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Uncoupling endosomal CLC chloride/proton exchange causes severe neurodegeneration

Stefanie Weinert, Niclas Gimber, Dorothea Deuschel, Till Stuhlmann, Dmytro Puchkov, Zohreh Farsi, Carmen F. Ludwig, Gaia Novarino, Karen I. López-Cayuqueo, Rosa Planells-Cases, Thomas J. Jentsch

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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 10th Oct 2019

Thank you for the submission of your manuscript (EMBOJ-2019-103358) to The EMBO Journal. Your manuscript has been sent to three reviewers, and we have in the meantime received reports from all of them, which I enclose below.

As you will see, the referees acknowledge the potential interest and novelty of your results, although they also express a number of issues that will have to be addressed before they can support publication of your manuscript in The EMBO Journal. In more detail referee #2 is concerned that definitive proof or requirement of CLC3's exchanger activity is not given at this point, and complementary experimentation is required in his/her view to consolidate this point. Referee#3 asks you to corroborate CLC3 localisation and function in vesicle fractions and points to data discrepancies in the CLC3 versus CLC4 loss-of-function phenotypes, which need clarification. In addition, the reviewers raise a number of points related to data presentation, terminology and appropriate discussion of the results that would need to be conclusively addressed to achieve the level of robustness needed for The EMBO Journal.

I judge the comments of the referees to be generally reasonable and given their overall interest, we are happy to invite you to revise your manuscript experimentally to address the referees' comments.

REFEREE REPORTS:

Referee #1:

- general summary and opinion about the principle significance of the study, its questions and findings

The study departs from the still surprising discovery that intracellular CLC proteins function as secondary active transporters rather than ion channels. It tackles the fundamental and poorly understood question whether and how the transport mode as an exchanger is required for the physiological function of vesicular CLCs. The case of the specific family member under investigation here, ClC-3, is particularly intriguing because of the many physiological functions that were assigned to this family member and the severe neurodegenerative phenotype associated with its knockout in mouse.

Following an elegant strategy that the Jentsch lab has used in transgenic mouse models to test and demonstrate the key importance of the chloride/proton exchange activity of ClC-5 and ClC-7, the manuscript first describes an unexpected result, i.e. that in the case of ClC-3 uncoupling the exchanger and converting it to a channel does not recapitulate the ko phenotype.
Based on this puzzling finding the authors address the formation of heterodimers between ClC-3 and ClC-4 as a possible mechanism behind this observation. And, indeed, this line of thought and experimentation leads to the discovery that ClC-4 requires ClC-3 for efficient exit from the endoplasmic reticulum (ER). One major value of this study is that it firmly shows one of the very few examples of obligatory heteromer formation between channel subunits in native tissues. In the presence of the uncoupled variant of ClC-3, ClC-4 still provides enough chloride/protein exchange activity in the form of heteromers. This is convincingly tested by knockout of the gene encoding ClC-4 from the transgene expressing uncoupled ClC-3. The experiment unequivocally demonstrates the suppression of the ClC-3 uncoupled phenotype by ClC-4. This is another major step in clarifying the physiological relevance of the secondary active transport mode of vesicular CLCs. At the same time, it identifies a new and interesting case of assembly-dependent trafficking.

- specific major concerns essential to be addressed to support the conclusions
There are no major concerns. All conclusions are based on extremely carefully conducted, complementary experiments. All the usual pitfalls that jeopardize so many studies at the intersection of physiology and cell biology are systematically avoided (such as over-expression and the use of poorly characterized antibodies). This study is rich in novel insights but it also provides guidance in a field riddled with unreliable evidence or over-interpretation. The quality and breadth of the methodology can only be called impressive.

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For Fig. S3d I would remove the brackets from the gel and mark the Endo H-sensitive form and the de-glycosylated form outside rather than on the blot

In the discussion I would not refer to ClC-3 as a "chaperone" for ClC-4. You could either look into the article by Herrmann, Malkus and Schekman (1999, Trends in Cell Biology) and refer to the concepts of "escorts" or "guides" or talk about assembly-dependent ER exit of ClC-4, which I find most appropriate since both proteins are functional transporters.

You might consider not to highlight senior authors or groups/laboratories when discussing the literature. Each first author has taken responsibility for their articles by being the first author.

Referee #2:

The proton pump activity of V-type ATPase in endosomes, lysosomes and synaptic vesicles (SVs) needs a neutralizing counter current provided by ClCs serving as 2Cl-/H+ antiporters or Cl- channels. Since disruption of endosomal ClC-3 induces severe neurodegeneration, in the present study, the authors elucidated the roles of 2Cl-/H+ antiport activity of ClC-3 by making good use of Clcn3unc/unc mice in which the E224A mutation was introduced into ClC-3 and is thereby expected to be converted its activity from a 2Cl-/H+ antiporter to a Cl- channel. They intended to address following two questions: whether the roles of ClC-3 in brain endosomes and lysosomes depend on its 2Cl-/H+ exchange transport or its Cl- channel conductance, and whether ClC-3 is also important in SVs. Then, they deduced three important and interesting conclusions: (1) the antiport activity, but not the channel conductance, is essential in brain endosomes and lysosomes, (2) ClC-3 has little significant role in SVs, and (3) ClC-3 forms a heteromer together with ClC-4. The second and third

conclusions are well verified by the data obtained through a variety of elegant experiments. However, the first conclusion is not directly evidenced.
This study is premised that the E224A mutant of ClC-3 is solely functioning as a Cl- channel, but not an antiporter, just as the cases of ClC-5 (Novarino et al. 2010) and ClC-7 (Weinert et al. 2010). Although its Cl- channel activity was shown in HeLa cells expressing E224A/ClC-3 (in Supplementary Fig. 2), lack of the antiport activity was not demonstrated. This must be shown in brain endosomes and lysosomes by directly monitoring intravesicular pH and Cl- concentration using WT/ClC-3 as a control. Since ClC-3 was shown to exist normally as a heteromer formed with ClC-4, further studies should be performed to determine whether the E224A mutant of ClC-3 in the heteromer is also functioning as a Cl- channel but not as a 2Cl-/H+ antiporter.

Referee #3:

The manuscript by Jentsch and colleagues investigates the roles of ClC-3 and ClC-4 mediated Cl-/H+ exchange in acidification of endolysosomes. They find that animals expressing a defective ClC-3 mutant that uncouples Cl- transport from H+ flux are normal. This is due to compensation of ClC-4/ClC-3unc heteromers, which provide sufficient Cl- and H+ fluxes to maintain normal homeostasis. Indeed, mice lacking ClC-4 are also normal, while Clcn3unc/unc/Clcn4-/- or Clcn3-/-/Clcn4-/- animals show progressively more severe phenotypes. The authors then use a wide array of complementary approaches to investigate whether ClC-3 localizes to synaptic vesicles (SV) and if it regulates their pH. This second topic is important, as numerous contrasting reports have indicated that ClC-3 is essential for SV acidification or that it is not present in these compartments. The authors' data suggests that ClC-3 is present in a non-negligible fraction of SV (5-15%), an estimate that is higher than some reports which indicated as low as 0.05% the population of ClC-3 positive SV. Notably, the lack of ClC-3 does not cause changes in acidification of SV or in the miniature postsynaptic currents, before neurodegeneration sets in.

Overall, this is an excellent, well written manuscript that thoroughly tackles a number of important questions. The conclusions are well-grounded in the data and of broad general interest. As such, I have only relatively minor comments and requests of the authors.

Pg. 7/Fig. 2, the authors show that ClC-3 localizes to vesicles that are partially positive for Lamp-1, an endo-lysosomal marker. Given the importance of the localization of ClC-3, I think that the authors should estimate what fraction of the total vesicle population is positive for Lamp-1 and ClC-3 and which is positive only for one or the other. This is important to understand what fraction of ClC-3 is in endo-lysosomes. Does this fraction change upon expression of ClC-3 with ClC-4? And in the ClC-3unc/unc mice?

Pg. 12/Fig 5. I am confused by the description and inferences drawn from the experiments in Fig. 5. The authors state that the decrease of VGLUT1 correlates with the disruption of Clcn3. How can the presence of ClC-3 in <10% of SV affect the abundance of VGLUT1 in the whole population of these vesicles? This should be at least discussed.
Similarly, the authors show that ClC-3 localizes to Tfn-positive vesicles in neurons and that the pH of these compartments is not altered in ClC-3 -/- cells. What fraction of Tfn-positive vesicles are also ClC-3 positive? If the overlap is 5-15% (which would be consistent with the estimate of ClC-3 positive SV), then the analysis should be repeated for the separate vesicle populations. If the overlap is significantly higher, then I think it is important to clarify why.

The authors show that that in the Clcn3-/- mice ClC-4 does not leave the ER, and they claim that is degraded. I am a bit puzzled by the latter claim, since in overexpression systems ClC-4 traffics to the PM better than ClC3. Aren't these two observations somewhat in contrast?

The authors state that ClC-3 and ClC-4 have largely overlapping functions. However, their data shows that Clcn3-/- mice have severe degeneration phenotypes while Clcn4-/- ones don't. Wouldn't this argue that ClC-3 alone is able to function while ClC-4 is not, indicating that the two homologues have distinct roles, with ClC-3 playing the most critical one?

Minor

Pg. 8 The authors conclude that the different phenotypes of the Clcn3unc/unc/Clcn4-/- compared to Clcn3-/-/Clcn4-/- mice is due to the uncoupled conductance of ClC-3 mutant. However (as they mention in the discussion), this could also reflect the presence or absence of the protein itself. Maybe this could also be mentioned in the results in addition to the discussion.

Please show in the supplementary information the data supporting the claim that in the Clcn3unc/unc mice there is no retina generation (Page 5).

Pg. 5, Please use the ClC-ec1 or ecClC nomenclature; the ecClC-1 name is confusing as it suggests that this protein is the E. coli homologue of the ClC-1 channel.

I suggest the authors tone down a bit how they make some of their points. While I agree with the concepts expressed, in some instances, they are unnecessarily aggressive (i.e. last sentence in pg. 15, or in pg 16 where they discuss the expression on ClC-3 at the plasma membrane).

When discussing the possible pitfalls of the various approaches used to estimate the abundance of ClC-3 in SV, the authors mention that past work might have overestimated VGLUT1 levels due to epitope shielding in calibration experiments. Is there a literature reference for this? If not, what are the basis for this hypothesis?

1st Revision - authors' response 19th Dec 2019

Point by Point Response to the reviewers (Weinert et al.)

We thank all three reviewers for their insightful, constructive and positive comments. Our detailed responses and changes in the manuscript are detailed below.

Referee #1:

- general summary and opinion about the principle significance of the study, its questions and findings

The study departs from the still surprising discovery that intracellular CLC proteins function as secondary active transporters rather than ion channels. It tackles the fundamental and poorly understood question whether and how the transport mode as an exchanger is required for the physiological function of vesicular CLCs. The case of the specific family member under investigation here, ClC-3, is particularly intriguing because of the many physiological functions that were assigned to this family member and the severe neurodegenerative phenotype associated with its knockout in mouse.

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Based on this puzzling finding the authors address the formation of heterodimers between ClC-3 and ClC-4 as a possible mechanism behind this observation. And, indeed, this line of thought and experimentation leads to the discovery that ClC-4 requires ClC-3 for efficient exit from the endoplasmic reticulum (ER). One major value of this study is that it firmly shows one of the very few examples of obligatory heteromer formation between channel subunits in native tissues. In the presence of the uncoupled variant of ClC-3, ClC-4 still provides enough chloride/protein exchange activity in the form of heteromers. This is convincingly tested by knockout of the gene encoding ClC-4 from the transgene expressing uncoupled ClC-3. The experiment unequivocally demonstrates the suppression of the ClC-3 uncoupled phenotype by ClC-4. This is another major step in clarifying the physiological relevance of the secondary active transport mode of vesicular CLCs. At the same time, it identifies a new and interesting case of assembly-dependent trafficking.

We thank the reviewer for appreciating the importance of our work.

- specific major concerns essential to be addressed to support the conclusions
There are no major concerns. All conclusions are based on extremely carefully conducted, complementary experiments. All the usual pitfalls that jeopardize so many studies at the intersection of physiology and cell biology are systematically avoided (such as over-expression and the use of poorly characterized antibodies). This study is rich in novel insights but it also provides guidance in a field riddled with unreliable evidence or over-interpretation. The quality and breadth of the methodology can only be called impressive.
Thank you for stressing the quality of our study.

- minor concerns that should be addressed
For Fig. S3d I would remove the brackets from the gel and mark the Endo H-sensitive form and the de-glycosylated form outside rather than on the blot

We have removed the brackets in Fig. S3d (now EV Fig. 2D) and have indicated the EndoH-sensitive form by small arrows at the left and right of the blot.

In the discussion I would not refer to ClC-3 as a "chaperone" for ClC-4. You could either look into the article by Herrmann, Malkus and Schekman (1999, Trends in Cell Biology) and refer to the concepts of "escorts" or "guides" or talk about assembly-dependent ER exit of ClC-4, which I find most appropriate since both proteins are functional transporters.

We agree that chaperone is a misleading term here. The only place where we have used it is a page 14, where we have now replaced 'chaperone' by 'binding partner': 'In the absence of the ClC-3 binding partner, a large fraction of ClC-4 remains in the ER*...* '

You might consider not to highlight senior authors or groups/laboratories when discussing the literature. Each first author has taken responsibility for their articles by being the first author.

This comment is well-taken. We had highlighted laboratories/senior authors a few times for D. Nelson, C. Fahlke, and once for S. Takamori, always followed by the reference which in EMBO J style is (first author, year), thus duly acknowledging the first author. We now directly mention the senior author in all three cases only once, mentioning the first authors instead. We believe that duly acknowledging the group once is justified and helps the reader.

Referee #2:

The proton pump activity of V-type ATPase in endosomes, lysosomes and synaptic vesicles (SVs) needs a neutralizing counter current provided by ClCs serving as 2Cl-/H+ antiporters or Cl- channels. Since disruption of endosomal ClC-3 induces severe neurodegeneration, in the present study, the authors elucidated the roles of 2Cl-/H+ antiport activity of ClC-3 by making good use of Clcn3unc/unc mice in which the E224A mutation was introduced into ClC-3 and is thereby expected to be converted its activity from a 2Cl-/H+ antiporter to a Cl- channel. They intended to address following two questions: whether the roles of ClC-3 in brain endosomes and lysosomes depend on its 2Cl-/H+ exchange transport or its Cl- channel conductance, and whether ClC-3 is also important in SVs. Then, they deduced three important and interesting conclusions: (1) the antiport activity, but not the channel conductance, is essential in brain endosomes and lysosomes, (2) ClC-3 has little significant role in SVs, and (3) ClC-3 forms a heteromer together with ClC-4.

We thank the reviewer for appreciating the importance of our work.

The second and third conclusions are well verified by the data obtained through a variety of elegant experiments.

We are pleased that the reviewer is convinced by our experiments and the conclusions drawn from them.

However, the first conclusion is not directly evidenced.
This study is premised that the E224A mutant of ClC-3 is solely functioning as a Cl- channel, but not an antiporter, just as the cases of ClC-5 (Novarino et al. 2010) and ClC-7 (Weinert et al. 2010). Although its Cl- channel activity was shown in HeLa cells expressing E224A/ClC-3 (in Supplementary Fig. 2), lack of the antiport activity was not demonstrated. This must be shown in brain endosomes and lysosomes by directly monitoring intravesicular pH and Cl- concentration using WT/ClC-3 as a control.

We indeed did not show directly that ClC-3 (which gives only small transport rates) is a Cl^-/H^+-antiporter and that the E224A mutant uncouples Cl^- from H^+-transport, but believe that there is overwhelming evidence for this notion from published work from several laboratories including our own, and that this notion is further supported by the present work.

In detail:
(1) ***Direct and convincing evidence*** *(using pH- and electrophysiological measurements) for ClC-3 being a Cl^-/H^+-exchanger, and for the E224A mutation uncoupling Cl^- from H^+-transport,* ***has been published*** *rather recently by Rohrbough and Lamb (J. Physiol 2018,* PMID: 29917234*) using an expression construct that apparently gives larger transport rates than the one we used. We have cited this paper several times in our manuscript, including the Introduction and the first section of results (and legend of Fig. EV1) in which we describe the uncoupling of the ClC-3 exchanger by the E224A mutation.*
(2) Our own experiments (Fig. EV1B,C), as acknowledged by the reviewer, show that the E224A mutation converts ClC-3 currents from outwardly-rectifying to linear, a hallmark of these uncoupling mutations.
(3) Equivalent 'gating glutamate' mutations uncouple CLC exchangers not only with ClC-4 and ClC-5 (Picollo & Pusch, Nature 2005 PMID: 16034421; Scheel…Jentsch, Nature 2005 PMID: 16034422), which belong to the same narrow homology branch as ClC-3, but also with ClC-6 (Neagoe…Jentsch, JBC 2010 PMID: 20466723), ClC-7 (Leisle…Stauber, EMBO J. 2011 PMID: 21527911), i.e. all mammalian vesicular CLCs. These papers are cited in the introduction and the legend to Fig. EV1B. However, the role of the gating glutamate in coupling Cl^- to H^+-countertransport is much more general. It was first described for the E. coli exchanger ecClC (Accardi & Miller, Nature 2004 PMID: 14985752) and later also for plant (Arabidopsis) CLCs (Bergsdorf, Zdebik, Jentsch, JBC 2009 PMID: 19261613).

Thus, published work provides very strong direct and indirect evidence for the notion that ClC-3 is a Cl^-/H^+-exchanger that can be uncoupled by the E224A mutation, obviating the need to replicate the work of Rohrbough et al. (2018).

Furthermore, the experiment suggested by the reviewer (measuring intravesicular pH and Cl concentration in endosomes and lysosomes from brain of WT and $ClC\text{-}3^{unc}$ mice), apart from being technically extremely challenging (in particular the Cl part, nobody has done this before), is unable to resolve this question since these vesicles (which never represent a pure population of a specific endosomal compartment) will also contain late-endosomal/lysosomal ClC-6 and lysosomal ClC-7, both of which are Cl^-/H^+-exchangers. With such a background transport activity, it is very unlikely that these experiments will reveal a difference between the genotypes.

Since ClC-3 was shown to exist normally as a heteromer formed with ClC-4, further studies should be performed to determine whether the E224A mutant of ClC-3 in the heteromer is also functioning as a Cl- channel but not as a 2Cl-/H+ antiporter.

As detailed below, previous structure-function studies very strongly suggest that this is the case, but experiments directly showing this are very difficult and to our mind unnecessary.

CLC chloride channels and transporters always function as dimers, with the ion translocation pathway entirely contained within each of its subunits, as convincingly shown by mutagenesis (Middleton, Pheasant, Miller, Nature 1996 PMID: 8894974; Ludewig, Pusch, Jentsch, Nature 1996 PMID: 8848047; Weinreich & Jentsch, JBC 2001 PMID: 11035003) and crystal and cryoEM structures (e.g. Dutzler et al, Nature 2002 PMID: 11796999). Mutations in the ion permeation pathway of one subunit only affect the permeation properties of that particular subunit in both

homodimeric and mutant/WT heterodimeric channels. This was *shown e.g. for the ClC-0 S123T mutation, which reduced single channel conductance and ion selectivity of the mutated subunit irrespective of whether the attached subunit is WT or mutant (Ludewig, Pusch, Jentsch, Nature 1996 PMID: 8848047). Concatemers between different CLC isoforms (e.g. ClC-0 and ClC-2, or ClC-0 and ClC-1) showed that the permeation properties (single channel conductance) of the individual subunit was independent from the attached subunit (Weinreich & Jentsch, JBC 2001). The crystal structure of the E. coli ecClC protein (Dutzler et al, Nature 2002 PMID: 11796999) later showed that the serine equivalent to ClC-0 S123 co-ordinates a chloride ion in the pore and is located close to the gating glutamate (E224 in ClC-3) which we have mutated in the present study. The role of this gating glutamate has been addressed extensively in both crystallographic studies (e.g. Dutzler, Campbell, McKinnon, Science 2003 PMID: 12649487), various transport models, molecular dynamic simulations, and mutagenesis studies. There is very strong evidence that protonation of the glutamate side chain (located in the center of the pore formed by a single subunit) is crucial for Cl-/H+ exchange. Thus, there is no reason to doubt that the exchange activity (or channel activity of the mutant) is independent from the exchange or channel activity of the attached subunit. Only a specific gating process, the 'common' gating (or slow gating for the Torpedo channel) depends on both subunits, as shown also a Cl-/H+-exchanger (ClC-7) (Ludwig…Jentsch, JBC 2013* PMID: 23983121).

Given the overwhelming evidence that the ion transport pathway is entirely enclosed within a single subunit, and hence not influenced by the other subunit in the dimer, experiments directly showing the independence of both translocation pathways for ClC-3 are not warranted. (Besides, the two other reviewers did not have this difficulty, but rather stated that our work is very convincing).

Demonstrating directly this independence for ClC-3, as suggested by the reviewer, is technically extremely challenging. It would require concatemers of WT and mutant subunits (simple co-expression will be 'contaminated' by homodimeric mutant and WT transporters). Whereas investigations of CLC concatemers are feasible in principle, as shown in our own previous work (e.g. Ludewig et a., 1996, and Weinreich et al., 2001), the expression efficiency of concatemers is low. This would be a major problem with the poorly expressing ClC-3.

After reading the comments of reviewer 2, we realized that we should have better explained the independence of the two pores / transport pathways more explicitly in our text. We have therefore expanded the paragraph describing the independence of the two permeation pathways of CLC proteins, and of the gating glutamate, on page 16 in Discussion, and now already inform the reader of the dimeric 2-pore structure of CLCs in the introduction (end of first paragraph).

Referee #3:

The manuscript by Jentsch and colleagues investigates the roles of ClC-3 and ClC-4 mediated Cl-/H+ exchange in acidification of endolysosomes. They find that animals expressing a defective ClC-3 mutant that uncouples Cl- transport from H+ flux are normal. This is due to compensation of ClC-4/ClC-3unc heteromers, which provide sufficient Cl- and H+ fluxes to maintain normal homeostasis. Indeed, mice lacking ClC-4 are also normal, while Clcn3unc/unc/Clcn4-/- or Clcn3-/-/Clcn4-/- animals show progressively more severe phenotypes. The authors then use a wide array of complementary approaches to investigate whether ClC-3 localizes to synaptic vesicles (SV) and if it regulates their pH. This second topic is important, as numerous contrasting reports have indicated that ClC-3 is essential for SV acidification or that it is not present in these compartments. The authors' data suggests that ClC-3 is present in a non-negligible fraction of SV (5-15%), an estimate that is higher than some reports which indicated as low as 0.05% the population of ClC-3 positive SV. Notably, the lack of ClC-3 does not cause changes in acidification of SV or in the miniature postsynaptic currents, before neurodegeneration sets in.

Overall, this is an excellent, well written manuscript that thoroughly tackles a number of important questions. The conclusions are well-grounded in the data and of broad general interest.

We thank the reviewer for appreciating the importance and quality of our work.

As such, I have only relatively minor comments and requests of the authors.

Pg. 7/Fig. 2, the authors show that ClC-3 localizes to vesicles that are partially positive for Lamp-1, an endo-lysosomal marker. Given the importance of the localization of ClC-3, I think that the authors should estimate what fraction of the total vesicle population is positive for Lamp-1 and ClC-3 and which is positive only for one or the other. This is important to understand what fraction of ClC-3 is in endo-lysosomes. Does this fraction change upon expression of ClC-3 with ClC-4? And in the ClC-3unc/unc mice?

While we can detect co-localization of ClC-3^{Venus} with Lamp-1 and other markers in brain sections as shown in Fig. 4, the signal-to-noise background is unfortunately quite bad and therefore does not allow a reliable quantification of co-localization with marker proteins. However, such an analysis is possible in primary cultures of neurons. We therefore newly bred enough homozygous ClC-3^{Venus} mice, established hippocampal neuronal cultures, and quantified the co-localization of ^{Venus}ClC-3 with three markers of the endosomal-lysosomal system, i.e. EEA1, the transferrin receptor (TfR), and Lamp-1. We found that in neuronal somata about 30% of ^{Venus}ClC-3 co-localizes with TfR, 20% with EEA1, and 10% with Lamp-1. Conversely, ~80% of TfR-positive vesicles express ^{Venus}ClC-3, compared to ~50% of EEA1-positive and ~30% of Lamp-1-positive vesicles. These new data, including example images and diagrams showing the quantitative evaluation, are shown in the new Appendix Figure S6, and are referred to on page 10 and 12 in Results.

Although it might be interesting to investigate the subcellular localization of ClC-3 in Clcn4$^{-/-}$ or Clcn3$^{unc/unc}$ mice, we assume that changes in localization will be minor because of the lack of a neurodegenerative phenotype in either mouse model. Moreover, such a potential change in localization will not alter the conclusions of our paper (endosomal Cl/H exchange is essential) in any way.
Further, such experiments would require newly crossing our Clcn3^{Venus} with Clcn4^{-} mice to obtain mice homozygous for each allele. It would take us roughly 9 months to obtain a sufficient number of animals for these experiment. To investigate a putative effect of the unc mutation on the localization of ClC-3 would even require us to newly generate homozygous Clcn3^{unc,Venus} mice since the E224A exchange and the epitope tag have to be on the same protein. Thus, these experiments, which are beyond the scope of our paper, are not feasible within a reasonable time span.

Pg. 12/Fig 5. I am confused by the description and inferences drawn from the experiments in Fig. 5. The authors state that the decrease of VGLUT1 correlates with the disruption of Clcn3. How can the presence of ClC-3 in <10% of SV affect the abundance of VGLUT1 in the whole population of these vesicles? This should be at least discussed.

Indeed, the loss of VGLUT1 over time cannot be due to a loss of the small fraction of SVs that contain ClC-3. The loss of VGLUT1 is gradual and correlates with the development of neurodegeneration which eventually leads to a complete loss of the hippocampus. Since this brain region is particularly rich in glutamatergic vesicles, loss of hippocampal neurons will entail a more pronounced loss of VGLUT1 compared to other neuronal and synaptic markers (see Fig. 5A). However, we cannot exclude an additional effect of ClC-3 on the trafficking of VGLUT1.
As suggested by the reviewer, we address this point in our discussion and write on page 18 to 19, Discussion: 'Since the loss of VGLUT1 roughly correlated with the degree of neurodegeneration, it might be owed to a preferential loss of mainly glutamatergic neurons or, less likely, from a trafficking defect of VGLUT1'.

Similarly, the authors show that ClC-3 localizes to Tfn-positive vesicles in neurons and that the pH of these compartments is not altered in ClC-3 -/- cells. What fraction of Tfn-positive vesicles are also ClC-3 positive? If the overlap is 5-15% (which would be consistent with the estimate of ClC-3 positive SV), then the analysis should be repeated for the separate vesicle populations. If the overlap is significantly higher, then I think it is important to clarify why.

Using primary neuronal cultures from Clcn3$^{Venus/Venus}$ mice (see above), we have now quantified the percentage of vesicles in neuronal somata in cultured hippocampal neurons that are positive for ^{Venus}ClC-3 and three endosomal/lysosomal markers. Roughly 80% of TfR-positive vesicles express ClC-3. These results are shown in the new Appendix Figure S6 and referred to in Results (page 12) where we describe these endosomal pH measurements. The fact that 80% of the vesicle population, which was used to measure pH (with a transferrin-receptor targeted probe), are ClC-3 positive fully validates our measurements.

We don't see any contradiction to the finding that only 5-15% of synaptic vesicles are positive for ClC-3. The biogenesis of these vesicles is different and an important conclusion of our work is that ClC-3 is mainly on endosomes, and only on a small portion of synaptic vesicles.

The authors show that that in the Clcn3-/- mice ClC-4 does not leave the ER, and they claim that is degraded. I am a bit puzzled by the latter claim, since in overexpression systems ClC-4 traffics to the PM better than ClC3. Aren't these two observations somewhat in contrast?

Of course, the reviewer is right: if all ClC-4 would be stuck in the ER, it would not be able to reach the plasma membrane after transfection. However, localization of ClC-4 at the plasma membrane is most likely an artifact of overexpression, and electrophysiological methods are sensitive enough to detect it there. It remains essentially unclear why ClC-3 gives much less plasma membrane currents compared to ClC-4 – it possibly transports less ions per transporter protein (Guzman… Alekov, ACS Chem Neurosci 2013 PMID: 23509947).
The essential point is that, without ClC-3, the bulk of ClC-4 is stuck in the ER. It needs ClC-3 to reach its normal localization in endosomes. We had therefore written in Discussion on page 14, avoiding any overstatement: 'In the absence of the ClC-3 binding partner, a large fraction of ClC-4 remains in the ER where it is probably degraded.'

The authors state that ClC-3 and ClC-4 have largely overlapping functions. However, their data shows that Clcn3-/- mice have severe degeneration phenotypes while Clcn4-/- ones don't. Wouldn't this argue that ClC-3 alone is able to function while ClC-4 is not, indicating that the two homologues have distinct roles, with ClC-3 playing the most critical one?

While the phenotypes of Clcn3$^{-/-}$ and Clcn4$^{-/-}$ mice suggest that ClC-3 plays a more critical role, this may be largely due to the fact that ClC-4 levels are substantially reduced in the ClC-3 KO, but not vice-versa – an observation we have attributed to a role of ClC-3 in facilitating the exit of ClC-4 from the ER by forming ClC-3/ClC-4 heteromers (see Fig. 1C-E, Fig. 2, Fig. EV2).

We changed 'largely overlapping' to 'partially overlapping' on page 8 and, to remind readers of this crucial observation, added after 'partially overlapping function':
'The occurrence of neurodegeneration in *Clcn3$^{-/-}$*, but not *Clcn4$^{-/-}$* mice might be explained by the fact that ClC-4 levels are strongly decreased in *Clcn3$^{-/-}$* mice, whereas ClC-3 abundance does not depend on ClC-4.'

Minor

Pg. 8 The authors conclude that the different phenotypes of the Clcn3unc/unc/Clcn4-/- compared to Clcn3-/-/Clcn4-/- mice is due to the uncoupled conductance of ClC-3 mutant. However (as they mention in the discussion), this could also reflect the presence or absence of the protein itself. Maybe this could also be mentioned in the results in addition to the discussion.

Following the suggestion of the reviewer, we have now also added a sentence in Results, page 9:
'However, we cannot exclude that the more severe phenotype of the double knock-out is in part owed to the lack of the ClC-3 protein that might bind to other unknown interaction partners'.

Please show in the supplementary information the data supporting the claim that in the Clcn3unc/unc mice there is no retina generation (Page 5).

We now show the retina on Clcn3$^{unc/unc}$ mice in comparison to WT and Clcn3$^{-/-}$ mice in Fig 1B.

Pg. 5, Please use the ClC-ec1 or ecClC nomenclature; the ecClC-1 name is confusing as it suggests that this protein is the E. coli homologue of the ClC-1 channel.

We have replaced Ec-ClC-1 by ecClC on page 5.

I suggest the authors tone down a bit how they make some of their points. While I agree with the concepts expressed, in some instances, they are unnecessarily aggressive (i.e. last sentence in pg. 15, or in pg 16 where they discuss the expression on ClC-3 at the plasma membrane).

On page 16 we have eliminated the possibly too aggressive 'is easily dismissed' by: 'The hypothesis that ClC-3 facilitates acidification of endosomes by increasing the capacitance of their membrane (Guzman et al, 2013) is not viable when luminal buffering of H^+ is taken into account (Jentsch & Pusch, 2018).'
In the paragraph mentioning the previous suggestions that ClC-3 is a plasma membrane Cl- channel (page 16), however, we did not find anything that could be interpreted as being aggressive. No change made here.

When discussing the possible pitfalls of the various approaches used to estimate the abundance of ClC-3 in SV, the authors mention that past work might have overestimated VGLUT1 levels due to epitope shielding in calibration experiments. Is there a literature reference for this? If not, what are the basis for this hypothesis?

The basis for this statement was a personal communication by Shigeo Takamori at a meeting several years ago. We again contacted Dr. Takamori and obtained his permission to cite him in a personal communication on page 18 and in the legend to Fig. EV4.

2nd Editorial Decision 17th Jan 2020

Thank you for submitting your revised manuscript for consideration by The EMBO Journal. Please accept my apologies for the delay in processing your revised manuscript due to protracted referee input. Your revised study was sent back to the three referees for re-evaluation, and we have received comments from two of them, which I enclose below. Please note that while referee #2 was unfortunately not able to re-assess the study at this time, we have considered your response to his-her raised critique and found the issues to be reasonably responded to. As you will see the other referees find that their concerns have been sufficiently addressed and they are now broadly in favour of publication.

Thus, we are pleased to inform you that your manuscript has been accepted in principle for publication in The EMBO Journal, pending some minor issues related to formatting and data representation points listed below, which need to be adjusted at re-submission.

Referee #1:

This is a revised manuscript that addresses all (minor) concerns I had regarding the originally submitted version. In this version all comments are carefully discussed and appropriately addressed. The manuscript makes a substantial contribution to the field of cellular ion homeostasis.

Referee #3:

The authors have addressed all of my concerns and suggestions in a very satisfactory manner. I have no further comments or requests.

2nd Revision - authors' response 24th Jan 2020

The authors performed the requested changes.

3rd Editorial Decision 27th Jan 2020

Thank you for submitting the revised version of your manuscript. I have now evaluated your amended manuscript and concluded that the remaining minor concerns have been sufficiently addressed.

Thus, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal.

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: Thomas J. Jentsch
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2019-103358R

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 $\chi 2$ 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?	The sample size was chosen based on our previous experience with ClC-3 KO mice as published in Stobrawa …. Jentsch, Neuron 29: 185-96 (2001) (PMID: 11182090).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The sample size was chosen based on our previous experience with ClC-3 KO mice as published in Stobrawa …. Jentsch, Neuron 29: 185-96 (2001) (PMID: 11182090).
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Samples were excluded when their preparations was questionable, e.g. when slices for electrophysiology did not appear healthy, when patches in patch-clamp recordings were unstable, or when Western blots indicated protein degradation. Animals were re-genotyped and mice carrying a not matching genotype were excluded.
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.	Animals were partially randomized, using the number assigned to an animal instead of the genotype.
For animal studies, include a statement about randomization even if no randomization was used.	Animals were partially randomized, using the number assigned to an animal instead of the genotype.
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.	In most experiments, blinding was not necessary as results were qualitative, or, in the case of slice physiology, no differences between the genotypes were detected.
4.b. For animal studies, include a statement about blinding even if no blinding was done	In most experiments, blinding was not necessary as results were qualitative, or, in the case of slice physiology, no differences between the genotypes were detected
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.	Statistical methods are described in Methods section of the manuscript, with more details given for the individual experiments in the respective figure legend.
Is there an estimate of variation within each group of data?	Yes

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Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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).	The antibodies used in this study are described in detail in the Methods section, including the catalog number of the commercial antibodies were have used. These widely used antibodies have been controlled by the companies and, more importantly, have been used and validated in many published studies. The anti-GFP labeling of the Venus-protein in Clcn3Venus mice was controlled by staining WT mice as negtive controls, using the same primary and secondary antibodies. The primary antibodies against CIC-3, CIC-4 and CIC-5 have been generated in our own laboratory and were extensively tested using respective KO mice (Maritzen..Jentsch, 2008; Günther...Jentsch, 1998; this study), as also detailed in the Methods section.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	HeLa, HEK and COS-7 cells (from ATCC) were used for transfection. They were not recently authenticated and sporadically checked for mycoplasma contamination.

* 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.	Mus musculus, male & female, animals of all ages. Mice were genetically modified by knock-in mutations. All animal experiments and housing are approved by the competent local authority "LAGeSo" and supervised by veterinarians of the MDC. The Clcn3 and Clcn4 KO mice have been published previously (Stobrawa...Jentsch, Neuron 2001). Clcn3unc and Clcn3Venus mice have been newly generated and are described in detail in the mansucript, together with the genetic background.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were approved by the competent local authority "LAGeSo".
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.	We confirm.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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.	NA
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.	NA

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	NA We have not generated new DNA/RNA or protein data in this study . The accession codes of the cDNAs and proteins used in this study are: mouse CIC-3 DNA: NM_007711.3 human CIC-3 cDNA NM_001243372.1 VGLUT1 cDNA: NP_892038.2
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G- Dual use research of concern

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