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PTEN is recruited to the postsynaptic terminal for NMDA receptor-dependent long-term depression

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1st Editorial Decision 05 November 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. First of all, please accept my sincere apologies for the considerable delay in getting back to you with a decision in this case (which was owed to delays both in finding a sufficient number of suited and available expert reviewers and in receiving all of their recommendations). In the meantime, we have now finally gotten the comments of all three reviewers (see below), who on the whole consider your approaches and findings potentially interesting and thus in principle suited for publication in The EMBO Journal. Nevertheless, especially referees 1 and 2 also raise a number of substantive issues, which would need to be decisively addressed before publication may eventually be warranted. Given that the major criticism refer mostly to experimental or technical (rather than conceptual) problems, I am inclined to give you the possibility to respond to the referees' comments in the form of a revised manuscript. Should you be able to adequately address the main issues, including the important comments on PSD95-PTEN interaction raised by referees 1 and 2, we should be able to consider a revised manuscript for eventual publication. As it is EMBO Journal policy to allow a single round of major revision only, it will however be important to diligently answer to all the various points raised at this stage if you wish the manuscript ultimately to be accepted. When preparing your letter of response, please also bear in mind that this will form part of the Review Process File, and will therefore be available online to the community in the case of publication (for more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html). Finally, from an editorial point of view, I suggest you also consider a somewhat less vague and complex title for the study (I'll be happy to discuss alternative proposals). In any case, please do not hesitate to get back to us should you need feedback on any issue regarding your revision.

Thank you for the opportunity to consider your work for publication. I look forward to your

revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

Jurado et al. proposed a molecular mechanism for PTEN-mediated synaptic depression. Authors found that NMDA stimulation increased interaction between PTEN and PSD-95. Furthermore, overexpression of PTEN impaired AMPAR-mediated synaptic transmission by PTEN phosphatase activity and both PTEN antagonist and dominant negative forms diminished NMDAR-mediated LTD, but not LTP or mGluR-mediated LTD. From these results, authors proposed a mechanism for NMDAR-induced LTD by regulated synaptic localization of PTEN and its activity.

Overall, PTEN is a signaling molecule and could induce many phenotypes including altered synaptic development and plasticity observed in PTEN KO heterozygous or knockout mice previously published. However, mechanisms for altered synaptic transmission and plasticity remain unclear. Therefore, a direct link between PTEN and AMPAR-mediated synaptic transmission is interesting. However, this manuscript lacks some critical information to establish a model proposed.

Criticism.
1, It is well established that 1 % Triton X-100 authors used does not solubilize PSD-95 at the PSD, suggesting that increased interaction between PSD-95 and PTEN seems occurring outside of the PSD. Although authors showed robust enhancement of PTEN at synapses by EM, because of nature of EM (rather qualitative, but not quantitative), it is better to show more simple quantitative assay, for example, biochemical PSD fractionation and immunofluorescence staining of PTEN, PSD-95 and NMDAR before and after NMDA stimulation in Fig 1. Notably, NMDA stimulation varied among figures.

2, One of two major findings is regulated interaction of PTEN with PSD-95. However, it remains unclear specificity of PSD-95 binding to PTEN. Authors must show interaction between PSD-95 and other PSD-95 protein binding protein, for example, NMDAR, before and after NMDA stimulation. In addition, Fig 1A should be quantitated.

3, Overexpression of PTEN impaired AMPAR-mediated transmission. This manuscript proposed links between regulated PTEN interaction with PSD-95 and PTEN-mediated AMPAR regulation. Therefore, it is necessary to show requirements of synaptic PTEN for regulation of AMPAR-mediated transmission, for example, measurement of AMPAR-mediated synaptic currents in neurons overexpressing PTENdeltaPDZ (Fig. 4).

4, Authors showed that PTEN impaired AMPAR-mediated basal transmission without changes in NMDAR-mediated basal transmission. How about synaptic plasticity in PTEN overexpressed neurons? By showing this, authors can segregate roles of PTEN in basal and regulated transmission. In addition, others have previously proposed roles of PTEN in LTP, it would be beneficial to show LTP and mGluR1 dependent LTD with PTEN antagonist.

5, Many groups reported that overexpression of PSD-95 increases AMPAR-mediated synaptic transmission. However, here authors proposed that overexpression of PSD-95 binding protein, PTEN, depress AMPAR-mediated synaptic transmission. Authors should discuss this controversy.

Referee #2 (Remarks to the Author):

In this ms, Jurado et al use a combination of biochemistry, imaging and electrophysiology to examine the effects of PTEN on hippocampal synaptic physiology. The first observation is that NMDA receptor activation increases the biochemical association of PSD95 and PTEN. The PDZ binding motif in PTEN mediates this interaction. FRAP data are provided which intend to show that PTEN is anchored in the spine and immunoEM data further establishes that some PTEN is located in the postsynaptic density. Slice recordings show that PTEN expression down regulates AMPA receptors and that blocking PTEN interferes with LTD but not LTP (or mGluR dependent LTD). According to their model, a paired stimulation protocol for LTD recruits PTEN and depletes PIP3, leading to activation of GSK3beta and down regulation of AMPA receptors. The findings are topical and of interest to neuroscientists in general, providing new clues into the regulation of AMPA receptors by lipids in the brain. The general finding that PTEN regulates AMPA receptors is novel and exciting. However, serious shortcomings for some of the experiments and the lack of insights into the mechanism weaken the manuscript.

1. In Figure 1, immunoprecipitation of PTEN pulls down PSD95 after NMDA receptor activation. There are several issues that come up with this experiment. First, the IP experiment uses total protein extracts. However, to show association of PSD95 with PTEN in the PSD, a technique for isolating the PSD fraction should be used. Without this, the interaction of PSD95 and PTEN could be elsewhere, such as in the somatic regions of the neuron. A detailed description of the protocol is also needed. Second, the reverse IP (IP of PSD95) is needed to support the conclusion that the two proteins specifically associate. Third, the IP of MAGI 2 is not convincing. A better example should be shown. Fourth, implicating NMDA receptor activation is very interesting but additional pharmacological experiments are needed. For example, extracellular Ca2+ should be required for this. What is the effect of a NMDA receptor antagonist (to block endogenously released glutamate)? Can this explain why the control band is so variable (sometimes detectable, sometimes not)?

2. In Figure 2, GFP PTEN deltaPDZ does not associate with PSD95, implicating the PDZ binding motif. This is fine. The FRAP experiments show a biphasic response, with fast recovery after 5' and slower recovery after 15' and 25'. Again, a few points need to be addressed for these experiments. First, without a protein protein binding assay, it cannot be concluded that the PTEN PDZ binding motif interacts directly with PSD95. It is possible that another protein mediates this association. Second, the timing of NMDA receptor activation is not consistent between experiments. In Figure 1, a short 5' exposure was used. However, this length of time is only sufficient to increase the mobility. The appropriate co ip experiment should be done after 25'. Lastly, it is concluded that after 15' or 25' "a larger fraction of PTEN is retained in spines in a long lasting manner after NMDAR activation". After photobleaching the entire spine, the recovery represents diffusion of unbleached fluorophores into the spine. Thus, the slower rate of recovery reflects anchoring of the PTEN outside of the spine, which is the opposite of the conclusion in the manuscript.

3. In Figure 4, expression of PTEN leads to smaller evoked AMPA currents but no change in NMDA currents. If NMDA receptor activation (or LTD) is needed to anchor PTEN to PSD95 and down regulate AMPA receptors, then why does expression of PTEN alone have any effect? Information on the mechanism (point 8) would be helpful. An important control that would strengthen the conclusion that a PDZ interaction is required is to show that PTEN deltaPDZ has no effect on the evoked AMPA current.

4. In Figure 5, an inhibitor of PTEN (bpV(HO)pic) or dominant negative PTEN prevents the maintenance of LTD. These experiments show that AMPA receptors can still be downregulated, hence the immediate decrease in current, but that this loss cannot be maintained. Is this an effect on the induction or maintenance of LTD? Again, the time course of the interaction seems to be important here. The FRAP and biochemical data indicate that changes occur after 15 - 25'. However, 15 25' after 1Hz stimulation seems to occur after the loss of LTD. This either argues that earlier time points 5 10' NMDA activation are involved more in the LTD, or that the two events are not really coupled.

Minor point. Perhaps more time points would help. It is important to clarify the function of either increased (5') or decreased (15 25') mobility of PTEN with respect to LTD. Lastly, it is mentioned that "voltage clamp whole cell recordings were obtained from nearby infected and uninfected CA1 pyramidal neurons". This means there should be a uninfected (control) and infected group for each experiment. However, the control group appears to be replicated in Figure 5 and 7. The statistics

need to be carried out on the paired controls - not the pooled controls.

5. In Figure 6, PTEN C124S appears to have no role in mGluR dependent LTD. However, there is too much variability to be certain that PTEN C124S has no effect. Is it known why the variability is higher for the PTEN C124S? Would a larger sample size show a significant change?

6. According to the study by Horne and Dell'Acqua (J. Neurosci., 2007), decreases in PIP2 are needed for LTD ("NMDAR activation of PLC is also necessary for decreases in spine PSD95 levels and AMPAR internalization"). However, PTEN would be expected to counteract this decrease by increasing levels of PIP2 through depletion of PIP3. Please explain.

7. In addition, spine levels of PSD95 decrease (Horne and Dell'Acqua, J. Neurosci., 2007), raising the possibility that PSD95 may move out of the spine to PTEN in the shaft. How can this be ruled out?

8. What is the mechanism underlying the NMDA receptor dependent increase in association of PSD95 and PTEN? Some experimental link here would strengthen the manuscript.

9. According to the model, paired stimulation produces LTD by recruiting PTEN to the spine, which depletes PIP3 levels and activates GSK3beta. However, are there additional effects of increased levels of PIP2 and/or lower PIP3 on AMPA receptor stability/targeting?

10. If PTEN is needed for removal of AMPA receptors during LTD, then it seems that blocking this with PTEN C124S might facilitate LTP. No change in LTP was reported. Please comment in the discussion.

Referee #3 (Remarks to the Author):

This is an excellent paper that defines a mechanism of regulation of PTEN by synaptic activity. It also defines a PTEN function in LTD. The work was performed carefully and there are appropriate controls for most experiments. The data are excellent and the work is focused and will be interesting for EMBO Journal readers.

I have the following comments:

1. The paper makes creative use of photobleaching to analyze the fraction of PTEN that is anchored under different conditions (Figure 2). In this experiment, it is very easy to see how anchorage could be controlled by PDZ interaction. But what might be the mechanism of PTEN mobilization seen during the first few minutes after NMDA treatment? A comment could be helpful.

2. What is the substrate for PTEN-induced decreases in AMPAR currents?

3. In Figures 5A, B and C, why does AMPA current go down and then come backup again, when PTEN is inhibited? It is clear that long lasting LTD is blocked when PTEN is inhibited, but what causes the transient decrease?

4. The Discussion considers the possible role of GSK3-beta in PTEN pathways. These are interesting speculations, but should they be incorrect, they would detract form an otherwise excellent paper. The authors could either qualify their speculations or provide some data.

1st Revision - authors' response 16 April 2010

We are pleased to send you this substantially revised version of our manuscript on the role

of PTEN in LTD. As you will see, we have been very responsive to the reviewers' comments. Particularly, we have extended our biochemical characterization of the association between PTEN and the postsynaptic scaffolding machinery, by including pharmacological controls, PSD fractionations and a better characterization of the kinetics of the interaction. We have also included a considerable number of new electrophysiological experiments (AMPA/NMDA responses, LTP, NMDAR LTD and mGluR LTD), which strongly corroborate our previous conclusions on the role of PTEN specifically in NMDA receptor-dependent LTD versus LTP or mGluR-dependent LTD. In all, we have included 10 new figure panels.
In summary, we consider this is a significantly improved manuscript, which provides novel mechanistic information on the role of PTEN in synaptic plasticity.

Referee #1

1. As requested by the reviewer (see also referee 2, point 1), we have carried out PSD fractionations to quantitatively evaluate the association of endogenous PTEN with the PSD scaffold before and after NMDA receptor activation. As shown in new Fig. 3, there is a two-fold increase in PTEN accumulation at the PSD fraction upon NMDA receptor activation. Importantly, the abundance at the PSD fraction of other synaptic proteins, such as PSD-95, NMDARs or CaMKII, remained constant or slightly decreased, ruling out non-specific effects such as protein aggregation or precipitation after NMDA receptor activation.
This experiment was carried out using purified synaptosomes as the starting material (see Supplementary Methods for details). Therefore, this new result reinforces the notion that PTEN is redistributed locally, within the spine (or within the synaptosome) to associate with the PSD scaffold. We have also confirmed this interpretation by carrying out quantitative confocal fluorescence microscopy of GFP-PTEN accumulation in spines before and after chemical LTD induction. As shown in new Supplementary Fig. 2B, GFP-PTEN distribution between spines and the adjacent dendritic shaft did not change upon NMDA receptor activation. This result argues that PTEN is not mobilized into the postsynaptic compartment (the spine) during LTD; instead, a fraction of the pre-existing PTEN molecules within the spine are recruited to the postsynaptic density. According to this interpretation, we would not expect to see changes in the immunofluorescence colocalization of PTEN and PSD-95 (or NMDA receptors), since confocal fluorescence cannot resolve subsynaptic compartments within the spine.
As for the amount of PTEN-PSD95 association upon NMDA receptor activation, it is indeed variable, as noted by the reviewer (see point 2, and also reviewer 2, point 1). We believe that this variability is related to the physiological state of the slices before LTD induction (that is, their previous history of synaptic activation). By quantifying the extent of PTEN-PSD95 co-precipitation before and after NMDA receptor activation across multiple experiments, we have estimated the induction factor as about 2 fold (new Fig. 1E).

2. As mentioned above, we have quantified the induction of PTEN-PSD95 association after NMDAR activation across multiple experiments. This is now shown in Fig. 1E. Unfortunately, we were unable to evaluate the interaction between PSD-95 and other synaptic partners by immunoprecipitation of PSD-95. All commercially available anti-PSD-95 antibodies we have found are raised against PDZ domains, and according to our results, they appear to interfere with PDZ-dependent interactions. In fact, we were not able to co-precipitate NMDARs with PSD-95 using these antibodies. To note, the association we report between PTEN and PSD-95 was detected using anti-PTEN and anti-GFP (for GFP-PTEN) antibodies.

3. As requested by the reviewer, we have carried out recordings of AMPA and NMDA receptor-mediated synaptic transmission in neurons overexpressing PTEN- PDZ. As shown in new Fig. 5G-I, AMPA (but not NMDA) receptor responses were depressed by the PTEN mutant. Although initially counterintuitive, this result suggests that overexpressed PTEN can reach the synaptic compartment in the absence of PDZ interactions. This interpretation is in agreement with our previous results with wild-type PTEN (new Fig. 5A-C), which is able to depress basal AMPAR transmission when overexpressed, although it does not significantly interact with PSD-95 in the absence of NMDAR activation (Fig. 1). Therefore, protein overexpression appears to overcome the requirement for NMDAR activation and PDZ-dependent interactions. This interpretation is now explicitly stated in the revised manuscript (end of page 11, beginning of page 12).

4. We have carried out the three experiments requested by the reviewer. As shown in new Fig.

7, neither LTP (panels B, C) nor mGluR-dependent LTD (panels E, F) was affected by the PTEN antagonist. These results perfectly replicate our previous observations using the PTEN dominant negative mutant (Fig. 7, panels A, C, D, F). We believe that the previous report of altered LTP in the PTEN knock-out (Fraser et al., Neuroscience 151, 476-488, 2008) was due to pleiotropic alterations caused by the absence of PTEN throughout development. By using semi-acute blockade of PTEN activity (overnight expression of dominant negative mutants or pharmacological inhibition), we believe we are revealing direct functions of PTEN at otherwise unperturbed synapses.
In addition, we have found that NMDAR-dependent LTD is not altered in neurons overexpressing wild-type PTEN (Supplementary Fig. 7). Since PTEN overexpression leads to a reduction in AMPAR-mediated responses (Fig. 5A, B), this new result suggests either that PTEN-induced depression does not saturate subsequent LTD expression, or alternatively, that overexpressed PTEN acts on a different pool of AMPARs from those removed during synaptically induced LTD. This interpretation is now stated in the revised manuscript (page 14, last paragraph).

5. It is well established in the literature that PSD-95 overexpression leads to AMPAR potentiation. However, according to our experiments, PTEN overepxression does not change the levels of PSD-95 (input lanes in Figs. 1G-H and 2A). It is also unlikely that overexpressed PTEN will drive more PSD-95 into synapses under basal conditions, since the association of PTEN and PSD-95 is triggered by NMDAR activation. Therefore, it does not seem that overexpressing a PSD-95 binding protein will have similar effects as the overexpression of PSD-95 itself. In addition, and separate from the potentiating effect of PSD-95 overexpression, there is also abundant evidence for a critical role of PSD-95 in LTD (Kim et al., Neuron 56, 488-502, 2007; Ehrlich et al. PNAS 104, 4176-4181, 2007; Xu et al. Neuron 57, 248-262, 2008; Carlisle et al. J Physiol 586, 5885-5900, 2008; Bhattacharyya et al. Nat Neurosci, 12, 172-181, 2009). In conclusion, although the role of PSD-95 in synaptic function is far from clear, we believe our results provide new evidence for PSD-95 as a central signaling organizer during bidirectional plasticity. These considerations are now explicitly presented in the Discussion (end of page 18, beginning of page 19).

Referee #2:

1. First. As requested by the reviewer, we have carried out PSD fractionations to evaluate the association of endogenous PTEN with the postsynaptic scaffold (see also referee 1, point 1). As shown in new Fig. 3, PTEN is significantly enriched in the PSD fraction upon NMDAR activation, and this effect is specific for PTEN versus other synaptic proteins such as PSD-95, NMDARs or CaMKII. The details of this new experiment are included in Supplementary Methods.
Second. As discussed above (referee 1, point 2), we attempted the reverse IP (IP of PSD-95 and detection of PTEN) using several anti-PSD-95 antibodies, with negative results. We believe this is due to the interference of these antibodies with PDZ-dependent interactions, because we were not able to detect NMDARs in the PSD-95 IPs either. Nevertheless, based on the reviewer's comment, we have toned down our interpretation of these experiments. We now claim that NMDAR activation leads to the association of PTEN with the PSD-95 scaffolding complex (supported by the PSD fractionations and electron microscopy experiments), although we cannot prove a direct interaction between these two proteins (page 18, end of second paragraph).
Third. We have also attempted several western blots for MAGI-2 using different antibodies, with similar results. We believe the expression of MAGI-2 is rather low in hippocampus, making very difficult to show a clear interaction with PTEN. Nevertheless, we have considered it is worth showing this example, as representative of a different pattern from the one obtained with PSD-95.
Fourth. We now show that the association between PSD-95 and PTEN upon NMDAR activation is indeed blocked by the NMDAR antagonist AP5 (Fig. 1, new panel B). We agree with the reviewer in that the variability of the association between PTEN and PSD-95 under basal conditions may reflect the release of endogenous glutamate before the experiment (that is, the previous history of synaptic activation in the slices; see also referee 1, point 2). We have now quantified the extent of PSD95-PTEN association before and after NMDAR activation across multiple experiments, yielding an estimated induction factor of about 2-fold (see quantification in Fig. 1E).

2. First. We agree with the reviewer that neither the FRAP nor the co-IP experiments prove a direct interaction between PTEN and PSD-95. We have now toned down this interpretation in the

revised version of the manuscript (page 18, end of second paragraph).
Second. We have now extended the time course of the co-IP experiments, as requested by the reviewer (Fig. 1, new panels D and E). Based on the average result from these time courses, we now conclude that the association of PTEN with PSD-95 persists after the end of the NMDAR activation, although it gradually declines by 30 min. There is indeed a mismatch between the biochemical association between PTEN and PSD-95, and the anchoring of PTEN in the spine, as noted by the reviewer. One possibility is that PSD-95 only participates in the initial phases of PTEN recruitment to the postsynaptic membrane, whereas its maintenance may be dependent on other components of the postsynaptic scaffold. This possibility is now explicitly acknowledged in the revised manuscript (page 18, end of second paragraph). Nevertheless, we should also point out that the technical differences between the FRAP and co-IP experiments make very difficult to exactly match their timing. In general, biochemical experiments are expected to follow faster kinetics that live-imaging ones. This is because slices are immediately transferred from one solution to the next for co-IP experiments, whereas solution exchange is rather slow under the continuous flow of perfusion solution at the imaging chamber of the microscope.
Third. It is true that fluorescence recovery in the spine is due to the diffusion of unbleached molecules from the adjacent dendritic shaft. However, this recovery reflects a bidirectional exchange of bleached and unbleached molecules. Otherwise there would be a net increase in PTEN amount in the spine after each FRAP experiment. To clarify this point, we have now tested whether the net amount of PTEN in the spine (versus the adjacent dendrite) is altered after LTD induction. As shown in Supplementary Fig. 2B, GFP-PTEN does not accumulate at (or is removed from) the spine upon NMDAR activation, arguing that LTD does not lead to a net redistribution of PTEN molecules between the spine and the dendrite. This is now stated in the revised version of the manuscript (page 8, third paragraph).

3. We have now included the experiment requested by the reviewer. As shown in new Fig. 5G-I, overexpression of PTEN- PDZ specifically depressed AMPA (but not NMDA) receptor responses. Although initially counterintuitive, this result argues that overexpressed PTEN is able to reach the synaptic compartment in the absence of PDZ interactions (see also referee 1, point 3). This interpretation also explains why overexpressed wild-type PTEN depresses AMPARs in the absence of LTD induction (and PSD-95 association), as noted by the reviewer. We now discuss this interpretation in the revised manuscript (end of page 11, beginning of page 12).

4. In an attempt to explore the role of PTEN in LTD induction, we have carried out additional LTD experiments in which PTEN activity was only blocked around the period of LTD induction: the PTEN inhibitor, bpV(HO)pic, was added to the slices 5 min before induction, and was removed 5 min after the end of the induction period. As shown in Supplementary Fig. 6, LTD expression was also blocked under these conditions, arguing that PTEN plays a role at least during LTD induction. As discussed below (referee 3, point 3), it is difficult to ascertain what is the cause (or causes) of the transient depression of synaptic transmission when PTEN activity is blocked. Perhaps AMPARs are initially removed after LTD induction, but they return to synapses if PTEN is not active. Nevertheless, we should point out that some initial depression is always expected from the fact that LTD induction is achieved by transient depolarization of the postsynaptic cell to 40 mV (which will reduce the driving force for current entry through AMPARs) and low frequency stimulation of the Schaffer collaterals (which may produce a transient presynaptic depression; Hvalby et al., J. Physiol. 571, 75-82, 2006).
Minor point: as requested by the reviewer, we have split the control data in Figs. 6 and 8, to plot each set of infected (or treated) neurons with its corresponding control of uninfected (or untreated) neurons. Statistics were always done with respect to the matching controls.

5. We have expanded the sample size of our mGluR LTD experiments, as requested by the reviewer (new Fig. 7D, F). mGluR LTD is now virtually identical in control and in PTEN-C124S-expressing neurons. In addition, we have carried out similar experiments blocking PTEN activity with the specific inhibitor bpV(HO)pic. As shown in new Fig. 7E, F, mGluR LTD was also unaffected. We believe we have now convincing evidence supporting our previous interpretation that mGluR-dependent LTD does not require PTEN activity.

6. We now cite this previous work from Dell'Acqua's laboratory in the revised version of the manuscript (end of page 21), since it is pertinent for our study. We believe the requirement for PIP2 turnover described by Horne and Dell'Acqua, and the requirement for PIP3 turnover we describe

here are not contradictory. This is because basal PIP2 levels are likely in large excess over PIP3 levels (Vanhaesebroeck et al., Annu. Rev. Biochem. 70, 535-602, 2001). Therefore, the turnover of PIP3 into PIP2 is not expected to significantly increase the basal concentration of PIP2. Nevertheless, taking together, Dell'Acqua's study and ours suggest an interesting relay of phosphoinositide metabolism during LTD, which would require degradation of PIP3 into PIP2 (via PTEN) and subsequent action of PLC for PIP2 turnover. This interpretation is now stated in the revised version of the manuscript (page 20, last paragraph).

7. We agree with the reviewer in that LTD leads to a decrease in PSD-95 levels at spines, as described by Horne and Dell'Acqua and others (Bhattacharyya et al., Nat. Neurosci. 12, 172-181, 2009; Sturgill et al., J Neurosci. 29, 12845-12854, 2009). One possibility is that PSD-95 only participates in the initial events of PTEN recruitment to the synaptic scaffold (point 2, second). Alternatively, PTEN may be anchored by means of the PSD-95 molecules remaining in the spine. This is possible, since PSD-95 is not completely depleted from spines after LTD. We now explicitly state these possibilities in the revised manuscript (page 18, end of second paragraph).

8. We are indeed working on potential mechanisms that may control the association of PTEN to the synaptic scaffold. However, PTEN is subject to multiple regulatory modifications (a complex pattern of phosphorylation, acetylation, oxidation, etc.; Tamguney and Stokoe, J Cell Sci. 120, 4071-4079, 2007). Therefore, we believe that an evaluation of these potential mechanisms is out of the scope of this manuscript.

9. As suggested by the reviewer, we have now evidence that lowering PIP3 levels has direct consequences on the stability/targeting of AMPARs at synapses (see also referee 3, point 2). Specifically, AMPARs appear to diffuse from the postsynaptic membrane into the extrasynaptic membrane of the spine when PIP3 is downregulated. This local redistribution would lead to synaptic depression, as the receptors would no longer be within the reach of synaptically released glutamate (Arendt et al., Nat. Neurosci. 13, 36-44, 2010). This new evidence is now mentioned in the Discussion (end of page 19).

10. By using both a dominant negative mutant (new Fig. 7A) and a pharmacological inhibitor (new Fig. 7B), we believe we have obtained convincing evidence that blocking PTEN activity does not alter LTP. This is not incompatible with the requirement of PTEN function during LTD. In fact, there are multiple studies where the magnitude of LTP and LTD can be modified independently of each other. These are some recent examples of genetic manipulations that impaired LTD without altering LTP: Lee et al., J Neurophysiol. 103, 479-489 2010; Ehrlich et al., PNAS 104, 4176-4181, 2007; Brown et al., Neuron 45, 81-94, 2005; Brown et al., J Neurosci 27, 13311-13315, 2007.

Referee #3:

1. This is indeed an interesting problem, on which we are currently working. PTEN is subject to multiple forms of regulation, including phosphorylation, acetylation, oxidation, etc., which modulate its ability to bind different protein partners (Gericke et al., Gene 374, 1-9, 2006; Tamguney and Stokoe, J Cell Sci. 120, 4071-4079, 2007). In addition to protein-protein interactions, PTEN binds phospholipids in a very dynamic manner (Vazquez et al., PNAS 103, 3633-3638, 2006; Rahdar et al., PNAS 106, 480-485, 2009). Therefore, there are multiple potential mechanisms that may regulate the release of PTEN from a retaining interaction, leading to an increased mobilization. This interpretation is mentioned in the revised version of the manuscript (end of page 18, beginning of page 19).

2. As discussed before (referee 2, point 9), we have now evidence that a reduction in PIP3 levels directly alters AMPAR stability and/or targeting at the synapse. Specifically, AMPARs appear to diffuse from the postsynaptic membrane into the extrasynaptic membrane of the spine when PIP3 is downregulated (Arendt et al., Nat. Neurosci. 13, 36-44, 2010). This local redistribution of AMPARs may be the initiating event leading to LTD. This interpretation is now mentioned in the Discussion (end of page 19).

3. This is an interesting point, which relates to the role of PTEN in LTD induction, expression or both. In order to further explore this issue, we have carried out new experiments in which PTEN activity was only blocked around the time of LTD induction (from 5 min before to 5 min after). Under these conditions, long-lasting depression was still blocked (new Supplementary Fig. 6). This result would argue that PTEN plays a role at least during LTD induction. It is difficult to ascertain what is the cause (or causes) of the transient depression of synaptic transmission when PTEN activity is blocked. Perhaps AMPARs are initially removed after LTD induction, but they return to synapses if PTEN is not active. Nevertheless, we should point out that some initial depression is always expected from the fact that LTD induction is achieved by transient depolarization of the postsynaptic cell to 40 mV (which will reduce the driving force for current entry through AMPARs) and low frequency stimulation of the Schaffer collaterals (which may produce a transient presynaptic depression; Hvalby et al., J. Physiol. 571, 75-82, 2006).

4. As discussed before (point 2), we believe that a reduction in PIP3 levels may be directly related with the redistribution of AMPARs away from the postsynaptic membrane (Arendt et al., Nat. Neurosci. 13, 36-44, 2010). It is still unknown whether or not this effect involves regulation of GSK-3beta. We now discuss this new evidence in the revised version of the manuscript (end of page 19).

Additional correspondence 09 May 2010

Thank you for submitting your revised manuscript for our consideration. We have now received comments on the revision from the original reviewers 1 and 2, who had both raised a significant number of critical concerns during the initial round of review. I am afraid that despite the significant amount of work you have invested into this revision, neither of them appears fully convinced that the major issues have been satisfactorily addressed, making an editorial decision anything but straightforward. As you will see from the reports I am enclosing below, the referees do not feel that your new experiments have been able to clarify the mechanisms underlying the PSD95-PTEN-glutamate receptor connection at the synapse - in fact, they indicate that some of the new data may have even confounded the original interpretations. Appreciating how much work has gone into this already very comprehensive study, and noticing the strengths of other parts of the study (especially towards physiological functions of PTEN in LTD, although their relation to the mechanistic data remains not fully clear), I would at this stage like to give you the opportunity to respond and comment on the referees' opinions and criticisms, before taking a final decision on how to proceed further with this manuscript. It would be helpful in this respect if you could also propose/imagine a limited number of further experiments to tackle some of the most pertinent points (e.g. the alternative IP approaches proposed by referee 1?). Depending on the contents of your response, which I may possibly discuss further with an expert editorial advisor or one of the referees, I hope we will then be able to reach an informed decision on your revised manuscript.

I am looking forward to receiving your comments.

Best regards,

Editor
The EMBO Journal

Referee 1 - comments on revised version

This is a revised ms, which I previously reviewed. The title is "PTEN is recruited to the postsynaptic terminal for NMDA receptor-dependent long-term depression". As described in the original review, PTEN is a signaling molecule and could induce many phenotypes including altered synaptic development and plasticity observed in PTEN KO heterozygous or knockout mice previously published. However, mechanisms for altered synaptic transmission and plasticity remain unclear. Therefore, a direct link between PTEN and AMPAR-mediated synaptic transmission is interesting. However, the molecular mechanism of LTD and physiological relevance remain uncertain in this revised manuscript.

Related to #1,2 in my comments, one of main finding "regulated interaction between PSD-95 and PTEN" is still unclear. In the original ms, I requested to show specificity of PTEN and PSD-95 interaction because of two reasons. A, this molecular mechanism is novel. B, proteins containing PDZ binding domains bind to PDZ domain proteins in transfected cells, but not necessarily in vivo. For example, historically so many proteins are reported as PSD-95 binding proteins including NMDARs. Indeed, authors agreed that overexpressed PTEN behaved differently from endogenous PTEN in the comment #3. Therefore, a result showing specific interaction of endogenous proteins is one of key experiments. In the rebuttal letter, authors described difficulty in PSD-95 antibody for immunoprecipitation. However, authors can use alternative approach, anti NMDAR antibody etc. In addition, authors can perform silver staining of immunoprecipitants with anti PTEN antibody used. As I stated, "PTEN is a signaling molecule and could induce many phenotypes", and lack of molecular mechanism will diminish impact and significance of this paper.

Related to #3,4 in my comments, another main finding is "LTD regulates PTEN interaction with PSD-95 to reduce AMPA receptor activity. This molecular link also remains unclear. In the original review, I asked to show requirements of synaptic PTEN for regulation of AMPAR-mediated transmission using PTEN-dPDZ. Unexpectedly, authors found PTEN-dPDZ depressed synaptic AMPA receptor as PTEN wild type. This result indicates that overexpressed PTEN is artifact as authors described. Therefore, I don't see any evidence indicating synaptic localization/activity of PTEN, possibly regulated by PSD-95 interaction, is required for LTD as the title indicated.

Referee 2 - comments on revised version

Authors: In this revised manuscript, the authors have nicely addressed most of my points by providing a significant amount of new data and discussion. It is unfortunate the IP of PSD95 did not work. The remaining concern relates to how the story comes together and the conclusions. The initial experiments look at the NMDA-dependent association of PTEN and PSD95; this association relies on the PDZ binding interaction (which is only examined with total protein). The surprising result is that both PTEN and deltaPTEN reduce the AMPA currents - according to the model, I expected deltaPTEN to have little effect, since this protein is unable to bind PSD95. As the authors suggest, this could be due to simple diffusion into the spine but this makes the conclusion of 'regulated' interaction more problematic since these two forms of PTEN seem to affect AMPA receptors. Also, if deltaPTEN readily diffuses into the spine to modulate AMPA, then deltaPTEN-C124S should also do the same - yet only PTEN-C124S interferes with LTD. This demonstration of the down-regulation of AMPA receptors in Figure 5 is critical for directly implicating PTEN mechanistically in LTD for Figure 6, but with the new data the interpretation is not straightforward. These gaps in the experiments need to be addressed. The additional slice physiology experiments are very nice and convincingly show that PTEN activity is important for LTD but not LTP or m-GluR LTD - this is one of the strengths of the manuscript. The issue here is the specificity of PTEN, which can affect many different proteins, and whether these changes are due to the regulated association of PTEN with PSD95 - there do not seem to be any experiments that address this in slices with 1Hz stimulation. Therefore, the slice physiology results are significant but do not have definitive links with the first half of the paper using NMDA and co-IP data. The authors, on the other hand, conclude "NMDAR activation triggers a PDZ dependent association between PTEN and the synaptic scaffold, which anchors PTEN at the postsynaptic terminal... and ... this PDZ-dependent interaction is required for PTEN's action during LTD". This over states the conclusions since the links between 1 Hz stimulation, NMDA-R activation, PTEN association with PSD95, and decrease in AMPA currents are more speculative. The authors need to provide a more balanced discussion, which includes some of the gaps in their experiments and does not over state the results.

Additional correspondence 13 May 2010

I sincerely appreciate the opportunity you are offering us to address the remaining concerns of the reviewers. I believe you appreciate that this manuscript reports a truly novel and relevant study on

the role of PTEN in synaptic plasticity. Particularly, we show that PTEN is involved in a specific form of synaptic plasticity, namely NMDAR-dependent LTD, and that it does so via its recruitment to the postsynaptic scaffold. Therefore, we are providing not only physiological data, but also mechanistic information on how PTEN is regulated during plasticity. This dissection of a specific function of PTEN at synapses is unprecedented in the field. We are glad that the three reviewers basically have agreed with these conclusions.

As reviewer #3 had no further concerns, and reviewer #2 suggests mostly revisions in the Discussion section, the pending issue is the comment from reviewer #1 on the specificity of the interaction between PTEN and PSD-95. In order to further address this remaining concern, we have planned new immunoprecipitation experiments with another battery of antibodies obtained from Richard Huganir's laboratory. There is no guarantee that these antibodies will work. However, as they were raised against different regions of PSD-95, there is a possibility we will still find an adequate one. We will also pursue the immunoprecipitations with NMDAR antibodies, as suggested by reviewer 1. We are also willing to provide a more balanced discussion, as requested by reviewer 2, in which the role of a specific PSD95-PTEN interaction is proposed, rather than established.

Nevertheless, I would like to point out a perhaps misleading observation from reviewer 1. In the second paragraph of his/her comments, it is stated: "Therefore, a result showing specific interaction of endogenous proteins is one of the key experiments". We do already demonstrate interaction between endogenous proteins (Figure panels 1A to 1E). In addition, we present data for the recruitment of endogenous PTEN to the postsynaptic scaffold, using biochemical (Fig. 3) and electron microscopy (Fig. 4) methods. Therefore, we believe we are providing strong mechanistic information that supports our physiological evidence for the role of PTEN in LTD. Indeed, within the context of the novel functions and regulations we are reporting for PTEN, we consider that the particular interaction between PSD-95 and PTEN is a rather minor point, which can be accordingly reflected in a more balanced discussion, as requested by reviewer 2.

Please let us know whether you consider this experimental plan appropriate, and we will go ahead with the new experiments. We certainly appreciate your help and advice in this matter.

2nd Editorial Decision 16 May 2010

Thank you for your response to the reviews on your revised manuscript. After careful consideration of your points and of the concerns brought up by the referees, I have decided to allow for an exceptional additional round of major revision in this case, to allow you to deal with the most pressing outstanding issues. Your suggestions how to address them sounds reasonable to me, and I would specifically encourage you to direct your efforts to the proposed co-IP experiments with anti-NMDAR antibodies, as well as tests with additional PSD-95 antibodies as also proposed. With respect to discussion and presentation of the conclusions, especially regarding the issues of causality and/or disconnect between the mechanistic and physiological data, I also agree that following the guidelines suggested by referee 2 should be fine. Therefore, I hope you will be able to resubmit a re-revised manuscript as soon as possible, using the hyperlink below. I would appreciate if you could kindly keep me informed of the progress of the additional revision work. And of course, please don't hesitate to get back to me should you require feedback on any other issue connected to this revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS:

(already provided – see above)

2nd Revision - authors' response 22 June 2010

We are pleased to send you this revised version of our manuscript, in which we have addressed the remaining concerns of the reviewers. Specifically, we have carried out new immunoprecipitation experiments to evaluate the specificity of the enhanced interaction between PSD-95 and PTEN versus other PDZ partners. We also provide a more balanced discussion to acknowledge the uncertainties in the causal relationship between the regulated association of PTEN to the synaptic scaffold and its role in LTD. Please find below our detailed response to the reviewers.

Referee #1

The most important remaining concern for this referee was the specificity of the regulated interaction between PSD-95 and PTEN. To address this issue, it was necessary to immunoprecipitate PSD-95 after LTD induction and evaluate whether the interaction with other PDZ partners, besides PTEN, was also affected. After testing a battery of anti-PSD-95 antibodies, we have found one that immunoprecipitates PSD-95 without interfering with its PDZ-dependent interactions. A limited amount of this antibody was made available from Richard Huganir's laboratory. As shown in the new Supplementary Fig. 1A (upper panel, anti-PTEN), immunoprecipitation of endogenous PSD-95 from hippocampal slices co-precipitated a small amount of endogenous PTEN, which roughly doubled by 15 min after NMDA application and then gradually declined by 25-60 min. This enhanced interaction required NMDA receptor activation, as it was blocked by the NMDA receptor antagonist AP5 (right-most lanes). These results are consistent with our previous immunoprecipitations using anti-PTEN antibodies (Fig. 1A-E).
Most notably, the enhanced interaction with PTEN was accompanied by a simultaneous decrease in the association of PSD-95 with NMDA receptors (Supplementary Fig. 1A, lower panel, anti-GluN1). That is, the NMDA receptor-PSD95 complex was present under basal conditions, but it reversibly dissociated upon NMDA application. Nevertheless, we should also mention that the decrease in the interaction between PSD95 and NMDA receptors was not consistently observed. For example, in the experiment shown in panel B, the enhanced interaction between PSD95 and PTEN was again visible, whereas the PTEN-NMDA receptor complex remained unaffected. However, we should emphasize that the enhanced interaction with PSD95 was specific for PTEN, and never observed for the PSD95-NMDA receptor complex.
In summary, these new data strongly reinforce our interpretation that NMDA receptor activation specifically regulates the interaction between PSD95 and PTEN, and not with other PDZ-dependent partners. To note, these new experiments were carried out by immunoprecipitating endogenous proteins from hippocampal slices.

Referee #2:
As discussed above, we have finally managed to immunoprecipitate the PSD-95/PTEN complex using an anti-PSD-95 antibody generously provided by Richard Huganir's laboratory. Using this antibody, we have been able to demonstrate that the enhanced interaction of PSD-95 upon NMDA application is specific for PTEN versus another PDZ-dependent partner, such as the NMDA receptor (new Supplementary Fig. 1).
The referee also points out to the fact that the PTEN mutant lacking the PDZ motif (PTEN- PDZ) is able to depress AMPA receptor responses (Fig. 5G-I), yet its catalytically inactive counterpart (PTEN-C124S- PDZ) cannot interfere with LTD (Fig. 8). We interpret this result as if overexpressed proteins are able to access the synaptic membrane in the absence of PDZ-dependent interactions (this is why the PTEN- PDZ depresses transmission). Nevertheless, in order to act as a dominant negative for LTD expression, the catalytically inactive mutant has to compete for relevant interactions with endogenous PTEN. We believe that PDZ-dependent interactions are the basis for this competition. Hence, overexpressed PTEN-C124S- PDZ is able to reach spines d presumably the synaptic membrane, but it does not interfere with endogenous PTEN during LTD because it is unable to engage in PDZ-dependent interactions.
Nevertheless, we acknowledge that the mechanistic connection between PDZinteractions and the physiological role of PTEN in LTD is rather indirect. We have now modified the revised version of the manuscript throughout the text to avoid overinterpreting our results. The specific places in the text where changes have been introduced are:

Abstract, line 9; page 4, last paragraph, line 5; page 16, heading of the second paragraph; page 17, line 6; page 17, end of second paragraph; page 18, second paragraph, line 1; page 19, end of third paragraph; page 32, title of Figure 8 legend.

