THE
EMBO
JOURNAL

Manuscript EMBO-2011-79807

CaMKII Binding to GluN2B is critical during memory consolidation

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Review timeline:

Submission date:	12 October 2011
Editorial Decision:	16 November 2011
Revision received:	29 November 2011
Accepted:	01 December 2011

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 16 November 2011

Thank you for submitting your manuscript to the EMBO journal. Your study has know been seen by three referees and their comments are provided below. As you can see the referees appreciate the work and support publication in the EMBO Journal. The referees raise minor comments that shouldn't involve too much additional work to address. Given these comments I would like to invite you to submit a suitably revised manuscript.

Also we 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 single PDF file comprising the original, uncropped and unprocessed scans of all or key gels used in the figures? These should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. This PDF will be published online with the article as a supplementary "Source Data" file. If you have any questions regarding this just contact me.

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REFEREE REPORTS

Referee #1

The paper by Halt et al. is an ultimate confirmation of the finding by the senior author that NR2B and CaMKII interacts at their C-terminus as well as a further elaboration that was difficult make in other system. The experiments are solidly carried out and the results are well presented. Because this study uses knock-in animal, there is minimum possibility of artifact or side effect of, for example, overexpression or culturing. One caveat is that it is rather confirmatory than presenting novel ideas. However, overall, I trust this paper contains sufficient important findings to the field, which justify publication in EMBO journal.

I do not see so many issues.

Specific comments.
The discussion is rather lengthy. Avoid repetition of results. Also, the authors spend half page to discuss about PPF, which is a negative result.

Figure 4F. The base line in control group is declining. Go back to the individual data and reject experiment which shows unstable baseline. If N is not enough, need to repeat more experiments.

Minor comments.
The title says "memory consolidation". But formally, one cannot distinguish whether consolidation is wrong or recall process is wrong.

Referee #2

This study explores the role of CaMKII-NR2B binding in synaptic plasticity and learning. The authors generated mutant KI mice with mutations which impair this binding in vivo. Detailed biochemical analysis demonstrated that these mutations resulted in 50% decrease in binding of CaMKII to both NR2B and NR1 in the membrane brain fraction and 50% decrease in LTP in hippocampal slices. Interestingly the total amount of CaMKII in that fraction did not change indicating that other CaMKII binding partners were sufficient to retain normal amount of CaMKII in bound state. Importantly, upon NMDAR activation of forebrain slices CaMKII binding to ether NR2B or NR1 was completely abolished, increase in phosphorylation of S831 in GluR1 was completely prevented, while 286P of total CaMKII maintained intact. Hippocampal slices from KI mice after cLTP treatment of hippocampal slices resulted in CaMKII accumulation in the PSD fraction and only transient increase in 286P. Behavioral analysis revealed that learning in Morris maze was not affected but memory retention decreased significantly.

Altogether the study complements existing data and advances the field in very important ways. The paper is clearly written and data are well presented and discussed. I have only few minor comments.

Minor comments

P3, L1-2 "This mechanism supports selective enrichment of CaMKII at synapses that are undergoing LTP (Lee et al, 2009; Zhang et al, 2008)."

The preceding sentences to which "mechanism" refers describes the role of CaMKII translocation and NMDAR binding in LTP-----but that data does not apply to actual LTP but to models of LTP and so is only suggestive. This should be made clear. Also, Refs on Bayer et a., 2006 and Barria, Malinow 2005 needs to be added here as the best (although still suggestive) support for the statement that synaptic accumulation of CaMKII in LTP is dependent on CaMKII binding to NMDAR. Also, Tom Oertner's work should be referenced in the sentence in italics above.

P5 L2 and after: "In KI neurons, the newly formed CaMKII clusters were mostly not co-localized with or juxtaposed to synapsin puncta and therefore mainly extrasynaptic (Figure 2K,L,P2,P3)."

I do not see what data brought the authors to this conclusion. Both figures 2Q and 2R show that neither Pierson coefficient nor the number of CaMKII clusters, that were colocalized with synapsin clusters, changed in KI mice cultures. There is some contradiction here. Please clarify.

Fig 3F. Although NMDAR EPSP slope does not chance in KI mice the total charge seems to decrease. This could be either due to change in a polysynaptic response contribution or due to change in the duration of the NMDAR current. Also, GABA antagonist was not included in these experiments and therefore the measurements of NMDAR fEPSP slope could be inaccurate. Whole-cell voltage clamp experiments in the presence of PTX (GABA A antagonist) should be used for these experiments in order to be definitive. There may be some previous work on this issue in Barria and Malinow; if so, this should be discussed. As it stands, there is a small possibility that the loss of LTP might result from less Ca entry through the NMDAR.

Fig 1B shows that in KI, the binding of NR1 to CaMKII decreased to almost the same extent as the binding of NR2B. Similarly after treatment of slices with NMDA both NR1 and NR2B binding to CaMKII were blocked completely! This finding requires mention in the discussion. There is evidence for a presynaptic component of LTP---perhaps what was blocked was only the postsynaptic contribution to LTP.

P7, l5 from the bottom. Lee et al., 2000 ref is not correct

P7, l2 from the bottom. The reference on Kopec et al, 2006 is incorrect. The correct ref is (Makhinson et al, 1999) and (lu et a., 2007) as indicateed in the supplemental materials.

P9. Please check refs in the second paragraph. I did not check all but the ref on Matsuzaki paper did not mention TARP .

P14 L1-2 from the bottom

"We conclude that KI mice have a highly selective defect in CaMKII-dependent forms of LTP and not basal transmission". In the paper of Sanhueza et a., 2010 a peptide that decreased interaction of CaMKII and NR2B did affect basal synaptic transmission. Could you suggest a possible reason for the differences?

In the introduction, the authors said that the mutant mouse strain in Zhou et al (2007) "had deficits in addition to disruption of CaMKII binding (see below)". Please clarify what are these deficits.

In Fig. 1, the authors concluded that "co-IP of the NMDAR complex with CaMKII was reduced by ~50% in KI mice", but the figure shows that co-IP of GluN2B with CaMKII was reduced by ~40%, and co-IP of GluN1 with CaMKII reduced by ~30%. Is there a difference between co-IP results for GluN1 and GluN2B?

In the methods section, please define what criteria were used to
determine colocalization of synapsin and CaMKII.

It was difficult as a reader to keep track of the multiple types of fractions used in biochemical work. It would be helpful to describe these in the Results section so that the reader would have an understanding of these preparations without having to flip back to METHODS.

Referee #3

In their report, Halt and colleagues investigated the role of CAMKII binding to GluN2B subunit in memory formation. The authors generated mice with two point mutations that impair CaMKII binding to the NMDAR GluN2B subunit. In these mice, calcium-triggered postsynaptic accumulation is abrogated for CaMKII, LTP is reduced by 50%, and calcium-triggered phosphorylation of the AMPAR GluA1 subunit by CaMKII is impaired. The mutant mice learn the Morris water maze to the same degree as WT but show deficiency during the period of early memory consolidation. The authors thus conclude that the activity-driven interaction of CaMKII

with the NMDAR is important for imprinting memory traces. The topic and the results are clearly of great interest for the field and the procedures used to tackle this important question are interesting and well-performed. The manuscript is of great quality and is thus fully adequate for publication in EMBO J. As a minor comment, I would be curious to see either an electrophysiological profile or surface distribution of 2B/2A-NMDA in KI mice. Also, should the glutamate synapses normally mature in KI mice since this process supposedly require an activity-dependent change in 2A/2B-NMDAR trafficking. This could be comment in the discussion.

1st Revision - authors' response 29 November 2011

Referee #1

1. The discussion is rather lengthy. Avoid repetition of results. Also, the authors spend half page to discuss about PPF, which is a negative result.
We shortened the Discussion by eliminating repetitions and condensing our discussion of PPF. We still list the factors that can influence PPF as the lack of effect of GluN2B KI on PPF for various inter-stimulus intervals suggests that these parameters are all unaffected in the KI mice.

2. Figure 4F: the base line in control group is declining. Go back to the individual data and reject experiment which shows unstable baseline. If N is not enough, need to repeat more experiments.
We took out the two WT recordings with strongly declining baseline, which leaves sufficient N (9). The resulting averages reflect now stable baseline recordings for the rest.

3. The title says "memory consolidation". But formally, one cannot distinguish whether consolidation is wrong or recall process is wrong.
Given that recall is normal 2 h after training (Fig. 8B, C, E, F) the impaired performance 24 h after last training (Fig. 8D, G, H) is more likely due to impaired consolidation. However, it is impossible to differentiate between impaired access to vs impaired maintenance of memory traces. One could argue that both, continued accessibility to and the engraving of memory traces during consolidation are part of consolidation but this is a question of definition of the word 'consolidation.' As we cannot differentiate between altered access vs engraving we changed the title and discussion to indicate that it is possible that access rather than or in addition to engraving was affected.

Referee #2

1. P3, L1-2 "This mechanism supports selective enrichment of CaMKII at synapses that are undergoing LTP (Lee et al, 2009; Zhang et al, 2008).”-*but that data does not apply to actual LTP but to models of LTP and so is only suggestive. This should be made clear. Also, Refs on Bayer et a., 2006 and Barria, Malinow 2005 needs to be added here as the best (although still suggestive) support for the statement that synaptic accumulation of CaMKII in LTP is dependent on CaMKII binding to NMDAR. Also, Tom Oertner's work should be referenced in the sentence in italics above.*
We modified this sentence to read: “This mechanism supports selective enrichment of CaMKII at synapses that are undergoing potentiation upon repeated glutamate uncaging, a model for LTP.” We added Bayer et al. 2006 to the preceding sentence as this work shows that application of glu+gly leads to postsynaptic accumulation of CaMKII in agreement with the other citations but it does not directly study LTP or LTP-like paradigms (glu+gly does not lead to LTP) and thus does not support that LTP induction leads to postsynaptic CaMKII accumulation. Barria and Malinow (2005) also do not provide images that would support this claim but had nicely shown the importance of CaMKII binding to GluN2B in LTP, which is thus cited in the next sentence. The relevant work by Oertner and coworkers is actually already referenced with Zhang et al., 2008.

2. P5 L2 and after: "In KI neurons, the newly formed CaMKII clusters were mostly not co-localized with or juxtaposed to synapsin puncta and therefore mainly extrasynaptic (Figure 2K,L,P2,P3)." *I do not see what data brought the authors to this conclusion. Both figures 2Q and 2R show that neither Pierson coefficient nor the number of CaMKII clusters, that were colocalized with synapsin clusters, changed in KI mice cultures. There is some contradiction here. Please clarify.*
To clarify we now rephrased the main text: “In KI neurons, the lack of increase in Pearson’s coefficient as well as in the number of CaMKII immunofluorescent puncta that colocalizes with the

synaptic marker synapsin (Mander's coefficient) indicates that, contrasting the CaMKII clusters that were present under basal conditions, the numerous newly formed CaMKII clusters were mostly not co-localized with or juxtaposed to synapsin puncta and were, therefore, formed mainly outside synapses (Figure 2K,L,P2,P3)." Also we believe that visual inspection of Fig. 2I vs L and Fig. 2O vs P give a clear optical impression of a much more yellow vs. green tone indicative of more CaMKII-synapsin colocalization in GluN2B WT vs KI neurons. Finally, to ensure that the two different methods of determining colocalization are clearly defined in our manuscript we added to MATERIAL AND METHODS in the main manuscript: "Colocalization of CaMKII and GluN2B with synapsin and was determined using the Image J (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA; http://imagej.nih.gov/ij/, 1997-2011) plugin JACoP (Bolte, 2006) to determine colocalization coefficients (Pearson's coefficient and Manders coefficient) for 10 neurons per treatment condition. Pearson's coefficient is an estimate of the fit of the intensity correlation between two channels to a straight line; a value of 0 describes no, 1 complete positive, and -1 complete negative correlation. Mander's coefficient (fraction of CaMKII or GluN2B signals colocalized with synapsin signals) is similar to Pearson's coefficient, but signal intensity is not considered; it describes the fraction of one signal that overlaps with the other."

3. Fig 3F. Although NMDAR EPSP slope does not change in KI mice the total charge seems to decrease. This could be either due to change in a polysynaptic response contribution or due to change in the duration of the NMDAR current. Also, GABA antagonist was not included in these experiments and therefore the measurements of NMDAR fEPSP slope could be inaccurate. Whole-cell voltage clamp experiments in the presence of PTX (GABA A antagonist) should be used for these experiments in order to be definitive. There may be some previous work on this issue in Barria and Malinow; if so, this should be discussed. As it stands, there is a small possibility that the loss of LTP might result from less Ca entry through the NMDAR.
The question whether abrogating CaMKII binding to GluN2B affects its biophysical properties or functional availability at postsynaptic sites is a very interesting one. However, we believe such a characterization is beyond the scope of the current work because it would take much more time, substantially delaying the publication of the data at hand. Whole cell patch recordings of evoked EPSCs varies a lot from recording to recording because they are very sensitive to precise positioning of stimulus electrodes and many other factors. Importantly, we show that CaMKII activation upon pharmacological activation of NMDAR is normal in GluN2B KI (Fig. 1G,H) and so is LTD (Fig. 3F), another function that depends on Ca^{2+} influx through NMDARs. Accordingly, Ca^{2+} signaling and its influx at postsynaptic sites are normal in the KI mice. Further, Barria and Malinow (2005) ectopically express in organotypic hippocampal slices GluN2B with two point mutations in the CaMKII binding site (R1300Q/S1303D) that are similar to ours (L1298A/R1300Q) and also abrogate CaMKII binding. The resulting NMDARs in this article displace endogenous NMDARs from the synapse but I/V curves and decay time constants are identical between NMDARs that contain WT vs mutant GluN2B, as now stated in the text.

4. Fig 1B shows that in KI, the binding of NR1 to CaMKII decreased to almost the same extent as the binding of NR2B. Similarly after treatment of slices with NMDA both NR1 and NR2B binding to CaMKII were blocked completely! This finding requires mention in the discussion.
We now state in the Dicussion more explicitely: "Stimulus-induced association of CaMKII with the NMDAR complex as reflected by CoIP of GluN2B as well as GluN1 with CaMKII is also abrogated in KI mice (Figure 1E,F). This latter finding is, once more, unexpected given the activity-induced CaMKII binding to NR1 in vitro but indicates that the NR1 interaction plays a modest if any role in activation-induced recruiting CaMKII to the NMDAR complex in vivo, including complexes consisting of GluN1/N2A."

5. There is evidence for a presynaptic component of LTP---perhaps what was blocked was only the postsynaptic contribution to LTP.
It is possible that a presynaptic component of LTP was mainly spared in the KI mice. However, a presynaptic component in LTP maintenance is still controversial and we have no evidence for this hypothesis and rather not speculate in the manuscript.

6. P7, l5 from the bottom. Lee et al., 2000 ref is not correct
We removed this reference.

7. P7, l2 from the bottom. The reference on Kopec et al, 2006 is incorrect. The correct ref is

(Makhinson et al, 1999) and (Lu et al., 2007) as indicated in the supplemental materials.
We are now citing (Makhinson et al, 1999) and (Lu et al., 2007), which in fact used the same chemLTP protocol as we did in this manuscript at hand. We retained the citation of Kopec et al, 2006 as this paper describes an electrophysiological characterization of a variation of the chemLTP protocol we use, although this variation was from a physiological point of view quite substantial.

8. P9. Please check refs in the second paragraph. I did not check all but the ref on Matsuzaki paper did not mention TARP .
It seems to us that all references are appropriate (most are review articles on molecular mechanisms of LTP). Matsuzaki et al., 2004, used glutamate uncaging to map whether AMPAR responses are increased at spines that underwent enlargement upon repeated glutamate uncaging, a model for LTP. This finding strongly supports at least a functional increase in AMPAR availability at postsynaptic sites upon LTP and we feel it thus supports the model that LTP is at least to a substantial degree mediated by an increase in postsynaptic AMPAR. This point was the main point we wanted to make here.

9. P14 L1-2 from the bottom "We conclude that KI mice have a highly selective defect in CaMKII-dependent forms of LTP and not basal transmission". In the paper of Sanhueza et a., 2010 a peptide that decreased interaction of CaMKII and NR2B did affect basal synaptic transmission. Could you suggest a possible reason for the differences?
I assume this comment refers to Sanhueza et al, 2011 (to which our laboratory contributed). The authors used a peptide derived from the endogenous CaMKII inhibitor protein. This peptide displaces CaMKII not only from GluN2B but also densin-180 (our recent preliminary data) and likely other binding partners that bind to the so-called T-site. Accordingly, it can be expected to have effects in addition to our KI mutations in GluN2B. We now discuss this issue thoroughly as follows:

"The membrane permeant peptide tatCN21 inhibits CaMKII at 5 μM (Buard et al, 2010). It also displaces CaMKII from GluN2B at 20 but not 5 μM in acute hippocampal slices (Sanhueza et al, 2011). It blocks LTP induction at both concentrations but reverses LTP during its maintenance phase only at 20 μM, consistent with the hypothesis that CaMKII binding to GluN2B is important for LTP to last. It also decreases basal synaptic transmission at 20 but not 5 μM (Sanhueza et al, 2011). Thus we first tested whether GluN2B KI mice have a defect in basal synaptic transmission. (...) The lack of defect in KI mice in basal transmission contrasts the decrease in transmission by 20 μM tatCN21 (see above) (Sanhueza et al, 2011), suggesting that this tatCN21 effect might be via a target other than CaMKII binding to GluN2B. In fact, our preliminary results suggest that tatCN21 also disrupts the CaMKII-densin180 interaction, which can compensate for loss of NMDAR interaction with respect to basal CaMKII targeting (Carlisle et al, 2011). Nevertheless, several forms of LTP were reduced by half in the KI mice indicating that CaMKII binding to GluN2B is important for maintenance of a portion of but not full LTP (Figure 4A,D,E)."

10. In the introduction, the authors said that the mutant mouse strain in Zhou et al (2007) "had deficits in addition to disruption of CaMKII binding (see below)". Please clarify what are these deficits
We re-worded this paragraph to indicate that the ectopic expression of the whole C-terminus of GluN2B in the mice studied in Zhou et al., 2007 causes a strong learning impairment when our KI mice don't at all-accordingly our new findings indicate that functions in addition to those that require the GluN2B – CaMKII interaction are affected in the mice in Zhou et al., 2007.

11. In Fig. 1, the authors concluded that "co-IP of the NMDAR complex with CaMKII was reduced by ~50% in KI mice", but the figure shows that co-IP of GluN2B with CaMKII was reduced by ~40%, and co-IP of GluN1 with CaMKII reduced by ~30%. Is there a difference between co-IP results for GluN1 and GluN2B?
We now state more accurately that the coIP was reduced by 35-40% (coIP of GluN1 (36%) and GluN2B (41%) with CaMKII is statistically not different).

12. In the methods section, please define what criteria were used to determine colocalization of synapsin and CaMKII.
We added this information to MATERIAL AND METHODS in the main manuscript (see #2 above)

13. It was difficult as a reader to keep track of the multiple types of fractions used in biochemical work. It would be helpful to describe these in the Results section so that the reader would have an understanding of these preparations without having to flip back to METHODS.
We integrated this methodological information into Figure legends or, when better fitting, into the main text of the Result section.

Referee #3

1. As a minor comment, I would be curious to see either an electrophysiological profile or surface distribution of 2B/2A-NMDA in KI mice.
Please see #3 of Referee 2 and Supplemental Figure 2I-R where we quantitatively show that synaptic localization of GluN2B is normal in the KI mice.

2. Also, should the glutamate synapses normally mature in KI mice since this process supposedly require an activity-dependent change in 2A/2B-NMDAR trafficking. This could be comment in the discussion.
As stated above it appears that in hippocampal cultures with mature synapses GluN2B distribution is normal in the KI mice. Together with a lack of change in NMDAR-mediated fEPSPs this finding argues against a substantial change in GluN2A/2B ratio. We thus would not expect a strong effect if any on basal transmission and in fact found that many parameters are normal including mEPSC decay tau which otherwise could have hinted at a change in AMPAR subunit composition. Gray et al. (2011: Neuron 71, 1085) finds a strong effect of GluN2A and 2B elimination in a few neurons during development on mEPSC amplitude and frequency, respectively, but not vice versa. Our modification is much more subtle and we do not see any alterations in mEPSC amplitude or frequency. To make this point clearer we now state in the Discussion: “Unaltered transmitter release was further indicated by lack of changes in the readily and totally releasable synaptic vesicle pools (Figure 3B,C) and in mEPSC frequency (Figure 3D). The mEPSC amplitude and decay tau was also unaffected (Figure 3D) indicating that AMPAR function and composition is not overtly affected.”

