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CaMKII-dependent phosphorylation of GluK5 mediates plasticity of kainate receptors

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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 14 September 2012

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

As you can see below, both referees find the analysis interesting and suitable for publication in The EMBO Journal. I will discuss in more detail below the referees comments, but given the comments provided I would like to invite you to submit a suitably revised manuscript. Referee #1 raise relative minor concerns and I suspect that you should be able to resolve them fairly easily. However, referee #2 raises more significant ones. The first major point concerns the LTP recording in figure 1 and 2, referee #2 finds that the recordings are off too low quality and that an improved analysis is needed. The referee has provided us with two files to illustrate this point, which I have attached. I would like you to address this issue in the revised version. In major point 2, referee #2 asks for further data on the function of the different phosphorylation sites. I see that this would add to the paper, but also recognize that this issue is not straightforward to address and that it involves a large amount of work. If you have any data on hand to address this issue, it would be good to incorporate it, but it is not needed for publication here. The last major issue raised by referee #2 concerns the need for some more data on endogenous proteins. As for point #2, I see that this would add to the paper, but also considering the data already in the manuscript to support the findings this is not a must for publication here. So as for point 2, if you have data on hand to address this issue then please include it, if not then we will go ahead with the paper without it. Please address the minor issues raised by this referee as well.

I should also add that I have discussed these points with referee #1.

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.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The authors describe a CamKII-dependent depression of kainate receptor synaptic currents at mossy fiber-CA3 synapses. Interestingly, this reduction in synaptic strength appears to rely on altered stability in the postsynaptic density. A number of mechanistic studies complement the central finding, and a sophisticated molecular replacement strategy reconstitutes phosphomutant GluK5 receptors in knockout mice to test several predictions. In all it is a very strong study with a range of techniques mobilized to address the impact of CamKII on GluK2-containing kainate receptors in vitro and in vivo. The conclusion that synaptic untrapping underlies timing-dependent plasticity of synaptic kainate receptors is a novel one that will be of interest to many in the neuroscience community.

I have minor criticisms on a few points in the study.

Minor criticisms

1. Figure 1c: There is some mismatch between the traces shown and the PPR data in the graph below. The PPR values appears to be over 3, but the amplitude of the second EPSC seems to be <2-fold larger than the first in the traces. How was the PPR calculated in these experiments? Also, KAR-EPSCs have been reported to show significant run-down in amplitude in analogous experiments, which might introduce some confounding interpretation into the data shown in Figure 1. Did the authors compare the time course of EPSC/P amplitudes with and without the train stimulation to ensure that the depression was due to induced plasticity rather than run-down?

2. Discussion of the results is somewhat out of order, which unnecessarily creates confusion. For example, the first panel of Figure 2 that is discussed in the Results section is Fig. 2G, which contains the BAPTA data. Why is it necessary to mention this before all the other results shown in Fig. 2? Also, on p. 5, last paragraph, "blockers" is inappropriately plural given that only one L-VGCC blocker (nifedipine) is identified.

3. Supplementary Figure 3 does not show a control condition where induction pairing is omitted, contrary to the statement on p. 6, line 8. The choice to put the kynurenate data in the supplemental information is a strange one given that this is a very important point in the study.

3. Supplemental Fig. 3D shows data for the PKC inhibitors Ro318220 and chelerythrine, which both prevent LTD. This is an important observation that should be discussed in the primary text. How do the authors interpret the simultaneous requirement for PKC and CamKII? Also, it raises questions about the rather unequivocal distinction drawn between the STDP-LTD and previously described PKC-dependent LTD that appears in the text (e.g., on p. 6).

4. Statistical tests used to analyze physiological data in Supp. Fig 4 should be ANOVAs, not unpaired t-tests. Also, how were the two-component fits of decay tested for significance, given that the proportional contributions were not given in the figure? The deactivation rates shown here appear to be somewhat faster than those reported by Barberis et al., 2008 (J. Neurosci.), but it is

difficult to tell without the actual mean data. Can the authors include this useful information for comparative purposes, and if indeed the GluK2/K5 currents are faster decaying than their previous reports perhaps speculate as to why?

5. What was the rationale for using a non-parametric statistical test to analyze the lateral diffusion data shown in Figure 5? It is not obvious that the data should diverge from a normal distribution.

6. Chamberlain et al. citation on p. 13, line 2, is incomplete. This is also the case for Yan et al. on p. 14, line 5.

Referee #2

The paper by Carta et al tested the hypothesis that the phosphorylation of KA receptor subunit GluK5 (KA2) can mediate its plasticity. They found that the CaMKII-mediated phosphorylation of GluK5 in three sites located in C-terminus detaches GluK5 containing KA receptor, thereby inducing LTD of KA receptor mediated transmission. This is an interesting work and potentially suitable for EMBO journal. However, there are much to be improved before it can be considered further.

Major comment.

1. The LTD recording in Figure 1 is too low quality. In B, C, and E, the baseline is decaying. Also in F, the baseline is going up. If one extrapolate the baseline following the trend, it will cancel out the LTD in B, C, E and reveal LTP. I am attaching evidence in png files separately sent to the journal office. The same applies to Figure 2. The authors should go back to the individual data, reject the data which shows unstable baseline, and if N is not sufficient, add more data. This may change the conclusion of the story.

2. The authors did not make it experimentally clear whether the mechanism to bring the receptor to the surface and those to exclude from the synapse are the same or different. In the case of GluA1, phosphorylational regulation depends on its phosphorylation sites. Phosphorylation at Ser845 regulates surface expression whereas Ser831 regulates its stability on synaptic site. It would be natural to think that the effect on interaction with PSD-95 is mediated by the disruption of interaction with PSD-95. But how about the mechanism to bring to the cell surface? It would be very important to address how the three CaMKII phosphorylation sites the authors differentially work to orchestrate these process. The authors should investigate three sites individually. Therefore, I suggest to do experiment in Figure 3C, 4A, B, D, E, 7, and 8 using D mutants individually change one of possible phosphorylation sites. Also Figure 6 using phosphorylated protein (where only one S/T are left while two others are mutated into alanine) similar to Figure 3B rather than with D mutants.
The interaction between KA2 and PSD-95 is postulated to be mediated by an interaction between SH3 domain and proline rich sequence (912-925 and 948-972) (Garcia, 1998). Only T976 is marginally close to the polyproline. Given this, it is very important to understand which phosphorylation site is involved and how the phosphorylation can affect the interaction with PSD-95.

3. Many experiments were performed by recombinant protein. Experiments, for example Fig 3C, can be accomplished also by endogenous protein. Authors should test whether the story authors demonstrated is consistent with the result from endogenous unphosphorylated and phosphorylated GluK5. The phosphorylation may be induced, for example, by glycine treatment known to increase intracellular Ca2+ and activate CaMKII.

Minor comments.
Figure 1C-E. There are small lettering saying wt and VC. This is confusing. All are wt recording here. If authors want to say VC in C and E, B and D should be CC (for current clamp). Also, information how they isolated KA component is missing. I presume they used GYKI.
Figure 2E and F. Organize the figure in the order appearing in the text.
Figure 3C. Expression level of Wt and two mutants are different. It is difficult to accept the conclusion of this data. They should abandon the data in C and redo the experiment. Also, GluK2

blot should be also included.
Figure 3B, C, 6A, B: The result of quantitative immunoblots should be shown as complete image, not as combined image.
Figure 4A and D. Include color code in Figure. D. Show images for all constructs and indicate synapses at much higher magnification.
Figure 5C and D. The authors seem to did separate sets of experiment with and without CaMKII for K5DDD and K5AAA (as evidenced by slightly different K5wt data in each of them). They should repeat the experiment in interleaved manner to draw statistically significant conclusion. Also why there was difference between K5wt and K5DDD in the presence of CaMKII?
Figure 7. The time course of KA-epsc in this figure is very different from Figure 1 and 8. The width at half-maximum point is ~50 msec in this figure while in other Figures, it is around 100-150 msec or less. As a matter of fact, this may be more closer to what other group report.
Figure 8B. Given the kynurenic acid blocks LTD and KAR activation itself is important for KAR-LTD, it is difficult to conclude this apparent blockade of KAR-LTD in K5DDD mutant expressing cells is occlusion or blockade of induction. This should be noted in the text.

1st Revision - authors' response 28 October 2012

The main changes in the text are indicated in bold fonts.

Referee #1

The authors describe a CamKII-dependent depression of kainate receptor synaptic currents at mossy fiber-CA3 synapses. Interestingly, this reduction in synaptic strength appears to rely on altered stability in the postsynaptic density. A number of mechanistic studies complement the central finding, and a sophisticated molecular replacement strategy reconstitutes phosphomutant GluK5 receptors in knockout mice to test several predictions. In all it is a very strong study with a range of techniques mobilized to address the impact of CamKII on GluK2-containing kainate receptors in vitro and in vivo. The conclusion that synaptic untrapping underlies timing-dependent plasticity of synaptic kainate receptors is a novel one that will be of interest to many in the neuroscience community.

I have minor criticisms on a few points in the study.

Minor criticisms

1. Figure 1c: There is some mismatch between the traces shown and the PPR data in the graph below. The PPR values appears to be over 3, but the amplitude of the second EPSC seems to be <2-fold larger than the first in the traces. How was the PPR calculated in these experiments? Also, KAR-EPSCs have been reported to show significant run-down in amplitude in analogous experiments, which might introduce some confounding interpretation into the data shown in Figure 1. Did the authors compare the time course of EPSC/P amplitudes with and without the train stimulation to ensure that the depression was due to induced plasticity rather than run-down?

Referee 1 is correct and indeed there was some apparent discrepancy between the PPR values and the traces showed in Figure 1C and 1E. In the original submission we had analyzed the PPR (r2/r1) by measuring both r1 and r2 from the baseline before r1. As indicated by Referee 1, in order to avoid confusion and mismatch between the values in the graphs and the traces, we reanalyzed the experiments calculating now the r2 from the baseline immediately before the stimulation artifact that elicited the second current. Values are now closer to previously reported ones (i.e Ito et al 2004).

Referee 1 raises a question about the appropriate controls for long lasting recordings of KAR mediated synaptic responses (see also Referee 2, point # 1). In the original version of the manuscript, we have performed experiments in which we measured KAR-EPSP amplitudes without any stimulation train to ensure that the depression was due to activity-dependent plasticity rather than rundown. The results were presented as bar graphs, which, we agree, was likely not appropriate. In the original submission these experiments were shown as a bar graph in Figure S2 panel C. In the revised manuscript we are now presenting this important control as a proper time course graph in Figure S2 panel C, D, E and as summary in F, and we refer to this in the main text.

As Referees can appreciate, no significant rundown of the KA-mediated synaptic responses was observed over time in the absence of the induction protocol (Figure S2 C). Similarly, no rundown was observed if postsynaptic or presynaptic stimuli where applied separately (Figure S2 D and S2

E). In general, after entering in the whole cell mode, we waited for at least 10 minutes, and then we recorded stable responses for 5-10 minutes before starting the experiment, to avoid recordings with unstable KA-mediated synaptic responses. We would like to point out that recent studies (Selak et al 2009; Chamberlain et al 2012) have also shown that synaptic KAR responses could be measured over 40-45 minutes without significant rundown.

2. Discussion of the results is somewhat out of order, which unnecessarily creates confusion. For example, the first panel of Figure 2 that is discussed in the Results section is Fig. 2G, which contains the BAPTA data. Why is it necessary to mention this before all the other results shown in Fig. 2? Also, on p. 5, last paragraph, "blockers" is inappropriately plural given that only one L-VGCC blocker (nifedipine) is identified.

As suggested by Referee 1, as well Referee 2, we have now changed the order of the Figures to match the data discussed in the Result section.

We have used the plural ("blockers") because KAR-LTD was also inhibited by blocking VGCC with nimodipine (Figure S3 panel D). We have now corrected the inaccuracy by referring to this additional experiment in the main text.

3. Supplementary Figure 3 does not show a control condition where induction pairing is omitted, contrary to the statement on p. 6, line 8. The choice to put the kynurenate data in the supplemental information is a strange one given that this is a very important point in the study.

We would like to clarify that we did include the kynurenate data in the main text and in main Figure 2 C. The control experiment where induction pairing is omitted while kynurenate is applied in the bath, is shown in Figure S3, panel B and referred to in the main text. In page 6, line 8 of the original version of the manuscript we were already referring to the control of the kynurenate experiment presented in Figure 2 C.

We have kept the control experiment in Figure S3 (panel B in the revised version), not to overload Figure 2. However, following the Referee's comment, we have specified in this figure that "no stimulation" was applied during kynurenate application.

3. Supplemental Fig. 3D shows data for the PKC inhibitors Ro318220 and chelerythrine, which both prevent LTD. This is an important observation that should be discussed in the primary text. How do the authors interpret the simultaneous requirement for PKC and CamKII? Also, it raises questions about the rather unequivocal distinction drawn between the STDP-LTD and previously described PKC-dependent LTD that appears in the text (e.g., on p. 6).

As suggested by Referee 1, in the revised version of the manuscript we have now moved to the primary text and primary figure the data showing that PKC inhibitors (Ro318220 and chelerythrine) prevent LTD (results section: page 6, last paragraph; discussion: page 14, last paragraph; Figure 2E and S3C). As already mentioned in the discussion, PKC may be involved in the activation of CaMKII itself as previously reported (Yan et al, 2011 JBC). Importantly, although KAR-LTD induced by the STDP-like protocol presented here is also blocked by PKC inhibitors, the mechanisms between previously reported LTD are likely to be different. Indeed STDP-LTD does not primarily depend on mGluRs and on internalization of KARs, but depends on calcium entry via VGCC. Altogether the activity of PKC is required, however the target of PKC is unknown. In contrast our data clearly indicate the critical role of CaMKII-dependent phosphorylation of GluK5. Altogether, it is possible that, as reported for other forms of plasticity, various signalling pathways may coexist to finely tune synaptic transmission.

4. Statistical tests used to analyze physiological data in Supp. Fig 4 should be ANOVAs, not unpaired t-tests. Also, how were the two-component fits of decay tested for significance, given that the proportional contributions were not given in the figure? The deactivation rates shown here appear to be somewhat faster than those reported by Barberis et al., 2008 (J. Neurosci.), but it is difficult to tell without the actual mean data. Can the authors include this useful information for comparative purposes, and if indeed the GluK2/K5 currents are faster decaying than their previous reports perhaps speculate as to why?

Referee 1 is correct regarding the statistical test to use in Figure S4. In the revised manuscript we have analyzed the data presented in Figure S4 with one-way ANOVA test followed by Dunnett's multiple comparison test. Using this new statistical test we have found that the amplitude value for K5wt and K5DDD are not anymore significantly different ($p=0.074$), although there is a clear tendency. This is likely due to the large variability of the current amplitude from cell to cell, and is

consistent with the small difference seen in biochemical experiments in Cos7 cells, as shown in Figure 3C (EC/IC, no difference for K2a/K5, and from 7 to 14 % for K2b/K5). Figure S4A has been modified.

We have also reanalyzed the kinetics of the currents presented in Figure S4C, as in Barberis et al, 2008. We have again found no significance difference between different constructs tested. Moreover, the apparent difference in deactivation rates between our study and the one in Barberis et al. is now minimal (in this paper 38.5 ± 3.5 ms; in Barberis et al. was 46 ± 2.5 ms). The remaining difference could be due to the fact that kinetics were studied in outside-out patches (Figure S4 B and C), contrary to Barberis et al., where kinetics were quantified in whole cell recordings. Moreover it should be noted that the two sets of experiments were performed on two different set-ups, with slight differences in the fast application device. We have added to the methods section the details of the analysis performed and we have modified Figure S4C.

5. What was the rationale for using a non-parametric statistical test to analyze the lateral diffusion data shown in Figure 5? It is not obvious that the data should diverge from a normal distribution.

As Referee 1 rightly observed, we used non-parametric statistical analysis because the diffusion data does not follow a normal (Gaussian) distribution. In fact, we have repeatedly found that the surface diffusion of transmembrane receptors (Kainate, AMPAR, NMDAR, and GABA receptors) is best described by a bi-modal distribution whose peaks represent the mean values of the mobile and immobile population (please see Figure 3 in the previous study by Bats et al., Neuron (2007) 53:719-734 and Figure 3-4 by Opazo et al. Neuron (2010) 67:239-252).

Because of this, we have indicated in the material and methods section (in the "Receptor tracking and Analysis" subsection) that "Non-Gaussian distributed data were tested by Mann-Whitney t-test".

6. Chamberlain et al. citation on p. 13, line 2, is incomplete. This is also the case for Yan et al. on p. 14, line 5.

We apologize for the inaccuracy, which has been corrected.

Referee #2

The paper by Carta et al tested the hypothesis that the phosphorylation of KA receptor subunit GluK5 (KA2) can mediate its plasticity. They found that the CaMKII-mediated phosphorylation of GluK5 in three sites located in C-terminus detaches GluK5 containing KA receptor, thereby inducing LTD of KA receptor mediated transmission. This is an interesting work and potentially suitable for EMBO journal. However, there are much to be improved before it can be considered further.

Major comment.

1. The LTD recording in Figure 1 is too low quality. In B, C, and E, the baseline is decaying. Also in F, the baseline is going up. If one extrapolate the baseline following the trend, it will cancel out the LTD in B, C, E and reveal LTP. I am attaching evidence in png files separately sent to the journal office. The same applies to Figure 2. The authors should go back to the individual data, reject the data which shows unstable baseline, and if N is not sufficient, add more data. This may change the conclusion of the story.

We are not sure that Referee 2 really refers to the quality of the recordings, which we think are more than acceptable. We do understand the point however, that the relatively short baseline recording shown in the time course graphs, may give the impression that the recordings are unstable (like for all plasticity protocols with whole-cell recordings, long baselines may prevent plasticity expression). As suggested by the Referee 2, we went back to the individual original raw data and reanalyzed the baseline period in a majority of experiments. From the data presented in the first submission, we have removed experiments in which the 4 last points of the baseline varied by more than 25% with respect to the first 5. When necessary we performed additional experiments to increase the n. Consequently, the baseline variability was reduced, but the overall result of each single experiment did not change. We hope that Referee 2 can now appreciate the figures in the revised version of the manuscript.

In addition, it should be noted that the control experiments clearly exclude the possibility of rundown (see answer to referee 1, point #1). These control experiments may not have been clearly

presented in the original version. We do apologize for this. We have now presented these important controls as time course graphs in Figure S2 C, D, E and F.

2. The authors did not make it experimentally clear whether the mechanism to bring the receptor to the surface and those to exclude from the synapse are the same or different. In the case of GluA1, phosphorylational regulation depends on its phosphorylation sites. Phosphorylation at Ser845 regulates surface expression whereas Ser831 regulates its stability on synaptic site.

It would be natural to think that the effect on interaction with PSD-95 is mediated by the disruption of interaction with PSD-95. But how about the mechanism to bring to the cell surface? It would be very important to address how the three CaMKII phosphorylation sites the authors differentially work to orchestrate these process. The authors should investigate three sites individually. Therefore, I suggest to do experiment in Figure 3C, 4A, B, D, E, 7, and 8 using D mutants individually change one of possible phosphorylation sites. Also Figure 6 using phosphorylated protein (where only one S/T are left while twoothers are mutated into alanine) similar to Figure 3B rather than with D mutants.

The point raised by Referee 2 is interesting for a complete understanding of the regulation of trafficking of KARs to synaptic sites. Indeed it would be certainly interesting to know the relative role of each individual phosphorylation site, although functionally, these three sites may also be phosphorylated at the same time following activation of CaMKII. However, re-doing the experiments with all three single mutants represents a very sizeable amount of additional work, and does not change the main message about the CaMKII dependent mechanism of STDP-LTD. In agreement with the editor, we did not perform these additional experiments which may be the subject of future studies.

The interaction between KA2 and PSD-95 is postulated to be mediated by an interaction between SH3 domain and proline rich sequence (912-925 and 948-972) (Garcia, 1998). Only T976 is marginally close to the polyproline. Given this, it is very important to understand which phosphorylation site is involved and how the phosphorylation can affect the interaction with PSD-95.

The referee is perfectly correct in mentioning the earlier data by Garcia et al., which identifies proline rich sequences as responsible for the interaction between GluK5 and PSD-95. Here, we do not think that any of the CaMKII phosphorylation sites is directly involved in the binding between PSD-95 and GluK5. Indeed phospho mutations of all three sites (GluK5DDD), although greatly decreasing the interaction (see Figure 6B), does not abolish this interaction. Rather, our current interpretation is that of a screening effect: phosphorylation of these sites induces changes in the folding of the C-ter domain of GluK5, making the interaction sites much less accessible. We have added this hypothesis in the discussion (Page 16, first paragraph).

3. Many experiments were performed by recombinant protein. Experiments, for example Fig 3C, can be accomplished also by endogenous protein. Authors should test whether the story authors demonstrated is consistent with the result from endogenous unphosphorylated and phosphorylated GluK5. The phosphorylation may be induced, for example, by glycine treatment known to increase intracellular Ca2+ and activate CaMKII.

In order to study the role of CaMKII phosphorylation on endogenous GluK5 proteins we have invested a lot of efforts to obtain phospho-specific antibodies for each of the three sites. Unfortunately we were not successful with any of these. Hence we were not able to study the plasma membrane localization of endogenous phospho-GluK5 in neurons. We have not tested whether a chemical plasticity protocol can work to induce STDP-LTD in cell cultures, however the referee may be aware that is has not yet been possible to record evoked KAR-EPSCs to test any form of synaptic plasticity of KARs in cell cultures.

Minor comments.

Figure 1C-E. There are small lettering saying wt and VC. This is confusing. All are wt recording here. If authors want to say VC in C and E, B and D should be CC (for current clamp). Also, information how they isolated KA component is missing. I presume they used GYKI.

The Referee 2 is right and the small lettering may generate some confusion. In the revised version of the manuscript we indicate more clearly both recording conditions. We are now more explicit regarding the pharmacological isolation of KAR mediated responses in the methods section and in the figure legends.

Figure 2E and F. Organize the figure in the order appearing in the text.

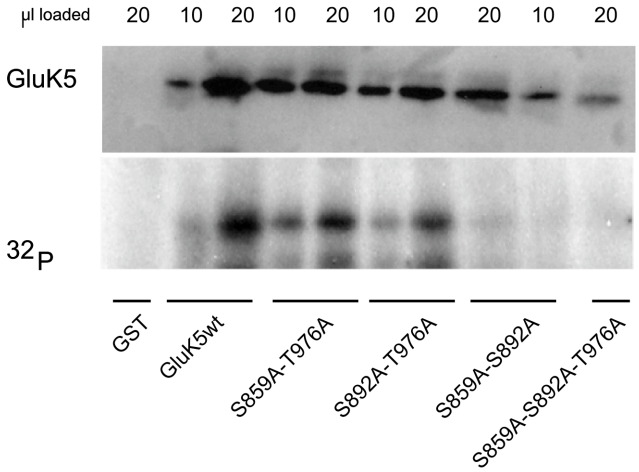
In the revised manuscript, we have now changed the order of the figures to match the discussed data in the text of the Result section (see answer to comment 2 of Referee 1).

Figure 3C. Expression level of Wt and two mutants are different. It is difficult to accept the conclusion of this data. They should abandon the data in C and redo the experiment. Also, GluK2 blot should be also included.

Following the suggestion of the referee, we have performed again the set of experiments in figure 3C in order to better analyze the trafficking of GluK5 and GluK2 expressed in COS-7 cells. In the figure we have included the images and quantification for all subunits and splice variants. The plasma membrane localization of GluK2a is not influenced by co-expression with any of the GluK5 mutants. This is probably due to the strong export motif on the C-terminal of GluK2a subunit which readily drives GluK2a to the surface (Ren et al, 2004; Jaskolski, et al; 2004). The plasma membrane localization of GluK2b is significantly higher when expressed with the GluK5DDD mutant.

Figure 3B, C, 6A, B: The result of quantitative immunoblots should be shown as complete image, not as combined image.

Figures 3C, 6A and 6B are now shown as complete images. Figure 3B corresponds to radioactive western blots. These blots were loaded with different amounts of samples in order to better quantify them. We have chosen to show only one amount for each mutant in order to simplify the figure. However, for the referee's own appreciation, we provide below the whole image, with the different amounts indicated



.

Figure 4A and D. Include color code in Figure. D. Show images for all constructs and indicate synapses at much higher magnification.

We have included a color code in the Figure. We have included a panel (Figure 4E) in order to show images for the different constructs as suggested by the referee. We have increased the magnification of synapses shown in Figure 4D.

Figure 5C and D. The authors seem to did separate sets of experiment with and without CaMKII for K5DDD and K5AAA (as evidenced by slightly different K5wt data in each of them). They should repeat the experiment in interleaved manner to draw statistically significant conclusion. Also why there was difference between K5wt and K5DDD in the presence of CaMKII?

We agree with the reviewer that the diffusion values for K5wt differed between Figure 5C and 5D. In the past, we have found that diffusion values for any given receptor can slightly vary from week to week depending on fluctuations in neuronal cultures, Qdot batch, antibody batch, etc.

Due to this variation, we always include a control condition (K5wt) so that each experiment can be represented as a stand-alone figure. In fact, even though figure 5C and D have slightly different values for K5wt (as expected since they were done in different periods of time) and thus the different conditions can not be directly compared between figures, we still found that the results and

the conclusions drawn from these figures were remarkably similar, thus strengthening the idea that phosphorylation increase the lateral mobility of GluK5-containing receptors.

We thank the reviewer for this nice observation. Although at the moment we have no conclusive answer, a possible explanation is that CaMKII only partially phosphorylates K5wt (e.i. it might have reached an equilibrium with protein phosphatases) whereas K5DDD represent a fully phosphorylated receptor. This is an interesting possibility as it implies that the number of phosphorylated residues might have an additive effect and thus gradually increase the physiological output in a rheostat-like manner as it has been previously observed for other proteins. Although we found this issue extremely interesting, we believe it is out of the scope of the current study as it will evidently require extensive biochemical, imaging and physiological experimentation to reach any robust conclusion.

Figure 7. The time course of KA-epsc in this figure is very different from Figure 1 and 8. The width at half-maximum point is ~50 msec in this figure while in other Figures, it is around 100-150 msec or less. As a matter of fact, this may be more closer to what other group report.

The decay rates of synaptic responses presented in figure 7 are much faster then the one presented for instance in figure 1 and 8 because are EPSPs and others are EPSCs. Notably synaptic potentials are slower than synaptic currents because of membrane capacitance. We have now clearly stated the recording mode in each figure legend.

Following the constructive comment of Referee 2, we reanalyzed the decay time of KA-EPSCs (figure 7 and S5) using a two components fit. In the original submission we have used a single exponential fitting that gave substantial variability, and apparent decay time values lower (faster) then the ones previously observed. The values are now included in the figure legend of Figure S5, and the criteria for analysis has been added to the methods section. As the Referees can appreciate we now have values that are similar to the one previously reported (Contractor et al 2003 for instance). We are grateful to the comments of Referee 1 and Referee 2 (see minor comment of Fig 7) that helped us to improve our data interpretation and clarified this important issue.

Figure 8B. Given the kynurenic acid blocks LTD and KAR activation itself is important for KAR-LTD, it is difficult to conclude this apparent blockade of KAR-LTD in K5DDD mutant expressing cells is occlusion or blockade of induction. This should be noted in the text.

Referee 2 is correct. Given that we found that KAR activation plays a crucial role in STDP-LTD, a possible interpretation of the lack of STDP-LTD is the decrease of the amplitude of KAR-EPSC by itself. This cannot be excluded and we now mention this point in the results section (page 12, last paragraph). However our interpretation is fully consistent with the lack of LTD with GluK5AAA, with the decrease of GluK5 amplitude when GluK5DDD replaces wt GluK5, and with the role of CaMKII in LTD.

2nd Editorial Decision 05 November 2012

Thanks for submitting your revised manuscript to the EMBO Journal. I asked referee #1 to look at the revised version. I have now heard back from the referee who appreciate the introduced changes and is supportive of publication in the EMBO Journal. There are just a few things to be clarified before acceptance here.

-) The referee notes that there are some typos in the text - please go careful through the text and correct any that you may find.

-) Lastly we also now encourage the publication of source data for, particularly for electrophoretic gels and blots. Would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels? 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.

Once we get these last issues resolved we will proceed with the acceptance of the paper for publication here.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORT

Referee #1:

I had a read through the revised manuscript and the rebuttal and find both quite reasonable. There are a fair number of typos, but in terms of novelty and technical quality the revised manuscript is impressive. It also seems a markedly improved and clearer report, resulting from the attention to both sets of criticisms. My minor criticisms were effectively addressed. The other referee's criticisms were also taken seriously (particularly with respect to the LTD baseline stability) and the consequence additions and amendments to the text should more than satisfy.

