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Acute knockdown of AMPA receptors reveals a trans-synaptic signal for presynaptic maturation

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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 31 August 2010

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments to the authors are provided below. As you can see the referees express interest in the study, but also bring up a number of important issues that have to be resolved. The likely most difficult concern to address is that we need further molecular insight into how the extracellular domain of GluA2 affects presynaptic function. I recognize that this issue might not be easy to resolve, but referee #3 offers a number of constructive suggestions. Should you be able to address the concerns raised, including the major points raised by referee #3, we would consider a revised manuscript. I would like to add that it is EMBO Journal policy to allow a single major round of revision only and it is therefore important to address the concerns raised at this stage. I also recognize that to address the concerns raised in full might take some extra time and I can extend the revision duration if that is helpful.

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

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The paper by Tracy et al. found that the down regulation of postsynaptic AMPA receptor decreased release of transmitter release from opposing presynaptic terminus. This was due to decrease in readily releasable pool but the functionality of remaining pool appears normal. The effect does not require activation of AMPA receptor. In heterologous cells, AMPA receptor subunits increase the proportion of active synapse when they are coexpressed with Neuroligin and cocultured with neurons. The authors proposes structural role of AMPA receptor extracellular domain that induce accumulation of releasable synaptic vesicles on the presynaptic side.

Overall, this paper is well conducted and potentially suitable for publication in EMBO J but there are clearly issues that require additional works and clarification.

There is one very strange thing in this paper. The authors used GluR2 subunit to rescue the phenotype. GluR2 homomer has very low conductance and it is very strange that it shows a rescue of the effect of RNAi exactly comparable to the control level. The authors should check the identity of clone they are using. If it has R/Q mutation in the channel pore, it may explain the observed results.

The major weak aspect of this paper is that the structural role of AMPA receptor extracellular domain has been already suggested by Passafaro and Sheng. GluR2 binds cadherin, a transsynaptic cell adhesion molecule. Therefore, it is not too surprising that AMPA receptor has a structural role that instructs presynaptic functionality or structure. The interaction with AMPA receptor and cadherin is mostly mediated by GluR2 subunit and the authors ruled out cadherin based on this. But the authors should devote more effort to test cadherin and other potential molecules that may or may not be involved in this process.

The reviewer is a puzzled with the authors' conclusion that the effect of AMPA receptor down regulation is to down regulate readily releasable pool without changing release probability. These two parameters are tightly coupled and I wonder how it is possible to separate them. In fact, the experiments based on which the authors concluded there is not change in release probability are not well conducted. The experiments of PPF (Figure 5A, B) show very strange trace for AMPA receptor component. The decay is very slow. Probably because the authors used extracellular local field stimulation, it caused bursting of the neurons and there are neurons fired asynchronously. The authors should think of better way to stimulate the preparation. Under such condition, I do not think the authors are measuring accurate PPF, which often change only very subtly. MK801 (Figure 5C, D) is also strange. If one looks the decay time course, the RNAi group decayed significantly faster. Depending on the statistics and number of observation, the conclusion may change. The response to repetitive pulses is indeed a measure of readily releasable pool and total pool size (Figure 5E and F). This should be more carefully analyzed, for example, by the method of Schneggenburger et al. (Neuron, Vol. 23, 399-409, June, 1999).

Specific comments.
- Figure 7 GluR1 and GluR2 only should be tested.
- Figure 7B and G. This count is counterintuitive. Why not plotting total number of puncta and proportion of active one among them?
- GluRA2 ecto. Explain why it has GluR1 tail. GluR1-ecto should be tested.
- "Total pool" and "readily releasable pool" are different terminology. The wording "total readily-releasable vesicle pool" (p13) is confusion. Also, what sucrose experiment defines is "readily releasable pool". In view of this, the first paragraph of discussion should be reworded.

Referee #2 (Remarks to the Author):

Tracy et al. studied the effects of AMPA receptor knock-down in neurons. The key finding is that AMPA receptor knock-down (GluA2/3) has collateral effects on NMDA receptor responses. Based on a series of experimental data, the authors conclude that this collateral effect is actually due to presynaptic silencing. Additional experiments are taken to indicate that AMPA receptors signal to

presynapses through an as yet unknown activity-independent mechanism.

Comments:

1. Experiments involving the application of exogenous NMDA indicate that AMPA receptor knock-down cells have normal NMDA receptor surface expression, and based on immunostaining for NMDA receptors, the authors conclude that synaptic NMDA receptor density is also normal in AMPA receptor knock-down cells. The problem here is that the resolution of light microscopy is not sufficiently high to differentiate synaptic receptors from perisynaptic receptors that may have diffused away from the actual synaptic contact but are still in the spine. Given that the key conclusion of this study hinges on the notion that synaptic NMDA receptor density is not changed after AMPA receptor knock-down, I think the authors need to test more directly if synaptic NMDA receptor density is really normal in the knock-down cells. This could be done by measuring NMDAergic mEPSC amplitudes. Better even would be to perform the MK-801 experiment (Fig. S2) in a modified manner, comparing untreated and knock-down cells: (i) Assess response to exogenous NMDA, (ii) then block synaptic NMDA receptors by stimulation in the presence of MK-801, (iii) then assess NMDA receptors that were not blocked by MK-801 with a second application of exogenous NMDA. If NMDA receptor distribution and synaptic clustering were normal in AMPA receptor knock-down cells, the ratio of MK-801-blockable NMDA receptors relative to non-blockable receptors would be the same as in untreated cells.

2. The authors use the density and fluorescence intensity of immunostained synaptic marker puncta as a readout to conclude that AMPA receptor knock-down cells have normal synapse numbers. What is missing - as far as I can see - are tests to show that the cell morphology and geometry is normal in the knock-down cells (i.e. dendrite length and branching). This should be shown.

3. The authors show that PPR, Pvr, Pr, and synaptic depression during high frequency stimulation are normal in AMPA receptor knock-down cells, yet RRP sizes as determined by sucrose stimulation of NMDAergic responses are smaller. The most trivial explanation for this finding would be a loss of postsynaptic sensitivity. The authors exclude this possibility, but see my comments above. If this were a presynaptic effect, it could only be explained by a total silencing of a subpopulation of presynapses in the AMPA receptor knock-down cells as otherwise an RRP reduction would be predicted to affect PPR, Pvr, Pr, and synaptic depression. Unfortunately, the authors did not test the notion of presynaptic silencing in the AMPA receptor knock-down cells but addressed this issue only in HEK/neuron co-culture assays. This is not sufficient - the co-culture assay is known to report phenomena that are not seen in vivo. The authors should directly test for presynaptically silent synapses in their neuron culture preparation using a combination of fixable FM staining, e.g. after KCl stimulation (to assess active synapses), and post-hoc immunostaining of presynapses in order to determine the ratio of active synapses in control and AMPA receptor knock-down neurons. Using lumenal Syt-1 staining instead of fixable FM might also work.

4. Likewise, I think the experiments using the ectodomain constructs of GluA2 in the HEK/neuron co-culture assay are not convining. This should also be done in neurons.

5. Direct transsynaptic interactions of postsynaptic receptors with presynaptic (adhesion) proteins is a phenomenon that has been described recently in another system (GABA receptors). The present study falls a bit short of this previous study as it remains at the phenomenological level, and the presynaptic interaction partner remains unknown.

Overall, I think my point 5 above would not preclude publication of this work in EMBO J., but I think the authors should address my points 1-4 above.

Referee #3 (Remarks to the Author):

This manuscript reports the intriguing finding that AMPA receptors not only alter postsynaptic maturation but also select presynaptic properties of excitatory neurotransmission. Using cultured neurons, the authors show that knockdown of AMPA receptor subunits reduces NMDA receptor currents, probably via altering release at a subset of excitatory synapses. In addition, they find that extracellular interactions of AMPA receptors increase the fraction of presynaptic terminals induced

in a co-culture system that contain recycling vesicles. The experiments are well performed and the authors carefully rule out a number of other explanations. However, additional experiments will further clarify these findings. Notably, a more detailed molecular analysis will substantially strengthen this study as outlined below.

1. One major point is the question whether presynaptic N-cadherin is the binding partner of GluA2 (and possibly the other GluA subunits) that mediates their trans-synaptic effects at a subset of synapses. This would be consistent with the reported effects of N-Cadherin on presynaptic maturation. If confirmed, this finding would add substantial mechanistic insight to this study.

2. Another major question is whether the postsynaptic knockdown of GluA subunits reduces the synaptic localization and/or expression level of presynaptic priming factors and of postsynaptic scaffolding molecules other than PSD-95. The results would provide additional molecular information on AMPA receptor-mediated presynaptic maturation.

3. To exclude indirect postsynaptic effects of AMPA receptor knockdown on NMDA receptors, have the authors measured whether NMDA receptor phosphorylation is altered? This can be addressed by quantitative immunostaining.

4. The authors use a physiological analysis to conclude that the pool of vesicles available for release is impaired if postsynaptic AMPA receptor levels are reduced. This intriguing observation is unexpected and would be strengthened if the authors can complement it with an EM analysis of vesicle pool organization of terminals formed atop neurons targeted for knock-down.

5. The authors should discuss why the knockdown of three GluA subunits can be fully rescued by expression of a single subunit in all experiments. This extent of compensation is unexpected. Does it indicate that all three subunits share the same trans-synaptic partner?

6. Why was it necessary to bolster the miniature recordings by performing them in 200 mM sucrose?

7. Is Figure 1B representative of the immunostainings after knockdown of GluA1-3? It shows a lack of staining, differing from the still detectable staining in Figure 2A at higher magnification.

8. What does the grey line in Figure 6E indicate?

9. The clarity of the figures can be improved by replacing "GluA RNAi" with "GluA1-3 RNAi".

10. In Figure 4B,C, what is the difference of quantifying integrated puncta density vs. average intensity?

11. There is a typo in the legend of Figure 7B, reading "presyanptic".

12. The reference for Cabezas et al is incomplete.

1st Revision - authors' response 28 December 2010

Response to reviewers for “Acute knockdown of AMPA receptor reveals a trans-synaptic signal for presynaptic maturation” by Tracy et al.

We appreciate the reviewers’ constructive comments of our manuscript – they have been most helpful. As a result, we have performed multiple new experiments to address the concerns raised by the reviewers. We completely agree with the reviewers that this fundamental observation we report, namely the finding that a knockdown of AMPA-type glutamate receptors (AMPARs) causes profound changes in presynaptic release properties, is surprising, as it suggests that AMPAR function may be broader than currently envisioned. Although we cannot at present, despite a major effort, delineate a mechanistic pathway mediating these AMPAR-dependent presynaptic changes, we believe that the phenomenon as such is now supported by so many lines of independent evidence that it is hopefully not in doubt, and its importance is self-evident.

Some of the reviewers' comments aimed at a more detailed molecular understanding of the AMPAR-dependent changes we describe. Specifically, the reviewers suggested based on considerable pre-existing work that N-cadherin may be involved in the mechanism underlying our fundamental observation. There is no doubt that N-cadherin is a major regulator of spine dynamics and synaptogenesis; however, we found no tangible link with the AMPAR-dependent changes in presynaptic properties we describe (see detailed description of the experiments below). We don't think our negative results cast doubt on the importance of N-cadherin, but they do suggest that N-cadherin is not essential for the particular process we identified. Our inability, despite considerable effort (see below), to obtain evidence for the involvement of N-cadherin in the AMPAR-dependent modulation of presynaptic release prompted us to survey the literature for other possible mechanisms. We found that in addition to N-cadherin, a number of other synaptic cell-adhesion molecules could tangibly be involved in the process we describe, e.g. neurexins (which have been reported to directly or indirectly interact with all postsynaptic neurotransmitter receptors), ephrins/eph receptors (which reportedly interact with at least some glutamate receptors), synaptic pentraxins (known to interact with AMPARs), SynCAMs, LRRTMs (again, reported to interact with AMPARs), and receptor phosphotyrosinephosphatases, to name just a few. Following up on all of these candidate interactors for AMPARs is simply beyond the scope of the present paper. We hope that the reviewers will agree that given the difficulties in sorting out which trans-synaptic interactions may control what synaptic properties, it is unrealistic for us to try to determine the AMPAR trans-synaptic binding partner and its signaling mechanism that underlies the fundamental observation we describe in this paper. It should be noted that although many separate, often outstanding and pioneering papers described these synaptic cell-adhesion molecules and suggested interactions with glutamate receptors for the adhesion molecules, none of the papers actually tested the potential role of post-synaptic AMPARs in shaping presynaptic properties. Thus, we hope that the reviewers will acknowledge and support the conceptual advance embodied in the identification of a trans-synaptic AMPAR-mediated regulation of presynaptic strength that we report, and agree that the depth of our controls unequivocally establishes the validity of this surprising and yet fundamental observation.

The following new experiments were included in the revised manuscript:

1) To address the potential involvement of N-cadherin in mediating the retrograde signaling from postsynaptic AMPARs to influence presynaptic vesicle release, we performed two sets of experiments. First, we knocked down N-cadherin in neurons with a lentivirally delivered shRNA that was previously validated, and achieved more than 90% knockdown efficiency. We found that knocking down N-cadherin in neurons did not alter the ability of AMPARexpressing HEK cells to induce active synapses in crossing axons from co-cultured neurons (see Figure 10D). Please note that N-cadherin is normally already expressed in HEK293 cells (Okuda et al., PNAS, 2007; Hogan et al., MCB 2004; also see Figure 10A). Thus, this result suggests that presynaptic N-cadherin is not the mediator for the retrograde signal initiated by postsynaptic AMPARs. Second, we knocked down N-cadherin in all neurons with lentivirally delivered shRNA, and performed Syt1 antibody uptake assay at these neuronal synapses that lack N-cadherin both pre- and post-synaptically to monitor synaptic vesicle exo- and endocytosis under basal activity. We found that the mean Syt1puncta intensity and the portion of inactive excitatory synapses (Syt1-negative, VGluT1-positive) are not different between control synapses and those that lack N-cadherin (see Figures 10E-F). This result, taken together with the neuron/HEK cell co-culture result mentioned above, suggests that postsynaptic N-cadherin is also not involved in mediating AMPAR retrograde signaling to promote presynaptic maturation of vesicle release.
2) To address the concern that recurrent activity may contaminate excitatory synaptic responses evoked by field stimulation, we recorded AMPAR EPSCs from synaptically connected pairs of neurons and measured their paired-pulse ratio (PPR). We found no change in the PPR by AMPAR knockdown. This result is presented in Figures 6C-D.
3) To further strengthen the conclusion that synaptic NMDARs are not affected by the AMPAR knockdown, we recorded dual component mEPSCs and measured the NMDAR-mediated component, as described by Roger Nicoll's pioneering work. We found no difference in the NMDAR mEPSC amplitude between control and AMPAR knockdown neurons, although the AMPAR mEPSC amplitude was significantly reduced in the same AMPAR knockdown neurons. This data is presented in the new Figures 4D-4F.
4) To examine whether knockdown of alters the cell morphology and geometry, we measured

total dendrite length and dendritic branching in neurons and found no difference between control and AMPAR knockdown neurons. These data are presented in Supplementary Figure 3.
5) To further test the hypothesis that the AMPAR knockdown increases the number of functionally inactive presynaptic terminals on neurons, we performed additional Syt1 antibody uptake experiments as suggested by the reviewers. We found that knocking down AMPARs in the postsynaptic neurons increased the occurrence of inactive excitatory synapses (Syt1-negative), which can be rescued by postsynaptic overexpression of either the GluA1 or GluA2 subunit. This result is now included in the new Figures 8A-C and the new Supplementary Figures 5C and D**.**
6) We examined whether AMPAR knockdown affects the synaptic localization and expression of other presynaptic active zone proteins and postsynaptic scaffolding molecules. Specifically, we analyzed the synaptic localization and expression of GRIP1, bassoon, piccolo, syntaxin, synapsin, and liprin-alpha. Surprisingly, we found that AMPAR knockdown causes a significant increase in synaptic liprin-alpha, whereas no other protein was significantly changed. This new data is included in Figure 5D.
7) We performed additional mEPSC recordings under normal ACSF condition as suggested by the reviewers, and replaced the data from sucrose-evoked mEPSCs with this new data (Figures 2E-2G).
8) Reviewer 1 suggested that GluA1 and GluA2 controls in the neuron/HEK cell co-culture experiment might strengthen the conclusions. We agree, and performed these experiments. We found that as expected, HEK cells expressing only AMPARs without NL1 did not induce a significant amount of synaptic contacts in crossing axons as indicated by VGluT1 staining (VGluT1 puncta density (No./μm2): NL1, 0.157 +/- 0.017; GluA1 alone, 0.025 +/- 0.004; GluA2 alone, 0.024 +/- 0.009; n = 8 cells/group; $p < 1 \times 10\text{-}5$). This data is now mentioned in the text.
9) In the original manuscript, we only described the GluA2 rescue of synaptic transmission following AMPAR knockdown performed with concurrent CNQX treatment, which was to show that the rescue was not mediated by the channel activity of GluA2 as such. We have now extended these experiments to include GluA1 as well. The results further strengthen the notion that the observed trans-synaptic signaling by AMPAR is not subunit specific, but equally performed by GluA1 and GluA2. This new data is included in Figure 8D.

Specific reviewers' concerns:

Referee 1:
1. There is one very strange thing in this paper. The authors used GluR2 subunit to rescue the phenotype. GluR2 homomer has very low conductance and it is very strange that it shows a rescue of the effect of RNAi exactly comparable to the control level. The authors should check the identity of clone they are using. If it has R/Q mutation in the channel pore, it may explain the observed results.
Agreed – we should have addressed this point. We used the unedited form of GluA2 (GluA2Q) for all experiments; this is now clearly stated in the text.

2. The major weak aspect of this paper is that the structural role of AMPA receptor extracellular domain has been already suggested by Passafaro and Sheng. GluR2 binds cadherin, a transsynaptic cell adhesion molecule. Therefore, it is not too surprising that AMPA receptor has a structural role that instructs presynaptic functionality or structure. The interaction with AMPA receptor and cadherin is mostly mediated by GluR2 subunit and the authors ruled out cadherin based on this. But the authors should devote more effort to test cadherin and other potential molecules that may or may not be involved in this process.
We agree with the reviewer that a structural role of AMPAR extracellular domain has been suggested by Passafaro and Sheng's previous work. As described in the introductory comments, we have now performed extensive additional experiments to probe the possible role of Ncadherin in the process we describe (Figure 10). We believe that our data conclusively rules out the involvement of the particular interaction that Passafaro and Sheng describe. Specifically, we show that our effect is not GluA2-specific; exogenous GluA1 expression can completely rescue the effect induced by AMPAR knockdown, which suggests that a different mechanism may be involved in mediating the trans-synaptic signaling. Moreover, we now directly examined the involvement of N-cadherin in this process (experiment #1 described above in the list of new

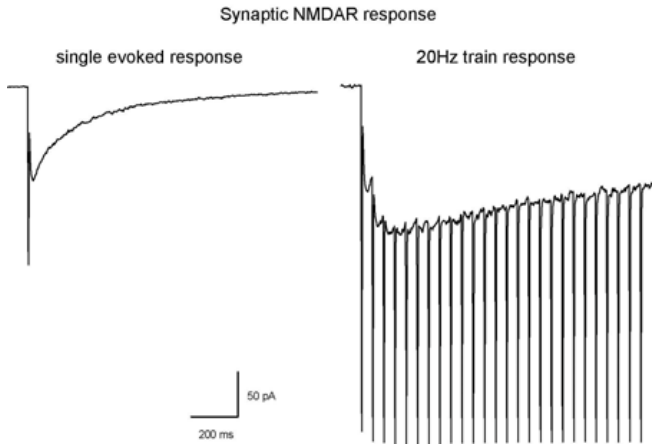
experiments) and found that N-cadherin is not required for the observed effect of AMPARs on presynaptic maturation.

3. The reviewer is a puzzled with the authors' conclusion that the effect of AMPA receptor down regulation is to down regulate readily releasable pool without changing release probability. These two parameters are tightly coupled and I wonder how it is possible to separate them.
We apologize for not addressing this point more clearly in the previous version of the manuscript. The size of the readily releasable pool (RRP) at *individual* synapses is indeed tightly coupled to the release probability of the synapse. However, sucrose-evoked response reflects the *total* RRP size, which is the sum of the individual RRPs from *all active* synapses on a neuron. Our data showing a reduced total RRP with unchanged release probability by AMPAR knockdown means that the loss of AMPARs results in more presynaptically inactive synapses, without affecting the release probability of the synapses that remain active. We have revised our text to further clarify this point.

4. The experiments of PPF (Figure 5A, B) show very strange trace for AMPA receptor component. The decay is very slow. Probably because the authors used extracellular local field stimulation, it caused bursting of the neurons and there are neurons fired asynchronously. The authors should think of better way to stimulate the preparation. Under such condition, I do not think the authors are measuring accurate PPF, which often change only very subtly.
Agreed – to avoid the potential recurrent activation problem by local field stimulation, we performed whole-cell patch clamp recordings from connected pairs of neurons. The eEPSCs were evoked through direct stimulation of a presynaptic neuron, which elicited an action potential in a single axon. We found that knocking down AMPARs from the postsynaptic neurons did not affect the paired-pulse ratio of eEPSCs, suggesting that the release probability of the active synapses is not affected by AMPAR knockdown. This result is now included in Figures 6C-D.

5. MK801 (Figure 5C, D) is also strange. If one looks the decay time course, the RNAi group decayed significantly faster. Depending on the statistics and number of observation, the conclusion may change.
We agree that MK-801 measurements of release probability are tricky, but feel that since we quantified the decay time constant from more than 20 cells in each group (Figure 6F inset), and found no significant change in the decay time constants by AMPAR knockdown, our results are reliable. Additionally, we have positive control experiments in which we validated the sensitivity of this assay to changed release probability in our hands by reducing external calcium concentration (Supplementary Figure 4). In fact, the RNAi group showed a trend of decreased decay time course, at best suggesting an increase in the release probability, which cannot explain the observed decrease in NMDAR eEPSC amplitude.

5. The response to repetitive pulses is indeed a measure of readily releasable pool and total pool size (Figure 5E and F). This should be more carefully analyzed, for example, by the method of Schneggenburger et al. (Neuron, Vol. 23, 399-409, June, 1999).
Schneggenburger et al., performed an elegant series of experiments to estimate the RRP size at the Calyx; note that similar experiments were also performed by Wu and Borst (Neuron 1999), and that the approach was adapted for slice recordings by Mennerick and colleagues. However, all of these experiments are based on the assumption that postsynaptic AMPAR responses are not changed. We cannot perform the same type of experiments because our manipulation (AMPAR knockdown) affects the number of postsynaptic AMPARs. The difference in eEPSC amplitude during a high frequency train reflects both changes in RRP and changes in postsynaptic AMPAR numbers. Since postsynaptic NMDARs are unchanged by AMPAR knockdown, we thought about using the NMDAR eEPSCs for the same analysis, but due to the slow kinetics of the NMDAR response, we cannot resolve individual eEPSC peaks even at 20 Hz (see figure left). Because inter-stimulus intervals shorter than the time required for recovery from depression must be used in order to estimate RRP size (Schneggenburger et al. used 100 Hz), using NMDAR response for this type of experiment is not feasible. Thus, the method of Schneggenburger, or Wu and Borst or Mennerick cannot be easily applied to our experimental configuration.



6. Figure 7 GluR1 and GluR2 only should be tested.
Agreed – although we initially did not perform the GluA1 and GluA2 alone control because AMPARs by themselves are not expected to induce synapse formation. We have now performed the experiment, and show that indeed HEK cells expressing AMPARs only without NL1 do not have a significant synaptogenic effect on crossing axons (VGluT1 puncta density (No./μm2): NL1, 0.157 +/- 0.017; GluA1 alone, 0.025 +/- 0.004; GluA2 alone, 0.024 +/- 0.009; n = 8 cells/group; $p < 1 \times 10\text{-}5$). These data are now mentioned in the text.

7. Figure 7B and G. This count is counterintuitive. Why not plotting total number of puncta and proportion of active one among them?
We did the quantification this way because we aimed to emphasize the effect of AMPAR knockdown in neurons, which is to induce more presynaptically inactive synapses.

8. GluRA2 ecto. Explain why it has GluR1 tail. GluR1-ecto should be tested.
Agreed – we have now mentioned that we included the GluR1 tail to ensure proper synaptic targeting. As regards GluA1 ecto, we actually did initially make this construct. However, the GluA1 ecto chimera did not traffic to the cell surface in HEK cells or neurons, suggesting that it does not fold properly. The GluA2 ecto chimera with the GluA1 C-terminal expresses well on the cell surface in HEK cells, but not in neurons (see Supplementary Figure 8 – a phenomenon not infrequently observed with expression of proteins in HEK293 cells and neurons, because the latter are much more finicky in terms of protein folding). We have also tried to express the pore-dead GluA2 construct (GluA2 R586E) (Shi et al., 2001, Cell) for rescue. Unfortunately, the expression level of GluRA2 R586E in neurons is too low to be detectable because we had to use a transfection method in order to deliver it together with three other GluA shRNAs. Shi et al. used Sindbis virus for expression, which can achieve exceedingly high levels of protein expression. Unfortunately, we cannot deliver four constructs with the Sindbis virus method because of the cellular toxicity of the Sindbis virus.

9. "Total pool" and "readily releasable pool" are different terminology. The wording "total readily-releasable vesicle pool" (p13) is confusion. Also, what sucrose experiment defines is "readily releasable pool". In view of this, the first paragraph of discussion should be reworded.
We apologize, and have revised the text related to this experiment.

Referee 2:

1. Experiments involving the application of exogenous NMDA indicate that AMPA receptor knock-down cells have normal NMDA receptor surface expression, and based on immunostaining for NMDA receptors, the authors conclude that synaptic NMDA receptor density is also normal in AMPA receptor knock-down cells. The problem here is that the resolution of light microscopy is not sufficiently high to differentiate synaptic receptors from perisynaptic receptors that may have diffused away from the actual synaptic contact but are still in the spine. Given that the key conclusion of this study hinges on the notion that synaptic NMDA receptor density is not changed after AMPA receptor knock-down, I think the authors need to test more directly if synaptic NMDA receptor density is really normal in the knock-down cells. This could be done by measuring NMDAergic mEPSC amplitudes.

Agreed – we performed dual component mEPSC recordings based on the pioneering method used by Roger Nicoll (Gomperts et al., 1998; Gomperts et al., 2000) and measured the NMDAR mEPSC amplitude. We found no change in the NMDAR mEPSC amplitude, which confirmed our conclusion that the number of NMDARs at synapses is not affected by the GluA RNAi. This data is included in Figure 4D-F.

2. Better even would be to perform the MK-801 experiment (Fig. S2) in a modified manner, comparing untreated and knock-down cells: (i) Assess response to exogenous NMDA, (ii) then block synaptic NMDA receptors by stimulation in the presence of MK-801, (iii) then assess NMDA receptors that were not blocked by MK-801 with a second application of exogenous NMDA. If NMDA receptor distribution and synaptic clustering were normal in AMPA receptor knock-down cells, the ratio of MK-801-blockable NMDA receptors relative to non-blockable receptors would be the same as in untreated cells.
This experiment suggested by the reviewer would provide an elegant approach for measuring postsynaptic NMDAR distribution and synaptic clustering, but is difficult to interpret if presynaptic properties are changed. Specifically, the conclusion of this experiment is based on the assumption that there is no change in the presynaptic release because the MK-801 blockade of synaptic NMDARs is dependent on whether the presynaptic terminals are active. In our case, knocking down AMPARs inactivates a subset of presynaptic terminals, therefore the experimental results would be difficult to interpret because changes in both the presynaptic function and the synaptic NMDAR localization can contribute to the change in the ratio of MK-801-blockable NMDAR relative to non-blockable ones.

3. The authors use the density and fluorescence intensity of immunostained synaptic marker puncta as a readout to conclude that AMPA receptor knock-down cells have normal synapse numbers. What is missing - as far as I can see - are tests to show that the cell morphology and geometry is normal in the knock-down cells (i.e. dendrite length and branching). This should be shown.
Agreed - We have performed the analysis for both total dendritic length and branching. The new data is included in Supplementary Figure 3.

4. The authors show that PPR, Pvr, Pr, and synaptic depression during high frequency stimulation are normal in AMPA receptor knock-down cells, yet RRP sizes as determined by sucrose stimulation of NMDAergic responses are smaller. The most trivial explanation for this finding would be a loss of postsynaptic sensitivity. The authors exclude this possibility, but see my comments above.
We have now addressed this issue as described above.
If this were a presynaptic effect, it could only be explained by a total silencing of a subpopulation of presynapses in the AMPA receptor knock-down cells as otherwise an RRP reduction would be predicted to affect PPR, Pvr, Pr, and synaptic depression. Unfortunately, the authors did not test the notion of presynaptic silencing in the AMPA receptor knock-down cells but addressed this issue only in HEK/neuron co-culture assays. This is not sufficient - the coculture assay is known to report phenomena that are not seen in vivo. The authors should directly test for presynaptically silent synapses in their neuron culture preparation using a combination of fixable FM staining, e.g. after KCl stimulation (to assess active synapses), and post-hoc immunostaining of presynapses in order to determine the ratio of active synapses in control and AMPA receptor knock-down neurons. Using lumenal Syt-1 staining instead of fixable FM might also work.
We took this reviewer's advice and did the suggested experiment. We indeed found using the Syt1 antibody uptake method that knocking down AMPARs increases the number of inactive presynaptic terminals at neuronal synapses. The results are included in the new Figures 8A-C and new Supplementary Figures 5C and D.

5. Likewise, I think the experiments using the ectodomain constructs of GluA2 in the HEK/neuron co-culture assay are not convining. This should also be done in neurons.
As mentioned above (point #8 to reviewer 1), the GluA2 ecto construct unfortunately does not traffic to the neuronal cell surface when expressed by transfection, which makes the neuronal rescue experiment impossible. We have now included the immunostaining data in Supplementary Figure 8 to demonstrate this. We have also tried to express the pore-dead GluA2 construct (GluA2 R586E) (Shi et al., 2001, Cell) for rescue. Unfortunately, the expression level

of GluRA2 R586E in neurons is too low to be detectable because we had to use a transfection method in order to deliver it together with three other GluA shRNAs. Shi et al. used Sindbis virus for expression, which can achieve exceedingly high levels of protein expression. Unfortunately, we cannot deliver four constructs with the Sindbis virus method because of the cellular toxicity of the Sindbis virus.

6. Direct transsynaptic interactions of postsynaptic receptors with presynaptic (adhesion) proteins is a phenomenon that has been described recently in another system (GABA receptors). The present study falls a bit short of this previous study as it remains at the phenomenological level, and the presynaptic interaction partner remains unknown.
Please see introductory comments.

Referee 3:

1. One major point is the question whether presynaptic N-cadherin is the binding partner of GluA2 (and possibly the other GluA subunits) that mediates their trans-synaptic effects at a subset of synapses. This would be consistent with the reported effects of N-Cadherin on presynaptic maturation. If confirmed, this finding would add substantial mechanistic insight to this study.
Agreed – we have performed N-cadherin knockdown in neurons with lentivirally delivered shRNA and performed the Syt1 antibody uptake assay at both heterologous synapses and neuronal synapses. Our results exclude a role of N-cadherin in the retrograde AMPAR signaling that we observed. Please see introductory comments and new Figure 10 for details.

2. Another major question is whether the postsynaptic knockdown of GluA subunits reduces the synaptic localization and/or expression level of presynaptic priming factors and of postsynaptic scaffolding molecules other than PSD-95. The results would provide additional molecular information on AMPA receptor-mediated presynaptic maturation.
Agreed – following up on this we observed a large increase in one particular scaffolding protein, liprin-α, that is linked to AMPAR trafficking (Wyszynski et al., Neuron 2002) but may also perform presynaptic functions (Zurner and Schoch, Genomics 2009; Olsen et al., JCB 2005) at mammalian synapses. Strikingly, no change in any other protein was detected, especially no changes in several presynaptic proteins that were analyzed. These data are now shown in Figure 5D.

3. To exclude indirect postsynaptic effects of AMPA receptor knockdown on NMDA receptors, have the authors measured whether NMDA receptor phosphorylation is altered? This can be addressed by quantitative immunostaining.
To our knowledge, only two relatively old papers in the literature use phospo-NMDA receptor antibodies for immunocytochemistry/immunohistochemistry in neurons (Zou et al., J Neurosci 2000; Tingley et al., JBC, 1997). These important papers show how difficult it is to probe synaptic NMDA receptor phosphorylation by immunostaining; in fact, none of these papers (or any other paper in the literature) achieves a quantification of the phospho-NMDA receptor signal at synapses. We nonetheless tried a commercial phospho-NR1antibody that is selective for Ser-897 for immunocytochemistry from Upstate (now Millipore) (Zou et al., J Neurosci 2000). Unfortunately, although this antibody works well for immunoblotting, the quality of the immunostaining is not sufficient to allow us to resolve synaptic NR1 specifically (the immunostaining signal reported by the above reference is mostly somatic with very little synaptic labeling). Since we achieve AMPAR knockdown by the calcium phosphate-mediated co-transfection of the three GluA shRNA constructs, only a small fraction of neurons in the culture are affected. Therefore, any change in the NMDAR phosphorylation state cannot be detected by immunoblotting. Thus, we feel that there is no realistic avenue available to directly address the question raised by the reviewer, namely whether NMDA receptor phosphorylation is altered by the AMPAR knockdown. As a consequence, we decided to address the issue indirectly but functionally, and we performed dual component mEPSC recordings (Figures 4D-F). These recordings measure the amplitude of the NMDAR component of mEPSCs, which would be altered by phosphorylation, and found that it is not changed by AMPAR knockdown. Because phosphorylation of NMDARs increases its current (MacDonald et al., 1989), we conclude that the phosphorylation state of the NMDARs is most likely not altered.

4. The authors use a physiological analysis to conclude that the pool of vesicles available for release is impaired if postsynaptic AMPA receptor levels are reduced. This intriguing observation is unexpected and would be strengthened if the authors can complement it with an EM analysis of vesicle pool organization of terminals formed atop neurons targeted for knockdown.
We do agree with the reviewer that it would be interesting to look at the ultrastructure of presynaptic terminals using EM to investigate the effect of postsynaptic AMPAR knockdown on vesicle pool organization. However, our transfection of GluA shRNAs only hits a small fraction of neurons in the culture, and the potential reorganization of the vesicle pools could be expected to occur at a fraction of presynaptic terminals that innervate the transfected neurons. Thus, finding these terminals on transfected neurons at the EM level will be extremely difficult, rendering such an experiment unfeasible.

5. The authors should discuss why the knockdown of three GluA subunits can be fully rescued by expression of a single subunit in all experiments. This extent of compensation is unexpected. Does it indicate that all three subunits share the same trans-synaptic partner?
We agree that the single subunit rescue results are highly informative in the potential mechanism underlying the observation. First, they suggest that N-cadherin may not be involved because the previously reported effect of N-cadherin and AMPAR interaction is GluA2-specific. Second, they indicate that these AMPAR subunits share the same presynaptic partner, and also that it is the common extracellular domains of the AMPAR subunits that mediate the interaction, which can be further investigated in future studies. We have now discussed this in more detail in the revised paper.

6. Why was it necessary to bolster the miniature recordings by performing them in 200 mM sucrose?
Sucrose was not really necessary, but was simply used to accelerate the speed of the experiments. We have now redone the mEPSC recordings without sucrose and confirmed the initial result (Fig. 2E-G). Specifically, we found that the AMPAR knockdown again significantly decreased both amplitude and frequency of mEPSCs. We therefore replaced the old sucrose mEPSC data with these new data.

7. Is Figure 1B representative of the immunostainings after knockdown of GluA1-3? It shows a lack of staining, differing from the still detectable staining in Figure 2A at higher magnification.
Yes, the images in Figure 1B are representative of immunostaining after GluA RNAi and they were taken with a 60X objective to highlight the degree of total protein knockdown at soma. In Figure 2A, we took images with a 100x objective to better visualize synaptic AMPAR puncta in dendrites.

8. What does the grey line in Figure 6E indicate?
We apologize – the grey line most likely arose as a glitch generated during PDF file conversion online, and was absent from the original figures.

9. The clarity of the figures can be improved by replacing "GluA RNAi" with "GluA1-3 RNAi".
We agree that the now officially adopted ‘GluA’ terminology can be confusing, as it may erroneously indicate that only GluRA=GluR1=GluA1 is targeted here. To ensure that the reader understands the figures more immediately as suggested, and to allow us at the same time to actually fit the labels into the figures, we have now added a clear description of the label to the Figure 1 legend as well as in the main text. Should the reviewer feel this is not sufficient, we would be happy to try to decrease the label size to fit the full description into the figures.

10. In Figure 4B,C, what is the difference of quantifying integrated puncta density vs. average intensity?
The integrated puncta intensity is a measure of the sum of the pixel intensities within each puncta. The average puncta intensity is a measure of the average pixel intensity within each puncta. We have now explained this better in the legend.

11. There is a typo in the legend of Figure 7B, reading "presyanptic".
Thanks – this has been corrected.

12. The reference for Cabezas et al is incomplete.

Again, we have corrected this mistake.

2nd Editorial Decision 20 January 2011

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original three referees to review the revised version and I have now received their comments. As you can see below, the three referees appreciate the added data and find that the finding that loss of postsynaptic AMPA receptors affects presynaptic vesicle release well supported by the data. Referee #3 is still not persuaded that the manuscript is a good fit for publication in the EMBO Journal as we gain limited molecular insight into how this trans-synaptic mechanism works. However, both referees #1 and 2 find that the finding interesting and insightful enough and recommend publication in the EMBO Journal. I recognize that you have tried to add more mechanistic insight by looking if N-cadherin is involved in mediating this effect and that the data supports that this is not the case. Given the comments and support provided by both referees #1 and 2, I am going to go with their overall recommendation and accept the manuscript for publication in the EMBO Journal. Before doing so, referee #1 has a few minor remaining concerns that should be resolved before acceptance here. As you can see referee #1 suggests removing figure 3C, 6A and B from the paper. Lets discuss this issue further by email or phone.

As soon as we receive the revised version we will proceed with the acceptance of the study for publication here.

Thank you for submitting your interesting study to the EMBO Journal.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The paper by Tracy et al. added new experiment. Overall, I really like this paper and judge it suitable for publication in EMBO J. except for one point that still requires the author's xeplanation.

In Figure 8, the authors found that there was a change of inactive synapse from 21% to 26% by GluA RNAi (by the way, the 8B and 8C say GluR, which should be corrected), which is a reduction of active synapse from 79% to 74%, which is only 6% (=5/79). However, the author found about 40% reduction of NMDAR mediated synaptic current.

Minor comments.
Figure 3C. Given the reply to my point 4, which admits there might be recurrent activation problem happening, the quantification of decay time constant is not useful. It may reflect the decay of NR but also contaminated with the recurrent activation. If the authors want to estimate the change in NR2 subunit involved, a positive control is required showing that this method actually can detect that. I would simply suggest to remove Figure.

Figure 6A and B. From the same reason, this should be removed. 6C and D are sufficient for presentation. If there is any recurrent activation, it is very difficult to accurately measure PPR. Reduction in apparent PPR in 1.0 mM Ca2+ may indicate a real change in PPR or simply a reduction in recurrent activation due to reduced p.

Referee #2 (Remarks to the Author):

The authors made a major effort to implement my previous comments. I now support publication.

Referee #3 (Remarks to the Author):

The key finding of this study, that the loss of postsynaptic AMPA receptors inactivates presynaptic vesicle release in a subset of synapses, is intriguing and well supported by this work. Notably, the authors employed a large number of control experiments to exclude a number of alternative explanations, such as changes in NMDA receptor surface expression or function. They can further corroborate in new rescue experiments that this function of AMPA receptors is independent of their channel activity. This revised manuscript also includes additional experiments that further support the key finding by measuring vesicle endocytosis in neuronal cultures. Unfortunately, however, this revised study still lacks sufficient molecular insight into the underlying trans-synaptic mechanism, despite the successful efforts of the authors to test - and rule out - a contribution of N-cadherin to this effect. This revised study is therefore in my opinion more appropriate for a more physiologically oriented journal.

2nd Revision - authors' response 03 February 2011

Response to reviewers:
We thank the overall very positive response from the reviewers. We removed the Figures 3C, 6A and 6B as reviewer #1 suggested. We also agree with this reviewer that the discrepancy between the 40% reduction in NMDAR eEPSC and the much smaller increase in the inactive synapses observed with syt1 uptake assay is somewhat surprising. We think this is likely due to the dynamic vesicle trafficking between terminals – syt1-labeled vesicles can travel from active terminals to inactive ones within the 20 minute labeling period, leading to an underestimate of inactive terminals. We have added a sentence in the text to address this possibility.

