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Hypothermia increases aquaporin 4 (AQP4) plasma membrane abundance in human primary cortical astrocytes via a calcium/ transient receptor potential vanilloid 4 (TRPV4)- and calmodulin-mediated mechanism

Mootaz M. Salman, Philip Kitchen, M. Nicola Woodroofe, James E. Brown, Roslyn M. Bill, Alex C. Conner and Matthew T. Conner

Review timeline:

Submission date:	15 May 2017
Editorial Decision:	05 June 2017
Revision received:	26 August 2017
Editorial Decision:	12 September 2017
Revision received:	14 September 2017
Accepted:	15 September 2017

Editor: Masahiko Watanabe

1st Editorial Decision 05 June 2017

Dear Dr. Conner,

Your manuscript was reviewed by external reviewers as well as by the Section Editor, Prof. Masahiko Watanabe, and ourselves.

The reviewers collectively indicated that your experiments generated new and important information. However, there are several important issues that need to be resolved before we can further consider your manuscript for publication. Specifically, Reviewer 1 considers that the manuscript should be reorganized to focus on the significance and novelty of your findings. Reviewer 2 points out that AQP4 surface localization experiments need to be corroborated with other methods including immunoprecipitation and immunoblotting. Accordingly, the revisions would need to resolve these important issues and other minor points.

We also noted the following points.
- qPCR: PPIA and CDKN1B primer sequences (or manufacturer's catalog numbers) needed
- Methods might be easier to read if broken down into sub-sections
- Several single-sentence paragraphs in the discussion.
- Author contributions should be a bit more detailed
- Please supply a data statement
- Bar charts should be replaced with much more informative scatter plots or similar (see Author Guidelines).
- It would probably be better to reduce the number of abbreviations in the title and abstract.

If you are able to respond fully to the points raised, we would be pleased to receive a revision of your paper within 12 weeks.

Thank you for submitting your work to EJN.

Kind regards,

Paul Bolam & John Foxe
co-Editors in Chief, EJN

Reviews:

Reviewer: 1 (Masanori Tachikawa, Tohoku University, Japan)

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Comments to the Author
Mootaz et al. demonstrate the increased plasma membrane translocation of aquaporin 4 (AQP4) in primarily cultured human astrocytes under hypothermic conditions. The data and the technical quality of the work appear convincing. This work fits the scopes of EJN. This reviewer raises the following questions and concerns throughout the manuscript.

1. Page 6 line 15: Figure 1A did not show the changes in AQP4 protein expression. Please correct.
2. Page 7 line 1-2: The cell surface membrane protein localization of EAAT1 tends to be decreased under hypothermic conditions. The description of "the hypothermia-induced relocalization is specific to AQP4" appears to be over interpretation by referring only the case of EAAT1.
3. Page 7 line 6: Please refer here the authors' previous report.
The authors describe that "We previously identified a novel regulatory mechanism for AQP4 surface localization in an in vitro model of astrocytic cell swelling." In contrast, in the Introduction section, they describe that "The specific effect of hypothermia on AQP4 in human astrocytes are not known". This leaves the impression that the present concept has already been reported elsewhere. The authors should specify the novelty of the present study with careful attentions.
4. Page 7 line 13: Please explain more clearly about "initial" distribution of AQP4.
5. Page 8 line 1-3: Please add the appropriate references.
6. Page 8 line 15-16: "some of the deleterious effects of hypothermic intervention in oedema that have been reported" Please add the appropriate reference(s).
7. This reviewer wonders whether the astrocytic swelling could be inhibited in the presence of calmodulin and/or TRPV4 inhibitors as well as the inhibitor of plasma membrane protein translocation. These lines of evidence would support the authors' belief that the enhancement of the APQ4 plasma membrane localization play pivotal role in the astrocytic swelling in stoke and traumatic injury.

Reviewer: 2 (Koji Shibasaki, Gunma University Graduate School of Medicine, Japan)

Comments to the Author
In this manuscript, the authors sought to determine the effects of TRPV4 and calmodulin on surface targeting of AQP4 in hypothermia. The results are potentially important and of general interest, but some data and its discussion/conclusion are not clear. Specific comments are indicated below.

Major concerns;

They only used recording of 450 nm absorbance by microplate reader for the quantification of surface biotinylation. However, they cannot exclude the possibility that their anti-AQP antibody (abcam) detected background biotin, which binds to other proteins in addition to AQP4. Therefore, they MUST show the immunoprecipitation and immunobliotting-based biotinylation results (in both Fig 2 and 3). Otherwise, we cannot evaluate their results.

Only two results (TRPV4 inhibitor and calmodulin inhibitor) suggest the involvement of TRPV4 and calmodulin for surface targeting of AQP4 in hypothermia. It is not enough to prove the involvement of two molecules. They MUST perform Ca2+-free, EGTA-AM and TRPV4 agonist experiments.

In discussion, they described that cell swelling might activate TRPV4 in hypothermia, however, TRPV4 is activated by warmth stimuli (>34°C). Their hypothesis have huge gap, since hypothermia should inactivate TRPV4. They should read and cite the following paper (Shibasaki et al. Pflügers Archiv. 467(12):2495-2507, 2015). If they would like to state the cell swelling in hypothermia, they MUST show the results how much the astrocytes were swelled. They MUST also add hypotonic stimulation to induce the cell swelling and AQP4 surface targeting. They should cite the following paper, too (Sci Rep. 2017 May 23;7(1):2295), since this report found the hypothermia/TRPV4 structural change affects the intracellular signaling.

Their discussion is not clear. Without their own cell swelling data, they described about TBI and stroke a lot. They should re-organize discussion.

Minor comments;

In Abstract (p2, line 7), "4h mild hypothermic" should be changed as "Four h mild hypothermic".

In Discussion (p8, line 4), "surface localiastion" should be changed as "surface localization".

Authors' Response 26 August 2017

We would like to thank the reviewers for their careful reading of our short communication manuscript and their detailed comments and suggestions, which have informed the changes in the manuscript. Our

responses to each of the reviewer's comments are included below (reviewers' comments in italics, our responses in regular font). We have also included a version of the manuscript with the resulting major changes highlighted in red.

The editors' comments and suggestions:

The reviewers collectively indicated that your experiments generated new and important information. However, there are several important issues that need to be resolved before we can further consider your manuscript for publication. Specifically, Reviewer 1 considers that the manuscript should be reorganized to focus on the significance and novelty of your findings. Reviewer 2 points out that AQP4 surface localization experiments need to be corroborated with other methods including immunoprecipitation and immunoblotting.

We thank the referees for their helpful and supportive comments. We can confirm that the manuscript has been reorganized to reflect the novelty of this work which is submitted as a short communication. With respect to the comment by Reviewer 2, we apologize for any confusion over how we presented the experimental work for the cell-surface biotinylation ELISA assay. This has now been re-written in the revised manuscript (page 5, lines 120-127). However, we are not entirely clear what the reviewer means by "anti-AQP antibody (abcam) detected background biotin". Since we have now clarified the method, we trust that this will no longer be of concern. Briefly we used a 96-well Pierce™ NeutrAvidin™ coated plate (Thermo Scientific; Cat. No. 15129), which binds all biotinylated proteins in the astrocyte cell lysate in the first incubation step. After washing off unbound proteins (i.e. non biotinylated proteins), the plate is then incubated with the anti-AQP4 antibody, which only binds biotinylated AQP4 protein bound to the avidin coated plate; cytosolic AQP4 proteins will not be biotinylated and hence are washed off the plate. If the question from the reviewer is about the specificity of the antibody, we have previously shown (Kitchen et al, 2015, JBC), using western blotting, that a single band is present at the correct molecular mass for AQP4 in whole cell lysates. Additionally, we have appended an example western blot to this letter, along with validation data showing the linearity of the OD_{450} signal on the ELISA with increasing levels of AQP4 overexpression.
The supplementary data for the anti-AQP4 antibody validation have been uploaded to Figshare. A clear data-sharing statement has been added to the revised manuscript, DOI 10.6084/m9.figshare.5293672 (page 10, line 261-263).
As this is a short communication, we are limited by the number of figures we can include. Since we have previously published a paper using this antibody, we are confident in its specificity.

Other minor points:
- qPCR: PPIA and CDKN1B primer sequences (or manufacturer's catalog numbers) needed
Details are provided (Page 4, Line 107-110).

- Methods might be easier to read if broken down into sub-sections
This a good point. We have updated Materials and Methods according to the editors' suggestions (page 4-6, line 99-146).

- Several single-sentence paragraphs in the discussion.
The discussion has been entirely re-written to avoid the short sentences, focusing on the novelty of our work and addressing reviewers' comments (page 8-9, line 208-249).

- Author contributions should be a bit more detailed
We have provided a more detailed Author contributions (page 10, line 255-260).

- Please supply a data statement
Datasets have been uploaded to Figshare. A clear data sharing statement has been added to the revised manuscript. DOI 10.6084/m9.figshare.5293672 (page 10, line 261-263).

- Bar charts should be replaced with much more informative scatter plots or similar (see Author Guidelines).
We recognise that our description was not sufficient to make clear the graphical representation of the data. Figure legends have been updated to better reflect the content of revised graphs (page 12-14, line 345-401).

- It would probably be better to reduce the number of abbreviations in the title and abstract.
We thank the editors for the valid suggestion. The paper has been re-titled according to the provided suggestion to "**Hypothermia increases aquaporin 4 (AQP4) plasma membrane abundance in human primary cortical astrocytes via a calcium/ transient receptor potential vanilloid 4 (TRPV4)- and calmodulin-mediated mechanism"** which best reflects the updated content and the intended message of this manuscript (page 1, line 1-3).

Reviewer 1

Comments to the Author
Mootaz et al. demonstrate the increased plasma membrane translocation of aquaporin 4 (AQP4) in primarily cultured human astrocytes under hypothermic conditions. The data and the technical quality of the work appear convincing. This work fits the scopes of EJN. This reviewer raises the following questions and concerns throughout the manuscript.

1. Page 6 line 15: Figure 1A did not show the changes in AQP4 protein expression. Please correct.

We thank the reviewer for pointing out this error. We have rewritten this paragraph so that the panels of figure 1 are now correctly referred to in the text (page 6, line 148-155).

2. Page 7 line 1-2: The cell surface membrane protein localization of EAAT1 tends to be decreased under hypothermic conditions. The description of "the hypothermia-induced relocalization is specific to AQP4" appears to be over interpretation by referring only the case of EAAT1.

We agree and our point here was that there is not a global increase in the membrane localization of all membrane proteins by e.g. mass vesicle evacuation. We have reworded this section to now read "the modest decrease in surface localization of the membrane protein, excitatory amino acid transporter, EAAT1, indicates that the hypothermia-induced increase in AQP4 plasma membrane localization is not a global cellular response, associated with all membrane proteins." We hope this clarifies the point (page 6, line 163-166).

3. Page 7 line 6: Please refer here the authors' previous report.

We have added this reference (page 3, line 81).

The authors describe that "We previously identified a novel regulatory mechanism for AQP4 surface localization in an in vitro model of astrocytic cell swelling." In contrast, in the Introduction section, they describe that "The specific effect of hypothermia on AQP4 in human astrocytes are not known". This leaves the impression that the present concept has already been reported elsewhere. The authors should specify the novelty of the present study with careful attentions.

We apologize for any confusion in how we presented the data. In the present manuscript, we show, for the first time, relocalization in response to **hypothermia** (hypothermia has only been shown by others to induce astrocyte swelling) in **human** astrocytes. We have previously shown that AQP4 relocalizes to the plasma membrane of **rat** astrocytes in response to cell swelling induced by extracellular **hypotonicity**. This is the "*in vitro* model of astrocytic cell swelling" that we refer to.

We agree and have clarified by rewriting section as follows: "We previously identified a novel regulatory mechanism for AQP4 surface localization in an *in vitro* model of rat astrocyte cell swelling in response to a reduction in extracellular osmolality" (page 7, line 174-176)

4. Page 7 line 13: Please explain more clearly about "initial" distribution of AQP4.

'Initial' here refers to the basal level of surface-localized AQP4 before swelling-induced relocalization. We have clarified in the text: "initial (*i.e.* pre- swelling-induced relocalization) distribution of AQP4 in these cells" (page 7, line 181-184).

5. Page 8 line 1-3: Please add the appropriate references.

We have added references for calcium activation of calmodulin and AQP4 mediation of brain oedema.

Vella, J., Zammit, C., Di Giovanni, G., Muscat, R. and Valentino, M., 2015. The central role of aquaporins in the pathophysiology of ischemic stroke. *Frontiers in cellular neuroscience*, *9*.

6. Page 8 line 15-16: "some of the deleterious effects of hypothermic intervention in oedema that have been reported" Please add the appropriate reference(s).

We have added the requested reference.
Lazaridis, C. and Robertson, C.S., 2016. Hypothermia for Increased Intracranial Pressure: Is It Dead?. *Current neurology and neuroscience reports*, *16*(9), p.78.

7. This reviewer wonders whether the astrocytic swelling could be inhibited in the presence of calmodulin and/or TRPV4 inhibitors as well as the inhibitor of plasma membrane protein translocation. These lines of evidence would support the authors' belief that the enhancement of the APQ4 plasma membrane localization play pivotal role in the astrocytic swelling in stoke and traumatic injury.

We agree with the reviewer; this study focuses on the novel hypothermic induction of AQP4 relocalisation and refers to previous work from Plesnila *et al.* (2000) that showed hypothermia-induced astrocytic swelling in rat astrocytes. We have previously shown that hypotonic stimulation induces cell swelling and increases AQP4-GFP surface targeting, while inhibition of this swelling occurs with the calmodulin inhibitors, trifluoperazine and W7 (Conner *et al.* 2012). We have included the following statement to clarify these points: line 243, "Further work showing inhibition of human astrocytic swelling in the presence of calmodulin and/or TRPV4 inhibitors would further support the role of AQP4 in astrocytic swelling in stoke and traumatic injury." We have also added new data showing hypotonicity-induced relocalization of AQP4 in these cells (see updated figure 3 and new figure 4; page 14).

Reviewer 2

Comments to the Author
In this manuscript, the authors sought to determine the effects of TRPV4 and calmodulin on surface targeting of AQP4 in hypothermia. The results are potentially important and of general interest, but some data and its discussion/conclusion are not clear. Specific comments are indicated below.

Major concerns;

They only used recording of 450 nm absorbance by microplate reader for the quantification of surface biotinylation. However, they cannot exclude the possibility that their anti-AQP antibody (abcam) detected background biotin, which binds to other proteins in addition to AQP4. Therefore, they MUST show the immunoprecipitation and immunobliotting-based biotinylation results (in both Fig 2 and 3). Otherwise, we cannot evaluate their results.

This has been addressed in the initial response to the editors above.

Only two results (TRPV4 inhibitor and calmodulin inhibitor) suggest the involvement of TRPV4 and calmodulin for surface targeting of AQP4 in hypothermia. It is not enough to prove the involvement of two molecules. They MUST perform Ca2+-free, EGTA-AM and TRPV4 agonist experiments.

These are all excellent suggestions. We have added EGTA-AM and TRPV4 agonist data (see updated figure 3 and new figure 4; page 14). This new data supports the involvement of calcium in the targeting of AQP4 to the plasma membrane and the TRPV4 agonist recapitulates the effects of hypothermia in regulating AQP4 surface expression.

We did assess the effect of chelating extracellular Ca^{2+} with EGTA,however exposure of the cells to media without extracellular calcium was very toxic to the primary astrocytes, reducing the proportion of viable cells (measured using CellTiter Glo) from 92% to 62%. Therefore, we have not included the Ca^{2+}-free data in the manuscript.

In discussion, they described that cell swelling might activate TRPV4 in hypothermia, however, TRPV4 is activated by warmth stimuli (>34°C). Their hypothesis have huge gap, since hypothermia should inactivate

TRPV4. They should read and cite the following paper (Shibasaki et al. Pflügers Archiv. 467(12):2495-2507, 2015).

We agree that cold inactivates TRPV4. It is also well-established that cell swelling activates TRPV4. In cells in which cold induces swelling, it is not clear which of the responses to either temperature or to swelling will over-ride the other. This is what we speculate on in the manuscript. We have added the following paragraph to the discussion section in order to expand on this:

"Interestingly, it has been shown that TRPV4 activity is inhibited by cold (temperature < 34°C) in mouse neurons. It is also well-established that TRPV4 opens in response to cell swelling, but the calcium signalling responses are different in neurons (fast, inactivating currents) and glia (slow currents, with sustained $[Ca]^{2+}_i$ elevation). In astrocytes, where hypothermia induces cell swelling, it is not obvious whether TRPV4 will be activated by the swelling, inactivated by the cold, or have intermediate activity, depending on the temperature and cell volume. Our data suggest that TRPV4 retains at least some activity in human cortical astrocytes at 32°C, as the AQP4 relocalization that we observe in response to hypothermia is blocked by a TRPV4 antagonist and recapitulated by a TRPV4 agonist." (page 9, line 223-236)

If they would like to state the cell swelling in hypothermia, they MUST show the results how much the astrocytes were swelled. They MUST also add hypotonic stimulation to induce the cell swelling and AQP4 surface targeting.

We have addressed part of this concern in the response to point 7 from reviewer 1. Additionally, we have also added new data showing hypotonicity-induced relocalization of AQP4 in these cells (see updated figure 3 and new figure 4; page 14).

They should cite the following paper, too (Sci Rep. 2017 May 23;7(1):2295), since this report found the hypothermia/TRPV4 structural change affects the intracellular signaling.

We are grateful for the valid suggestion. We have cited the indicated paper and added the following paragraph to the discussion "The proposal that hypothermia influences important cellular signalling mechanisms is supported by recent work, which shows that hypothermia/TRPV4-mediated effects on intracellular signaling (Zou et al., 2015)" (page 8, line 216-218).

Their discussion is not clear. Without their own cell swelling data, they described about TBI and stroke a lot. They should re-organize discussion.
The discussion has been entirely re-written to avoid the short sentences, focusing on the novelty of our work and addressing reviewers' comments in relation to TBI and stroke (page 8-9, line 208-249).

Minor comments;

In Abstract (p2, line 7), "4h mild hypothermic" should be changed as "Four h mild hypothermic".

We have changed "4 h" to "Four-hour" (page 2, line 36)

In Discussion (p8, line 4), "surface localisation" should be changed as "surface localization".

We have corrected this mistake (page 9, line 215).

We would like to thank the reviewers again for agreeing to review our manuscript and hope that the changes we have made as a result of their comments will make this manuscript suitable for publication in EJN.

2nd Editorial Decision 12 September 2017

Dear Dr. Conner,

Your re-submitted manuscript has been reviewed by external reviewers as well as by the Section Editor, Prof. Masahiko Watanabe and ourselves. We are pleased to inform you that we expect that your manuscript will be accepted for publication in EJN after you have addressed a few minor issues. Please add the title page describing the author information and refer to supplementary figure in the

text. Note also that it is policy of EJN not to publish simple bar charts; one simple solution is to overlay the individual data points on the bar charts.

If you are able to respond fully to the points raised, we shall be pleased to receive a revision of your paper within 30 days.

Thank you for submitting your work to EJN.

Kind regards,

Paul Bolam & John Foxe
co-Editors in Chief, EJN

Reviews:

Reviewer: 1 (Masanori Tachikawa, Tohoku University, Japan)

Comments to the Author
This reviewer's questions and concerns have been adequately addressed by adding the new experimental results.

Reviewer: 2 (Koji Shibasaki, Gunma University Graduate School of Medicine, Japan)

Comments to the Author
The resubmitted manuscript by Mootaz et al. is now much improved. Additional results would convince readers of the reliability of this study. However, there are several careless mistakes in the manuscript need to be corrected.

Minor comments;

There is spell-miss. In page 5 (line 114) “normalized” should be normalized. In Supplementary Fig. 1 legend, AQP4 DNA should be changed as AQP4 cDNA.

Authors’ Response 14 September 2017

Dear Professors Foxe and Bolam,

We hereby resubmit the final version of the manuscript EJN-2017-05-24576.R1 “Hypothermia increases aquaporin 4 (AQP4) plasma membrane abundance in human primary cortical astrocytes via a calcium/ transient receptor potential vanilloid 4 (TRPV4)- and calmodulin-mediated mechanism”) by Salman et al. for publication by the European Journal of Neuroscience.
We would like to thank the reviewers and editors for their acceptance of our manuscript for publication subject to the minor changes highlighted in red on the manuscript and detailed below. (Reviewer’s and editor’s comments in italics, our responses in regular font).

Editors:
Please add the title page describing the author information and refer to supplementary figure in the text. Note also that it is policy of EJN not to publish simple bar charts; one simple solution is to overlay the individual data points on the bar charts.

We have added the title page describing the author information and have referred to supplementary figure in the text.

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Following an email discussion with Professor Foxe it was agreed that in addition to the bar charts the same data would be presented as scatter plots (box-and-whisker plots) in a supplementary figure which is referred to in the main text body.

Reviewer: 1

Comments to the Author

This reviewer's questions and concerns have been adequately addressed by adding the new experimental results.

No action required

Reviewer: 2

Comments to the Author

The resubmitted manuscript by Mootaz et al. is now much improved. Additional results would convince readers of the reliability of this study. However, there are several careless mistakes in the manuscript need to be corrected. Minor comments; There is spell-miss [sic]. In page 5 (line 114) "normalized" should be normalized. In Supplementary Fig. 1 legend, AQP4 DNA should be changed as AQP4 cDNA.

This minor spelling error has been corrected and has been made consistent throughout. Supplementary Fig. 1 legend, AQP4 DNA has been changed to AQP4 cDNA