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Munc18-1 mutations that strongly impair SNARE-complex binding support normal synaptic transmission

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Transaction Report:

(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 14 February 2012

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. I should add that referee 1 chose to sign his report. As you will see, all three referees are rather positive about the paper and would support its publication here after appropriate revision. I would like to invite you to submit a revised version of the manuscript that addresses the referees' criticisms in an adequate manner, in particular the issues with respect to a more balanced and mildened discussion of the discrepancies with other studies in the literature.

I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Peer 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

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

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

revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This paper investigates the role of interactions between Munc18-1 and the neuronal SNARE complex in neurotransmitter release. Munc18-1 and the neuronal SNAREs, syntaxin-1, synaptobrevin and SNAP-25, play crucial roles in release, but it is still unclear how their functions are coupled. Munc18-1 binds to the so-called closed conformation of syntaxin-1 and also to SNARE complexes containing open syntaxin-1, and both types of complexes involve interactions with a short sequence at the N-terminus of syntaxin-1 called the N-peptide. In this paper, the authors examine three different mutations that impair binding to the SNARE complex and none of them yield significant disruptions of neurotransmitter release as assessed by electrophysiological measurements in autapses. These results suggest that Munc18-1 binding to the SNARE complex is not important for neurotransmitter release, which contradicts some previous studies but agrees with other published results. The data presented in these paper are of high quality and I believe they make an important contribution to this area. Hence, I certainly recommend publication in EMBO Journal, although I have several concerns that authors may want to address.

1. My main concern is that the strong tone of some parts of the paper will contribute to confuse an already confused field. This is not a strong criticism because the authors try to give a balanced view on the contradictions with other papers, but I still think that the authors should be cautious in concluding that binding of Munc18-1 to the SNARE complex is not physiologically important. I believe that the data presented in this paper do suggest that impairing SNARE complex binding does not have strong effects in release. However, given the other data available in the literature, it is plausible that impairing this interaction does have some physiological effects that depend on the system and may be too subtle to be detected with the tools used in the present study (see points 2 and 3). With this interpretation, the results of this paper are still very important, but they can be reconciled better with other available data.

2. In general, the paper presents high quality ITC data and I agree with the authors that this is indeed a rigorous technique to study protein-protein interactions (see statement in the middle of page12). However, ITC has its limitations, particularly when the enthalpies of the interaction are small and the protein concentrations cannot be increased due to solubility issues. For instance, previous ITC data described by the Fasshauer lab could not detect binding of Munc18-1 to the SNARE four-helix bundle (Burkhardt et al. 2008), but binding can be clearly detected by other methods and the affinity is on the order of 6-10 micromolar (Xu et al. 2010), which is not very tight but is not very weak either. Hence, I suggest that the authors do not use the term 'abolish' when referring to the effects of the Munc18-1 mutations on SNARE complex binding, and instead use less absolute terms such as 'impair'.

3. I am not an expert in electrophysiology but from my view of the electrophysiological data (all of which appear to be of high quality) I also would suggest the authors to be cautious with the interpretation of these data. For instance, while the data clearly show that there are no significant differences between the EPSC amplitudes measured for WT Munc18-1 and the mutants, there could be differences within the error of the measurements. In addition, it seems that the paired pulse ratios are increased somewhat for all the mutants, which if I am not mistaken indicates a decrease in the release probability.

4. In page 4, the authors state that Munc18-1 binds weakly to SNARE complexes, despite reporting in Table 1 that the Kd is 719 nM. Of course, this is a semantic issue and what 'weakly' means is relative, but the authors should be aware that most biophysicsts would not consider such an affinity

to be weak.

5. The errors in Table 1 seem unrealistically small and they appear to be the errors yielded by the fitting program, which are generally much smaller than the errors observed in repeated ITC experiments. The errors from multiple experiments should ideally be reported but, if the authors did not repeat them, they should at least state very clearly in Table 1 that the errors come from fitting the data and the real errors are expected to be larger.

6. The Kd measured for binding of the Munc18-1 E59K mutant to syntaxin-1 (48 nM) is considerably smaller than the value we reported in Deak et al. 2009 (12 nM). As we mentioned in that paper, we had difficulty getting consistent ITC data when using syntaxin-1(2-253) because of its tendency to aggregate, and we obtained much better data with syntaxin-1(2-243), which aggregates much less (Chen et al. 2008, J. Biomol. NMR 41, 43-54). Since the authors are using syntaxin-1(2-262), which aggregates even more, they may want to point out in the manuscript that differences in the Kds may result from using different fragments. In addition, it is important to report the concentrations used for the ITC experiments, since the aggregation depends on the protein concentration.

Josep Rizo

Referee #2 (Remarks to the Author):

In this article, the authors explored the role of Munc18 by a combination of biochemical assays to characterize interaction with synaptic SNARE complexes and syntaxin and cell biological assays in cultured neurons to characterize functional properties of WT and mutant forms.

The question addressed by this article is timely and critical in the field of membrane fusion and secretion because the function of Munc18 is still unclear: role as a chaperone for the transport of syntaxin, role prior to fusion in a priming process, or role in fusion via binding to assembled SNARE complexes or a combination of the three? Similarly to other articles, the data presented here take advantage of testing mutants in biochemical assays and complementation assays in Munc18 KO neurons. The main finding is that three point mutants unable to bind SNARE complexes still complement function. The experiments presented here were rigourously carried out and controlled. Nevertheless, the conclusion may appear contradictory to that of Shi et al in a recent article in MBoC, as well as several other studies, at the same time that it agrees with others. In the Shi et al article, the authors used a Munc18-1 quadruple mutant and showed that this mutant reduced interaction with syntaxin 1A and impaired chaperone function, but still bound to assembled SNARE complexes and promoted liposome fusion and secretion in neuroendocrine cells. In order to help the community reach a consensus on Munc18's function, the authors should test the Munc18-1 quadruple mutant in their assays, then discuss in detail differences between their experimental conditions and those of previous work (I compared some of the concentrations used in different articles' biochemical assays and found that they were large enough to account for discrepancies). This may lead the authors to a more balanced conclusion.

Referee #3 (Remarks to the Author):

The manuscript by Meijer et al. investigates a potential post-docking role of Munc18-1 in synaptic vesicle exocytosis. The authors use a combination of sophisticated biochemical (isothermal titration calorimetry, fluorescence-based anisotropy), morphological (chemical-fixation electron microscopy) and electrophysiological (postsynaptic currents in autaptic and continental neurons) methods to study the role of three point mutations in Munc18-1, L130K, F115E and E59K. It was shown previously that the first two mutations affect the binding to the N-peptide of syntaxin1, while the latter one affects the binding to the Habc domain of syntaxin1. The authors now show in ITC experiments that all mutants do not bind to the assembled SNARE complex and have a greatly reduced affinity to monomeric syntaxin1. Furthermore, L130K and F115E can still inhibit SNARE complex assembly (albeit at a much reduced rate compared to wt Munc18-1), while E59K could not.

In neuronal cultures the overall morphology was largely unchanged. Since most electrophysiological parameters were unchanged as well (compared to wt Munc18-1 rescue), the authors conclude that Munc18-1 is involved in SNARE complex assembly, but it's continued binding to the assembled SNARE complex is neither required for nor is it affecting synaptic transmission.
The strength of this paper is that it uses a clean genetic background (Munc18-1 KO) and performs structure-function studies in a realistic physiological setting, i.e. intact neurons in culture. This is in strong contrast to many liposome fusion assays which are, due to their ease of use, published at an alarming frequency. As correctly pointed out by the authors in the discussion, liposome fusion assays might lead to artificial results, at least for the in vivo situation of an intact synapse where all potential interactors and modulators are present. Therefore, the conclusion that Munc18-1 does not have a post-docking/post-priming role drawn by Meijer et al. solves an important controversy in the field.

This is a manuscript I already reviewed for Neuron for which I recommended publication after minor revisions. At that time I raised three major and five minor points which have been all adequately addressed in this version. Therefore, I do not have any remaining criticism about this well written manuscript.

1st Revision - authors' response 23 February 2012

We thank the referees for their positive and conscientious reports. We are pleased to see that all reviewers approved of the quality of the experiments conducted in this study and the relevance of the question addressed. Referee #1 and #2 both state that the manuscript will benefit from a more balanced discussion. In this revised manuscript, we followed this suggestion and made the Discussion more balanced. We have also addressed the remaining concerns raised by referee #1 and #2. Please find a point-by-point reply below.

Referee #1:

1. Referee #1's main concern is that *"the strong tone of some parts of the paper will contribute to confuse an already confused field"*. The referee notes that some effects might be too subtle to be detected with the tools used in our study and that acknowledging this will reconcile our results better with other available data. We agree with the reviewer. Therefore, we included an alternative explanation of the data in our proposed model (P14, L12-13) and changed the formulation in the result section from 'does not affect synaptic transmission' to 'does not have a detectable effect on synaptic transmission using our assays' (P7, L33-34).

2. The reviewer points out that "*ITC has its limitations",* that some interactions *"can be clearly detected by other methods"* and suggests "*that the authors do not use the term 'abolish' when referring to the effects of the Munc18-1 mutations on SNARE complex binding, and instead use less absolute terms such as 'impair'".* We can only agree to this point. If interactions cannot be detected by ITC, it does not necessarily mean that the interaction does not take place at all. In fact, it is almost impossible to conclusively disprove an interaction. We agree that 'impair' is indeed a better term than 'abolish'. Since the impairment is >10fold we suggest to use the term "strong impairment".

3. The reviewer advises: *"I also would suggest the authors to be cautious with the interpretation of* [the electrophysiological] *data"* We have changed the tone of the manuscript to allow for subtle undetected effects on synaptic transmission (see point 1 of referee #1). The reviewer also expressed concerns about the small increase in the paired pulse ratio. To accommodate these concerns, we added a short discussion on the potentially decreased release probability (P13, L22-26). However, since the potential difference on paired pulse ratio in the N-terminal mutants is not statistically significant and is not reflected in other parameters like EPSC size or rundown kinetics, strong conclusions are not justified.

4.The reviewer points out that we have used the word 'weakly' in a potentially misleading way (*"most biophysicsts would not consider such an affinity to be weak"*). The reviewer is again

completely right. Of course we meant *relatively* weak. And we only wanted to highlight the huge difference in affinities (about four orders of magnitude) between Munc18-1 and syntaxin 1a (≈ 1 nM) and Munc18-1 and the SNARE complex (Kd =719 nM). We have changed the terms "weakly" and "more weakly" into "lower affinity as compared to monomeric Syntaxin" and "even lower affinity" (P4, L9-10 and L12-13).

5.The reviewer states that "*the errors in Table 1 seem unrealistically small and they appear to be the errors yielded by the fitting program, which are generally much smaller than the errors observed in repeated ITC experiments. The errors from multiple experiments should ideally be reported but, if the authors did not repeat them, they should at least state very clearly in Table 1 that the errors come from fitting the data and the real errors are expected to be larger.*
Generally, the ITC experiments in which Munc18-1 mutants were mixed with syntaxin 1a have been carried out more than once (with very similar results), but indeed the errors reported in Table 1 are the numbers obtained from fitting the data of a single run. Hence, the magnitude of the error is probably larger as correctly pointed out by the reviewer. This has now been stated in the legend of Table 1.

6. The reviewer points out that "*The Kd measured for binding of the Munc18-1 E59K mutant to syntaxin-1 (48 nM) is considerably smaller than the value [ ] reported in Deak et al. 2009 (12 nM)",* points out that different syntaxin constructs used in the two studies might explain this difference and suggests "*to point out in the manuscript that differences in the Kds may result from using different fragments. In addition, it is important to report the concentrations used for the ITC experiments, since the aggregation depends on the protein concentration."* It is indeed correct that longer fragments of the soluble portion of syntaxin exhibit a higher tendency to oligomerize at high protein concentration. We have observed this tendency as well, but usually have not encountered the severe problems reported (e.g. Dulubova et al. 1999, EMBO J. 18, 4372-82; Lerman et al. 2000, Biochemistry 39, 8470-9). We observed, for example, that the H3 domain of syntaxin 1a has a much stronger tendency to oligomerize compared to the entire cytosolic domain. Moreover, we noticed in our earlier study that the LE mutant (L165A, E166A) is more prone to oligomerize compared to wild-type syntaxin 1a (Burkhardt et al. 2008, EMBO J. 27, 923-33). In fact, an estimate of the dissociation constant of oligomerized syntaxin fragments can be obtained by ITC experiments in which syntaxin is injected into buffer as the change is accompanied by a sharp heat uptake, indicating that oligomerization of syntaxin is readily reversible (e.g. Wiederhold et al. 2009, JBC, 284, 13143-52). For example, for the E59K mutant of Munc18 we have carried out ITC titrations at different protein concentrations (20µM, 57µM, and 82µM of syntaxin) and have observed clear heat uptake peaks due to dissociation only at the highest syntaxin concentration used. Similarly, gel filtration experiments showed that only a portion of syntaxin is usually oligomerized. Still, we cannot rule out that the slightly different affinities observed between different labs (see also the ITC data published in Malintan et al. 2009, JBC, 284, 21637-46; Han et al. 2009, Mol Biol Cell 20, 4962-75; Han et al. 2011, Mol Biol Cell 22, 4134-49; Shi et al. 2011, Mol Biol Cell 22, 4150-60) are caused by the tendency of syntaxin to aggregate. In fact, it is likely that subtle differences in protein quality & activity, protein concentration, and buffer conditions between labs cause the observed differences in affinities. On the other hand, it should be noticed that we had observed no significant difference in the affinity between syntaxin 1a and Munc18 when using syntaxin aa1-240 or syntaxin aa1-262 in our earlier study (Burkhardt et al. 2008, EMBO J. 27, 923-33). Moreover, the different affinities of wild-type Munc18-1 and the E59K mutant for syntaxin are corroborated by our off-rate analysis that shows that the E59K mutant dissociates much more quickly from syntaxin. The exact protein concentrations used for the ITC experiments have now been added to the Figure S1 legend in the supplementary information section.

Referee #2:
This reviewer points out that a previous study (Shi et al. 2011) examined a quadruple mutant Munc18-1 protein that partially overlap with mutations that we have used in our study. The reviewer states that *"the authors should test the Munc18-1 quadruple mutant in their assays, then discuss in detail differences between their experimental conditions and those of previous work".* Generally, we certainly adhere to the obligation to try and reproduce data from other labs, but we have to point out that in our current manuscript we specifically addressed a different question: what are the consequences for synaptic transmission if Munc18-1 binding to SNARE-complexes is specifically impaired. The quadruple mutant mentioned by the reviewer cannot help to answer this question.

First, we feel we should make as few as possible changes to the natural protein to achieve our goal (strong impairment of SNARE-complex binding). We have achieved that goal in 3 single mutants (F115E, L130K and E59K), which we subsequently studied in synapses. Second, such multiple mutations produce loss of other aspects of Munc18-1 function. Shi et al. show that the Munc18-1 quadruple mutant affects interaction with monomeric Syntaxin and Syntaxin trafficking to the plasma membrane in PC12 cells. These additional defects will undoubtedly confound data obtained on synaptic transmission, complicate the interpretation and divert from our original aim. For these reasons, we are convinced that introduction such multiple mutations will not strengthen our message and the manuscript.

Referee #3:

"I do not have any remaining criticism about this well written manuscript"
We are pleased to hear that we succeeded in improving the manuscript based on previous comments from this reviewer. We thank this reviewer for his/her previous suggestions and support for our manuscript.

