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CSPα Knockout Causes Neurodegeneration by Impairing SNAP-25 Function

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(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 22 August 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. Three referees have now evaluated it, and their comments are shown below. You will be pleased to see that the referees are generally very positive about publication of the paper here. Still, referee 3 thinks that some further work needs to be done before the data are strong enough to fully justify all conclusions drawn. I would thus like to invite you to submit a revised version of the manuscript, in which the points raised by referees 3 and 1 are addressed in an adequate manner. I should add that it is EMBO Journal policy to allow only a single round of revision and that 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:

Since loss of CSP leads to neuronal degeneration investigation of its mechanism of action is of general interest as it could provide insights into mechanism of neuroprotection form neurodegenerative diseases. Earlier work from this group identified SNAP-25 as a target for the chaperone activity of CSP. This work left open the important question of whether the loss SNAP-25 and SNARE complexes was the key aspect in neurodegeneration in CSP KO mice. This new study elegantly addresses this issue and by using of genetic manipulation of SNAP-25 levels in the absence of CSP and by knock-down of SNAP-25 and its overexpression the authors now demonstrate that SNAP-25 is the key target for the neuroprotective role of CSP.

Overall this is an excellent and comprehensive study that provides important conclusions and should be of general interest to the EMBO readership.

Since this manuscript was submitted a key paper has now been published on line (Noskova er al Am J Hum Gen 2011) indicating that mutations in CSP in humans leads to neuronal ceroid lipofuscinosis and neurodegeneration. This adds to the general significance of the current paper and the authors should include discussion of the new paper and how these findings fit with the role of CSP as a SNAP-25 chaperone.

Referee #2:

This is a follow up study from the group of Tom Südhof, where the authors continue to investigate the molecular mechanism underlying neurodegeneration produced by the lack of cysteine string protein (CSP) alpha. Having established in previous studies that lack of CSP alpha destabilizes SNARE complexes and enhances the degradation of SNAP-25, they now test whether reduced or enhanced levels of SNAP-25 can worsen or protect against the neurodegeneration phenotype, respectively. Using a combination of mouse genetic/molecular genetic tools (including in vivo, virus-based RNAi experiments), they provide unambiguous evidence for a central role of SNAP-25 deficiency in CSPalpha deletion-induced neurodegeneration. Overall, this is a solid study that addresses an important question using multidisciplinary approaches, including an impeccable biochemical analysis of SNARE proteins/complexes. Importantly, this study provides an excellent example of how a destabilization of a single presynaptic SNARE protein (with important consequences on the overall stability of SNARE complexes) can lead to neurodegeneration in the context of a defective folding machinery, providing a potential molecular basis for neurodegenerative processes in human disease.

Referee #3:

Sharma et al report on the role of the chaperone CSPα in regulating the function of the exocytic SNARE SNAP-25 and its relationship to neurodegeneration. Capitalizing on the available repertoire of knockout mice it is shown that loss of SNAP-25 in heterozygous KO mice or reduction of its expression induced by lentiviral knockdown exacerbates neurodegeneration and defective SNARE complex assembly seen in CSPα-KO mice. Most importantly, overexpression of wt but not truncated mutant SNAP-25 rescues SNARE complex assembly deficits and enhanced neurodegeneration induced by loss of CSPα. From these data a model is postulated suggesting that the main function of CSPα is to chaperone SNAP-25 and, thus SNARE complex assembly.

Neurodegeneration in the absence of CSPα is hypothesized to be the result of the accumulation of defective SNAP-25 rather than impaired neurotransmission or loss of function of other unidentified substrates.

The question of how defective SNARE assembly and/ or loss of SNAP-25 seen in CSPα KO mice may cause neurodegeneration has wide implications for our understanding of presynaptic physiology and worthy of publication in The EMBO J. Overall, the data are of high quality. However, I also feel that the main hypothesis needs to be further substantiated. From the model presented it appears that increased misfolding of SNAP-25 in the absence of CSPα should result in impaired neurotransmission. Whether or not this is the case cannot be judged from the data (see my specific comments below). Furthermore, the authors claim that neurodegeneration is the consequence of the accumulation of defective SNAP-25. If this were the case then underexpression rather than overexpression of SNAP-25 should cure the defect. In fact, all data seem more consistent with the view that sustained loss-of-function rather than gain-of-malfunction is causing neurodegeneration. Hence, the interpretation of the data and the model should be re-considered.

Specific comments:

1. An important point in the model put forward by Sharma et al is the claim that neurodegeneration is not the result of altered neurotransmission. The data in this respect are incomplete. KD of SNAP-25 does not seem to produce any overt alterations in evoked transmission. What should have been looked at, however, is neurotransmission in neurons or slices from CSPα -/-; SNAP-25 -/+ mice, i.e. under conditions of exacerbated neurodegeneration and more severe defects in SNARE complex assembly.
Moreover, it is possible that the phenotype arises from differences in spontaneous fusion events rather than evoked release. This should be checked.

2. Rescue of SNARE complex formation (and neurodegeneration) by overexpressed SNAP25 is expected if the primary defect indeed results from loss of SNAP-25, even if CSPα has other targets. The observed rescue in my opinion strongly suggests that the phenotype is caused by SNAP-25 loss-of-function rather than accumulation of misfolded "bad" molecules as proposed. Either additional evidence for the hypothesis that defective SNAP-25 is causing the phenotype is required or else the model ought to be modified.

3. The expression level (judged by GFP fluorescence) of SNAP-25-eGFP WT appears to be much higher than that of all other constructs including GFP. This seems peculiar. Is this the result of altered degradation, synthesis, or does this merely reflect inefficient axonal targeting of SNAP-25 mutants? If so, I wonder how meaningful these controls are.

4. Is SNARE complex formation induced by overexpression of SNAP-25 in CSPα KO mice comparable to the level of SNARE complexes detected in WT or CSPα -/+ mice? This would seem an important piece of information to underscore the claim that OE of SNAP-25 indeed rescues loss of CSPα function.

5. Do the authors actually see biochemical signs of neuronal cell death in CSPα -/-; SNAP-25 -/+ mice, i.e. by staining with apoptotic markers such as caspase?

6. The discussion makes no clear distinction between synapse formation and synapse maintenance. In fact work mostly from the authors' laboratory has demonstrated that synapses form in the absence of neurotransmisson or presynaptic SNARE complexes. The defect seen rather appears to reflect a maintenance problem. This should be discussed.

1st Revision - authors' response 18 October 2011

Response to the reviewers' comments for Sharma et al., "CSPα Knockout Causes Neurodegeneration by Impairing SNAP-25 Function"

We thank the reviewers for their outstandingly helpful comments. Reviewers #1 and #2 accepted the paper pending text changes, which we instituted – needless to say, we are very grateful to these reviewers for their comments. Reviewer #3 made a series of suggestions that were helpful, but that required extensive new experiments which we have now performed. These experiments included:

1. Extensive additional electrophysiological measurements to document the surprisingly small effect of the loss of most SNAP-25 from neurons on synaptic transmission
2. Detailed quantifications of the expression levels of GFP-SNAP-25^{WT}, GFP-SNAP-25$^{1-197}$, and SNAP-25$^{1-180}$
3. Examination of apoptosis in CSPα KO neurons using measurements of caspase-3 cleavage, and determination of the effect of SNAP-25 overexpression or knockdown on the level of caspase-3 cleavage.

As a result of these experiments, the figures have grown significantly in size, but we do believe that the paper is now a more thorough and convincing study.

In the following, we cite the reviewers' comments in italic typeface, and describe our response in bold typeface.

Referee #1:
Since loss of CSP leads to neuronal degeneration investigation of its mechanism of action is of general interest as it could provide insights into mechanism of neuroprotection form neurodegenerative diseases. Earlier work from this group identified SNAP-25 as a target for the chaperone activity of CSP. This work left open the important question of whether the loss SNAP-25 and SNARE complexes was the key aspect in neurodegeneration in CSP KO mice. This new study elegantly addresses this issue and by using of genetic manipulation of SNAP-25 levels in the absence of CSP and by knock-down of SNAP-25 and its overexpression the authors now demonstrate that SNAP-25 is the key target for the neuroprotective role of CSP. Overall this is an excellent and comprehensive study that provides important conclusions and should be of general interest to the EMBO readership. Since this manuscript was submitted a key paper has now been published on line (Noskovaer al Am J Hum Gen 2011) indicating that mutations in CSP in humans leads to neuronal ceroidlipofuscinosis and neurodegeneration. This adds to the general significance of the current paper and the authors should include discussion of the new paper and how these findings fit with the role of CSP as a SNAP-25 chaperone.

We thank the referee for the careful evaluation of our paper and for the constructive comments, and have now cited the new paper the reviewer mentions in the revised manuscript. This new paper indeed adds further significance to our work, and we are grateful for the information!

Referee #2:
This is a follow up study from the group of Tom Südhof, where the authors continue to investigate the molecular mechanism underlying neurodegeneration produced by the lack of cysteine string protein (CSP) alpha. Having established in previous studies that lack of CSP alpha destabilizes SNARE complexes and enhances the degradation of SNAP-25, they now test whether reduced or enhanced levels of SNAP-25 can worsen or protect against the neurodegeneration phenotype, respectively. Using a combination of mouse genetic/molecular genetic tools (including in vivo, virus-based RNAi experiments), they provide unambiguous evidence for a central role of SNAP-25 deficiency in CSPalpha deletion-induced neurodegeneration. Overall, this is a solid study that addresses an important question using multidisciplinary approaches, including an impeccable biochemical analysis of SNARE proteins/complexes. Importantly, this study provides an excellent example of how a destabilization of a single presynaptic SNARE protein (with important consequences on the overall stability of SNARE complexes) can lead to neurodegeneration in the context of a defective folding machinery, providing a potential molecular basis for neurodegenerative processes in human disease.

We also thank this referee for her/his consideration of our paper, which has been most helpful.

Referee #3:
Sharma et al report on the role of the chaperone CSPa in regulating the function of the exocytic SNARE SNAP-25 and its relationship to neurodegeneration. Capitalizing on the available repertoire of knockout mice it is shown that loss of SNAP-25 in heterozygous KO mice or reduction of its expression induced by lentiviral knockdown exacerbates neurodegeneration and defective SNARE complex assembly seen in CSPa-KO mice. Most importantly, overexpression of wt but not truncated mutant SNAP-25 rescues SNARE complex assembly deficits and enhanced neurodegeneration induced by loss of CSPa. From these data a model is postulated suggesting that the main function of CSPa is to chaperone SNAP-25 and, thus SNARE complex assembly. Neurodegeneration in the absence of CSPa is hypothesized to be the result of the accumulation of defective SNAP-25 rather than impaired neurotransmission or loss of function of other unidentified substrates.

The question of how defective SNARE assembly and/ or loss of SNAP-25 seen in CSPa KO mice may cause neurodegeneration has wide implications for our understanding of presynaptic physiology and worthy of publication in The EMBO J. Overall, the data are of high quality. However, I also feel that the main hypothesis needs to be further substantiated. From the model presented it appears that increased misfolding of SNAP-25 in the absence of CSPa should result in impaired neurotransmission. Whether or not this is the case cannot be judged from the data (see my specific comments below). Furthermore, the authors claim that neurodegeneration is the consequence of the accumulation of defective SNAP-25. If this were the case then underexpression rather than overexpression of SNAP-25 should cure the defect. In fact, all data seem more consistent with the view that sustained loss-of-function rather than gain-of-malfunction is causing neurodegeneration. Hence, the interpretation of the data and the model should be re-considered.

We very much appreciate this referee's careful assessment of our paper, and her/his very positive overall comments. Moreover, we completely agree with her/his ideas about how to interpret our results.

Unfortunately, an important fundamental misunderstanding of our model arose in the referee's comments, a misunderstanding that is probably due to insufficient clarity in our description of the model. As described in the Discussion, our model exactly conforms to what the reviewer suggests. Our model also suggests that the neurodegeneration is most likely not caused by a toxic SNAP-25 conformer, but that in the absence of sufficient levels of functional SNAP-25, there is a relative excess of the other synaptic SNARE proteins (syntaxin-1 and synaptobrevin-2/VAMP2), and SNARE-complex assembly is impaired. Our model hypothesizes that excess SNARE proteins that do not have sufficient interaction partners (because functional SNAP-25 is lost) are the cause of neurodegeneration. Although we cannot rule out the possibility that impaired SNARE-complex assembly is to blame more directly, the electrophysiology data weaken this argument. Nowhere in the paper do we propose that accumulation of misfolded SNAP-25 is the most likely cause of the neurodegeneration, although we do mention it in the Discussion as an alternative possibility that cannot be ruled out. The referee's misunderstanding may have occurred in part because in earlier papers, we actually did consider the toxic SNAP-25 confomer idea, but have since discarded it.

Specific comments:
1. An important point in the model put forward by Sharma et al is the claim that neurodegeneration is not the result of altered neurotransmission. The data in this respect are incomplete. KD of SNAP-25 does not seem to produce any overt alterations in evoked transmission. What should have been looked at, however, is neurotransmission in neurons or slices from CSPa -/-; SNAP-25 -/+ mice, i.e. under conditions of exacerbated neurodegeneration and more severe defects in SNARE complex assembly. Moreover, it is possible that the phenotype arises from differences in spontaneous fusion events rather than evoked release. This should be checked.

We somewhat disagree with the reviewer that electrophysiological recordings should be done in slices instead of cultured neurons, at least if the purpose is to analyze baseline synaptic transmission events as is the goal in the present experiments. Quantification of synaptic response sizes is much more reliable in cultured neurons, making them a better preparation for the present experiments. Also, in brain slices from mice that exhibit severe neurodegeneration (like CSP$^{-/-}$ or CSP$^{-/-}$/SNAP-25$^{-/+}$), it is difficult to decide whether a synaptic transmission phenotype is primary or an effect of neurodegeneration. However, we do agree that monitoring spontaneous synaptic transmission is useful, and have now performed such experiments (Figs. 2 and S2). The results conform to those obtained for evoked release.

2. Rescue of SNARE complex formation (and neurodegeneration) by overexpressed SNAP25 is expected if the primary defect indeed results from loss of SNAP-25, even if CSPa has other targets. The observed rescue in my opinion strongly suggests that the phenotype is caused by SNAP-25 loss-of-function rather than accumulation of misfolded

"bad" molecules as proposed. Either additional evidence for the hypothesis that defective SNAP-25 is causing the phenotype is required or else the model ought to be modified.

We completely agree with the reviewer – indeed, our model exactly states that the phenotype is caused by a SNAP-25 loss-of-function.

3. The expression level (judged by GFP fluorescence) of SNAP-25-eGFP WT appears to be much higher than that of all other constructs including GFP. This seems peculiar. Is this the result of altered degradation, synthesis, or does this merely reflect inefficient axonal targeting of SNAP-25 mutants? If so, I wonder how meaningful these controls are.

The reviewer is right. There is a significant increase in overall GFP fluorescence in sections expressing GFP-SNAP-25^{WT}, which rescues the loss of neurons in CSPα KO mice. We have now determined whether this change indicates increased number of GFP-SNAP-25^{WT} expressing neurons (due to the rescue of neurodegeneration) or increased expression of GFP-SNAP-25^{WT} per neuron. We used the following methods in the revised paper to clarify this issue:

1. **We measured the expression levels of GFP-SNAP-25^{WT}, GFP-SNAP-25$^{1-197}$, and SNAP-25$^{1-180}$ by quantitative immunoblotting of protein levels (Fig. 5C) and by fluorescence density detection (total fluorescent pixels/fluorescent area; Fig. S5B) in cultured CSPαKO neurons. All three proteins were expressed at similar levels under conditions of no neurodegeneration. GFP alone is expressed at a higher level per neuron than any of the GFP-SNAP-25 fusion proteins, possibly because the confocal slicing of the cell captures more of the cytosolic GFP than the membrane-bound GFP-SNAP-25 chimeras, or because it is more stable.**
2. **We measured the total fluorescence density (total fluorescent pixels/fluorescent area) in neurons of CSPα KO brain sections expressing GFP alone, GFP-SNAP-25^{WT}, GFP- SNAP-25$^{1-197}$, or GFP-SNAP-25$^{1-180}$ (Fig. S6B). Again, we found no difference between the various GFP-SNAP-25 fusion proteins, suggesting that the overall increase in fluorescence per section is due to more CSPα KO neurons rescued by GFP-SNAP-25^{WT}. In the brain sections, the levels of GFP alone are not higher than the GFP-SNAP-25 fusion proteins, possibly because epifluorescence microscopy was used here which does not distinguish between cytosolic vs. membrane bound fluorescence.**
3. **Changes in overall fluorescence per section correlate well with neuron survival, which is measured by NeuN:DAPI ratio, in either SNAP-25 knockdown experiments or GFP-SNAP-25 overexpression experiments (Figs. 4, S4, 6 and S6).**

The overall level of fluorescence in brain sections thus indicates the number of fluorescent neurons rather than protein expression levels per neuron.

4. Is SNARE complex formation induced by overexpression of SNAP-25 in CSPa KO mice comparable to the level of SNARE complexes detected in WT or CSPa -/+ mice? This would seem an important piece of information to underscore the claim that OE of SNAP-25 indeed rescues loss of CSPa function.

We agree, but believe that this information is fully available. Previous studies (Chandra et al., 2005; Sharma et al., 2011) and the current data (Figures 1 and 2) demonstrate that SNARE-complex assembly in CSPα KO mice is reproducibly reduced by 50 ± 5%, dependent on age and experimental conditions. This decrease is observed both in cultured neurons and in brain homogenates. Since in the present experiments we show that SNAP-25 overexpression increases SNARE-complex assembly approximately 2-fold, there appears to be a good overall match.

5. Do the authors actually see biochemical signs of neuronal cell death in CSPa -/-; SNAP-25 -/+ mice, i.e. by staining with apoptotic markers such as caspase?

To address this question, we have now examined neurons in CSPα KO mice under various conditions for evidence of caspase-3 cleavage as a marker of apoptosis. We find that compared to WT brains, CSPα KO brains exhibited a marked increase in cleaved caspase-3 at P50. Since we no longer have CSP KO/SNAP-25+/- mice available or brain sections from such mice (as suggested by the reviewer), we measured apoptosis in CSPα KO brains injected with lentiviruses expressing either SNAP-25 shRNA or expressing GFP-SNAP-25 chimera. Rescue of neuronal cell death in CSPα KO brains by GFP-SNAP-25 overexpression correlated with a decrease in cleaved caspase-3 (Figs. 6 and S6), whereas increased neuronal cell death due to the SNAP-25 knockdown resulted in an increased level of cleaved caspase-3 (Figs. 4 and S4). We thank the reviewer for suggesting these experiments, which added another mechanistic aspect to the study.

6. The discussion makes no clear distinction between synapse formation and synapse maintenance. In fact work mostly from the authors' laboratory has demonstrated that synapses form in the absence of neurotransmisson or presynaptic SNARE complexes. The defect seen rather appears to reflect a maintenance problem. This should be discussed.

We completely agree, and have done so in the revised manuscript.

We thank the reviewers for their valuable comments, and hope the paper can now be accepted for publication.

2nd Editorial Decision 02 November 2011

Thank you for sending us your revised manuscript. Our original referee 3 has now seen it again, and you will be pleased to learn that in his/her view you have addressed all criticisms in a satisfactory manner.

Prior to acceptance, there is one editorial issue that needs further attention. We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. Would you be willing to provide files comprising the original, uncropped and unprocessed scans of all gels used in the figures? We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source Data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.

Thank you very much again for considering our journal for publication of your work and for your kind cooperation.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #3:

The authors have done an excellent job in revising their Ms. I strongly endorse publication of this important piece of work, which for the first time reveals the molecular mechanistic basis for neurodegeneration caused by loss of synaptic protein complexes.

