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Modulation of Synaptic Function by VAC14, a Protein that Regulates the Phosphoinositide PI(3,5)P_2 and PI(5)P

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1st Editorial Decision 01 November 2011

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

While the referees appreciate the findings describing a role for PI(3,5)P2 in neurotransmission, they also find that a significant extension of the work would be needed in order to consider publication in the EMBO Journal. As you can see, the referees find that more molecular insight into how VAC14 affects AMPAR trafficking is needed as well as some more support for that the phenotypes observed are mediated by reduction of PI(3,5)P2. Should you be able to significantly extend the findings along the lines as suggested by the referees then we are willing to look at a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses in this revised version. I also recognise that a lot of additional work is needed, but I can extend the revision time to 6 months should that be needed.

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, please visit our website: http://www.nature.com/emboj/about/process.html

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

The manuscript by Zhang et al. developed new VAC14 antibody and found that VAC14 is localized in multiple organelles such as endosome, lysosome and recycling endosome in fibroblast, using Vac14-/- as a negative control. In neuronal cells, they showed that VAC14 is localized in the spines as well as dendrites and axons.
mEPSC amplitude, but not frequency and decay time course, is enhanced in Vac 14-/- mice than wild-type cells. This increase in mEPSC amplitude is caused by the increase in the number of AMPA receptor on the synapses.

I find that the experiment in this manuscript is overall acceptable quality. But I cannot avoid feeling that it lacks excitement required for publication in EMBO J. The manuscript is too descriptive rather than going mechanistic details of their finding. Overall, more specialized journal would be better home for this paper.

1. VAC14 is a scaffolding protein, which may also binds other cellular molecules. Indeed the authors mention that it also binds NOS. Besides PI(3,5)P2, there might be other things which have changed. They need to demonstrate that the phenotype they saw are indeed mediated by the reduction of PI(3,5)P2, but not other mechanisms. If they cannot, they should largely change the conclusion.

2. The author shows that VAC14 partially colocalizes with multiple endocytic organelles. However I am not sure which organelle VAC14 dominantly localizes in. Quantitative evaluation of localization in early endosome, late endosome, lysosome, autophagosomes and others is required.

3. The authors speculate from immunostaining experiments that VAC14 plays some roles at early endsome, lysosome, late endosome and others. However the function and localization are different. The authors need to do following experiments to examine the detail role of VAC14 in membrane trafficking pathways in fibroblasts.
A. Uptake of transferrin to examine endocytic pathway.
B. Use of fluorescent sphingomyelin (NBD-SM) to examine recycling pathway.
The following paper is going to help you:
Molecular Biology of the cell vol. 18, 2667-2677, July 2007.

4. In order to examine whether VAC14 is important for the function of the vesicle release in from the presynaptic terminus, the authors need to do electrophysiologically test that.

5. In last paragraph in page 13, there is Fig 6 D-E. But I cannot find these figures.

Referee #2

Modulation of Synaptic Function by VAC14, a Protein that Regulates the Phosphoinositide PI(3,5)P2, by Zhang et al.

The authors describe synaptic phenotypes induced by a null allele of the VAC14 protein, a scaffold for the lipid kinase Fab1 in primary hippocampal neurons. The most striking finding is the increased mEPSP amplitude that correlates with an increased level of an AMPA receptor subunit in the cell surface of null neurons. The authors propose a model of either decreased endocytosis and/or increased recycling of AMPA receptors to explain the phenotypes observed in VAC14 deficient neurons.
The manuscript is well crafted and careful data interpretation adds a plus to this story. Well executed experiments makes a significant contribution to our understanding of the biology PI(3,5)P2 in neuronal cells and to the biology of this lipid in the endocytic pathway. The implications for the

presence of VAC14 in early, late endosomes and lysosome are quite important as they suggest unsuspected roles for PI(3,5)P2 in multiple compartments along the endocytic route. I'm quite positive about this work, however there are some details that the authors should consider.
1) Fig 9 model proposes an increased recycling or decreased endocytosis of AMPA receptors to explain VAC14-/- synaptic phenotypes, yet the authors do not explored whether VAC14 is localized in recycling endosomes or whether Vac14 localization is affected by recombinant reagents that perturb recycling endosomes, such as mutants of rab11 or some of its effectors. If this evidence were to be provided it would strengthen the model presented in Fig 9. Alternatively, this model could gain support if cell surface AMPA determinations were to be complemented with a determination of the intracellular pools of the receptor. In this type of experimental design in increased recycling and decreased endocytosis make precise predictions as to the internal levels of AMPA receptor.
Minor comments
2) One interesting hypothesis is that VAC14 deficiency may trigger excitotoxicity. To what extent does the vacuolation in processes and cell bodies obey to excitotoxicity? May be a simple yet illuminating experiment would be to assess vacuolation of VAC14 null neurons in the presence of AMPA blockers.
3) The localization images would greatly benefit from quantitative analyses of colocalization.
4) The progression of the results section is interrupted by the jump from Figure 4 to 7. This should be corrected.
5) Synaptic vesicle diameters in Supp. Fig 8. How many animals? How many terminals and how many vesicles? I would also suggest that the diameter data are presented as a cumulative distribution or a frequency histogram as there may be small number of organelles that are larger in VAC14 nulls.
6) There are some typos...For example in the abstract "neurodegeneration"

Referee #3

In this report Zhang et al. describe a role for PI(3,5)P2 (abbr. PIP2) in excitatory neurotransmission. Their results implicate that deletion of VAC14, a scaffolding protein required for PIP2 biosynthesis and turn-over, results in increased postsynaptic function mediated by increased levels of GluA2-containing AMPA-Rs.

First, they generated an antibody against VAC14 facilitating subcellular localization of the protein. They detect co-labeling of early- and late endosomes/ lysosomes in fibroblasts and in neurons; in neurons localization to synapses is evident. Moreover, the protein localizes to axons and dendrites and co-localizes with pre- and post-synaptic markers.

When recording mEPSCs they notice an increase in amplitude in the absence of VAC14 (Vac14-/-) relative to the wild type, which is lost upon VAC14 transfection into Vac14-/- neurons. Together with increased levels of surface GluA2 in Vac14-/- it is suggested that VAC14 plays an inhibitory role by modulating AMPAR levels at synapses.

Overall, this paper is well written and features interesting data. However, a couple of issues require further clarification. For example, Fig 8 shows an increase in surface GluA2. As total GluA2 levels are similar between wt and VAC14-/- (Fig S8), the increase in surface GluA2 should be shown by normalizing surface to total GluA2 levels i.e. by permeabilizing after the life stain and probing for total GluA2.

In Fig. 7 a significant decrease in excitatory synapse number is shown for Vac14-/- neurons, which doesn't quite match the apparent increase of GluA2 density shown in Fig 8A. If the size of GluA2 puncta increases in Vac14-/- , which would match the increase in mEPSC amplitude, then this should be quantified in both, Figs 7 and 8, as this is not apparent from Fig. 7A. Wouldn't Fig 7 suggest a decrease in mEPSC frequency?

Finally, the interesting observation that Vac14 (PIP2) affects aspects of AMPAR endocytic cycling warrants a more direct assessment, for example the potential impairment of AMPAR endocytosis in VAc14-/- should be tested using standard antibody-feeding/acid stripping protocols.

Minor comments:
- Image analysis protocols should be described in greater depth

- P. 13 last line Fig 5 D-E instead of '6 D-E'.

1st Revision - authors' response 02 June 2012

Reviewer #1:

1. *VAC14 is a scaffolding protein, which may also binds other cellular molecules. Indeed the authors mention that it also binds NOS. Besides PI(3,5)P2, there might be other things which have changed. They need to demonstrate that the phenotype they saw are indeed mediated by the reduction of PI(3,5)P2, but not other mechanisms. If they cannot, they should largely change the conclusion.*

   The above comment raises two critical points. First, are the effects of loss of VAC14 due solely to its function as a scaffolding protein for the lipid kinase PIKfyve, and its regulator FIG4? Second, is $PI(3,5)P_2$ the major lipid affected by loss of VAC14? To address the first issue, we tested and found that neurons cultured from the *Fig4*$^{-/-}$ mouse mutants exhibited a similar increase in miniature EPSC amplitude (Figure 3F-H). In fibroblasts from *Fig4*$^{-/-}$ mice, the levels of VAC14 are normal, but there is a 3-fold reduction in the level of $PI(3,5)P_2$ (Chow et al, 2007) and, interestingly, a 2.5 fold reduction in PI(5)P (Zolov, et al, submitted). This strongly suggests that the elevation in minis is due to defects in the PIKfyve/VAC14/FIG4 pathway rather than as yet unknown VAC14-dependent, FIG4-independent pathways. Notably, in *Vac14*$^{-/-}$ mice, the loss of VAC14 equally impacts the levels of $PI(3,5)P_2$ and PI(5)P (Zhang et al, 2007). Consistent with a role for PIKfyve in regulation of both phospholipids, Sbrissa et al. recently found that a PIKfyve inhibitor (YM201636) reduced both $PI(3,5)P_2$ and PI(5)P (Sbrissa et al, 2012). Thus, though PIKfyve was historically thought to function mainly through $PI(3,5)P_2$ biosynthesis, we now feel it is important to consider the reduction of both phospholipids in our interpretation of these data and have revised the manuscript accordingly.

2. *The author shows that VAC14 partially colocalizes with multiple endocytic organelles. However I am not sure which organelle VAC14 dominantly localizes in. Quantitative evaluation of localization in early endosome, late endosome, lysosome, autophagosomes and others is required.*

   We quantified the relative distribution of VAC14 in fibroblasts and found that 20% of VAC14 puncta colocalize with early endosomal marker EEA1, 30% with late endosomal and lysosomal marker LAMP1, and 19% with both markers. To quantify the colocalization of Vac14 puncta with late endosomes and lysosomes separately, we tested and found that 15% of VAC14 puncta colocalize with late endosomal marker LBPA and 23% with lysosomal Dextran. Additionally, we found 17% of VAC14 puncta colocalize with the autophagy marker LC3. These results are summarized in Figure S4F-G. In neurons, we tested the degree of colocalization of VAC14 with EEA1 and LAMP1 in dendrites, where VAC14 puncta could be easily identified, and found that 14% of VAC14 puncta colocalized with EEA1, 20% with LAMP1, 57% with neither, and 9% with both markers. These results are summarized in Figure S7C.

3. *The authors speculate from immunostaining experiments that VAC14 plays some roles at early endsome, lysosome, late endosome and others. However the function and localization are different. The authors need to do following experiments to examine the detail role of VAC14 in membrane trafficking pathways in fibroblasts.*
   A. Uptake of transferrin to examine endocytic pathway.
   B. Use of fluorescent sphingomyelin (NBD-SM) to examine recycling pathway.
   The following paper is going to help you:
   Molecular Biology of the cell vol. 18, 2667-2677, July 2007.

   We agree that determination of the effect of loss of VAC14 on known membrane trafficking pathways within the endomembrane system is critical to the field. These

questions have been partially addressed by us and others (Ikonomov et al, 2003; Rutherford et al, 2006; Zhang et al, 2007; de Lartigue et al, 2009). While the specific questions of transferrin and NDB-SM trafficking have not yet been addressed, the focus of this manuscript is on the localization and role of VAC14 at the postsynaptic membrane. Therefore we tested the effects of loss of VAC14 on three steps in trafficking of AMPA receptors: 1) endocytosis, 2) traffic from early endosomes to late endosomes, 3) recycling from endosomes to the plasma membrane. We found that the excess AMPA receptors at the cell surface in *Vac14*$^{-/-}$ neurons are due to a defect in endocytosis (Figures 7 and 8). This defect was observed using two independent methods: antibody feeding of live neurons (Figure 7) and pHluorin-GluA1 trafficking in live imaging assays (Figure 8).

In order to examine whether VAC14 is important for the function of the vesicle release in from the presynaptic terminus, the authors need to do electrophysiologically test that.

Thank you for this suggestion. Using electrophysiology, we measured the probability of vesicle release in presynaptic terminals by recording the rate of use-dependent blockade of NMDA receptors with the NMDA receptor antagonist, MK801. NMDA receptor currents were isolated by recording in the presence of CNQX and bicuculline to block AMPA and $GABA_A$ receptors, respectively. Mg^{2+} was excluded from the extracellular solution to relieve the Mg^{2+} block of NMDA receptors, and currents were evoked repeatedly with local stimulation using an extracellular electrode. We found that the rate of progressive blockade occurred more quickly in *Vac14*$^{-/-}$ neurons (Figure 4E, F), which is consistent with an increase in probability of vesicle release (Rosenmund et al, 1993).

4. *In last paragraph in page 13, there is Fig 6 D-E. But I cannot find these figures.*

Thank you for catching this typo. Figure 6 D-E should have read "Figure 5 D-E." In the revised version, these results may be found in Figure 3 D-E.

Reviewer #2:

1. *Fig 9 model proposes an increased recycling or decreased endocytosis of AMPA receptors to explain VAC14-/- synaptic phenotypes, yet the authors do not explored whether VAC14 is localized in recycling endosomes or whether Vac14 localization is affected by recombinant reagents that perturb recycling endosomes, such as mutants of rab11 or some of its effectors. If this evidence were to be provided it would strengthen the model presented in Fig 9. Alternatively, this model could gain support if cell surface AMPA determinations were to be complemented with a determination of the intracellular pools of the receptor. In this type of experimental design in increased recycling and decreased endocytosis make precise predictions as to the internal levels of AMPA receptor.*

We have now determined the ratio of surface to total GluA2, and show that the proportion of GluA2 at the surface is higher in *Vac14*$^{-/-}$ neurons (Figure 6C-D). Using antibodies against GluA2 that target different epitopes, one on the surface and one on the intracellular C-terminus, we labeled surface receptors, then permeabilized with 0.1% Triton, probed for total receptors, and visualized the staining in dendrites with confocal imaging. The ratio of surface to total was increased in *Vac14*$^{-/-}$ neurons. We also directly measured AMPA receptor trafficking using two independent methods (described above) and found that the observed accumulation of AMPA receptors on the surface is a result of decreased endocytosis without any changes in recycling rates (Figures 7 and 8).

2. *One interesting hypothesis is that VAC14 deficiency may trigger excitotoxicity. To what extent does the vacuolation in processes and cell bodies obey to excitotoxicity? May be a simple yet illuminating experiment would be to assess vacuolation of VAC14 null neurons in the presence of AMPA blockers.*

   We used TTX, APV and CNQX in combination to block synaptic transmission. However, vacuolation in *Vac14*$^{-/-}$ was not rescued (Figure S2). It is likely that the PIKfyve/VAC14/FIG4 pathway has multiple functions. Vacuolation is the most visually striking phenotype, but may not be directly linked to defects observed in synaptic function.

3. *The localization images would greatly benefit from quantitative analyses of colocalization.*

   This was also suggested by reviewer #1. The results are summarized in Figure S4F-G for fibroblasts and Figure S7C for neurites.

4. *The progression of the results section is interrupted by the jump from Figure 4 to 7. This should be corrected.*

   In this revision the figures are described in the correct order.

5. *Synaptic vesicle diameters in Supp. Fig 8. How many animals? How many terminals and how many vesicles? I would also suggest that the diameter data are presented as a cumulative distribution or a frequency histogram as there may be small number of organelles that are larger in VAC14 nulls.*

   Three wild-type and three *Vac14*$^{-/-}$ animals were used in the EM study. In total, 33 terminals and 576 vesicles were measured in wild-type hindbrain; 29 terminals and 388 vesicles were measured in *Vac14*$^{-/-}$ hindbrain. 33 terminals and 433 vesicles were measured in wild-type hippocampus; 15 terminals and 66 vesicles were measured in *Vac14*$^{-/-}$ hippocampus. This information is now included in the manuscript.

6. *There are some typos...For example in the abstract "neurodegneration"*

   Thank you for catching this typo.

Reviewer #3:

1. *Fig 8 shows an increase in surface GluA2. As total GluA2 levels are similar between wt and VAC14-/- (Fig S8), the increase in surface GluA2 should be shown by normalizing surface to total GluA2 levels i.e. by permeabilizing after the life stain and probing for total GluA2.*

   Thank you for this suggestion. We have performed this experiment and the results are reported in Figure 6C-D.

2. *In Fig. 7 a significant decrease in excitatory synapse number is shown for Vac14-/- neurons, which doesn't quite match the apparent increase of GluA2 density shown in Fig 8A. If the size of GluA2 puncta increases in Vac14-/- , which would match the increase in mEPSC amplitude, then this should be quantified in both, Figs 7 and 8, as this is not apparent from Fig. 7A. Wouldn't Fig 7 suggest a decrease in mEPSC frequency?*

We agree that the reduced density of synapses would predict a reduction in mEPSC frequency. Notably, this is not what we observed. However, since mEPSC frequency is determined by both the number of synapses and the probability of synaptic vesicle release, we have examined the possibility that *Vac14*$^{-/-}$ neurons exhibit an increase in release probability. Indeed, in new experiments, we find that use-dependent block of evoked NMDA receptor currents with MK801 (an established electrophysiological measure of release probability) (Rosenmund et al, 1993) is accelerated in *Vac14*$^{-/-}$ neurons, suggesting an increase in release probability that likely cancels out the effect of synapse number with respect to the frequency of mEPSCs (Figure 4E-F).

3. *Finally, the interesting observation that Vac14 (PIP2) affects aspects of AMPAR endocytic cycling warrants a more direct assessment, for example the potential impairment of AMPAR endocytosis in VAc14-/- should be tested using standard antibody-feeding/acid stripping protocols.*

We measured GluA2 endocytosis using the antibody-feeding/acid stripping method, in dendrites and in the soma (Figure 7). We also measured endocytosis of fluorescent pHluorin-GluA1 in cultured neurons (Figure 8). In both assays, endocytosis was reduced in the *Vac14*$^{-/-}$ mutant.

4. *Minor comments:*
- Image analysis protocols should be described in greater depth

These details have now been added.

- P. 13 last line Fig 5 D-E instead of '6 D-E'.

Thank you for catching this typo. In the revised version, these results may be found in Figure 3D-E.

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Zhang Y, Zolov SN, Chow CY, Slutsky SG, Richardson SC, Piper RC, Yang B, Nau JJ, Westrick RJ, Morrison SJ, Meisler MH, Weisman LS (2007) Loss of Vac14, a regulator of the signaling lipid phosphatidylinositol 3,5-bisphosphate, results in neurodegeneration in mice. *Proceedings of the National Academy of Sciences of the United States of America* **104:** 17518-17523

Acceptance letter 27 June 2012

Thank you for submitting your revised manuscript to the EMBO Journal. Your revision has now been seen by referees #2 and 3.

As you can see below, both referees appreciate the introduced changes and support publication here. I am therefore very pleased to 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

The authors have addressed all comments and added significant amounts of elegant and important data. In its present form this is a great piece of work and an important contribution to membrane traffic, cellular neuroscience and membrane PIP metabolism.

Referee #2

This manuscript has been revised satisfactorily, the authors have addressed all queries. This work is now suitable for publication.

