EMBO *reports*

$A\beta_{1-42}$ triggers the generation of a retrograde signaling complex from sentinel mRNAs in axons

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Review timeline:

Submission date:	3 November 2017
Editorial Decision:	11 December 2017
Revision received:	27 March 2018
Editorial Decision:	19 April 2018
Revision received:	20 April 2018
Accepted:	24 April 2018

Editor: Esther Schnapp

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 11 December 2017

Thank you for the submission of your manuscript to EMBO reports. I apologize for the unusual delay in getting back to you; we have only now received the full set of referee reports that is pasted below.

As you will see, all referees acknowledge that the findings are potentially novel and interesting. However, they also raise some concerns that would need to be addressed in order to strengthen the data and conclusiveness of the study.
Given that most concerns are raised by more than one referee, I think that all of them should be addressed. I have not initiated the cross-commenting of the referees on each others' reports, given the delay, but my understanding is that all concerns are sensible and can be addressed. Please let me know in case you disagree and we can discuss the revisions further.

Given the constructive comments, we would thus like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss this further. You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when

discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

Regarding data quantification, please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends. Please also include scale bars in all microscopy images.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where in the manuscript the requested information can be found. The completed author checklist will also be part of the RPF (see below).
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

The present study "Oligomeric Aβ1-42 triggers the generation of a retrograde signaling complex from sentinel mRNAs in axons" builds on an earlier publication from this lab on locally translation of ATF4 as a neurodegenerative signal transmitted from axons to cell bodies. This previous study, while identifying an interesting mechanism, also raised intriguing questions. From the previous study it was not clear how the local insult (Aβ1-42) led to increased Atf4 mRNA levels in axons.

This new study attempts to answer this important question by using a candidate approach to test known signaling mechanisms identified in nerve injury, namely STAT3 activation and vimentin expression. Main findings are 1) Locally applied Aβ1-42 rapidly induces axonal protein synthesis

measured with ribopuromycylation, 2) Aβ1-42-treatment induced activation of S6 phosphorylation is Ca2+ signaling dependent and is blocked by BAPTA-AM treatment, 3) Aβ1-42-dependent localization of Atf4 transcript requires axonal signaling via retrograde transport, 4) full Aβ1-42-dependent upregulation of axonal ATF4 protein levels requires transcription, 5) Aβ1-42 induces phosphorylation/activation of STAT3, but not its local translation, and 6) Aβ1-42-dependent axonal Atf4 translation requires local translation of axonal vimentin mRNA. Taken together, these data lead the authors to propose a model where exposure to Aβ1-42 triggers rapid activation of STAT3 (but not its local translation) and the rapid local synthesis of vimentin, which is retrogradely transported to the soma, causing a rapid and transient transcription-dependent increase in somatic Atf4 transcript levels, followed by its transport into axons and local translation. Interestingly, a local role for STAT3 and stathmin after activation via CNTF has been shown in a previous study to rescues axon degeneration in the pmn (mutant TBCE) model of motoneuron disease. The authors propose that CNS neurons react to neurodegenerative insults via the axonal translation of 'sentinel mRNAs' encoding vimentin and perhaps other components of a retrograde signaling complex that transmit the information about the event to the neuronal soma, and triggers either neurodegeneration or a regenerative response.

There is a growing consensus that local translation in axons occurs not only during development but also in mature neurons. This asymmetry serves 2 purposes: 1) to supply the growth cone with material required for axon outgrowth, pathfinding, and regeneration, and 2) to provide a means for the axon in highly polarized neurons to communicate with the cell body. In this study the authors provide a compelling link between the signaling function of local translation, and human neurodegenerative disease.

In summary, this is an interesting and original study that addresses an important question, using a sophisticated in vitro approach that has been very well established in this lab. The authors are using their preferred system of primary hippocampal neurons grown in compartmentalized microfluidic chambers, and combined with local treatment and transfection of axons. This stucy can be expected to be of considerable interest for the field but also the wider readership of this journal. While it addresses basic and fundamental questions of signaling in highly polarized cells, it has also the potential to impact translational research. It will be of interest for the future to find out how signaling that causes neurodegeneration vs. regeneration differs on a molecular level.

While the manuscript is very well written and technically sound, there are several issues that should be addressed:

Major point:
The important ultimate finding presented in Figure 6 appears less well fleshed out than the other results for ATF4 and STAT3, ad thus less convincing. Specifically, the authors do not validate the presence of 'sentinel' Vim transcripts in the axon, by using FISH. The authors demonstrate that STAT3 protein levels remain unchanged, but do not investigate vimentin protein levels in the cell soma. Since the actual retrograde transport of vimentin is not shown, there is also the possibility that decreased vimentin protein levels are caused by the UPS, which would be easily addressed by using local application of proteasome inhibitors.

Minor points:

The type of STAT3 phosphorylation should be described in the text. After all, there is Y705 and S727 described in the literature.

Figure legends are not consistent. Sometimes they describe the model used ("Dissociated hippocampal neurons were cultured...."), sometimes they do not ("Axons were treated....").

Referee #2:

In this manuscript, the authors build upon their previous work addressing the contribution of local axonal protein synthesis in the propagation of the neurodegenerative signal in response to axonal exposure to Aβ1-42 peptide. Here, they demonstrate that there is an early local translation response, which is important for generating the retrograde signal that results in the axonal recruitment of Aft4

mRNA. They further show that this is a Ca2+-dependent event, and requires the local translation of vimentin protein from Vimentin mRNA that exists in the axon prior to Aβ1-42 peptide exposure. Overall, this is a generally clearly-written and interesting paper building on this lab's longstanding interest in the role of local axonal translation in the role that it plays in both normal and pathological neuronal processes. Study of local axonal protein synthesis is an emerging field and insight into the role that these locally synthesized proteins play in both normal and pathological conditions are of critical importance and are therefore likely to be of high interest to Journal readers. I have several questions, comments, and additions, that if adequately addressed would strengthen the manuscript and make it suitable for publication.

Major Issues:

1. In the experiments in Figure 1, is axonal translation increasing with bath applied Aβ1-42? You show that bath application does not alter cell body translation, but if you include a higher magnification of one of the axons in each image, we could determine if bath application increases axonal translation in a manner similar to axonal-only application. In other words, does the Aβ1-42 signal need to be exclusively axonal (without accompanying cell body signaling)?

2. Why not show the puromycin analysis at 60 minutes to show that the increase in translation is truly transient?

3. In the experiments in Figure 4, what happens to the Atf4 mRNA levels in the axon when cell bodies are treated with actinomycin D? Does the axonal Aβ1-42 signal drive axonal localization of pre-existing cell body Atf4 mRNA or new synthesis of Atf4 mRNA?

4. What happens to cell body degeneration when axonal synthesis of vimentin is blocked in response to Aβ1-42? The authors show that this local synthesis is necessary to drive Aft4 mRNA localization into the axon, but not that it has an impact on neuronal survival in response to Aβ1-42.

5. Is the Aβ1-42-induced vimentin retrograde signal erk-dependent, similar to the injury-signal? Also, you implicate calcium as being critical for this process, but have you shown that Aβ1-42 stimulation of the axon results in a rise in axonal calcium levels?

Minor Issues:

1. I don't think the Table contributes much beyond what is already in the text.

2. Overall, many of the images are not of the quality that this group usually produces. They seem over-processed or thresholded for both the immunostaining and the FISH, unlike their previous studies on this topic. Also, in general the quality of some of the staining is not particularly good (Figures 3A, 5A, 6C as examples).

3. I don't understand how the phosphor-S6/S6 analyses in Figure 2 are done. The images don't appear to be representative of the quantification shown. Is the ratio of phospho-S6 to total S6 really approximately 0.3 in the BAPTA-AM treated? I see no apparent phospho-S6 staining.

4. In the final paragraph of the Discussion, the authors state that their results further support the notion that mature neurons contain transcripts and are capable of protein synthesis. I don't really think these results add support to this idea. I do, however, think they lend considerable support to the idea that, similar to peripheral neurons (as in the Fainzilber retrograde-injury studies), axonal translation in central nervous system axons is important for relaying injurious signals to the cell body.

Referee #3:

This manuscript provides experimental evidence to show that when Aβ1-42 is added to axon terminals it elicits localized translation that gives rise to a retrograde signaling complex that eventually leads to axon degeneration. The results show that addition of Aβ1-42 causes the rapid translation of vimentin mRNA in axons and that specific knock-down of axonal vimentin protein

synthesis prevents the formation of the retrograde signaling complex and rescues axon degeneration. In addition, inhibition of dynein transport is shown to prevent the retrograde movement of the signaling complex and prevents the Aβ1-42-induced axon degeneration. The authors conclude that axons react to neurodegenerative insults such as Aβ1-42 by local translation of 'sentinel' mRNAs followed by transport to the soma and new transcription.

This interesting study follows up on Hengst's previous pioneering work showing that Aβ1-42 causes axon degeneration via axonal synthesis of the transcription factor ATF4. The ATF4 synthesis, however, does not occur until several hours after Aβ1-42 treatment raising the question of what comprises the immediate retrograde signal? Here, the authors report that the later transport/translation of ATF4 in axons is dependent on immediate early axonal protein synthesis (within 30 mins of Aβ1-42 treatment) and on retrograde transport by blocking retrograde transport with ciliobrevin. The results, therefore, provide novel and important insight into the signaling mechanism of Aβ1-42-induced axon degeneration. The manuscript would benefit from considering the points below and adding further experimental evidence to strengthen the results, particularly with respect to vimentin axonal translation and retrograde transport.

Specific comments:

1. Vimentin levels are reported to decrease 30 mins after Aβ1-42 treatment (Figure 6A). The authors conclude that this loss is consistent with retrograde transport of nascent vimentin to the soma. This is supported by data showing that a dynein inhibitor prevents the loss of Aβ1-42-induced axonal vimentin. However, it seems possible that this loss reflects degradation of the protein rather than retrograde trafficking. There is evidence in the literature that dynein affects proteasomal transport (e.g. Kreko-Pierce and Eaton, JCB 2017) so it is conceivable that ciliobrevin interferes with the function of the ubiquitin proteasome system and, therefore, inhibits degradation. It could be informative to test whether the vimentin loss still occurs in the presence of inhibitors of the UPS.

2. Did the authors examine shorter timepoints such as 5-10 mins after Aβ1-42 treatment to see if the levels of vimentin increase (reflecting local protein synthesis) before they decrease? At the moment, the evidence for axonal synthesis of vimentin rests on a rather convoluted result showing that the decrease in axonal vimentin at 30 mins does not occur when vimentin translation is inhibited by axonal siRNA. While this is certainly consistent with the data, other interpretations are possible.
3. No direct evidence is presented in the manuscript demonstrating the presence of vimentin mRNA in axons. This should be validated with FISH and/or RT-PCR.

4. It would be valuable to provide direct visualization of nascent vimentin being trafficked retrogradely. This could be done with a vimentin-GFP reporter and FRAP.

5. Was an experiment done without cell bodies to confirm the new vimentin was translated locally?

6. The study does not provide any insight into what vimentin does in the retrograde signaling complex. Can the authors at least speculate on its role?

7. Some of the fluorescent puromycylation signal looks saturated in the images in Fig. 1 B & C. Since accurate quantitation requires non-saturated samples, how was the quantitation done? There is minimal information regarding the exact methods of quantitation of the immunofluorescence and what was done to avoid pixel saturation. Details along these lines should be included.

8. The result that BAPTA-AM reduces translation as indicated by phospho-S6 levels does not seem surprising given calcium's potential involvement in many possible steps of the signaling cascade. Could it even prevent the initial activation step or entry of Aβ1-42 across the membrane? In which case, how relevant is this result? The authors' conclusion "that Aβ1-42 regulates immediate-early axonal translation via Ca2+ signaling" while broadly correct is rather simplistic and does not to acknowledge the full complexity of the effects of calcium inhibition.

9. Previous work by Jaffrey's group has shown that axonally translated CREB acts as a retrograde signal in NGF-dependent axon survival. How do the CREB findings relate to the present findings? Is CREB also present in the signaling complex? Much of the discussion focuses on STAT3 but there is no mention of CREB.

10. The subtitle "Transcription is required for Atf4 translation in axons" seems too strong given that axonal ATF4 was only partially inhibited by actinomycin D treatment.

11. Fig. 4C: Was the increase in Atf4 mRNA in cell bodies seen after Aβ1-42 axonal treatment (Fig. 4D) inhibited by actinomycin D? It is possible that the increase was due to retrograde transport of axonal Atf4. The Atf4 levels were normalised against Gapdh transcripts. Is it possible that Gapdh levels change with addition of Aβ1-42 in a way that could confound these results?

1st Revision - authors' response 27 March 2018

Referee #1:

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This new study attempts to answer this important question by using a candidate approach to test known signaling mechanisms identified in nerve injury, namely STAT3 activation and vimentin expression. Main findings are 1) Locally applied Aβ1-42 rapidly induces axonal protein synthesis measured with ribopuromycylation, 2) Aβ1-42-treatment induced activation of S6 phosphorylation is Ca2+ signaling dependent and is blocked by BAPTA-AM treatment, 3) Aβ1-42-dependent localization of Atf4 transcript requires axonal signaling via retrograde transport, 4) full Aβ1-42-dependent upregulation of axonal ATF4 protein levels requires transcription, 5) Aβ1-42 induces phosphorylation/activation of STAT3, but not its local translation, and 6) Aβ1-42-dependent axonal Atf4 translation requires local translation of axonal vimentin mRNA. Taken together, these data lead the authors to propose a model where exposure to Aβ1-42 triggers rapid activation of STAT3 (but not its local translation) and the rapid local synthesis of vimentin, which is retrogradely transported to the soma, causing a rapid and transient transcription-dependent increase in somatic Atf4 transcript levels, followed by its transport into axons and local translation. Interestingly, a local role for STAT3 and stathmin after activation via CNTF has been shown in a previous study to rescues axon degeneration in the pmn (mutant TBCE) model of motoneuron disease. The authors propose that CNS neurons react to neurodegenerative insults via the axonal translation of 'sentinel mRNAs' encoding vimentin and perhaps other components of a retrograde signaling complex that transmit the information about the event to the neuronal soma, and triggers either neurodegeneration or a regenerative response.

There is a growing consensus that local translation in axons occurs not only during development but also in mature neurons. This asymmetry serves 2 purposes: 1) to supply the growth cone with material required for axon outgrowth, pathfinding, and regeneration, and 2) to provide a means for the axon in highly polarized neurons to communicate with the cell body. In this study the authors provide a compelling link between the signaling function of local translation, and human neurodegenerative disease.

In summary, this is an interesting and original study that addresses an important question, using a sophisticated in vitro approach that has been very well established in this lab. The authors are using their preferred system of primary hippocampal neurons grown in compartmentalized microfluidic chambers, and combined with local treatment and transfection of axons. This stucy can be expected to be of considerable interest for the field but also the wider readership of this journal. While it addresses basic and fundamental questions of signaling in highly polarized cells, it has also the potential to impact translational research. It will be of interest for the future to find out how signaling that causes neurodegeneration vs. regeneration differs on a molecular level.

While the manuscript is very well written and technically sound, there are several issues that should be addressed:
We thank the reviewer for these positive comments.

Major point:

The important ultimate finding presented in Figure 6 appears less well fleshed out than the other results for ATF4 and STAT3, and thus less convincing. Specifically, the authors do not validate the presence of 'sentinel' Vim transcripts in the axon, by using FISH.
In the revised manuscript, we are presenting in new Fig 5A FPKM values and qRT-PCR results for vimentin mRNA in axons. We believe that those methods are more quantitative than FISH, especially for a low abundance mRNA.
The authors demonstrate that STAT3 protein levels remain unchanged, but do not investigate vimentin protein levels in the cell soma. Since the actual retrograde transport of vimentin is not shown, there is also the possibility that decreased vimentin protein levels are caused by the UPS, which would be easily addressed by using local application of proteasome inhibitors.
The reviewer suggests to test whether the decrease in vimentin levels is prevented by UPS inhibitors. We have performed this experiment multiple times, but unfortunately MG132 had a strong effect on the baseline of vimentin levels in axons that confounded the interpretation of this experiment. We decided not to include this inconclusive experiment.

Minor points:

The type of STAT3 phosphorylation should be described in the text. After all, there is Y705 and S727 described in the literature.
We used an antibody directed against the pTyr705 epitope of STAT3. This information is now included in the Experimental Procedures section and the Figure Legend.

Figure legends are not consistent. Sometimes they describe the model used ("Dissociated hippocampal neurons were cultured...."), sometimes they do not ("Axons were treated....").
We have updated the figure legends to consistently describe the model used.

Referee #2:

In this manuscript, the authors build upon their previous work addressing the contribution of local axonal protein synthesis in the propagation of the neurodegenerative signal in response to axonal exposure to Aβ1-42 peptide. Here, they demonstrate that there is an early local translation response, which is important for generating the retrograde signal that results in the axonal recruitment of Aft4 mRNA. They further show that this is a Ca2+-dependent event, and requires the local translation of vimentin protein from Vimentin mRNA that exists in the axon prior to Aβ1-42 peptide exposure. Overall, this is a generally clearly-written and interesting paper building on this lab's longstanding interest in the role of local axonal translation in the role that it plays in both normal and pathological neuronal processes. Study of local axonal protein synthesis is an emerging field and insight into the role that these locally synthesized proteins play in both normal and pathological conditions are of critical importance and are therefore likely to be of high interest to Journal readers. I have several questions, comments, and additions, that if adequately addressed would strengthen the manuscript and make it suitable for publication.
We thank the reviewer for her/his positive comments.

Major Issues:

1. In the experiments in Figure 1, is axonal translation increasing with bath applied Aβ1-42? You show that bath application does not alter cell body translation, but if you include a higher magnification of one of the axons in each image, we could determine if bath application increases axonal translation in a manner similar to axonal-only application. In other words, does the Aβ1-42 signal need to be exclusively axonal (without accompanying cell body signaling)?
We have performed the requested experiment (new Fig EV1B). The $A\beta_{1-42}$ signal does not need to be exclusively axonal.

2. Why not show the puromycin analysis at 60 minutes to show that the increase in translation is truly transient?

We have updated Fig 1B to include the 60 minutes time point as suggested. The puromycylation signal reverts back to baseline.

3. In the experiments in Figure 4, what happens to the Atf4 mRNA levels in the axon when cell bodies are treated with actinomycin D? Does the axonal Aβ1-42 signal drive axonal localization of pre-existing cell body Atf4 mRNA or new synthesis of Atf4 mRNA?
In response to a comment by reviewer 3 we have now reanalyzed our data (see Fig EV3 in the revised manuscript). The reviewer is correct: the axonal $A\beta_{1\text{-}42}$ signal drives the axonal localization of pre-existing *Atf4* mRNA.

4. What happens to cell body degeneration when axonal synthesis of vimentin is blocked in response to Aβ1-42? The authors show that this local synthesis is necessary to drive Aft4 mRNA localization into the axon, but not that it has an impact on neuronal survival in response to Aβ1-42.
The reviewer is right that we do not show that immediate-early translation of vimentin is required for cell loss at 48 hrs. We did not perform the proposed experiments, because vimentin mRNA was part of the axonal transcriptome found at 24 hrs in $A\beta_{1\text{-}42}$-treated axons. Vimentin is likely part of both retrograde signaling complexes: the immediately formed described here and the ATF4-containing one we reported earlier in Baleriola et al. The proposed siRNA approach would necessarily affect both complexes, and as we already know that the ATF4 complex is required for cell loss, the expected results could not be unambiguously interpreted in support of immediate-early vimentin synthesis in $A\beta_{1\text{-}42}$-treated axons.

5. Is the Aβ1-42-induced vimentin retrograde signal erk-dependent, similar to the injury-signal?
We are now presenting data in Fig EV5 indicating that cell body ERK levels and ERK phosphorylation are not affected by axonally applied $A\beta_{1\text{-}42}$, suggesting that the retrograde signal is ERK independent or that the levels of (phospho)-ERK are too low to be detected by immunocytochemistry.
Also, you implicate calcium as being critical for this process, but have you shown that Aβ1-42 stimulation of the axon results in a rise in axonal calcium levels?
In the revised manuscript we are now presenting in Fig 2 and Fig EV2 calcium imaging data showing that $A\beta_{1\text{-}42}$ triggers an increase in intra-axonal calcium levels within seconds.

Minor Issues:

1. I don't think the Table contributes much beyond what is already in the text.
We kept the table as it might be helpful for the readers.

2. Overall, many of the images are not of the quality that this group usually produces. They seem over-processed or thresholded for both the immunostaining and the FISH, unlike their previous studies on this topic. Also, in general the quality of some of the staining is not particularly good (Figures 3A, 5A, 6C as examples).
In the revised version of the manuscript, we included a description in the Materials and Methods section of how we performed the image analysis. All quantification was done on non-saturated images. We provide better images for the Figures mentioned by the reviewer.

3. I don't understand how the phosphor-S6/S6 analyses in Figure 2 are done. The images don't appear to be representative of the quantification shown. Is the ratio of phospho-S6 to total S6 really approximately 0.3 in the BAPTA-AM treated? I see no apparent phospho-S6 staining.
The quantification of the phospho-S6/S6 was done on un-saturated images; details for how images have been quantified have been added to the Materials and Methods section. We are now presenting better images in Fig 2C.

4. In the final paragraph of the Discussion, the authors state that their results further support the notion that mature neurons contain transcripts and are capable of protein synthesis. I don't really think these results add support to this idea. I do, however, think they lend considerable support to the idea that, similar to peripheral neurons (as in the Fainzilber retrograde-injury studies), axonal translation in central nervous system axons is important for relaying injurious signals to the cell body.
We have changed the last paragraph of the Discussion to incorporate to reviewer's suggestion.

Referee #3:

This manuscript provides experimental evidence to show that when Aβ1-42 is added to axon terminals it elicits localized translation that gives rise to a retrograde signaling complex that eventually leads to axon degeneration. The results show that addition of Aβ1-42 causes the rapid translation of vimentin mRNA in axons and that specific knock-down of axonal vimentin protein synthesis prevents the formation of the retrograde signaling complex and rescues axon degeneration. In addition, inhibition of dynein transport is shown to prevent the retrograde movement of the signaling complex and prevents the Aβ1-42-induced axon degeneration. The authors conclude that axons react to neurodegenerative insults such as Aβ1-42 by local translation of 'sentinel' mRNAs followed by transport to the soma and new transcription.

This interesting study follows up on Hengst's previous pioneering work showing that Aβ1-42 causes axon degeneration via axonal synthesis of the transcription factor ATF4. The ATF4 synthesis, however, does not occur until several hours after Aβ1-42 treatment raising the question of what comprises the immediate retrograde signal? Here, the authors report that the later transport/translation of ATF4 in axons is dependent on immediate early axonal protein synthesis (within 30 mins of Aβ1-42 treatment) and on retrograde transport by blocking retrograde transport with ciliobrevin. The results, therefore, provide novel and important insight into the signaling mechanism of Aβ1-42-induced axon degeneration. The manuscript would benefit from considering the points below and adding further experimental evidence to strengthen the results, particularly with respect to vimentin axonal translation and retrograde transport.
We are grateful to the reviewer for the positive and thoughtful comments.

Specific comments:

1. Vimentin levels are reported to decrease 30 mins after Aβ1-42 treatment (Figure 6A). The authors conclude that this loss is consistent with retrograde transport of nascent vimentin to the soma. This is supported by data showing that a dynein inhibitor prevents the loss of Aβ1-42-induced axonal vimentin. However, it seems possible that this loss reflects degradation of the protein rather than retrograde trafficking. There is evidence in the literature that dynein affects proteasomal transport (e.g. Kreko-Pierce and Eaton, JCB 2017) so it is conceivable that ciliobrevin interferes with the function of the ubiquitin proteasome system and, therefore, inhibits degradation. It could be informative to test whether the vimentin loss still occurs in the presence of inhibitors of the UPS.
The reviewer suggests to test whether the decrease in vimentin levels is prevented by UPS inhibitors. We have performed this experiment multiple times, but unfortunately MG132 had a strong effect on the baseline of vimentin levels in axons that confounded the interpretation of this experiment. We decided not to include this inconclusive experiment.

2. Did the authors examine shorter timepoints such as 5-10 mins after Aβ1-42 treatment to see if the levels of vimentin increase (reflecting local protein synthesis) before they decrease? At the moment, the evidence for axonal synthesis of vimentin rests on a rather convoluted result showing that the decrease in axonal vimentin at 30 mins does not occur when vimentin translation is inhibited by axonal siRNA. While this is certainly consistent with the data, other interpretations are possible.
We are now providing data for 15 min $A\beta_{1-42}$ treatment (new Fig EV4) that reveal a statistically non-significant increase in vimentin levels.

3. No direct evidence is presented in the manuscript demonstrating the presence of vimentin mRNA in axons. This should be validated with FISH and/or RT-PCR.
In Fig 5A we are now presenting FPKM values and qRT-PCR results for vimentin mRNA in axons.

4. It would be valuable to provide direct visualization of nascent vimentin being trafficked retrogradely. This could be done with a vimentin-GFP reporter and FRAP.
We agree that this experiment would potential strengthen our conclusions. It is technically more challenging than it appears to be: to get locally translated, we would have to deliver the tagged vimentin in the context of its normal 5' and 3'UTRs. The standard approach of exogenously overexpressing mRNAs to study axonal translation events is in our opinion/experience prone to artifacts as overexpression is often overwhelming he endogenous mRNA localization machinery.

Moreover, the endogenous levels of locally synthesized vimentin are very low; it is unclear whether a reporter approach would yield a sufficient signal at these relevant levels.

5. Was an experiment done without cell bodies to confirm the new vimentin was translated locally?
We have not performed this experiment, because the enucleation would necessarily involve injuring the axons. Axonal injury is sufficient to induce local vimentin production, confounding the interpretation of such an experiment. In our puromycylation experiments, puromycin was added exclusively to axons, supporting our interpretation that $A\beta_{1\text{-}42}$ induces local, intra-axonal protein synthesis.

6. The study does not provide any insight into what vimentin does in the retrograde signaling complex. Can the authors at least speculate on its role?
In the first paragraph of the revised manuscript we now provide a discussion of vimentin likely role as a scaffolding protein in the retrograde signaling complex.

7. Some of the fluorescent puromycylation signal looks saturated in the images in Fig. 1 B & C. Since accurate quantitation requires non-saturated samples, how was the quantitation done? There is minimal information regarding the exact methods of quantitation of the immunofluorescence and what was done to avoid pixel saturation. Details along these lines should be included.
All image analysis and quantification were done on not saturated images. Details how the quantification was performed is now included in the Experimental Procedures section.

8. The result that BAPTA-AM reduces translation as indicated by phospho-S6 levels does not seem surprising given calcium's potential involvement in many possible steps of the signaling cascade. Could it even prevent the initial activation step or entry of Aβ1-42 across the membrane? In which case, how relevant is this result? The authors' conclusion "that Aβ1-42 regulates immediate-early axonal translation via Ca2+ signaling" while broadly correct is rather simplistic and does not to acknowledge the full complexity of the effects of calcium inhibition.
We are now presenting new data in Fig 2 that provide more insight into the role of source of calcium in the activation of local translation. The relevant passages in the manuscript have been updated accordingly.

9. Previous work by Jaffrey's group has shown that axonally translated CREB acts as a retrograde signal in NGF-dependent axon survival. How do the CREB findings relate to the present findings? Is CREB also present in the signaling complex? Much of the discussion focuses on STAT3 but there is no mention of CREB.
The reviewer suggests an interesting possibility. However, we did not specifically investigate CREB in the context of local $A\beta_{1\text{-}42}$ signaling, primarily because we did not detect CREB mRNA in the transcriptome data set for axons from hippocampal neuron (Baleriola et al. Cell, 2014). The previous CREB-related work from the Jaffrey lab was done in dorsal root ganglion cells.

10. The subtitle "Transcription is required for Atf4 translation in axons" seems too strong given that axonal ATF4 was only partially inhibited by actinomycin D treatment.
We have changed the subheading to ‘Inhibition of transcription reduces Atf4 translation in axons’.

11. Fig. 4C: Was the increase in Atf4 mRNA in cell bodies seen after Aβ1-42 axonal treatment (Fig. 4D) inhibited by actinomycin D? It is possible that the increase was due to retrograde transport of axonal Atf4. The Atf4 levels were normalised against Gapdh transcripts. Is it possible that Gapdh levels change with addition of Aβ1-42 in a way that could confound these results?
In the revised manuscript, the old Fig 4 is now Fig EV3. The reviewer is absolutely correct: we reanalyzed our data and the originally reported change in *Atf4* levels was driven by changes in *Gapdh* levels. When we normalized to the input, the levels for *Atf4* mRNA in the cell bodies do not change, indicating that the transcription of another target rather than *Atf4* is required for *Atf4* localization to the axons. We are very grateful to the reviewer for this especially helpful comment!

2nd Editorial Decision 19 April 2018

Thank you for the submission of your revised manuscript. We have now received all referee reports that are pasted below, and I am happy to say that all referees support the publication of your work. Referee 1 has a few more minor suggestions that I would like you to address before we can proceed with the official acceptance.

The information on the statistics needs to be clarified. You mention technical replicates, biological replicates per group and independently performed experiments. "n" should be used for independently performed experiments only, but the total number of cells or axons can be mentioned too. My impression is that the biological replicates per group you mention are in fact technical replicates. Can you please explain what was done and use the same wording for all figure legends? IF less than 3 independent experiments are performed no error bars should be calculated, but all data points from the experiments should be shown. And no error bars should be calculated for technical replicates either. If data from a single experiment are shown, this needs to be explained in the figure legend. It seems that figures 4C and EV3 show error bars for technical replicates (but you also mention biological replicates), figures 5A and EV2A do not specify the bars, error bars and "n", figures EV1B and EV4 seem to be single experiments, and data in figure EV2B are from 2 experiments but do show error bars. I attach a commentary on replicates and repeats to this email for your information.

The manuscript currently has 5 main figures and should thus be formatted as a scientific report, with combined Results and Discussion sections. If you prefer to keep the 2 sections separate, please add one more main figure to the manuscript so that it becomes a full article. Please let me know if you have any questions.

I look forward to seeing a final version of your manuscript as soon as possible.

REFEREE REPORTS

Referee #1:

The authors have adequately responded to reviewer comments, improving the quality of this strong manuscript.

Minor points are listed below.

Figure legend 2A does not state experimental model

Page 5/82: "thus axons were exposed to 3 μM oligomeric Aβ1-42, which we previously found to be equivalent to approximately 250 nM [7]." This seems to refer to the effective concentration and influences of small volume and hydrophobicity in microfluidic chambers. "....250 nM in regular cultures" should be added

Page 8/158: "Atf4 levels in cell bodies of Aβ1-42-treated axons were indistinguishable significantly from controls (Fig EV3C),"
-> perhaps "not significantly different"?

Page 10/195: "The FPKM value for Vim in our previously reported transcriptome of untreated hippocampal axons was lower than the Actb" -> lower than the level for Actb?

Page 10/200: "treatment. After 15 minutes we detected a non-significant trend towards increased axonal vimentin levels (Fig EV4), while we observed a significant decrease in axonal vimentin levels compared to vehicle-treated axons (Fig 5B)." -> while after 30 minutes we observed?

In this reviewer's opinion, Fig. 5 would be easier to understand, if more information was added to label the figure. The difference between 5C and D (treatment and timing) is unclear without reading the legend.

Referee #2:

In this manuscript, the authors build upon their previous work addressing the contribution of local axonal protein synthesis in the propagation of the neurodegenerative signal in response to axonal exposure to Aβ1-42 peptide. Here, they demonstrate that there is an early local translation response, which is important for generating the retrograde signal that results in the axonal recruitment of Aft4 mRNA. They further show that this is a Ca2+-dependent event, and requires the local translation of vimentin protein from Vimentin mRNA that exists in the axon prior to Aβ1-42 peptide exposure. This is a revision of a previous submission, and the authors have done a good job addressing questions and concerns of the reviewers. In particular, the additional details regarding quantification of fluorescent images, inclusion of better representations of these images, additional experiments regarding the components necessary for generating the retrograde signal, and new analysis of existing data based on suggestions from the reviewers has strengthened their manuscript and the validity of the conclusions that they draw. As such, this clearly-written and interesting paper is likely to be of broad interest to readers.

Referee #3:

The authors have addressed all my comments in a satisfactory way. The manuscript reports a very interesting and novel set of findings that will be of broad interest to readers.

2nd Revision - authors' response 20 April 2018

Referee #1:

The authors have adequately responded to reviewer comments, improving the quality of this strong manuscript.

Minor points are listed below.

Figure legend 2A does not state experimental model
We have added ‘of hippocampal neurons’ to the figure legend.

Page 5/82: "thus axons were exposed to 3 μM oligomeric Aβ1-42, which we previously found to be equivalent to approximately 250 nM [7]." This seems to refer to the effective concentration and influences of small volume and hydrophobicity in microfluidic chambers. "....250 nM in regular cultures" should be added
We have added ‘in regular cultures’.

Page 8/158: "Atf4 levels in cell bodies of Aβ1-42-treated axons were indistinguishable significantly *from controls (Fig EV3C),"*
-> perhaps "not significantly different"?
We have deleted ‘significantly’.

Page 10/195: "The FPKM value for Vim in our previously reported transcriptome of untreated hippocampal axons was lower than the Actb" -> lower than the level for Actb?
We have added ‘level for’.

Page 10/200: "treatment. After 15 minutes we detected a non-significant trend towards increased axonal vimentin levels (Fig EV4), while we observed a significant decrease in axonal vimentin levels compared to vehicle-treated axons (Fig 5B)." -> while after 30 minutes we observed?
We have added ‘after 30 minutes’.

In this reviewer's opinion, Fig. 5 would be easier to understand, if more information was added to label the figure. The difference between 5C and D (treatment and timing) is unclear without reading the legend.
We have added treatment and timing details for B, C, and D to the figure.

Referee #2 and #3 had no further questions or concerns.

Editor's comments:

The information on the statistics needs to be clarified. You mention technical replicates, biological replicates per group and independently performed experiments. "n" should be used for independently performed experiments only, but the total number of cells or axons can be mentioned too. My impression is that the biological replicates per group you mention are in fact technical replicates. Can you please explain what was done and use the same wording for all figure legends? IF less than 3 independent experiments are performed no error bars should be calculated, but all data points from the experiments should be shown. And no error bars should be calculated for technical replicates either. If data from a single experiment are shown, this needs to be explained in the figure legend. It seems that figures 4C and EV3 show error bars for technical replicates (but you also mention biological replicates), figures 5A and EV2A do not specify the bars, error bars and "n", figures EV1B and EV4 seem to be single experiments, and data in figure EV2B are from 2 experiments but do show error bars. I attach a commentary on replicates and repeats to this email for your information.
In the original manuscript we used 'biological replicates' to mean 'independently performed experiments'. We have now updated all figure legends, replacing 'biological replicates' with 'independently performed experiments'.
Figures 4C (now 5C), EV3C (now 4C) – We have changed the figures to represent the means ± SEM of six independent experiments each with triplicate qRT-PCR measurements and updated the figure legend accordingly.
Figure EV3B (now 4B) – We already had plotted the means of nine independent experiments each with duplicate measurements. We have updates the figure legend accordingly.
Figure 5A (now 6A)– We have updated the figure legend to include the missing information.
Figures EV1B & EV4 (now EV3) – These data were sampled from several cover slips that were independently processed per condition. All primary neurons originated from the same pregnant rat; we followed the editor's advice and present the data as a scatter plot without error bars.
Figures EV2A & B – We have removed the error bars in A and added the requested information to the figure legend. B is now presented as a scatter plot with means.

The manuscript currently has 5 main figures and should thus be formatted as a scientific report, with combined Results and Discussion sections. If you prefer to keep the 2 sections separate, please add one more main figure to the manuscript so that it becomes a full article.
We have changed Expanded View Figure 3 to a main text figure (Fig 4). The numbering of the following figures has been changed accordingly: EV3: Fig 4; EV4: EV3; EV5: EV4; Fig 4: 5; Fig 5: Fig 6.

We have added an accidentally omitted funding source in the Acknowledgements section: 'L.K.R. was supported by a training grant from the Eunice Kennedy Shriver National Institute of Child Health and Human Development (T32HD007430)'

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Ulrich Hengst
Journal Submitted to: Embo Reports
Manuscript Number: EMBOR-2017-45435

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	not applicable
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	not applicable
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	described in Materials and Methods section under subheading 'Image Acquisition and Analysis'
For animal studies, include a statement about randomization even if no randomization was used.	not applicable
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	described in Materials and Methods section under subheading 'Image Acquisition and Analysis'
4.b. For animal studies, include a statement about blinding even if no blinding was done	not applicable
5. For every figure, are statistical tests justified as appropriate?	yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Data are symmetric as assessed by scatter plots.
Is there an estimate of variation within each group of data?	Standard errors of the mean are included in each figure.
Is the variance similar between the groups that are being statistically compared?	Yes, see SEM (erros bars)

C- Reagents

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Information for all antibodies is provided in Materials and Methods
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	not applicable

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	not applicable
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	not applicable
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	not applicable

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	not applicable
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	not applicable
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	not applicable
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	not applicable

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	not applicable
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	not applicable
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	not applicable
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G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No, no dual use releant research