THE EMBO JOURNAL

Manuscript EMBO-2009-73188

Opposing functions of two sub-domains of the SNARE-complex in neurotransmission

Jens P. Weber, Kerstin Reim and Jakob B. Sørensen

Corresponding author: Jakob B. Sørensen, Faculty of Health Sciences, University of Copenhagen

Review timeline:

Submission date:	18 November 2009
Editorial Decision:	07 January 2010
Revision received:	12 May 2010
Accepted:	27 May 2010

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 07 January 2010

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below. As you will see from their reports the referees express interest in the study but raise a number of concerns that need to be addressed.

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

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):

Weber and colleagues present a study of the SNARE complex responsible for neurotransmitter release. They show that mutations of the C-terminal and N-terminal parts of the complex have opposing effects on several characteristics of vesicle priming/fusion. The manuscript is well written and the data are convincing. There is a certain lack of mechanistic insights and I would suggest to check, at least, binding of synaptotagmin to the mutated complexes.

Referee #2 (Remarks to the Author):

This is an elegant manuscript that addresses the relationship between SNARE core complex stability and distinct forms of neurotransmitter release. Overall, the authors present an interesting data set, which include key SNAP-25 mutations that significantly suppress spontaneous neurotransmission but leave evoked neurotransmission largely intact. I think the experiments are conducted extremely well and I see few caveats that would preclude me to accept the validity of these observations. However, I have some concerns in organization of the manuscript and interpretation of the results that I hope the authors can address before I can enthusiastically recommend this manuscript for publication.

1. Are all the SNAP-25 mutants targeted to synapses (or axons) equally? The authors have already used an EGFP tagged SNAP-25 for their experiments, and therefore it should be straightforward to document their localization.

2. The bars in Figure 3J should be scaled accordingly. It is hard to judge the remaining level of mEPSC activity in L+7/+8 and Cdelta9 mutants.

3. I disagree with the authors' interpretation that their results show correlated changes in evoked and spontaneous neurotransmission. If anything, the same data set can be used to argue just the opposite. L+7/+8 and Cdelta9 mutants seem to suppress spontaneous release down to very low levels (Fig 3J) but the same mutants cause only a mild decrease in release probability (Fig 6E) (it would be nice to quantify this data using exponential fits for better comparison). Along the same lines, L-1 and L+2 mutants cause a dramatic increase in spontaneous neurotransmission (Fig. 4K) without causing a similarly dramatic change (to the same degree) in evoked release probability (Fig. 6F). I agree that the two forms of transmission are affected negatively (in the same direction) and they highly likely rely on the same (or similar) SNARE components. But this fact and the presented data do not amount to a "correlation" between the two forms of release as the authors propose. I hope the authors can discuss this issue in a more balanced fashion with stricter adherence to the quantitative profile of their observations.

4. Although it is true that SNAP-25 and synaptobrevin mutants show significant spontaneous release, the amount of remaining release is typically 6 to 10-fold reduced compared to wild type neurons. Therefore, these canonical SNARE components are definitely involved in maintenance of spontaneous release. The only exception is the SNAP-25 knockout phenotype seen in the neuromuscular junction (Washbourne et al., 2002). However, in this case loss of SNAP-25 causes substantial synaptic sprouting which may over-compensate the decrease in spontaneous release seen per bouton basis.

Referee #3 (Remarks to the Author):

This is an interesting study that probes the SNARE motif of SNAP-25 and tests the role of this protein in several aspects of synaptic transmission. The abundance of diverse effects presented in this paper offers a great deal of food for thought and in some instances clear interpretations are possible. However, I disagree with the major conclusion that authors draw regarding the idea that a single form of SNARE complex assembly can explain both spontaneous and evoked release. The

fact that some mutations impair both processes in the same way makes for a weak argument. One could just as easily argue that because mutations in other loci alter these two forms of release differently that different assembly processes are involved. The authors have over interpreted these results and not given the matter sufficiently objective consideration.

The authors have also not done justice to the prior work on this fundamental question of the relationship between spontaneous and evoked release. They stated on p. 4
"A single concept, which can account for both evoked and spontaneous neurotransmission is currently missing."
I believe that Lou et al. (Nature, 435:497, 2005) made a significant contribution to this point and their work merits consideration. Another paper (Finley et al. J. Neurosci. 22:1266, 2002) is relevant for different reasons. Finley et al did similar experiments to investigate role of SNAP-25 in synaptic transmission and this paper should also be discussed.
Specific points:
P. 5. The comment that the SNAP-25 mutants compete with native SNAP-25 is not valid if complexin is in excess.
P. 8. An 'escape' hypothesis is proposed to explain N-terminal and mid-SNARE motif mutations. This was very unclear and needs another few sentences to spell it out more explicitly. Do the authors really think that syntaxin can jump from a mutant SNAP-25 to another isoform in the middle of the fusion process?
Figs. 3-5. It seems odd that the sucrose recovery plots had a linear time axis and the 40 Hz train recovery plots had a logarithmic time axis. Why were these choices made?
Fig. 6G. The Ndelta24 plot shows two clear components. Please comment.
P. 12. The authors suggest that alternative sensors account for the increase in spontaneous release in the syt I KO. But this well-known result is usually interpreted as indicating that in the absence of Ca syt I acts as a clamp to block fusion. More broadly through this papers, the authors seem dismissive of the role of syt I as a fusion clamp and this hypothesis deserves a more balanced discussion.
P. 15. The authors discuss the hypothesis that mutations in the middle of SNAP-25 destabilize the primed state but this would slow down priming and alter some of the kinetic processes that depend on the priming rate. If there are no such manifestations in their other data then this hypothesis is not reasonable.
P. 16. The authors state
"This also indicates that part of the extremely high stability of the SNARE-complex (Fasshauer et al, 2002) is used for stabilizing the primed vesicle state, thereby preventing spontaneous neurotransmitter release".
I think I know what the authors are trying to say but this sentence, and possible the underlying thoughts, are very unclear. The authors should spell out there point more completely. Are they referring to a partially assembled state of the SNARE complex? If mutants that disrupt this part of the complex promote spontaneous release, does this mean that the SNARE complex disassembles for spontaneous fusion? This would mean that zipping drives evoked release but not spontaneous release. It seems that the authors only relate their results to the zipper hypothesis when it is convenient and ignore the implications of results that do not fit with the zipper hypothesis.
As a final minor point the authors should mention in the abstract that their experiments were done in cultured hippocampal neurons.

1st Revision - authors' response 12 May 2010

Reviewer #1
We would like to thank the reviewer for his/her encouraging remarks regarding the quality of writing and the data.

" There is a certain lack of mechanistic insights..."
The mechanistic insight of the paper comes – in our opinion – from the functional experiments, when seen together with the published crystal structure of the SNARE-complex, and previously published data concerning the biochemical/biophysical properties of mutants (Sørensen et al., 2006, EMBO J. 25, 955-966). However, we realize that the reader might not have those papers at hand;

therefore, in the revised version we have tried to more clearly reiterate the main points, when they are needed. The conclusion of the paper regarding the role of the SNARE-complex in spontaneous and evoked release is mechanistic in nature, and this has been made clear in the revised Fig. 8. However we realize that not everybody agree with us on the particular interpretation.

"...I would suggest to check, at least, binding of synaptotagmin to the mutated complexes."

This is a very good idea, and we went to some length trying to answer it. We expressed our mutants in cultured neurons, prepared protein extracts by pooling many cultures over the course of several weeks and attempted GST-pulldowns with a GST-C2AB construct (like we had done with GST-complexin). However, this was unsuccessful, and we therefore in another attempt performed a co-IP using GFP-trap system to pull down our overexpressed (GFP-tagged) SNAP-25. However, even though we clearly succeeded in pulling down our GFP-SNAP-25 constructs, the syt-Western showed a positive signal even with control beads (see inserted Fig. 1). Thus, these experiments were unsuccessful. When going through the literature, we noticed that very few investigators have recently attempted to show binding of synaptotagmin to SNAP-25/SNARE-complex under these conditions (in the crude protein extract. Instead most labs have used purified proteins (for instance Dai et al., 2006, J. Mol. Biol. 367, 848-863; Lynch et al., 2007, Mol. Biol. Cell 18, 4957-4968; Bai et al., 2004, Neuron 41, 929-942). In our case we have very limited amounts of protein, because we work in culture, and pursuing this question further would therefore require us to move to bacterially expressed proteins, something that none of the authors have experience with. Therefore, we have not pursued this further. It is our understanding from the literature that synaptotagmin is an extremely 'sticky' protein, which is very hard to work with, and we would therefore have to approach other groups to carry out this experiment, if the reviewer still feels it is needed. We would not want to deliver a sub-par dataset. As it is, we note that none of our conclusions/arguments rest on the notion that the mutations do not change binding to synaptotagmin – indeed, we discuss that this might contribute to, but cannot explain, the phenotypes seen.

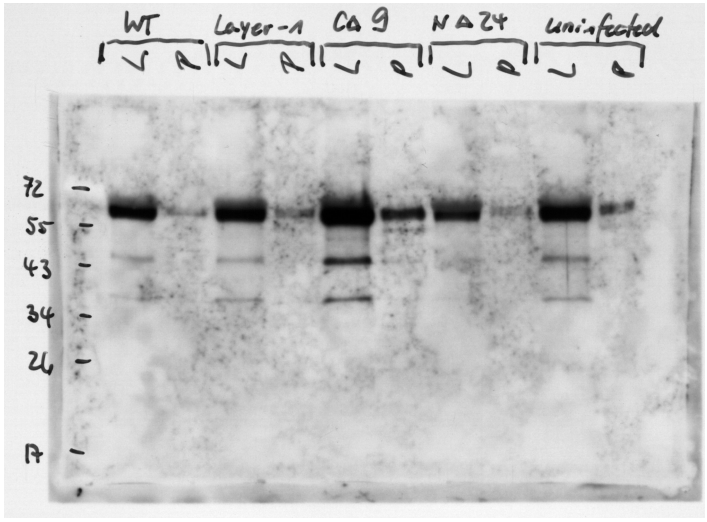

Figure 1 Co-IP between EGFP-SNAP-25 and synaptotagmin-1. Th GFP-Trap system from Chromotek was used to pull down EGFP-SNAP-25 from the protein extract from several pooled cell cultures infected with the virus shown (WT=EGFP-SNAP-25). Following extensive washing protein attached to the beads wer analyzed by SDS-PAGE and Western blotting using standard procedures. Immunoreactive proteins were visualized ECL (Amersham Biosciences). A Synaptotagmin 1 antibody was used for immunodetection (clone 41.1, 1:1000 all from Synaptic System). In a separate experiment, it was shown that EGFP-SNAP25 itself is pulled down with this procedure, together with the cognate partner SNAREs (syntaxin and synaptobrevin). The band around 65 kDa is specific for synaptotagmin-1. L=load; P=pellet (bound to beads). This band is present even in beads incubated with uninfected neurons, indicating that the band represents unspecific binding to the beads, rather than specific binding to EGFP-SNAP-25.

Reviewer #2

We would like to thank the reviewer for his/her enthusiastic comments on our manuscript!

1. Are all the SNAP-25 mutants targeted to synapses (or axons) equally? The authors have already used an EGFP tagged SNAP-25 for their experiments, and therefore it should be straightforward to document their localization.

We performed this experiment using confocal imaging of tagged SNAP-25 and staining against synaptobrevin-2 and included the data into the revised manuscript as a new Supplementary Fig. 2. As it can be seen – and this has been reported before both for overexpressed and native SNAP-25 – SNAP-25 in fact decorates both the cell body and the neurites, both axons and dendrites. It is present in the synapse as shown by colocalization of the EGFP signal with the synaptobrevin-2 specific signal. This distribution is not changed by any of the mutations we have studied.

2. The bars in Figure 3J should be scaled accordingly. It is hard to judge the remaining level of mEPSC activity in L+7/+8 and Cdelta9 mutants.

The axes have been rescaled.

3. I disagree with the authors' interpretation that their results show correlated changes in evoked and spontaneous neurotransmission. If anything, the same data set can be used to argue just the opposite. L+7/+8 and Cdelta9 mutants seem to suppress spontaneous release down to very low levels (Fig 3J) but the same mutants cause only a mild decrease in release probability (Fig 6E) (it would be nice to quantify this data using exponential fits for better comparison). Along the same lines, L-1 and L+2 mutants cause a dramatic increase in spontaneous neurotransmission (Fig. 4K) without causing a similarly dramatic change (to the same degree) in evoked release probability (Fig. 6F). I agree that the two forms of transmission are affected negatively (in the same direction) and they highly likely rely on the same (or similar) SNARE components. But this fact and the presented data do not amount to a "correlation" between the two forms of release as the authors propose. I hope the au thors can discuss this issue in a more balanced fashion with stricter adherence to the quantitative profile of their observations.

Upon reading the comments of the reviewers #2 and #3 – and re-reading our manuscript – we realize that we have over-interpreted our data as far as the relationship between spontaneous and evoked release is concerned. We thank the reviewers for pointing this out; we are very happy to have this opportunity to improve our manuscript! As a result of the comments of both reviewers, we have:
a. Removed the very specific interpretation (previous Fig. 8) (see also comments to reviewer #3). Instead, we now discuss the implications in words in the last paragraphs of the Discussion section. Our general point is that there are two parts to the SNARE-complex, which appear to affect release probabilities (especially for spontaneous release) in opposite directions. The interpretation of this data is entirely consistent with the recent hypothesis on complexin action on the SNARE-complex. We have illustrated this particular interpretation in a new, simpler, Fig. 8, which makes no explicit statements about a correlation between evoked and spontaneous release.
b. Pointed out in several places that spontaneous release was affected more than evoked release and removed statements about a ‘correlation’.
c. Changed the Introduction to align it better with the Discussion, putting less emphasis on the origin of spontaneous release. We have also changed the Abstract and title of the paper to avoid making implicit statements about a correlation between spontaneous and evoked neurotransmission.
d. Discussed better the relationship between our data and previous data using Botulinum Neurotoxin or mutagenesis (this especially applies to the C-terminal mutations, which were not thoroughly discussed before).
e. Discussed the deviations between the data obtained in neurons and those obtained in chromaffin cells.

We think that the manuscript has improved considerably, and we hope the reviewer will agree!

4. Although it is true that SNAP-25 and synaptobrevin mutants show significant spontaneous release, the amount of remaining release is typically 6 to 10-fold reduced compared to wild type neurons. Therefore, these canonical SNARE components are definitely involved in maintenance of spontaneous release. The only exception is the SNAP-25 knockout phenotype seen in the neuromuscular junction (Washbourne et al., 2002). However, in this case loss of SNAP-25 causes substantial synaptic sprouting which may over-compensate the decrease in spontaneous release seen per bouton basis.

Yes, we agree. This was not clear from the Introduction. In rewriting the manuscript, this has now been pointed out: “The SNARE-complex is involved in both evoked and spontaneous neurotransmission. However, removal of a SNARE-component typically eliminates evoked release, whereas spontaneous release persists at a reduced rate, indicating that the requirements for spontaneous release are less strict (Bronk et al, 2007; Deitcher et al, 1998; Delgado-Martinez et al, 2007; Schoch et al, 2001; Washbourne et al, 2002).”

Reviewer #3

This is an interesting study that probes the SNARE motif of SNAP-25 and tests the role of this protein in several aspects of synaptic transmission. The abundance of diverse effects presented in this paper offers a great deal of food for thought and in some instances clear interpretations are possible.

We thank the reviewer for his/her encouraging remarks!

However, I disagree with the major conclusion that authors draw regarding the idea that a single form of SNARE complex assembly can explain both spontaneous and evoked release. The fact that some mutations impair both processes in the same way makes for a weak argument. One could just as easily argue that because mutations in other loci alter these two forms of release differently that different assembly processes are involved. The authors have over interpreted these results and not given the matter sufficiently objective consideration.

Upon reading the comments of the reviewers #2 and #3 – and re-reading our manuscript – we realize that we have over-interpreted our data as far as the relationship between spontaneous and evoked release is concerned. We thank the reviewers for pointing this out; we are very happy to have this opportunity to improve our manuscript! As a result of the comments of both reviewers, we have:
a. Removed the very specific interpretation (previous Fig. 8) (see also comments to reviewer #2). Instead, we now discuss the implications in words in the last paragraphs of the Discussion section. Our general point now is that there are two parts to the SNARE-complex, which appear to affect release probabilities in opposite directions. The interpretation of data is entirely consistent with the recent hypothesis on complexin action on the SNARE-complex. We have illustrated this particular interpretation in a new, simpler, Fig. 8, which makes no statements about a correlation between evoked and spontaneous release.
b. Pointed out in several places that spontaneous release was affected more than evoked release and removed statements about a 'correlation'.
c. Changed the Introduction to align it better with the new Discussion, putting less emphasis on the origin of spontaneous release. We have also changed the Abstract and title of the paper to avoid making implicit statements about a correlation between spontaneous and evoked neurotransmission.
d. Discussed better the relationship between our data and previous data using Botulinum Neurotoxin or mutagenesis (this especially applies to the C-terminal mutations, which were not thoroughly discussed before).
e. Discussed some deviations between the data obtained in neurons and those obtained in chromaffin cells.

The authors have also not done justice to the prior work on this fundamental question of the relationship between spontaneous and evoked release. They stated on p. 4 "A single concept, which can account for both evoked and spontaneous neurotransmission is currently missing."
I believe that Lou et al. (Nature, 435:497, 2005) made a significant contribution to this point and their work merits consideration. Another paper (Finley et al. J. Neurosci. 22:1266, 2002) is relevant for different reasons. Finley et al did similar experiments to investigate role of SNAP-25 in synaptic transmission and this paper should also be discussed.

Thank you for pointing it out! The Finley paper has now been included in the discussion. The statement on p. 4 has been removed and the focus shifted, therefore we have not cited the Lou paper (we now cite another Lou et al paper from the same group, which has to do with the effect of Munc13-1 on evoked and spontaneous release).

Specific points:
P. 5. The comment that the SNAP-25 mutants compete with native SNAP-25 is not valid if complexin is in excess.
We agree and the comment has been removed.

P. *8. An 'escape' hypothesis is proposed to explain N-terminal and mid-SNARE motif mutations. This was very unclear and needs another few sentences to spell it out more explicitly. Do the authors really think that syntaxin can jump from a mutant SNAP-25 to another isoform in the middle of the fusion process?*

Answer: This was unclear. What we mean is that if SNAP-25 competes with other Qb/Qc-SNAREs in the cell (SNAP-23, SNAP-29, SNAP-47), then expressing a SNAP-25 version with less affinity for syntaxin might cause the other SNAREs to 'win' and bind to syntaxin instead. Exocytosis might then be driven by another complex (for instance SNAP-23:syntaxin-1), which might be more prone to spontaneous release. This is a worry for more N-terminal mutations, since assembly starts in this end. So, we do not suggest that syntaxin can jump, but that it might assemble to another SNAP-25 isoform in the first place. Thus, it was important to confirm that in the presence of N-terminal mutations, spontaneous release was still driven by the mutant, not by some other isoform. We solved this problem by combining the N-terminal deletion with a C-terminal mutation. The combined mutant eliminated spontaneous release, confirming that competition is not the reason for the N-terminal phenotype. We have followed the advice of the reviewer and spelled this out more clearly in the text.

Figs. 3-5. It seems odd that the sucrose recovery plots had a linear time axis and the 40 Hz train recovery plots had a logarithmic time axis. Why were these choices made?
In the previous version of the manuscript, we plotted the 40Hz recovery plots on a logarithmic time axis, because the data cover >2 orders of magnitude, and some points are therefore not clearly visible on a linear scale. The sucrose data cover only a single order of magnitude and can therefore be better represented on a linear scale. However, we realize that this can cause confusion. Therefore, in the new version of the manuscript, both data sets are plotted on linear time axes.

Fig. 6G. The Ndelta24 plot shows two clear components. Please comment.
Yes, there are two components. Two components to the MK-801 induced decay of the NMDA-driven EPSCs were observed already in the two original descriptions of that method (Rosenmund et al., 1993; Hessler et al., 1993). They are usually ascribed to two different populations of synapses with different release probabilities. We have now pointed this out in the text. In fact, all of the traces are fitted better with a double exponential function. Since the two components do not always appear to have the same relative amplitudes, we have not attempted to treat this quantitatively (calculating the release probabilities), since in doing this, it is usually assumed that the two synapse populations do not change relative amplitudes.

P. 12. The authors suggest that alternative sensors account for the increase in spontaneous release in the syt I KO. But this well-known result is usually interpreted as indicating that in the absence of Ca syt I acts as a clamp to block fusion. More broadly through this papers, the authors seem dismissive of the role of syt I as a fusion clamp and this hypothesis deserves a more balanced discussion.

Both hypotheses have been put forward to account for the increase in spontaneous release in the Syt 1 KO, and we did not wish to favor one over the other. In writing these sections we were (maybe too) influenced by the recent Südhof paper (Xu et al., 2009, Nat. Neurosci. 12, 759-767), where they showed that the spontaneous release changes its calcium dependence according to the apparent calcium-affinity of Syt-1, but in the absence of Syt-1 there is a massive increase in spontaneous release, which they ascribe to a different calcium sensor (confusingly, they state that syt-1 'clamps' the second sensor). We have now rewritten to leave the question open.

P. 15. The authors discuss the hypothesis that mutations in the middle of SNAP-25 destabilize the primed state but this would slow down priming and alter some of the kinetic processes that depend on the priming rate. If there are no such manifestations in their other data then this hypothesis is not reasonable.

We agree with the reviewer – there was a logical flaw in that argument and the Fig. 8. We are happy to be able to correct it! In the new version of the manuscript, this interpretation/hypothesis has been removed. In fact, we realized that the middle mutations cannot have changed the energy level of the primed vesicle state (for the reason mentioned by the reviewer), but must have changed the energy level of the final fusion barrier. This is partly implicit in the new discussion in the last part of the discussion, and also the new Fig. 8. However, we chose not to discuss this at length, but to make a simpler, more illustrative point about the different roles of the two parts of the SNARE-complex.

P. 16. The authors state

"This also indicates that part of the extremely high stability of the SNARE-complex (Fasshauer et al, 2002) is used for stabilizing the primed vesicle state, thereby preventing spontaneous neurotransmitter release".
I think I know what the authors are trying to say but this sentence, and possible the underlying thoughts, are very unclear. The authors should spell out there point more completely. Are they referring to a partially assembled state of the SNARE complex? If mutants that disrupt this part of the complex promote spontaneous release, does this mean that the SNARE complex disassembles for spontaneous fusion? This would mean that zipping drives evoked release but not spontaneous release. It seems that the authors only relate their results to the zipper hypothesis when it is convenient and ignore the implications of results that do not fit with the zipper hypothesis.

We have now spelled out our point much more completely. The last paragraph of the Discussion is devoted to discussing the implications of having two different domains of the SNARE-complex, which have opposing effects on neurotransmission. What we were trying to say (but it didn't come across clearly enough) was that tight assembly of the N-terminal end might put up an obstacle for the C-terminal end to assemble. So, indeed, we were referring to the partially assembled state of the SNARE complex. Conversely, loosening up the N-terminal end might allow the C-terminal end to assemble more easily, triggering spontaneous release. This might seem odd, but in fact it fits exactly with the current hypothesis for how complexin clamps the SNARE-complex for release. According to this hypothesis, complexin binds around the middle of the SNARE-complex, in order to put the accessory helix close to the C-terminal end, where it blocks C-terminal assembly and release. Since complexin can only bind to the assembled SNARE-complex, this indeed means that N-terminal assembly of the SNARE-complex puts up a barrier for C-terminal assembly. However, since we could not show any difference in complexin binding with our mutants, we suggest that this interdependence between the N- and the C-terminal end is an intrinsic feature of the SNARE-complex, which is exacerbated or exploited by complexin. This also fits better with our phenotype for evoked release (see Discussion). This has been illustrated in a new, simpler, Fig. 8.

We showed that spontaneous release still depends on the C-terminal end of the SNARE-complex, even in the presence of N-terminal mutants. We conclude, therefore, that the C-terminal end still has to assemble, even when spontaneous release is unclamped – this agrees with the zipper hypothesis. Thus, we do not feel that we ignore implications of our data. However, we also note that while we think our data overall agree with the zippering hypothesis (and we note this once towards the end of the Result section), it is not the aim of the paper to make this point again.

As a final minor point the authors should mention in the abstract that their experiments were done in cultured hippocampal neurons.
This has now been done.

