THE EMBO JOURNAL

Manuscript EMBO-2012-82900

PKCλ Is Critical in AMPA Receptor Phosphorylation and Synaptic Incorporation during LTP

Si-Qiang Ren, Jing-Zhi Yan, Xiao-Yan Zhang, Yun-Fei Bu, Wei-Wei Pan, Wen Yao, Tian Tian and Wei Lu

Corresponding author: Wei Lu, Nanjing Medical University

Review timeline:

Submission date:	07 August 2012
Editorial Decision:	17 September 2012
Revision received:	15 January 2013
Acceptance Letter:	13 February 2013
Accepted:	13 February 2013

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.)

Editor: Karin Dumstrei

1st Editorial Decision 17 September 2012

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 the referees find the analysis interesting. However they also indicate that some of the findings are too preliminary and that further analysis would be needed to substantiate the conclusions. Should you be able to address the raised concerns in full then I would like to invite you to submit a revised version of the manuscript. I should add that it is EMBO Journal policy to allow only a single major round of revision, and that it is therefore important to address the raised concerns at this stage.

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, 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.

REFEREE REPORTS

Referee #1

The authors provide evidence that PKC lambda is necessary for standard LTP in CA1. They find that injection of active PKC lambda or stimulation of its upstream regulator PI3K results in an increase in postsynaptic responses. They also show results that suggest that the atypical PKC - targeting protein p62 is necessary for LTP.

Major Concerns

1. The use of 0.5 vs 2 microM of the PKM zeta-derived ZIP peptide, which the authors call Myr-aPKC-PS, does not allow to unequivocally differentiate between inhibition of lambda vs. zeta. One reason is that the difference of these concentrations is too small to use that as a clear cut tool in slices, where drug access is usually reduced, typically requiring higher concentrations than in vitro for drug effects. Accordingly, IC50 values from in vitro experiments are not immediately applicable. A second reason is that ZIP / Myr-aPKC-PS titrations on PKC lambda vs. PKM zeta phospho-transfer activity in Suppl. Fig. 2 is not convincing (see below #2). It is thus imperative that the authors repeat certain key experiments performing lambda knock down and perform in parallel a rescue experiment to exclude off target effects. This should include experiments shown in Fig. 1C, either Fig. 2E or F, and either Fig. 3C/D or Fig. 3E/F. The authors should test thoroughly whether lambda KD affects basal synaptic transmission (PPF, AMPAR-EPSC or mEPSC, NMDAR-EPSC or AMPAR/NMDAR ratio if AMPAR mEPSC is unaltered). It would also be nice to see that lambda knock down occludes selected Myr-aPKC-PS effects. Also, to increase confidence in the lambda knock down shRNA it would be re-assuring to see that this shRNA does in fact knock down lambda (possibly in recombinant form) in a system other than the brain - e.g., lambda ectopically expressed in HEK293 cells. The latter would also help to lay to rest the concern that the lambda antibody used to monitor knock down of lambda in the hippocampus (and to IP lambda for ZIP / Myr-aPKC-PS titration in Suppl Fig. 2) might not be specific for lambda, which is otherwise a further issue the authors would have to carefully address.

2. Data in Suppl. Fig. 2 do not convincingly show that 0.5 microM selectively inhibit PKM zeta but not lambda whereas 2 microM inhibit both isoforms. This is in part because the authors use IP of PKC isoforms for their in vitro kinase assays with either a supposedly lambda specific antibody (but see above) or an antibody that recognizes PKC lambda and PKM zeta, and, not to be overlooked, PKC zeta. The authors argue that in the absence of the lambda activating PIP3 they should only see PKM zeta activity. It is not clear why PIP3 would activate purified PKC lambda as I believe the main mechanism of PIP3 is to recruit and activate PDK, which then in turn phosphorylates and activates PKC lambda and also PKC zeta (although the truncated PKM zeta isoform is constitutive active). In any case, the assay should be repeated with truly pure PKC lambda, PKC zeta (which likely is also affected by ZIP although PKC zeta is largely ignored in this MS) and PKM zeta. This is the more important as Wu-Zhang et al (2012) report that ZIP does not inhibit PKM zeta inside cells, a finding the authors need to address and discuss (JBC 287, 12879-85).

3. p62, also named ZIP (not to be confused with the above ZIP/ Myr-aPKC-PS peptide), can act as a targeting protein for atypical PKC isoforms. The authors find that PI3K stimulation increases in parallel to S818 phosphorylation also the association of GluA1 with p62 and PKC lambda. Knock down of p62 as well as peptides that appear to interfere with p62 binding to GluA1 or to PKC lambda prevent LTP. As for the PKC lambda knock down experiments, it is necessary to perform knock down and rescue experiment in parallel experiments. The peptides are not very convincing as they reduce coIPs only marginally or modestly. They would have to be better characterized as well. Also, for the co-IP experiments non-solubilized material needs to be removed by ultracentrifugation- otherwise any coIP could reflect colocalization in non-solubilized membrane fragments rather than a true specific direct or indirect interaction.

Modest Concern

The authors argue that the fact that the PI3K activating 740Y-P increases pThr563 of PKC lambda but does not change total amount of then constitutively active PKM zeta means that it acts mainly

via the former. They ignore that it could act via PKC zeta or by causing redistribution of PKM zeta without altering total amount of PKM zeta. They should monitor activation status of PKC zeta especially if it can be done with a phospho-specific antibody analogous to what they used for PKC lambda.

Minor Concerns

1. Figure legends should make it clear when and how treatments occurred-for instance, that 740Y-P was injected via the patch pipette as it seems to be the case based on the Supplemental material.

2. Claiming that 740Y-P and PI3K injections induce LTP should be substantiated by showing in some sort of wash out experiments that the potentiation is permanent and lasting at least 1 hr after removal of the original stimulus-if that cannot be done the authors should not refer to these potentiations as LTP

3. The authors prepare a Triton X-100 - insoluble fraction with the assumption that proteins from the postsynaptic density (PSD) including AMPAR would be enriched. This is reasonable (although the enrichment will be much lower than for truly purified PSDs) but using tubulin as loading control is not very useful because tubulin is not part of the PSD (if it is present in some preparations it is considered a contamination by many PIs). It would make much more sense to use PSD-95 as loading control for normalization of GluA1 content. However, this is a minor issue because the authors also monitor GluA1 localization and mEPSC in cultured neurons.

4. The authors use a peptide derived from the PKC phosphorylation site S818 on GluA1 to further test the role of PKC lambda. They find that the Ser 818 but not the Ala 818, in which Ser is replaced with Ala, prevents the 740Y-P - induced potentiation. However, whether this peptide affects specifically PKC lambda or PKM zeta, PKC zeta, or even other PKC isoforms and whether it specifically prevents S818 phosphorylation or acts as a general PKC inhibitor is unclear. Also, substituting a phosphorylated Ser with Ala does not necessarily change the efficacy of a peptide to block PKC activity or targeting (and the Ala peptideshould not be called "invalid"). The authors should test with pure PKC zeta, PKC lambda and PKM zeta and perhaps other isoforms whether these two peptides affect activity of PKC lambda and zeta and of classic and novel PKC isoforms and whether it specifically coutneracts S818 phosphorylation (S831, another prominent PKC site in GluA1 could act as control for the latter).

Suppl Fig. 6 somewhat indicates that the phosphor-S818 antibody is specific for phosphorylated GluA1 (although the blot is not overly convincing and should be repeated and quantified) but it does not show pSer818-the phosphor-Ser 818 peptide could block binding of this antibody to other phosphorylated sites including S831. That should be specifically evaluated.

The above #4 issues are listed under minor concerns because the authors could take the S818 peptide data out as their paper is already very extensive and work out details of these peptides for another paper. However, if the authors want to present results with these peptides they need to thoroughly address these issues.

Referee #2

In this manuscript Ren et al. showed that PKCλ acts downstream of phosphatidylinositol 3-kinase (PI3K) and is critical in AMPAR phosphorylation and subsequent incorporation into postsynaptic site during LTP expression. In addition, they showed that P62 forms trimeric complex with both AMPAR and PKCλ and facilitates AMPAR phosphorylation by PKCλ.
Based on these data, the authors suggest that PKCλ is the critical signaling molecule responsible for GluA1-containing AMPAR phosphorylation and synaptic incorporation at activated synapses during LTP expression.

Whereas some of their findings are of potential interest, they are in preliminary stages, and several of the conclusions drawn are not fully substantiated by the data presented. Also, important controls are missing and there are some technical concerns (see below).
Specific points:

(1): The authors should include in Figure 1 as control the results with PKCλ-shRNA scrambled. Moreover rescue experiments should be included.

(2) The neurons of Figure 3C should be replaced by a more convincing images. The data is not very convincing, especially the surface staining of GluA1! These data should be complemented by the standard surface biotinylation assay. Also the authors should be analyze the surface level of GluA2.

(3) The authors should be included the inputs in the IPs and IgGs (as negative control for the IPs).

(4) Again, the surface staining of GluA1 should be replaced by a more convincing images. The quality of the images is very poor.

(5) The discussion is rather poorly written and contains a number of over-interpretations.

Referee #3

The manuscript "PKC lambda is critical in AMPA receptor phosphorylation and synaptic incorporation during LTP" sheds new light on the intracellular pathways involved in the regulation of synaptic plasticity. The authors show in this study that PKC lambda activation through PI3Kinase is necessary for early phase LTP. Furthermore, the authors could show that PKC lambda increases phosphorylation of GluA1 at Ser818, leading to an increase in synaptic GluA1 content. Finally, they could demonstrate that p62 is serving as an adaptor protein for PKC lambda and GluA1 in the LTP signaling cascade. The study uses various peptide constructs in a clever way to elucidate the role and interplay of different signaling molecules necessary for LTP maintenance.

Major criticism:

1.Figure 1c: The authors show that 2 weeks PKC lambda-shRNA expression inhibits LTP. It is necessary to know whether the expression of PKC lambda-shRNA over two weeks is changing AMPA- and NMDA-receptor dependent baseline transmission, potentially leading to impaired LTP induction?

2.The experiment leading to figure 2a and b needs a better description. What kind of cells were used (dissociated neurons or slice cultures)? Was the incubation with 740Y-P permanent or transient? If transient, how long before the samples were collected?

3.Figure 3c and 7c: The authors state that they stain for surface GluA1. However, they use a methanol fixation protocol, which to my knowledge does not allow differentiating between intracellular and extracellular epitopes (methanol fixes and permeabilizes cells). The authors should repeat this experiment under proper conditions, i.e. using non-permeabilized conditions like for example incubating dissociated neurons with GluA1 antibody before fixation, e.g. Passafaro et al, Nat. Neurosci. 2001 Sept; 4(9) page 917.

4.In the context of figure 3e and f, the authors state that "PI3K activation by 740Y-P treatment induced enhancement in both mEPSC amplitude and frequency" and that this result provides "compelling evidence that AMPAR incorporation upon PI3K activation was achieved". Given that AMPAR incorporation is a postsynaptic event, the increase in miniature frequency cannot be seen as "compelling evidence", since changes in frequency could hint to a presynaptic effect. In order to tighten their arguments, the authors should at least perform paired-pulse facilitation to show that the effect is postsynaptic and the increase in frequency maybe due to an un-silencing of synapses.

5.The authors show that phosphorylation of S818 in GluA1 is necessary for LTP and that this phosphorylation could be achieved through activation of PKC lambda. However, it would be interesting to know whether this phosphorylation through PKC lambda is also sufficient for LTP induction (i.e. PKC lambda has no targets other than GluA1 for inducing LTP). This could be achieved by inducing LTP in the presence of PKC lambda (or PI3K) inhibitors in cells that express a GluA1-S818 phosphomimetic mutation.

Minor criticism:

1.The manuscript contains several grammatical errors

2.In the introduction:
- The term "aPKC" should be explained as "atypical PKC".
- The sentence mentioning aPKC for the first time (second paragraph ninth line) lists twice PKC zeta as a member of the aPKC group.
- The protein p62 should be introduced with a few words (adaptor protein etc.).

3.The western blot images appear hazy

4.In the context of some of their 740Y-P experiments, the authors write that they have induced a "chemical form" of LTP. This is somewhat misleading since 740Y-P is applied through the recording pipette and therefore permanently present. It is perhaps better to claim an "increase in synaptic transmission" than to have induced LTP (which would be a transient manipulation leading to permanent increase in synaptic transmission).

1st Revision - authors' response 15 January 2013

Reply to Reviewers:

Referee #1

The authors provide evidence that PKC lambda is necessary for standard LTP in CA1. They find that injection of active PKC lambda or stimulation of its upstream regulator PI3K results in an increase in postsynaptic responses. They also show results that suggest that the atypical PKC - targeting protein p62 is necessary for LTP.

Major Concerns

1. The use of 0.5 vs 2 microM of the PKM zeta-derived ZIP peptide, which the authors call Myr-aPKC-PS, does not allow to unequivocally differentiate between inhibition of lambda vs. zeta. One reason is that the difference of these concentrations is too small to use that as a clear cut tool in slices, where drug access is usually reduced, typically requiring higher concentrations than in vitro for drug effects. Accordingly, IC50 values from in vitro experiments are not immediately applicable. A second reason is that ZIP/Myr-aPKC-PS titrations on PKC lambda vs. PKM zeta phospho-transfer activity in Suppl. Fig. 2 is not convincing (see below #2). It is thus imperative that the authors repeat certain key experiments performing lambda knock down and perform in parallel a rescue experiment to exclude off target effects. This should include experiments shown in Fig. 1C, either Fig. 2E or F, and either Fig. 3C/D or Fig. 3E/F. The authors should test thoroughly whether lambda KD affects basal synaptic transmission (PPF, AMPAR-EPSC or mEPSC, NMDAR-EPSC or AMPAR/NMDAR ratio if AMPAR mEPSC is unaltered). It would also be nice to see that lambda knock down occludes selected Myr-aPKC-PS effects. Also, to increase confidence in the lambda knock down shRNA it would be re-assuring to see that this shRNA does in fact knock down lambda (possibly in recombinant form) in a system other than the brain - e.g., lambda ectopically expressed in HEK293 cells. The latter would also help to lay to rest the concern that the lambda antibody used to monitor knock down of lambda in the hippocampus (and to IP lambda for ZIP / Myr-aPKC-PS titration in Suppl Fig. 2) might not be specific for lambda, which is otherwise a further issue the authors would have to carefully address.
Answer: Thanks for your very nice suggestion. This time we repeat key experiments by performing PKC lambda knock down experiment and a rescue experiment in parallel to exclude off target effects (please see Figures 1A-1C, Figures 2E, Figures 3G-3H and Supplementary Figure S2). We packaged rAAV2/1-mCherry-mt-PKCλ which has silent mutations resistant to RNA knockdown by PKCλ-shRNA and injected this virus with rAAV2/1-EGFP-PKCλ-shRNA simultaneously into hippocampus. We found that PKC lambda rescue indeed reverses the changes in LTP and AMPAR incorporation caused by PKC lambda. In addition, the hippocampal pyramidal cells infected with rAAV2/1-EGFP-scrambled-PKCλ-shRNA can express normal LTP. We also test the possible effect of PKC lambda KD on basal synaptic transmission (PPF, AMPAR-EPSC and NMDAR-EPSC). We

fail to detect any significant effect by PKC lambda KD on these currents and thus exclude its contribution to impaired LTP induction (please see Supplementary Figure S3). Moreover, our new results indicate that PKC lambda knock down occludes selected Myr-aPKC-PS effects (Supplementary Figure S5). Finally, to increase confidence in the lambda knock down shRNA, we repeat knock down and rescue experiment in a system other than the brain - e.g., lambda ectopically expressed in HEK293T cells and confirm that this shRNA does in fact knock down lambda (Supplementary Figure S2), as you suggest here. Taken together, these new results provide further support to the notion that the effects by the PKC lambda knock down are indeed caused by decrease of PKC lambda function.

2. Data in Suppl. Fig. 2 do not convincingly show that 0.5 microM selectively inhibit PKM zeta but not lambda whereas 2 microM inhibit both isoforms. This is in part because the authors use IP of PKC isoforms for their in vitro kinase assays with either a supposedly lambda specific antibody (but see above) or an antibody that recognizes PKC lambda and PKM zeta, and, not to be overlooked, PKC zeta. The authors argue that in the absence of the lambda activating PIP3 they should only see PKM zeta activity. It is not clear why PIP3 would activate purified PKC lambda as I believe the main mechanism of PIP3 is to recruit and activate PDK, which then in turn phosphorylates and activates PKC lambda and also PKC zeta (although the truncated PKM zeta isoform is constitutive active). In any case, the assay should be repeated with truly pure PKC lambda, PKC zeta (which likely is also affected by ZIP although PKC zeta is largely ignored in this MS) and PKM zeta. This is the more important as Wu-Zhang et al (2012) report that ZIP does not inhibit PKM zeta inside cells, a finding the authors need to address and discuss (JBC 287, 12879-85).
Answer: Thanks for your nice suggestion. This time we repeat the in vitro assay of kinase activity with truly pure PKCλ/ι and PKMζ and obtain results that is consistent to previous data performed with antibodies (Supplementary Figure S4). Because only PKCλ/ι and PKMζ are expressed in the hippocampus, and there are no PKCζ mRNA and protein existing in hippocampus and most cortex region (Hernandez *et al*, 2003; Oster *et al*, 2004), we ignore PKCζ in this study. We also address and discuss Wu-Zhang's (JBC 2012) finding that ZIP does not inhibit PKM zeta inside cells in Discussion section, as you suggest here.

3. p62, also named ZIP (not to be confused with the above ZIP/ Myr-aPKC-PS peptide), can act as a targeting protein for atypical PKC isoforms. The authors find that PI3K stimulation increases in parallel to S818 phosphorylation also the association of GluA1 with p62 and PKC lambda. Knock down of p62 as well as peptides that appear to interfere with p62 binding to GluA1 or to PKC lambda prevent LTP. As for the PKC lambda knock down experiments, it is necessary to perform knock down and rescue experiment in parallel experiments. The peptides are not very convincing as they reduce coIPs only marginally or modestly. They would have to be better characterized as well. Also, for the co-IP experiments non-solubilized material needs to be removed by ultracentrifugation- otherwise any coIP could reflect colocalization in non-solubilized membrane fragments rather than a true specific direct or indirect interaction.
Answer: Thanks for your nice suggestion. This time we perform p62 knock down and rescue experiment in parallel to further confirm that the effects we observed in the association of GluA1 with p62 and PKC lambda are indeed caused by decrease of p62 function (please see Figure 4G, 4I and Supplementary Figure S2).We also repeat the co-IP experiments and obtain results that help to confirm the peptide's effect (please see Figure 4A, 4C and 4E). In addition, in order to reflect a true specific direct or indirect interaction between p62 and other proteins, this time we use ultracentrifugation to remove non-solubilized material (please see Supplementary Figure S14) and repeat those Co-IP experiments, as you suggest here.

Modest Concern

The authors argue that the fact that the PI3K activating 740Y-P increases pThr563 of PKC lambda but does not change total amount of then constitutively active PKM zeta means that it acts mainly via the former. They ignore that it could act via PKC zeta or by causing redistribution of PKM zeta without altering total amount of PKM zeta. They should monitor activation status of PKC zeta especially if it can be done with a phospho-specific antibody analogous to what they used for PKC lambda.
Answer: Only PKCλ/ι and PKMζ are expressed in the hippocampus, and there are no PKCζ mRNA and protein existing in hippocampus and in most cortex region (Hernandez *et al*, 2003; Oster *et al*, 2004). That's why we do not put emphasis on PKC zeta. To test whether PKMζ can redistribute

after PI3K activator 740Y-P treatment, we detect the amount of PKMζ in the postsynaptic density (Triton-insoluble fraction), we found that there are no change of PKMζ in PSD after 740Y-P treatment (Figure 2C). Furthermore, we also transfected EGFP-labeled PKMζ into primary hippocampal pyramidal neuron, and we found there are no change in the EGFP fluorescence ratio between spine and dendritic shaft before and after 740Y-P treatment (Supplementary Figure S8). These data help us to exclude the possible PKM zeta redistribution caused by PI3K activation.

Minor Concerns

1. Figure legends should make it clear when and how treatments occurred-for instance, that 740Y-P was injected via the patch pipette as it seems to be the case based on the Supplemental material.
Answer: Sorry for the confusion. This time we clarify when and how treatments occurred in the figure legends. For example, we emphasize that 740Y-P is applied via the patch pipette (see figure legends in Figure 2)

2. Claiming that 740Y-P and PI3K injections induce LTP should be substantiated by showing in some sort of wash out experiments that the potentiation is permanent and lasting at least 1 hr after removal of the original stimulus-if that cannot be done the authors should not refer to these potentiations as LTP
Answer: Thanks for your nice suggestion. This time we perform new experiment showing that after 10 min 740Y-P application and wash out, the potentiation can last for more than 40 min after wash out (Supplementary Figure S9). Because these experiments are performed with whole-cell patch-clamp recording, the 1 hour recording time for the cell is a standard duration for detecting LTP.

3. The authors prepare a Triton X-100 - insoluble fraction with the assumption that proteins from the postsynaptic density (PSD) including AMPAR would be enriched. This is reasonable (although the enrichment will be much lower than for truly purified PSDs) but using tubulin as loading control is not very useful because tubulin is not part of the PSD (if it is present in some preparations it is considered a contamination by many PIs). It would make much more sense to use PSD-95 as loading control for normalization of GluA1 content. However, this is a minor issue because the authors also monitor GluA1 localization and mEPSC in cultured neurons.
Answer: Thanks for your nice suggestion. This time we perform new experiments by using PSD95 as loading control for normalization of GluA1 content (please see Figure 3A and Figure 6A).

4. The authors use a peptide derived from the PKC phosphorylation site S818 on GluA1 to further test the role of PKC lambda. They find that the Ser 818 but not the Ala 818, in which Ser is replaced with Ala, prevents the 740Y-P - induced potentiation. However, whether this peptide affects specifically PKC lambda or PKM zeta, PKC zeta, or even other PKC isoforms and whether it specifically prevents S818 phosphorylation or acts as a general PKC inhibitor is unclear. Also, substituting a phosphorylated Ser with Ala does not necessarily change the efficacy of a peptide to block PKC activity or targeting (and the Ala peptideshould not be called "invalid"). The authors should test with pure PKC zeta, PKC lambda and PKM zeta and perhaps other isoforms whether these two peptides affect activity of PKC lambda and zeta and of classic and novel PKC isoforms and whether it specifically coutneracts S818 phosphorylation (S831, another prominent PKC site in GluA1 could act as control for the latter).

Suppl Fig. 6 somewhat indicates that the phosphor-S818 antibody is specific for phosphorylated GluA1 (although the blot is not overly convincing and should be repeated and quantified) but it does not show pSer818-the phosphor-Ser 818 peptide could block binding of this antibody to other phosphorylated sites including S831. That should be specifically evaluated.

The above #4 issues are listed under minor concerns because the authors could take the S818 peptide data out as their paper is already very extensive and work out details of these peptides for another paper. However, if the authors want to present results with these peptides they need to thoroughly address these issues.
Answer: Thanks for your nice suggestion. We would like to take the S818 peptide data out as this paper and work out more details of these peptides (for example, the questions you point out here) for another paper, as you suggest here. Nevertheless, we test phosphorylation levels at GluA1 Ser818 and Ser831 sites under various treatments including 740-YP treatment and find that only phosphorylation at GluA1 Ser818, but not at Ser831 sites, changes under this treatment

(Supplementary Figure S11).

Referee #2

In this manuscript Ren et al. showed that PKCλ acts downstream of phosphatidylinositol 3-kinase (PI3K) and is critical in AMPAR phosphorylation and subsequent incorporation into postsynaptic site during LTP expression. In addition, they showed that P62 forms trimeric complex with both AMPAR and PKCλ and facilitates AMPAR phosphorylation by PKCλ. Based on these data, the authors suggest that PKCλ is the critical signaling molecule responsible for GluA1-containing AMPAR phosphorylation and synaptic incorporation at activated synapses during LTP expression.

Whereas some of their findings are of potential interest, they are in preliminary stages, and several of the conclusions drawn are not fully substantiated by the data presented. Also, important controls are missing and there are some technical concerns (see below).

Specific points:
(1) The authors should include in Figure 1 as control the results with PKCλ-shRNA scrambled. Moreover rescue experiments should be included.
Answer: Thanks for your very nice suggestion. This time we perform new experiments to show results with PKCλ-shRNA scrambled (Please see Figure 1C). In addition, we repeat key experiments by performing PKC lambda knock down experiments and rescue experiments in parallel to exclude off target effects (please see Figures 1A-1C, Figures 2E-2F, Figures 3G-3H and Supplementary Figure S2 for PKCλ rescue data).

(2) The neurons of Figure 3C should be replaced by a more convincing images. The data is not very convincing, especially the surface staining of GluA1! These data should be complemented by the standard surface biotinylation assay. Also the authors should be analyze the surface level of GluA2.
Answer: Thanks for your very nice suggestion. This time we perform new experiments to repeat the results in Fig. 3C and now provide more convincing images (Please see Figure 3C). These data is complemented by the new data on standard surface biotinylation assay (Figures 4E and 4F). In addition, we also analyze the surface level of GluA2 (Please see Supplementary Figure S10), as you suggest here.

(3) The authors should be included the inputs in the IPs and IgGs (as negative control for the IPs).
Answer: Thanks for your nice suggestion. This time we add inputs and IgGs in the co-IPs (Please see Figure 4 and Figure 5).

(4) Again, the surface staining of GluA1 should be replaced by a more convincing images. The quality of the images is very poor.
Answer: Thanks for your nice suggestion. This time we perform new experiments to show surface staining of GluA1 with images with better quality (Please see Figure 6).

(5) The discussion is rather poorly written and contains a number of over-interpretations.
Answer: Thanks for your nice suggestion. This time we rewrite the discussion and delete some unnecessary over-interpretations (Please see Discussion section).

Referee #3

The manuscript "PKC lambda is critical in AMPA receptor phosphorylation and synaptic incorporation during LTP" sheds new light on the intracellular pathways involved in the regulation of synaptic plasticity. The authors show in this study that PKC lambda activation through PI3Kinase is necessary for early phase LTP. Furthermore, the authors could show that PKC lambda increases phosphorylation of GluA1 at Ser818, leading to an increase in synaptic GluA1 content. Finally, they could demonstrate that p62 is serving as an adaptor protein for PKC lambda and GluA1 in the LTP signaling cascade. The study uses various peptide constructs in a clever way to elucidate the role and interplay of different signaling molecules necessary for LTP maintenance.

Major criticism:

1.Figure 1c: The authors show that 2 weeks PKC lambda-shRNA expression inhibits LTP. It is necessary to know whether the expression of PKC lambda-shRNA over two weeks is changing AMPA- and NMDA-receptor dependent baseline transmission, potentially leading to impaired LTP induction?
Answer: Thanks for your nice suggestion. This time we perform new experiments to test the possible effect of PKC lambda KD (e.g., the expression of PKC lambda-shRNA over two weeks) on basal synaptic transmission (PPF, AMPAR-EPSC and NMDAR-EPSC). We fail to detect any significant effect by PKC lambda KD and thus exclude its contribution to impaired LTP induction (Please see Supplementary Figure S3).

2.The experiment leading to figure 2a and b needs a better description. What kind of cells were used (dissociated neurons or slice cultures)? Was the incubation with 740Y-P permanent or transient? If transient, how long before the samples were collected?
Answer: Thanks for your nice suggestion. This time we describe the experiment process in figure 2a and 2b in more detail. The western blot assay was performed on acute hippocampal slices. The slices were incubated with 740Y-P for 20 min before they were collected.

3.Figure 3c and 7c: The authors state that they stain for surface GluA1. However, they use a methanol fixation protocol, which to my knowledge does not allow differentiating between intracellular and extracellular epitopes (methanol fixes and permeabilizes cells). The authors should repeat this experiment under proper conditions, i.e. using non-permeabilized conditions like for example incubating dissociated neurons with GluA1 antibody before fixation, e.g. Passafaro et al, Nat. Neurosci. 2001 Sept; 4(9) page 917.
Answer: Thanks for your nice suggestion. This time we used paraformaldehyde fixation protocol to repeat the experiments in Figure 3C and 7C (now Figure 6C). We first treat cultured neurons with different drugs, and then fixed cells with 4% paraformaldehyde. For labeling of surface GluA1, neurons were then incubated with an N-terminal antibody (1:50) for 12 hrs and a Cy5-conjugated anti-mouse secondary antibody (1:200) for 2 hrs. Cells were then labeled with a synaptophysin antibody (1:100) for 10 hrs and TRITC-conjugated anti-rabbit secondary antibody (1:200) for 2 hrs with 0.1% Triton X-100 in PBS (please see new data in Figure 3C and Figure 6C).

4.In the context of figure 3e and f, the authors state that "PI3K activation by 740Y-P treatment induced enhancement in both mEPSC amplitude and frequency" and that this result provides "compelling evidence that AMPAR incorporation upon PI3K activation was achieved". Given that AMPAR incorporation is a postsynaptic event, the increase in miniature frequency cannot be seen as "compelling evidence", since changes in frequency could hint to a presynaptic effect. In order to tighten their arguments, the authors should at least perform paired-pulse facilitation to show that the effect is postsynaptic and the increase in frequency maybe due to an un-silencing of synapses.
Answer: Thanks for your nice suggestion. This time we perform paired-pulse facilitation experiment to clarify whether the change in frequency is due to pre- or post-synaptic effect. We find that 740Y-P treatment does not display any effect on paired-pulse facilitation (Please see Supplementary Figure S13), suggesting the increase in frequency is more likely due to an un-silencing of synapses.

5.The authors show that phosphorylation of S818 in GluA1 is necessary for LTP and that this phosphorylation could be achieved through activation of PKC lambda. However, it would be interesting to know whether this phosphorylation through PKC lambda is also sufficient for LTP induction (i.e. PKC lambda has no targets other than GluA1 for inducing LTP). This could be achieved by inducing LTP in the presence of PKC lambda (or PI3K) inhibitors in cells that express a GluA1-S818 phosphomimetic mutation.
Answer: Thanks for your nice suggestion. We are very sorry we don't do this experiment. One of the other reviewers suggest to "take the S818 peptide data out as their paper is already very extensive and work out details of these peptides for another paper". Although our preliminary data suggest S818 phosphorylation is important for LTP, we clearly need much more data to establish the causal link between PKC lambda activation and S818 phosphorylation. These experiments can not be totally finished in 3 months. So we decide to follow that reviewer's suggestion and take most of data about S818 phosphorylation data out. We plan to further study the role of S818 phosphorylation by PKC lambda in LTP in the next paper. We are very sorry for not well addressing your question.

Minor criticism:

1.The manuscript contains several grammatical errors
Answer: Sorry for the grammatical errors. This time we carefully go through the whole manuscript and correct those grammatical errors.

2.In the introduction:
- The term "aPKC" should be explained as "atypical PKC".
Answer: Thanks. We have made the change.

- The sentence mentioning aPKC for the first time (second paragraph ninth line) lists twice PKC zeta as a member of the aPKC group.
Answer: Thanks. We have made the change.

- The protein p62 should be introduced with a few words (adaptor protein etc.).
Answer: Thanks. The protein p62 is now introduced in the Result section.

3.The western blot images appear hazy.
Answer: Sorry for the hazy images. This time we repeat all these western blot experiments and provide new images with better quality (Please see Figures 1-3 and Figure 6).

4.In the context of some of their 740Y-P experiments, the authors write that they have induced a "chemical form" of LTP. This is somewhat misleading since 740Y-P is applied through the recording pipette and therefore permanently present. It is perhaps better to claim an "increase in synaptic transmission" than to have induced LTP (which would be a transient manipulation leading to permanent increase in synaptic transmission).
Answer: Thanks. It's true that 740Y-P induced changes in EPSCs can be better described as "increase in synaptic transmission" than to have induced LTP, because 740Y-P is applied through the recording pipette and therefore permanently present. This time we have made the correction. In addition, we also perform new experiment by perfusing slices with 740Y-P (mM) for 10 minutes and find that increase in EPSCs can still be induced and persist for at least 40 min (Please see Supplementary Figure S9). Under this condition, "increase in synaptic transmission" may also be termed as LTP.

Acceptance Letter 13 February 2013

Thank you for submitting your revised manuscript to The EMBO Journal. Your revision has now been re-reviewed by referees 1 and 2. As you can see below, both referees appreciate the introduced changes and support publication here. I am therefore very pleased to accept the paper for publication here.

A few remaining comments:

- Referee # 3 points out that there are some typos in the text. Please go through the text one more type and correct any typos.

- I would like to suggest that you remove all the statistical information from the result section and add that to the corresponding figure legend. I think that would improve the readability of the result section. Let me know if you wish to discuss this.

- Lastly, we also now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this just contact me.

You can send the source data and the modified MS to this email address and we will upload it for

you.

Please also see below for important information on how to proceed. Make sure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

Thank you for contributing to the EMBO Journal

REFEREE REPORTS

Referee #1

The authors have thoroughly and carefully addressed the Critiques and the results are very convincing. The implication of PKClambda in LTP is strong given that the authors used both, pharmacological inhibition as well as knock down. The evidence implicating p62 in docking PKClambda at the AMPA receptor in LTP both further increases confidence in the role of PKC lambda in LTP and expands the molecular mechanisms the authors unravel in this manuscript.

Taking the S818 peptide data out as suggested by Reviewer 1 is appropriate.

Referee #2:

The manuscript by Ren et al. improved significantly. The authors have addressed my concerns with the appropriate experiments and changes in the manuscript. Furthermore, the most recent findings concerning the specificity of the ZIP inhibitor (Lee et al. and Volk et al, Nature Vol. 493) are discussed.
Unfortunately the manuscript contains again many typographical errors (e.g. on page 10: "Wortammin" instead of Wortmannin).

