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Assembly of a 2 Adrenergic Receptor - GluR1 Signaling Complex for Localized cAMP Signaling

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Transaction Report:

(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 26 January 2009

Thank you for submitting your manuscript to the EMBO Journal. I have now had the opportunity to read the manuscript carefully and to discuss it with the other members of our editorial team and I am sorry to say that we cannot offer to publish it.

Your analysis reports that the beta2 adrenergic receptor (b2AR) forms a large complex with the AMPA receptor (GluR1), adenylyl cyclase, G proteins and PKA. The b2AR -GluR1 complex formation is mediated via PSD-95 and Stargazin. Furthermore, b2AR activation leads to phosphorylation of b2AR-associated GluR1, which affects GluR1 surface expression and AMPA receptor responses. We appreciate that the findings report on the interplay between b2AR and AMPA receptor signaling and provides a characterization of a large b2AR -GluR1 signaling complex. However, previous work has also reported that PKA mediated GluR1 phosphorylation affects it surface expression levels and norepinephrine signaling has also been shown to lead to GluR1 phosphorylation and to facilitate GluR1 trafficking. Given these considerations, I am afraid that we are unfortunately not persuaded that the overall conceptual advance and novel insight provided is sufficient to consider publication in the EMBO Journal.

Please note that we publish only a small percentage of the many manuscripts that we receive at the EMBO Journal, and that the editors have been instructed to only subject those manuscripts to external review which are likely to receive enthusiastic responses from our reviewers and readers. As in our carefully considered opinion, this is not the case for the present submission, I am afraid

our conclusion regarding its publication here cannot be a positive one. I am sorry to have to disappoint you on this occasion.

Yours sincerely,

Editor
The EMBO Journal

Additional Correspondence – Authors' Response   29 January 2009

We appreciate all the work you and your colleagues do to ensure the highest standard for EMBO J. Also, we agree that it is not novel that beta-adrenergic signaling regulates AMPA receptors. However, please allow us to point out that this is not the most important finding of our manuscript. Rather we show for the first time that the beta2 adrenergic receptor (b2AR) forms a complete signaling complex with the AMPA R GluR1 subunit that includes constitutive associated trimeric Gs protein, adenylyl cyclase, and, as shown earlier, PKA. This is only the second such complex that contains all elements of a cAMP signaling cascade (the first one was the b2AR-L-type Calcium channel complex, published by us earlier: Davare et al., 2001: Science 293, 98-101). However, in this previous paper we did not show that the association of the b2AR with the L-type channel is required for signaling from the b2AR to the channel. This is a question of central importance because a single cell (including neurons and cardiomyocytes) often contain several GPCR that act by stimulating adenylyl cyclase to produce the diffusible cAMP yet cause different effects within a cell. The idea that signaling complexes such as the b2AR-GluR1 or b2AR-L-type channel complex exist to allow for highly localized signaling has been raised by several investigators already a number of years ago but remained untested.
We now show for the first time that signaling from the b2AR REQUIRES association with one of its downstream targets (here GluR1) in two different ways: 1. Only GluR1 that is associated with the b2AR (10-20% of total GluR1) becomes phosphorylated upon b2AR activation (Fig. 3C,D). 2. Two different peptides that disrupt the b2AR-GluR1 complex prevent GluR1 phosphorylation upon b2AR activation (Fig. 3G-J). We then show with our peptides that increases in GluR1 at the synapse observed upon b2AR stimlation depend on the b2AR-GluR1 interaction (we use state of the art surface labeling for immunofluorescent microscopy and whole cell patch clamp electrophysiology).

Perhaps I should add that only the importance of beta-adrenergic signaling for certain forms of synaptic plasticity in the hippocampus has been systematically studied. In all of these papers (which are discussed in our manuscript) authors acknowledge that there is a (though variable) effect of b-adrenergic stimulation on basal synaptic transmission (which could explain at least in part the role of b-adrenergic signaling in synaptic plasticity but was always ignored; however, this effect on basal synaptic transmission was never studied). Our systematic analysis confirms a variable yet statistically clearly relevant effect of b2 AR signaling in the hippocampus (Suppl. Fig. 3). We further find that b2AR stimulation has a more robust and nearly invariable effect in the prefrontal cortex. In the last part of our manuscript we show that the b2AR-induced increase of basal synaptic transmission (which again has not been systematically studied before) DEPENDS ON THE b2AR BEING LINKED TO GluR1. The role of the prefrontal cortex in regulating motivation of behavior and in drug seeking and the importance of the beta-adrenergic system in arousal and directing attention further add to the relevance of our work.

For the above reasons I would appreciate if you would reconsider you decision.

Thank you very much for your time and efforts.

Additional Correspondence   30 January 2009

Thank you for your email asking us to reconsider our decision and to send the manuscript out for peer-review. I have now discussed the matter again with my colleagues, and we have no objections to seeking advice from experts in the field. I will get back to you as soon as we hear back from the referees.

2nd Editorial Decision   05 March 2009

Thank you for submitting your manuscript to the EMBO Journal. We have now heard back from three referees, whom we asked to evaluate your manuscript. As you can see from the comments below there is a clear interest in your study. However, the referees also raise different concerns with the analysis that would have to be resolved in order for further consideration here. I will not repeat all their individual points of criticism in this letter, but in particular referee #2 finds the present analysis too incomplete and indicate that a much further extension of the work would be needed to consider publication in the EMBO Journal. Should you be able to address the raised concerns in full, we would consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will 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.

REFEREE REPORTS

Referee #1 (Remarks to the Author):

This study by Joiner et al investigates the interaction between β2 adrenergic receptors and GluR1 AMPA receptor subunits. They show that the two receptors are located in the same complex, probably linked via PSD-95 and TARPs. 2AR activation increases GluR1 phosphorylation and surface expression in hippocampal neurons. In prefrontal cortex, β 2AR activation increases EPSC amplitude.

The study is generally well performed and clearly presented. It is an interesting study that adds to the evidence that βARs can regulate AMPA receptor phosphorylation and expression. However, I have some concerns/comments that the authors should address.

1. The authors have used hippocampal slices and cultured neurons for the imaging and western blot analyses, yet prefrontal cortex slices were used for electrophysiology. Although the reasons for using prefrontal cortex for electrophysiology are clear, it does raise the question of consistency throughout the study. Would the same effects of ISO observed in cultured hippocampal neurons be seen in prefrontal neurons - indeed the observed effects may be larger. Is it possible for the authors to perform some imaging or biochemistry experiments on prefrontal cortex neurons/slices to better link together the different techniques?

2. It is known that the β1 AR can also regulate GluR1 phosphorylation of Ser845 - this is not currently mentioned in the manuscript and should be discussed.

3. The authors have concentrated on the GluR1 subunit. Have they any information on possible interaction between β2ARs and the GluR2 AMPA receptor subunit?

4. Information on n values is sometimes lacking. Thus, for figure 1 please give n values for puncta/slices/animals. For Figure 2, details of number of timres each experiments was performed is lacking for parts A and E. For Fig 3- 5 I assume the number in each bar is the n value but this needs to be stated at least for the first of these figures.

5. The concentration of ISO used varies between experiments, from 1 to 10 µM. Why was the same concentration not used in each experiment?

6. Why was IBMX used in experiments presented in figure 4 but not other experiments? What was the effect of ISO alone in these experiments?

7. Some basic statements in the Introduction are incorrect. Thus to say that "Norepinephrine acts via β1AR and β2AR" is to ignore completely the α receptors. NE is also a much weaker agonist of β2 receptors than the other ARs.

Referee #2 (Remarks to the Author):

The report by Joiner et al. is studying the effect of beta2 adrenergic receptor (ADRs) stimulation on the increase in GluR1 surface expression and activity.
The main point is that this ADRs effect requires their clustering with GluR1 via association of both ADRs and the dimer stargazin-GluR1 with the PDZ domains of PSD-95. This clustering is necessary for phosphorylation of GluR1 on Ser 845 known to be implicated in GlUR1 surface expression. Thus, clustering constitutes the originality of the paper.
Minor:
1) The authors citations for cAMP localization are quite old. Recent papers (see for example Richter et al EMBO.J 2008 and papers cited within) should be quoted.
2) in the abstract the important role of stargazin is not mentioned ???. The author are speaking of "beta2 ADRs-GluR1 association" without stating that it is an "indirect" association.
Major:
1) one puzzling observation is that IP of beta-2 ADRs co-immunoprecipitated PSD-95 but not NMDA receptors. MNDA receptors are well known to bind PSD-95. The authors should immuno-precipitate PSD-95 and see if beta-2 and NMDA receptors are co-immunoprecipated. More important immunoprecipitation of NR2A should not immunoprecipitate beta-2 ADRs. This will define two populations of PSD-95 , one which bind beta2 ADRs and GluR1 the other which bind NMDA receptor and (perhaps GluR1). This is very important to know. The specificity claimed here between association of beta2 ADRs-PSD-95 and GluR1 is not based on strong data.
2) Figure 2E: why this experiment done with forebrain??? the authors are claiming that in the forebrain (at least cortex) it is not stragazin which is needed for beta2-ADRs-GluR1 association???the experiment should be done in cerebellum.
3) The increase in EPSC is blocked by non-specific blockers of exocytosis. Is the increase blocked by PKA inhibitors ?? Why is it irreversible??? is phosphorylation persistent??
4)The authors seem to ignore that PKA also phosphorylate the C-terminal of stargazin (J.Biol.Chem 277: 12359 2002) and this is affecting its interaction with PSD-95. Are beta2 ADRs able to phosphorylate stargazin ? is it sensitive to the interaction of beta2 ADRs with PSD-95 ??
In conclusion the study is far for complete.

Referee #3 (Remarks to the Author):

Noradrenergic and cAMP signaling in the brain has been shown to be important in a number of cognitive processes, including learning. In the current manuscript, Joiner et al have investigated the molecular mechanisms that may underlie β2 adrenergic receptor (β2AR) mediated signaling in hippocampal and cortical neurons. The authors demonstrate that β2AR forms a complex, via PSD-95 and stargazin, with GluR1, and that specific activation of β2AR results in GluR1 phosphorylation at S845. Moreover, the authors nicely demonstrate that only GluR1 associated with β2AR can be phosphorylated as a result of β2AR activation. Furthermore, β2AR activation leads to an increase in the surface expression of GluR1 and an increase in EPSCs and AMPAR mEPSCs in PFC slices. Finally the authors demonstrate that this increase in EPSCs is blocked by inhibiting GluR1 exocytosis. The authors conclude that this study establishes a supramolecular complex that allows highly localized signaling complex for β2AR/cAMP signaling in hippocampal neurons.
This study is well thought out and provides convincing evidence for a β2AR mediated increase in AMPAR responses and moreover, provides a compelling argument for the concept of localized β2AR/cAMP signaling in neurons. However, a number of experimental and text-based changes are

required before this manuscript is suitable for publications.

Major concerns:

1) The authors demonstrate that β2AR and GluR1 form a complex and state that PSD-95 is an intermediate in this complex. However, the authors do mention that other scaffold proteins can interact with GluR1 (eg SAP97). As β2ARs contain a type 1 PDZ domain, the authors should be careful not to claim that the interaction between β2AR and GluR1 is only mediated via PSD-95, and that other scaffold proteins may also mediate this interaction. Furthermore, although the authors demonstrate that the use of peptides that mimic the C-terminal of β2AR (DSPL) and that block the PDZ1 and 2 domains of PSD-95 (ESDV) do attenuate β2AR mediated effects on AMPAR responses, the authors should demonstrate that these peptides do in fact disrupt the complex formed between β2AR and GluR1. This is especially important for the ESDV peptide, as it would indicate whether a substantial portion of the β2AR/GluR1 complex is mediated via PSD-95 and not another scaffold protein. This can be easily tested by coimmunoprecipitation studies.

2) It is not readily clear from the text what concentration of ISO is used for a number of experiments in this study. Furthermore, it is also not clear how long the agonist was incubated for, before GluR1 phosphorylation was tested. This should be clearly stated in the text and not only in the methods and figure legends for all treatments. It seems that 15 min of ISO increased GluR1 phosphorylation; however, as these experiments were performed in the absence of IBMX, one would expect that the GluR1 phosphorylation at S845 would be time dependent - endogenous PDEs would act to reduce cAMP activity, thereby reducing PKA activity, and therefore, a time dependent effect on GluR1 phosphorylation would be expected. The authors should test the time course of GluR1 phosphorylation. This would also be in agreement with the electrophysiological studies that the authors have performed, where the authors state that a time dependent increase in AMPAR mEPSCs and EPSCs is observed.

3) The authors demonstrate that Gs and adenylyl cyclase is part of the complex formed between β2AR and GluR1. However, they do not provide any evidence that they are required for the phosphorylation or insertion of GluR1. The authors could test this by blocking adenylyl cyclase activity using an inhibitor such as SQ22536, or blocking Gs activity and subsequently testing GluR1 phosphorylation following β2AR activation. This would provide evidence that β2AR is acting via a Gs/adenylyl cyclase dependent pathway, and not via a Gβ/γ, or G-protein independent pathway, leading to GluR1 phosphorylation/insertion, and provide further evidence for a localized cAMP signaling supramolecular complex.

4) The peptides DSPL and ESDV, and their inactive analogs, is utilized in a number of experiments throughout the manuscript. However, a number of important controls are required. In addition to demonstrating that these peptides do disrupt the β2AR/GluR1 complex, the authors should demonstrate whether or not these peptides have any effect on the localization of related and unrelated proteins. For example, does treatment for 2 hours with DSPL lead to a reduction of β2AR from its synaptic localization? Similarly, does ESDV have a similar effect on GluR1 or other synaptic proteins (NR1, NR2A etc)? This is important considering the fact that these peptides reduce basal EPSC amplitudes. In addition, further explanation and discussion of the effects of these peptides on basal EPSC amplitudes is required.

5) In supplemental figure 2, the authors show that ICI 118551 and DSPL inhibit ISO insertion of surface GluR1. However, does either treatment reduce basal levels of surface GluR1? This could in part explain the reduction in basal EPSC amplitudes seen after incubation with these antagonists/peptides. The authors should compare GluR1 puncta/10 m between untreated and treated (with peptide/antagonist) cells. Furthermore, does incubation with either peptide or ICI 118551 reduce basal level of GluR1 phosphorylation? Fig 3 A suggest that the latter may do this. One might expect this to be the case, indicating that β2AR has some levels of constitutive activity.

6) In Figure 6, Joiner et al show that ISO increases AMPAR mEPSCs, however, they do not investigate whether ISO has any effect on mEPSC kinetics. This is important as phosphorylation of GluR1 at S845 increases their open probability. Furthermore, have the authors tested to see if blocking β2AR-depdent phosphorylation of GluR1 attenuates the increase in AMAPR mEPSCs?

The graph in Fig. 6E is somewhat confusing. The authors should show the amplitude before and after treatment with ISO separately and not as a % increase. This should also be shown for AMPAR mEPSC frequency.

7) The initial biochemical characterization of the β2AR/GluR1 complex is performed in hippocampal neurons, but subsequent electrophysiological studies are performed in PFC slices. Although hippocampal neurons and cortical neurons are very similar, certain differences in their molecular make up can be described. Therefore, the authors should at least demonstrate that β2AR and GluR1 do form a complex in cortical neurons - ideally, the authors would also show that ISO treatment can lead to GluR1 phosphorylation at S845 in cortical neurons.

Minor points:
1) In Fig. 1, the authors state that majority of β2AR is synaptic, is there any β2AR in the shaft?

2) It is unclear what antibody(ies) are used in Fig 2A, this show be clearly labeled in the figure.

3) The blot in Fig 3G is not convincing and should be replaced.

4) Fig. 4 is not clearly labeled and maybe somewhat confusing to readers.

5) In addition to % of GluR1 puncta, the total number of puncta per 10 m should be graphed in Fig. 5.

6) Do the authors have the number of puncta, or the % change in the puncta number for supplemental Fig. 2?

1st Revision - authors' response   21 September 2009

We thank the Reviewers for their insightful comments and are encouraged by the positive tenor of the review. We undertook extensive efforts to thoroughly address the concerns by the Reviewers. Their statements are repeated for convenience below in *italics*.

Reviewer 1

This study is generally well performed and clearly presented. It is an interesting study …

1. *Would the same effects of ISO observed in cultured hippocampal neurons be seen in prefrontal cortex* (PFC)*? Is it possible for the authors to perform some imaging or biochemistry experiments on prefrontal cortex neurons/slices?*
1.1 We performed immunogold EM for the β_2 AR in PFC with exquisite results, i.e., gold labels are largely localized at the postsynaptic densities as would be expected for selective postsynaptic localization (new Fig. 1C).
1.2 New co-IPs from PFC confirm that GluR1, β_2AR, adenylyl cyclase (AC) and Gs form a complex in PFC (new Suppl. Fig. 1A).
1.3 We treated acute PFC slices with ISO, which resulted in nearly full GluR1 S845 phosphorylation within 2 min and lasted more than 15 min following wash out (new Suppl. Fig. 8).
These results illustrate the existence of a β_2 AR-AC-Gs-GluR1 complex at postsynaptic sites that regulates S845 phosphorylation.

2. *Discuss that β_1AR also regulates S845 phosphorylation.*
We now add the following statement to the Discussion: "One study suggests that the β_1AR can also regulate S845 phosphorylation (Vanhoose, 2003). The differences between this and our study that lead to this contrasting outcome could be related to differences in the exact experimental system, though quite similar, or the high concentrations of the β_1 selective blocker betaxolol (10 µM) use in the other study at which isotype specificity could have been lost (e.g., Smith and Teitler, 1999: Cardiovasc Drugs Ther 13, 1230126; Sharif and Xu, 2004: J Ocul Pharmacol Ther 20, 93-99). Also, there was no evidence presented that the ICI118551 batch was active in the other study." We would

like to add that there was no additional evidence in that study that would have supported their conclusion that S845 phosphorylation had been induced by β_1 AR activation, in contrast to our work (e.g., only GluR1 that was associated with the β_2 AR became phosphorylated upon ISO treatment; our Fig. 3C,D). It is thus possible that the effect by Vanhoose and Winder could have been mediated by the β_2 AR.

3. *Have they* (the authors) *any information on possible interaction between the β_2 AR and GluR2?*
Whereas a number of publications clearly show that PKA phosphorylates and regulates GluR1, there is no evidence for GluR2, hence our focus on GluR1. However, we looked for GluR2 in the IPs from PFC and cerebellum. There is clear coIP with the β_2AR from cerebellum and perhaps (though less clear) from PFC (new Suppl. Fig. 1A).

4. *Information on n values is sometimes lacking.*
Fig. 1: We added: "a total of 329 synapses in 15 fields in stratum radiatum 25-100 μm away from the pyramidal cell layer from 3 adult SD rats (5 fields per rat) were analyzed."
Fig. 3A: We added: "Comparable results were obtained in three other experiments."
Fig. 3E: We added: "In each case similar results were obtained in 3-7 independent experiments."
Fig. 3-5: We added "n" to the left of the boxes within bar diagrams to indicate the meaning of the numbers in Fig. 3 and 5 and state the numbers in the legend to new Fig. 4.

5. *The concentration of ISO varies between experiments from 1 to 10 μM. Why was the same concentration not used in each experiment?*
As I was initiating the collaborations with Drs. El-Husseini, Yan, and Law, I indicated to them that ISO is typically used between 1 and 10 μM rather than just indicating a singe exact concentration, which admittedly would look in retrospect more consistent. However, because ISO is a very selective and after several decades of use in fact still considered specific βAR agonist even at 10 μM, this should in my opinion not constitute a serious concern.

6. *Why was IBMX used in* (original) *Fig. 4* (imaging of SEP-tagged GluR1 over time) *but not other experiments? What was the effect of ISO alone in these experiments?*
In initial experiments we wanted to first established a robust ISO effect by inhibiting cAMP degradation with this phosphodiesterase inhibitor. We now tested in 3 independent experiments the effect of ISO alone and found that it increases within 5 min SEP-GluR1 surface expression (new Fig. 4; original Fig. 4 with IBMX is now Suppl. Fig. 3).

7. *To say that "norepinephrin acts via β_1AR and β_2AR" is to completely ignore the αARs. NE is also a much weaker agonist of the β_2AR than the other ARs.*
We modified our respective introductory statement to "Norepinephrin acts via αAR and bAR. Stimulation of the b_1AR and b_2AR activates G_s, adenylyl cyclase, and PKA" to explicitly indicate that other pathways exist. Further discussion of αAR signaling should not be necessary as we have no evidence that any of our effects involve αAR.
It is true that norepinephrin is less potent towards b_2AR than b_1AR but I want to emphasize that it is a full agonist (i.e., can elicit maximal responses at saturating concentrations) for the b_2AR.

Reviewer 2
Minor:
1. *The authors' citations for cAMP localizations are quite old. Recent papers should be quoted.*
We consider the Levitzki, 1988, a classic cornerstone publication that is still fully valid and provides an additional perspective from more recent work on localized signaling by GPCR. However, following the Reviewer's suggesting, we added three newer citations: Zaccolo and Pozzan, 2002; Fischmeister et al., 2006; and Richter et al., 2008.

2. *In the abstract the important role of stargazin is not mentioned?*
We now state in the abstract that GluR1 "is linked to the b_2AR via complexes formed by stargazin and PSD-95 or their homologues."

Major:
1. *one puzzling observation is that IP of b_2AR co-immunoprecipitated PSD-95 but not NMDA receptors. NMDA receptors are well known to bind PSD-95. The authors should immuno-precipitate PSD-95 and see if b_2AR and NMDA receptors are co-immunoprecipated. More important immunoprecipitation of NR2A should not immunoprecipitate b_2AR. This will define two populations*

of PSD-95, one which bind b₂AR and GluR1 the other NMDA receptor and (perhaps GluR1). This is very important to know. The specificity claimed here between association of b₂AR-PSD-95 and GluR1 is not based on strong data.

We now show that IP of the NMDAR-PSD-95 complex does not result in co-IP of the b_2AR, which was otherwise detectable in lysate as positive control for probing the immunoblot (new Fig. 1F). PSD-95 does coIP with the NMDAR. Together with our finding that neither NR1 nor NR2A nor NR2B co-precipitated with the b_2AR in several independent experiments (Fig. 2E, right panels) we feel the evidence is now compelling that the b_2AR can associate with GluR1/stg/PSD-95 complexes but not with NMDAR-PSD-95 complexes. Also, PSD-95 was present in GluR1 IPs and b_2AR IPs (Fig. 2F, left panels). Collectively these results do in fact define two different PSD-95 pools, one associated with GluR1 and the b_2AR but not the NMDAR and one associated with the NMDAR but not the b_2AR. Accordingly, the interaction of the b_2AR with PSD-95 or it homologues is governed by other interactions of PSD-95 (or homologues). In other words, it is controlled by the molecular environment of PSD-95. That only certain combinations of binding partners for complex formation with PSD-95 are realized in vivo appears to be critical in order to avoid physiologically undesirable assemblies or, worse, chaos by random complex formation. This notion is now included in the text. We appreciate the Reviewer pointing out that this is an important aspect of our work that we had not discussed in the previous manuscript.

2. *Figure 2E: why is this experiment done with forebrain? The authors are claiming that in the forebrain (at least cortex) it is not stragazin which is needed b₂AR-GluR1 association? The experiment should be done in cerebellum.*

Please note that we believe stg is one of several "TARPs" that mediate the GluR-PSD-95 association in forebrain. Accordingly, stg contributes in forebrain to the GluR-PSD-95 complexation but other TARPs do so too and can substitute there. In cerebellum stg is the main TARP. Other TARPs are not sufficiently present (or not present in the relevant neuronal cell types) to substitute for stg there. To confirm that GluR1 and the b_2AR interact with each other and with Gs and adenyl cyclase in cerebellum we followed the advice by the Reviewer and repeated the respective immunoprecipitation experiments with cerebellar extracts (new Suppl. Fig. 1A).

3. *The increase in EPSC is blocked by non-specific blockers of exocytosis. Is the increase blocked by PKA inhibitors ? Why is it irreversible? Is phosphorylation persistent?*

We now show that the highly specific PKA inhibitory PKI peptide prevents the upregulation of EPSCs and mEPSC by ISO (new Fig. 6 F,G and new Suppl. Fig. 7). We further find that in acute PFC slices 15 min ISO treatment leads to an increase in phosphorylation of GluR1 on S845 that lasts at least another 15 min following wash-out of ISO (new Suppl. Fig. 8). This finding is consistent with the electrophysiological observation that within 15 -20 min starting wash-out of ISO the EPSC response is still elevated. We do not know the molecular basis for this lasting increase, which seems to be beyond the scope of this article.

4. *The authors seem to ignore that PKA also phosphorylates the C-terminal of stargazin (J.Biol.Chem 277: 12359, 2002) and this is affecting its interaction with PSD-95. Are β₂ARs able to phosphorylate stargazin? Is it sensitive to the interaction of β₂AR with PSD-95?*

We obtained the phosphospecific antibody from Dr. E. Kim, the senior author of this study on stg phosphorylation at Thr321, and repeated ISO stimulation in 3 experiments. We did not observe an obvious increase in phosphorylation as detected with this antibody. This negative result is not surprising because the hydroxyl group of the C-terminal Thr phosphorylated in the above study typically forms a hydrogen bridge with a histidine in its PDZ domain binding partners. Thr321 would thus not easily be accessible and available for phosphorylation and only after that hydrogen bond is broken, which would lead to dissociation of PSD-95 from stg. Conversely, it is highly likely that this phosphorylation would disrupt PDZ binding (prevent PSD-95 interaction) as does the analogous phosphorylation of the NMDAR NR2B subunit by casein kinase 2 (Chung et al., 2004: J Neurosci 24, 10248). Furthermore, mutating Thr321 to Phe prevents its PDZ interaction further suggesting that this Thr is critical for PSD-95 binding. The mutation also reduces AMPAR surface expression contrasting the increase in GluR1 we see upon stimulation of the b₂AR-PKA pathway. Also, Choi et al. as cited by Reviewer 3 above demonstrated that the Thr321-phosphorylated stg is depleted from the postsynaptic site contrary to what we find for GluR1 and the β₂ARs. Please note that they never provided any evidence that it is actually PKA that phosphorylates Thr321 in brain or

intact neurons. Because the result of this experiment is negative we do not include it into the manuscript.

Reviewer 3

This study is well thought out and provides convincing evidence for a β_2AR-mediated increase in AMPAR responses and more over provides a compelling argument for the concept of localized β_2AR/cAMP signaling… .

Major:

1. The authors demonstrate that b$_2$AR and GluR1 form a complex and state that PSD-95 is an intermediate in this complex. However, the authors do mention that other scaffold proteins can interact with GluR1 (e.g., SAP97). As b$_2$AR contain a type 1 PDZ domain, the authors should be careful not to claim that the interaction between b$_2$AR and GluR1 is only mediated via PSD-95, and that other scaffold proteins may also mediate this interaction. We appreciate this remark and state right away in the Abstract "GluR1, which is linked to the β_2AR via complexes formed by stargazin and PSD-95 or their homologues" and added corresponding statements throughout the text. *Furthermore, although the authors demonstrate that the use of peptides that mimic the C-terminal of b$_2$AR (DSPL) and that block the PDZ1 and 2 domains of PSD-95 (ESDV) do attenuate b$_2$AR mediated effects on AMPAR responses, the authors should demonstrate that these peptides do in fact disrupt the complex formed between b$_2$AR and GluR1. This is especially important for the ESDV peptide, as it would indicate whether a substantial portion of the b$_2$AR /GluR1 complex is mediated via PSD-95 and not another scaffold protein. This can be easily tested by coimmunoprecipitation studies.* We now show that the DSPL peptide specifically disrupts coimmunoprecipitation of the β_2AR but not PSD-95 with GluR1 whereas the ESDV peptide disrupts both coimmunoprecipitations. These results are consistent with the model that GluR1 associates via stg (or homologues) with PDZ1 and 2 of PSD-95, which should not be affected by DSPL but only by ESDV (new Suppl. Fig. 1B). The interaction of GluR1 with the β_2AR should of course be affected by both because it depends not only on its C-terminal binding to PDZ3 of PSD-95, which is blocked by DSPL, but also on the intact GluR1/stg – PSD-95 interaction.

2. It is not readily clear from the text what concentration of ISO is used for a number of experiments in this study. Furthermore, it is also not clear how long the agonist was incubated for, before GluR1 phosphorylation was tested. This should be clearly stated in the text and not only in the methods and figure legends for all treatments. We added corresponding statements throughout the text. *It seems that 15 min of ISO increased GluR1 phosphorylation; however, as these experiments were performed in the absence of IBMX, one would expect that the GluR1 phosphorylation at S845 would be time dependent - endogenous PDEs would act to reduce cAMP activity, thereby reducing PKA activity, and therefore, a time dependent effect on GluR1 phosphorylation would be expected. The authors should test the time course of GluR1 phosphorylation. This would also be in agreement with the electrophysiological studies that the authors have performed, where the authors state that a time dependent increase in AMPAR mEPSCs and EPSCs is observed.* As stated in response to request #3 by Reviewer 2, we find that in acute PFC slices 15 min ISO treatment leads to an increase in phosphorylation of GluR1 on S845 that lasts at least another 15 min following wash-out of ISO (new Suppl. Fig. 8). We do not know at this point why the phosphorylation and upregulation of EPSCs persist (which we feel is beyond the scope of the current manuscript) but these new biochemical phosphorylation experiments are in perfect agreement with our electrophysiological recordings.

3. The authors demonstrate that Gs and adenylyl cyclase is part of the complex formed between b$_2$AR and GluR1. However, they do not provide any evidence that they are required for the phosphorylation or insertion of GluR1. The authors could test this by blocking adenylyl cyclase activity using an inhibitor such as SQ22536, or blocking Gs activity and subsequently testing GluR1 phosphorylation following b$_2$AR activation. This would provide evidence that b$_2$AR is acting via a Gs/adenylyl cyclase dependent pathway, and not via a G-protein independent pathway, leading to GluR1 phosphorylation/insertion, and provide further evidence for a localized cAMP signaling supramolecular complex. We now show that the highly specific PKA inhibitory PKI peptide prevents the upregulation of EPSCs and mEPSC by ISO (new Fig. 6 and new Suppl. Fig. 7). These findings exclude a

membrane-delimited pathway as they show that the b_2AR acts via its classic Gs-AC-PKA pathway and not a membrane-delimited or other, non-conventional pathways or Gi (the latter should, of course, inhibit PKA).

Please note that we had already shown that direct stimulation of adenylyl cyclase with forskolin also leads to increased S845 phosphorylation in parallel to the ISO effect (Fig. 3E,F). We now tested the effect of 20 μM SQ22536 on ISO-induced S845 phosphorylation in PFC slices and found that it inhibited this phosphorylation by more than 50%. A respective statement is added to the text.

4. *The peptides DSPL and ESDV, and their inactive analogs, is utilized in a number of experiments throughout the manuscript. However, a number of important controls are required. In addition to demonstrating that these peptides do disrupt the b_2AR/GluR1 complex, the authors should demonstrate whether or not these peptides have any effect on the localization of related and unrelated proteins. For example, does treatment for 2 hours with DSPL lead to a reduction of b_2AR from its synaptic localization? Similarly, does ESDV have a similar effect on GluR1 or other synaptic proteins (NR1, NR2A etc)? This is important considering the fact that these peptides reduce basal EPSC amplitudes.*

Please note that we had already shown in Fig. 5F that DSPL (though not ESDV) has a clear tendency towards reducing surface GluR1 puncta number, possibly reflecting constitutive b_2AR activity (see #5 below). We now analyzed the effect of all four peptides on NR1, PSD-95, and b_2AR localization. None of the parameter we tested indicated any change for any of these 3 proteins (new Suppl. Fig. 5). This negative result for PSD-95 and NMDAR is not surprising as it is consistent with a number of studies over the last 10 years that indicate that PSD-95 has only a very modest role in controlling postsynaptic NMDAR localization (e.g., El-Husseini et al, 2001: Science 290, 1364; Schlueter et al., 2006: Neuron 51, 99). At the same time these results are re-assuring because they indicate that the peptides do not have overt widespread effects. Why does ESDV not more strongly influence GluR1 localization as knock-down of PSD-95 clearly reduces synaptic GluR1 targeting (citations as before)? We don't know the answer but should point out that a fundamental difference between our study and this other work is that the latter used long-term methods to inhibit PSD-95 function (mainly knock-down over several days). Perhaps a deficiency in GluR1 targeting upon impaired interaction with PSD-95 develops only over some extended time periods.

5. *In supplemental figure 2* (now Suppl Fig. 4)*, the authors show that ICI 118551 and DSPL inhibit ISO insertion of surface GluR1. However, does either treatment reduce basal levels of surface GluR1? This could in part explain the reduction in basal EPSC amplitudes seen after incubation with these antagonists/peptides. Furthermore, does incubation with either peptide or ICI 118551 reduce basal level of GluR1 phosphorylation? Fig 3A suggest that the latter may do this. One might expect this to be the case, indicating that b_2AR has some levels of constitutive activity.*

We found in some but not all experiments that ICI118551 reduced basal S845 phosphorylation (Fig. 3A) but the effect did not reach statistical significance (Fig. 3B), all of which is now stated in the text. We did not test the effect of the peptides only on basal S845 phosphorylation due to time limitations (these are very time consuming and difficult experiments). ICI118551 and DSPL (but not ESDV) showed a strong tendency towards reducing GluR1 surface labeling under basal conditions without any ISO treatment, which reached statistical significance when synaptic surface GluR1 puncta frequency was analyzed (new Fig. 5D) but not quite for analysis shown in Fig. 5F and G. This is now stated in the text. We also found that ICI118551 reduced basal EPSC amplitude in PFC by 14.4±2.6%, which is now added to the text.

Taken together most but not all phosphorylation and cell surface labeling experiments indicate some basal effect of ICI118551 and the GluR1-b_2AR disrupting peptides whereas the whole cell patch peptide injection experiments clearly show a reduction of synaptic AMPAR responses. The more distinguished response in the PFC could be due to the fact that it is more heavily innervated by noradrenergic projections and responds stronger to bAR regulatory mechanisms than the hippocampus, the source for our phosphorylation and imaging experiments.

6. *In Figure 6, Joiner et al show that ISO increases AMPAR mEPSCs, however, they do not investigate whether ISO has any effect on mEPSC kinetics. This is important as phosphorylation of GluR1 at S845 increases their open probability. Furthermore, have the authors tested to see if blocking b_2AR-depdent phosphorylation of GluR1 attenuates the increase in AMPAR mEPSCs? The graph in Fig. 6E is somewhat confusing. The authors should show the amplitude before and after*

treatment with ISO separately and not as a % increase. This should also be shown for AMPAR mEPSC frequency.

6.1. We now provide information on mEPSC kinetics ("ISO also significantly increased mEPSC amplitude (Figure 6D,E) but had no effect on mEPSC frequency (Figure 6E), decay time constant (control: 4.7±0.5 ms, n=6; ISO: 4.8±0.32 ms, n=5; wash: 4.7±0.36 ms, n=5), or the 10-90% rise time (control: 2.7±0.05ms, n=6; ISO: 2.7±0.05ms, n=5; wash: 2.8±0.07ms, n=5).").

6.2. We tested whether injection via patch pipette of the PKA-inhibitory PKI peptide affects upregulation of mEPSC (and also EPSC) amplitude in several experiments. Accordingly PKI but not a scrambled control peptide prevents the ISO-triggerd increase in mEPSC (and EPSC) amplitude (new Fig. 6F,G; new Suppl. Fig. 7).

6.3. PKI itself decreases the amplitude of EPSC consistent with the above notion that constitutive b_2AR activity keeps PKA activity and S845 at an elevated level for somewhat enhanced postsynaptic responses under basal condition (new Fig. 6F,G; new Suppl. Fig. 7).

6.4. We now provide bar diagrams that show the actual values for mEPSC amplitude and frequency under control and ISO conditions (revised Fig. 6E)

7. *The initial biochemical characterization of the b_2AR/GluR1 complex is performed in hippocampal neurons, but subsequent electrophysiological studies are performed in PFC slices. Although hippocampal neurons and cortical neurons are very similar, certain differences in their molecular make up can be described. Therefore, the authors should at least demonstrate that b_2AR and GluR1 do form a complex in cortical neurons - ideally, the authors would also show that ISO treatment can lead to GluR1 phosphorylation at S845 in cortical neurons. (See also Reviewer 1, #1).*

7.1 New co-IPs from PFC confirm that GluR1, β_2AR, adenylyl cyclase (AC) and Gs form a complex in PFC (new Suppl. Fig. 1A).

7.2 We treated acute PFC slices with ISO, which resulted in nearly full GluR1 S845 phosphorylation within 2 min and lasted more than 15 min following wash out (new Suppl. Fig. 8).

These results illustrate the existence of a β_2 AR-AC-Gs-GluR1 complex in PFC that regulates S845 phosphorylation.

Minor:

1. *In Fig. 1, the authors state that majority of β_2ARs is synaptic, is there any β_2AR in the shaft?*
Our new immunogold labeling clearly shows that β_2ARs are mainly concentrated at postsynaptic site (new Fig. 1C). However we also see some label in dendritic shaft and neuronal cell bodies and state in the main text now: "Gold particles concentrated over the postsynaptic density close to the postsynaptic membrane. Particles could also be seen within spines, in large dendritic shafts, where they were typically associated with microtubules, and in the cytoplasm of neuronal somata (data not shown)."

2. *It is unclear which antibodies are used in Fig. 2A.*
We now state in the legend and Materials that we used for IP two different antibodies against the β_2AR named H20 and M20 (from Santa Cruz as given in Materials) and for probing an antibody against PSD-95 [produced by myself but] first described in (Sans et al, 2000) as "JH62092" which results in a very specific PSD-95 band on immunoblots from brain extracts.

3. *The blot in Fig 3G is not convincing and should be replaced.*
As we did not have time to perform all requested experiments even with a 3 months extension of the resubmission deadline (in part due to my move to UC Davis in late August) and the Reviewer categorized this request as "minor point" we did not repeat these very difficult and time consuming experiments. We should add that the results had been similar for 3 independent experiments and are completely consistent with all other data.

4. *Fig. 4 is not clearly labeled and maybe somewhat confusing to readers.*
We replaced previous Fig. 4 with a completely new one. The old Fig. 4 is now Suppl. Fig. 3 with improved figure legend, which clarifies now the lay-out of this figure.

5. *In addition to % of GluR1 puncta, the total number of puncta per 10 μm should be graphed in Fig. 5.*
We now show total number of GluR1 puncta per 10 μm (new Fig. 5C).

6. *Do the authors have the number of puncta, or the % change in the puncta number for supplemental Fig. 2 (now supplemental Fig. 4)?*

We added new panel E, which gives the puncta quantification for these panels A-D as surface GluR1 puncta number per 10 μm (new Suppl. Fig. 5D). Results from panels F-K in Suppl. Fig. 5 are given in quantitative form in Fig. 5G.

3rd Editorial Decision 12 October 2009

Thank you for submitting your revised manuscript to the EMBO Journal. The revised version has now been seen by the three original referees and their comments to the authors are provided below. As you can see, the three referees really appreciate the carried out revisions and find that you have addressed the initially raised concerns very well. There are, however, some issues that need to be resolved before publication here. The frst one concerns the reproducibility of the data and referee #1 finds that the n values need to be clearly indicated throughout the manuscript. I also noted that you have a supplemental experimental procedure section, I think that there is room enough to add this section to the materials and methods part of the main manuscript. Finally I also noted that that on some of the gels (Fig 1E and Fig 3A) that it looks like that some the images have been pasted together without proper indication (IgG lane on fig 1E and ICI118551+ lane on fig 3A). I would like to ask you to clearly indicate on the figures (white space on black line) where images have been pasted together. I would also appreciate if you could send me by email the original scans for both those figures. I should point out that this is our standard procedure in such cases. Please also carefully check the other figures.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.

Thank you for the opportunity to consider your work for publication.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors have completed additional experiments and revised the manuscript. Most of my original concenrs have been addressed.

There is still a lack of information on n values in portions of the nmanuscript. For example, how many times were the immunoprecipitaiton experiments shown in Figures 1D-F performed? Likewise the EM shown in Figure 1C? The reader must be sure that results are reproducible. Please ensure that n values are given throughout the manuscript.

Referee #2 (Remarks to the Author):

The authors have correctly answered to my criticism

Referee #3 (Remarks to the Author):

The authors have completely satisfied all of my points with some excellent work. I very much look forward to seeing this article published.

2nd Revision - authors' response   22 October 2009

Thank you so much for the positive feedback on our manuscript entitled "A β_2 Adrenergic Receptor - AMPA Receptor Signaling Complex" by Joiner et al. We revised the manuscript as follows.

1. *n values need to be clearly indicated throughout the manuscript*

We added n values to all sub-figure legends to cover all data shown in figures (see red high lights). For Fig. 3 and 5 *n* is given inside bars and number of independent experiments stated in legend. Quantitative data that are mentioned specifically in the text received n values in the main text (see red high lights).

2. *Add Supplemental Exp. Procedures to main text*. This is not possible without exceeding the 55,000 character count (currently 54,912 including spaces but without references). Also, the Supplemental Procedures apply only to Suppl. Fig. 6 and no Fig. in the main manuscript.

3. *Fig. 1E and 3A look like they have been pasted together*

As I moved my lab from U Iowa to UC Davis ~ 6 weeks ago, we cannot locate the original blot for Fig. 1E. However, we have other comparable experiments and substituted 1E with one of those without any pasting. To illustrate the original data for that blot we attach the file "Fig. 1E."

The original blots for Fig. 3A (see "Fig. 3A") contained 2 additional lanes that were not relevant for this MS. We used same exposures for pasting. We added a line and a corresponding statement to the legend.

I think these actions will satisfy your concerns but if you have any further questions please do not hesitate to contact me. Also I would like to express my appreciation that EMBO J is publishing neuroscience articles as it provides a great venue for publishing work 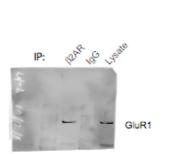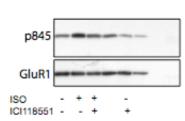with strong emphasis on molecular mechanisms.