To
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Dear [redacted]

Thank you for your reply regarding our manuscript # MCN-06-240; entitled P ICK1 INTERACTS W ITH alpha7 NEURONAL NICOTINIC ACE TYLCHOLINE RECEPTORS AND CONTROLS THEIR CLUSTERING.

We are grateful for your and the reviewer's comments, and the positive evaluation of our work. We have revised and modified the text and figures according to the referees' critiques. As a consequence we provide new data about sequence comparisons between alpha7 and other PICK1-binding proteins; redesigned Figures 4, 5 and 6 (4 and 6 by repeating experiments); and added many new and clarifying statements in all parts of the paper. These changes have improved the manuscript considerably and we hope that it can be published without delay.

Sincerely,

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Detailed response: we have addressed your editorial comments and responded to the comments by the reviewers as follows.

Response to Editor only (not for reviewers)

Reviewer #1:

The reviewer appears positive and has two points that come to mind. The second about RNAi is a good idea and we are pursuing it at present. As so often with RNAi, experiments prove more difficult than planned, as effective sequences producing interfering RNA have to be found, tested with negative controls, and introduced into neurons – all very difficult. Thus this approach would delay the manuscript massively and we think it is beyond the scope of this paper.

Reviewer #3:

The reviewer appears positive. But 14 points are brought up, each asking, if one reads carefully, for multiple experiments, resulting in over 30 additional experiments requested. For obvious reasons it is not possible to do all of them, unless the paper is massively delayed by at least one year. We have picked out the points that we judged to be the best, most relevant and doable within the three months that you granted us for the revision. As a result, we answer several points with new data derived from additional experiments, while other points are discussed without additional data. We feel, in summary, that we have answered the reviewer's thoughts thoroughly.

Detailed response: w e have addressed ed itorial comments and responded to the comments by the reviewers as follows.

Response to Reviewers

Reviewer #1:

The reviewer states that we present an interesting study demonstrating evidence that PICK1 interacts with nicotinic alpha7 receptors and inhibits their clustering on hippocampal interneurons. This reviewer evaluates that our experiments are carefully done, and the findings are reasonably interpreted. This reviewer has two points which we address in the following. Numbers refer to numbers used by the reviewer.

1. We thank this reviewer for this suggestion. We do not know an antibody sensitive enough to precipitate alpha4 nAChR from brain (allowing an analysis for associated PICK1 in immunoblots). However, we feel that the evidence presented in Fig. 1(lack of interaction of the alpha4 loop in the yeast two-hybrid experiments) and Fig. 2C (lack of interaction of the alpha4 loop in the COS lysate pull-down experiments) is strong enough to make it very unlikely that an interaction would occur *in vivo*. We are well aware of the risk of false positive interactions in YTH screens. Therefore, we have verified the positive alpha7 loop interaction using two biochemical assays, and have confirmed the control of the alpha4 loop in the GST assays. This is a standard experimental approach routinely followed, and we think it gives sufficient proof for both, the positive alpha7, and the negative alpha4 loop data. We have added an explaining sentence on page 6 (end of middle section).

2. We agree with the reviewer that the role of the PICK1- alpha7 interaction remains a key question. We present one of these roles (control of alpha7 nAChR clustering) and further roles remain to be investigated. Downregulating endogenous PICK1 could identify further mechanisms. We currently pursue these experiments using RNAi technology as suggested by the reviewer, but consider these data too preliminary to include. Although the mechanism by which PICK1 influences alpha7 clustering remains unclear, we think our present findings are important enough to justify publication at this point. We have included a statement in the Discussion (page 15, first paragraph) that future experiments will address these issues.

Reviewer #3:

This reviewer states that we present novel and interesting results and suggests additional experiments to strengthen the paper. We thank the reviewer for the extensive evaluation and are happy to include the suggestions. Numbers refer to the numbers used by the reviewer.

Introduction

1. We fully agree with the reviewer and have changed this sentence accordingly (page 3).

2. We thank the reviewer for asking these important questions. We did not introduce these important issues due to text limitation constraints but did mention them in the

discussion (page 13). Yes, there are other examples of PICK1 inducing a decrease of receptor clustering for other receptors. PICK1 causes decreased clustering of GluR2-containing AMPA-Rs in hippocampal pyramidal cells, apparently by favouring receptor internalization (Perez et al., 2001; Terashima et al., 2004). At the same time, PICK1 causes AMPA-R clustering when expressed in heterologous cells (Xia et al., 1999). In contrast, PICK1 increases surface levels of dopamine transporters in neurons (Torres et al., 2001). Thus, the situation is very complex in neurons and heterologous cells, as PICK1 can play differential roles in regulating surface clustering of these receptors. We have extended a sentence in the introduction to mention this complexity (page 4).

Results

1. The aim of our screen was to identify novel interactors of alpha7 nAChR and not to assess interaction strength of various domains of PICK1 or alpha7. Interactions in yeast as such always remain somewhat artificial (the proteins must meet in the yeast nucleus), bringing up question marks about attempts to render these interactions quantitative. We think it more important to test yeast-interactions in other systems (such as recombinant and native protein pulldowns as done in Fig. 2 and 3). Therefore we do not see a need to provide an additional assessment of our yeast two-hybrid data, but agree that additional information evaluating the strength of interactions in general can be advantageous under certain circumstances. The approach we have selected is routinely used, for example in the following high-profile publications:

Boudin H, Doan A, Xia J, Shigemoto R, Huganir RL, Worley P, Craig AM. Presynaptic clustering of mGluR7a requires the PICK1 PDZ domain binding site. Neuron. 2000. 28(2):485-97.

Staudinger J, Lu J, Olson EN. Specific interaction of the PDZ domain protein PICK1 with the COOH terminus of protein kinase C-alpha. J Biol Chem. 1997. 272(51):32019-24.

Xia J, Zhang X, Staudinger J, Huganir RL. Clustering of AMPA receptors by the synaptic PDZ domain-containing protein PICK1. Neuron. 1999. 22(1):179-87.

We have added a statement about the nature of our presentation of yeast interaction data referring to these papers in the Results (page 4).

2. We agree with the reviewer about the importance of such a sequence alignment analysis. Therefore we have performed the suggested alignments and include them in Fig.1. We aligned the bait 9 sequence of alpha7 first with the corresponding amino acid sequences of the following proteins: nAChR alpha3, alpha 4, beta2, beta 4. This comparison reveals a variable degree of conservation, with no region that would show a particularly low identity. A stretch towards the C-terminus of the loop shows high conservation, but this cannot be the binding motif, as we show that alpha4 and beta2 do no interact with PICK1. The motif EVRY also shows high conservation (but again cannot alone be the interacting motif for the same reasons as mentioned; we demonstrate this explicitly in Fig. 1D). The motif ESEV is less conserved but again is not critical (Fig. 1D).

Second, we compared bait 9 of alpha7 to the PICK1-interacting regions at the C-termini of Arf1, Arf3, EphB2, GluR2, GluR3, $GluR5_{2b}$, GluR6, mGluR7a, and PKCalpha. The alignments demonstrate the low degree of conservation as shown below the aligned sequences. No particular motifs can be recognized. The C-termini of all these proteins but Arf1 and Arf3 correspond to class I or II motifs for PDZ

domain interaction (according to Nourry et al., 2003). Arf1 and Arf3 are clearly different from these motifs and thus particularly interesting for us, because the PICK1-binding region of alpha7 is also different from known motifs.
Third, we therefore aligned alpha 7 specifically with Arf1 and Arf3. Again, no particular conservation or motifs were obvious.
We have changed Fig.1, Legend (page 30), Results (page 5, 6) and Discussion (page 12) to incorporate these findings.

3. We agree with the reviewer that determining the minimal sequence required for interaction with PICK1 would be of great interest. The sequence comparison done in Fig. 1 shows no obvious region of interest within the 38 aa of bait 9. Thus, defining a region or a motif would require systematic deletions and amino acid replacements, which is very time-consuming and could possibly be inconclusive, e.g. when binding is based on the three-dimensional structure, making the localization of a simple binding motif impossible. Therefore, we preferred to focus on the question whether the interaction of this alpha7 domain with PICK1 *per se* has a functional significance at all. We have included a statement in Results about what would be necessary to further narrow down the PICK1-binding region in alpha7 (page 6).

4. We think that our yeast two-hybrid data as shown in Fig. 1 and the functional results presented in Fig. 7 convincingly demonstrate that i) the PDZ domain of PICK1 is needed for the alpha7 loop-PICK1 interaction, and that ii) the functional PDZ domain of PICK1 is needed for an effect on α7 nAChR clusters. The AA mutation within the PDZ domain is well known from the literature to inactivate the binding capacity of this domain completely (Xia et al., 1999; Boudin et al., 2000). We show that overexpression of the mutant PICK1-AA protein did not change the pattern of alpha7 surface clusters *in vivo*, therefore we think it is neither necessary nor well invested to go back to the yeast two-hybrid analysis and test the PICK1-AA mutant further. The reviewer questions a direct binding, but a "go-between" protein would have to be expressed in COS cells as in bacteria, and it would have to survive the protein purification on glutathione-sepharose (for GST) – all very unlikely scenarios. We have included a statement in the Discussion (page 12).

5. We disagree with the reviewer and think that the data shown in Fig. 2 are complete and contain all the controls needed to demonstrate the specificity of the novel interaction. First, with all controls shown and mentioned, we are certain that we detect interaction of alpha7 nAChR and PICK1, and demonstrate specificity of precipitation and detection. Given all the controls we did, we feel it unnecessary to perform the suggested additional pull-down experiments. We agree that it would be a further negative control to use some of the alpha7 bait constructs for pull-down experiments, but again as outlined above, our focus is on elucidating the functional relevance of this novel interaction; we prefer to invest our time and resources in a functional analysis.

6. The reviewer correctly describes what can be seen from Fig. 3, and we are grateful for bringing up the point. As with all biochemical precipitations, a protein in solution is precipitated specifically, by interacting with its appropriate binding partner (=alpha7 for PICK1; bungarotoxin coupled to sepharose for alpha7), and also, to a low degree, non-specifically, by simply "sticking" to the resin (=sepharose beads). To distinguish specific from non-specific precipitation of PICK1, excess bungarotoxin or

nicotine are used, which eliminate alpha7 from precipitation. PICK1, nonetheless, shows some signal, illustrating its low non-specific sticking to the resin. This amounts to some 15% of the total PICK1 signal precipitated. Thus, most of PICK1 in Fig. 3A binds to alpha7, and a low percentage sticks to the beads. Therefore the data clearly show that in hippocampal tissue, PICK1 associates with alpha7, although in a precipitation some PICK1 is pulled down non-specifically. We have incorporated this statement in the Results (page 7).

7. We did the experiments in Fig. 3 to show that PICK1 can not only be co-precipitated specifically with alpha 7 using bungarotoxin (Fig. 3A, B), but also using antibodies against alpha 7 (Fig. 3C, D). This combination of approaches results in much better overall evidence that PICK1 associates with alpha7 in brain tissue than if only one approach (bungarotoxin as in Fig. 1A, B) was used. We have strengthened this thought by a statement in the Results. The non-immune IgG bands are not a problem, because they run at slightly higher molecular weight than PICK1. The antibody against alpha7 runs at still higher apparent MW. This is nothing to worry about, because the non-immune IgG is a control antibody mix, while the alpha7-antibody is a monoclonal antibody specifically against alpha7 – and monoclonal antibodies can have slightly varying MWs. In summary: in all controls of Fig. 3C, the molecular weight range of PICK1 is clean and free from signal, because the antibody band runs above it. Thus the Figure does show specific co-precipitation of PICK1 with alpha7. We have included a statement in the Results (page 7).

8. We thank the reviewer for this positive comment and confirm that the blots shown in Fig. 3D were done in parallel with those of Fig. 3C, even using exactly the same samples. We split the immunoprecipitation samples in two parts: one part was loaded on one gel to produce blots of Fig. 3C, the other part was loaded on a parallel gel, which was used to produce blots in Fig. 3D. We include a statement in the legend of Fig. 3 (page 31) and the Results (page 7). Taken together, Fig. 3 shows very strong evidence that PICK1 and alpha7 specifically associate in hippocampal neurons, but alpha7 does not associate with PSD95 proteins or GluR2.

9 and 10. We apologize for the lack of clarity of this set of data. We have now changed Fig. 4 and clarified why we used different cell lines and detection systems to evaluate the interaction of PICK1 with transfected and constitutively expressed $\alpha 7$ nAChR. We have taken the reviewer's suggestions into account and have modified Fig. 4 and associated texts accordingly (page 8, 9, 31).

11. We agree with the reviewer that alpha7 cell surface expression might be modulated by ligand and that PKC is one of the first candidates that could be assessed in this context (see new statement in Discussion; page 15, 16). Modulation of alpha7 clustering by ligand is however hard to study using currently available reagents: to visualize the alpha7 clusters, cells are incubated with fluorescent bungarotoxin (which inactivates the receptor), then processed for microscopy. Ligand binding competes with bungarotoxin-binding. Unfortunately, antibodies against alpha7 nAChR suitable for immunofluorescence experiments on endogenous alpha 7 receptors in neurons are not available. Regarding the PKC pathway, we have started some experiments but these are too preliminary to include in this paper within reasonable time.

12. We now provide images that were derived in the same fashion. We have replaced the original Fig. 5d-f with a maximal projection of confocal stacks, so that both set of images are now consistent and easier to interpret. In addition, in Fig. 5a-c, we show inserts (boxes) at higher magnification. The new Fig. 5 shows individual alpha7 clusters in dendritic areas, just as the new Fig. 6 does. We have changed the Figure legend accordingly (page 32).

13. We agree with the reviewer and have repeated the experiments for Fig. 6 using cultures of hippocampal primary neurons. We repeatedly performed triple-stainings detecting endogenous alpha7 nAChR (using α-BT), endogenous PICK1 protein (using the polyclonal antibody from Upstate), and VGAT as a marker for interneurons. For image acquisition, we used a new high-resolution epifluorescence microscope (Zeiss Imager with ApoTome, Zeiss), which has a higher sensitivity than our confocal microscope, notably for detection of PICK1 immunofluorescence. Remarkably, the new set of data showed that rather than being colocalized, PICK1 and alpha7 nAChR clusters tend to be adjacent to each other or even apposed (new Fig. 6). This finding is in excellent agreement with the results from the SH-SY5Y cell transfection experiments (revised Fig. 4B) and is consistent with our conclusion that PICK1 negatively regulates alpha7 nAChR clustering. We have made the necessary changes in Results (page 10), Figure 6 and Legend (page 32), and Discussion.

14. We thank the reviewer for the positive evaluation of the results presented in Fig. 7 and 8. The reviewer has several comments which we address as follows:

a. We used these two different approaches to i) confirm the results with independent techniques; and ii) to confirm a healthy morphology of the interneurons overexpressing PICK1 to ascertain that PICK1 viral expression does not harm the cells. We think that the combination of these two approaches allows more solid conclusions. We have added a statement about this in Results (page 11). The greater variability of the transfection experiments is probably due to a more varying level of expression of EYFP-PICK1. This, together with the lower number of cells analyzed, may explain why the statistical significance is reduced in Fig. 8.

b. Our conclusion is derived from the observation and quantitative analysis of an apparent reduction in the number of α-bungarotoxin-labeled clusters in cells overexpression PICK1 with an intact PDZ domain. We are well aware that multiple mechanisms might explain this observation, as discussed in detail on page 15. However, the specificity of this effect is supported by the multiple controls shown in Fig. 7 and 8. We have added statements in the Discussion to explain this better (page 15).
In addition, data presented in Fig. 9 show that the $GABA_A$ receptor alpha1 subunit signal is not downregulated in interneurons after EYFP-PICK1 expression compared to control EYFP expression. This finding indicates that overexpression of PICK1 in hippocampal GABAergic interneurons does not have a general effect on surface receptors, but specifically reduces surface clusters of alpha7 nAChRs.

c. This is an excellent suggestion. Unfortunately, all our attempts to perform this experiment have failed. We have generated EGFP-constructs containing the whole loop or the bait 9 region of alpha7. Expression in heterologous cells (SH-SY5Y) was possible and we managed to express the fusion proteins in cultured hippocampal

neurons. In all cases, EGFP signals were diffusely distributed with no signs of clustering. However, the hippocampal neurons overexpressing the bait 9 or loop sequence of alpha7 consistently died shortly after expression started, indicating toxicity of the overexpressed sequences, therefore preventing us to draw further conclusions. We do not feel that this information should be added to the manuscript.

d. We think that overexpression of PICK1 only decreases alpha7 clustering in interneurons and not SH-SY5Y or other cell types, because we hypothesize that currently unknown interneuron-specific factors are needed for this mechanism. This mechanism may depend on one or several proteins expressed in interneurons that bind(s) to the alpha7-PICK1 complex and effects its targeting. In such a manner, alpha7-PICK1 complexes may have a defined molecular composition in these cells. Consistent with this, alpha7 clusters in populations of spinal cord neurons differently colocalize with cytoskeletal and lipid rafts components (Roth and Berg, 2003) (Discussion, page 13, 14).

Discussion

We have added a paragraph to discuss how PICK1-PKC interaction could have a potential effect on the novel interaction between alpha7 and PICK1 which we report in this study, as suggested (page 15, 16).
To our knowledge, there are no reports of PKC modulating alpha7 nAChR gene expression, clustering or surface expression. No interaction partners have yet been identified for alpha7 nAChRs, underlining the importance of this current study, but among other nAChRs, members of the PSD95 family interact with alpha3 and beta4 subunits (Conroy et al., 2003; Parker et al., 2004).

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Reviewers' original comments:

Reviewer #1: This is an interesting study presenting evidence that Pick1 interacts with nicotinic alpha7 receptors and inhibits their clustering on hippocampal interneurons. Pick1 was isolated by yeast-2-bybrid using the large intracellular loop of alpha7 as bait. Pull-down experiments and immunoprecipitations were used to gain additional evidence of specific association, and fluorescence was used to assess co-distribution of Pick1 and alpha7 after heterologous expression in cell lines or in hippocampal interneurons in dissociated cell culture. The results support the view that Pick1 interacts directly, or possibly indirectly, with alpha7. The interaction is not very tight in that the two components often form independent clusters. And what Pick1 does to alpha7 receptor distribution is not entirely clear, despite the arguement for a negative effect on receptor clusters. Nonetheless, the results are interesting, the experiments are carefully done, and the findings are reasonably interpreted.
Two points come to mind.

1) A number of permutations were carried out to demonstrate specific association between Pick1 and alpha7. Perhaps most stringent, however, would have been a negative control showing that alpha4 nicotinic receptors did not co-immunoprecipitate with Pick1 from synaptosomal preps, in contrast to that seen with alpha7. They

showed earlier that the alpha4 loop was not recognized in the yeast-2-hybrid experiments, making this control a reasonable expectation.

2) A key question that remains is the role of Pick1-alpha7 interactions.
The authors propose that Pick1 prevents alpha7 clustering by any of several mechanisms, but they have to contend with an incomplete co-distribution of Pick1 and alpha7, and use overexpression of Pick1 to show reduced alpha7 clustering. More telling would be experiments to downregulate endogenous Pick1 levels, e.g. with RNAi, to see if changes occurred in alpha7 levels or distribution. This would be most relevant and could provide stronger evidence for a specific effect.

Reviewer #3: The manuscript by Baer et al describes a novel interaction between PICK1 and alpha7 neuronal nicotinic acetylcholine receptors and provides evidence that this putative interaction results in decreased alpha7 clustering in cultured hippocampal neurons. While the results are novel and interesting, additional experiments are required to strengthen the paper.
There are also redundancies and inconsistencies in the experimental approach that need to be addressed.

Introduction

1. On line 23 the phrase "linking alpha7 to pathogenesis of schizophrenia" is too strongly worded. A better phrase would be "suggest that alpha7 might play a role in the pathogenesis of schizophrenia".

2. Are there any examples of PICK1 inducing a decrease of receptor clustering of other receptors? In other words, is the inhibition of receptor clustering a novel role for PICK1?

Results

1. In the YTH studies in Figure 1, it would be informative to provide an assessment of the strength of interaction between the bait and prey (provide beta-gal protein or activity numbers). When doing this, include a positive control (AMPA or mGlur7a) that will demonstrate the relative strength of PICK 1 interaction with alpha7. This is particularly important for the experiments in Figure 1C where the mutation of known PDZ binding site does not abolish the interaction (based on the rather arbitrary +/- terminology).

2. Also include in Figure1, amino acid sequence alignment of alpha7-PICK1 interacting domain (aa 429 to aa 467) and PICK1-interacting domains present in other PICK1 interacting proteins (for example AMPA, mGlur7a, PKC etc.if known). It will also be useful to include in this alignment the analogous loop regions of alpha3, alpha4, beta4 and beta2 acetylcholine receptors.
Such an sequence alignment analysis will provide useful information about potential amino acid residues that defines PICK1 interacting regions.

3. It is not clear why the authors did not further narrow down the region in

alpha7 necessary for interaction with PICK1. The 38 aa region should be further narrowed down. The precise identification of a smaller region or even amino acid residues within the 429 to 467 region would greatly enhance the significance of the paper and is strongly recommended. On the other hand if attempts were made to narrow the 38 aa region, but without success, this should be mentioned.

4. Similarly the authors should have narrowed down the PDZ domain region necessary for interaction with alpha7. In particular, a strong control would be to test the PICK1 AA mutant that is used in Figure 7, in the YTH system, and show that it fails to interact with alpha7. This would provide strong and compelling evidence that the results in figure 7 are due to direct PICK1-alpha7 interaction and not due to secondary effects of PICK1 over expression.

5. The results in Figure 2 are incomplete- control pull down experiments that used PICK1 (1-126), PICK1 (126-417) and the PICK1 AA mutant are necessary. Similarly control experiments with some of the positive and negative alpha7 baits used in Figure 1A and 1C will be informative.

6. In Figure 3A, no explanation is provided for the (faint but consistent) pull down of PICK1 seen in experiments where excess toxin/nicotine is used during the toxin or nicotine mediated pull down. Taken together with the alpha7 blots which is clean, this suggests that PICK1 interacts (albeit weakly) with a receptor/protein that is pulled down by toxin or nicotine. Is this another subunit or isoform of acetylcholine receptor? If so this should be mentioned and/or tested.

7. In the experiments shown in Figure 3 C&D why weren't the alpha7 pulled down by toxin or nicotine done as in Figure 3A? This would have provided better signal to noise in 3C. The antibody bands and, in particular, the bands in the non-immune IgG lanes make it difficult to make any quantitative comparison.

8. The negative controls shown in 3D are nice but were these done on the blots shown in 3C?

9. Figure 4 is a good example of redundancy and poor experimental planning. It is not clear why the authors did not use confocal microscopy for all the experiments- A&C are conventional whereas B is confocal. Different methods are used to detect alpha7 expression. Why not just use alpha-BT consistently? Having said that why don't you see membrane labeling of alpha7 in COS and HEK cells? Without cell surface expression, these experiments are completely irrelevant as you cannot assess the effect of PICK1 on alpha7 clustering or surface expression. Also the in vitro experiments in Figure 2 show robust interaction between PICK1 and alpha7- then why is it that you only see partial co localization in Figure 4A? Are there any data on co localization of PICK1 with AMPA or mGlur7a in COS cells? These would serve as a nice control.

10. Figure 4 needs to be completely revamped. You could show COS cells with alpha7 and with and without the EYFP-PICK1 construct used in Figures 7 & 8 (preferably with the mutant PICK EYFP construct too). The alpha7 expression

should be detected using alpha-BT. The experiment with the SH-SY5Y should be repeated using the same approach and with better looking cells.
Preferably use confocal for all experiments. The HEK figure is redundant and irrelevant and should be removed.

11. Another issue with the imaging experiments is that PICK1-alpha7 interactions and its functional consequence on alpha7 cell surface expression might be modulated by ligand or induced (for example by the PKC pathway). Have you considered treating your co-expressed cells with acetylcholine or inhibitors/activators of the PKC pathway? After all, PICK1 is a PKC-interacting protein.

12. Figure 5 is difficult to interpret. The images that are shown for VGAT and GAD are not easily comparable- one is a maximal projection of confocal stacks and the other is a single confocal section. Comparing the high resolution images provided, I would argue that alpha7 clusters overlap with GAD more than they do with VGAT. The authors need to be consistent and provide images that were derived similarly.

13. The images provided in Figure 6 are yet another example of poor presentation. All I see are some dots that overlap. Those dots/specks could just be background noise. Why don't you provide a confocal image similar to the one shown in figure 5 (where one can clearly see the soma and the dendrites)? This figure 6 is not a convincing demonstration of
PICK1/alpha-7 co localization.

14.Figures 7&8 represent experiments that are very nicely executed and presented. But there are several issues with regard to rationale and interpretation.

a. The authors need to explain why two different transfection approaches (viral versus magnetofection) were used. The two methods gave very different statistical outcomes- the magnetofection results in Fig 8 have a $p=0.03$ whereas for the viral infection they were several magnitudes lower. Any reason why?

b. The authors claim that PICK1 over expression reduces clustering is not supported by the conventional fluorescence images in Figures 7 & 8- over expression of PICK1 appears to decrease the overall surface expression of
alpha7 - not just clustering. This raises the possibility that PICK1 over expression might have nonspecifically reduced the gene expression of alpha7 in these neurons or alternatively induced receptor internalization. Without experiments to rule out these two possibilities, it is premature to claim that PICK1 decreases alpha7 receptor clustering.

c. PICK1 interacts with multiple proteins in the neuron (in particular
PKC) and thus over expression of WT PICK1 could potentially disrupt multiple signaling pathways. Therefore the better way to approach the issue would be to over express the alpha7 domain that you have shown in Figure 1 interacting with PICK1. This domain (bait 9) should be introduced into the neurons using the methods that you have used with the PICK1 over expression experiments. If PICK1-alpha7 interaction modulates receptor clustering or surface expression in vivo, such an experiment would provide more direct evidence. The over expressed bait 9 should interfere with

the ability of endogenous PICK1 to specifically interact with endogenous alpha7 receptor.

d. Why doesn't PICK1 over expression decrease clustering in heterologous cells (specifically SH-SY5Y)?

Discussion

You need to discuss the potential effect of PICK1-PKC interaction. Are there any reports of PKC modulating alpha7 receptor gene expression, clustering or surface expression? Your discussion needs to include a better analysis/discussion of your results.