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Autophagy regulates neuronal excitability by controlling cAMP/Protein Kinase A signaling at the synapse

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Dear Prof. Kononenko,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by two referees and their comments are provided below.

As you can see from the comments, the referees find your study interesting and suitable for publication here. They raise constructive comments that I would like to ask you to address in a revised version. I think it would be helpful to discuss the raised points further and I am happy to do so via email or video.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

Thank you for the opportunity to consider your work for publication. I look forward to discussing the revisions further.

Yours sincerely,

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

I have attached a PDF with helpful tips on how to prepare the revised version.

Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (13th Jun 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The manuscript "Autophagy regulates neuronal excitability by controlling cAMP/Protein Kinase A signaling" by Overhoff et al., addresses the role of autophagy in regulation of synaptic function. The main claim of the manuscript suggests a novel function of autophagy that does not depend on the turnover of damaged proteins and organelles. The study adds to growing evidence that neuronal autophagy has adapted to specific requirements of proteostasis imposed by synaptic neurotransmission. In brief, the authors propose that in excitatory neurons autophagy is triggered by starvation and that this type of autophagy in particular contributes to the turnover of the inhibitory R1α/β subunits of PKA and thereby indirectly promotes neuronal PKA signalling. The manuscript contains an impressive amount of data and the authors have used a broad array of different techniques to analyse ATG5 functions in neurons including whole animal experimentation. Seen from this perspective the paper deserves publication in a high-impact journal. However, I have several concerns that leave me lukewarm with a decisive recommendation for publication. While the evidence that alterations in PKA signalling, cAMP-dependent remodelling of cytoskeletal phosphoproteome, increased thickness of the PSD, alteration of AMPAR and augmented excitatory neurotransmission occur following ATG5-deletion is convincing, the different parts of the study are only loosely connected and the central hypothesis as stated in the last sentence of the Introduction 'Our findings identify a previously unknown role of starvation-induced autophagy in the regulation of PKA-dependent signaling in the brain, where it functions as a modulator of synaptic activity in cortical excitatory neurons.' is not really supported by the data to an extend that allows for a definite conclusion. Although this is potentially an important finding emphasizing the role of autophagy in regulation of signaling at synapses, several data sets are preliminary and important controls are lacking.

General comments
- The manuscript is difficult to read. In part because it contains a lot of data that do not really support the main story. On the other hand, the Introduction and the Discussion are very long and not always to the point.
- I don't like that dispensable data sets are intermingled with core experiments that provide novelty to the field. I sometimes had

the impression that available data were put together to give the impression that the amount of evidence in support of the central hypothesis is broad and substantial. See my specific comments below.
- In several Figures it is unclear what the N-numbers reflect. In many panels I see only three data points per group. This raises doubts on the validity and appropriateness of the statistical tests.
- Taken together, this is an interesting study that puts forward an interesting hypothesis. However, I suggest to reorganize the manuscript, to remove data that do not contribute to the main conclusion and to test more stringently test the hypothesis that starvation-induced autophagy targets prominently inhibitory R1α/β subunits of PKA, which results in synaptic scaling processes.

Specific comments
- I don't understand the logic of the behavioral phenotyping. First, differences in rearing are only obvious in Atg5flox:Slc32a1-Cre KO mice with a deletion in inhibitory neuron. The corresponding knockout in excitatory neurons has no effect. Why then testing for the startle response in both lines? If seizures are present only in Atg5flox/flox:CamKIIα-Cre mice why do the authors conclude that autophagy in general regulates intrinsic excitability. There are no direct measures of intrinsic excitability and why should moderate increases in surface expression of AMPAR cause seizures? Any published evidence for that? Wouldn't one expect an effect on cognitive behavior; i.e. hippocampus-dependent learning and memory? All these parts are loosely and not convincingly connected.
- Why two different proteomic approaches? On my opinion the SILAC data are hardy quantitative. How good is the overlap between SILAC and label-free proteomics? I see only GABARAPL2 and two other proteins consistently regulated. Is this believable? If the latter one based on FACS-sorting is superior why did the authors start with SILAC? The differences are relatively small and it is surprising how different the results are as compared to previously published studies on autophagosome content profiling. I don't see how different methods of purification can account for these discrepant results and if yes this has to be discussed.

- Neuronal autophagosome content profiling was not done under starvation conditions in this study. If starvation triggers selective autophagy of inhibitory R1α/β subunits of PKA it is hard to understand why those proteins are most prominently regulated in the ko mice. Any mechanistic idea? Isn't it surprising given the tremendous upregulation of p62 that other changes are relatively mild?

- It should be clearly stated that proteomics with Atg5flox:Slc32a1-Cre KO was done with striatal tissue, which is hidden in the Figure legends. Thus, the analysis was done with medium spiny neurons, which are different from interneurons. It is important to discuss this. Moreover, if one compares different brain regions a direct comparison of excitatory and inhibitory neurons is not meaningful. It was difficult to follow up on this and I am not sure but it looks like that all proteomic experiments were done with neurons of different age. The question arises what do we learn from these data?

- I have some concerns related to pictorial evidence for starvation-induced autophagy in neurons. Several reports have shown that autophagy in neurons is a constitutive process and starvation protocols involving nutrient deprivation have little or no effect on the rate of autophagosome formation (Maday S, Holzbaur EL (2014); Maday S, Holzbaur EL (2016); Lee S, Sato Y, Nixon RA (2011). The lack of classical excitatory and inhibitory neuronal markers, as well as glial markers makes it difficult to exclude the role of starvation in upregulation of inhibitory subunits of PKA in glia cells.

- In Figure 1A and B I am surprised to see a complete downregulation of ATG5 in either excitatory or inhibitory neurons in comparison to cortical brain lysates from wild type animals. It's important to load samples onto the same gel to allow for a direct comparison on the ATG5 expression levels in all mouse lines. Why have the authors used b-actin in panel A and GAPDH in panel B for normalization?

- In the Results section under the heading "PKA R1α/β levels are highly upregulated in autophagy-deficient excitatory and inhibitory neurons" the authors conclude that autophagy controls neuronal excitation by regulating PKA signaling. Augmented excitatory neurotransmission in mice lacking ATG5 was already reported. The gain in excitatory neurotransmission was shown to be a consequence of elevated calcium release from ER stores at presynaptic sites due to selective accumulation of tubular ER (Kuijpers et al., 2021) that is a major structural alteration and regulation of PKA signaling under these conditions might not have a causative role. Clear and conclusive evidence that PKA signaling is instrumental for the gain in excitatory transmission is lacking.

- An intriguing finding appears be that mTORC1 plays no role in the induction of the starvation-induced neuronal autophagy investigated in this study. However, the presented data are rather preliminary and the current data set does not add much to the story without further mechanistic insights.

- Fig. 2 panels E-H. Neuron-specific markers (inhibitory and excitatory) are missing making it difficult to judge the neuron-specific upregulation of the inhibitory subunits of PKA. I am also surprized that upregulation of PKA- R1α/β is largely somatic. Can the authors comment on that? In general, the manuscript would profit from quantitation of immunocytochemical data showing enrichment of PKA- R1α/β at specific cellular compartments. As mentioned above, glial markers and quantification of PKA-R1α/β within glial cells should be done to support the conclusions.

- Fig. 2J-K. The increase in Pearson`s or Manders co-localization coefficient between PKA- R1β/Bassoon and PKA- R1β/PSD95

upon the different treatments should be indicated.

- Fig.2 panel L show occasional single spots of PKA-R1α in several subcellular compartments. Also, one or two gold particle per image are not convincing to demonstrate upregulation in ATG5 deficient mice. The manuscript would profit from quantification that shows enrichment of PKA-R1α at synaptic compartments based on the number of particles per compartment. It is not clear how the mean of gold particle/volume fraction was quantified (panel M).

- Alterations in PKA signaling are shown indirectly by alterations is total phosphorylation of several substrates. To make a claim that PKA signaling in ATG5 deficient mice is altered at synaptic compartments, the authors should consider to isolate synaptosomes or to perform live imaging with a PKA sensor (PKA-FRET probe) targeted to postsynaptic compartments.

- Fig. 4K Changes in pCREB levels should be indicated with the gradient LUT to visualize differences in CREB activation. Neuron-specific marker should be added.

- Given the large number of transcripts regulated in response to ATG5 deletion (Fig. 4G, Suppl. Table 2) it is highly likely that the phenotype might also be caused by changes in gene expression and PKA-signaling at synaptic sites. The authors appear to ignore this possibility.

- Fig. 5K It looks like that synapses characterized by increased volume of PSDs have reduced numbers of presynaptic vesicles. Can the authors comment on that? Finally, I don't see any causative relationship between changes in PSD-size and the proposed mechanism.

- The pictorial evidence in Fig. 6C is of rather poor quality. The GLUR1 channel looks saturated. Puncta are very broad and cover the entire dendrite. How was this analysis performed? What is the ratio of synaptic GluR1 to those present in the dendritic shaft. Without a volume fill it will be difficult ta make assessment. Pictorial evidence for the quantitation in Fig. 6 E and F is lacking. How were expression levels of eGFP-ATG5 determined? Quantitation for fig. S6L is mandatory.

- Fig. 6J Why only three peaks in knockout neurons following tetanic stimulation (not the case in k)? How were the data normalized? Peaks are very broad (>3-5 seconds). This looks odd to me. Are these somatic/nuclear calcium waves? How about dendrites? What is the biological meaning of the comparison in Fig. 6L? The most striking differences in deltaF/F are in peak amplitude. How are AMPAR coupled to calcium release to allow for such a pronounced difference if this is not an artifact of buffer equilibration? This phenotype is similar to the one reported in Kuijpers et al.. Does it require RyR-dependent ER-calcium release?

- I found it very hard to understand what is shown in Fig. 6P-S. These are supposedly MEA-recordings. If yes, the cultures have a high baseline activity. What are the culture conditions? The knockout effect on spiking is tremendous. I would expect a huge increase in surface expression of AMPAR to explain this finding.

- Fig. 6 T and U show increased sAMPAR frequency and amplitude in some but not all cells. Statistical analysis is lacking.

Referee #2:

The study by Overhoff et al. describes a new unexpected function of autophagy in neurons, where autophagy controls the levels of PKA activity by degrading PKA regulatory subunits. The authors demonstrate that impairment of autophagy through genetic ablation of Atg5 inhibits PKA signaling, causing on the one hand changes in CREB1-mediated gene expression, and on the other hand diminished phosphorylation of postsynaptic scaffolding proteins. This in turn leads to altered AMPA-receptor trafficking and to neuronal hyperexcitability, revealed by seizures at the organismal level.
The findings are novel and exciting, the study is very comprehensive, uses an impressive spectrum of in vivo and in vitro techniques, and dissects the molecular mechanism very thoroughly.

Main comments:
1. Fig. 4A, C and N: it looks like pCREB and total CREB levels were probed on different blots. This should be done on the same blot. In addition, the forskolin-induced pCREB enhancement in KO MEFs appears to be of a similar magnitude as in WT (about 2.5-fold, Fig. 4D), so it is difficult to agree with the conclusion that ATG5 is important for regulating PKA signaling in these cells. As this piece of data is not crucial for the story, the authors could consider removing it.

2. While pCREB activation measurements by high content screening microscopy (Fig. 4E-F) were conducted in cortical cells, cAMP levels shown in Fig. S4A were measured in the hippocampus. These analyses should be conducted in the same tissue to be comparable.

3. Fig. S6L: the authors should perform a quantification of GluR1/PSD-95 colocalization in vivo.

4. Measuring the area of the postsynaptic density (Fig. 5K, L) is quite a crude and indirect way of showing altered localization of postsynaptic proteins. Ideally, it would be great to use super-resolution microscopy to convincingly demonstrate a change in localization of one or two candidate scaffolding proteins. Or this limitation should at least be acknowledged.

5. I think some conclusions and claims are overstated:
The conclusion of the first section of results (p. 5): "...ATG5 functions in ... neurons to regulate their intrinsic excitability" is not supported by data, as other reasons of network rearrangement beyond neurodegeneration have not been explored and cannot be excluded. I recommend rephrasing the conclusion based on the presented data.
p. 7: "In agreement with robust upregulation of PKA subunits..." - especially in the FACS-sorted samples, the upregulation is not robust.
p. 15: "...Our data suggest that ATG5-dependent protein phosphorylation can serve as a crucial regulator of postsynaptic protein localization and trafficking" - at this point in the manuscript, altered localization or trafficking has not yet been demonstrated for any postsynaptic protein.

6. Some of the figures are extremely packed, and the labeling on the panels very difficult to read (e.g., Suppl. Fig. S1 panels O-R, Fig. 4G, Fig. S5E, Fig. S6K). Could you please make these panels more reader-friendly? I recommend dividing Fig. 1 and Fig. S1 into two (phenotypic analysis of knockout mice / proteomics).

Minor points:
7. It would be useful to mention that the majority of neurons in the cortex are glutamatergic, while the majority in the striatum are GABAergic, to provide a rationale for doing the biochemical assays with these brain regions in the respective knockout mice.

8. Fig. 1A-B and S1A, D - Figure legends mention Atg5, but Western blots and graphs are labeled with Atg5-Atg12. Please clarify.

9. It is confusing that the occurrence of seizures in Atg5flox:Slc32a1-Cre mice is shown twice (Fig. 1H and S1N), with different results. I think one piece of data in the supplementary figure would be sufficient.

10. Legend to Fig. 3C: Level of PKA R1alpha in "starvation + BafA1" is indicated as 48.52% - should probably be 148.52%?

11. Fig. 3I-J: please show control images

12. Fig. 3L and 4M: please start the y-axis from zero.

13. Please explain the boxes in Fig. 4N in the figure legend.

14. Panel S6O uses the abbreviation VGAT, not used anywhere else in the paper. Please make consistent.

Further suggestions:
15. I think it would be very helpful to include a graphical summary of the described molecular mechanism as an additional panel in the last figure.

Point by Point Response to Reviewers.

We would like to thank the reviewers for their constructive comments and are pleased that they both noted
that our observations are novel and of great interest. Below, we address all of the concerns that were expressed by both reviewers.

Referee #1:

The manuscript "Autophagy regulates neuronal excitability by controlling cAMP/Protein Kinase A signaling" by Overhoff et al., addresses the role of autophagy in regulation of synaptic function. The main claim of the manuscript suggests a novel function of autophagy that does not depend on the turnover of damaged proteins and organelles. The study adds to growing evidence that neuronal autophagy has adapted to specific requirements of proteostasis imposed by synaptic neurotransmission. In brief, the authors propose that in excitatory neurons autophagy is triggered by starvation and that this type of autophagy in particular contributes to the turnover of the inhibitory R1α/β subunits of PKA and thereby indirectly promotes neuronal PKA signalling. The manuscript contains an impressive amount of data and the authors have used a broad array of different techniques to analyse ATG5 functions in neurons including whole animal experimentation. Seen from this perspective the paper deserves publication in a high-impact journal.

Response: We thank reviewer 1 for this positive comment on the novelty of our work.

However, I have several concerns that leave me lukewarm with a decisive recommendation for publication. While the evidence that alterations in PKA signalling, cAMP-dependent remodelling of cytoskeletal phosphoproteome, increased thickness of the PSD, alteration of AMPAR and augmented excitatory neurotransmission occur following ATG5-deletion is convincing, the different parts of the study are only loosely connected and the central hypothesis as stated in the last sentence of the Introduction 'Our findings identify a previously unknown role of starvation-induced autophagy in the regulation of PKA-dependent signaling in the brain, where it functions as a modulator of synaptic activity in cortical excitatory neurons.' is not really supported by the data to an extend that allows for a definite conclusion. Although this is potentially an important finding emphasizing the role of autophagy in regulation of signaling at synapses, several data sets are preliminary and important controls are lacking.

Response: We greatly appreciate the reviewer's comment that "the evidence that alterations in PKA signalling, cAMP-dependent remodelling of cytoskeletal phosphoproteome, increased thickness of the PSD, alteration of AMPAR and augmented excitatory neurotransmission occur following ATG5-deletion is convincing". We have now introduced the necessary controls and performed new experiments that support our central hypothesis

General comments
- The manuscript is difficult to read. In part because it contains a lot of data that do not really support the main story. On the other hand, the Introduction and the Discussion are very long and not always to the point.

Response: Following the reviewer's suggestion, we have now made a great effort to simplify the results. We have also removed some data and/or moved them to suppl. figures. We have also shortened the length and improved the content of the introduction and discussion.

- I don't like that dispensable data sets are intermingled with core experiments that provide novelty to the field. I sometimes had the impression that available data were put together to give the impression that the amount of evidence in support of the central hypothesis is broad and substantial. See my specific comments below.

Response: We have now simplified the results, divided the data into seven main figures instead of six, and deleted or moved some data into suppl. material.

- In several Figures it is unclear what the N-numbers reflect. In many panels I see only three data points per group. This raises doubts on the validity and appropriateness of the statistical tests.

Response: We always included the N numbers (which are independent experiments) and the corresponding number of neurons in the respective figure legend (e.g., WT=30 and KO=40 neurons in total per condition from N=3 independent experiments, where N would reflect the independent experiments). We have now followed the reviewer's suggestion and consistently replotted all graphs to visualize the distribution of the data and to account for the adequacy of the statistical tests. Statistical significance applies to both conditions. Additionally, we have also increased the number of N for some WB experiments, which contained only N=3 data points.

- Taken together, this is an interesting study that puts forward an interesting hypothesis.

Response: We thank reviewer 1 for this enthusiastic comment on our work.

However, I suggest to reorganize the manuscript, to remove data that do not contribute to the main conclusion and to test more stringently test the hypothesis that starvation-induced autophagy targets prominently inhibitory R1α/β subunits of PKA, which results in synaptic scaling processes.

Response: We thank the reviewer for this suggestion. We have now restructured the manuscript by removing some data that do not contribute to the main conclusion. We have also performed three new experiments to support our hypothesis: 1) analysis of PKA signaling at spines using a PSD-confined PKA-FRET probe, 2) STED microscopy of scaffold protein localization to the PSD in WT and KO, and 3) analysis of GLUR1 localization to spines and shafts of excitatory CamKIIα+ neurons in vivo.

- I don't understand the logic of the behavioral phenotyping. First, differences in rearing are only obvious in Atg5flox:Slc32a1-Cre KO mice with a deletion in inhibitory neuron. The corresponding knockout in excitatory neurons has no effect. Why then testing for the startle response in both lines?

Response: Autophagy has been previously shown to promote memory formation (Glatigny et al, 2019). Here wanted to further characterize the effects of ATG5 deletion in either excitatory or inhibitor neurons on mouse behavior. Behavioral analysis was performed using the SHIRPA-based phenotyping protocol (Hatcher et al., 2001), which includes a series of tests such as rearing and startle response, as well as locomotor activity, defecation, urination, and others. Each mouse line was subjected to SHIRPA-based phenotyping in the same manner. We apologize for not making this aspect more explicit in our original manuscript version. We now provide an elaborated table describing the SHIRPA assessment of both

mouse lines (Suppl. Figs. 1H,I), as well as the description of SHIRPA phenotyping in the Methods section.

If seizures are present only in Atg5flox/flox:CamKIIα-Cre mice why do the authors conclude that autophagy in general regulates intrinsic excitability. There are no direct measures of intrinsic excitability and why should moderate increases in surface expression of AMPAR cause seizures? Any published evidence for that?

Response: We apologize for not clearly explaining our rationale for the statement that "autophagy regulates intrinsic excitability." Increased excitability of GABAergic neurons would result in the absence of seizures due to increased network inhibition. However, we agree that the sentence quoted by the reviewer is misleading. Therefore, we have deleted this statement in the current version of the manuscript.

Several studies have reported a link between surface AMPA receptors (particularly the GLUR1 subunit, which mediates Ca2+ permeability of AMPA receptors) and epilepsy. For example, Liu et al. (2016, Sci. Reports) show that mice lacking TFR in neuronal progenitors develop seizures due to decreased endocytosis of GLUR1 and GLUR2 from the plasma membrane. In this work, the increase in surface fraction is approximately 1.2-1.3-fold, which is comparably low to our studies in which we find 1.5-fold more surface GLUR1. We have now normalized the data to WT to better illustrate this in the figure S8K. We believe this increase is substantial considering that approximately 50% of all AMPA receptors in KO neurons are now stranded at the surface. Furthermore, our new data indicate that KO neurons have increased numbers of synaptic GLUR1 receptors in vivo (Fig. 7F). Our gene ontology analysis in Fig. 7A also shows that autophagy-dependent PKA signaling may contribute to the seizure phenotype not only by regulating GLUR1 transport per se but also by altering the phosphorylation of a substantial fraction of postsynaptic cytoskeletal components that, when mutated, are known to cause EEG abnormalities associated with seizures in humans. We have now made this point more clearly in our manuscript.

Other studies reporting the association between surface AMPAR and seizures are:

1. Wang et al., 2021 Cell Death and Disease
2. Joshi & Kapur, 2018, Epilepsia

Also reviewed in Guo & Ma, 2021 Front in Neural Circuits. This work is cited in the manuscript.

Wouldn't one expect an effect on cognitive behavior; i.e. hippocampus-dependent learning and memory? All these parts are loosely and not convincingly connected.

Response: We thank reviewer 1 for this suggestion. As mentioned above, autophagy has previously been shown to promote memory formation (Glatigny et al., 2019). Here, we sought to perform global phenotyping of mice with ATG5 deletion in excitatory or inhibitory neurons. Our behavioral phenotyping was performed using the SHIRPA protocol, which is now available in Suppl. Fig. 1H,I.

-Why two different proteomic approaches? On my opinion the SILAC data are hardy quantitative. How good is the overlap between SILAC and label-free proteomics? I see only GABARAPL2 and two other proteins consistently regulated. Is this believable? If the latter one based on FACS-sorting is superior why did the authors start with SILAC? The differences are relatively small and it is surprising how different the results are as compared to previously published studies on autophagosome content profiling. I don't see how different methods of purification can account for these discrepant results and if yes this has to be discussed.

Response: SILAC- and FACS-based proteomics capture changes in different cellular compartments and were used as complementary approaches in the current study. FACS-based proteomics identifies cellular soma-intrinsic changes, whereas SILAC-based proteomics reports on bulk changes in brain lysates and

may additionally reflect differences in dendritic/axonal compartments. The overlap between the two would only report similarities in the soma. We believe it was beneficial to combine both approaches to obtain an unbiased view of proteomic changes in autophagy-deficient brains.
All published data describe content profiling of autophagosomes isolated from the total brain (for instance Goldsmith et al., 2022). Glia cells make up at least half of all brain cells (in rodents, about 50% of all brain cells are glial cells, Herculano-Houzel, 2014), and thus the published data report autophagosome cargo from a mixed neuron/glia population. We believe this is a major discrepancy between the published data and our work describing proteome changes, specifically in autophagy-deficient excitatory or inhibitory forebrain neurons. We have included reference to this discrepancy in the Discussion of our current manuscript. We are not aware of any data reporting autophagosome profiling from these neuron-only populations.
As mentioned below, FACS and SILAC are two complementary approaches that must be performed at two different time points due to technical limitations. Therefore, we believe that the overlap of the deregulated proteome from SILAC and FACS is not beneficial. We believe that these data taken together demonstrate that PKA inhibitory subunits are upregulated across ages, compartments, and neuronal classes under ATG5-deficient conditions.

- Neuronal autophagosome content profiling was not done under starvation conditions in this study. If starvation triggers selective autophagy of inhibitory R1α/β subunits of PKA it is hard to understand why those proteins are most prominently regulated in the ko mice. Any mechanistic idea? Isn't it surprising given the tremendous upregulation of p62 that other changes are relatively mild?

Response: We thank the reviewer for this comment, which raises an important point that we did not fully address in the previous version of the manuscript. Our original data revealed that starvation decreases R1α levels independently of mTORC1 activity. Early work in Aplysia has established that cAMP levels per se are crucial regulators of PKA R1α/β degradation, which causes the holoenzyme to dissociate, thus freeing R subunits that are then targeted for ubiquitin-mediated degradation (Chain et al, 1999). To test whether starvation regulates PKA R1α/β degradation by upregulating cAMP levels per se we have now analyzed cAMP levels in cortical primary neurons subjected to 16h starvation. We found that starvation increased cAMP levels by about 20% (Fig. 4D,E), indicating that the effect of starvation on PKA R1α degradation is likely not mediated by its effect on autophagy induction. In support of this hypothesis are our data that PKA R1α levels can be stabilized in NSC34 cells cultured in normal media and treated with bafilomycin A1 (Fig. 4H,I), suggesting that basal autophagy is likely directly responsible for R1α degradation.

We therefore speculate that it is not recruitment to autophagosomes but the dissociation of the PKA complex that is triggered by starvation in neurons (also presented as a scheme in Fig. 8). Consistent with our new data, we hypothesize that starvation may trigger PKA signaling by facilitating dissociation of the PKA complex, for example, by upregulating intracellular cAMP levels. The starvation-induced increase in cAMP levels causes dissociation of the PKA holoenzyme, thereby releasing the C and R subunits. Subsequently, the PKA R1a/b subunits are sequestered by autophagosomes constitutively formed at synapses. We have now carefully re-written the manuscript to precisely state this hypothesis.

- It should be clearly stated that proteomics with Atg5flox:Slc32a1-Cre KO was done with striatal tissue, which is hidden in the Figure legends. Thus, the analysis was done with medium spiny neurons, which are different from interneurons. It is important to discuss this. Moreover, if one compares different brain regions a direct comparison of excitatory and inhibitory neurons is not meaningful. It was difficult to follow up on this and I am not sure but it looks like that all proteomic experiments were done with neurons of different age. The question arises what do we learn from these data?

Response: The reason for using cortical and striatal tissue is that most neurons in the cortex are glutamatergic, whereas most in the striatum are GABAergic. Using these two distinct regions of the mouse forebrain, we were able to link changes in the measured proteome to KO neurons. Because we are working with conditional KO mice, only by using these brain regions could we assume that the proteomic changes were due to the loss of ATG5 in our samples. We agree with the reviewer that medium spiny neurons are not interneurons. However, we would like to note that we do not mention the term "interneurons" in our manuscript. Our intention was to show the proteome changes in autophagy-deficient excitatory and inhibitory neurons of the forebrain. We have now included a statement in the current version of the manuscript outlining the rationale for using these two brain areas for proteomic analysis.

For SILAC experiments, both mouse lines were always analyzed at 12-13 weeks of age, because seizures in animals lacking ATG5 in forebrain excitatory neurons begin at approximately 10 weeks of age. FACS-based analysis was always performed with 3-week-old animals. As indicated in the manuscript, 3-week-old brain tissue is the age at which the contribution of debris is still relatively low. FACS sorting of neurons from older mouse brains is technically challenging.

- I have some concerns related to pictorial evidence for starvation-induced autophagy in neurons. Several reports have shown that autophagy in neurons is a constitutive process and starvation protocols involving nutrient deprivation have little or no effect on the rate of autophagosome formation (Maday S, Holzbaur EL (2014); Maday S, Holzbaur EL (2016); Lee S, Sato Y, Nixon RA (2011).

Response: We fully agree that the role of starvation-induced autophagy in neurons is controversial. We have now rewritten the introduction to reflect this point (also citing the work suggested by the reviewer).

The lack of classical excitatory and inhibitory neuronal markers, as well as glial markers makes it difficult to exclude the role of starvation in upregulation of inhibitory subunits of PKA in glia cells.

Response: We thank the reviewer for this suggestion. The current version of the manuscript includes the controls suggested by the reviewer (see details below).

- In Figure 1A and B I am surprised to see a complete downregulation of ATG5 in either excitatory or inhibitory neurons in comparison to cortical brain lysates from wild type animals. It's important to load samples onto the same gel to allow for a direct comparison on the ATG5 expression levels in all mouse lines. Why have the authors used b-actin in panel A and GAPDH in panel B for normalization?

Response: Fig. 1A shows ATG5 levels in the cortex (CamKIIα-Cre line), whereas 1C shows ATG5 levels in the striatum (Slc32a1-Cre line). The reason for using cortical and striatal tissue (also noted by the second reviewer) is that the majority of neurons in the cortex are glutamatergic, whereas the majority in the striatum are GABAergic. Because we are working with conditional KO mice, only by using these brain regions could we can assume that the proteomic changes were due to the loss of ATG5 in our samples. We did not use the same loading control because we quantified ATG5 levels between WT and KO per line and not for comparison between lines. We have now followed the reviewer's suggestion and quantified ATG5 levels in cortical and striatal WT/KO lysates run on the same gel normalized to the same loading control (b-actin). These data are included in Figs. 1 A-D.

- In the Results section under the heading "PKA R1α/β levels are highly upregulated in autophagy-deficient excitatory and inhibitory neurons" the authors conclude that autophagy controls neuronal excitation by regulating PKA signaling. Augmented excitatory

neurotransmission in mice lacking ATG5 was already reported. The gain in excitatory neurotransmission was shown to be a consequence of elevated calcium release from ER stores at presynaptic sites due to selective accumulation of tubular ER (Kuijpers et al., 2021) that is a major structural alteration and regulation of PKA signaling under these conditions might not have a causative role. Clear and conclusive evidence that PKA signaling is instrumental for the gain in excitatory transmission is lacking.

Response: We appreciate and understand the reviewer's concerns. We agree that the conclusion is misleading because the data supporting the fact that autophagy controls neuronal excitability by controlling PKA signaling are presented later in the manuscript (Fig. 7). Therefore, we have corrected it to avoid misunderstanding. This sentence now reads as follows:

"This established role of PKA in regulating synaptic function together with our findings indicating an increase in protein levels of inhibitory R1α- and R1β-subunits of the PKA complex in autophagy-deficient neurons prompted us to further investigate the link between autophagy, PKA signaling and neuronal function".

Of course, we are also aware of the Kuijpers et al. 2021 study, which we have cited several times in our manuscript. Our study differs from the 2021 study by Kuijpers et al. in several aspects such as:

1. The proteomic analysis of Kuijpers et al. was performed with cultured cerebellar granule neurons (not in vivo and not in the cortex, or striatum). These data cannot be directly projected to our work. We did not detect changes in tubular ER (ie, reticulon 3, VapB, and the ryanodine receptor (RyR)) in ATG5-deficient cortical excitatory neurons in-vivo.
2. Electrophysiological analysis in Kuijpers et al. was performed in hippocampal slices from mice in which ATG5 deletion was driven by the EMX1 promoter. EMX1-expressing progenitors produce projection neurons, oligodendrocytes, and astrocytes. Therefore, the hyperexcitability phenotype described in Kuijpers et al cannot be attributed to changes in excitatory glutamatergic neurons and cannot be directly compared with our study reporting changes in hyperexcitability in mice lacking ATG5, specifically in postmitotic neurons.
3. The authors of Kuijpers et al. conclude that "the increased excitatory neurotransmission in ATG5-cKO mice is a presynaptic phenotype." Our work describes the hyperexcitability in ATG5-cKO mice that originates from the postsynaptic side of the synapse. We believe that our data do not contradict this but instead, add a perspective in which hyperexcitability in ATG5KO mice may also arise from postsynaptic dysfunction.
4. Finally, in contrast to our work, Kuijpers et al. do not report seizures in mice in which the EMX1 promoter drives ATG5 deletion.

We have now carefully rewritten the manuscript to address this important point raised by the reviewer.

Furthermore, we have now included new data indicating that the GCAMP7 excitability phenotype in dendrites of ATG5 KO excitatory neurons is primarily a consequence of extracellular Ca^{2+} entry (fig. S8N,O), suggesting that the contribution of Ca^{2+} from intracellular stores i.e. ER is small under this condition.

- An intriguing finding appears be that mTORC1 plays no role in the induction of the starvation-induced neuronal autophagy investigated in this study. However, the presented data are rather preliminary and the current data set does not add much to the story without further mechanistic insights.

Response: We thank the reviewer for this comment. We agree that this result is intriguing. Indeed, our new data suggest (as also mentioned above) that the effect on PKA R1a/b degradation is not mediated by starvation-induced autophagy per se but is rather a result of the upregulation of cAMP levels under starvation conditions. The starvation-induced increase in cAMP levels causes dissociation of the PKA holoenzyme, releasing the C and R subunits. Subsequently, the PKA R1a/b subunits are sequestered by

autophagosomes, which are constitutively formed at synapses. The absence of mTORC1 inhibition on the rate of PKA R1a/b degradation is consistent with this hypothesis.

- Fig. 2 panels E-H. Neuron-specific markers (inhibitory and excitatory) are missing making it difficult to judge the neuron-specific upregulation of the inhibitory subunits of PKA. I am also surprised that upregulation of PKA- R1α/β is largely somatic. Can the authors comment on that?

Response: Our reason for not showing IHC analyses with neuron-specific markers was that FACS-based analyses with CamKIIa-expressing or VGAT (Scl32a1)-expressing neurons from tdTomato reporter mice show neuron-intrinsic upregulation of PKA R1 subunits in KO neurons, suggesting that these changes are specific to neurons. We have now followed the reviewer's suggestion, and the current version of the manuscript includes the following controls:

1. Examples of PKA R1a/b immunostainings in brains carrying tdTomato additionally as a neuronal reporter, which reveal confinement of PKA R1 either to excitatory or inhibitory neurons (corresponds to the FACS-based proteomics data). Please see Fig. 2K,L.
2. Quantification of PKA R1a in brains additionally co-stained with neuronal marker NeuN, and glial markers GFAP and MBP. Please see Fig. 2M-R and Suppl. Fig. 3O-R.

The upregulation of PKA R1 alpha/beta looks largely somatic due to a different ratio of soma/neuronal processes volume. However, we would like to note that PKA R1 is also enriched in the neuropil and synapses (Fig. 3). One possibility for somatic enrichment of PKA R1 in autophagy-deficient neurons is that type 1 PKA is mostly soluble, whereas type 2 can be bound to AKAPs. Thus, it is likely that "undegraded" PKA R1a will freely diffuse in the neuron, ultimately accumulating in the cell body due to its larger volume.

In general, the manuscript would profit from quantitation of immunocytochemical data showing enrichment of PKA- R1α/β at specific cellular compartments. As mentioned above, glial markers and quantification of PKA- R1α/β within glial cells should be done to support the conclusions.

Response: We have followed the reviewer's suggestion and included the requested controls in the current version of the manuscript, as described above. We now also report the quantification of the Mander's colocalization coefficient of PKA R1a/b with Bassoon and PSD95 (Fig. 3).

- Fig. 2J-K. The increase in Pearson`s or Manders co-localization coefficient between PKA-R1β/Bassoon and PKA- R1β/PSD95 upon the different treatments should be indicated.

Response: As mentioned above, we have now presented the analysis of Mander's colocalization coefficients of PKA R1a/b with Bassoon and PKA R1alpha with PSD95 (Fig. 3).

- Fig.2 panel L show occasional single spots of PKA-R1α in several subcellular compartments. Also, one or two gold particle per image are not convincing to demonstrate upregulation in ATG5 deficient mice. The manuscript would profit from quantification that shows enrichment of PKA-R1α at synaptic compartments based on the number of particles per compartment. It is not clear how the mean of gold particle/volume fraction was quantified (panel M).

Response: EM analysis was performed using a standard stereology-based procedure in which the amount of gold dots per compartment is normalized to the volume fraction of that compartment in the grid. This is described in M&M as follows: "The reference area of sub-neuropil structures was determined by superimposing a grid over the neuropil images (volume fraction estimation). The number of gold particles found at those structures was normalized to the volume fraction corresponding structures in the neuropil". We have now followed the reviewer's suggestion and re-plotted the data as the number of gold particles per compartment and present this as a new quantification in Fig. 3J.

- Alterations in PKA signaling are shown indirectly by alterations is total phosphorylation of several substrates. To make a claim that PKA signaling in ATG5 deficient mice is altered at synaptic compartments, the authors should consider to isolate synaptosomes or to perform live imaging with a PKA sensor (PKA-FRET probe) targeted to postsynaptic compartments.

Response: We thank the Reviewer for this suggestion. Unfortunately, the isolation of brain synaptosomes will not work since we work with conditional KO mice (and thus isolation of pure KO synaptosomes is not possible). However, we have now performed imaging of PKA activity in synaptic compartments using the PKA-FRET/FLIM sensor targeted to PSD-95 (AKARet-PSD). This data are now presented in Fig. 6I,J.

- Fig. 4K Changes in pCREB levels should be indicated with the gradient LUT to visualize differences in CREB activation. Neuron-specific marker should be added.

Response: We thank the reviewer for this suggestion. We have now indicated a LUT gradient to visualize the difference in CREB activation. We have also added DIC images to reveal the neuronal morphology of cells with active CREB in the nucleus. We identified neurons by their polarized morphology in DIC for analysis. This point is now stated in the current version of M&M in the manuscript and DIC pictures provided in the figure panels.

- Given the large number of transcripts regulated in response to ATG5 deletion (Fig. 4G, Suppl. Table 2) it is highly likely that the phenotype might also be caused by changes in gene expression and PKA-signaling at synaptic sites. The authors appear to ignore this possibility.

Response: Although the heat map plot indicates changes in gene expression, our differential gene expression (DGE) analysis shows only 8 genes significantly deregulated in ATG5 KO condition (with FDR<0.05) (see Fig. S6D). The expression of the remaining genes is not significantly altered between genotypes. None of these 8 genes, to our knowledge, is directly involved in the regulation of PKA signaling at synapses. For this reason, we did not consider the possibility that gene expression per se is a regulator of PKA signaling in KO neurons. Moreover, our proteomic analysis did not reveal any changes in the expression of proteins encoded by these genes.

- Fig. 5K It looks like that synapses characterized by increased volume of PSDs have reduced numbers of presynaptic vesicles. Can the authors comment on that? Finally, I don't see any causative relationship between changes in PSD-size and the proposed mechanism.

Response: We thank the reviewer for this suggestion. We have now analyzed SV density in WT and KO neurons, and our analysis shows no changes between genotypes (fig. S7K). As we discuss in the Results section, “the postsynaptic density of synapses contains cytoskeletal scaffold proteins, receptors, and ion channels, and the phosphorylation state of these components is known to be central to synaptic transmission (Esteban et al., 2003). The size of postsynaptic density is a common characteristic of neuronal excitability per se, where the larger size of the PSD is positively correlated with higher numbers of AMPA-type glutamate receptors (AMPARs) (Takumi et al., 1999; Tao-Cheng, 2019). For instance, the anchoring of DLG at synapses is optimal when DLG is in the dephosphorylated state (Koh et al., 1999)”. Thus, decreased phosphorylation of postsynaptic scaffolding proteins could increase their localization at the synapse and thus increase postsynaptic density area in the EM. In fact, our new data on STED-based analysis of CASKIN1 and SHANK3 colocalization with PSD95 are in line with this hypothesis (Fig. 6K-M).

The pictorial evidence in Fig. 6C is of rather poor quality. The GLUR1 channel looks saturated. Puncta are very broad and cover the entire dendrite. How was this analysis performed? What is

the ratio of synaptic GluR1 to those present in the dendritic shaft. Without a volume fill it will be difficult ta make assessment.

Response: We have provided analysis description in our previous Methods chapter: “Colocalization of AMPA receptor subunit GLUR1 and postsynaptic density marker protein PDS95 were assessed in Atg5flox:CAG-Cre^{Tmx} neurons in a semi-automated fashion using a macro-tool. For colocalization analysis an ROI were set in an area devoid of somata. Each channel was individually smoothened and binarized with a user-defined threshold before calculating the overlap area by dividing the overlap between PSD95 and GLUR1 by total GLUR1 signal (custom written Image J script is available as Data S2”.

We now followed the reviewer's suggestion and analyzed the colocalization of GLUR1 and PSD95 separately for the spine and dendritic shafts in WT and ATG5-KO excitatory neurons in vivo, additionally having tdTomato as "volume filling" (Figs. 7C-F). We also replaced images for the data set for cultured neurons (fig. S7G).

Pictorial evidence for the quantitation in Fig. 6 E and F is lacking. How were expression levels of eGFP-ATG5 determined?

Response: We apologize for not including the images supporting the data in Figs. 6E and F. These are now included as part of Fig. 7 (G,I). The expression level of ATG5 was determined by the eGFP signal. The functionality of this construct has been demonstrated in overexpression studies (Negrete et al., 2020, Nat. Comm). We have now included this information in the current version of the manuscript.

Quantitation for fig. S6L is mandatory.

Response: We performed the analysis of GLUR1 on spines and shafts in WT and KO excitatory neurons in vivo, additionally having tdTomato as "volume fill" (Fig. 7C-F).

- Fig. 6J Why only three peaks in knockout neurons following tetanic stimulation (not the case in k)? How were the data normalized? Peaks are very broad (>3-5 seconds). This looks odd to me. Are these somatic/nuclear calcium waves?

Response: Fig. 6J (new Fig. 7K) shows the responses of WT and KO neurons to four tetanic stimulations of 1 second duration each with an interval of 3 seconds. Peaks in the WT and KO are normalized to the first peak, which is set to 1 ($\Delta F/F_{peak1}$). Thus, in both the WT and KO, there are four peaks (we have now moved to the front the orange color to make it more visible), but KO neurons facilitate more, resulting in a higher difference between the fourth peak and the first peak (Fig. 7M). Application of CNQX completely abolishes this facilitation.
The peaks represent GCAMP7 responses to electric field stimulations and were measured at the cell soma. We used somatic calcium signals as a proxy for spike activity (Ali & Kwan, 2019). These differences between genotypes are completely abolished under Ca2+-free extracellular conditions (new data set Fig. S7N,O), suggesting that these signals are likely not nuclear calcium waves. However, we cannot exclude the possibility that changes in intracellular Ca2+ due to extracellular influx may cause the appearance of nuclear calcium waves in KO.

How about dendrites?

Response: We thank the reviewer for this suggestion. We have now added quantification of GCAMP7f responses in dendrites of excitatory WT and ATG5 KO neurons (which would represent the changes in calcium due to AMPA/NMDA receptor activity or reflect the backward propagating action potential) (fig. S7L, N,O). An increase in calcium response in autophagy-deficient dendrites can be blocked by performing experiments with 0 mM extracellular Ca2+ and using AMPAR antagonist CNQX. This suggests that the observed phenotype is the result of increased extracellular Ca2+ entry (likely via

AMPA/NMDA receptors and/or voltage-gated extracellular Ca2+ channels). AMPA receptors may either directly contribute to increased intracellular calcium levels (since GLUR1-containing AMPARs are themselves permeable to calcium, or their depolarization-generated activation reverses the magnesium blockade of the NMDA receptor).

What is the biological meaning of the comparison in Fig. 6L? The most striking differences in deltaF/F are in peak amplitude. How are AMPAR coupled to calcium release to allow for such a pronounced difference if this is not an artifact of buffer equilibration? This phenotype is similar to the one reported in Kuijpers et al.. Does it require RyR-dependent ER-calcium release?

Response: As we noted in the old version of the discussion and now in the results section: “In contrast to the majority of AMPARs, which contain GLUR2, GLUR1-containing AMPARs are Ca2+ permeable (Jonas & Burnashev, 1995)”. Thus, increased density of GLUR1-containing AMPAR may alter calcium signaling and cause neuronal hyperexcitability in KO neurons. This scenario is in agreement with the fact that application of CNQX completely rescues the GCAMP7 phenotype in the soma and dendrites of KO cells. Furthermore, we have now included new data indicating that the GCAMP7 excitability phenotype in dendrites of ATG5 KO excitatory neurons is primarily a consequence of extracellular Ca^{2+} entry (fig. S8N,O), suggesting that the contribution of Ca^{2+} from intracellular stores i.e. ER is small under this condition.

As stated above, we are also aware of the Kuijpers et al. 2021 study. Our study differs from the 2021 study by Kuijpers et al. in several aspects such as:

1. The proteomic analysis of Kuijpers et al. was performed with cultured cerebellar granule neurons (not in vivo and not in the cortex, or striatum). These data cannot be directly projected to our work. We did not detect changes in tubular ER (ie, reticulon 3, VapB, and the ryanodine receptor (RyR)) in ATG5-deficient cortical excitatory neurons in-vivo.
2. Electrophysiological analysis in Kuijpers et al. was performed in hippocampal slices from mice in which ATG5 deletion was driven by the EMX1 promoter. EMX1-expressing progenitors produce projection neurons, oligodendrocytes, and astrocytes. Therefore, the hyperexcitability phenotype described in Kuijpers et al cannot be attributed to changes in excitatory glutamatergic neurons and cannot be directly compared with our study reporting changes in hyperexcitability in mice lacking ATG5 specifically in postmitotic neurons.
3. The authors of Kuijpers et al. conclude that "the increased excitatory neurotransmission in ATG5-cKO mice is a presynaptic phenotype." Our work describes the hyperexcitability in ATG5-cKO mice that originates from the postsynaptic side of the synapse. We believe that our data do not contradict this, but instead add a perspective in which hyperexcitability in ATG5KO mice may also arise from postsynaptic dysfunction.
4. Finally, in contrast to our work, Kuijpers et al. do not report seizures in mice in which the EMX1 promoter drives ATG5 deletion.

All in all, our results differ from those of Kuijpers et al. in that i) we demonstrate the "postsynaptic" effect of autophagy deletion at synapses and ii) we do not detect changes in tubular ER components, including RyR, in cortical glutamatergic neurons.

We have now carefully rewritten the Results and Discussion secitons to address this important point raised by the reviewer.

- I found it very hard to understand what is shown in Fig. 6P-S. These are supposedly MEA-recordings. If yes, the cultures have a high baseline activity. What are the culture conditions? The knockout effect on spiking is tremendous. I would expect a huge increase in surface expression of AMPAR to explain this finding.

Response: We apologize for not indicating more clearly that the example traces in Fig. 6P-S (new Fig. 7P) represent network activity of primary cortico-hippocampal WT/KO neurons analyzed with the MEA system. We now clearly state this in the results section. The mean frequency of neuronal firing in WT neurons is 0.589±0.285 Hz (also see Fig, 7Q), which is rather on the lower side of baseline neuronal activity. The culturing conditions are described in detail in M&M:
"Cortico-hippocampal neurons from postnatal Atg5flox:flox mice were isolated as described before (Kononenko et al., 2017) and plated in 24-well plates with gold electrodes on epoxy (24W700/100F-288, Multi Channel Systems). At DIV5, the KO was induced by transduction with CamKIIα-Cre (AAV9.CamKII.HI.eGFP-Cre.WPRESV40, Penn Vector Core). CamKIIα-eGFP (AAV9.CamKII0.4.eGFP.WPRE.rBG, Penn Vector Core) was used for the control group. MEA recordings were acquired at DIV15 using the Multiwell-Screen software of the Multiwell-MEA-System (Multi Channel Systems). Recordings were taken for 3 min with a sampling rate of 20000 Hz and automatic threshold estimation".

As mentioned earlier, several studies have reported the link between surface AMPA receptors (particularly the GLUR1 subunit, which mediates Ca2+ permeability of AMPAR) and epilepsy. For example, Liu et al. (2016, Sci. Reports) show that mice lacking TFR in neuronal progenitors develop seizures due to decreased endocytosis of GLUR1 and GLUR2 from the plasma membrane. In this work, the increase in surface fraction is approximately 1.2-1.3-fold, which is comparatively small to our studies in which we find 1.5-fold more surface GLUR1 in KO (32% in WT, 48% in KO). We believe this increase is substantial considering that approximately 50% of AMPA receptors in KO neurons are now stranded at the surface.

Moreover, our Gene Ontology analysis in Fig. 7A shows that PKA likely contributes to the seizure phenotype not only by regulating GLUR1 transport per se but also by altering the phosphorylation of a significant portion of postsynaptic cytoskeletal components that, when mutated, are known to cause EEG abnormalities that are accompanied by seizures in humans. We have now made this point clear in our manuscript.

- Fig. 6 T and U show increased sAMPAR frequency and amplitude in some but not all cells. Statistical analysis is lacking.

Response: Statistical analysis was shown in the original legend for the Fig. 6 (current Fig. 7S,T).

"(WT: 7.75± 0.72 pA, KO: 9.79± 2.75 pA; nWT=11, nKO=9; unpaired mean difference between WT and KO (ΔWT-KO): 2.04 [95%CI: 0.629, 4.22]; pWT/KO=0.0152 (two-sided permutation t-test)).
and WT (WT: 5.27± 2.66 Hz, KO: 9.47± 5.16 Hz; nWT=11, nKO=9; ΔWT-KO: 4.19 [95%CI: 0.57, 7.56]; pWT/KO=0.0326 (two-sided permutation t-test))".

Referee #2:

The study by Overhoff et al. describes a new unexpected function of autophagy in neurons, where autophagy controls the levels of PKA activity by degrading PKA regulatory subunits. The authors demonstrate that impairment of autophagy through genetic ablation of Atg5 inhibits PKA signaling, causing on the one hand changes in CREB1-mediated gene expression, and on the other hand diminished phosphorylation of postsynaptic scaffolding proteins. This in turn leads to altered AMPA-receptor trafficking and to neuronal hyperexcitability, revealed by seizures at the organismal level.
The findings are novel and exciting, the study is very comprehensive, uses an impressive spectrum of in vivo and in vitro techniques, and dissects the molecular mechanism very

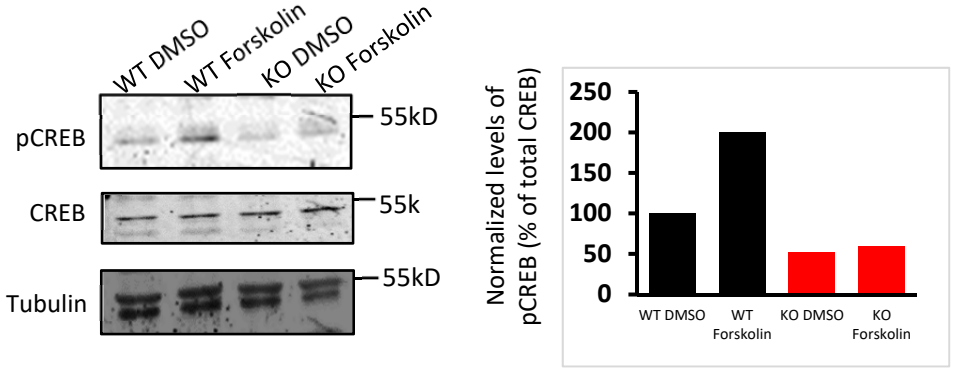
thoroughly.

Response: We thank the reviewer 2 for this positive comment on our work.

Main comments:

1. Fig. 4A, C and N: it looks like pCREB and total CREB levels were probed on different blots. This should be done on the same blot. In addition, the forskolin-induced pCREB enhancement in KO MEFs appears to be of a similar magnitude as in WT (about 2.5-fold, Fig. 4D), so it is difficult to agree with the conclusion that ATG5 is important for regulating PKA signaling in these cells. As this piece of data is not crucial for the story, the authors could consider removing it.

Response: Thank you for this suggestion. Because of very similar molecular weight, this can be done using the LI-COR Odyssey Imaging System. We followed the suggestion and performed an experiment in which the pCREB/CREB and tubulin signals were imaged on the same blot using LI-COR. The results are shown below. The results reflect the data in Fig. 5A. To repeat all experiments using the LI-COR Odyssey Imaging System, we would need to prepare new lysates from our primary neurons by sacrificing 3 mice per culture dish for each N of the experiment (at least 12 mice in total for N=4). This would be incompatible with performing all other experiments for the revision of this manuscript.



We would like to mention that for quantification of the data set shown in the manuscript, pCREB and CREB levels were analyzed using the same protein lysate and treated in parallel on the gels and membranes. Each of the signals for pCREB and CREB was first normalized to the respective loading control (to account for the loading error) before calculating the pCREB/CREB ratio. We apologize that this was not mentioned in the previous version of the manuscript. The current version now contains this statement. In addition, we would like to point out that pCREB levels were also significantly reduced in high-content screening microscopy.

We also followed the reviewer's comment and removed the analysis of CREB signaling in MEFs cells from the manuscript.

2. While pCREB activation measurements by high content screening microscopy (Fig. 4E-F) were conducted in cortical cells, cAMP levels shown in Fig. S4A were measured in the hippocampus. These analyses should be conducted in the same tissue to be comparable.

Response: We would like to note that cAMP was also analyzed in cortical neurons using high-content screening microscopy, and these data were shown in Fig. S4C (new: Fig. S6B,C).

3. Fig. S6L: the authors should perform a quantification of GluR1/PSD-95 colocalization in vivo.

Response: We performed the analysis of GLUR1 on spines and shafts in WT and KO excitatory neurons in vivo, additionally expressing tdTomato as "volume fill" (Fig. 7C-F).

4. Measuring the area of the postsynaptic density (Fig. 5K, L) is quite a crude and indirect way of showing altered localization of postsynaptic proteins. Ideally, it would be great to use super-resolution microscopy to convincingly demonstrate a change in localization of one or two candidate scaffolding proteins.

Response: We thank the reviewer for this excellent suggestion. We have now performed STED-based superresolution microscopy to demonstrate the changes in localization of two proteins, CASKIN1 and SHANK3, at the PSD of KO neurons (Fig. 6K-M).

5. I think some conclusions and claims are overstated:
The conclusion of the first section of results (p. 5): "...ATG5 functions in ... neurons to regulate their intrinsic excitability" is not supported by data, as other reasons of network rearrangement beyond neurodegeneration have not been explored and cannot be excluded. I recommend rephrasing the conclusion based on the presented data.

p. 7: "In agreement with robust upregulation of PKA subunits..." - especially in the FACS-sorted samples, the upregulation is not robust.

p. 15: "...Our data suggest that ATG5-dependent protein phosphorylation can serve as a crucial regulator of postsynaptic protein localization and trafficking" - at this point in the manuscript, altered localization or trafficking has not yet been demonstrated for any postsynaptic protein.

Response: We thank the reviewer for these suggestions. We have now changed the text according to the reviewer's suggestions.

6. Some of the figures are extremely packed, and the labeling on the panels very difficult to read (e.g., Suppl. Fig. S1 panels O-R, Fig. 4G, Fig. S5E, Fig. S6K). Could you please make these panels more reader-friendly? I recommend dividing Fig. 1 and Fig. S1 into two (phenotypic analysis of knockout mice / proteomics).

Response: We have now rearranged the panels as suggested by the reviewer. We have now created 7 main and 8 supplementary figures.

Minor points:
7. It would be useful to mention that the majority of neurons in the cortex are glutamatergic, while the majority in the striatum are GABAergic, to provide a rationale for doing the biochemical assays with these brain regions in the respective knockout mice.

Response: We thank the reviewer for this comment. We have now incorporated this information into the manuscript.

8. Fig. 1A-B and S1A, D - Figure legends mention Atg5, but Western blots and graphs are labeled with Atg5-Atg12. Please clarify.

Response: We apologize for this mistake. In fact, the figure legend should say ATG5-ATG12, since the observed band represents the ATG5-ATG12 conjugate. We have corrected now the figure legend.

9. It is confusing that the occurrence of seizures in Atg5flox:Slc32a1-Cre mice is shown twice (Fig. 1H and S1N), with different results. I think one piece of data in the supplementary figure would be sufficient.

Response: Our intention was to clarify the fact that although young adult Atg5flox:Slc32a1-Cre mice do not show seizures (new Fig. 1L), older KO animals at approximately 1 year of age (new Fig. S1J) show minor seizure events. We apologize that this point was not clearly presented in the previous version of the manuscript. We have now reorganized the data to more accurately illustrate this fact.

10. Legend to Fig. 3C: Level of PKA R1alpha in "starvation + BafA1" is indicated as 48.52% - should probably be 148.52%?

Response: We apologize for this mistake. We have now corrected it in the figure legend.

11. Fig. 3I-J: please show control images

Response: We have now added control images to Fig. S5D-F.

12. Fig. 3L and 4M: please start the y-axis from zero.

Response: We have now corrected the y-axis so that it starts at zero.

13. Please explain the boxes in Fig. 4N in the figure legend.

Response: This data set is now removed from the manuscript to focus on the main conclusions of the work, as suggested by reviewer 1.

14. Panel S6O uses the abbreviation VGAT, not used anywhere else in the paper. Please make consistent.

Response: We now use the designation Slc32a1 throughout the manuscript.

Further suggestions:

15. I think it would be very helpful to include a graphical summary of the described molecular mechanism as an additional panel in the last figure.

Response: We thank the reviewer for this suggestion and present a graphical summary as the final Fig. 8.

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Dear Natalia,

Thank you for submitting your revised manuscript to The EMBO Journal. Your study has now been seen by the two referees and the comments are provided below.

As you can see both referees appreciate that the introduced changes.

Referee #1 finds that there is still room for improvement regarding the writing. The referee also suggests that some of the data can be removed. I think fine to leave the data in but take a careful look at the text and make sure that it flows well, avoid any over statements and make sure that you have a balanced discussion.

Referee #2 raises some good points that I would like to ask you to address in a revised version.

When you submit the revised version will you also please take care of the following points:

- Please correct heading "Data and materials availability" to "Data availability". Make sure to add the accession numbers to the datasets as well.

- Conflict of Interest should be corrected to Disclosure and Competing Interests

- The CRediT contributor roles taxonomy is used to specify the contributions of each author in the journal submission system. Please use the free text box in the 'author information' section of the manuscript submisssion system to provide more detailed descriptions (e.g., 'X provided intracellular Ca++ measurements in fig Y'). CRediT replaces the 'Author Contributions' section..

- Please check the reference formal. Citations with more than 10 authors should be cut after 10 authors et al.

- Tables S1-S4 should be named Dataset EV1-EV4 and the colors should be removed from the tables. The legends should be add to each file on the first sheet.

- For the appendix: for the ToC please include only the data that is provided in the appendix file. Tables S5-S9 should be named Appendix Tables S1-S5. Please also correct callout in text. The references should be listed alphabetically.

- I wonder if you should include DataS1 in the appendix.

- The movie needs to be zipped together with its legend. The name should be Movie EV1.

- We encourage the publication of source data for electrophoretic blots and quantitative data. Take a look at this document for how to upload and organize

https://www.embopress.org/pb-assets/embo-site/Guide%20for%20SourceData%20Submission-1656066810500.pdf

- Our publisher has also done their pre-publication check on your manuscript. When you log into the manuscript submission system you will see the file "Data Edited Manuscript file". Please take a look at the word file and the comments regarding the figure legends and respond to the issues.

- "Methods" needs correcting to Materials and Methods.

- Heading "Main Figures" should be corrected to Figure Legends.

- The Acknowledgement and COI sections should be above the Reference section.

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels).

That should be all let me know if we need to discuss anything further

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The authors have addressed most of my concerns. Most important, they have included new experiments that support the hypothesis that PKA signaling in particular at synapses is affected following ATG5-gene deletion. The authors also discuss now why content profiling of autophagosomes in other studies revealed vastly different results. They explain the differences in protocols, transgenic mouse models and purification schemes. However, I still have the impression that the different parts of the manuscript are not well connected and the manuscript is still difficult to read. Some data sets are on my opinion dispensable and do not add much to the principal findings. The observed behavioral phenotypes are very mild and the behavioral seizures are not really substantiated by the other data. There is no real link of this analysis to PKA signaling. The data can be removed. I still believe that one would expect phenotypes in cognitive behavior; i.e. hippocampus-dependent learning and memory. But such experiments were not done. The link to the seizure phenotype is still far fetched. The in vivo electrophysiological analysis is not quantitative. Without a rescue and further analysis I don't see much of a point to include these data in the manuscript in particular without a convincing connection to a PKA-dependent and rather modestly increased surface expression of presumably Ca2+-permeable AMPAR. This is simply lacking. The references that the authors cite to back up their notion were difficult to find. None of them actually provides this causal link.

Referee #2:
The authors have been very responsive and have addressed the reviewers' comments thoroughly. I appreciate the new STED analyses, the additional controls and additional quantifications of the imaging data, as well as the figure rearrangements. I also think the manuscript text has gained in clarity. Overall, I am very enthusiastic about the paper.

A few comments on the revised version:

1. The biochemical analyses of cortical and striatal lysates in the excitatory and inhibitory neuronal knockout mice, respectively, are already presented in Fig. 1A-D, while the explanation about the predominantly glutamatergic/GABAergic nature of the cortical/striatal neurons only comes on p. 6, in connection with Fig. 2/S2. It would be helpful to move the explanation to the very beginning of results.

2. Although the figures have clearly improved, some of the panels still contain very small text and are difficult to read, e.g. Fig. 2E, Fig. 5E, Fig. S2B and Fig. S7B-D.

3. The new data on the role of cAMP in the degradation of PKA regulatory subunits is very interesting, but I do not fully understand how the data fits to the proposed model: if cAMP is "upstream" of autophagic degradation of PKA R1alpha/beta (first cAMP acts to dissociate the tetrameric enzyme complex, then the free regulatory subunits are targeted for degradation in the autophagosomes), why does elevation of cAMP concentration by forskolin not rescue PKA signaling in Atg5 KO cells at least to some extent (Fig. 5A-B)? At least a fraction of the catalytic subunits should be transiently liberated by elevating cAMP concentration, even if the regulatory subunits are then not properly degraded. In fact, there seems to be a mild increase in PKA signaling readouts upon Forskolin application in the blots shown in Fig. 5A and 6A. Could the authors provide an explanation?

4. A suggestion regarding the title: it would be good to specify the synaptic location of the described mechanism by saying e.g. "...controlling cAMP/Protein Kinase A signaling at the synapse".

Minor:
5. There seems to be a typo on p. 8: "...nor in the brains of Atg5flox:Slc32a1-Cre KO mice (Fig. S2F)" - should be "...(Fig. S3F)".

6. The look-up table in Fig. 7E does not look correct (red should be high?)

Point by Point Response to Reviewers.

We would like to thank the reviewers for their constructive comments. Below, we address all remaining concerns that were expressed by reviewers.

Referee #1:

The authors have addressed most of my concerns. Most important, they have included new experiments that support the hypothesis that PKA signaling in particular at synapses is affected following ATG5-gene deletion. The authors also discuss now why content profiling of autophagosomes in other studies revealed vastly different results. They explain the differences in protocols, transgenic mouse models and purification schemes.

Response: We thank the reviewer for this positive comment on our work.

However, I still have the impression that the different parts of the manuscript are not well connected and the manuscript is still difficult to read. Some data sets are on my opinion dispensable and do not add much to the principal findings. The observed behavioral phenotypes are very mild and the behavioral seizures are not really substantiated by the other data. There is no real link of this analysis to PKA signaling. The data can be removed. I still believe that one would expect phenotypes in cognitive behavior; i.e. hippocampus-dependent learning and memory. But such experiments were not done. The link to the seizure phenotype is still far fetched. The in vivo electrophysiological analysis is not quantitative. Without a rescue and further analysis I don't see much of a point to include these data in the manuscript in particular without a convincing connection to a PKA-dependent and rather modestly increased surface expression of presumably Ca2+-permeable AMPAR. This is simply lacking. The references that the authors cite to back up their notion were difficult to find. None of them actually provides this causal link.

Response: We have now made a strong effort to simplify the results section, which has now been shortened by almost one page. We have also added the following sentence to the Discussion to avoid exaggeration between the seizure phenotype and PKA signaling:

“Whether the occurrence of spontaneous recurrent seizures in mice with autophagy deficiency in the excitatory forebrain neurons reported in the current study and previously (McMahon et al, 2012) is a direct consequence of dysfunctional PKA signaling needs to be determined in further studies. Based on our current data, we propose a model whereby autophagy contributes to neuronal excitability by regulating PKA-dependent phosphorylation of postsynaptic proteome, a function crucial for AMPA-mediated glutamatergic neurotransmission”.

Referee #2:

1. The biochemical analyses of cortical and striatal lysates in the excitatory and inhibitory neuronal knockout mice, respectively, are already presented in Fig. 1A-D, while the explanation about the predominantly glutamatergic/GABAergic nature of the cortical/striatal neurons only comes on p. 6, in connection with Fig. 2/S2. It would be helpful to move the explanation to the very beginning of results.

Response: We thank the reviewer for this suggestion, which has now been incorporated in the Results section.

2. Although the figures have clearly improved, some of the panels still contain very small text and are difficult to read, e.g. Fig. 2E, Fig. 5E, Fig. S2B and Fig. S7B-D.

Response: We have now increased the font size in figures specified by the reviewer.

3. The new data on the role of cAMP in the degradation of PKA regulatory subunits is very interesting, but I do not fully understand how the data fits to the proposed model: if cAMP is "upstream" of autophagic degradation of PKA R1alpha/beta (first cAMP acts to dissociate the tetrameric enzyme complex, then the free regulatory subunits are targeted for degradation in the autophagosomes), why does elevation of cAMP concentration by forskolin not rescue PKA signaling in Atg5 KO cells at least to some extent (Fig. 5A-B)? At least a fraction of the catalytic subunits should be transiently liberated by elevating cAMP concentration, even if the regulatory subunitsare then not properly degraded. In fact, there seems to be a mild increase in PKA signaling readouts upon Forskolin application in the blots shown in Fig. 5A and 6A. Could the authors provide an explanation?

Response: We thank the reviewer for this comment. In fact, this point is reflected in our data in Fig. EV6C. We observed that PKA signaling was not completely abolished upon forskolin stimulation of Atg5flox:CamKIIα-Cre acute KO slices, likely as a result of the release of the sequestered catalytic subunit by elevated cAMP levels. This sentence is now included in the results part of the Results section in the manuscript.

We believe that the lack of complete rescue with forskolin in KO neurons is due to the fact that each of the PKA R1 subunits is upregulated more than twofold under this condition. This suggests that rescue of PKA signaling (by release of its catalytic subunit) would require a significantly higher concentration of cAMP compared with controls. However, the exact cAMP concentration could not be deteremined until the presice stochiometry of the PKA complex subunits in ATG5 KO neurons is known.

4. A suggestion regarding the title: it would be good to specify the synaptic location of the described mechanism by saying e.g. "...controlling cAMP/Protein Kinase A signaling at the synapse".

Response: We thank the reviewer for this suggestion. The title is changed accordingly.

Minor:
5. There seems to be a typo on p. 8: "...nor in the brains of
Atg5flox:Slc32a1-Cre KO mice (Fig. S2F)" - should be "...(Fig. S3F)".

Response: We have corrected the typo.

6. The look-up table in Fig. 7E does not look correct (red should be high?)

Response: We have corrected the look-up table.

2nd Revision - Editorial Decision **14th Sep 2022**

Dear Natalia,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now looked at the introduced changes and all looks good.

I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a nice study!

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Corresponding Author Name: Kononenko Natalia
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This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
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Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	All used materials are commercially available (Data availability section). No restrictions apply.

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	All antibody information can be found in the supplementary material table S8.

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Primer information used for genotyping is provided in supplementary material table S5.

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	NSC34 cells (CLU140, Cedarlane) were obtained from Prof. B. Wirth (University Hospital, Cologne) (suppl. Material and Methods)
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Information about primary cultures are provided in the Method section and in the suppl. Material and Methods.
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Cell lines were authenticated by their morphology (Material and Methods)

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Laboratory animal strains and genetic modifications are listed in the M&M section.
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	No animals from the field are used in this study.
Please detail **housing and husbandry conditions**.	Yes	Housing information are provided in the Material and Method section.

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	No plants are used in this study.
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	No microbes are used in this study.

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	No human reaseach participant data are included in this study.

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	The service of core facilities is mentioned in the acknowledgements.

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	This study was not pre-registered.
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	No clinical trial study.

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	All experiment protocol are described in the method section of the main manuscript or the M&M section of the supplementary material.

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	In Material and Methods section: sample sizes were not chosen based on pre-specified effect size. Instead, multiple independent experiments were carried out using severalsample replicates as detailed in the figure legends.
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	In Material and Methods section: mice were genotyped for allocation into control (Cre negative) or experimental (Cre positive) groups. Within each group, all animals were generally used for experimental procedures, so no randomization was applied
Include a statement about **blinding** even if no blinding was done.	Yes	In Material and Methods section: Animals were genotyped prior to experiments, i.e. no blinding was used to allocate experimental groups. However, we assessed data and were not blinded to the primary
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Yes	In Material and Methods section: data was only excluded when the quality of the sample was not optimal. Predefined quality criteria were: in living samples- vacuolization orother signs of cellular degeneration, normal cell morphology and protein transport (which is usually hampered inunhealthy cells) and image streams that are in focus; proper sample preparation and mounting in fixed samples
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	In Material and Methods section: normality test using GraphPad Prism version 9 (Shapiro-Wilk test) was perform to determine the parametric and non-parameteric statistical tests used for analysis (for most of data sets with more than 4 data points).

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	The number of experiments is indicated in the figure legend.
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	All data represent biological replicates (indicated in In Material and Methods section).

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	Study does not include human participants.
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	Study does not include human participants.
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	Study does not include human participants.
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	The authority granting ethics approval of the performed animal experiments are listed in the M&M section.
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	Study does not include specimen and field samples.

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	This study does not fall under dual use.
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	This study does not fall under dual use.
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	This study does not fall under dual use.

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The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	This study is not suitable for the mentioned community standards.
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	This study is not suitable for the mentioned community standards.
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	This study is no clinical trail study.

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data availabilty section is provided in the supplementary material.
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	No human clinical genomic datasets were used.
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	No computational models are included in this study.
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	No publicly available datasets are used in this study.