EMBO reports

Manuscript EMBOR-2013-37101

Physiological release of endogenous tau is stimulated by neuronal activity

Emma C Phillips, Dawn HW Lau, Wendy Noble, Diane P Hanger and Amy M Pooler

Corresponding author: Amy M Pooler, King's College London

Review timeline:

Submission date:	24 August 2012
Editorial Decision:	28 September 2012
Revision received:	20 December 2012
Editorial Decision:	12 January 2013
Resubmission date:	23 January 2013
Accepted:	28 January 2013

Editors: Alejandra Clark, Barbara Pauly

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 28 September 2012

Thank you for the submission of your research manuscript to EMBO reports. We have now received the full set of reports on it.

As you will see, although all referees find the study potentially interesting they raise concerns pertaining to the interpretation that tau is physiologically released from neurons. Specifically Referee 3 suggests that the described tau release may be a response to cellular stress evoked by the in vitro treatments in cell culture, rather than a physiological response. He/she feels that additional experiments, including the use of initiator caspase inhibitors and Abeta should be carried out to distinguish between these possibilities. Referee 1 has specific methodological concerns regarding the identity of the tau molecule that is released, including the size, phosphorylation state and localisation. In addition Referee 2 feels that several points should be further clarified, such as the proposed role for extracellular tau under physiological conditions.

Given the potential interest of the novel findings and considering that all referees provide constructive suggestions on how to move the study forward, I would like to give you the opportunity to revise the manuscript, with the understanding that the main referees concerns have to be addressed and that acceptance of the manuscript would entail a second round of review. I would like to point out that it is EMBO reports policy to allow a single round of revision and thus, acceptance or rejection of the manuscript will depend on the outcome of the next final round of peer-review.

Revised manuscripts should be submitted within three months of a request for revision; they will

otherwise be treated as new submissions. If you feel that this period is insufficient for a successful submission of your revised manuscript I can potentially extend this period slightly. Also, the length of the revised manuscript should not exceed roughly 30,000 characters (including spaces). Should you find the length constraints to be a problem, you may consider including some peripheral data in the form of Supplementary information. However, materials and methods essential for the repetition of the key experiments should be described in the main body of the text and may not be displayed as supplemental information only.

We have also started encouraging authors to submit the raw data for western blots (i.e. original scans) to our editorial office. These data will be published online as part of the supplementary information. This is voluntary at the moment, but if you agree that this would be useful for readers I would like to invite you to supply these files when submitting the revised version of your study.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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."

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

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1

In this work, Pooler et al have reported a novel and interesting observation about the release of intracellular tau to the extracellular medium. They have described a physiological secretion regulated by activation of AMPA receptors in primary neuron cultures.
The work is sound but I have some methodological questions.

Specific points:

- It is not clear the nature of the tested extracellular tau. In Material and Methods is indicated that exosomes were obtained but also in Methods it was indicated that to obtain extracellular tau the medium was centrifuged at low speed and the supernatant, after that centrifugation, was taken. In that supernatant soluble tau and vesicle-associated tau could be present. Thus, to distinguish both fractions, and to concentrate (without Amicon) vesicle associated tau, it could be advisable to do a fractionation by high speed centrifugation (that reported for exome preparation) and test whether extracellular tau is in the supernatant (soluble tau) or in the pelleted fraction (vesicle-associated tau). On the other hand, the pelleted fraction may include also the presence of beta actin that usually is associated to exosomes. These points should be clarified.
- The nature of the primary neuron cultures (hippocampal, cortex, ...) should be indicated in Methods.
- Figure 1D shows dendritic compartment (MAP2 immunostaining), however the manuscript is focused on tau protein (mainly an axonal protein). It would be interesting to show tau immunostaining in those primary neuron cultures.
- FIG. 1E seems to suggest that tau protein found in the media is unphosphorylated but also with a low molecular weight. Is there some truncation?
- Most of the figures are improperly cited in the text.

Referee #2

The manuscript on "Physiological release of endogenous tau is stimulated by neuronal activity" by Pooler et al., describes the mechanisms involved in intracellular release of tau. It has been proposed that this may has been the initial step in neuropathological deposition of the cytoskeletal protein tau spread progressively from the entorhinal cortex to anatomically connected brain regions. Authors have shown that neuronal stimulation by membrane depolarization in the presence of high potassium or by receptor activation e.g. S- AMPA induces intracellular calcium is responsible for the intracellular tau release from healthy, mature cortical neurons.

The findings are important, however, authors should provide the explanations of some the following comments.

1. Tau is an intracellular cytoskeletal protein why intracellular release of tau is a physiological process, if it is, what is its extracellular role under physiological conditions.
2. Why NMDA failed to induce tau release.
3. Data presented in figure 4 do not rule out the possibility that the other synaptic mechanisms are not involved in tau release in addition to pre-synaptic.
4. Non- phosphor tau is associated with MTs, phosphor-tau is released intracellularly, and therefore, there is a better possibility of phospho-tau compared to non- phosphor -tau to be released under the conditions described in the manuscript. Comment should be made on this statement.

Referee #3

Pooler et al show that tau can be released in primary neurons in the absence of measurable cell death and that neuronal activity can modulate that release. They show that the mechanism is AMPA dependent and relies on calcium and exocytosis of pre-synaptic vesicles. Furthermore, they characterize secreted tau as non-exosomal. The implication is that tau release may occur as part of a physiological pathway and that, in neurodegenerative conditions, that pathway may become aberrant and contribute to pathogenesis.

The experiments are well designed and the results are solid. However, I am not convinced that the best interpretation of the results is that the release of tau is physiological and regulatable by neuronal activity, as the authors put it. In my view, it remains formally possible that tau release may be a response to cellular stress linked to the in vitro treatments in culture, rather than being part of a bona fide physiological pathway. Although MTS and live/dead assays show no apparent cell death, neither one is informative regarding the activation of early stages of apoptosis. This is important because neuronal cultures are subject to low levels of stress and might be primed by a wide range of treatments to generate or potentiate stress responses without necessarily ending in apoptosis.

In short, whether neuronal tau release is physiological or part of a response to upstream cytotoxic events is a conceptually important question. Changes in tau homeostasis are well known to occur under stress conditions, and the changes in tau release patterns described here might simply be a representative example.

Additional experiments would need to be included to address that issue. For example, do initiator caspase inhibitors prevent tau release? Conversely, does exposure to a range of Abeta concentrations (from neurotrophic picomolar to toxic micromolar amounts) elicit changes in tau release patterns?

Response to referees' comments

Referee #1

This referee notes that our work presents a novel and interesting observation and raises the following points:

It is not clear the nature of the tested extracellular tau. In Material and Methods is indicated that exosomes were obtained but also in Methods it was indicated that to obtain extracellular tau the medium was centrifuged at low speed and the supernatant, after that centrifugation, was taken. In that supernatant soluble tau and vesicle-associated tau could be present. Thus, to distinguish both fractions, and to concentrate (without Amicon) vesicle associated tau, it could be advisable to do a fractionation by high speed centrifugation (that reported for exome preparation) and test whether extracellular tau is in the supernatant (soluble tau) or in the pelleted fraction (vesicle-associated tau). On the other hand, the pelleted fraction may include also the presence of beta actin that usually is associated to exosomes. These points should be clarified.
As this reviewer mentions, we have prepared exosomes from neurons to determine whether these structures make a significant contribution to extracellular tau. However, as we showed in Fig 4C, the amount of tau in exosomes comprises only a minor proportion of the total tau in neurons, indeed exosomal tau was at the limit of detection by this method. In comparison, we estimate that Amicon column concentration resulted in at least 10-fold more tau than we obtained from pelleted exosomes. A recent report using M1C neuroblastoma cells stably transfected with a four-repeat tau isoform has shown both non-exosomal and exosomal tau release, although the relative proportions of tau in each fraction were not stated (Saman et al, 2012). The exosomal tau in that study contained the N-terminus and microtubule-binding repeat region but, unike the extracellular tau detected in our study (see Fig. 1F), it appeared to be C-terminally truncated. Moreover, in contrast to non-exosomal tau, exosomal tau released from transfected cells is phosphorylated (Saman et al, 2012). Notably, neuron and neuroblastoma cells expressing GFP-tau have been reported not to release significant amounts of tau in exosomes (Faure et al, 2006; Santa-Maria et al., 2012). We conclude that extracellular tau released from neurons is, for the most part, soluble and not vesicle-associated. This point is clarified on page 8 of the manuscript.

The nature of the primary neuron cultures (hippocampal, cortex, ...) should be indicated in Methods.
We have now indicated in the Methods section that cortical neurons were used in this study (page 10, Cell Culture).

Figure 1D shows dendritic compartment (MAP2 immunostaining), however the manuscript is focused on tau protein (mainly an axonal protein). It would be interesting to show tau immunostaining in those primary neuron cultures.
We have immunostained neurons to visualize tau in the LIVE/DEAD assay. Representative images of this new experiment are now included, in place of MAP2, in Figure 1D. The legend to this figure has been amended to reflect this change, as has the text on pages 11 (Methods) and 13 (Figure Legends) of the manuscript.

FIG. 1E seems to suggest that tau protein found in the media is unphosphorylated but also with a low molecular weight. Is there some truncation?
We have examined truncation of extracellular tau by probing western blots of neuronal culture medium with antibodies directed against tau N- and C-termini (TP007 and TP70, respectively). We found that both of these antibodies label tau in culture medium and have therefore added western blots of concentrated extracellular tau labeled with TP007 and TP70 to Fig 1F. The lack of labeling of released tau by PHF-1, and detection of tau by Tau-1 (Fig 1F), indicates that released tau is not phosphorylated at these epitopes. Therefore,

we conclude that tau released by neurons contains intact N- and C-termini and that the most likely explanation for the tau species exhibiting faster electrophoretic migration is due to decreased phosphorylation relative to intracellular tau. This point is now included on page 6 of the revised manuscript.

Most of the figures are improperly cited in the text.
We apologise for this labeling error. All of the figures are now correctly cited in the text.

Referee #2

This referee notes the importance of our findings and raises a number of queries, which we have addressed below:

Tau is an intracellular cytoskeletal protein why intracellular release of tau is a physiological process, if it is, what is its extracellular role under physiological conditions.
Our data suggest that tau release from neurons may have a physiological role. The fact that tau release is modulated by receptor signaling suggests that this likely involves interneuronal signaling. In support of this view, a previous report has shown that extracellular tau binds to and activates muscarinic acetylcholine receptors (Gomez-Ramos et al., 2008). Taken together, this indicates a potential signaling role for extracellular tau, this point is included on page 9 of the revised manuscript.

Why NMDA failed to induce tau release.
Treatment of neurons with NMDA did not stimulate neuronal release of tau, despite previous demonstration that similar concentrations of NMDA elicit calcium influx into neurons via NMDA receptors (Zhang et al., 2012). There is recent evidence for differential signalling between AMPA and NMDA receptors with respect to downstream events. For example, internalization of AMPA receptors follows two distinct pathways, depending upon whether AMPA or NMDA receptors are stimulated (Schwarz et al., 2010). We conclude that neuronal tau release is mediated by an AMPA receptor-, and not NMDA receptor-dependent pathway. In order to clarify this point, we have amended the Results section (page 7) to include the following: “In contrast, NMDA receptor stimulation (NMDA; 100 μM) for 30 min did not induce tau release (Fig 2C), although a similar concentration of NMDA was shown to activate NMDA receptors in cultured neurons.”

Data presented in figure 4 do not rule out the possibility that the other synaptic mechanisms are not involved in tau release in addition to pre-synaptic.
We agree with the reviewer that tau may be released by additional mechanisms; however, the partial blockade of AMPA-stimulated tau release by tetanus toxin indicates that at least one of the mechanisms by which neurons secrete tau involves pre-synaptic activity. This point is clarified on page 8 of the manuscript.

Non- phosphor tau is associated with MTs, phosphor-tau is released intracellularly, and therefore, there is a better possibility of phospho-tau compared to non-phosphor -tau to be released under the conditions described in the manuscript. Comment should be made on this statement.
In a previous study, we demonstrated that ~10% of neuronal tau is associated with the plasma membrane and this tau is largely dephosphorylated (Pooler et al., 2012). We hypothesize, therefore, that this pool of dephosphorylated, non-microtubule-associated tau may be released by neurons. A comment to this effect is included on page 6 of the manuscript.

Referee #3

This referee comments that our “experiments are well designed and the results are solid”. Below we address the query from this referee relating to the interpretation of our results.

In short, whether neuronal tau release is physiological or part of a response to upstream cytotoxic events is a conceptually important question. Changes in tau

homeostasis are well known to occur under stress conditions, and the changes in tau release patterns described here might simply be a representative example. Additional experiments would need to be included to address that issue. For example, do initiator caspase inhibitors prevent tau release? Conversely, does exposure to a range of Abeta concentrations (from neurotrophic picomolar to toxic micromolar amounts) elicit changes in tau release patterns?
The referee infers here that the observed changes in the amount of tau released from neurons result from stress-inducing, potentially cytotoxic, treatments. We agree with the reviewer that it is important to determine whether activation of initiator caspases might be involved in tau release from neurons. We have now performed additional experiments to determine the effects of (*S*)-AMPA on the activation of initiator caspase-8. We found that the amount of active caspase-8 (p18 fragment) in neurons was unchanged following treatment with (*S*)-AMPA (100μM, 4h). These results indicate that caspase-8 activity is not altered by (*S*)-AMPA, and that stimulation of tau release by this treatment is not due to activation of initiator caspases. We have included this data as an additional panel (E) in Figure 1.

Experiments measuring release of tau in response to treatment with a range of Abeta concentrations require careful optimization, including controls to monitor cell death, since the latter will also result in release of extracellular tau. Furthermore, the results obtained are unlikely to add to our original finding that tau release is a normal physiological process in neurons. Undertaking the required experiments is not trivial and, for the reasons above and also in the interests of the timely dissemination of our novel data, we do not consider these experiments to be within the scope of this study.

References

Davis et al. (1995) Biochem J. 309:941-9.
Faure J, et al. (2006) Mol Cell Neurosci. 31:642-648.
Gomez-Ramos et al (2008) Mol Cell Neurosci. 37:673-81.
Maas et al. (2000) J Biol Chem. 275:15733-40
Pooler et al. (2012) Neurobiol Aging. 33:431.e27-38.
Saman et al (2012) J Biol Chem. 287:3842-9.
Santa-Maria et al. (2012) J Biol Chem. 287:20522-33.
Schwarz LA et al. (2010) J Neurosci. 30:16718-29.
Wasilewska-Sampaio et al. (2005) FASEB J. 19:1627-36.
Zhang et al. (2012) Neurosci Bull. 28:550-60.

2nd Editorial Decision 12 January 2013

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the referee reports, which I attach below.

As you will see, although referee #2 is now supportive of publication, referee #3's original concerns regarding the physiological release of tau by neurons remain after assessing the revised manuscript. Since this is a critical finding in the study, we seeked further advice from an external expert in the field who is also very familiar with the scope of our journal and who evaluated the manuscript and the comments of the referees. Our external advisor agreed with referee #3 that further experimental evidence, such as that of Abeta treatment of neurons, is required to conclusively demonstrate a physiological release of tau protein. In addition, he/she suggested that experiments to test whether neuronal activity in vivo affects tau release should also be tackled in order to make the study fully conclusive.

I appreciate your efforts to address the referee concerns, but in light of the nature of this significant criticism from referee #3 and the external advisor, I have no choice but to decide that we cannot

consider your manuscript for publication at this stage. Given the interest in the topic, however, if you feel that you can provide compelling evidence on the physiological release of tau that would fully address the comments of referee #3, we would be glad to consider a new submission of your work. I would like to clarify that the last experiment suggested by the advisor, -assaying the in vivo effects of neuronal activity on tau release- would not be required for a new submission. I would also like to add, that this would be treated as a new submission rather than a revision and would be editorially afresh, especially with respect to novelty at the time of resubmission

I am very sorry not to bring better news this time. I hope that the referee comments are helpful in your continued work in this area and thank you for considering EMBO reports for publication.

REFEREE REPORTS:

Referee #2

Authors have adequately answered the comments raised by this reviewer

---

Referee #3

My initial concern about the manuscript's conclusions remains after this revised version. As I mentioned in my original comments, whether neuronal tau release is physiological, which is the key claim of the manuscript, or part of a response to upstream cytotoxic events, is a conceptually important question, one that the authors need to answer reasonably well, and I do not think that they have succeeded.

Unless proven otherwise, the most parsimonious explanation for the manuscript results is that tau release is occurring as a consequence of the stress that is inherent to the in vitro conditions used in the study. While it does not appear that such stress initiates apoptosis (new panel in figure 1), it remains possible that it may trigger tau release, and that amyloid, the best-known upstream modulator of tau homeostasis, may influence such response further.

In their reply to my comments, the authors state that testing the impact of Abeta exposure "will not add to the original finding that tau release is a normal physiological process in neurons". This statement is incorrect, as it assumes that their hypothesis (i.e. tau release is a novel physiological process) is already proven. To reiterate, whether tau release is physiological or not is precisely the key question that the authors are trying to answer, and in order to do that, they need to discard Abeta as the most likely driver of tau release (which is a very reasonable assumption, given what's currently known about amyloid and tau biology).

2nd Revision - authors' response 23 January 2013

We are submitting our manuscript, entitled "**Physiological release of endogenous tau is stimulated by neuronal activity**", for consideration for publication in EMBO reports. Following our previous submission of this manuscript (EMBOR-2012-36558V2), we were encouraged by Editor Dr. Alejandra Clark to resubmit a revised version in light of the high interest and novelty surrounding our findings, noted by the reviewers. In this revised version, we have added new data in relation to the release of tau from neurons in response to beta-amyloid peptide (Suppl Fig 1). This additional work is included in response to the remaining point from reviewer 3 that we should "discard Abeta as the most likely driver of tau release". We believe that we have now addressed this issue and therefore we would appreciate our work being re-considered for publication in EMBO reports.

The salient points of this manuscript are our novel findings that:

- Endogenous tau is released from healthy neurons in a **physiological process** that is stimulated by neuronal activity, including AMPA receptor stimulation.
- Neuronal release of tau can be **regulated** by modulating calcium and neuronal activity.
- Regulated tau release from neurons occurs **without cell death**.
- Tau release is dependent, at least in part, on a **pre-synaptic** mechanism.
- Endogenous tau released from neurons is **not highly phosphorylated**.
- This previously **undescribed process** positions tau release at the forefront of the search for new targets for treating Alzheimer's disease and related disorders in which tau pathology plays a prominent role.

Spread of tau pathology throughout the brain in Alzheimer's disease follows a specific anatomical pathway. However, the mechanism underlying this spread of tau is not understood. Our finding that tau release from neurons is regulated by a physiological process provides a novel model as to how tau pathology progresses in Alzheimer's disease. Therefore, our work is expected to have a very high impact in the field of Alzheimer's research.

3rd Editorial Decision 28 January 2013

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: 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."

Finally, we provide a short summary of published papers on our website to emphasize the major findings in the paper and their implications/applications for the non-specialist reader. To help us prepare this short, non-specialist text, we would be grateful if you could provide a simple 1-2 sentence summary of your article in reply to this email.

Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

REFEREE REPORT:

Referee #3:
The new data added to the manuscript strengthens it considerably. This is now a very solid body of work. I'm satisfied that the authors have ruled out reasonably well the possibility of tau release occurring as a result of 1) cell culture artifacts and 2) the pathogenic process that links amyloid to tau.

