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AT1R-CB_1R heteromerization reveals a new mechanism for the pathogenic properties of angiotensin II

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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 11 November 2010

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see below, the referees express an interest in the study, but also raise many different concerns with the data and the conclusions drawn. One main concern raised is that the data provided do not sufficiently support the conclusion that ATR1-CB1R receptor heteromerization mediates the observed signaling potentiation. Another explanation could also be that functional crosstalk between signaling pathways could lead to the observed enhanced signaling. In other words, more data is needed to show that the ATR1-CB1R interaction is physiological relevant. With such significant reservations from all three referees, I am afraid that I cannot offer to commit to a revised version at this stage. As there is an interest I can offer that should you be able to address the concerns raised in full, by the inclusion of additional data, and provide further evidence for that ATR1-CB1R receptor heteromerization is important for the observed effects then we would be willing to consider a resubmission. I should add that for resubmissions we consider the novelty of data at the time of resubmission and may, if needed, bring in new referee(s).

For the present submission, I am sorry that I cannot be more positive, but I hope nevertheless that you will the referees comments useful.

Yours sincerely,

Editor

The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The paper entitled "AT1R-CB1R heterodimerization reveals a new mechanism for the pathological properties of angiotensin II" by Rozenfeld et al. deals with the heterodimerization of these 2 GPCRs and the new functional properties aquired by AT1 in this dimerization, ie coupling to Gi and cAMP inhibition, which may participate to the profibrotic actions of angiotensin II. This is a comprehensive and well designed paper with extensive new information about the possible physiological consequences of GPCR heterodimerization. The 2 strengths of the paper include (i) the convincing demonstration that AT1R couples to Gi in presence but not in absence of CB1R likely as a result of heterodimerization and (ii) the interesting comparison of a cell model which overexpressed AT1R artificially (or not) to a hepatic primary culture where CB1R expression is induced "naturally" as the result of a pathological process. This paper has however some weaknesses including (i) the absence of investigation of whether these observations are an exception or a more general process and (ii) the choice of Erk as a specific pathway activated by AngII since this pathway is activated by many others factors, including CB1R activation and is activated by AngII via multiple signaling pathways which are either G-protein dependent or independent (β-arrestin etc...). This does not simplify the analysis of the results. Therefore the results should be analyzed with caution and the controls should be multiplied. Many are already present in the paper but some are missing.

Therefore, I have several concerns about the data:
1. Many other GPCRs are coupled to Gi. Could the major observation made in this paper with CB1R, ie AngII-Erk activation via Gi acivation by the heterodimer, be made with another Gi-coupled receptor?
2. Since CB1R also activates Erk pathway, the potentiation of Erk activation by AngII in the presence of CB1R activation (fig.1) could be simply due to a "presensitization" of the Erk pathway. It is important to know if the same potentiation of the AngII response could also be observed in the presence of a "subphysiological" dose of a growth factor, the receptor of which being expressed in these cells.
3. I have some concerns about your "heterodimer specific " monoclonal antibody. Since its selection is based on the presence of the epitope in Neuro 2A-AT1 cells but not the Neuro2A cells, it is not surprising that it recognizes the 1st but not the 2nd in an ELISA! More interesting is the disappearance of the signal when CB1R expression is knock-down. However, the demonstration that an immunoprecipitate of the Neuro2A-AT1 cells is recognized by both AT1R and CB1R antibodies in Western blot will be a lot more convincing.
4. As you explored in parallel the possible implication of AT1R (and its activation) in CB1R signaling, it will be important to demonstrate that
• AngII is able to modify Erk activation induced by a CB1R agonist,
• CB1R agonist is able to stimulate calcium,
in the context of Neuro2A-AT1 cells but not Neuro 2A cells.
5. Finally, since AngII-induced Erk activation also involves G-independent signaling pathway, starting with the recruitment of β-arrestin, it will be important to demonstrate that the observed effet of the heterodimer on Erk has nothing to do with β-arrestin recruitment.

Other comments
1. It is not clear whether your cells are grown in the presence or the absence of FCS (which contains AngII) and at which time after AngII application the Erk measurements were done etc.. Minimal information should be given in M&M section.
2. Confirm that THL, SR141716 and Hu210 are doing nothing on AngII induced Erk response, when CB1R is knock down.
3. Images of Figure 6D are really not convincing of colocalization
4. The concept of activating but non-signaling dose of agonist cannot be accepted. A molecule of receptor which is activated signals. You should talk about "an agonist dose which does not induce a measurable signal".
5. Figure 2D, 2E and 2F are not correctly located in the text.
6. The discussion could be shortened a little, but not the pathophysiological relevance of the

heterodimer. Besides the liver, the list of the known tissues which physiologically or pathologically may coexpress the 2 receptors is an important information, which is missing.

Referee #2 (Remarks to the Author):

In the manuscript entitled "AT1R-CB1R heteromerization reveals a new mechanism for the pathogenic properties of angiotensin II", the authors have examined the possible consequences of heteromerization of two GPCRs on the activation of different signaling pathways. They conclude that coexpression of AT1 and CB1 receptors leads to receptor interactions resulting in a potentiation of AT1R signaling. Phosphorylation of ERK by AT1R activation, which is Gq dependent in the absence of canabinoid receptor, becomes Gi dependent in the presence of CB1R. Moreover the coupling of AT1R to Gq also increases when CB1R is co-expressed. Finally the authors considered that this receptor heteromerization constitutes a molecular basis to explain what happens in fibrotic liver and more specifically in hepatic stellate cells when CB1R is up-regulated.

The manuscript is very well written and easy to follow. The fact that the up-regulation of one receptor can modify the signaling properties of a second receptor by forming heteromers is of course a very interesting question, especially when this could corresponds to what has been observed in a pathophysiological context. Unfortunately the authors over-interpreted some of their data and the demonstration is not completely convincing.

Major concerns
On the one hand the authors have demonstrated that receptors are interacting and they bring sufficient evidence to validate the demonstration although the negative control based on the use of eGFP-tagged endothelin converting enzyme-2 is not a good negative control. To be relevant, successful BRET experiments have to be performed between this protein and a GPCR in order to prove that such a BRET signal is possible otherwise one can suspect that because of distance or orientation constraints of luciferase and GFP, BRET is not possible even if these molecules are in close proximity.
On the other hand, the authors have demonstrated that potentiation of the AT1R signalling pathways are dependent on CB1R expression. This has been quite well demonstrated and the data are rather convincing.
However, the authors established a correlation between these two observations but their present no data directly supporting this proposal. Of course the hypothesis formulated by the authors is credible but a few others hypotheses can also be proposed. One can imagine that potentiation is due to crosstalk between the signaling pathways, as this has been reported in many cases between a Gq-coupled and a Gi-coupled receptors (see for example Carroll Curr Biol 1995; Rives EMBO J 2009; Philip Curr Biol 2010). The authors mentioned that by contrast to what has been observed on class C GPCRs, the class A receptors are interacting and that "it suggest that different regulatory mechanisms may apply to class A and class C GPCRs". They are right but they have to establish the link between heteromerization and signaling potentiation before reaching a conclusion (see the title) The best way to demonstrate the authors proposal would be to identify mutants AT1R or CB1R that no longer interact, and show that the potentiation is no longer observed, even though the receptors are expressed at the same level. Another alternative could be the co-expression of AT1R with another GPCR coupled to the same signalling pathway as CB1R but that does not interact with AT1R. Is the potentiation still observed under such conditions? Did they stimulate a AT1R non-interacting receptor coupled to Gi/o in hepatic stellate cells? If the interactions of CB1R and AT1R are really specific the activation of another Gi/o-coupled receptor should not induce the same potentiation.

As I mentioned before, demonstrating such a link between receptor heteromerization and signaling potentiation is really important and will be in accordance with the title. If the authors cannot establish that the potentiation is really the consequence of the interaction, they have to consider and discuss all possibilities, including GPCR heteromerization, and most importantly they will have to change the title.

Few other published manuscripts on other physiological models have claimed to establish that GPCR heteromerization affects GPCR signaling but this has rarely been definitely proved. It would be worthwhile to demonstrate it on the model the authors are using.

Minor concerns

Page 6: colocalization of GPCRs do not really support receptor interaction. The resolution of microscopy image is in the range of 400 nm ... but relocalization of GPCRs when coexpressed is much stronger. I suggest the authors modify "we also examined the changes in CB1R ..." in "Indeed we examined ..."

I think that references to the various panels of figure 2 are not correct (page8): "Fig2D" should be replaced by "Fig 2F" and references to Fig 2D and 2E should be made above.

Referee #3 (Remarks to the Author):

The authors attempted to demonstrate that angiotensin 1 receptor (AT1R) and endocannabinoid receptor CB1R form heterodimers with specific functional characteristics. While the data indicate that there is complex functional interaction between these two receptors in cells where AT1R is overexpressed and in hepatic stellate cells (HSCs) from rats that chronically received ethanol, the authors do not present direct proof that the formation of AT1R-CB1R heterodimer is behind all their observations. Several vital issues, such as receptor quantification, the mechanism involved in ERK activation, and the relevance of the signaling pathways examined for mitogenic signaling and profibrogenic gene expression in HSCs, were not addressed. The absence of these crucial components greatly weakens this study.

Main problems:

1. The authors do not present the data on expression levels of the two receptors in any of the experimental situations described. Having this information (in absolute numbers, fmol/mg protein) is vital: while one can envision the dominance of AT1R-CB1R heterodimer signaling when both receptors are expressed at similar levels, this becomes impossible to swallow if one expressed at 10 times higher level than the other. This is especially important as each of the receptors in question is known to function perfectly well by itself.

2. The authors use ERK activation as a readout in >80% of their experiments. It is well known that GPCRs can activate ERK via many different pathways: through PKC, PKA, Gbg, RTK transactivation, and b-arrestin-mediated scaffolding, to name just a few. The authors did not even test which pathways are involved. They also assume that the same pathways operate in transfected cells and HSCs endogenously expressing both receptors, but did not test this experimentally.

3. The authors also imply that ERK activation participates in mitogenic signaling and profibrogenic gene expression in HSCs, but do not present any proof of this. More than half of the study would only be relevant if that's the case.

4. Described procedure for subtractive immunization would generate antibodies to AT1R with higher probability than to putative heterodimer. The authors should present strong evidence indicating that the antibodies are specific to heterodimer and do not react with AT1R to justify their conclusions.

5. Although there exists extensive literature showing that BRET signal does not necessarily mean specific interaction, there are also papers claiming otherwise. In any case, the authors should add the data regarding the expression levels of both receptors (in fmol/mg protein) in BRET experiments and compare these levels to the ones that are in HSCs. This is very important, as many genuine interactions happen at 2-4 pmol/mg, but completely disappear when one gets to more physiological levels of 20-100 fmol/mg.

6. The authors need to use appropriate statistics throughout the paper. Student's t-test is only valid when one compares two groups. For the comparisons presented here the authors need to use more sophisticated statistics.

Presentation concerns:

7. Fig.1. Panels A and C. Statistical significance of the differences in ERK activation curves should be shown. Panel B. The extent of CB1R knockdown should be quantified and statistically analyzed. Panel D. The statistics used should be indicated. As six experimental groups are shown here, the analysis should include correction for multiple comparisons.

8. Fig.2. Panel B. Low magnification does not allow one to judge co-localization (with this image size every other pair of proteins out of 2,000-4,000 expressed in the cell would appear co-localized). Panels C, D, E, and F. Statistical analysis showing significance (with correction for multiple comparisons, where appropriate) is needed to evaluate these data.

9. Fig.3, Fig.4 and Fig.5E. The same statistical issues as in Fig.2 should be addressed.

10. Fig.6. Panel C. Statistics needed. Panel D. The same low magnification problem as in Fig.2B.

11. Fig.7. Statistical analysis should be described. The method should include correction for multiple comparisons. Mentioned Student's t-test is not an appropriate method of analyzing differences where more than two experimental groups are used (ANOVA or equivalent with appropriate post-hoc tests is needed).

12. Suppl Figs 1 and 2: the same statistical and magnification (suppl fig. 2B,C) issues should be addressed.

13. Misstatement on p.14. Actually, both GABAB and mGlu1a are constitutively dimeric class C GPCRs.

Resubmission 19 January 2011

Referee #1:

We thank the reviewer for the positive and encouraging remarks. We addressed the reviewer's concerns as detailed below:

1. Many other GPCRs are coupled to Gi. Could the major observation made in this paper with CB1R, ie AngII-Erk activation via Gi activation by the heterodimer, be made with another Gi-coupled receptor?
To address this question, we coexpressed AT1R with delta or mu opioid receptors (DOR or MOR), both Gi-coupled receptors. We did not observe any detectable cross-talk between these Gi-coupled receptors and AT1R. These experiments are detailed in Supplementary Figure 4D.

2. Since CB1R also activates Erk pathway, the potentiation of Erk activation by AngII in the presence of CB1R activation (fig.1) could be simply due to a "presensitization" of the Erk pathway. It is important to know if the same potentiation of the AngII response could also be observed in the presence of a "subphysiological" dose of a growth factor, the receptor of which being expressed in these cells.
We directly addressed this by examining the effect of a very low dose of PDGFbb on AngII-mediated ERK phosphorylation in the presence of a CB_1R antagonist. In these conditions, PDGFbb treatment did not rescue AT1R signaling, indicating that presensitization of the ERK pathway is not responsible for the increased ERK phosphorylation. These experiments are detailed in Supplementary Figure 4C.

In addition, to further assess the specificity of these effects, we examined the effect of blocking CB_1R with an antagonist on ERK phosphorylation induced by a more physiological concentration of PDGFbb. The CB_1R antagonist did not affect PDGFbb-mediated ERK phosphorylation in Neuro2A cells or in activated HSCs (Suppl. Fig 4A & 4B).
Together, these findings support the specificity of AT1R-CB_1R interaction in the regulation of AngII-mediated signaling.

3. I have some concerns about your "heterodimer specific " monoclonal antibody. Since its selection is based on the presence of the epitope in Neuro 2A-AT1 cells but not the Neuro2A cells, it is not

surprising that it recognizes the 1st but not the 2nd in an ELISA! More interesting is the disappearance of the signal when CB1R expression is knock-down. However, the demonstration that an immunoprecipitate of the Neuro2A-AT1 cells is recognized by both AT1R and CB1R antibodies in Western blot will be a lot more convincing.
We agree and are in the process of optimizing conditions of immunoprecipitation to obtain clear signal of recognized CB_1R and AT1R. Due to the detergent sensitivity of the recognition by the AT1R-CB_1R heteromer antibody, this has taken quite a bit of effort and we have yet to obtain publication-quality data from these studies. Due to the labor-intensive and time-consuming nature of these studies, we put our efforts into characterizing the specificity of the antibody by other assays. We used the recognition of an epitope in Neuro2A-AT1R cells and the lack of recognition in the same cells after CB_1R knock-down as evidence for the specificity of the antibody.
Second, we used cells coexpressing CB_1R with a variety of other GPCRs (MOR, DOR, KOR or CB_2R) to test the specificity of the CB_1R-AT1R antibody. We find that the antibody does immunoreact only with cells coexpressing CB_1R and AT1R.
Third, we used cells expressing different ratios of CB_1R and AT1R. We find that only when CB_1R and AT1R are expressed at similar levels (but not when expressed at 1:5 or 5:1 ratios), there is maximal recognition by the heteromer antibody.
Finally, we used the AT1R-CB_1R heteromer antibody to specifically block Ang II-mediated signaling and find this effect only in cells expressing AT1R-CB_1R (but not in cells expressing AT1R alone) (Figure 4D).
These evidences support the specificity of this antibody and the direct involvement of the heteromer in AngII-mediated signaling in cells coexpressing the two receptors.

4. As you explored in parallel the possible implication of AT1R (and its activation) in CB1R signaling, it will be important to demonstrate that AngII is able to modify Erk activation induced by a CB1R agonist, and test if a CB1R agonist is able to stimulate calcium in the context of Neuro2A-AT1 cells but not Neuro 2A cells.
The effect of Ang II on ERK activation induced by a CB_1R agonist is detailed in the manuscript (Figure 2G). In addition to that, we now provide data showing that the CB_1R agonist does not lead to detectable calcium signaling in Neuro2A nor in Neuro2A-AT1R. Interestingly, and in agreement with our previous data showing that blocking CB_1R leads to a decrease in calcium response, stimulation with a CB_1R agonist leads to a potentiation of AngII-mediated calcium signaling. These new data are detailed in revised Figure 5F and Supplementary Figure 6.

5. Finally, since AngII-induced Erk activation also involves G-independent signaling pathway, starting with the recruitment of beta-arrestin, it will be important to demonstrate that the observed effect of the heterodimer on Erk has nothing to do with beta-arrestin recruitment.
We examined the involvement of Arrestin3 in Ang II-mediated ERK phosphorylation in cells expressing AT1R alone or together with CB_1R. We find that, as previously reported (Ahn et al, 2004), Arrestin3 contributes to Ang II-mediated ERK phosphorylation in AT1R-expressing cells (Suppl. Fig 5B). In contrast, in AT1R-CB_1R expressing cells, downregulation of Arrestin3 leads to an increase in ERK phosphorylation (Suppl. Fig 5B), supporting that Arrestin3 contributes to desensitization of AT1R signaling in the context of the AT1R-CB_1R heteromer. Together, our data confirm that in the context of the heterodimer, Ang II-mediated pERK levels are higher than in AT1R cells, but this increase is differentially modulated by Arrestin3.

Other comments

1. It is not clear whether your cells are grown in the presence or the absence of FCS (which contains AngII) and at which time after AngII application the Erk measurements were done etc.. Minimal information should be given in M&M section.
Cells were serum-starved at least 4 hours prior to the Ang II stimulation. This has been added to the Methods section.

2. Confirm that THL, SR141716 and Hu210 are doing nothing on AngII induced Erk response, when CB1R is knock down.
We now provide evidence that the CB_1R ligands do not affect Ang II-mediated ERK response when CB_1R is knocked-down. This is now detailed in Supplementary Figure 1B.

3. Images of Figure 6D are really not convincing of colocalization

In order to show colocalization more convincingly, we now provide higher magnification images.

4. The concept of activating but non-signaling dose of agonist cannot be accepted. A molecule of receptor which is activated signals. You should talk about "an agonist dose which does not induce a measurable signal".
We have changed the text according to the reviewer's suggestion.

5. Figure 2D, 2E and 2F are not correctly located in the text.
We apologize and have addressed this in the revised manuscript.

6. The discussion could be shortened a little, but not the pathophysiological relevance of the heterodimer. Besides the liver, the list of the known tissues which physiologically or pathologically may coexpress the 2 receptors is an important information, which is missing.
We have shortened the discussion, and have added information regarding possible colocalization of CB_1R and AT1R in other tissues.

Referee #2:

We thank the referee for his/her enthusiasm and positive appreciation of our work.

Major concerns

On the one hand the authors have demonstrated that receptors are interacting and they bring sufficient evidence to validate the demonstration although the negative control based on the use of eGFP-tagged endothelin converting enzyme-2 is not a good negative control. To be relevant, successful BRET experiments have to be performed between this protein and a GPCR in order to prove that such a BRET signal is possible otherwise one can suspect that because of distance or orientation constraints of luciferase and GFP, BRET is not possible even if these molecules are in close proximity.
In order to address this valid concern, we examined changes in BRET between tagged AT1R and CB_1R in the presence of untagged endothelin converting enzyme-2. Overexpression of the enzyme does not decrease the BRET ratio, whereas overexpression of untagged AT1R decreases the BRET ratio, supporting the specificity of the energy transfer obtained between tagged CB_1R and AT1R.

On the other hand, the authors have demonstrated that potentiation of the AT1R signalling pathways are dependent on CB1R expression. This has been quite well demonstrated and the data are rather convincing.
We appreciate this comment.

However, the authors established a correlation between these two observations but they present no data directly supporting this proposal. Of course the hypothesis formulated by the authors is credible but a few others hypotheses can also be proposed. One can imagine that potentiation is due to crosstalk between the signaling pathways, as this has been reported in many cases between a Gq-coupled and a Gi-coupled receptors (see for example Carroll Curr Biol 1995; Rives EMBO J 2009; Philip Curr Biol 2010). The authors mentioned that by contrast to what has been observed on class C GPCRs, the class A receptors are interacting and that "it suggests that different regulatory mechanisms may apply to class A and class C GPCRs". They are right but they have to establish the link between heteromerization and signaling potentiation before reaching a conclusion (see the title) The best way to demonstrate the authors proposal would be to identify mutants AT1R or CB1R that no longer interact, and show that the potentiation is no longer observed, even though the receptors are expressed at the same level.
This is an excellent suggestion, and we undertook such studies using site-directed mutagenesis to identify transmembrane residues involved in AT1R dimerization. We found that the majority of transmembrane mutants did not exhibit plasma membrane localization, and were retained in the endoplasmic reticulum, presumably because they did not fold properly (Pignatari et al, 2006). While additional studies are underway to identify mutants that would exhibit plasma membrane localization but would be unable to dimerize with CB_1R, it is not clear if we will be able to identify such mutants in a timely manner. We believe that waiting for such mutants would delay the publication of this manuscript. Due to the timely nature of the studies presented here and due to the evidence that we provide for AT1R-CB_1R heteromerization, (using the heteromer-specific

antibody), we believe that the results we report here are highly relevant and would be of interest for a wide readership.

Another alternative could be the co-expression of AT1R with another GPCR coupled to the same signalling pathway as CB1R but that does not interact with AT1R. Is the potentiation still observed under such conditions?
Did they stimulate a AT1R non-interacting receptor coupled to Gi/o in hepatic stellate cells? If the interactions of CB1R and AT1R are really specific the activation of another Gi/o-coupled receptor should not induce the same potentiation.
To address this question, we coexpressed AT1R with DOR or MOR, both Gi-coupled receptors. We did not detect any cross-talk between these Gi-coupled receptors and AT1R.
In addition, we show that there was no cross-talk between AT1R or CB_1R with PDGFR. These experiments are detailed in Supplementary Supplementary Figure 4.

As I mentioned before, demonstrating such a link between receptor heteromerization and signaling potentiation is really important and will be in accordance with the title. If the authors cannot establish that the potentiation is really the consequence of the interaction, they have to consider and discuss all possibilities, including GPCR heteromerization, and most importantly they will have to change the title.
Through the introduction of these new experiments, we address the possibility that mechanisms other than heteromerization may be responsible for the cross-talk between AT1R and CB_1R, and show that such mechanisms do not play a role in the cross-talk. Therefore, our new data strongly support a role for heteromerization in the cross-talk.

Minor concerns

Page 6: colocalization of GPCRs do not really support receptor interaction. The resolution of microscopy image is in the range of 400 nm ... but relocalization of GPCRs when coexpressed is much stronger. I suggest the authors modify "we also examined the changes in CB1R ..." in "Indeed we examined ..."
I think that references to the various panels of figure 2 are not correct (page8): "Fig2D" should be replaced by "Fig 2F" and references to Fig 2D and 2E should be made above.
Thank you for pointing this out; we addressed these points in the revised manuscript.

Referee #3

We thank the reviewer for his/her observations, and for suggesting studies that strengthen the manuscript.

1. The authors do not present the data on expression levels of the two receptors in any of the experimental situations described. Having this information (in absolute numbers, fmol/mg protein) is vital: while one can envision the dominance of AT1R-CB1R heterodimer signaling when both receptors are expressed at similar levels, this becomes impossible to swallow if one expressed at 10 times higher level than the other. This is especially important as each of the receptors in question is known to function perfectly well by itself.
We agree with the reviewer and have added information regarding receptor expression levels, in Supplementary Table 1. We find that in Neuro2A-AT1R, AT1R is expressed at a level quite similar to that of CB_1R.

2. The authors use ERK activation as a readout in >80% of their experiments. It is well known that GPCRs can activate ERK via many different pathways: through PKC, PKA, Gbg, RTK transactivation, and b-arrestin-mediated scaffolding, to name just a few. The authors did not even test which pathways are involved. They also assume that the same pathways operate in transfected cells and HSCs endogenously expressing both receptors, but did not test this experimentally.
We have directly addressed this by characterizing the pathway of ERK activation using inhibitors of PLC, PKC, Src and PDGFR (Suppl. Fig 5A) and Arrestin3 siRNA (Suppl. Fig 5B). We find that as previously reported (Ahn et al, 2004), Arrestin3 contributes to ERK phosphorylation in AT1R-expressing cells (Suppl. Fig 5B). In contrast, in AT1R-CB_1R expressing cells, downregulation of Arrestin3 leads to an increase in ERK phosphorylation (Suppl. Fig 5B), supporting the switch in AT1R signaling induced by CB_1R.

Together, our data confirm that in the context of the heterodimer, Ang II-mediated pERK levels are higher than in AT1R cells, but this increase is differentially modulated by Arrestin3.
In addition, we expanded the examination of AT1R-CB_1R-mediated signaling by showing that Ang II-mediated p38 and JNK activation is also attenuated by treatment with a CB_1R antagonist (Suppl. Fig 2A).
Together, our results show that AT1R activity, regardless of the signaling pathway examined (ERK, G protein activation, calcium response), is dependent upon CB_1R activity.

3. The authors also imply that ERK activation participates in mitogenic signaling and profibrogenic gene expression in HSCs, but do not present any proof of this. More than half of the study would only be relevant if that's the case.
In order to directly test the contribution of ERK activation to Ang II-mediated profibrogenic response, we used the MEK inhibitior PD98059, and find that this treatment prevents Ang II-mediated increase in profibrogenic gene expression. This is now included in the revised manuscript, in revised Figure 7F.

4. Described procedure for subtractive immunization would generate antibodies to AT1R with higher probability than to putative heterodimer. The authors should present strong evidence indicating that the antibodies are specific to heterodimer and do not react with AT1R to justify their conclusions.
We have included additional controls for the specificity of the AT1R-CB_1R antibody, in Suppl. Fig 3.
In addition, we now provide evidence that the heteromer-specific antibody blocks Ang II-mediated signaling in AT1R-CB_1R expressing cells, but not in cells expressing AT1R alone (Figure 2E), supporting the specificity of the antibody.

5. Although there exists extensive literature showing that BRET signal does not necessarily mean specific interaction, there are also papers claiming otherwise. In any case, the authors should add the data regarding the expression levels of both receptors (in fmol/mg protein) in BRET experiments and compare these levels to the ones that are in HSCs. This is very important, as many genuine interactions happen at 2-4 pmol/mg, but completely disappear when one gets to more physiological levels of 20-100 fmol/mg.
We agree with the reviewer and now indicate the expression levels of the receptors for the BRET experiments, which were close to the physiological expression level (~400-500 fmol/mg protein).

6. The authors need to use appropriate statistics throughout the paper. Student's t-test is only valid when one compares two groups. For the comparisons presented here the authors need to use more sophisticated statistics.
We now indicate the use of ANOVA followed by Bonferroni test when comparing multiple groups. This is detailed in the last paragraph of the Methods section.

Presentation concerns:

7. Fig.1. Panels A and C. Statistical significance of the differences in ERK activation curves should be shown. Panel B. The extent of CB1R knockdown should be quantified and statistically analyzed. Panel D. The statistics used should be indicated. As six experimental groups are shown here, the analysis should include correction for multiple comparisons.
8. Fig.2. Panel B. Low magnification does not allow one to judge co-localization (with this image size every other pair of proteins out of 2,000-4,000 expressed in the cell would appear co-localized). Panels C, D, E, and F. Statistical analysis showing significance (with correction for multiple comparisons, where appropriate) is needed to evaluate these data.
9. Fig.3, Fig.4 and Fig.5E. The same statistical issues as in Fig.2 should be addressed.
10. Fig.6. Panel C. Statistics needed. Panel D. The same low magnification problem as in Fig.2B.
11. Fig.7. Statistical analysis should be described. The method should include correction for multiple comparisons. Mentioned Student's t-test is not an appropriate method of analyzing differences where more than two experimental groups are used (ANOVA or equivalent with appropriate post-hoc tests is needed).
12. Suppl Figs 1 and 2: the same statistical and magnification (suppl fig. 2B,C) issues should be addressed.

We have carefully analyzed all of the data in figures, included statistical significance as recommended, and modified the presentation accordingly. Hence, all the figures have been modified in the revised manuscript.

13. Misstatement on p.14. Actually, both GABAB and mGlu1a are constitutively dimeric class C GPCRs.
This statement has been reworded. The current wording in the revised manuscript is as follows:
"Cross-talk between Gai- and Gaq-coupled receptors has been described for several receptor pairs, and typically leads to a potentiation of Gaq signaling (Carroll, 1995; Hilairet, 200; Rives, 2009), found often to be heteromerization-independent (Rives, 2009)."

2nd Editorial Decision 21 February 2011

Thank you for submitting your manuscript to the EMBO Journal. This is an invited resubmission of MS 76240. I asked the original referees to review the resubmission and their comments are provided below - the referees are listed in the same order as on the previous submission.

As you can see, the referees appreciate the introduced changes and find the dataset significantly improved. There are a number of remaining issues that should not involve too much additional work to address. Given the positive response from the referees, I would like to invite you to submit a suitably revised manuscript that takes the remaining concerns into consideration. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The revised version of the manuscript by Rozenfeld et al. adds a lot of new data and information, which answer most of my initial concerns. It is clear, comprehensive and the conclusions are strongly supported by an impressive number of convincing experiments.

Briefly, I still have a concern about the specificity of the Gi coupling of AT1R in the AT1-CB1 receptor heterodimer and the link between this coupling and heterdimerization. You demonstrate clearly that other Gi coupled GPCR (DOR or MOR) do not confer this property to AT1R when they are coexpressed. It would be also very informative to know if in parallel these receptors dimerize or not with AT1R. If not, it will be a strong argument for the specificity and the link between AT1/CB1 heterodimerization and the new acquired properties of AT1R. If DOR or MOR dimerize with AT1R, this will indicate that an additional structural event is necessary.

Minor points:
1.Supp Fig.4: AM251 should be defined.
2. Suppl. Fig.5 A is difficult to understand in absence of indication of AngII treatment. If I understand well lanes 1 and 7 are without AngII and the other lanes with AngII.
3. In this same figure (Suppl Fig.5A) the effect of PLC inhibitor is surprising in Neuro2A-AT1R cells (lane 3) and in contradiction with what is observed in Figure 3 indicating that in these cells, the Ang II induced Erk activation is independent of Gq. A commentary is necessary in order to avoid any confusion of the reader.

Referee #2 (Remarks to the Author):

The new version of the manuscript entitled "AT1R-CB1R heteromerization reveals a new mechanism for the pathogenic properties of angiotensin II" written by Rosenfeld and his colleagues has been substantially modified and the authors have brought answers to some points raised by the reviewers but two points still need attention.

1{degree sign}) I am still not convinced by the BRET experiments and more specifically by the negative controls. In the new version of the manuscript, the authors now show that ECE does not compete with any of the BRET partners, leaving intact the BRET signal despite over-expression of ECE. Is there any indication in the literature of a possible interaction between GPCRs and ECE ? Then what really support the relevance of such a control? Are these molecules targeted into the same membrane sub-domains?
Only a BRET bar graph is presented and saturation curves which are usually performed to support the specificity of interactions between two partners in BRET experiments were not carried out. If, in addition, the negative control is not relevant, the strength of the BRET data is questionable.
Can delta and Mu opioid receptors constitute negative control for BRET experiments? Indeed the authors have reported that co-expression of opioid receptors and AT1R does not result in the switch in G protein coupling to the AT1R, by contrast to the co-expression of AT1 and CB1 receptors. Therefore it suggests that opioid and AT1 receptor do not interact with each other, such that opioid receptors should constitute good negative controls.
Getting a convincing negative control is often difficult in BRET or FRET experiments, though these are critical. The authors must indicate in which cell type the BRET experiments were carried out (Neuro 2a cell?).

2{degree sign}) The authors have provided rather convincing data to suggest that AT1 G protein coupling switching observed after co-expression of CB1, is not due to crosstalk between signalling pathways (no effect of opioid receptor expression). They also mentioned that co-expression of CB1 and AT1 also induces a decrease in the Gq signalling pathway of AT1. Does it also result from receptor interactions or to crosstalk between signalling pathways? Have the authors analyzed the effect of opioid receptors on the AT1 Gq coupling properties? Indeed, different types of crosstalk between AT1 and CB1 receptors may co-exist.

Minor revision

The authors have to choose between "Mu and Delta receptors", "mu and delta receptors" or "MOR and DOR".

Referee #3 (Remarks to the Author):

The authors show that angiotensin 1 receptor (AT1R) and endocannabinoid receptor CB1R form heterodimers with specific functional characteristics. Importantly, the authors find that in hepatic stellate cells (HSCs) from rats that chronically received ethanol injcresed expression of CB1R leads to the type of angiotensin signaling observed in model cells expressing both receptors, and that it likely represents mitogenic signaling and profibrogenic gene expression in HSCs.
In the revised manuscript the authors presented significantly strengthened set of data with many necessary controls. All three remaining issues relate to receptor measurements by radioligand binding and should be easily addressable:

1. Radioligand binding assays used to generate data in Suppl Table 1 should be described. Expression of both receptors in normal HSCs (mentioned in the text) should be included for comparison. Also, the numbers should have error bars. As these data are crucial for establishing the case for heterodimer-specific signaling, they should be presented in the main manuscript (in the text or in Fig. 6 for both types of HSCs), whereas actual Scatchard plots with error bars would be most welcome in the supplement.

2. Fig. 1. Was the extent of CB1 knockdown measured by radioligand binding? This would be much more reliable and precise than inherently semi-quantitative Western.

3. Suppl Fig. 3C. These data are very important, but their interpretation depends on the actual expression levels of the two receptors, especially considering that anti-receptor antibodies indicate ~2-fold change in the expression, whereas anti-tag antibodies suggest ~5-fold difference. E.g., if compared to 1:1 case, the amount of one of the receptors was reduced to ~20% of the original, the data suggest the limited sensitivity of anti-heterodimer antibody more than its specificity. However, if the expression of one receptor was increased 5-fold while the other expressed at the same level, the data would suggest that both receptors preferentially form homodimers, so that either receptor expressed at higher level successfully out-competes heterodimerization partner. Also, what was the control (background) optical density with each antibody? Was it subtracted? Panel A suggests that "blank" noise of heterodimer-specific antibody is about 20% of the signal. Is that so? The authors should explain these experiments better and clearly discuss possible implications.

1st Revision - authors' response 20 March 2011

Referee #1 (Remarks to the Author):

The revised version of the manuscript by Rozenfeld et al. adds a lot of new data and information, which answer most of my initial concerns. It is clear, comprehensive and the conclusions are strongly supported by an impressive number of convincing experiments.

Briefly, I still have a concern about the specificity of the Gi coupling of AT1R in the AT1-CB1 receptor heterodimer and the link between this coupling and heterdimerization. You demonstrate clearly that other Gi coupled GPCR (DOR or MOR) do not confer this property to AT1R when they are coexpressed. It would be also very informative to know if in parallel these receptors dimerize or not with AT1R. If not, it will be a strong argument for the specificity and the link between AT1/CB1 heterodimerization and the new acquired properties of AT1R. If DOR or MOR dimerize with AT1R, this will indicate that an additional structural event is necessary.
In order to directly address this concern, we carried out additional experiments and investigated dimerization between MOR and AT1R using the BRET assay. We did not observe any significant BRET signal between MOR-rLuc and AT1R-GFP suggesting that these two receptors are not in close enough proximity to physically interact (this is included in the revised figure 2C).
In addition, and in response to referee #2, we examined the effect of coexpression of opioid receptors with AT1R on coupling to Gq, using Ang II-induced intracellular calcium release as a read-out. We found that stimulation of DOR did not potentiate Ang II-induced calcium response. These results suggest that the functional interaction between Gi- and Gq-coupled receptors is receptor specific. This further shows the specificity of the functional interaction between CB_1R and AT1R, affecting both Gi- and Gq-mediated responses, in contrast to interactions between DOR and AT1R, which do not lead to measurable modulation of AT1R responses.

Minor points:

1.Supp Fig.4: AM251 should be defined.
AM251 is now defined in the legend of suppl. Fig 5 of the revised manuscript.

2. Suppl. Fig.5 A is difficult to understand in absence of indication of AngII treatment. If I understand well lanes 1 and 7 are without AngII and the other lanes with AngII.
We apologize for the omission. The figure now indicates the lanes corresponding to Ang II treatment.

3. In this same figure (Suppl Fig.5A) the effect of PLC inhibitor is surprising in Neuro2A-AT1R cells (lane 3) and in contradiction with what is observed in Figure 3 indicating that in these cells, the Ang II induced Erk activation is independent of Gq. A commentary is necessary in order to avoid any confusion of the reader.
The involvement of PLC in Gi-coupled receptor-mediated ERK activation in Neuro2A cells has been recently described (Ma'ayan A, et al., Sci Signal. 2009). It is therefore not in contradiction with the lack of involvement of Gq. We have expanded the discussion of this point in the revised manuscript, (page 12, first paragraph).

Referee #2 (Remarks to the Author):

The new version of the manuscript entitled "AT1R-CB1R heteromerization reveals a new mechanism for the pathogenic properties of angiotensin II" written by Rosenfeld and his colleagues has been substantially modified and the authors have brought answers to some points raised by the reviewers but two points still need attention.

1- I am still not convinced by the BRET experiments and more specifically by the negative controls. In the new version of the manuscript, the authors now show that ECE does not compete with any of the BRET partners, leaving intact the BRET signal despite over-expression of ECE. Is there any indication in the literature of a possible interaction between GPCRs and ECE ? Then what really support the relevance of such a control? Are these molecules targeted into the same membrane sub-domains?
We have now added data with appropriate negative controls. As described earlier, we did not find significant BRET signal between AT1R and MOR. This is now included in the revised Figure 2C.

Only a BRET bar graph is presented and saturation curves which are usually performed to support the specificity of interactions between two partners in BRET experiments were not carried out. If, in addition, the negative control is not relevant, the strength of the BRET data is questionable.
We have added a saturation curve that supports the specificity of the interaction between CB_1R and AT1R. This is now in Figure 2C in the revised manuscript.

Can delta and Mu opioid receptors constitute negative control for BRET experiments? Indeed the authors have reported that co-expression of opioid receptors and AT1R does not result in the switch in G protein coupling to the AT1R, by contrast to the co-expression of AT1 and CB1 receptors. Therefore it suggests that opioid and AT1 receptor do not interact with each other, such that opioid receptors should constitute good negative controls.
As described above, opioid receptors do constitute good negative controls.

Getting a convincing negative control is often difficult in BRET or FRET experiments, though these are critical. The authors must indicate in which cell type the BRET experiments were carried out (Neuro 2a cell?).
The BRET experiments were carried out in HEK293 cells. This is now indicated in the revised manuscript.

2- The authors have provided rather convincing data to suggest that AT1 G protein coupling switching observed after co-expression of CB1, is not due to crosstalk between signalling pathways (no effect of opioid receptor expression). They also mentioned that co-expression of CB1 and AT1 also induces a decrease in the Gq signalling pathway of AT1. Does it also result from receptor interactions or to crosstalk between signalling pathways? Have the authors analyzed the effect of opioid receptors on the AT1 Gq coupling properties? Indeed, different types of crosstalk between AT1 and CB1 receptors may co-exist.
We carried out experiments to directly address this by examining the effect of coexpression of MOR or DOR with AT1R on coupling to Gq, using Ang II-induced increased intracellular calcium release as a read-out. Interestingly, we found that stimulation of MOR potentiates Ang II-induced calcium response, whereas stimulation of DOR had no effect. These results suggest that the functional interactions between Gi- and Gq-coupled receptors are receptor specific, and further demonstrates the specificity of the direct interaction between CB_1R and AT1R, affecting both Gi- and Gq-mediated responses, as opposed to interactions between MOR and AT1R that affect only Gq-mediated response, and between DOR and AT1R, which does not lead to measurable modulation of AT1R responses for either Gi- or Gq-mediated signaling.

Minor revision

The authors have to choose between "Mu and Delta receptors", "mu and delta receptors" or "MOR and DOR".
We have now unified this and use MOR and DOR throughout the manuscript.

Referee #3 (Remarks to the Author):

The authors show that angiotensin 1 receptor (AT1R) and endocannabinoid receptor CB1R form heterodimers with specific functional characteristics. Importantly, the authors find that in hepatic stellate cells (HSCs) from rats that chronically received ethanol injcresed expression of CB1R leads to the

type of angiotensin signaling observed in model cells expressing both receptors, and that it likely represents mitogenic signaling and profibrogenic gene expression in HSCs.
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1. Radioligand binding assays used to generate data in Suppl Table 1 should be described. Expression of both receptors in normal HSCs (mentioned in the text) should be included for comparison. Also, the numbers should have error bars. As these data are crucial for establishing the case for heterodimer-specific signaling, they should be presented in the main manuscript (in the text or in Fig. 6 for both types of HSCs), whereas actual Scatchard plots with error bars would be most welcome in the supplement.
We now describe the binding experiments in the "Methods" section, and included receptor expression data (with error bars) in the main text, Table I.

2. Fig. 1. Was the extent of CB1 knockdown measured by radioligand binding? This would be much more reliable and precise than inherently semi-quantitative Western.
We could not measure CB_1R knockdown using radioligand binding because the quantity of material necessary to carry out ligand binding is prohibitively large (~100 times more material than for Western blot). This would also require a change in the protocol for siRNA transfection that is likely to affect the siRNA transfection efficiency. We have found the use of fluorescent secondary antibodies to quantify receptor levels using the Licor system (Odyssey) to be fairly reliable in yielding quantitative data as compared to enzyme conjugated secondary antibodies that provides semi-quantitative data on relative receptor levels by the chemiluminescence assay.

3. Suppl Fig. 3C. These data are very important, but their interpretation depends on the actual expression levels of the two receptors, especially considering that anti-receptor antibodies indicate ~2-fold change in the expression, whereas anti-tag antibodies suggest ~5-fold difference. E.g., if compared to 1:1 case, the amount of one of the receptors was reduced to ~20% of the original, the data suggest the limited sensitivity of anti-heterodimer antibody more than its specificity. However, if the expression of one receptor was increased 5-fold while the other expressed at the same level, the data would suggest that both receptors preferentially form homodimers, so that either receptor expressed at higher level successfully out-competes heterodimerization partner. Also, what was the control (background) optical density with each antibody? Was it subtracted? Panel A suggests that "blank" noise of heterodimer-specific antibody is about 20% of the signal. Is that so? The authors should explain these experiments better and clearly discuss possible implications.
These studies were carried out to see if changing the ratio of the plasmids for the two receptors (that should lead to changes in receptor abundance) would lead to changes in heteromer abundance. Since the efficiency of transcription/translation of each plasmid/receptor is likely to be different and the levels of detection by different antibodies are likely to vary, a direct comparison between absolute levels of each receptor abundance is not possible. However, one can make a relative comparison between the ratio of the receptor pair. We find that when cells are transfected with a greater amount of plasmid for one or the other receptor, the relative level of heteromer is lower than when comparable amount of the two plasmids are transfected. This was included as an additional support of the specificity of the heteromer antibody. Taken with the other controls, we hope the reviewer agrees that these data support the $AT1R\text{-}CB_1R$ antibody heteromer-specificity.
If the reviewer judges this figure to be confusing, we will be happy to remove it from the manuscript.

3rd Editorial Decision 05 April 2011

Thank you for submitting your revised manuscript to the EMBO Journal. Your revised version has now been seen by the original referee #1 and 2. As you can see below both referees are satisfied with the revised version and support publication here. I am therefore very pleased to proceed with the acceptance of your paper for publication here. You will receive the formal acceptance letter shortly.

Best regards

Editor

The EMBO Journal

REFEREE REPORTS

Referee #1:

The authors have answered correctly to all my queries. No further question. Ok for publication.

Referee #2:

The authors modified their ms as requested, I have no further comments. In my opinion, the ms can now be published in EMBO J

