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PIKE-mediated PI 3-kinase Activity is Required for AMPA Receptor Surface Expression

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 22 February 2011

Thank you for submitting your manuscript to the EMBO Journal. Three referees have now reviewed your manuscript and their comments are provided below.

As you can see, the referees appreciate that the analysis provides new insight into a role of PIKE in regulating AMPA-R surface expression and activity. However, they also raise significant concerns with the manuscript that I am afraid preclude its publication here. Many of the experiments require proper controls and/or different approaches to test the hypothesis. Given that significant concerns are raised and as it is unclear if these can be resolved, I am afraid that I cannot offer to consider the manuscript further at this stage. Should you future work enable you to address the concerns raised in full, then we would be willing to consider a resubmission. I should point out that for resubmissions we consider the novelty of the data at the time of resubmission and may, if needed, bring in new referee(s).

I am sorry that I do not have more positive news on this occasion, but hope that you might find these comments useful.

Yours sincerely,
Editor
The EMBO Journal

Referee reports:

Referee #1 (Remarks to the Author):

In this manuscript, the authors report that PIKE-L is involved in LTP in the hippocampus. LTP has been extensively investigated but the molecular mechanisms involved are only partially understood. A key issue is how the activation of NMDARs results in the insertion of AMPARs into the synaptic membrane. Previous work has identified PI3K in this respect. The present work builds upon this by showing a critical role for the PI3K enhancer PIKE. It is shown that PIKE-L (one of 3 isoforms) binds directly to, and forms a tertiary complex with, GluA2 and GRIP. Interestingly, PIKE-L is required for activity-dependent activation of GluA2-associated PI3K and for the associated insertion of GluA2 subunits into the plasma membrane. As a consequence inhibition of PIKE-L activity, or knockout of the protein, results in a loss of LTP.
There is a lot of information contained within this manuscript and much of this is likely to be of interest to workers in the field. I am therefore supportive of publication but have a number of points to be addressed.

1. The role of PI3K in LTP is somewhat controversial. It has been claimed by at least two groups that its involvement is dependent on the strength of induction (e.g., Peineau et al Neuron 2007). This point should be acknowledged. It would be useful to know whether LTP can be induced in the PIKE-L knockout also with stronger induction protocols, as would be predicted if its involvement is only via PI3K.
2. In relationship to LTP, while it is true to say that AMPAR trafficking is likely to be a major part of the process, it is not the only mechanism that is probably involved. This should be clarified in the text.
3. With respect to the LTP experiments, there is always an issue of interpretation if LTP is affected when baseline transmission is altered too. The authors need to control carefully for the reduction in synaptic transmission.
4. The paired-pulse experiments do not simply reflect presynaptic changes, since these are field recordings that are sensitive to GABA-inhibition etc.
5. I don't believe that the alterations in seizure susceptibility etc really fits well in the ms. My suggestion would be to focus on the trafficking in relationship to LTP for this study.
6. It would be nice to see a scheme of what the authors think is happening.
7. The English needs tidying up in places.

Referee #2 (Remarks to the Author):

PI3K plays a key role in regulating synaptic AMPAR trafficking during LTP and LTD. In this manuscript, Ye and colleagues reported that PIKE-L, a GTPases that interacts and enhances the activity of PI3K and Akt, controlled synaptic GluA2 trafficking during LTP. They argued that PIKE-L stimulated surface GluA2 expression, which promoted LTP. They concluded that association of PIKE-L and AMPARs substantiated PI3K activity, which was the pre-requisite for subsequent GRIP1 association and cell-surface retention of AMPARs.

In my opinion many experiments reported here are poorly designed. This study is incomplete, lack mechanistic insight. The major claim is not supported by the data.

Here are my major concerns:
1) The main conclusion that association of PIKE-L and AMPARs substantiated PI3K activity, which was the pre-requisite for subsequent GRIP1 association and cell-surface retention of AMPARs is not supported by experiments. The authors neither tested whether the association or PI3K activity was required for the subsequent GRIP1 association nor examined whether delivery or retention of AMPARs was changed.

2) The authored concluded that PIKE-L/GluA2 association was critical for activity-dependent AMPAR insertion during LTP based on the reduced TBS-induced potentiation in PIKE KO neurons. However, this experiment was poorly designed and problematic. First, because synaptic transmission was reduced in KO neurons, the extracellular TBS stimuli should induce a smaller potentiation. Second, it is likely that NMDA transmission was also altered in KO neurons. The

reduced NMDA transmission would reduce potenitation. Finally, it is possible that the properties of LTD were changed in KO neurons. Therefore, the TBS stimuli might induce both LTP and LTD in KO neurons and the authors actually measured a mix of LTP and LTD in the experiment.

3) The regulations of synaptic AMPAR trafficking, LTP and LTD by PI3K have been extensively studied. The authors failed to discuss many of the previously published mechanisms, including the well established notions that PI3K is involved in the mGlu/homer-mediated LTD, that synaptic trafficking of GluA2/GluA3 AMPARs mediates LTD, and that GRIP controls synaptic trafficking of GluA2/GluA3 AMPARs. Although the authors noted the associations of PIKE with both mGlu and homer, and GluA2 with both GluA1 and GluA3. It is not clear why the authors chose not to test whether PIKE associates with GluA3, mediates LTD, and controls synaptic trafficking of GluA2/GluA3.

4) The authors seemed to assume the synaptic trafficking of GluA1/GluA2 behaves the same in cultured cells and slices. In fact, the mechanism in cultured neurons is very different from that in slices and intact brains. Many results made from cultured cells should be repeated in the slice preparation.

5) The author reported that AMPA transmission was rectified in PIKE KO neurons. Why did the rectification start at 10 mV instead of zero? Also, could the rectification result from the secondary effect of the prolonged KO of PIKE gene? Acute manipulations are needed to confirm the results obtained from KO neurons.

6) In figure 3, why did adenovirus alone affect surface receptor expression? Could the differences in surface expression be explained by different toxic effects of different viral constructs? Independent approaches are needed to confirm the results.

Other points:
7) The efficiency of PIKE knockdown shRNA should be documented.

8) Many experiments were not rigorously analyzed. For example, figure S3 and the elevated surface GluA2 expression after PIKE-L KS expression should be quantified.

9) The statement that "while incorporation of GluA2 to AMPAR renders the passage of Ca2+, AMPAR with Q/R edited GluA2 is calcium permeable" is simply incorrect.

Referee #3 (Remarks to the Author):

PIKeE-mediated PI3-kinase activity is regulated for AMPA receptor surface expression (by Chan et al.).
In this study, the authors show that PIKE- GTPases regulate neuronal AMPAR activity by promoting GluA2/GRIP association. They showed that ablation of PIKE or disruption of PIKE-L/GluA2 interaction in neurons caused a reduction of GluA2 surface expression and LTP. The authors further show that the reduction of GluA2 surface expression increases the Ca2+ permeability trigged by kainate in PIKE(-/-) neurons, suggesting a higher susceptibility to neurotoxic insults. Whereas some of their findings are of potential interest, however, there are a number of additional controls necessary for me to support this manuscript for publication in EMBO Journal.

Specific comments:

Figure 1. The authors need to show the % of input in panels :B, C, G, and I.
The myc blot in figure 1I lane 4 should be replaced by a more convincing blot. Based on the image Figure 1E the co-localization between PIKE-L and GRIP it appear not convincing, should be changed and quantified.

Figure 2. Also here the % of inputs are not shown. In Fig B, the authors claimed that PIKE-L co-localizes with GluA2. This image is not convincing at all .

The localization of PIKE-L based on the image it appears to be diffuse. The co-localization with GluA2 should be quantified.

Figure 3-4. The authors demonstrated that knockdown of PIK-L by shRNA abolished the glycine-provoked PI3K activity and reduction of GluA2 surface expression . These experiments need refinement in the form of rescue experiments.

Figure 5. Again, the rescue conditions in these experiments should be included.

Resubmission 17 June 2011

Thank you for your email on 2/22/11 regarding the decision on our manuscript 'PIKE-mediated PI 3-kinase activity is required for AMPA receptor surface expression (EMBOJ-2011-77045)'. We are grateful to the constructive comments from the reviewers who think our manuscript contains 'lot of information' that 'is likely to be of interest to workers in the field (Reviewer 1)' and some of (the) findings are of potential interest (Reviewer 3)'. The manuscript has been revised accordingly, which includes new biochemical, immunohistological and electrophysiological results that are able to address the concerns of the reviewers. Specific revisions are made as follow:

Reviewer 1:

1. The reviewer commented that the role of PI3K in LTP is controversial, which should be discussed in the manuscript.

As suggested, we have discussed that the controversial role of PI3K in the 'Discussion' section (p14) with new citations on the work that suggested its involvement depends on the strength of LTP induction.

2. The reviewer suggested studying if LTP can be induced in PIKE knockout mice with stronger induction protocol.

As suggested, we have induced LTP in PIKE knockout brain slides using high frequency stimulation (HFS) (Opazo et al., J Neurosci 2003 23:3679-3688). In contrast to the theta blast stimulation (TBS) that no LTP is induced in the absence of PIKE (Fig 5I), stronger induction protocol is able to induce LTP in PIKE -/- neurons, although the magnitude is smaller (Fig 5J), suggesting PIKE-L is partially involved in the process. These results also indicate that the presence of PIKE is necessary for physiological inducers (e.g. TBS or glycine) to trigger LTP. However, when the cells are stimulated with strong inducers (e.g. HFS), other signaling pathways is involved in addition to PIKE or PI3K, thus causing LTP with smaller magnitude. Indeed, Opazo et al. (J Neurosci 2003 23:3679-3688) have reported that inhibition of PI3K using LY294002 can only partially suppress HFS-induced LTP but almost completely abolish TBS-induced LTP, which is in good alignment with our conclusion that PIKE/PI3K is important to input-dependent LTP formation.

3. The reviewer suggested clarifying the AMPAR trafficking is not the only mechanism causing LTP.

As suggested, we have stated in the 'Introduction' that LTP induction/expression can also be modulated by other signaling cascades leading to changes of transcription and synapse remodeling (p4).

4. The reviewer commented that the interpretation of LTP experiments is difficult when the baseline transmission is altered in PIKE -/- mice.

We agree that it is difficult to interpret if LTP is affected when baseline transmission is altered too. However, there is no positive correlation between baseline transmission and the LTP. Indeed, Hung et al have shown a weak synaptic transmission but normal LTP and enhanced spatial learning in mice lacking Shank1 at SC- CA1 synapse (J Neurosci, 2008 28:1697-708). And our recent research have also shown that enhanced LTP can be induced in PV-Cre; ErbB4 -/- mice that have decreased

synaptic basal transmission at SC-CA1 synapse (Chen et al., PNAS, 2010 107: 21818-21823).

5. The reviewer commented that the paired-pulse experiments do not simply reflect presynaptic changes since there are field recordings that are sensitive to GABA-inhibition

To exclude this possibility, we measured evoked EPSC by holding the membrane potential at -70 mV in the presence of the 20 μM GABAA receptor antagonist bicuculline (Fig S7). Similar to our previous data, we found that the paired-pulse ratio of evoked EPSCs was normal in PIKE-/- mice, compared with control mice.

6. The reviewer criticized that the study on seizure susceptibility observed in the PIKE -/- mice does not fit well in the manuscript and suggested focusing on the trafficking in relationship to LTP.

As suggested, the current study mainly focuses on the role of PIKE in regulating AMPAR trafficking and LTP formation and the data of the high calcium influx in PIKE -/- neurons leading to seizure and apoptosis have been removed from the manuscript.

7. The reviewer suggested including a scheme of the role of PIKE in the neurons during LTP formation.

We have included a proposed model in the revised manuscript (Fig 7). In this model, we propose that PIKE-L serves as a molecular linkage between GluA2 and GRIP1. Upon glycing/TBS stimulation, PIKE-L complexs with GluA1 and GRIP1, sustains the GluA2-associated PI3K activity and helps GluA2 to retain on the cell surface for LTP expression.

8. *The reviewer commented that the English needs tidying up in places.*

As suggested, we have edited the manuscript carefully to avoid possible typo and grammatical errors.

Reviewer 2:

1. The reviewer criticized the main conclusion that PIKE-L-mediated PI3K activation is pre-requisite for GRIP1/GluR2 association and cell surface retention of AMPAR is not supported by experiments, because the author neither tested whether PI3K activity is necessary for GRIP1/GluR2 association or delivery or retention of AMPAR.

As a matter of fact, we have already examined the importance of PI3K in GluA2/GRIP1 association after glycine stimulation in our original manuscript. In Fig 3A (1st panel), we have shown clearly that GluA2/GRIP1 association is enhanced after glycine stimulation. To further test if PI3K is important in this association, we pre-treated the neurons with PI3K inhibitor LY294002. Our result shows that the GluR2/GRIP1 binding is greatly diminished in the presence of LY294002 (Fig 3B, 1st panel). Moreover, we have shown that GluA2/GRIP1 interaction is reduced in PIKE-null brain, where PI3K activity is reduced (Fig 4A, 3rd panel, Fig 4C and Fig S4E), which fits well with the decreased cell surface expression of GluA2 in PIKE -/- neurons (Fig 4D and E). These data strongly suggest that both PI3K and PIKE-L are important for the GluA2/GRIP1 association. Indeed, the role of PI3K in the surface retention of GluA2 has already been reported by other research groups (Lu et al., 2001 Neuron 29: 243-254; Arendt et al., 2010 Nat Neurosci 13: 36-44), which we have already stated in the original manuscript (p.4 and p14).

2. The reviewer was concerned with the conclusion that PIKE-L/GluR2 association is critical for AMPAR insertion during LTP.

We respectfully disagree with the comment that TBS stimuli should induce a smaller potentiation in PIKE KO neurons as the synaptic transmission is reduced, because there is no positive correlation between baseline transmission and the LTP. For example, Hung et al have shown a weak synaptic transmission but normal LTP and enhanced spatial learning in mice lacking Shank1 at SC- CA1 synapse (J Neurosci, 2008 28:1697-708). We showed recently that enhanced LTP can be induced in PV-Cre; ErbB4 -/- mice that have decreased synaptic basal transmission at SC-CA1 synapse (Chen

et al., PNAS, 2010 107: 21818-21823). It is worthy pointing out that our conclusion that PIKE-L/GluR2 association is critical for AMPAR insertion during LTP is not based entirely on the TBS-induced experiments. We have also performed an array of biochemical, immunohistological and electrophysiological experiments to prove that PIKE-L is critical for glycine-induced LTP induction. Thus, our data provide strong evidence on the role of PIKE in both activity and chemical induced-LTP.

We have measured NMDA currents in PIKE -/- neurons to exclude the possibility that change of LTP in PIKE -/- neurons is caused by alteration of NMDA transmission. We characterized AMPA- and NMDA-mediated currents in pyramidal neurons of PIKE -/- mice in a whole cell configuration. Purified NMDA-EPSC was determined by subtracting the traces obtained in 100 M DL-AP5 at + 40 mV from those obtained before the treatment. The isolated AMPA-EPSC was measured at -60 mV in presence of DL-AP5, which could be inhibited by CNQX. As shown in Fig 5F to H, the NMDA current is comparable between PIKE -/- and the wild-type control. However, the amplitude of AMPA-EPSC was significantly reduced in PIKE -/- CA1 neurons, indicating the AMPA but not NMDA current is affected in the absence of PIKE-L.

While we could not exclude the possibility that the properties of LTD might be altered in PIKE -/- mice, however, further investigation on this topic is beyond the scope of this study as the present report aims at elucidate the role of PIKE-L in LTP formation. The identification of PIKE-Lës role in LTD involves extensive experiments, which are more suitable for an independent project.

3. The reviewer asked why the authors chose not to test whether PIKE associates with GluA3, mediates LTD, and control synaptic trafficking of GluA2/GluA3.

We agree that it will be interesting to study the role of PIKE in GluA3-mediated LTD formation and will be helpful in fully elucidating the role of PIKE in synaptic activities. Indeed, we have assessed the interaction between endogenous PIKE-L and different subunits of AMPAR in mouse brain using immuoprecipitation. While we found that PIKE-L interacts with GluA1 and GluA2 strongly, we could not detect any significant association between PIKE-L and GluA3. Therefore, we focused our study on PIKE-L-mediated GluR2 surface trafficking instead of studying its role in modulating the function of other AMPA subunits.

4. The reviewer suggested that results from cultured cells should be repeated in the slice preparation as the mechanism of synaptic trafficking of GluA1/GluA2 behaves differently in cultured cells and slices.

While we agree that the cellular activity of neurons in cell culture might be different from that of intact brain, we think the data presented in the manuscript are sufficient to support our conclusion as we have included both in vitro and in vivo experiments in our study. As shown in the manuscript, glycine-induced GluR2 surface expression is reduced in primary culture of rat neurons infected with shPIKE (Fig 3E and F), which is further supported by the identical results obtained from PIKE -/- neuron cultures (Fig 4C and D). To further demonstrate PIKE is critical in GluA2 surface expression in vivo, we performed biochemical assays to measure the amount of GluA2 in membrane surface of neurons. Fitting with the data in culture neurons (Fig 4C and D), the in vivo data clearly shown that the number of GluA2 on cell surface is significantly reduced in PIKE -/- cortex and hippocampus (Fig 4B). Moreover, electrophysiological studies indicated that the AMPA current is also reduced in PIKE -/- neuron slides (Fig 5A to H). All these data strongly support our conclusion that PIKE-L is critical in modulating the surface expression of GluA2.

5. The reviewer asked the reason of unique rectification of PIKE knockout neurons, which starts at 10 mV instead of 0 mV. Moreover, s/he suggested performing acute manipulations to confirm the results.

We thank the reviewer for pointing this out. The rectification of PIKE knock out neurons which start at 10 mV instead of 0 mV probably because the setting of liquid junction potentials was not strong enough to correct these offset errors. To confirm the rectification of the PIKE-/- neurons, we have determined the AMPA receptor medicated EPSCS/Voltage (I/V) relationship in acute hippocampus slices as suggested. Since reviewer 1 suggested removing these data from the manuscript, we thus attached these updated data with this letter but not in the revised manuscript. As shown in the

attached figure 1, an inwardly rectifying was obtained at positive membrane potentials in neurons of PIKE -/- mice slices, unlike a linear I/V relationship in control.

6. The reviewer asked why adenovirus alone affects surface receptor expression in Fig 3.

In the current study, we did not observe any viability, morphology or receptor surface expression changes in the cultured neurons infected with control adenovirus. We assume that it is the missing of bar labels that makes the reviewer misinterprets the data in Fig 3F. In this figure, the solid bars represent the expression of surface GluA2 that have been treated with glycine, whereas the open bars represent the expression of GluA2 in PBS-treated cells. Appropriate labels have been added in the revised manuscript.

7. The reviewer suggested documenting the efficiency of PIKE shRNA in knocking-down the PIKE.

In our original manuscript, we have already shown the efficiency of shPIKE in depleting PIKE-L in cultured neurons (Fig 3C, 3rd panel; Fig 3G, 2nd panel).

8. The reviewer criticized that the data in Fig S3 has not been rigorously analyzed.

The data shown in Fig S3 are actually the pictures showing the whole neurons that we used in analyzing the effect of PIKE overexpression or depletion on GluA2 surface expression in cultured neurons. We did include the quantification of the analysis in the original manuscript (Fig 3E and F). Also we have stated clearly this in the 'Result' section (p. 9 and 10).

9. The reviewer commented that the statement 'while incorporation of GluA2 to AMPA renders the passage of Ca2+, AMPAR with Q/R edited GluA2 is calcium permeable' is incorrect.

This is a typo mistake. The statement has been amended to 'while incorporation of GluA2 to AMPA renders the passage of Ca2+, AMPAR with Q/R unedited GluA2 is calcium permeable'.

Reviewer 3:

1. The reviewer requested showing the % of input in Fig 1B, 1C, 1G 1I, 2A, 2D, 2E and 2F.

As requested, the amounts of inputs shown in Fig 1 and Fig 2 have been clearly labeled.

2. The reviewer requested to replace the myc-blot in Fig 1I with a more convincing blot.

The myc immunoblotting in Fig 1I is now replaced with a new picture of better quality.

3. The reviewer suggested to change Fig 1E and Fig 2B with better pictures and quantify the co-localization of PIKE-L and GRIP1 or GluA2

We have replaced Fig 1E and Fig 2B with new pictures showing better co-localization of PIKE-L and GRIP1 or GluA2. Also, the Pearson's Correlation coefficient (Rr) has been included (p6 and 7).

4. The reviewer suggested including rescue experiments to refine the conclusion that PIKE-L is necessary for glycine-induced GluA2 surface expression.

As suggested, we have infected PIKE -/- neurons with adenovirus overexpressing PIKE-L and measured the GluA2 surface expression after glycine treatment. As shown in Fig 3E and F, expression of PIKE-L in PIKE-null neurons increases the surface expression of GluA2 in both basal and glycine-stimulated conditions. This data, together with other results presented in the manuscript, strongly demonstrated that PIKE-L is critical to GluA2 surface expression in neurons.

I hope our manuscript is now suitable for publication in the EMBO Journal with the above revisions and thank you for your reconsideration of our manuscript.

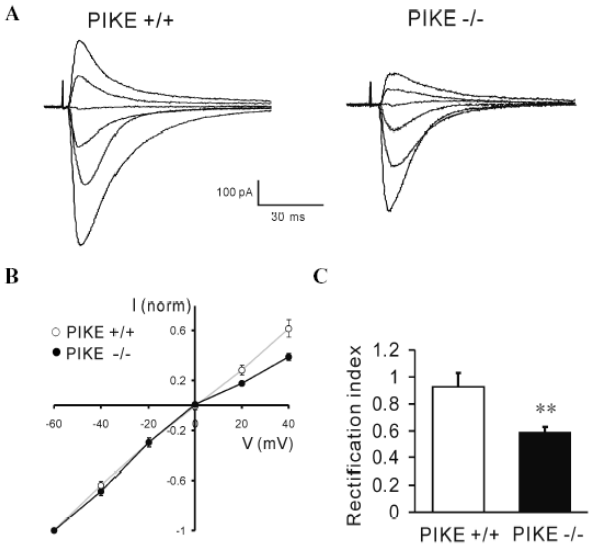

Fig 1

Rectification properties of synaptic AMPAR in PIKE -/- mice.

A. Representative traces showing AMPAR-medicated currents at different holding potentials in presence of NMDA receptor blocker (DL-AP5 100 μM) in the ACSF. Traces were obtained at holding the potentials from -60 mV to +40 mV, in 20 mV steps.

B. Summary of corresponding peak I/V relationship (n = 7 cells from 3 wild-type mice and n = 9 cells from 4 PIKE -/- mice).

C. The rectification index from B. The rectification index was calculated by the traces at theholding potentials of -60, 0, +40 mV by the following formula: RI = [(I +40 - I 0)/(I 0 - I 60)] x 3/2 (**: P<0.01, Studentís t-test).

Acceptance letter 13 July 2011

Thank you for submitting your manuscript to the EMBO Journal. This manuscript is an invite resubmission of MS 77045 that rejected post review earlier this year. The resubmission has now been re-reviewed by the original referees # 1 and 3 and their comments are provided below.

As you can see both referees appreciate the introduced changes and support the publication of the manuscript as is. I am therefore pleased to proceed with the acceptance of the paper. You will receive the formal acceptance letter shortly.

Sincerely

Editor
The EMBO Journal

Referee #1

In this revised manuscript the authors have addressed my initial concerns in a satisfactory manner. I have no additional comments.

Referee #3

The authors have carefully answered convincingly to points requested and the paper is suitable for publication in EMBO journal

