The referees also find that a more careful discussion of the discrepancies between your findings and the Meijer et al. 2012 paper should be included and alternative hypothesis should be better discussed. This is an important issue to resolve in the revised version.

Once the raised issues are resolved then I would like to invite your to submit a suitably revised manuscript to the EMBO Journal.

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, 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

In this article, the authors report the effect of N-terminal mutants of Syntaxin1A on neurotransmitter release, with the goal to precisely characterize the N-peptide and Habc domain. The authors rely on Syntaxin1A KO neurons in which Syntaxin1B is silenced and WT or mutant Syntaxin1A is simultaneously expressed using viral transduction in a complementation 'rescue' assay. They extensively characterize spontaneous and evoked neurotransmission in autaptic cultured neurons.

The experiments were well carried out and controlled. They represent a very rigorous functional study of Syntaxin1A N-ter mutants. The main findings are that Syntaxin1 N-peptide and Habc are essential for spontaneous synaptic vesicle fusion and maintenance of the RRP, but only N-peptide is essential for evoked release.

I have several questions which need to be addressed to get a clear cut message:
- the authors oppose their results and interpretation to that of Meijer et al 2012. Whilst it is worth discussing potential discrepancies, it remains that the present article addresses only the effect of Syntaxin1A mutants while Meijer studied only Munc18 mutants. Despite the fact that Munc18 interacts with Stx1 N-ter domain, the authors should be more cautious as many alternative hypothesis can be drawn to account for the discrepancies between the two studies, on top of expression level and remaining low affinity of some of Meijer's mutants. For instance, Stx1 lateral diffusion, dynamic interaction with SNAP25 and Munc18 may be affected by some mutations.
- Release as measured by electrophysiology represents the final functional output of a multi-step molecular process, which the authors do not characterize in full here. The reader should be kept aware of this complexity in the discussion and alternatives hypothesis should be exposed.
- the authors should show evoked EPSCs in all conditions or at least explain why they were not able to do so.

Minor comments:
I assumed from previous work of the authors, that the model consisted in autaptic cultures but the word is not even found in the whole article. A little more detail about the method would help as this article quotes a paper which quotes another one and another one until one can find some information.

Referee #2

Sudhof and colleagues analyze the function of the N-terminal region of syntxin 1 using a knockdown/rescue approach in cultured neurons lacking one of the syntaxin isoforms. As expected, knockdown of syntaxin 1B massively reduces both spontaneous and evoked release, which is rescued by parallel expression of wildtype syntaxin. The authors then use syt mutants for rescue that either lack the N-terminal peptide (DeltaN) or carry a deletion of the Habc-domain (DeltaHabc). Whereas DeltaN is completely non-functional, the DeltaHabc mutant largely rescues evoked release but not the reduction in the readily releasable pool or in mini frequency.

This interesting study adds yet another facet to the increasingly complicated picture of syntaxin activation. Furthermore, it contradicts a recent study from the Verhage group. It is not overly satisfying that no plausible explanation is available for this discrepancy as both studies appear to be of high quality (at least to an outside reader). Considering the intense and controversial discussion in the field concerning the mechanism of Munc18 in activating the plasma membrane SNAREs, I nevertheless support publication of this work pending minor revision.

Specific comments:
1. The authors show that expression of DeltaHabc does not rescue the reduction in Munc18 expression evoked by the knockdown (Fig. 1), and they suggest that this reduction is causing the reduction of spontaneous release. It would complement the data set if a similar analysis were carried out for the DeltaN mutant.
2. It is puzzling that in the DeltaHabc rescue evoked release is rescued but not spontaneous release or the RRP. Even more surprisingly, response to 10Hz stimulation also appears to be normal, at least in inhibitory neurons where the effect of the KD does not appear to be as dramatic as in excitatory neurons. In my opinion, it would strengthen the MS considerably if the authors carry out paired-pulse analysis with different delay times in order to assess whether the reduced RRP does indeed result in a more rapid depletion of vesicles available for evoked exocytosis.

Referee #3

The manuscript by Zhou et al. investigates the roles of the N-terminal peptide and the Habc domain of syntaxin in neurotransmission at synapses from the central nervous system. The experiments use neurons that are depleted in syntaxin 1a and 1b. Syntaxin depletion is achieved using neurons cultured from syntaxin1a knockout mice that are treated with lentivirus to introduce short hairpin RNAs designed to suppress syntaxin 1b. The approach is to compare electrophysiological measurements on these syntaxin depleted cells when syntaxin carrying changes or deletions in these two key domains is expressed in the syntaxin-depleted cells, also using lentivirus methods. The variants that are compared include deletion of the N-terminal peptide, deletion of the Habc domain, the so-called LE mutation that switches the preferred binding of Habc to generate 'closed' syntaxin, and the 5D mutation.

The main findings are a) syntaxin missing the N-terminal domain does not support normal spontaneous release, normal evoked release, nor the readily releasable pool size. The LE mutation does not rescue the N-terminal loss of function phenotype, suggesting the open/close regulation of syntaxin by Munc18 is not responsible for these changes; b) syntaxin with the Habc domain deleted does not support normal spontaneous release; c) Habc deleted syntaxin does support normal evoked release.

The conclusions derived from the results in this manuscript are important as they strengthen the case that the N-terminal peptide is essential for proper neurotransmitter release in central neurons, and they identify a novel role for the Habc domain in spontaneous release phenomena. It is an important contribution to detail the functions of these 2 domains in a cellular setting because they are key interaction targets for Munc18, which is essential for proper neurotransmission. The mechanistic details that underlie Munc18's essential role in proper synaptic function are being debated in current literature. This manuscript is a valuable contribution to that discussion. There is a significant conflict with Meijers et al. 2012, but this issue is carefully and fairly discussed. The experiments appear carefully performed and well thought out (to my knowledge) and this paper deserves publication.

Specific comments:

1) The importance of the Habc domain to spontaneous release processes is a most interesting finding. To address details of this finding, it would be nice to see experiments where both the N-terminal and Habc domains are removed from syntaxin using this syntaxin-depleted rescue preparation. The Habc domain determines the positioning of the N-terminal peptide relative to assembling SNARE domains when it is present. Therefore, it is difficult to completely isolate effects of deleting the Habc domain from those of the N-terminal peptide functions, especially if Munc18 were to interact simultaneously with the N-terminal peptide and parts of SNARE complexes or SNARE domains.

2) Similar to the previous point, the results that the Habc deletion mutant rescues evoked release suggests that if the Munc18-SNARE interaction involves the N-terminal syntaxin peptide, and if this interaction is essential for evoked release, then it accommodates a dramatically different configuration.

3) The RNA hairpins also target syntaxin 1a. I presume that the expression of the syntaxin 1a from the lentivirus induced system is stronger than the inhibition by the shRNA. It is not described how well this can be controlled. From the consistency across different experiments in the paper it seems that it is controlled well, but a comment about the reliability of this expression system would be useful. Perhaps this is related to the point in Fig 1b where it is clear that the syntaxin1b KD in the
syntaxin 1a KO neurons is expressed at ~40% normal levels. Evidently this background suggests that the inhibition of syntaxin 1a expression is modest. Modest inhibition would allow overexpression to overwhelm the population in the cell. Is this the authors' picture of how the balance of inhibition and expression works?

4) In the early part of the results, or the methods, it would be useful to make more explicit that all experiments are in syntaxin 1a KO. It is not always obvious that this is what is meant by the designation control. In the beginning of the discussion this fact is described unambiguously, but I would have appreciated this clear statement earlier.

5) The statement on page 8, "Together, these finding confirm and extend previous conclusions that binding of Munc18-1 to the N-terminal sequence of syntaxin-1 is essential for membrane fusion" is possibly slightly overreaching. Previous literature does support this conclusion, but the current observation is strictly that the N-terminal domain deletion alters spontaneous and evoked release properties. Even though no other N-terminal syntaxin interaction partners have been identified, perhaps the authors wish to consider possibly soften this statement since their work does not directly demonstrate Munc18 interactions.

We very much thank the reviewers for their insightful and helpful comments. As described below, we have tried to address all criticisms in the revised paper, and performed new experiments for that purpose. Specifically, we have determined the effect of the N-peptide deletion on the stabilization of Munc18-1 levels by syntaxin-1 (new Figure 2), analyzed the effects of N-peptide deletion and the LE-mutation alone and together on excitatory evoked synaptic transmission (new Fig. 5D), examined short-term plasticity (new Figs. S1 and S2), and investigated the effect of the double deletion of both the N-peptide and the H\textsubscript{abc}-domain (new Fig. S3). In addition, although not specifically requested, we have compared rescue of synaptic transmission in syntaxin-1 deficient neurons by syntaxin-1A and -1B to assure their functional equivalence (new Figs. 1D and 1E). Of these data, the new experiments that determine the role of the syntaxin-1 N-peptide in stabilizing Munc18-1 were particularly interesting because they show that surprisingly, syntaxin-1 lacking the N-peptide fully stabilizes Munc18-1, even though it cannot function in fusion. This result is consistent with the overall conclusion that the Munc18-1/syntaxin-1 N-peptide complex functions directly in fusion, whereas the Munc18-1/closed syntaxin-1 complex (which is abolished by the H\textsubscript{abc}-domain deletion or the LE mutation) chaperones both proteins, and regulates the speed of fusion. We hope that with these additional data and the changes made in the paper, the reviewers are now satisfied with the manuscript, and that the paper can be accepted for publication.

Reference #1
In this article, the authors report the effect of N-terminal mutants of Syntaxin1A on neurotransmitter release, with the goal to precisely characterize the N-peptide and H\textsubscript{abc} domain. The authors rely on Syntaxin1A KO neurons in which Syntaxin1B is silenced and WT or mutant Syntaxin1A is simultaneously expressed using viral transduction in a complementation 'rescue' assay. They extensively characterize spontaneous and evoked neurotransmission in autaptic cultured neurons. The experiments were well carried out and controlled. They represent a very rigorous functional study of Syntaxin1A N-ter mutants. The main findings are that Syntaxin-1 N-peptide and H\textsubscript{abc} are essential for spontaneous synaptic vesicle fusion and maintenance of the RRP, but only N-peptide is essential for evoked release.

We thank the reviewer for a careful assessment of our paper

I have several questions which need to be addressed to get a clear cut message:

1. -the authors oppose their results and interpretation to that of Meijer et al 2012. Whilst it is worth discussing potential discrepancies, it remains that the present article addresses only the effect of Syntaxin1A mutants while Meijer studied only Munc18 mutants. Despite the fact that Munc18 interacts with Stx1 N-ter domain, the authors should be more cautious as many alternative hypothesis can be drawn to account for the discrepancies between the two studies, on top of expression level and remaining low affinity of some of Meijer's mutants. For instance, Stx1 lateral
diffusion, dynamic interaction with SNAP25 and Munc18 may be affected by some mutations. Release as measured by electrophysiology represents the final functional output of a multi-step molecular process, which the authors do not characterize in full here. The reader should be kept aware of this complexity in the discussion and alternatives hypothesis should be exposed.

We agree, and have tried to express our view more cautiously in the Discussion.

2. the authors should show evoked EPSCs in all conditions or at least explain why they were not able to do so.

We have performed additional experiments to conform to this suggestion. Evoked EPSCs are more difficult to measure in cultured neurons than evoked IPSCs. In the revised paper, we now show rescue of evoked EPSCs for all key observations to document that the results apply not only to inhibitory synapses but also to excitatory synapses as suggested by the reviewer (see Figs. 5 and 7).

Minor comments:
I assumed from previous work of the authors, that the model consisted in autaptic cultures but the word is not even found in the whole article. A little more detail about the method would help as this article quotes a paper which quotes another one and another one until one can find some information.

We apologize for the confusion – we have now clearly explained in the manuscript that all of our measurements were performed in dissociated cultures by a method that we developed several years ago, and that has now also been widely adopted by others (Maximov et al., 2007).

Referee #2
Sudhof and colleagues analyze the function of the N-terminal region of syntaxin 1 using a knockdown/rescue approach in cultured neurons lacking one of the syntaxin isoforms. As expected, knockdown of syntaxin 1B massively reduces both spontaneous and evoked release, which is rescued by parallel expression of wildtype syntaxin. The authors then use syt mutants for rescue that either lack the N-terminal peptide (DeltaN) or carry a deletion of the Habc-domain (DeltaHabc). Whereas DeltaN is completely non-functional, the DeltaHabc mutant largely rescues evoked release but not the reduction in the readily releasable pool or in mini frequency.

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We very much thank the reviewer for the thoughtful comments and positive assessment of our paper

Specific comments:
1. The authors show that expression of DeltaHabc does not rescue the reduction in Munc18 expression evoked by the knockdown (Fig. 1), and they suggest that this reduction is causing the reduction of spontaneous release. It would complement the data set if a similar analysis were carried out for the DeltaN mutant.

This is a very important suggestion that we have followed. We now include data in the paper demonstrating that the ΔN mutant fully rescues the decrease in the expression level of Munc18, even though it does not rescue release (revised Fig. 2). Thus, the loss of Munc18-1 is due to a loss of the closed conformation of syntaxin-1 that forms a complex with Munc18-1, and not due to a loss of syntaxin-1 function as such. We were surprised by this finding which dissociates the function of Munc18-1 and syntaxin-1 in chaperoning each other from their function in fusion – the first time these two have been demonstrated to be independent of each other.

2. It is puzzling that in the DeltaHabc rescue evoked release is rescued but not spontaneous release or the RRP. Even more surprisingly, response to 10Hz stimulation also appears to be normal, at least in inhibitory neurons where the effect of the KD does not appear to be as dramatic as in excitatory neurons. In my opinion, it would strengthen the MS considerably if the authors carry out
paired-pulse analysis with different delay times in order to assess whether the reduced RRP does indeed result in a more rapid depletion of vesicles available for evoked exocytosis.

We agree that the phenotype of the ΔHabc-domain mutant may appear puzzling, but there is a rational explanation for most of it based on previous studies. We showed earlier (Gerber et al., 2008) that constitutively open syntaxin-1 produces an increase in vesicular release probability – probably because SNARE-complex assembly is facilitated – and a decrease in RRP – probably because Munc18-1 is decreased. The ΔHabc-domain mutant is equivalent to constitutively open syntaxin-1, and has the same phenotype. The only phenotypic difference between constitutively open syntaxin-1 and the ΔHabc-domain mutant is that constitutively open syntaxin-1 exhibits INCREASED spontaneous release whereas the ΔHabc-domain mutant exhibits DECREASED spontaneous release. At present, we have no mechanistic explanation for this observation, but believe it is related to the emerging notion that spontaneous release is fundamentally different from evoked release.

We have carried out the experiments suggested by the reviewer, and not only performed paired-pulse analyses but also short-term depression analyses (now included in the Supplementary Information). The results are not very informative, however, probably because of the complexity of the underlying process in which the decrease of the RRP that is caused by the ΔHabc-domain mutant (probably because of the decrease in Munc18-1) is associated with an increase in the refilling rate of the RRP (Gerber et al., 2008). Thus a more rapid depletion and a more rapid refilling balance each other, and therefore these experiments do not actually shed light on the interesting spontaneous release phenotype.

Referee #3
The manuscript by Zhou et al. investigates the roles of the N-terminal peptide and the Habc domain of syntaxin in neurotransmission at synapses from the central nervous system. The experiments use neurons that are depleted in syntaxin 1a and 1b. Syntaxin depletion is achieved using neurons cultured from syntaxin1a knockout mice that are treated with lentivirus to introduce short hairpin RNAs designed to suppress syntaxin 1a and 1b. The approach is to compare electrophysiological measurements on these syntaxin depleted cells when syntaxin carrying changes or deletions in these two key domains is expressed in the syntaxin-depleted cells, also using lentivirus methods. The variants that are compared include deletion of the N-terminal peptide, deletion of the Habc domain, the so-called LE mutation that switches the preferred binding of Habc to generate 'closed' syntaxin, and the 5D mutation.

The main findings are a) syntaxin missing the N-terminal domain does not support normal spontaneous release, normal evoked release, nor the readily releasable pool size. The LE mutation does not rescue the N-terminal loss of function phenotype, suggesting the open/close regulation of syntaxin by Munc18 is not responsible for these changes; b) syntaxin with the Habc domain deleted does not support normal spontaneous release; c) Habc deleted syntaxin does support normal evoked release.

The conclusions derived from the results in this manuscript are important as they strengthen the case that the N-terminal peptide is essential for proper neurotransmitter release in central neurons, and they identify a novel role for the Habc domain in spontaneous release phenomena. It is an important contribution to detail the functions of these 2 domains in a cellular setting because they are key interaction targets for Munc18, which is essential for proper neurotransmission. The mechanistic details that underlie Munc18’s essential role in proper synaptic function are being debated in current literature. This manuscript is a valuable contribution to that discussion. There is a significant conflict with Meijers et al. 2012, but this issue is carefully and fairly discussed. The experiments appear carefully performed and well thought out (to my knowledge) and this paper deserves publication.

We really appreciate the reviewer’s constructive and positive evaluation.

Specific comments:
1) The importance of the Habc domain to spontaneous release processes is a most interesting finding. To address details of this finding, it would be nice to see experiments where both the N-terminal and Habc domains are removed from syntaxin using this syntaxin-depleted rescue preparation. The Habc domain determines the positioning of the N-terminal peptide relative to assembling SNARE domains when it is present. Therefore, it is difficult to completely isolate effects
of deleting the Habc domain from those of the N-terminal peptide functions, especially if Munc18 were to interact simultaneously with the N-terminal peptide and parts of SNARE complexes or SNARE domains.

We have performed this experiment, and show the results in the Supplementary Materials (Fig. S3). The bottom line is that the phenotype of the N-peptide deletion dominates, and release is blocked.

2 Similar to the previous point, the results that the Habc deletion mutant rescues evoked release suggests that if the Munc18-SNARE interaction involves the N-terminal syntaxin peptide, and if this interaction is essential for evoked release, then it accommodates a dramatically different configuration.

We completely agree with this conclusion that reflects also our view.

3 The RNA hairpins also target syntaxin 1a. I presume that the expression of the syntaxin 1a from the lentivirus induced system is stronger than the inhibition by the shRNA. It is not described how well this can be controlled. From the consistency across different experiments in the paper it seems that it is controlled well, but a comment about the reliability of this expression system would be useful. Perhaps this is related to the point in Fig 1b where it is clear that the syntaxin1b KD in the syntaxin 1a KO neurons is expressed at ~40% normal levels. Evidently this background suggests that the inhibition of syntaxin1a expression is modest. Modest inhibition would allow overexpression to overwhelm the population in the cell. Is this the authors' picture of how the balance of inhibition and expression works?

We agree, and now point out in the text that all experiments were performed in Syntaxin-1A KO neurons, and that the Syntaxin-1A rescue construct used contains silent nucleotide changes that render its expression insensitive to the shRNA used.

4) In the early part of the results, or the methods, it would be useful to make more explicit that all experiments are in syntaxin1a KO. It is not always obvious that this is what is meant by the designation control. In the beginning of the discussion this fact is described unambiguously, but I would have appreciated this clear statement earlier.

We agree, and have followed this suggestion.

5) The statement on page 8, "Together, these finding confirm and extend previous conclusions that binding of Munc18-I to the N-terminal sequence of syntaxin-1 is essential for membrane fusion" is possibly slightly overreaching. Previous literature does support this conclusion, but the current observation is strictly that the N-terminal domain deletion alters spontaneous and evoked release properties. Even though no other N-terminal syntaxin interaction partners have been identified, perhaps the authors wish to consider possibly soften this statement since their work does not directly demonstrate Munc18 interactions.

Again, we agree and have reworded the cited sentence.

We thank the reviewers for their helpful comments.
and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this just contact me.

Once we get these last issues resolved we will proceed with the acceptance of the paper for publication here.

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REFeree COMMENTS

Referee #1

The authors have satisfactorily answered the reviewers' requests.

Referee #2

The authors have nicely addressed all issues raised in my report, and I thus recommend publication.