address the propensity of b2-adrenergic receptor ($ß_2$AR) to oligomerize using the most direct approach: reconstituting purified receptors labeled with relatively small fluorescent moieties into phospholipids and performing rigorous FRET analysis. The authors should be commended for performing very thorough controls for receptor functionality, orientation in lipid vesicles, distribution in vesicle population, etc. An important issue of "bystander FRET" is very well addressed by FRET saturation assays and reconstitution of the receptor at 10 times higher lipid/ $ß_2$AR ratio. These experiments exclude usual artifacts and make the data very reliable.
The authors show that purified $ß_2$AR spontaneously oligomerizes upon reconstitution into lipids, forming predominantly tetramers in the absence of ligands and in the presence of neutral antagonists or agonists. Another very important finding of this study is that in the presence of inverse agonists receptors form higher order oligomers and/or more stable tetramers.

The results of this excellent study are very important for GPCR field and would be of great interest to the broad readership of the EMBO Journal. The only possibly questionable experiments are GTPγS binding assays. A few presentation issues should be also addressed to improve the manuscript. In addition, the demonstration of the ability (or inability, as the case may be) of oligomeric ß$_2$AR to couple to G protein could make this work exceptionally strong. If feasible with the methods and reagents in hand, the authors should perform these decisive experiments.

Biological role of observed ß$_2$AR oligomers:
1. The authors should place their results into broader biological context. Their data suggest that ß$_2$AR "forced" into inactive conformation by inverse agonists forms the most stable and/or the largest oligomers. In the context of previous findings that dimeric rhodopsin (Bayburt et al, 2007) and neurotensin NTS1 receptor (Proc. Natl. Acad. Sci. U. S. A. 104, 12199-12204) have lower ability to activate G proteins than monomeric forms, the results are consistent with the idea that oligomers of at least some Family A GPCRs represent an inactive form, whereas monomers represent the signaling state of the receptor. The authors should discuss (or possibly refute) this interpretation.
2. In the same vein, it would be most important to show whether b2AR in oligomeric form efficiently couples to G protein and whether G protein binding affects the oligomerization of the receptor. If at all feasible, the authors should use FRET saturation experiments to test whether agonist-treated receptor in the presence of sufficient amount of accessible heterotrimeric G protein remains in the same oligomerization state as in its absence.

GTPγS binding assay is the only possible experimental weakness in this study. In particular:
3. The methods suggest that the authors used membrane-tethered Gas (Lee et al, 1999). In the original paper, the authors characterized this construct in Sf9 cell membranes containing endogenous bg-subunits. Did purified tet-Gas used here contain bg-subunits? If yes, the authors should show this and estimate the fraction of tet-Gas in heterotrimeric form. If not, the authors should address the relationship between receptor coupling to tet-Gas and to physiologically relevant heterotrimeric Gs.
4. It is well known that the initial rate of GTPγS binding (first linear part of the time course) reflects the activity of the receptor. In contrast, the plateau of GTPγS binding in this type of assay reflects the amount of functional G protein present. As the authors use 30 min incubation, they should show that the rate of GTPγS binding is linear from 0 to 30 min, and that in the range used here it is linearly proportional to the amount of ß$_2$AR added.
5. The authors should demonstrate whether the orientation of tet-Gas matches that of the receptor, i.e., whether all receptors in the sample had unimpeded access to G protein. Another important concern is whether all molecules of G protein had unimpeded access to GTPγS (which does not cross lipid bilayer). E.g., if the orientation of tet-Gas matches that of the receptor, only 10% of it (coupling to the ~10% of the ß$_2$AR in the inside-out orientation) has access to GTPγS. Although this does not undermine the validity of the functional test per se, it raises the question how the oligomerization state of the 90% of the receptors in outside-out orientation corresponds to the state of the 10% that activated G protein in this assay. The authors should clarify this important point.

Other issues:
6. The data presented here strongly suggest that monomers, dimers, tetramers, and possibly larger oligomers of ß$_2$AR are in equilibrium. The same was recently shown to be the case for D2 dopamine receptor (Fonseca JM, Lambert NA (2009)). The authors should discuss functional implications of this for G protein activation assay (where just a few receptors could yield a robust signal) and for their FRET studies, which by definition report on the state of the majority of receptors, ignoring small subpopulations.
7. Show the data on activation-induced TM6 movement in monomeric ß$_2$AR reconstituted into HDL particles and in predominantly tetrameric receptor in liposomes.
8. No evidence for very large oligomers of dark (inactive) rhodopsin reported in mica-adsorbed disc membranes (Liang et al, 2003) was found in the discs in the natural environment of the rod outer segment (J Biol Chem. 2008 Oct 31; 283(44):30015-24), even though rhodopsin occupies ~50% of the disc membrane. Does this affect authors' interpretation?
9. Suppl Fig.5 would be more appropriate as panel D in Fig.7.
10. In the introduction the authors lump together references to studies of Family A and Family C GPCRs. Considering that there is no doubt that Family C GPCRs are constitutive dimers, and that

the role of dimerization in the function of some of these receptors is very well established (in contrast to Family A receptors), this is hardly appropriate.
11. The reference to Fonseca JM, Lambert NA (2009) is incomplete.

Referee #3 (Remarks to the Author):

G-protein coupled receptor oligomerization is a mater of intense debate, and it is still not clear whether rhodopsin-like GPCRs assemble into dimers or higher order oligomers. In the present study, the authors examined this issue using purified $ß_2$AR reconstituted into lipid vesicles. By inserting specific donor and acceptor fluorophores at various positions in the receptors, they confirmed that $ß_2$AR spontaneously form oligomers in the total absence of additional proteins. Their data are more compatible with a tetramer than with a dimer, as based on the saturation kinetics. They went on and analyzed the effect of various ligands (agonist, antagonist and inverse agonist) on the FRET efficiencies, and found that only inverse agonists binding resulted in a change in FRET, compatible with a larger oligomeric state, and a likely more compact association between the protomers.
Some critical controls necessary for the interpretation of the data are well performed. 1) They demonstrated, using several complementary approaches, that most receptors are inserted in the lipid vesicles in the same orientation (extracellular part of the receptor exposed at the surface of the vesicles). 2) They also precisely determined the size of the vesicles and the number of receptors per vesicles. However, I am surprised that the authors considered that 10% occupancy of the vesicle surface by the receptor correspond to a low density. This means that, if monomeric, the receptors are only distant of 170 A, a distance that they can cover several times within the time frame of the FRET measurement. Although the observation that similar FRET is observed with a 10 fold higher ratio of lipid over receptor is consistent with the authors view, are the receptors similarly distributed in all vesicles under these conditions?
As it stands, this paper does not bring much new information to the field. Although the observation that the receptor may form spontaneous tetramers is interesting (and consistent with many recent papers dealing with class A receptor oligomerization), this would need further support (such as cross-linking experiments for example). The absence of effect of agonists and antagonists is well consistent with what has already been reported for the $ß_2$AR. The new information is then limited to the effect of inverse agonists that lead to an increase in FRET efficacy, likely because of the formation of larger oligomers. But this interpretation remains to be further validated.
One key issue when performing and interpreting FRET data is the proportion of labeled receptors. This must be quantified for each fluorescent-maleimide and each position since major differences may be observed between these different parameters.
The FRET efficacy values obtained need clarification. These are quite low, and compatible with distances larger than 60A between the labeled partners for the smallest, according to a Ro of 50-60 A. Such distances are not compatible with physically associated proteins.

1st Revision - authors' response 04 August 2009

Response to Referee comments:

We greatly appreciate the comments and suggestions from the referees. We have responded to all of their concerns and we believe the revised manuscript is significantly improved. New experimental data presented in Figure 8 and Supplementary Figures 2 and 8. The inclusion of the new experimental data as well as the need to perform these experiments in the 90-day time frame necessitated the help of several new contributors who have been included as authors in the revised manuscript.
In the following response, the referees' comments are given in italics. Our responses are given in regular font. It should be noted that space considerations limited the extent to which we were able to modify the text in response to comments of the referees.

Referee #1:
This manuscript describes a series of FRET-based studies examining receptor-receptor interactions between highly-purified beta-2-adrenergic receptors ($ß_2$AR) reconstituted into artificial

lipid bilayers. The authors report evidence that the receptors can associate into oligomers, most likely tetramers, and that the interactions between the receptors are altered by treatment with inverse agonists. The authors describe many rigorous control experiments they performed to convince themselves that the observed receptor-receptor interactions were not simply a result of random collisions or artifacts of absurdly high packing of receptors into the lipid bilayers. Overall, the data shown here are extremely convincing and the paper is very clearly written.
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In our revision we more specifically address previous studies of ligand regulation ß$_2$AR oligomers. In particular, previous energy transfer experiments from Michel Bouvier's group have suggested small agonist-induced changes in steady-state BRET (Angers et al. 2000). However, later studies from the same group using more sensitive energy transfer methods and BRET saturation (Mercier et al. 2002) did not observe significant agonist-induced changes in ß$_2$AR oligomerization. Instead, the authors suggest that previous reports of agonistinduced changes were most likely due to conformational changes in individual protomers. As noted in our original manuscript, this may also explain the small agonist-induced changes we observe for TM6/H8 labeling pairs. Thus our results are in agreement with cell-based studies on the ß$_2$AR. The following text has been added to page 10 of the revised discussion.

- "For the ß$_2$AR, Michelle Bouvier's lab reported a small agonist-induced increase in steady state BRET; however, the authors concluded that this could be due to a small change in the steady-state oligomers or to conformational changes in individual protomers(Angers & Bouvier, 2000). In subsequent BRET saturation studies from this lab (Mercier et al, 2002) and fluorescence recovery after photobleaching studies from the Bünemann lab (Dorsch et al, 2009), no significant agonist-induced effect was observed. Our results with the full agonist (isoproterenol) are in agreement with these cell-based studies. Isoproterenol causes a relatively minor change in intermolecular FRET, with the only significant change occurring in the TM6/H8 FRET pair (Figure 7A; Table II), but no change in FRET saturation (Figure 7B)."

The referee suggested the possibility that a high level of constitutive activity in our modified receptors may also explain the lack of agonist-induced change in FRET. This does not appear to be the case. In Figure 4C we compare the function of the three modified receptors with the wild-type $ß_2$AR in a Gs coupling assay employing the same reconstitution conditions used for FRET experiments.
Agonist-stimulation of all three single-cysteine mutants is not significantly different from wild-type $ß_2$AR. More importantly, we observe approximately 6-fold stimulation of [35S]- GTPγS binding over basal activity, demonstrating that the highly-purified receptor preparations used in our studies do not exhibit a level of basal (constitutive) activity that would interfere with observing agonist-induced
effects.

Referee #2:
Oligomerization of Family A GPCRs and its biological role is a hotly debated issue. Here the authors address the propensity of b2-adrenergic receptor ($ß_2$AR) to oligomerize using the most direct approach: reconstituting purified receptors labeled with relatively small fluorescent moieties into phospholipids and performing rigorous FRET analysis. The authors should be commended for performing very thorough controls for receptor functionality, orientation in lipid vesicles, distribution in vesicle population, etc. An important issue of "bystander FRET" is very well addressed by FRET saturation assays and reconstitution of the receptor at 10 times higher lipid/ $ß_2$AR ratio. These experiments exclude usual artifacts and make the data very reliable.
The authors show that purified $ß_2$AR spontaneously oligomerizes upon reconstitution into lipids, forming predominantly tetramers in the absence of ligands and in the presence of neutral antagonists or agonists. Another very important finding of this study is that in the presence of inverse agonists receptors form higher order oligomers and/or more stable tetramers.
The results of this excellent study are very important for GPCR field and would be of great interest to the broad readership of the EMBO Journal. The only possibly questionable experiments are GTPγS binding assays. A few presentation issues should be also addressed to improve the manuscript. In addition, the demonstration of the ability (or inability, as the case may be) of oligomeric $ß_2$AR to couple to G protein could make this work exceptionall strong. If feasible with the methods and reagents in hand, the authors should perform these decisive experiments.

Biological role of observed $ß_2$AR oligomers:

1. The authors should place their results into broader biological context. Their data suggest that $ß_2$AR "forced" into inactive conformation by inverse agonists forms the most stable and/or the largest oligomers. In the context of previous findings that dimeric rhodopsin (Bayburt et al, 2007) and neurotensin NTS1 receptor (Proc. Natl. Acad. Sci. U. S. A. 104, 12199-12204) have lower ability to activate G proteins than monomeric forms, the results are consistent with the idea that oligomers of at least some Family A GPCRs represent an inactive form, whereas monomers represent the signaling state of the receptor. The authors should discuss (or possibly refute) this interpretation.

As the referees noted out, our studies showing the effect of inverse agonists on $ß_2$AR oligomerization are compatible with the finding that oligomeric forms of NTS1 and rhodpsion are less active towards G protein activation. However, while higher order oligomerization may represent a mechanism for the effect of inverse agonists on G protein coupling, it is not the only mechanism, as inverse agonists are effective at preventing Gs coupling to $ß_2$AR monomers reconstituted into HDL particles (Yao et al, 2009). The revised manuscript includes the following statement on page 11 in the Discussion:

- "Thus, the more constrained structure of the inverse agonist bound receptor may be more compatible with closer packing of protomers within a tetramer (Fig. 9E), a higher-order packing (Fig. 9F), or more stable $ß_2$AR oligomers with fewer monomers. It has been observed that oligomers of rhodopsin (Bayburt et al, 2007) and NT1 receptor (White et al, 2007) couple less efficiently to G proteins than monomers, therefore higher order

packing induced by the inverse agonist may restrict access of receptor to G protein. However, this higher order packing is not required for the inverse agonist effect, as an inverse agonist can efficiently prevent coupling of monomeric 2AR to Gs (Yao et al, 2009)."

2. In the same vein, it would be most important to show whether ß$_2$AR in oligomeric form efficiently couples to G protein and whether G protein binding affects the oligomerization of the receptor. If at all feasible, the authors should use FRET saturation experiments to test whether agonisttreated receptor in the presence of sufficient amount of accessible heterotrimeric G protein remains in the same oligomerization state as in its absence.

It is very difficult to determine the effect of oligomerization on ß$_2$AR coupling to Gs in vesicles, since it is likely that oligomers are dynamic and exist in equilibrium with monomers. However, at the suggestion of Referee 2 we attempted to determine the effect of the G protein Gs on ß$_2$AR oligomerization. These experiments were very challenging because of the need to perform multiple reconstitutions with different ratios of Cy3 and Cy5 labeled receptor in the presence and absence of purified Gs. We reconstituted ß$_2$AR and Gs at a stoichiometry of 1 receptor to 3 Gs heterotrimers to guarantee that sufficient Gs was incorporated into the vesicles to observe ß$_2$AR -Gs coupling. We used a conformational reporter on the ß$_2$AR (previously described (Yao et al, 2009)) to confirm coupling under these conditions. FRET saturation experiments showed to a small but significant reduction in FRET saturation in the presence of Gs.
Therefore, Gs may preferentially interact with monomers or dimers, thereby reducing the fraction of higher order oligomers. This data has been included in Figure 8 and the experiment described on page 7.

GTPgS binding assay is the only possible experimental weakness in this study. In particular:
3. The methods suggest that the authors used membrane-tethered Gas (Lee et al, 1999). In the original paper, the authors characterized this construct in Sf9 cell membranes containing endogenous bg-subunits. Did purified tet-Gas used here contain bg-subunits? If yes, the authors should show this and estimate the fraction of tet-Gas in heterotrimeric form. If not, the authors should address the relationship between receptor coupling to tet-Gas and to physiologically relevant heterotrimeric Gs.

The GTP S binding assay was employed as a functional assay to determine the effects of mutations used for site-specific labeling and the effect of modifying receptors with Cy dyes on the ability of the *ß$_2$AR* to activate Gs. We did not intend to use these studies to infer anything about the effect of oligomerization on Gs activation. We used the tethered-Gs because it is more easily purified than Gs heterotrimer. Tet-Gs does not contain ßγ, as this is removed during purification by washing Tet-Gs immobilized on a Flag column with GDP-AlF. This information has been added to the revised methods. The membrane tether appears to be an adequate surrogate for G ßγ. Tet-Gs can support high affinity agonist binding; moreover, we observe ICI inhibitable basal activity as well as robust agonist-stimulated activity (Swaminath et al, 2005). Therefore we believe this assay is well suited for comparing the function of wild type and modified receptors.
Reconstitutions were done using the same Tet-Gs preparation at the same receptor protein concentrations. The only differences were the receptor modifications. Therefore, these assays are effective at evaluating the functional consequence of a ß$_2$AR mutation.

4. It is well known that the initial rate of GTPγS binding (first linear part of the time course) reflects the activity of the receptor. In contrast, the plateau of GTPγS binding in this type of assay reflects the amount of functional G protein present. As the authors use 30 min incubation, they should show that the rate of GTPγS binding is linear from 0 to 30 min, and that in the range used here it is linearly proportional to the amount of ß$_2$AR added.

Regarding the time course, we use 30 min as it exhibits the best signal to noise.
As we showed in Ratnala & Kobilka 2009 (Methods Mol Biol 552:67-77), Figure 2, GTPγS binding to tet-Gs increases in a receptor and agonist dependent manner and reaches a plateau around 30 min.

As we showed in Rasmussen et al 2007 (Nature 450:383-374), Supplementary Figure 4, receptor independent GTPγS binding is minimal at 30 min, relative to 2AR stimulated binding. Moreover, basal activity is low compared to agonist-stimulated activity. Therefore GTPγS binding at 30 min does reflect the amount of functional receptor. The initial rate of GTPγS binding is slow, possibly due to the low concentration of GTPγS used (1 nM) and the slow rate of diffusion of GTPγS into are relatively impermeable vesicles. The fact that GTPγS does cross the membrane is likely due to some residual detergent in the preparation. The permeability of vesicles containing 2AR and Tet-Gs is likely to be greater that vesicles containing Gs alone because of the additional detergent associated with Tet-Gs.

5. The authors should demonstrate whether the orientation of tet-Gas matches that of the receptor, i.e., whether all receptors in the sample had unimpeded access to G protein. Another important concern is whether all molecules of G protein had unimpeded access to GTPγS (which does not cross lipid bilayer). E.g., if the orientation of tet-Gas matches that of the receptor, only 10% of it (coupling to the ~10% of the b2AR in the inside-out orientation) has access to GTPγS. Although this does not undermine the validity of the functional test per se, it raises the question how the oligomerization state of the 90% of the receptors in outside-out orientation corresponds to the state of the 10% that activated G protein in this assay. The authors should clarify this important point.

Again, the GTP S binding assay was only employed as a functional assay to evaluate the ability of modified receptors to couple to Gs. It is clear from the results that sufficient β_2AR and Tet-Gs are in the proper orientation for efficient interactions. The issue of GTPγS access to Gs is discussed above.

Other issues:
6. The data presented here strongly suggest that monomers, dimers, tetramers, and possibly larger oligomers of β_2AR are in equilibrium. The same was recently shown to be the case for D2 dopamine receptor (Fonseca JM, Lambert NA (2009)). The authors should discuss functional implications of this for G protein activation assay (where just a few receptors could yield a robust signal) and for their FRET studies, which by definition report on the state of the majority of receptors, ignoring small subpopulations.

The referee raises an important point that was not adequately addressed in the original manuscript. Fonseca JM, Lambert NA (2009) Mol Pharm use FRAP to study the stability of D2 dopamine receptor dimers. Of interest, they show that intermolecular cysteine crosslinking used to study the dimer interface dramatically reduced the dynamic behavior. This has been discussed more fully on page 9 of the revised manuscript.

- "While a β_2AR monomer can activate Gs (Whorton et al, 2007), it is not known if higher order oligomers facilitate or impair coupling. Oligomers of rhodopsin (Bayburt et al, 2007) and NT1 receptor (White et al, 2007) couple less efficiently to G proteins than monomers. If higher order oligomers impair coupling, the dynamic character of β_2AR oligomers would ensure that a fraction of the β_2AR would exist as monomers or dimers competent for G protein activation. Under our experimental conditions, the co-reconstitution of Gs with β_2AR was associated with a small decrease in FRET saturation that was reversed by GTPγS (Figure 8). This is compatible with G protein coupling shifting the equilibrium to lower order oligomers."

7. Show the data on activation-induced TM6 movement in monomeric β_2AR reconstituted into HDL particles and in predominantly tetrameric receptor in liposomes.

This has been added as Supplementary Figure 8.

8. No evidence for very large oligomers of dark (inactive) rhodopsin reported in mica-adsorbed disc membranes (Liang et al, 2003) was found in the discs in the natural environment of the rod outer segment (J Biol Chem. 2008 Oct 31; 283(44):30015-24), even though rhodopsin occupies ~50% of the disc membrane. Does this affect authors' interpretation?

As the referee points out, oligomerization of rhodopsin is somewhat controversial. However, rhodopsin is a highly specialized GPCR that exists in a specialized cellular structure at a very high density. Its tendency to form stable oligomers may differ from that of other GPCRs. Wang et al state that the "relatively fast diffusion constant (for rhodopsin) would seem to argue against a significant fraction of rhosopsin … being confined to large arrays containing hundreds of rhodopsins in quasi-crystalline aggregates" as observed by Liang et al (2003). They do not exclude the possibility of smaller oligomers. Our results do not suggest very large oligomers. One could argue that the higher order oligomers observed by Liang et al result from the preparation used for AFM, and are therefore an artifact. However, it is also possible that, like crystallography, the AFM preparation traps and enriches a state that exists to some degree in native membranes. Thus, it is likely that for both rhodopsin and the $ß_2$AR ,oligomers are dynamic with monomers, dimers, and higher order oligomers existing in equilibrium.

9. Suppl Fig.5 would be more appropriate as panel D in Fig.7.

This has been done.

10. In the introduction the authors lump together references to studies of Family A and Family C GPCRs. Considering that there is no doubt that Family C GPCRs are constitutive dimers, and that the role of dimerization in the function of some of these receptors is very well established (in contrast to Family A receptors), this is hardly appropriate.

This section has been revised.

11. The reference to Fonseca JM, Lambert NA (2009) is incomplete.

This has been corrected.

Referee #3:

G-protein coupled receptor oligomerization is a mater of intense debate, and it is still not clear whether rhodopsin-like GPCRs assemble into dimers or higher order oligomers. In the present study, the authors examined this issue using purified $ß_2$AR reconstituted into lipid vesicles. By inserting specific donor and acceptor fluorophores at various positions in the receptors, they confirmed that $ß_2$AR spontaneously form oligomers in the total absence of additional proteins. Their data are more compatible with a tetramer than with a dimer, as based on the saturation kinetics. They went on and analyzed the effect of various ligands (agonist, antagonist and inverse agonist) on the FRET efficiencies, and found that only inverse agonists binding resulted in a change in FRET, compatible with a larger oligomeric state, and a likely more compact association between the protomers. Some critical controls necessary for the interpretation of the data are well performed.

1) They demonstrated, using several complementary approaches, that most receptors are inserted in the lipid vesicles in the same orientation (extracellular part of the receptor exposed at the surface of the vesicles).

2) They also precisely determined the size of the vesicles and the number of receptors per vesicles. However, I am surprised that the authors considered that 10% occupancy of the vesicle surface by the receptor correspond to a low density. This means that, if monomeric, the receptors are only distant of 170 A, a distance that they can cover several times within the time frame of

the FRET measurement. Although the observation that similar FRET is observed with a 10 fold higher ratio of lipid over receptor is consistent with the authors view, are the receptors similarly distributed in all vesicles under these conditions?

We show the specificity of interactions in three different ways. 1-The specificity of FRET efficiency for different FRET pairs. If FRET were due to random collisions, we should observe the same FRET efficiency for all pairs. 2-FRET saturation experiments. 3-The FRET efficiency does not change at higher lipid:protein ratios. As requested by Referee 3, we now show that increasing the lipid:receptor ratio to 10,000:1 does not alter the distribution of receptors in vesicles (new Supplementary Figure 2).

As it stands, this paper does not bring much new information to the field.

We respectfully disagree. We show that 2AR has a natural tendency to oligomerization, and that oligomerization does not require accessory or chaperone proteins. This has never been shown and cannot be determined from cell-based studies. We show that receptors exist in higher order oligomers, and that agonists and neutral antagonists have little effect on the oligomeric state. We show that inverse agonists promote the formation of higher order oligomers, while the G protein Gs reduces the complexity of oligomers.

Although the observation that the receptor may form spontaneous tetramers is interesting (and consistent with many recent papers dealing with class A receptor oligomerization), this would need further support (such as cross-linking experiments for example). The absence of effect of agonists and antagonists is well consistent with what has already been reported for the β_2AR. The new information is then limited to the effect of inverse agonists that lead to an increase in FRET efficacy, likely because of the formation of larger oligomers. But this interpretation remains to be further validated.

In the original manuscript we showed that inverse agonists led to more extensive crosslinking (Figure 7 and Supplementary Figure 6), supporting the FRET saturation experiments. While these crosslinking experiments are consistent with higher order oligomers, we observed relatively few protein bands representing dimers, trimers and tetramers. However, all crosslinking experiments must be interpreted with caution. Crosslinking reagents form irreversible covalent interactions between protomers. The efficiency of crosslinking depends on several factors including the type of crosslinker used (size and chemical reactivity), the number of available reactive sites on the protein, the duration of crosslinking, and the temperature. Therefore, depending on the conditions, it is possible to observe extensive crosslinking between proteins that associate transiently through random collisions, and relatively little crosslinking between protomers that form more stable oligomers. In fact, depending on the orientation of the protomers, crosslinks may be more likely to occur within a single protomer or between two protomers in two different oligomers during random collisions than between two protomers in an individual oligomer. This is because of the limited number of lysines on the extracellular side of the receptor (the outward face in vesicles) and the predicted distance between lysines. As shown in Fig. 1, there are only two l lysines in the extracellular loops (K97, and K305). Both of these lysines would be expected to be relatively constrained. In the β_2*AR* crystal structure K305 is involved in a salt bridge with K192 in the second extracellular loop, while K97 forms a hydrogen bond with the backbone carbonyl of L95. Depending on the orientation of protomers within a tetramer or dimer, these reactive amines may be too far away from each other to efficiently crosslink, and would more likely form intramolecular crosslinks, or crosslinks with lysines during transient interactions with another oligomer. The only other reactive groups are the amino terminus and a single lysine on the amino terminal FLAG epitope. The amino terminus is not resolved in the crystal structure; therefore we can't predict the orientation of these reactive groups. Considering these problems with performing and interpreting crosslink experiments, and the dynamic nature of oligomers, we don't believe additional crosslinking experiments are warranted. One key issue when performing and interpreting FRET data is the proportion of labeled receptors. This must be quantified for each fluorescent-maleimide and each position since major differences may be observed between these different parameters. We include this data in Supplementary Table I.

The FRET efficacy values obtained need clarification. These are quite low, and compatible with distances larger than 60A between the labeled partners for the smallest, according to a Ro of 50-60 A. Such distances are not compatible with physically associated proteins.

The relatively low FRET efficiencies are most likely explained by the fact that receptors exist in a dynamic equilibrium of monomers, dimers, tetramers and higher order oligomers. This is discussed on page 9 of the revised manuscript.
Other factors that may contribute to the lower than expected FRET efficiencies include a labeling stoichiometry that is less than 100%, and the fact that the Ro for Cy3-Cy5 has been reported to be as low as 37Å for some systems.

CITED LITERATURE.

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Decision letter 18 August 2009

I am pleased to inform you that your manuscript has been seen again by referee 2, who is satisfied with the revised version. Their comments are appended below. We are now ready to accept your manuscript for publication.

A formal letter of acceptance will be sent to you shortly.

With kind regards,

Editor
The EMBO Journal

Referee 2 comments:

Oligomerization of Family A GPCRs and its biological role is a hotly debated issue. Here the authors test the ability of b2-adrenergic receptor (b2AR) to oligomerize using the most direct approach: reconstituting purified receptors labeled with relatively small fluorescent moieties into phospholipids and performing rigorous FRET analysis. The authors present very thorough controls of receptor functionality, orientation in lipid vesicles, distribution in vesicle population, etc. An important issue of "bystander FRET" is very well addressed by FRET saturation assays and reconstitution of the receptor at 10 times higher lipid/b2AR ratio. Moreover, the authors use FRET between different positions in the receptor. Taken together, the data exclude usual artifacts and make the conclusions unusually reliable.
Unambiguous demonstration of several things makes this work particularly important. 1. The authors show that purified b2AR spontaneously oligomerizes upon reconstitution into lipids, demonstrating that other proteins present in the cell are not required (although they likely modulate this process). 2) The b2AR forms tetramers in the absence of ligands and in the presence of neutral antagonists or agonists (the data are compatible with monomer-dimer-tetramer equilibrium). 3) Inverse agonists induce the formation of higher order oligomers and/or more stable tetramers. 4) G protein apparently destabilizes receptor oligomers.
The results of this excellent study are very important for GPCR field and would be of great interest to the broad readership of the EMBO Journal.

