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PICK1 inhibition of the Arp2/3 complex controls dendritic spine size and synaptic plasticity

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

1st Editorial Decision 16 June 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are enclosed. As you will see, their recommendations vary widely: referee 2 finds the conceptual advance over your previous NCB paper to be limited and is not supportive of publication, while referees 1 and 3 are overall more positive, although still raise major concerns with the study in its current form. Both these referees highlight in particular the need to provide evidence as to how this function of PICK1 is regulated during LTD: you have previously shown that PICK1 activity is modulated by calcium, but do not incorporate this into your current study at all. The referees also highlight significant concerns as to the hippocampal data - both on a technical front, and also because over-expression of wild-type and mutant PICK1 give similar phenotypes. Constructive suggestions are made as to how to improve and extend these data and I would strongly encourage you to follow these.

Given the interest expressed by referees 1 and 3, we are willing to over-rule the negative recommendation of referee 2 and to invite you to submit a revised version of your manuscript. However, I would like to make clear that, for a positive outcome, you will need to significantly revise and extend your manuscript along the lines suggested by the referees - particularly to address the major criticism as to how the PICK1-Arp2/3 interaction is regulated and how this impacts on plasticity. I do understand that this will likely require a large amount of work, and recognise that you may prefer instead to take the manuscript elsewhere at this stage; in which case, please let me know. However, if you do feel able to undertake such an analysis, we would be happy to see a revised version.

I should add that it is EMBO Journal policy to allow only a single round of revision. Acceptance of your manuscript will thus depend on the completeness of your responses included in the next, final

version of the manuscript. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as a standard revision time, and as a matter of policy, we do not consider any competing manuscripts published during this period as negatively impacting on the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

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):

Pick1 is a synaptic scaffolding protein containing a PDZ domain that interacts with PKC-alpha, GluR2/3, mGluR7, and dopamine transporter, a BAR domain that interacts with phosphatydil inositol monophosphate, and acid-rich region.

Hanley's group previously found that Pick1 directly interacts with Arp2/3 complex and inhibit its activity to induce actin polymerization. They identified single amino acid W413 involved in the interaction. Using this mutant in combination with shRNA against Pick1, they showed that inhibition of Arp2/3 by Pick1 is required for normal development of dendritic arbors and NMDA-induced endocytosis of GluR2. Also, the activity of Pick1 to inhibit Arp2/3 is enhanced by interaction with GluR2.

In the new paper submitted to EMBO J., Nakamura et al. extended their analysis to dendritic spine structure and LTD. When they overexpressed Pick1 or Pick1 W413 mutant in GFP-actin expressing neurons, they found that the size of dendritic spine was increased when they expressed W413 mutant while decreased when they expressed WT. Downregulation of Pick1 with shRNA increased the size of spines, which was rescued by WT but not W413A mutant Pick1. This is consistent with the role of Pick1 as an inhibitor of Arp2/3. Furthermore, this interaction is required for NMDA-receptor dependent shrinkage of dendritic spines. Overexpression of Pick1 enhances AMPA receptor mediated synaptic transmission and reduces inhibits LTD. The mutant, in contrast, reduced the transmission and blocked LTD.

The authors concluded that Pick1 is a novel regulator of spine dynamics.

Overall, I feel this paper is interesting in proposing that Pick1 is involved in regulation of Arp2/3, which in turn regulates dendritic spine structure and synaptic response. There are several major issues, though.

- This paper heavily relies on single point mutation construct Pick1W413A to draw conclusion that Pick1-Arp2/3 complex formation is important. To complement this, a mutation on Arp2/3 side is very important to test if they can see if the interaction is specific.

- It is very unclear when this interaction happens, in other word, whether it is constitutive or activity dependent. This is very important to understand the data of Figure 6. As is now, the change in plasticity may be secondary to the changes caused by the overexpression of mutant. For example,

the mutant reduces the synaptic response. Under such condition, even the cells were depolarized at -40 mV, it may not allow enough Ca2+ in to the cell. If this is a case, one cannot conclude that Pick1-Arp2/3 interaction is involved in LTD process.

- It is an intrigueing possibility that Pick1 is a Ca2+ sensor as suggested by the senior author previously. This view may be integrated into this paper, which will highly strengthen the relevance of Pick1 in the activity induced shrinkage of dendritic spine and removal of AMPA receptor.

Minor comments

Figure 1. T-test and KS-test are used here and the rest of the study. I do not think they are appropriate for testing multiple data sets, although there exist many papers errorneously use them.

Overall, the electrophysiology is conducted very poorly as follows. The authors should carefully go through all data. This reviewer will not accept simple replacement of the sample traces, if this paper comes back for re-review.

Figure 6A. From the sample trace, I think the recording condition is not best. It appears that the Rs is changing. The authors should show average Rs and Ri in the graph and if they notice any unstable recording, they should reject those experiments and if needed, repeat experiments.

P9 line 4 from bottom. "In contrast, neurons..." and Figure 6C.
From Cii, the W413A mutant seems to reduce the synaptic transmission. The authors made only 8 recordings for this. Considering the variability of this assay, they should double or triple the N.

Figure 6D. Again, I doubt the author's recording condition. The transmission does not go back to the baseline completely. This is more severe in +40 mV. This indicate either NMDA-R component is remaining and/or the slice are bursting. Indeed, the authors did not use 2Cl-adenosine, which is often used in slice culture system to prevent the bursting. The sample trace for WT pick1 shows very fast decay. I doubt if this is a recording from a pyramidal cell. There are interneurons in PC layer which shows AMPA receptor kinetic like this but it can be distinguished from pyramidal cells from capacitance current, which shows a rapid decay. Interneurons typically lacks GluR2 and of course if the authors include interneurons, it will show small rectification index.

Figure 6E. Sample trace for control. Here again, the trace looks strange for AMPA receptor.

In the previous paper, the authors found that the Pick1 overexpression changes the arborisation of dendrite. This may change the total number of synapse formed on the dendrite and change the synaptic response. The authors should take this in to account.

P12 "We propose a model whereby during LTD, PICK1 strongly inhibits actin polymerisation when bound to GluA2 to drive the removal of surface AMPARs, and independently has a less localised effect on F-actin levels to shrink spines."
Interaction with other proteins such as PKCalpha, mGluR, dopamine transporter and pick1-Arp2/3 serves may also show similar change. In fact, GluR2 may not be very abundant protein as it is membrane protein compared with PKC, which is soluble protein. Do authors have evidence that other proteins are not involved in this process?

Referee #2 (Remarks to the Author):

Nakamura et al (PICK1 inhibition of the Arp2/3 complex controls dendritic spine size and synaptic plasticity) study the role of PICK1 in spine morphology and synaptic plasticity, in particular during hippocampal LTD. They show by knock-down and overexpression experiments that PICK1 regulates spine size via inhibition of the Arp2/3 complex. Mechanisms regulating spine actin dynamics is a topic of current and wide interest, and such efforts are therefore timely.

Although the manuscript is nicely written and the experiments are well performed, this study is an unambiguous follow up on the Rocca et al., study in Nature Cell Biology two years ago "Inhibition of Arp2/3-mediated actin polymerisation by PICK1 regulates neuronal morphology and AMPA

receptor endocytosis". This manuscript focuses more on structural (spine morphology) and functional (electrophysiology) experiments, however the same interaction is studied (PICK1-Arp2/3) using the same reagents/constructs. While reading through the discussion section I was wondering what is really new about the findings in the study. 1/ It is not really surprising that PICK1 as an inhibitor of Arp2/3 in spines regulates spine size (Fig. 1,2). Depletion of several Arp2/3 complex activators, including WAVE, WASP, Abi2 alter the morphology of spines (Haeckel et al., JNS 2008; Kim et al., Nature 2006; Soderling et al., JNS 2007; Wegner et al., JBC 2008). 2/ The data showing that PICK1 regulates spine size independently of GluR2 trafficking is nice but not shocking (Fig. 3). Several other studies have already challenged the idea that GluR2 itself is not involved in spine morphogenesis. 4/ The function of PICK1 in mediating LTD-induced spine shrinkage is interesting and completely novel (Fig. 4). 5/ The data showing that PICK1-Arp2/3 interactions are involved in synaptically-induced LTD in hippocampal CA1 (Fig, 6) is striking but again not fully unexpected given the role of PICK1 in AMPA receptor internalization (Rocca et al., NCB, 2008). Moreover, similar results were shown by a recent study from the Huganir lab using PICK1 knock-out mice (Clem et al., JNS, 2010).

Overall, the authors have put together what seems to be at first side an interesting story, but to my taste the new data are too much an extension of the Rocca et al., study. Most of the results were expected and others confirm the conclusions from their previous work. In general, most postsynaptic proteins identified up-to-date seem to both regulate synaptic plasticity and spine morphology. Therefore there is nothing wrong with the author's conclusion that PICK1 plays a central role not only in regulating AMPAR trafficking and functional plasticity (Rocca et al. NCB 2008), but also in controlling structural plasticity of dendritic spines (this study) by modulating Arp2/3-mediated actin polymerization. However, in my opinion more is needed to publish these data in a high profile journal, such as EMBO J.

Minor/technical concern:
It is surprising that a ~50% decrease in PICK1 expression (Figure S1; which is comparable with a heterozygous PICK1 knock-out mice) has such a strong phenotype in culture (e.q. increase in spine size Figure 2). Since the heterozygous mice don't seem to have a phenotype, what is the author's explanation? Did they ever tested other shRNAs or looked at spine morphology in shRNA treated hippocampal slices?

Referee #3 (Remarks to the Author):

This manuscript addresses a potentially important regulatory mechanism by which interaction of PICK1 with the Arp2/3 complex regulates activity-dependent remodeling of dendritic spines and synaptic plasticity. In a previous paper, the same research group found that PICK1 inhibits potently Arp2/3 complex-mediated actin polymerization, neuronal morphology and AMPA receptor endocytosis. In this new study, the authors suggest that PICK1 inhibition of the Arp2/3 complex controls dendritic spine size and hippocampal LTD.
Although the authors deal with potentially important observations, the experimental results, especially the ones arguing the role of PICK1 inhibition of Arp2/3 complex in regulation of hippocampal LTP, should be re-designed to strengthen the conclusions of the manuscript. In addition, several technical concerns should be addressed.

Major points

1. For hippocampal LTD experiment, the authors used overexpression strategy (wild-type of PICK1 versus W413A mutant) to address of the role of PICK1 interaction with Arp2/3 complex in hippocampal LTD. Unfortunately the authors observed the same phenotype in both wild-type and mutant PICK1-overexpressed neurons. It turned out that overexpression of wild-type PICK1 changed basal synaptic transmission. Thus, the interpretation of those results involves assumptions and the author's conclusion has not been supported clearly. Because a previous paper (Terashima et al., Neuron (2008) 57, 872-882) showed that knock-down with shRNA or knock-out approach did not change basal synaptic transmission, I suggest that the authors adopt the same approach in conjunction with add-back approach (wild-type versus mutant) to strengthen their conclusion.

2. In this manuscript, the neuronal activity-dependent interaction between PICK1 and Arp2/3

complex is an important biochemical parameter to be observed but is not shown. Authors should address NMDA-dependent physical interaction between PICK1 and Arp2/3 complex by Co-IP experiment, for instance in Figure 4.

Minor points

1. In Figure1, authors need to characterize spine length as well. Because the shrinkage of spines is likely to involve changes of spine length in addition to spine head size, it would be important to add the information.
2. Although authors say that the results with IRES-actinEGFP and IRES-EGFP constructs are the same, the cumulative frequency curves from Fig 1 (IRES-actin-EGFP) and Supplementary Figure 1A (IRES-EGFP) look different. Please clarify this point.
3. In the text and legend for Figure 1, the authors used *P<0.05, but ** is used for the graph of Figure 1. Please clarify this point.
4. In Figure 2B, the authors should describe the level of PICK1 after add-back overexpression of wt or W413A.
5. In general, images with actin-EGFP are different from images with EGFP. Signal of actin-EGFP is relatively enriched in dendritic spines than in dendritic shaft as shown in most representative images. However, some of the representative images used in this manuscript, especially Figure 4D, are not likely images from actin-EGFP. Instead those are likely the images of neurons expressing a volume marker such as EGFP. Authors should clarify the identity of the signals in each Figure.

1st Revision - authors' response 22 October 2010

In our revised manuscript, we have addressed all of the referees' concerns, and have added a considerable amount of new data, particularly in figure 1, figure 7, figure 8, and supplementary figure 3.
To incorporate these data, it has been necessary to increase the size of the manuscript to include 8 main figures, and 3 supplementary figures.

Response to Referees:

Referee 1

1. "This paper heavily relies on single point mutation construct Pick1W413A to draw conclusion that Pick1-Arp2/3 complex formation is important. To complement this, a mutation on Arp2/3 side is very important to test if they can see if the interaction is specific."
The referee is concerned about the use of the single point mutation in PICK1 to define a role for Arp2/3 interactions, and asks for further evidence that PICK1 is exerting its effect on spine size via the Arp2/3 complex. She/he suggests mutating Arp2/3 to block its interaction with PICK1.

We agree with the referee that, in principle, manipulation of each of the interacting proteins in turn would strengthen the conclusions. However, such an experiment is not feasible in this particular case because we have demonstrated previously that PICK1 competes with N-WASP for binding to the Arp2/3 complex (Rocca et al., 2008), indicating that they share the same binding site. Therefore, mutating Arp2/3 would interfere with regulation via N-WASP as well as PICK1, severely limiting any conclusions that could be drawn.

To circumvent this problem but still comply with the referee's overall suggestion we designed an alternative experiment, which we believe provides compelling evidence that PICK1 regulates spine size via inhibition of the Arp2/3 complex. The CA domain of N-WASP is known to inhibit Arp2/3 activity (Rohatgi et al, 1999), and overexpression of this peptide has been shown to lead to spine shrinkage in cultured neurons (Haeckel et al, 2008). If PICK1 is exerting its effects via Arp2/3 inhibition, then spine shrinkage induced by N-WASP CA should occlude that induced by PICK1 overexpression. On the other hand, if PICK1 functions by some other mechanism, additive effects would be expected, ie PICK1 and CA co-expression would lead to greater effects than either one

alone.
As shown in the revised Figure 1c, we find that, as shown previously (Haeckel et al, 2008), N-WASP CA expression results in spine shrinkage. Co-expression of PICK1 leads to no further reduction in spine size, indicating that PICK1 is acting via the same pathway, ie Arp2/3 inhibition.

Furthermore, evidence that the W413A mutation specifically influences binding to the Arp2/3 complex is provided in the supplementary figures of our previous study (Rocca et al, 2008), which demonstrate that W413A-PICK1 binds GluA2, GRIP, PKC, Actin, / -SNAP, and phospholipids in a manner that is indistinguishable from WT-PICK1.

2. "It is very unclear when this interaction happens, in other word, whether it is constitutive or activity dependent. This is very important to understand the data of Figure 6. As is now, the change in plasticity may be secondary to the changes caused by the overexpression of mutant. For example, the mutant reduces the synaptic response. Under such condition, even the cells were depolarized at -40 mV, it may not allow enough Ca2+ in to the cell. If this is a case, one cannot conclude that Pick1-Arp2/3 interaction is involved in LTD process."

The referee suggests that the PICK1-Arp2/3 interaction may be regulated during LTD.

This is an important experiment for this study, and we are grateful to the referee for suggesting it. We have carried out co-IPs from cultured neurons exposed to the chemical LTD protocol, and we find that the interaction between PICK1 and Arp2/3 is indeed regulated by NMDA receptor activation. Ten minutes following the start of the NMDA stimulus, PICK1 binding to Arp2/3 is enhanced approximately two-fold compared to untreated cultures. These data have been added as Figure 8A. This experiment strongly suggests that following LTD induction, Arp2/3 inhibition via PICK1 is transiently enhanced, providing the necessary trigger to drive spine shrinkage.

3. It is an intrigueing possibility that Pick1 is a Ca2+ sensor as suggested by the senior author previously. This view may be integrated into this paper, which will highly strengthen the relevance of Pick1 in the activity induced shrinkage of dendritic spine and removal of AMPA receptor.

The referee suggests that PICK1 may be acting as a calcium sensor in this process, presumably by mediating a calcium-sensitive interaction with Arp2/3.

PICK1 binds calcium ions with a KD of around 10 μM, and binds GluA2 in a calcium-sensitive manner with maximal binding at 15 μM [Ca2+]free, a property that is required for NMDA-induced AMPAR internalisation (Hanley & Henley, 2005). Therefore, we tested the possibility that the PICK1-Arp2/3 interaction is also sensitive to [Ca2+] free, and carried out Arp2/3-PICK1 co-IPs in a range of physiologically relevant [Ca2+] free. From these experiments, PICK1 binds Arp2/3 equally well in all [Ca2+] free tested, suggesting that the PICK1-Arp2/3 interaction is not calcium-sensitive. These data have been added as figure 8B.
This observation suggests that an alternative signaling pathway regulates the PICK1-Arp2/3 interaction during LTD, perhaps via kinase/phosphatase activation. We have added such a comment in the discussion section.

Minor points:

1. Figure 1. T-test and KS-test are used here and the rest of the study. I do not think they are appropriate for testing multiple data sets, although there exist many papers errorneously use them.

The referee is concerned that we are using inappropriate statistical tests. Specifically that t-tests and K-S tests should not be used for testing multiple data sets.

Whilst we understand the referee's concerns, we believe that the statistical tests that we have applied in our analysis are entirely appropriate. Multivariate statistical tests are adjustments (due to Bonferonni or Sidak for example) that are made to the P-value of the test to reduce the probability of type I errors (i.e. false positives). However, these kinds of adjustments are based on multiple tests on the same data set, and are well known to be conservative (i.e increase the rate of type II errors, or false negatives) for the typical problems one is trying to address.

In particular, they adjust for the family-wise error assuming that we are looking to compare each subset of our data with each other member, asking the question, "Are any of these subsets different from any others?" without asking specifically which, if any, are actually different.
Importantly, in our analyses, we are always interested in specific null hypotheses such as "Are treatment A and control the same?" and "Are treatment B and control the same?", and our results are always interpreted in this manner. We consequently believe that bivariate statistics, such as the t-test (for normally distributed data), and KS-test (as a non-parametric test), are the most appropriate tests that control both type I and type II errors most efficiently for our data.

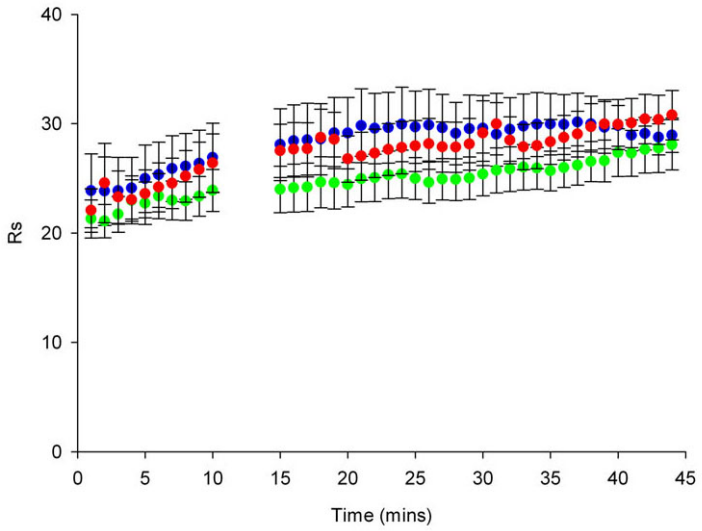
Overall, the electrophysiology is conducted very poorly as follows. The authors should carefully go through all data. This reviewer will not accept simple replacement of the sample traces, if this paper comes back for re-review.

We have addressed each of the specific concerns in turn, below.

2. Figure 6A. From the sample trace, I think the recording condition is not best. It appears that the Rs is changing. The authors should show average Rs and Ri in the graph and if they notice any unstable recording, they should reject those experiments and if needed, repeat experiments.

The referee is concerned that the series resistance (Rs) is unstable in Figure 6A, and suggests we reject those with unstable Rs.

We thank the reviewer for pointing out an omission to our methods section. We routinely discard experiments if Rs changes by >30% during an experiment and this fact is now clearly stated in the methods. We occasionally observe a rundown in EPSC amplitude even with a stable Rs and this is the reason why sometimes in control conditions the EPSC amplitude ends up below the baseline. However, to mitigate this problem we always perform 2 pathway experiments and assess LTD by comparison with the control pathway. We include the average Rs during these experiments for the reviewer in the figure below.



Pooled Series Resistance for LTD experiments
Pooled series resistance vs time for LTD experiments from virally transduced CA1 neurons. Points represent minute averages of series resistance values from EGFP (blue), WT-PICK1 (green) and W413A-PICK1 (red) transduced cells +/- SEM.

3. From Cii, the W413A mutant seems to reduce the synaptic transmission. The authors made only

8 recordings for this. Considering the variability of this assay, they should double or triple the N. The referee believes that the W413A mutant reduces synaptic transmission.

We apologise for a mislabelling of the y-axis in the previous version of Figure 6Cii, which suggested that synaptic transmission is reduced in W413APICK1 expressing neurons, and we thank the referee for highlighting this error. In fact, the W413A mutant does not reduce synaptic transmission as can be seen in figure 6Ci. In order to clarify this situation, we have presented the data in figure 6Cii a slightly different manner. For each condition, we have calculated the relative EPSC amplitude for transduced cells compared to neighbouring untransduced cells, and expressed this as a normalised value. We feel that this is a clearer way of presenting the data.
Given that the W413A mutant does not cause any change in synaptic transmission, it is unlikely that further experiments will change this result and so we do not feel this is necessary.

4. Figure 6D. Again, I doubt the author's recording condition. The transmission does not go back to the baseline completely. This is more severe in +40 mV. This indicate either NMDA-R component is remaining and/or the slice are bursting. Indeed, the authors did not use 2Cl-adenosine, which is often used in slice culture system to prevent the bursting. The sample trace for WT pick1 shows very fast decay. I doubt if this is a recording from a pyramidal cell. There are interneurons in PC layer which shows AMPA receptor kinetic like this but it can be distinguished from pyramidal cells from capacitance current, which shows a rapid decay. Interneurons typically lacks GluR2 and of course if the authors include interneurons, it will show small rectification index.

The referee is concerned that either there is an NMDAR component to our recordings, or our slices are bursting.

The use of 2Cl-adenosine is prevalent in recordings from organotypic slice cultures that are kept for longer than 5 days but we have found it unnecessary in our cultures, which are acutely prepared and then only kept for 24 hours (see also Terashima et al 2004, 2008). We do occasionally observe polysynaptic activity in the slices but we always discard experiments where this prevents us from reliably measuring the peak monosynaptic EPSC amplitude.
The traces in Figure 6D show a shorter time period than those in Figures 6A-C in which currents do return to baseline. The nature of recordings from these 24 hour cultured slices is that EPSCs are generally smaller and therefore noisier than those in acute slices (Terashima et al., 2004). This accounts for the variability in the appearance of our example traces. However, on the positive side, they do not suffer from the high levels of bursting activity found in longer term organotypic cultured slices. We use D-AP5 at a concentration of 50 M to exclude the possibility of any remaining NMDAR-mediated component of the EPSC at +40mV.

He/she also suggests that the change in rectification we observe may be a result of recording from interneurons rather than pyramidal cells.

The increased AMPAR EPSC rectification following WTPICK1 overexpression we observe is entirely consistent with previous reports (Terashima et al., 2004, 2008). It is highly unlikely that we are specifically recording from interneurons infected with WT-PICK1 instead of pyramidal cells because the same viral vector is used for the delivery of WT-PICK1, W413A-PICK1 and EGFP, and the position of infected and recorded cells in the slices was consistent for all conditions, in all experiments. Therefore, the observed change in rectification only with WT-PICK1 overexpression, but not with W413A or EGFP controls represents a specific effect of WT-PICK1 overexpression in pyramidal neurons.
We accept that our traces show a range of kinetic profiles (eg. the WT-PICK1 traces in figures 6B and 7 show a more normal decay), for the reasons discussed in the response to the previous point, above. We originally chose the example for 6D because it clearly shows the rectification change. The referee points out that the kinetic profile of this trace may lead readers to question our recording conditions, so we have replaced the example trace in 6D. We feel that this is reasonable given our specific responses that address the referee's concerns.

5. In the previous paper, the authors found that the Pick1 overexpression changes the arborisation of dendrite. This may change the total number of synapse formed on the dendrite and change the synaptic response. The authors should take this in to account.
The referee states that in our previous paper, we showed that PICK1 overexpression changes

dendritic arborisation.

In fact, this is not the case; we found that PICK1 knockdown in young neurons (and little effect in more mature neurons) results in aberrant dendritic branching, but we saw no such effect of PICK1 overexpression (Rocca et al., 2008).

6. Interaction with other proteins such as PKCalpha, mGluR, dopamine transporter and pick1-Arp2/3 serves may also show similar change. In fact, GluR2 may not be very abundant protein as it is membrane protein compared with PKC, which is soluble protein. Do authors have evidence that other proteins are not involved in this process?
The referee suggests that additional PICK1 interactors may be involved in the process of spine shrinkage. In particular she/he mentions PKC as a likely candidate.

This is certainly an insightful point, since PICK1 binds a number of additional proteins, including PKC. PICK1 specifically binds activated PKC and is involved in targeting this enzyme to dendritic spines (Perez et al, 2001). Furthermore, PKC activation has been shown to reduce spine size (Calabrese & Halpain, 2005). We therefore tested whether PKC inhibition inhibits PICK1-induced spine shrinkage. We found that pharmacological inhibition of PKC using chelerythrine has no effect on spine shrinkage induced by PICK1 overexpression. These data have been added as Supplementary Figure 3A.
In addition, we investigated whether PICK1 is involved in spine shrinkage induced by PKC activation. Using time-lapse live cell imaging, we confirmed previous reports that activation of PKC by PMA leads to a significant reduction in spine size. Knockdown of PICK1 expression by shRNA has no effect on this process, indicating that PICK1 is not required for PKC-induced spine shrinkage. These data have been added as Supplementary Figure 3B.
Taken together, these two experiments indicate that PKC is not involved in PICK1-induced spine shrinkage. The referee is correct in pointing out that other PICK1 interactors may play a role, and we have added a note in the discussion to this effect. However, to test all known interactors would be beyond the scope of this study.

Referee 2

The referee's main concern is that much of our data is "not really surprising", "not shocking", "not unexpected". We find this an unusual criticism, and can think of many papers published in high-profile journals that could fit this description. We feel that the important issue is to demonstrate a novel, important finding in a convincing manner. Indeed, this referee points out that "the function of PICK1 in mediating LTD-induced spine shrinkage is interesting and completely novel", and also that "the manuscript is nicely written and the experiments are well performed".

The referee mentions that a number of Arp2/3 activators have been identified as playing a role in spine morphology. Our paper is, to our knowledge, the first Arp2/3 inhibitor to be implicated in this process. Hence we feel our findings represent an important conceptual advance in our knowledge about the mechanisms that underlie spine dynamics.

The referee states that similar results were recently shown in Clem et al., 2010. This paper is completely different from our current study, so we are very confused about why the referee is making this comparison. This paper does not address the role of the PICK1-Arp2/3 interaction at all. It describes the role of PICK1 in regulating insertion of GluA2-lacking AMPARs in barrel cortex following alterations in whisker stimulation.

Minor point:

The referee is surprised that a 50% knockdown of PICK1 levels has such a profound effect on spines, and also states that the PICK1 knockout mouse has no spine phenotype.

As far as we are aware, dendritic spine size has not been analysed in PICK1 knockout mice. The referee does not provide a reference for this, but in the paper where this mouse was characterized, no such analysis was carried out (Steinberg et al, 2006).
Significant effects on dendritic spine size following a similar level of knockdown of the protein APT1 has been shown previously (Siegel et al, 2009).

Referee 3

Major Points:
1. For hippocampal LTD experiment, the authors used overexpression strategy (wild-type of PICK1 versus W413A mutant) to address of the role of PICK1 interaction with Arp2/3 complex in hippocampal LTD. Unfortunately the authors observed the same phenotype in both wild-type and mutant PICK1-overexpressed neurons. It turned out that overexpression of wild-type PICK1 changed basal synaptic transmission. Thus, the interpretation of those results involves assumptions and the author's conclusion has not been supported clearly. Because a previous paper (Terashima et al., Neuron (2008) 57, 872-882) showed that knock-down with shRNA or knock-out approach did not change basal synaptic transmission, I suggest that the authors adopt the same approach in conjunction with add-back approach (wild-type versus mutant) to strengthen their conclusion.

The referee is concerned about the explanation of why the same LTD phenotype is seen for WT and W413A mutant. The referee states that our interpretation of the data involves assumptions and that some of our conclusions were not fully supported by our results.

In this revised manuscript we have now included extensive additional experiments to further validate our conclusions (see below). Notwithstanding this new confirmatory data, we disagree that the first version of the manuscript was based on assumptions. It has been shown previously (Terashima et al, 2004) that PICK1 overexpression leads to an internalisation of surface GluA2 (resulting in altered basal EPSC properties), and we confirm this result here. Since LTD involves PICK1-mediated GluA2 internalisation, prior internalization of GluA2 as a result of PICK1 overexpression occludes LTD.
We show in the original version of the manuscript that the W413A mutation abolishes the effect of PICK1 overexpression on altered basal EPSC properties, indicating that in W413A expressing neurons, synaptic receptors are unchanged compared to untransfected neurons. We feel that this is a crucial observation, because it demonstrates that the absence of LTD in W413APICK1-expressing cells must represent a bona fide blockade of plasticity.

The referee's suggestion of using a knockdown-and-rescue approach is in theory an excellent idea and we have acted on this suggestion. However, transfection with the rescue constructs results in a higher level of PICK1 expression (approximately 40% higher) compared to untransfected endogenous levels (see Supplementary Figure 2). This "over-rescue" has net effects on spines that are similar to overexpression of WT PICK1 alone (see Figure 2B and also the additional figure 2 for referees, showing that WT-PICK1-rescue occludes NMDA-induced spine shrinkage). Therefore, LTD would also be occluded in WT-PICK1-rescued neurons.

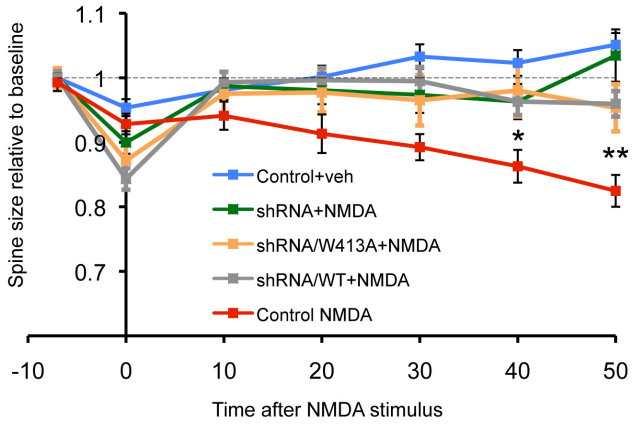


Figure 2 for referees.
Over-rescue by WT-PICK1 following endogenous PICK1 knockdown occludes NMDA-induced spine shrinkage.

Co-expression of sh-resistant W413A-PICK1 does not rescue the blockade of NMDA-induced spine shrinkage induced by PICK1 shRNA. Under basal conditions, co-expression of W413A-PICK1 has no effect on spine size compared to PICK1 shRNA alone (see figure 2B). These data support the conclusion that interaction with the Arp2/3 complex is necessary for PICK1-mediated spine dynamics.

Under basal conditions, co-expression of sh-resistant WT-PICK1 "over-rescues" total PICK1 expression levels, leading to a significant overexpression (see Supplementary Figure 2B). This consequently also over-rescues the change in spine size induced by PICK1 shRNA, resulting in smaller spines compared to controls (see figure 2B).
PICK1 overexpression occludes NMDA-induced spine shrinkage as tested in fixed cells (see figure 4B). Hence in the live cell imaging experiment described here, no change in relative spine size is observed following NMDAR activation, consistent with an occlusion.

Data are from 6-12 neurons, 73-186 spines per condition. *p<0.01, **p<0.001.

To circumvent this problem, we used an alternative strategy that provides additional evidence to support our conclusions. To achieve this, we took advantage of the fact that the effects of PICK1 overexpression on AMPAR trafficking and AMPAR EPSCs are NMDAR-dependent and NMDAR antagonists block the effects of PICK1 overexpression (Hanley & Henley, 2005; Terashima et al, 2008). Therefore, D-AP5-treated WT-PICK1-overexpressing neurons have the normal (same as untransfected) complement of synaptic AMPARs, with normal, non-rectifying EPSCs, as shown by the rectification and amplitude measurements (Figure 7A and B, also see Terashima et al, 2008). This phenomenon allows us to pharmacologically manipulate the activity of overexpressed PICK1 and monitor more acute effects of increased PICK1 expression by analysing the effects of D-AP5 washout on AMPAR EPSCs. Using this approach we observe a very rapid depression in AMPAR EPSC in WT-PICK1 overexpressing neurons in response to D-AP5 washout (and therefore to NMDAR activation). Crucially, this depression is completely blocked by the W413A mutation (Figure 7C).
This result confirms that disrupting the interaction between PICK1 and Arp2/3 blocks synaptic depression in response to NMDAR activation during basal activity conditions.

It is interesting that the acute effect (minutes) of PICK1 recruitment is different from the long-term (overnight) effect. The acute effect results in reduced AMPAR EPSC amplitude (see figure 7), indicating an internalization of GluA1/2 or GluA2/3 receptors. The long-term effect of PICK1 overexpression results in a selective internalization of GluA2 subunit and enhanced AMPAR EPSCs (see figure 6 and Terashima et al, 2004). This suggests that, following the PICK1-dependent

internalization of GluA1/2 or GluA2/3 receptors, GluA2-lacking AMPARs (which have higher conductance) are inserted at later time points.
We have added a section stating this in the discussion.

2. In this manuscript, the neuronal activity-dependent interaction between PICK1 and Arp2/3 complex is an important biochemical parameter to be observed but is not shown. Authors should address NMDA-dependent physical interaction between PICK1 and Arp2/3 complex by Co-IP experiment, for instance in Figure 4.

The referee suggests that the PICK1-Arp2/3 interaction may be regulated during LTD.

Please see response to the same comment from referee 1, above.

Minor points:

1. In Figure1, authors need to characterize spine length as well. Because the shrinkage of spines is likely to involve changes of spine length in addition to spine head size, it would be important to add the information.
The referee suggests analyzing spine length as well as area.

For this analysis, we used the software package NeuronStudio (Rodriguez et al, 2008) that automatically defines spines and provides parameters including length. Spine length is significantly reduced in WTPICK1 overexpressing cells, and significantly increased in W413APICK1 overexpressing cells, indicating that as the referee correctly predicts, PICK1 regulates spine length as well as area. These data have been added to figure 1B.

2. Although authors say that the results with IRES-actinEGFP and IRES-EGFP constructs are the same, the cumulative frequency curves from Fig 1 (IRES-actin-EGFP) and Supplementary Figure 1A (IRES-EGFP) look different. Please clarify this point.
The referee points out a difference between the cumulative frequency curve for figure 1 and Suppl Fig 1A.

The referee is correct in noticing this discrepancy; the cumulative curves in Supplementary figure 1A do not correspond to the histograms. We apologise for this error, and have added the correct cumulative graph that corresponds to the data shown.

*3. In the text and legend for Figure 1, the authors used *P<0.05, but ** is used for the graph of Figure 1. Please clarify this point.*
The referee points out an error in the use of asterisks in figure 1

It should be *p<0.05, and has been corrected in the new figure 1A.

4. In Figure 2B, the authors should describe the level of PICK1 after add-back overexpression of wt or W413A.
The referee asks for a quantification of PICK1 levels following knockdown of endogenous protein and rescue with recombinant sh-resistant WT or W413A.

We have carried out this analysis, and the data are displayed in Supplementary Figure 2B. Expression of shRNA-resistant WT- or W413A-PICK1 in conjunction with knockdown of endogenous PICK1 by shRNA results in PICK1 expression levels that are 40% higher than endogenous PICK1 expression.
Since mCherry is partially excited by the 633 nm laser, we were unable to use three different fluorophores (e.g. mCherry for shRNA, EGFP for rescue constructs and Cy5 for PICK1 immunocytochemistry). Therefore we used GFP versions of the shRNA plasmids in conjunction with the ñIRES-EFGP constructs for rescue, and used Cy3 for PICK1 staining. Independent experiments using different fluorescent reporter constructs verify that >90% of double-transfected cells are positive for both plasmids, hence we assume that GFP-expressing cells express both PICK1 shRNA and rescue constructs.

5. In general, images with actin-EGFP are different from images with EGFP. Signal of actin-

EGFP is relatively enriched in dendritic spines than in dendritic shaft as shown in most representative images. However, some of the representative images used in this manuscript, especially Figure 4D, are not likely images from actin-EGFP. Instead those are likely the images of neurons expressing a volume marker such as EGFP. Authors should clarify the identity of the signals in each Figure.
The referee is concerned about the identity of the GFP signal in Figure 4D.

All the GFP images presented in the main figures are actin^{EGFP}. We have added improved images for figure 4D.

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Terashima A, Cotton L, Dev KK, Meyer G, Zaman S, Duprat F, Henley JM, Collingridge GL, Isaac JT (2004) Regulation of synaptic strength and AMPA receptor subunit composition by PICK1. J Neurosci 24(23): 5381-5390

Terashima A, Pelkey KA, Rah JC, Suh YH, Roche KW, Collingridge GL, McBain CJ, Isaac JT (2008) An essential role for PICK1 in NMDA receptor-dependent bidirectional synaptic plasticity. Neuron 57(6): 872-882

2nd Editorial Decision 19 November 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2010-74769R. I sent your manuscript back to original referees 1 and 3, and have now received the comments from both of them. As you will see, while referee 3 is now satisfied with the revision, referee 1 still has significant concerns with the electrophysiology data. I do recognise that his/her tone is rather harsh here (particularly since some of your co-authors are expert electrophysiologists), but his/her points seem reasonable and would need to be addressed before we can consider publication of your study. The referee also requests further analysis using the CA-WASP construct, and I do agree that this would be valuable if and where possible. I do realise that these experiments can be technically challenging and time-consuming, but I hope that you should be able to address these remaining concerns without too much difficulty. Please don't hesitate to get in touch if you have any questions or comments about this additional round of revision.

I look forward to receiving the revised version of your manuscript.

Best wishes,
Editor

———

REFEREE REPORTS:

Referee #1 (Remarks to the Author):

I am sorry for the authors for delay in response due to a personal issue.

I went through the revised manuscript and see the authors' effort to improve their work. While my opinion that this paper should be eventually published in EMBO Journal remain unchanged, I still see much room to be improved.

Major comments.

1. In response to my comment that the paper heavily relies on W413A mutant, the authors used an additional construct N-WASP CA. This approach is informative but they used this only in the overexpression experiment in Figure. 1. The same construct should be tested more extensively in Figure Figure 4, 6, etc.

2. 3. These pointes are appropriately revised.

Minor comments.

This reviewer is still not satisfied the way they revised electrophysiology experiments. Overall, it is still poor. I wonder if the authors had chance to consult the specialists around them.

1. Statistics. I understand the authors point.
2. About Rs. From the sample trace of Figure 6A control cell, I see the time course of the event changed before and after LTD induction. This is often caused by change in Rs. An experienced electrophysiologist will not use such trace for sample.
3. New Figure 6Ci. Move the Y-axis so that it crosses X-axis at X=0. Also, plot in square and remove average value. The authors should know that the average value does not mean anything as the number of fibers they are stimulating is not identical between the slices, though I admit that there are such publications. The average value is dragged by the pairs which have a large response.
4. Figure 6D. I am still not satisfied the quality of the data. Again, I doubt the authors have sufficient knowledge and skill. Between groups, the traces at 0 mV go either outward (EGFP) or inward (WT-Pick1 and W413A mutant). I believe the latter is typical because voltage clamping is always not perfect. But I do not understand why current flows outward. Maybe GABA component is still remaining. Again, it is not a sample trace an experienced electrophysiologist will use. Also, I suspect there are residual NMDA component as the decay in trace is obviously slower in +40 mV. This is very obvious, for example, in the control trace in WT. This was pointed out in the first

review but the authors reply was that they put 50 uM AP5 and they even do not bother replacing the sample trace. Again I doubt the authors' knowledge in electrophysiological recording and quality control of the data.
5. I understand the author's point.
6. Adequately revised.

New comment:
1. Figure 7B. Again I see problems with recording quality. The sample trace in B appears to be overlap significantly with slow decay of the stimulation artifact and the measurement of rectification is not accurate. Also, this should include the W413A mutant.
2. Figure 7C. The baseline is going down in WT and in mutant. The authors should go back to the individual trace and reject the cells which do not show a stable baseline. If N is not sufficient, the authors should repeat the experiment.

Referee #3 (Remarks to the Author):

The authors have fully addressed the concerns brought up during the first review process. The current manuscript is significantly improved and I do not have any other concern.

Additional correspondence 24 November 2010

Thank you for passing on the referees' comments to us. We were pleased to see that referee 3 now considers our manuscript ready for publication. As you would have guessed, we were less pleased with the latest comments from referee 1, whose tone was indeed rather harsh. Many of the comments from this referee are inaccurate, inconsistent and unreasonable. Jack Mellor, in whose lab the electrophysiology was carried out, is confident that he can address all of the referee's comments regarding the electrophysiology, which we will do in the formal rebuttal.

I also wanted to query the referee's comment regarding the CA domain construct.

In the first review process, the referee asked for more evidence to demonstrate that PICK1 regulates spine size via Arp2/3 inhibition. This was a reasonable suggestion, and the experiment using N-WASP CA presented in figure 1C provides the necessary evidence to address this point.

The referee now suggests that the CA domain construct should be tested further, but does not specify what experiments should be carried out using this construct in figure 4, 6, etc.

Most importantly, the referee does not explain what would be gained by further experiments using this construct. Figures 4 and 6 demonstrate that PICK1-Arp2/3 interactions are involved in NMDA-induced spine shrinkage and LTD. It is hard to see quite how additional experiments using the CA construct would contribute any further information to these figures about the role or mechanism of action of PICK1, having successfully used it to support the conclusion that PICK1 shrinks spines via Arp2/3 inhibition in figure 1C.

It is important to clarify that N-WASP CA domain has been used previously to induce spine shrinkage (Haeckel et al., 2008), so any further characterisation of its action in this regard is unnecessary.

Of course we don't want to carry out redundant experiments. We would be grateful if you could consider our case for accepting this specific aspect of the paper as it stands.

Additional correspondence 26 November 2010

Many thanks for your message regarding the revision of this study. I have to say that I am inclined to agree with you that there would only be a limited benefit to extending the use of the N-WASP CA construct in other experiments - particularly given the earlier paper that you refer to. While clearly there is always an advantage to demonstrating the same results via multiple different approaches, we would therefore not insist on additional analysis using this construct.

I'm glad you feel you can resolve the remaining issues with the electrophysiology data, and I look forward to receiving the revised manuscript in due course.

With best wishes,

Editor

2nd Revision - authors' response 03 December 2010

Response to referees.

Referee 3 states that the manuscript is "significantly improved" and he/she does "not have any other concern".

Referee 1 is of the opinion that "this paper should be eventually published in EMBO Journal", however he/she still sees "room for improvement".

Major comments:
1. In response to my comment that the paper heavily relies on W413A mutant, the authors used an additional construct N-WASP CA. This approach is informative but they used this only in the overexpression experiment in Figure. 1. The same construct should be tested more extensively in Figure Figure 4, 6, etc.

In the first review process, the referee asked for more evidence to demonstrate that PICK1 regulates spine size via Arp2/3 inhibition. This was a helpful suggestion, and the experiment using N-WASP CA presented in figure 1C provides the evidence requested to address this point.
Surprisingly, the referee now suggests that the CA domain construct should be tested further, but does not specify what experiments should be carried out.
We do not see what substantive new information would be gained by further experiments using this construct. Figures 4 and 6 demonstrate that PICK1-Arp2/3 interactions are involved in NMDA-induced spine shrinkage and LTD. It is unclear how additional experiments using the CA construct could contribute any further information to these figures about the role or mechanism of action of PICK1, having successfully used it to support the conclusion that PICK1 shrinks spines via Arp2/3 inhibition in figure 1C.
Furthermore, the N-WASP CA domain has been used previously to induce spine shrinkage (Haeckel et al., 2008), so any further experiments would be confirmatory rather than providing new information.

Minor comments:

This reviewer is still not satisfied the way they revised electrophysiology experiments. Overall, it is still poor. I wonder if the authors had chance to consult the specialists around them.

Jack Mellor trained in the internationally renowned electrophysiology labs of Prof Roger Nicoll (post-doctoral) and Prof Andrew Randall (PhD) and has over 15 years of specialist electrophysiology experience. This is reflected in a large number of high quality publications in

journals such as Nature, Science, Neuron, Nature Neuroscience, Journal of Neuroscience and Journal of Physiology. We are confident that our rigorous experimental and analytical standards are second to none and this is supported by the following list of recent publications:

Buchanan KA, Petrovic MM, Chamberlain SEL, Marrion NV & Mellor JR (2010). Facilitation of Long-Term Potentiation by Muscarinic M1 Receptors is mediated by inhibition of SK channels. Neuron (in press).

Mistry, R., Dennis, S., Frerking, M. & Mellor, J.R. (2010). Dentate Gyrus Granule Cell Firing Patterns Can Induce Mossy Fiber Long-Term Potentiation In Vitro. Hippocampus (in press).

Isaac, J.T., Buchanan, K.A., Muller, R.U. and Mellor, J.R. (2009). Hippocampal place cell firing patterns can induce long-term synaptic plasticity in vitro. Journal of Neuroscience 29, 6840-6850.

Dixon, R.M., Mellor, J.R.*, and Hanley, J.G. (2009). PICK1-mediated Glutamate Receptor Subunit 2 (GluR2) Trafficking Contributes to Cell Death in Oxygen/Glucose-deprived Hippocampal Neurons. Journal of Biological Chemistry 284, 14230-14235.
*last equal authorship

Buchanan, K.A. & Mellor, J.R. (2007). The development of synaptic plasticity induction rules and the requirement for postsynaptic spikes in rat hippocampal CA1 pyramidal neurones. Journal of Physiology 585, 429-445.

Martin, S., Nishimune, A., Mellor, J.R.* & Henley, J.M. (2007). SUMOylation regulates kainate-receptor-mediated synaptic transmission. Nature 447, 321-325.
*last equal authorship

We have also discussed our work extensively with other electrophysiologists and they are entirely supportive of the analytical approaches and interpretation.

The reviewer does not make any substantive points about the experiments or the conclusions drawn from them. The problem seems to be with the example traces shown. We are aware that the example traces shown in this study are not the ideal traces often shown in other publications that use different slice preparations. This is because of the specific experimental conditions we have employed to look at expression of PICK1 constructs in slices cultured for only 24 hours. In these slices, EPSCs are smaller and therefore noisier with greater waveform fluctuations than those found in acute slices or long-term organotypic cultured slices. A good example of previous data where smaller EPSCs are also noisier can be seen in Shi et al, (1999), figure 2B. We have, however, applied the same rigorous quality control and statistical analysis that we would for data from any other slice preparation.

Specifically, these conditions require that we reject experiments if:
1) series resistance changes by >30% during the course of an experiment,
2) we observe burst firing in response to synaptic stimulation,
3) The control pathway amplitude during LTD experiments changes by >20%,
4) baseline for individual experiments changes significantly as tested by regression analysis.

In addition all experiments were performed in the presence of picrotoxin at a concentration (50 M) that completely blocks GABAA receptors and rectification experiments were performed in the presence of D-AP5 at a concentration (50 M) that we have previously shown to completely block NMDA receptors (Dixon et al, 2009), and is widely and routinely used to completely remove the NMDAR component of the EPSC. Given this rigorous quality control, we are entirely confident about the validity of our data and the conclusions drawn from them.

The main concern is therefore with the choice of example traces. In the previous review it was our impression that the reviewer did not want us to replace these. From the current review, it is now clear that we misunderstood his/her intention and so we have replaced the traces shown in figures 6A, 6D, 7A and 7B in line with the reviewer's comments.

In response to the reviewer's specific points:

2) About Rs. From the sample trace of Figure 6A control cell, I see the time course of the event changed before and after LTD induction. This is often caused by change in Rs. An experienced electrophysiologist will not use such trace for sample.

We apply a rigorous check on series resistance changes during our experiments and reject any where there is a change of >30%. The small change in time course observed in the sample trace could be due to a change in series resistance but equally could be due to the natural random fluctuation seen in EPSC waveform during any electrophysiological recording (for example see Shi et al, 1999, Terashima et al, 2004; Terashima et al, 2008) and particularly evident in recordings from slices cultured for 24 hours. We have now replaced the traces with ones where the waveforms contain less fluctuation.

3) New Figure 6Ci. Move the Y-axis so that it crosses X-axis at X=0. Also, plot in square and remove average value. The authors should know that the average value does not mean anything as the number of fibers they are stimulating is not identical between the slices, though I admit that there are such publications. The average value is dragged by the pairs which have a large response.

We thank the reviewer for pointing out the error in the y-axis position and have now moved it. The reviewer is correct to point out that it is not sensible to compare EPSC amplitudes between slices. This is why we perform the within slice comparisons represented in the figure where we record from a transfected and a non-transfected cell in the same slice using the same synaptic input. This is clearly stated in the methods section.

The important analysis is then to test whether the EPSC amplitudes recorded from each cell within the same slice are equal. We found a good way to represent this is to show the mean values and their relationship to the line of unity shown in the graph (see also Hayashi et al, 2000). However, we can equally show this by separating the two data sets and removing the mean values, which we have now done in line with the reviewer's suggestion (figure 6Ci and 6Cii).

4) Figure 6D. I am still not satisfied the quality of the data. Again, I doubt the authors have sufficient knowledge and skill. Between groups, the traces at 0 mV go either outward (EGFP) or inward (WT-Pick1 and W413A mutant). I believe the latter is typical because voltage clamping is always not perfect. But I do not understand why current flows outward. Maybe GABA component is still remaining. Again, it is not a sample trace an experienced electrophysiologist will use. Also, I suspect there are residual NMDA component as the decay in trace is obviously slower in +40 mV. This is very obvious, for example, in the control trace in WT. This was pointed out in the first review but the authors reply was that they put 50 uM AP5 and they even do not bother replacing the sample trace. Again I doubt the authors' knowledge in electrophysiological recording and quality control of the data.

The direction of AMPAR-mediated EPSCs at a holding potential of 0mV is dependent on the exact value of the reversal potential. For the internal and external solutions we have used we would expect the reversal potential to be ~0mV (without junction potential correction). However, there are always small fluctuations in the exact value of the reversal potential (e.g. Hayashi et al, 2000; Hestrin et al, 1990; Shi et al, 1999; Terashima et al, 2008) possibly due to voltage clamp differences depending on the precise dendritic location of activated synapses. This can lead to small inward, small outward or no current at all when recording at a holding potential that is very close to the reversal potential, in this case 0mV. This entirely explains the data shown in figure 6D and is consistent with data from many other labs, as referenced above.

We pointed out in our previous response that the traces shown in figure 6D are on a different timescale to those shown in other figures. This means that few of the responses return to baseline whether they are recorded at -70mV or +40mV. We accept that the original trace for WT control appears to have a longer timecourse at +40mV than -70mV and we have now replaced these traces as requested by the reviewer. We did not replace them in the previous revision because we were under the impression from the first review that the referee did not want us to replace them.

New comments:

1) Figure 7B. Again I see problems with recording quality. The sample trace in B appears to be overlap significantly with slow decay of the stimulation artifact and the measurement of rectification is not accurate. Also, this should include the W413A mutant.

The sample traces have now been replaced in Figure 7B. The peak response never overlaps with the stimulation artefact so we are confident that our rectification measurements are accurate.

Figures 7A and 7B confirm that D-AP5 blocks the observed changes in EPSC amplitude and rectification index following overexpression of WT-PICK1 (figure 6D). The reason for not including data for the W413A mutation in Figures 7A and 7B is that W413A overexpression has no effect on EPSC amplitude or rectification index (see figure 6D). Therefore there is no effect to block by D-AP5 application.

2) Figure 7C. The baseline is going down in WT and in mutant. The authors should go back to the individual trace and reject the cells which do not show a stable baseline. If N is not sufficient, the authors should repeat the experiment.

We have already applied a rigorous quality control and rejection of data based on the criteria given above. This includes a statistical regression analysis for the baseline of each experiment. The baseline shown has no significant rundown therefore we do not feel it is appropriate to reject any of the data on these grounds. We note that the initial one or two points in the baseline for the WT and W413A data in Figure 7C are higher than the others but this is within the overall variance of the baseline data.

References:

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Terashima A, Pelkey KA, Rah JC, Suh YH, Roche KW, Collingridge GL, McBain CJ, Isaac JT (2008) An essential role for PICK1 in NMDA receptor-dependent bidirectional synaptic plasticity. Neuron 57(6): 872-882

Acceptance letter 20 December 2010

Many thanks for submitting the latest version of your manuscript EMBOJ-2010-74769R1. It has been seen again by referee 1, whose comments are enclosed below. As you will see, he/she still has some concerns about the electrophysiology data, but at the end of the day has no further objections to publication. Therefore, I am pleased to be able to tell you that we can now accept your manuscript for publication in the EMBO Journal.

Thanks and best wishes,
Editor

Referee 1:
The authors replaced the sample traces. However, when I compared the data set presented in current and previous ones, they are identical. So the authors' reaction is only superficial without going into the individual data. They insist that they did enough quality control of the data. But given the quality of sample traces initially presented (meaning those used in the analysis), this reviewer has difficulty in accepting their words literally.

I think I, as a reviewer, did enough to improve this paper, which was initially judged as borderline rejection. I will leave the final decision to the discretion of the editor.

