EMBO reports

Carbonic anhydrase 7 bundles filamentous actin and regulates dendritic spine morphology and density

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1st Revision - Editorial Decision 2nd Mar 2020

Dear Dr. Ruusuvuori

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, the referees also point out several technical concerns and have a number of suggestions how the study could be strengthened. More evidence for the actin-bundling activity of CA-VII should be presented and the potential links to pH regulation - if existing - dissected and discussed. Moreover, the statistical analysis needs to be improved and evidence for the localization of endogenous CA-VII should be presented.

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

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

IMPORTANT NOTE: we perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages
https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

8) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

9) Regarding data quantification:
- Please ensure to specify the name of the statistical test used to generate error bars and P values, the number (n) of independent experiments underlying each data point (not replicate measures of one sample), and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
IMPORTANT: Please note that error bars and statistical comparisons may only be applied to data obtained from at least three independent biological replicates. If the data rely on a smaller number of replicates, scatter blots showing individual data points are recommended.
- Graphs must include a description of the bars and the error bars (s.d., s.e.m.).

- Please also include scale bars in all microscopy images.

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

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

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

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

Yours sincerely

Martina Rembold, PhD
Editor
EMBO reports

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Referee #1:

The manuscript by Bertling, Blaesse, Seja et al. is generally well-written. I am confident that it falls within the scope of EMBO Report. However, there are a few critical things that needs to be addressed.

The authors' conclusion that CA-VII bundles F-actin is weak. In Movie 2, I see the filament elongates and their brightness increased. It is indeed brighter the control experiment in Movie 1. But I am not sure how strong this is as an evidence of bundling. In the movie, I do not see a clear event of bunding of more than two fibers. Can the brightness change be the change in focus plane? More standard assay is low-speed centrifuge assay. Also, EM observation will give definite answer to this issue. These two assays should be conducted, if the authors wish to conclude bunding. Pyrene actin polymerization assay may be helpful to interpret the imaging results.

The result of gel filtration is also not very strong. It is fairly high concentration (33 uM) so it could be an artifact. In a related issue, generally it is not a good idea to use DsRed to make fusion protein and test the distribution because it is a tetramer. It will change the oligomerization status of the protein and can change the conclusion. Experiments in Figure 3B needs to be redone by using EGFP or mCherry fusion protein (note that EGFP itself is a weak dimer). Indeed, if homodimerization is required for the bundling, a comparison of tetrameric DsRed fusion and monomeric mCherry fusion will be interesting. The former should induce stronger bundling than the latter.

Figure 4D. Show structural features such as coil, sheet, and turns.

The effect of overexpression of WT and catalytic activity null mutant CA-VII may be discussed in relation to pH change. Does the overexpression of WT indeed change intracellular pH?

It should be noted that CA-II is not normally expressed in neuron. It is used as a marker for oligodendrocytes.

Throughout the manuscript, the authors use abbreviation CAII and CAVII (sometimes with a space). But for clarity, it might be less confusing to write CA2 and CA7 or CA-II and CA-VII.

Referee #2:

In this article, Bertling Blaesse, Seja et al. describe a novel role of carbonic anhydrase VII (CAVII) in the modulation of actin dynamics at dendritic spines. The authors convincingly show that CAVII co-localizes, binds to and bundles actin in cultured NIH3T3 fibroblasts and they describe a novel CAVII DDERIH surface motif which is crucial for actin binding. In addition, they demonstrate that in neurons, CAVII localizes to dendritic spines and that overexpression as well as knockout of CAVII alters dendritic spine properties, including spine morphology as well as spine density. Overall, the data presented are interesting and novel, the experiments seem thoroughly planned and performed. The article is well written and the story convincing. Nevertheless, in order to be ready for publication in EMBOreports major concerns raised below should be re-discussed in the manuscript and supported by additional data. Individual comments can be found below but I would like to summarize my general thoughts first:
One of the most interesting points of the paper is the novel role of CAVII as a potential linker between F-actin dynamics and activity-dependent (maybe even highly localized) control of pH transients in different neuronal compartments. However, from the data presented here it is not clear whether these two functions might be completely independent of each other (stabilizing F-actin versus modulating pH transients) or whether indeed CAVII might be able to link both phenomena. The data presented focus on the novel actin binding function (localization to F-actin and potential stabilization) which seems at least to be independent of the catalytical activity of CAVII. In my opinion the authors should clarify more which hypothesis they promote (independent functions versus linking actin dynamics to pH modulation). If it is indeed the latter even more interesting model they should try to dissect this in more experiments e.g. by monitoring pH in dendritic spines of overexpressing versus KO cells and correlating this to differences in actin dynamics using for instance fluorescence recovery after photobleaching experiments following expression of eGFP-actin, mutant constructs could be used in addition to discriminate between actin binding versus catalytic function). In my opinion this would very much strengthen the significance of the work.
In line with this point the introduction predominantly focuses on the pH buffering function of the two neuronally expressed carbonic anhydrases II and VI, while the results start with experiments in fibroblasts showing distinct localization patterns of CAII and CAVII due to interaction with F-actin and also the discussion is predominantly focused on the CAVII-actin interaction. One of the most interesting points which is the connection between actin and pH is discussed mainly at the end of the discussion. I belief that restructuring would help to clarify their hypothesis and to emphasize important parts of the paper.
Along this line of thinking the authors raised the very interesting point of the potential activity-dependent nature of CAVII function at the very end of the discussion. I strongly believe that the significance of the paper would profit from experiments showing at least some evidence in this

direction. Chemical induction of LTP in overexpression and KO cells might be used to monitor spine structural plasticity for instance in the absence of CAVII and moreover to detect translocations of the protein in and out of spines in an activity-dependent manner either by overexpression of tagged CAVII or by immunostaining against the endogenous protein in stimulated cultures. It needs to be emphasized in this respect that the authors do not provide evidence that the endogenous protein is localized to F-actin and enriched spines.

Detailed points are listed below:

- Page 4, Line 91, The authors write 'we demonstrate that the distinct subcellular localization of CAVII is due to direct interactions with filamentous actin'. The authors should phrase this with more care as they see in their experiments a colocalization of CAVII with F-actin, while they do not provide evidence for a direct interaction specifically with F-actin. It would be important to also show the localization of the endogenous protein.
- Page 9, Lines 178-180, I am missing a plausible explanation for the diffuse cytosolic pattern of the EGFP-CAII-revCAVII mutant.
- Page 10, Line 202ff. Bertling et al. speak of CAVII-expressing neurons, yet I would rather use the term 'CAVII-overexpressing' neurons as it might be misleading otherwise.
- Discussion- Page 14, Line 264/265, the authors write 'CAVII interacts only with a specific subset of actin filaments'. While this would be a really interesting finding I cannot find data to support this hypothesis. If the authors cannot provide data they should clarify that this is hypothetical.
- Page 17, Line 353/354, as the observation that the spine pH increases after induction of LTP (Diering et al., 2011) and in turn might lead to dissociation of CAVII from actin filaments (which might support F-actin destabilization) is perfectly fitting towards previous observations showing a remodeling of the actin cytoskeleton in the early phases of synaptic plasticity, I would suggest to discuss this point in greater detail.
- Figure 1, in the figure legend is reported that the n for eGFP expressing cells is only 2 so this should be raised at least to three independent replications
- Figure 2A, is the enhancement of CAVII binding by the decreased pH significant?
- Figure 2 supplement 2, only a small fraction of CAVII exists as dimers, however, later in the discussion the bundling of F-actin is attributed mainly to homodimers. The authors should at least discuss other mechanism of bundling as the fraction of dimers is really small or whether there could be different conditions promoting dimerization.
- Figure 3, how were the categories predefined to ensure an unbiased analysis, was the observer blind to the conditions? Are the differences in the abundance of the different categories significant?
- Figure 5 E, what are the statistical tests used?
- Figure 5 supplement 2, also here at least in some experiments only two repetitions were performed, I suggest to always use at least three. The authors describe that there is no significant difference between the constructs, however, at least the difference between mutant R223E and H96798C seems rather large to me.
- Figure 6, what is the difference between 6C and 6D, as only D shows the strong alterations in spine shape attributed to the overexpression of CAVII (also obvious in Figure 6 supplement 1 C) whereas spines in 6A and C look rather normal to me? Is this phenotype variable?
- Figure 6 supplement 1, what n was used for statistics?
- Figure 7, what is the n for statisitcs, why was the non-parametric Mann-whitney test used in addition to the T-test, non-parametric tests have to be performed when the criteria for parametric tests are not met, is this the case here? It is important to report always the exact p values, are they derived from the mann whitney test or the T-test. The authors should discuss in more detail

whether spine phenotypes in KO versus overexpressing cells are similar and what would be an explanation for this.

General Comment: I think the description of the statistical tests used (including statistics software) and the n used for the statistics (e.g. whether single spines were used or rather average of a dendrite) is in parts insufficient and not self-explanatory or easy to access for the reader. Therefore, I would suggest to either include a supplementary table including all relevant values and information about the statistics or to find a more elegant way to present the relevant statistical information.

Referee #3:

The manuscript by Bertling et al. "Carbonic anhydrase VII regulates dendritic spine morphology and density via actin filament bundling" reports novel aspects of CAVII as an actin binding protein and the effects on spine morphology.
The authors show that CAVII but not the close relative CAII binds to F-actin in a spin-down assay and in cells upon transfection of GFP/RFP-variants. In cells CAVII binds to F-actin bundles and stress fibres but not to the cortical F-actin and lamellipopdia. Expression of CAVII renders cells somewhat more stable toward latrunculin treatment. Structurally, the authors define domains which confer F-actin binding and distinguish CAVII and CAII. Expression of CAVII in rat neurons leads to increased spine density and morphological alterations. Similarly, in a knockout model for CAVII they show reduced spine density and altered spine morphology.
In summary this is a quite solid and nice piece of work, and the data are presented in a well equilibrated fashion without excessive over interpretation. Also the findings are novel and interesting and shed some new light on the dichotomy of this metabolic enzyme.

However there are a number of aspects which should be addressed or commented on:

The experiments in fibroblasts are quite stringent, however the possibility remains that the increased F-actin binding of CAVII is a mass effect of overexpression. Is CAVII expressed at all in fibroblasts (western blot) ? Is there a dependence of F-actin co-localization with respect to the amounts of CAVII expression ?
The bundling activity remains somewhat unexplained. I recommend to be cautious with the 'Gel filtration dimer' suggestion that implies that a bundling mode similar to a-actinin might exist. This is unnecessarily missleading. It has been shown that even peptides simply by charge action can bundle actin filament and most likely CAVII can bundle as a monomer. Can the authors give a more substantial reasoning?
Certainly CAVII is a metabolic enzyme which could influence the pH in cells and compartments. However, the authors show that the 'Enzyme dead' variant still binds F-actin. The actin related functions therefore seem to be independent from the catalytic activity. The authors should highlight this more and reduce the part that biases the reader towards pH regulation.
The expression of RFP-CAVII in neurons recruits it to spines. The physiological relevance would be more convincing if the authors could complement the overexpression with an antibody staining for endogenous CAVII.
Is there actually more F-actin in CAVII positive spines (phalloidin quantitation)? Are the overall F-actin fraction altered in the CAVII knockout brains (Triton-X fractionation in low speed and high speed fractions) ?
The major alterations in the knockout seems to be morphological parameters. An obvious question is if CAVII is also controlling cell migration. This aspect is not at all addressed in the manuscript, not

in fibroblasts and not in neurons. At least doing basic histology (which they might have done already) should reveal if neuronal migration is affected in the knockout. The cortex is probably the most sensitive area where defects in neuronal migration would be evident as defective layering or ectopic neurons.

Response to referee comments Bertling, Blaesse, Seja et al. / EMBOR-2020-50145V2
Please note that new text in revised manuscript is in blue.

Referee #1:

The manuscript by Bertling, Blaesse, Seja et al. is generally well-written. I am confident that it falls within the scope of EMBO Report. However, there are a few critical things that needs to be addressed.

Ref 1, comment 1: *The authors' conclusion that CA-VII bundles F-actin is weak. In Movie 2, I see the filament elongates and their brightness increased. It is indeed brighter the control experiment in Movie 1. But I am not sure how strong this is as an evidence of bundling. In the movie, I do not see a clear event of bunding of more than two fibers. Can the brightness change be the change in focus plane? More standard assay is low-speed centrifuge assay. Also, EM observation will give definite answer to this issue. These two assays should be conducted, if the authors wish to conclude bunding. Pyrene actin polymerization assay may be helpful to interpret the imaging results.*

 # Authors´ response: We understand now that we failed to visualize and report our F-actin bundling results convincingly. While the low-speed centrifugation assay and EM imaging as suggested by the referee are valid methods, we first decided to improve our reporting of the present methods and data.
Our quantification of filaments imaged using TIRF microscopy takes into account both thickness and intensity of the filament/bundle (see Figure 2C). Without mCA7 (or any other bundling factors) actin filaments polymerize, i.e. the length of the actin filament increases, but filaments do not bundle (intensity and thickness stay the same). With mCA7, filaments start forming bundles (starting from 1+1, then 2+1 etc) relatively quickly. The intensity analysis presented in 2C demonstrates that thickness/intensity of individual filaments shows very little change in control experiments whereas with mCA7, the intensity doubles or triples in 5 minutes (indicating 1+1 and 1+2 bundling), and in 23 minutes (our measurement end-point), the intensity is 4-6 fold higher than at start. We have now added an illustration showing how individual filaments bundle together (Figure 2 D&E), which correlates well with the original intensity measurements shown in 2C. We have added kymographs visualizing bundle formation to Figure 2D. Regarding the current TIRF analysis, it is not possible that the brightness changes are due to a shift in focal plane or laser angle. If the focal plane is lost, the whole imaging view is lost because light is not reflected back to the objective.
As there was a clear difference in filament/bundle elongation (see Figure 2C), we now followed the elongation of individual filaments/bundles in control and in the presence of mCA7. With mCA7, filaments/bundles became elongated in a stepwise manner and these steps were promoted by the addition of another short filament to the end (or close to the end) of the original filament/bundle (new Figure Extended View 2D,E). While we cannot exclude the possibility that mCA7 augments polymerization of individual filaments, our data clearly suggest that enhancement of filament/bundle elongation occurs through bundling of existing filaments/bundles together. As bundling can be clearly seen in the TIRF videos, we added four more videos to the supplementary data.

Amendments and additions in the manuscript:
1. New supplementary videos (2 PBS control and 2 mCA7; Movies EV3, 4, 6, and 7).
2. New coloring of filaments in Figure 2B (Fire, visualizes better intensity changes).
3. New kymograpghs of all analyzed videos (Figure 2D). Kymographs demonstrate clearly how bundles form in the presence of mCA7 but not in control experiments
4. New panel showing bundling of individual filaments together in the presence of mCA7 (Figs 2E and EV2D,E).
5. Text revised accordingly, see Results, page 6, lines 106-120 in the revised manuscript.

Ref 1, comment 2: *The result of gel filtration is also not very strong. It is fairly high concentration (33 uM) so it could be an artifact. In a related issue, generally it is not a good idea to use DsRed to make fusion protein and test the distribution because it is a tetramer. It will change the oligomerization status of the protein and can change the conclusion. Experiments in Figure 3B needs to be redone by using EGFP or mCherry fusion protein (note that EGFP itself is a weak dimer). Indeed, if homodimerization is required for the bundling, a comparison of tetrameric DsRed fusion and monomeric mCherry fusion will be interesting. The former should induce stronger bundling than the latter.*

 # Authors´response: Because referee #3 also raised critical comments on gel filtration results, we decided to remove these data from the manuscript as they are not essential for our main conclusions.

In addition, it is important to note that we used DsRed and EGFP-tagged CAs in parallel in many experiments, and, notably, DsRed and GFP-tagged CA7 produced similar results in all of these experiments. There are several examples of using both constructs in the manuscript: In Figure 1, strong expression of DsRed-CA7 (Fig 1A) or GFP-CA7 (Fig1H) results in stress-fiber bundling and generation of membrane protrusions. Likewise, the effect of CA7 on spine morphology in neurons correlated with expression levels (example on DsRed-CA7 in Figure 6A, and EGFP-CA7 in Figure 6, supplement 1C). We have used both constructs *in vivo* with similar results (EFGP-CA7 results shown in Fig 6 C and D). It is important to note that in the *in vitro* bundling assay, we used purified CA7 with a small C-terminal His-tag (Figure 2). Thus, the used tag does not explain the observed bundling effect in these experiments, either.

Amendments in the manuscript:
1) Statistical analysis of the latrunculin results (Figure EV5, legend)
2) New text on page 13, lines 262 - 267.

Ref 1, comment 3: *Figure 4D. Show structural features such as coil, sheet, and turns.*
Authors´s response: We have now illustrated these structural features in Figure 4D.

Ref 1, comment 4: *The effect of overexpression of WT and catalytic activity null mutant CA-VII may be discussed in relation to pH change. Does the overexpression of WT indeed change intracellular pH?*

 # Authors´ response: The effect of a CA on pH depends on acid-base dynamics, and a yes or no answer cannot be given without considering the time course and magnitudes of acid or alkaline loads. Baseline pHi is set close to neutral by the interplay of plasmalemmal acid-base transporters, and maintaining it close to this set point is crucial because of the high pH-sensitivity of numerous proteins. Indeed, studies on isolated hippocampal neurons show that the onset of CA7-expression does not change baseline pH (Ruusuvuori et al., 2004), but whether CA7 provides a significant effect on pHi dynamics during e.g. enhanced acid (or base) load (especially in the sub-plasmalemmal compartment) has not been studied so far.

It should be emphasized that, in the present work, we use CA overexpression with a background of endogenous CA in the NIH3T3 cells. These cells do not express CA7 but they do have CA activity, likely due to CA2 expression. Similarly, experiments on cultured neurons were done after the onset of endogenous CA7 expression at DIV14, to allow studies on spines.

We made a set of pH_i measurements in cultured hippocampal neurons expressing WT or H96/98C CA7, using the fluorescent pH indicator, BCECF (unpublished results). The baseline pH of DsRed-CA7-transfected cells was similar to control neurons, but their response to CO_2/HCO_3 withdrawal indicated that the speed of the pH response was enhanced, pointing to a contribution by CA7.

Ref 1, comment 5: *It should be noted that CA-II is not normally expressed in neuron. It is used as a marker for oligodendrocytes.*

Authors´ response: Here, we respectfully disagree with the reviewer: there is ample evidence for CA2 expression in neurons. Indeed, the strong expression of CA2 in oligodendrocytes (Cammer 1984), as well as its presence in other non-neuronal cell types in the CNS (choroid plexus: Halmi et al., 2006; Johansson et al., 2008 and astrocytes: Cammer & Tansey 1988), is well documented. There are, however, publications showing that CA2 is expressed in central neurons (e.g. Wang, Bradley and Richerson, 2002/ IHC on rat CA1-CA3 pyramidal neurons; Halmi et al., 2006/mRNA in rat CA1-CA3 pyramidal neurons; Kida et al., 2006/ protein in a subset of human CA1-CA3 pyramidal neurons). In addition to these molecular biological studies, our results on CA7 KO, CA2 KO and CA7-CA2 KO double KO mice provide direct functional data on the presence of CA2 in hippocampal pyramidal neurons, which is expressed after postnatal day 18 (Ruusuvuori et al., EMBOJ 2013). We'd like to add here that the fact that intrapyramidal CA2 is expressed about a week later than CA7 in mice, provides an excellent opportunity to examine CA7 functions in isolation within the time window P10-P18.

Ref 1, comment 6: *Throughout the manuscript, the authors use abbreviation CAII and CAVII (sometimes with a space). But for clarity, it might be less confusing to write CA2 and CA7 or CA-II and CA-VII.*

Authors´ response: The use of abbreviations is now uniform: we use CA2 and CA7 throughout the manuscript.

Referee #2:

In this article, Bertling Blaesse, Seja et al. describe a novel role of carbonic anhydrase VII (CA7) in the modulation of actin dynamics at dendritic spines. The authors convincingly show that CA7 co-localizes, binds to and bundles actin in cultured NIH3T3 fibroblasts and they describe a novel CA7 DDERIH surface motif which is crucial for actin binding. In addition, they demonstrate that in neurons, CA7 localizes to dendritic spines and that overexpression as well as knockout of CA7 alters dendritic spine properties, including spine morphology as well as spine density. Overall, the data presented are interesting and novel, the experiments seem thoroughly planned and performed. The article is well written and the story convincing. Nevertheless, in order to be ready for publication in EMBOreports major concerns raised below should be re-discussed in the manuscript and supported by additional data. Individual comments can be found below but I would like to summarize my general thoughts first:

Ref 2, comment 1: *One of the most interesting points of the paper is the novel role of CA7 as a potential linker between F-actin dynamics and activity-dependent (maybe even highly localized) control of pH transients in different neuronal compartments. However, from the data presented here it is not clear whether these two functions might be completely independent of each other (stabilizing F-actin versus modulating pH transients) or whether indeed CA7 might be able to link both phenomena.*
*The data presented focus on the novel actin binding function (localization to F-actin and potential stabilization) which seems at least to be independent of the catalytical activity of CA7. In my opinion the authors should clarify more which hypothesis they promote (**independent functions** versus linking actin dynamics to pH modulation). If it is indeed the latter even more interesting model they should try to dissect this in more experiments e.g. by monitoring pH in dendritic spines of overexpressing versus KO cells and correlating this to differences in actin dynamics using for instance fluorescence recovery after photobleaching experiments following expression of eGFP-actin, mutant constructs could be used in addition to discriminate between actin binding versus catalytic function). In my opinion this would very much strengthen the significance of the work.*

#Authors´response: Our original aim was indeed to find out whether CA7 might provide a link between pH regulation and actin dynamics. Actin binding is not affected by catalytic activity of CA7, as the CA7 mutant with loss of catalytic activity co-localizes with F-actin. Interestingly, CA activity might be required for CA7-

induced filopodia formation because a CA7 mutant with loss of catalytic activity did not induce filopodia (unpublished data); and acetazolamide treatment rescued filopodia formation induced by CA7 overexpression in NIH3T3 cells. Although these results suggest that catalytic activity of CA7 has an effect on the actin cytoskeleton, they do not provide solid evidence for a well-defined mechanism. Therefore, we did not include these pilot data into the present manuscript.

There are various explanations to account for this effect: 1) catalytic activity affects CA7 actin binding or 2) mutations in the catalytic pocket affects the conformation of CA7 and, thus, F-actin binding. Our present data do not distinguish between these two alternatives, which should be addressed in future work.

Our general hypothesis is (cf. comment by the reviewer) that CA7 has two interrelated functions: Actin binding/bundling and pH-buffering work together:
a) CA7 binds F-actin to protect F-actin from depolymerization if pH drops
b) CA7 binds F-actin promoting its localization within spines to buffer pHi during and after synaptic activity

An alternative possibility is that the catalytic activity of CA7 bears no functional relationship to its ability to bind to actin and to influence cytoskeletal dynamics. However, a number of factors, including the subcellular localization of CA7 and its proximity to transmembrane acid-base sinks and sources, provide support to our general working hypothesis. We'd like to point out here that hypotheses with a very broad scope such as the present one will require much more work to become properly tested. This is, notably, the very first study that raises these important questions.

Ref 2, comment 2: *In line with this point the introduction predominantly focuses on the pH buffering function of the two neuronally expressed carbonic anhydrases II and VI, while the results start with experiments in fibroblasts showing distinct localization patterns of CA2 and CA7 due to interaction with F-actin and also the discussion is predominantly focused on the CA7-actin interaction. One of the most interesting points which is the connection between actin and pH is discussed mainly at the end of the discussion. I belief that restructuring would help to clarify their hypothesis and to emphasize important parts of the paper.*

#Authors´response: We thank the referee for this very helpful comment. We have now restructured and rewritten a number of sections in the introduction and discussion.

Ref 2, comment 3: *Along this line of thinking the authors raised the very interesting point of the potential activity-dependent nature of CA7 function at the very end of the discussion. I strongly believe that the significance of the paper would profit from experiments showing at least some evidence in this direction. Chemical induction of LTP in overexpression and KO cells might be used to monitor spine structural plasticity for instance in the absence of CA7 and moreover to detect translocations of the protein in and out of spines in an activity-dependent manner either by overexpression of tagged CA7 or by immunostaining against the endogenous protein in stimulated cultures. It needs to be emphasized in this respect that the authors do not provide evidence that the endogenous protein is localized to F-actin and enriched spines.*

#Authors´ response: Again, we would like to emphasize the pioneering nature of our present study. We are of course very enthusiastic to examine the role of CA7 in synaptic plasticity and disease models in future work. However, we do hope the referee will appreciate the originality and scope of the data in the present work.

Ref 2, comment 4: *Page 4, Line 91, The authors write 'we demonstrate that the distinct subcellular localization of CA7 is due to direct interactions with filamentous actin'. The authors should phrase this with more care as they see in their experiments a colocalization of CA7 with F-actin, while they do not provide*

evidence for a direct interaction specifically with F-actin. It would be important to also show the localization of the endogenous protein.

#Authors´response: We agree, and have now changed this sentence to: "Specifically, we demonstrate that CA7 directly binds and bundles actin in vitro, and that it colocalizes with filamentous actin (F-actin) when overexpressed in fibroblasts and neurons". Please see our response to this point in the letter to the Editor: "A major problem which we failed to emphasize in our manuscript is the lack of a valid CA7 antibody that would work in immunocyto or immunohistochemistry (IHC). Using CA7-KO mouse brain tissue, we have tested the antibody which has been used in publications (Bootorabi et al., 2011, del Giudice et al., 2013, Viikila et al., 2016) for CA7 IHC, and found that it is not selective for CA7. Therefore, we have made numerous attempts to create a specific CA7 antibody using conventional immunization techniques, but these have failed. In addition, we have invested lots of work in developing a llama VHH nanobody selective for CA7 but, despite of about a hundred of monoclonal lines of these VHH nanobodies now available in our lab, there has been no success."

Ref 2, comment 5: *Page 9, Lines 178-180, I am missing a plausible explanation for the diffuse cytosolic pattern of the EGFP- CA2-revCA7 mutant.*

#Authors´response: With the mutant EGFP- CA2-revCA7 we tested if the diffuse expression of EGFP- CA2 could be modulated by introducing the actin-binding motifs identified in CA7. This mutation, however, did not affect the subcellular distribution of the mutant protein. Hence, destroying a crucial structural motif by mutating a couple of amino acids in CA7 (as in CA7 mutants1 and 2) seems to be easier than re-generating a structural motif in CA2. This is pointed out also in the Discussion p. 12/lines 242 - 245.

Ref 2, comment 6: *Page 10, Line 202ff. Bertling et al. speak of CA7-expressing neurons, yet I would rather use the term 'CA7-overexpressing' neurons as it might be misleading otherwise.*

#Authors´response: Indeed, these cells are CA7-overexpressing neurons and this has now been corrected (p. 9 / lines 187 – 189).

Before: CA7-expressing neurons had a high proportion of aberrant spines, i.e. thick, filopodia-like dendritic protrusions with no clear spine head (Figure 6-figure supplement 1C).
Corrected: EGFP-CA7-overexpressing neurons had a high proportion of aberrant spines, i.e. thick, filopodia-like dendritic protrusions with no clear spine head (Fig EV5C).

Ref 2, comment 7: Discussion- Page 14, Line 264/265, the authors write 'CA7 interacts only with a specific subset of actin filaments'. While this would be a really interesting finding I cannot find data to support this hypothesis. If the authors cannot provide data they should clarify that this is hypothetical.

#Authors´response: This was based on results presented on lines 106-107 and in Figure 1 –supplement 1 (now Figure EV1). We hope that the clarification of the results and conclusion as done now better reflects the results. We added a new video (MovieEV1) demonstrating the lack of CA7 in protruding lamellipodium. For this video, we used highly dynamic melanocyte cell line B16F1 to demonstrate that whenever lamellipodium protrudes forward (branched actin network polymerisation is the pushing force), CA7 is absent. Whenever forward protrusion stops or cell edge is retracting, CA7 is present at the cell edge. We now wrote this more carefully, not saying that CA7 interacts with specific subset of actin filaments but saying that CA7 is not present at protruding lamellipodium rich in branched actin network.

New text:

Results (p. 5 / lines 87-88): An exception to this were the outer edges of lamellipodia, mainly containing branched actin (Higgs, 2011), in which EGFP-CA7 was not present (Fig EV1 and Movie EV1).
Discussion (p. 12 - 13 / lines 247-251): Furthermore, while EGFP-CA7 strongly co-localized with F-actin in fibroblast stress-fibers, the edges of the highly dynamic lamellipodia were largely devoid of CA7. It should be noted here that in the lamellipodia of migrating fibroblasts, actin filaments are polymerized by Arp2/3 complex, resulting in strongly branched actin-filament network at the leading edge. Our results thus demonstrate that CA7 preferably interacts with non-branched actin filaments.

New video: Movie EV1

Ref 2, comment 8: *Page 17, Line 353/354, as the observation that the spine pH increases after induction of LTP (Diering et al., 2011) and in turn might lead to dissociation of CA7 from actin filaments (which might support F-actin destabilization) is perfectly fitting towards previous observations showing a remodeling of the actin cytoskeleton in the early phases of synaptic plasticity, I would suggest to discuss this point in greater detail.*

#Authors´response: The referee takes here up points which we also find important and interesting, as we have already stated above. However, after summarizing the feedback of all three Reviewers we concluded that it is best to focus on the novel CA7-actin interaction. The comments on the lack of spine pH measurements are absolutely relevant, and we are keen to work towards these aims in future studies. We have rewritten the discussion parts on the subject and hope that the current manuscript will evoke interest to the subject and experiments directly assessing this issue will be conducted.

Ref 2, comment 9: *By monitoring pH in dendritic spines of overexpressing versus KO cells and correlating this to differences in actin dynamics using for instance fluorescence recovery after photobleaching experiments following expression of eGFP-actin, mutant constructs could be used in addition to discriminate between actin binding versus catalytic function).*

#Authors´response: Spines: Monitoring pH in dendritic spines has been successfully carried out by one group only (Diering et al., 2011). We were able to monitor pH changes in NIH3T3 cells. **KO cells:** The CA7 KO cells turned out to be very difficult to culture and transfect. They seem to be very sensitive to pH changes. **FRAP**: We did FRAP experiments in CA7-overexpressing neurons (monitoring actin and CA7), but we did not correlate this to pH change. To monitor pH changes, we used de4GFPs, which display pKa values ranging from 6.8 to 8.0 and emission that switches from a green form (λmax ~515 nm) to a blue form (λmax ~460 nm) with acidifying pH. This means that to monitor de4GFP, we need emission wavelengths 515 and 460. For FRAP, the best wavelength is 488 (normally strongest lasers and fluorescence tags with the best properties), which is difficult to combine with de4GFP monitoring. Separating these three from each other is virtually impossible. A further challenge is that bleaching with 488 for FRAP will bleach also de4GFP. To solve all these technical issues and to design and conduct conclusive experiments has not been feasible as part of the revision.

Ref 2, comment 10: *Figure 1, in the figure legend is reported that the n for eGFP expressing cells is only 2 so this should be raised at least to three independent replications*

Authors´ response: We made new experiments and have added data from EGFP transfected cells from two additional independent replications to increase the number of replications to n= 4 (now total of 56 cells analyzed) for this control construct (see Figure 5 and Figure 5 Source Data).

Ref 2, comment 11: *Figure 2A, is the enhancement of CA7 binding by the decreased pH significant*?

#Authors´ response: We have now included statistical analysis of data. The analysis is indicated in the results (p 5 /lines 99-100) and in the figure legend of Figure 2A.

Ref 2, comment 12: *Figure 2 supplement 2, only a small fraction of CA7 exists as dimers, however, later in the discussion the bundling of F-actin is attributed mainly to homodimers. The authors should at least discuss other mechanism of bundling as the fraction of dimers is really small or whether there could be different conditions promoting dimerization.*

Authors´ response: As all three referees had similar comments on these data, we decided to remove it. In the revised manuscript we focus on the novel F-actin and CA7 interaction, and future work will address the more specific questions on the exact mechanisms of F-actin bundling.

Ref 2, comment 13: *Figure 3, how were the categories predefined to ensure an unbiased analysis, was the observer blind to the conditions? Are the differences in the abundance of the different categories significant?*

Authors´ response: In the experiment presented in Figure 3, categories were predefined based on our earlier work. (Hotulainen et al., 2005 Mol Biol Cell (doi: 10.1091/mbc.e04-07-0555).
Analysis of experiments, using two-way ANOVA, with Dunnett's multiple comparison test, show that cells transfected with DsRed-CA7 loose F-actin structures more slowly. The statistical difference is now indicated in the figure 3 legend.

Ref 2, comment 14: *Figure 5 E, what are the statistical tests used?*

 # Authors´ response: Statistical comparison against CA7 was done with the Kruskall-Wallis test corrected for multiple comparisons using GraphPadPrism. This is indicated in the Figure 5 legend (p. 33/ lines 791 – 793).

Ref 2, comment 15: *Figure 5 supplement 2, also here at least in some experiments only two repetitions were performed, I suggest to always use at least three. The authors describe that there is no significant difference between the constructs, however, at least the difference between mutant R223E and H96798C seems rather large to me.*

 # Authors´ response: For the illustrations of the transfections we indeed had EGFP controls from only two repetitions (two different days, four wells each day). However, for statistical analysis of immunoblots, which represent a separate data set, there were always four or more independent repetitions as indicated in the figure legend. To clarify this, we added the numbers to the bar diagram in Figure EV4. We decided to remove the EGFP-CA and DAPI pictures which were only illustrating the ratio of transfected cells to DAPI-stained nuclei.
Regarding the statistical difference between R223E and H96/898C, we apologize for the mistake in wording and analysis. The comparison of the mutants is against WT-CA7. This has been now been corrected. Furthermore, we originally did the statistical comparison against CA7 with Kruskal-Wallis, which indeed showed that none of the expression levels differed from CA7 (P values 0.99 – 0.066). However, the data set passed normality test and, hence, the correct test is one-way ANOVA and Dunnett's multiple comparison test. The P values, which now show a statistically significant difference in the expression level of CA7-mutant2 and CA7-H96/98C, are now included in the bar diagram and the statistical test is provided in the figure legend (p. 34 / lines 810 – 812).

Ref 2, comment 16: *Figure 6, what is the difference between 6C and 6D, as only D shows the strong alterations in spine shape attributed to the overexpression of CA7 (also obvious in Figure 6 supplement 1 C) whereas spines in 6A and C look rather normal to me? Is this phenotype variable?*

 # Authors´ response: Indeed, the spine phenotype, just like the fibroblast morphology (Fig 1), varies depending on the expression level of CA7. Neurons overexpressing CA2 (Fig 6A) or low levels of CA7 (Fig 6C) have mushroom, stubby, and thin spines. Strong overexpression of CA7 (6D) causes the spines to turn into aberrant, thick filopodia-like structures that lack the spine head (Fig 6D, and Fig 6-supplementary Fig 1C), likely due to the formation of rigid, linear F-actin bundles.

The text (p. 10 / lines 201 – 203) is now revised to better explain our results: "The spine phenotype of CA7-overexpressing neurons depended on the expression level. Strong CA7 overexpression induced the formation of abnormal, filopodia-like dendritic protrusions that lack a clear spine head (Figure 6D). This finding is in line with the observations made in cultured neurons (see Figs 6A and EV5C)."

Ref 2, comment 17: *Figure 6 supplement 1, what n was used for statistics?*

 # Authors´ response: The spine density is calculated per cell. We analyzed 10 cells for each construct with two independent repeats, as explained in the figure legend. Because of the low n, we did not do a statistical comparison.

Ref 2, comment 18: *Figure 7, what is the n for statisitcs, why was the non-parametric Mann-whitney test used in addition to the T-test, non-parametric tests have to be performed when the criteria for parametric tests are not met, is this the case here? It is important to report always the exact p values, are they derived from the mann whitney test or the T-test. The authors should discuss in more detail whether spine phenotypes in KO versus overexpressing cells are similar and what would be an explanation for this.*

 # Authors´ response: We thank the referee for pointing out the inconsistency in reporting n values and statistical analyses. Data were first tested for normality using D'Agostino & Pearson normality test. Since the data set for wild type apical dendrites did not pass the test, we used the non-parametric Mann-Whitney test for testing the difference in apical dendrites. In basal dendrites, both data sets passed the normality test, and we used Student's *t*-test. We agree on the importance of exact p-values, and we have added them to Figure legend. n-values used for statistics are now clearly stated in Results (p. 11) and Figure legend, and indicated in panels 7C and D:
Apical dendrite spine density: WT n=29 cells, KO n=28 cells
Basal dendrite spine density: WT n=30 cells, KO n=28 cells
Spine head diameter: WT: n=467 spines, KO n=421 spines

Changes in spine phenotype upon deletion/overexpression of CA7 and the possible underlying mechanisms are now included to Discussion (p. 14 – 15 / lines 280 – 294).

Referee #3:

The manuscript by Bertling et al. "Carbonic anhydrase VII regulates dendritic spine morphology and density via actin filament bundling" reports novel aspects of CA7 as an actin binding protein and the effects on spine morphology.
The authors show that CA7 but not the close relative CA2 binds to F-actin in a spin-down assay and in cells upon transfection of GFP/RFP-variants. In cells CA7 binds to F-actin bundles and stress fibres but not to the cortical F-actin and lamellipopdia. Expression of CA7 renders cells somewhat more stable toward latrunculin treatment. Structurally, the authors define domains which confer F-actin binding and distinguish CA7 and CA2. Expression of CA7 in rat neurons leads to increased spine density and morphological alterations. Similarly, in a knockout model for CA7 they show reduced spine density and altered spine morphology. In summary this is a quite solid and nice piece of work, and the data are presented in a well equilibrated

fashion without excessive over interpretation. Also the findings are novel and interesting and shed some new light on the dichotomy of this metabolic enzyme.

However there are a number of aspects which should be addressed or commented on:

***Ref 3, comment 1:** The experiments in fibroblasts are quite stringent, however the possibility remains that the increased F-actin binding of CA7 is a mass effect of overexpression. Is CA7 expressed at all in fibroblasts (western blot)? Is there a dependence of F-actin co-localization with respect to the amounts of CA7 expression?*

 # Authors´response: Indeed, CA7 expression is very restricted: Western blot data from cultured NIH3T3 fibroblasts show that they do not express CA7 endogenously (Figure I, below). However, the co-localization of CA7 with actin was not dependent on the expression level of EGFP-CA7 in these cells (Figure II, below).

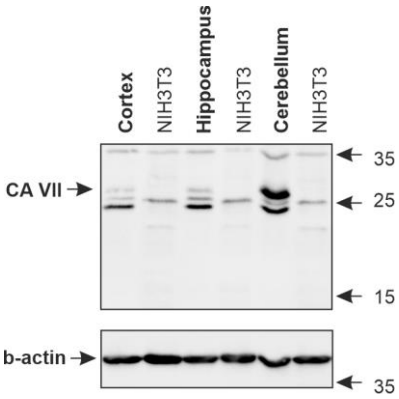

Figure I: NIH3T3 cells do not express endogenous CA7. Tissue lysate from adult mouse cortex, hippocampus and cerebellum served as positive controls. The predicted MW of CA7 is 30 kD. Equal amounts of total protein lysate (10 µg) were loaded per well.

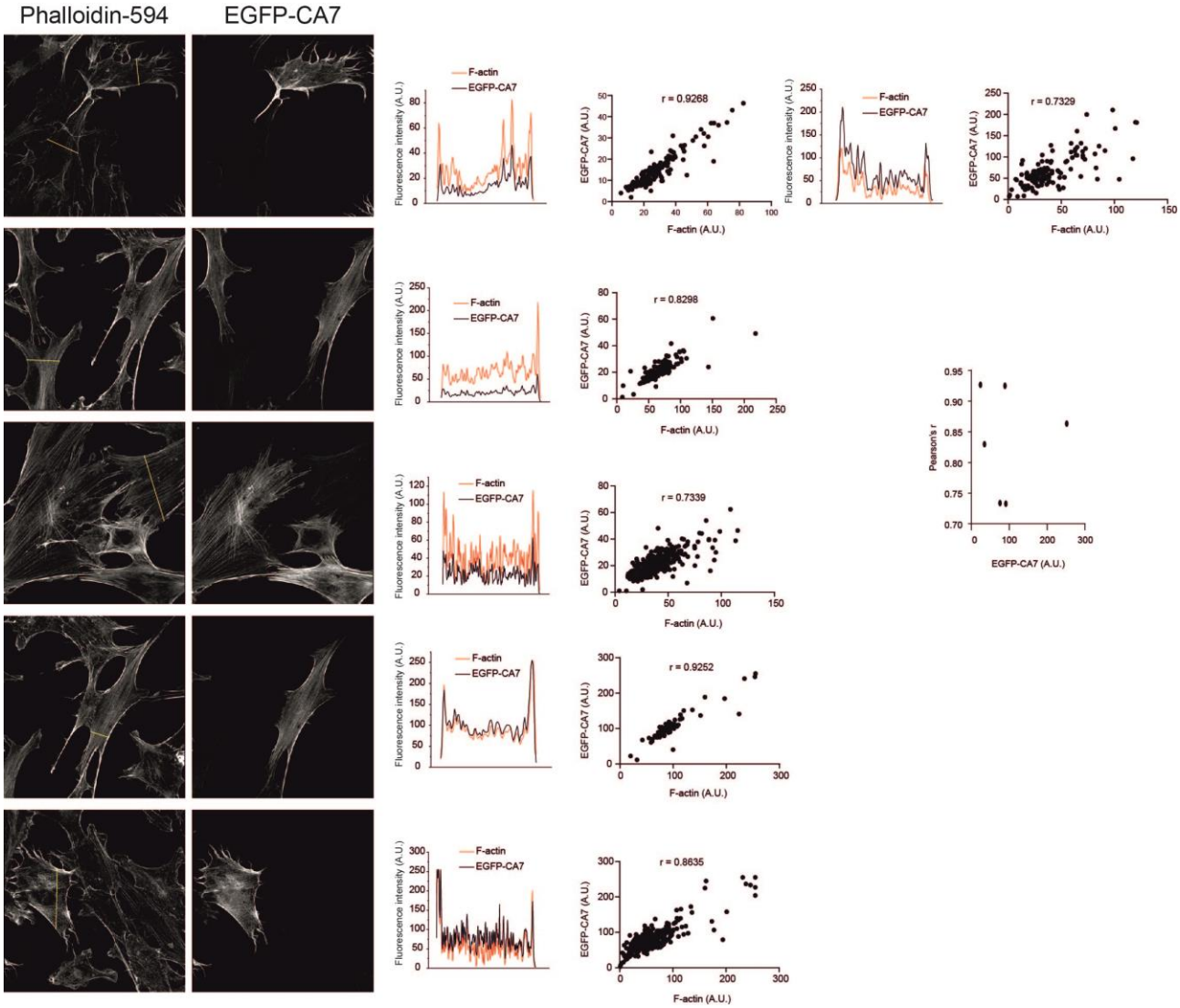

Figure II: The expression level of EGFP-CA7 does not affect its colocalization with f-actin. (A) NIH3T3 fibroblasts expressing EGFP-CA7, stained with Phalloidin-594 to visualize actin. Yellow lines indicate analyzed cross-section. (B) (left) Fluorescence intensity plots for actin (red) and EGFP-CA7 (black) along the yellow lines in (A). (right) Corresponding scatterplots of fluorescent intensities per pixel (EGFP-CA7 vs. Phalloidin-594) with pearson's r. (C) Pearson's r plotted against the respective EGFP-CA7 expression level per cell show no correlation between expression level and actin colocalization.

Ref 3, comment 2: *The bundling activity remains somewhat unexplained. I recommend to be cautious with the 'Gel filtration dimer' suggestion that implies that a bundling mode similar to a-actinin might exist. This is unnecessarily missleading. It has been shown that even peptides simply by charge action can bundle actin filament and most likely CA7 can bundle as a monomer. Can the authors give a more substantial reasoning?*

 # Authors´response: As referee #1 also raised critical comments on gel filtration results, we decided to remove this data from the manuscript. In the revised manuscript we focus on the novel F-actin and CA7 interaction and future work will address the more specific questions on the exact mechanisms of F-actin bundling.

Ref 3, comment 3: *Certainly CA7 is a metabolic enzyme which could influence the pH in cells and compartments. However, the authors show that the 'Enzyme dead' variant still binds F-actin. The actin*

related functions therefore seem to be independent from the catalytic activity. The authors should highlight this more and reduce the part that biases the reader towards pH regulation.

Authors´ response: We thank the referee for this insightful comment. As stated at the beginning of our response, the emphasis of the paper as a whole has been shifted to the CA7-actin interaction and its consequences.

Ref 3, comment 4: *The expression of RFP-CA7 in neurons recruits it to spines. The physiological relevance would be more convincing if the authors could complement the overexpression with an antibody staining for endogenous CA7.*

Authors´ response: We fully agree that this is a very important issue. Please see our response to this point in the letter to the Editor: "A major problem which we failed to emphasize in our manuscript is the lack of a valid CA7 antibody that would work in immunocyto or immunohistochemistry (IHC). Using CA7-KO mouse brain tissue, we have tested the antibody which has been used in publications (Bootorabi et al., 2011, del Giudice et al., 2013, Viikila et al., 2016) for CA7 IHC, and found that it is not selective for CA7. Therefore, we have made numerous attempts to create a specific CA7 antibody using conventional immunization techniques, but these have failed. In addition, we have invested lots of work in developing a llama VHH nanobody selective for CA7 but, despite of about a hundred of monoclonal lines of these VHH nanobodies now available in our lab, there has been no success."

Ref 3, comment 4: *Is there actually more F-actin in CA7 positive spines (phalloidin quantitation)? Are the overall F-actin fraction altered in the CA7 knockout brains (Triton-X fractionation in low speed and high speed fractions)*?

Authors´ response: This is an intriguing question. However, according to our radioactive in situ hybridization (RAISH) results, neuronal CA7 is expressed at very low levels in many brain areas. F-actin, in contrast, is abundant in all brain cells and thus, the CA7-F-actin complex makes a minor part of the total F-actin. It is therefore unlikely that we could detect a difference in the amount of F-actin in whole brain lysates from WT and CA7 KO animals.

Ref 3, comment 5: *The major alterations in the knockout seems to be morphological parameters. An obvious question is if CA7 is also controlling cell migration. This aspect is not at all addressed in the manuscript, not in fibroblasts and not in neurons. At least doing basic histology (which they might have done already) should reveal if neuronal migration is affected in the knockout. The cortex is probably the most sensitive area where defects in neuronal migration would be evident as defective layering or ectopic neurons.*

Authors´ response: This question is very interesting, especially since we have recently shown that another ion-regulatory protein, KCC2, affects neuronal morphology and apoptosis (Mavrovic et al., 2020 EMBOr). Notably, KCC2 activity as well as KCC2 protein can be detected already in late embryonic phase (Spoljaric et al., 2019). In contrast, CA7 functional activity can only be detected later, at around postnatal day 10 in hippocampal pyramidal neurons, correlating well with the onset of mRNA upregulation (Ruusuvuori et al., 2004, 2013). Our unpublished radioactive *in situ* hybridization results (from P5-P20 rats) show that in cortical neurons CA7 mRNA levels increase with similar timeline. Since neuronal migration and cortical layering are completed prenatally (Mavrovic et al, 2020, EMBOr), i.e. well before the expression of CA7 starts we do not expect that CA7 has an effect on cortical layering.

2nd Revision - Editorial Decision 7th Dec 2020

Dear Dr. Ruusuvuori

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, referee 1 and 3 are overall satisfied with the revision and support publication after a careful discussion of the limitations of the current dataset (referee 3). Referee 2 acknowledges that you convincingly show a novel actin-bundling function for CA7 but also points out that further mechanistic insight is missing and that the link to its role in pH regulation has not been further explored. The referee is also concerned that the localization of endogenous CA7 was not shown. I have discussed these reports further with the editorial team and also received further feedback from referee 3. Referee 3 overall agrees with the concerns raised by referee 2 but nevertheless considered the current dataset interesting for the field and supported publication after a careful editing of conclusions and a clear discussion of all limitations and points raised by referee 2.

Given the support from two of the referees and that the core dataset on CA7's actin bundling function was considered convincing by all three referees, we would therefore like to offer publication in EMBO Reports. Please discuss all concerns and aspects raised by referee 2 and 3 in the manuscript text, please phrase your conclusions on the role of CA7 in dendritic spines in the most careful and appropriate manner, point out all limitations and discuss alternative explanations. I also strongly suggest toning down the statement in the title, i.e., that spine morphology is causally related to filament bundling. Please also provide a point-by-point response.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

- Please add a 'Conflict of interest' section

- References: Please list only the first 10 authors followed by et al

- Please add a callout to Figure 4A, where appropriate.

- Please combine the two source data files for Figure 2 into one .xls file with two tabs.

- Please remove the legends for the Source Data files from the main article. You can provide this information in the .xls files themselves, e.g., by introducing a separate tab called 'legend'

- Please remove the movie legends from the manuscript and provide them as individual README.text files. Then zip each movie with its legend and upload the zipped files.

- I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission.

- During our routine image integrity check we noticed some inconsistencies in the following figure panels: EV4B, EV4H, EV5D-F. In order to avoid any ambiguities, could you please provide the raw unmodified source data for these?

- Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of

the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-600 pixels large (width x height) in .png format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

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

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

The authors adequately addressed my concerns. The manuscript should be published.

Referee #2:

The now re-submitted manuscript by Bertling, Blaesse, Seja et al. improved concerning the statistical analysis and the way statistics are reported. In the rebuttal letter the authors describe very intriguingly their hypothesis how the pH- and actin-dependent functions of CA7 might work hand-in-hand in dendritic spines. They even mention that the CA activity might be important for filopodia formation as the deficient mutant did not induce the formation of filopodia. However, these data seem to be too preliminary as they were not included in the manuscript. Rather the general pH regulatory aspect is now completely removed from the story. I agree with the fact that the authors can convincingly show that CA7 has an actin-binding domain and an actin-bundling function (which is indeed a novel finding) that seems to be independent of its pH-regulatory role and that the knockout as well as overexpression of CA7 is leading to alteration of dendritic spine morphology as well dendritic spine density in neurons. However, without further mechanistic detail on how actin dynamics are modulated by CA7 in neurons and/or the interplay with regulations in pH, I am not convinced that the novelty of the data presented is enough to justify publication in EMBO reports. The authors even mention that they performed FRAP experiments to study actin dynamics modulated by gain or loss of function of CA7 but report that pH monitoring in parallel was not possible. In my opinion even the FRAP experiments alone without pH-monitoring in KO cells or neurons expressing various mutants would have added significantly to the story by showing how CA7 might influence dynamic actin in spines and thereby would have revealed important mechanistic insight. It would have also been important to discuss the fact that CA7 is not located to the outer edges of lamellipodia of NIH3T3 cells which are characterized by highly branched actin, however, is described to be localized in dendritic spines (which are containing highly branched actin structures). How can this be explained, where is it localized in spines? Maybe even close to the neck region where indeed bundled actin is enriched. Super-resolution microscopy might be useful to reveal these details and thereby add again more insight into the role of CA7 in the CNS and more specifically at synapses. If currently the lack of a specific antibody makes it impossible to confirm that indeed also endogenous CA7 is localized in spines it would be even more important to reveal

whether and how spine actin dynamics are affected by gain- and loss-of-function experiments.
In my opinion the authors nicely show first evidence of a potentially exciting new role of CA7 for actin dynamics in spines which might be even linked to regulations in pH, however, at present the data presented a rather descriptive and lack more mechanistic insight to be published in the current form in EMBO reports. I believe that the story might reach this level once the authors can provide more details about CA7-dependent modulations in actin dynamics and of course if possible a link to pH modulations.

Referee #3:

The revised manuscript by Bertling et al. "Carbonic anhydrase VII regulates dendritic spine morphology and density via actin filament bundling" reports novel aspects of CAVII as an actin binding protein and the effects on spine morphology.

In my opinion the authors addressed the most critical issues raised by the referees. Some ambiguous data not relevant for the message were removed and the statistical analysis adjusted. Also new data and supplementary data were added to support the conclusions.
It is a very nice piece of data as it stands now.

I would have a final comment on the general approach of addressing protein function via fusion proteins. Based on this, the presented data are certainly solid and interesting enough to be presented to the community. However, a proof that authentic CAVII is behaving similarly is difficult at this juncture because of the lack of a working antibody. I appreciate that this problem is recognised by the authors and it does not diminish their interesting findings at all and it does not change my opinion that this work should be published.
However, I would find it important and appropriate if this issue is at least picked up in the discussion, just to offer the reader alternative interpretations. This will do no damage to the manuscript. Unfortunately it has become extremely rare in the scientific community that authors discuss potential alternative interpretations other than their strong opinion, although the data clearly allow this.

Point-to-point response letter/ EMBOR-2020-50145V3

Response to referee comments Bertling, Blaesse, Seja et al. / EMBOR-2020-50145V3
Please note that the text that has been revised in the manuscript is shown in blue.

Editorial changes:

1) Please add a 'Conflict of interest' section / *Section added p. 26 lines 552- 553.*

2) References: Please list only the first 10 authors followed by et al / *Corrected*.

3) Please add a callout to Figure 4A, where appropriate. / *Callout added p. 7, line 135*

4) Please combine the two source data files for Figure 2 into one .xls file with two tabs./*Source data for Figure 2 combined to one Excel.*

5) Please remove the legends for the Source Data files from the main article. You can provide this information in the .xls files themselves, e.g., by introducing a separate tab called 'legend' /F*or each Source Data file, a figure legend has been included to a separate tab named "Legend" and Figure Source Data legends have been removed from the text file.*

6) Please remove the movie legends from the manuscript and provide them as individual README.text files. Then zip each movie with its legend and upload the zipped files. / *Movie EV1 and Movies EV2-7 have been Zipped with corresponding legends and the legends were removed from the text file.*

7) I attach to this email a related manuscript file with comments by our data editors. Please address all comments and upload a revised file with tracked changes with your final manuscript submission. / *Responses are included as comments and changes in the text are indicated in blue.*

8) During our routine image integrity check we noticed some inconsistencies in the following figure panels: EV4B, EV4H, EV5D-F. In order to avoid any ambiguities, could you please provide the raw unmodified source data for these?
/ *Unmodified raw images are now included in the relevant Source data Excel's on separate tabs (i.e. Figure 5 Source Data 1 (EV4B included), Figure 5 Source Data2 (EV4H), and Figure 6 Source Data (EV5D-F)).*

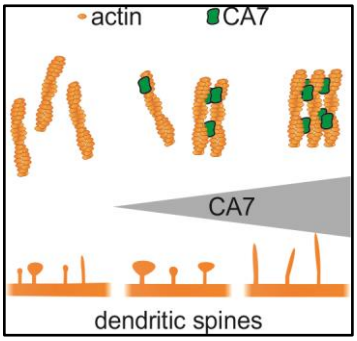
9) Finally, EMBO reports papers are accompanied online by A) a short (1-2 sentences) summary of the findings and their significance, B) 2-3 bullet points highlighting key results and C) a synopsis image that is 550x200-600 pixels large (width x height) in .png format. You can either show a model or key data in the synopsis image. Please note that the size is rather small and that text needs to be readable at the final size. Please send us this information along with the revised manuscript.

A) Carbonic anhydrase CA7 is a pH-regulatory molecule that is expressed in pyramidal neurons at the time of onset of dendritic spinogenesis. Here we show that CA7, but not CA2, binds and bundles actin filaments and has a morphogenetic role in cells.

B)

- CA7 binds and bundles actin filaments *in vitro*
- CA7-overexpression induces actin filament bundling and leads to aberrant spine morphology in neurons
- Knockout of CA7 leads to higher spine density and smaller spines *in vivo*

C) Synopsis, reflecting the findings outlined in bullet points, is now included.



Response to Referee comments:

Referee #2:

Editorial summary:
"Referee 2 acknowledges that you convincingly show a novel actin-bundling function for CA7 but also points out that further mechanistic insight is missing and that the link to its role in pH regulation has not been further explored. The referee is also concerned that the localization of endogenous CA7 was not shown."

Authors´response: We address these limitations now in detail in the Discussion.

The specific Referee comments on these issues are listed as a), b) and c):

Referee 2 comment a) "The authors even mention that they performed FRAP experiments to study actin dynamics modulated by gain or loss of function of CA7 but report that pH monitoring in parallel was not possible. In my opinion even the FRAP experiments alone without pH-monitoring in KO cells or neurons expressing various mutants would have added significantly to the story by showing how CA7 might influence dynamic actin in spines and thereby would have revealed important mechanistic insight."

Authors´response: As pointed out in our previous response, the surprisingly low viability of the KO cultures prevented us from performing a systematic series of these

experiments, which require time-pregnant TG mice, in reasonable timeframe during the present pandemic. See discussion on page 14, lines 287-290:

"In the future, it will be interesting to study whether CA7 affects actin filament turnover. The present latrunculin B treatment experiment (Fig 3) suggests that CA7 reduces the depolymerization rate of actin filaments, but direct measurement of the actin turnover rate by fluorescent recovery after photobleaching or photoactivation assays would give a more definitive answer."

Referee 2 comment b) "It would have also been important to discuss the fact that CA7 is not located to the outer edges of lamellipodia of NIH3T3 cells which are characterized by highly branched actin, however, is described to be localized in dendritic spines (which are containing highly branched actin structures). How can this be explained, where is it localized in spines? Maybe even close to the neck region where indeed bundled actin is enriched. Super-resolution microscopy might be useful to reveal these details and thereby add again more insight into the role of CA7 in the CNS and more specifically at synapses. If currently the lack of a specific antibody makes it impossible to confirm that indeed also endogenous CA7 is localized in spines it would be even more important to reveal whether and how spine actin dynamics are affected by gain- and loss-of-function experiments."

Authors´response: These are all relevant points, and we plan to address these questions in future work. We have now discussed these limitations on page 13, lines 270-276:

"The lack of CA7 in subcellular areas with branched actin is similar to the distribution of the actin-binding protein drebrin-A (Ludwig-Peitsch, 2017). Drebrin-A is known to stabilize actin filaments by binding along the actin double-helix (Mikati et al., 2013). Interestingly, drebrin-A localizes in the middle of the spine where the more stable actin filament pool (consisting of straight actin filaments) is located (Koganezawa et al., 2017). Because of the lack of a specific CA7 antibody, we are unable to study localization of endogenous CA7 in spines but, in light of its actin binding characteristics, it is possible that CA7 has a distribution within spines that is similar to drebrin-A. This is an interesting question for future studies."

Referee 2 comment c) In my opinion the authors nicely show first evidence of a potentially exciting new role of CA7 for actin dynamics in spines which might be even linked to regulations in pH, however, at present the data presented a rather descriptive and lack more mechanistic insight to be published in the current form in EMBO reports. I believe that the story might reach this level once the authors can provide more details about CA7-dependent modulations in actin dynamics and of course if possible a link to pH modulations.

Authors´response: The comments on the lack of spine pH measurement in Referee comments are absolutely relevant and we are keen to work towards these aims but our trials have shown that such experimental work is a project of its own, which would need sophisticated techniques at (or even beyond) the current cutting-edge level (see e.g. Sulis Sato e al., (2017) PNAS). We have now revised the discussion on actin modulation

and hope that our present study will facilitate experimental work directly assessing this interesting topic.

Referee #3:

Referee 3 asked to discuss better the lack of antibody and to remind that results are based on fusion-protein expression as he/she writes here: "*However, a proof that authentic CAVII is behaving similarly is difficult at this juncture because of the lack of a working antibody. I appreciate that this problem is recognised by the authors and it does not diminish their interesting findings at all and it does not change my opinion that this work should be published.*
However, I would find it important and appropriate if this issue is at least picked up in the discussion, just to offer the reader alternative interpretations. This will do no damage to the manuscript. Unfortunately it has become extremely rare in the scientific community that authors discuss potential alternative interpretations other than their strong opinion, although the data clearly allow this."

Authors´response: Thus, in addition to bringing this up in the Results page 9, lines 180–182 we have now added a paragraph about the antibody problems to the Discussion (page 13, lines 259-269):

"We have made numerous, but unsuccessful attempts to create a specific, CA7 antibody compatible in IHC using conventional immunization techniques. In addition, we have developed a llama VHH nanobody (cf. Paalanen et al., 2011) selective for CA7 in ELISA. Despite of about a hundred of monoclonal lines of these VHH nanobodies, we have not succeeded in developing a CA7-specific one that is suitable for IHC in brain tissue. In addition to our own CA7-KO validated antibody that works in immunoblots (Ruusuvuori et al., 2013), we have also tested a number of commercially available antibodies, as well as those used in previous publications (Bootorabi et al., 2010; Del Giudice et al., 2013; Viikila et al., 2016). Unfortunately, these antibodies showed unspecific staining in our experimental settings when CA7-KO mouse tissue was used as negative control. Hence, in the current study addressing the subcellular distribution of CA7, we were left with no other option than to express fluorescently tagged CA fusion proteins. We fully recognize the caveats in work conducted on fluorescently tagged proteins, especially those with DsRed, as discussed below.."

3rd Revision - Editorial Decision 14th Jan 2021

Manuscript number: EMBOR-2020-50145V3
Title: Carbonic anhydrase 7 bundles filamentous actin and regulates dendritic spine morphology and density
Author(s): Enni Bertling, Peter Blaesse, Patricia Seja, Elena Kremneva, Gergana Gateva, Mari Virtanen, Milla Summanen, Inkeri Spoljaric, Pavel Uvarov, Michael Blaesse, Ville Paavilalnen, Laszlo Vutskits, Kai Kaila, Pirta Hotulainen, and Eva Ruusuvuori

Dear Eva,

Thank you for submitting your revised manuscript and please apologize my delayed response, which is due to the Christmas and New Year holidays and the work that accumulated during it.

As discussed, I am now writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed.

The authors have addressed all minor editorial requests.

4th Revision - Editorial Decision 28th Jan 2021

Dr. Eva Ruusuvuori
University of Helsinki
Molecular and Integrative Biosciences, Faculty of Biological and Environmental Sciences
Helsinki
Finland

Dear Eva,

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

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Corresponding Author Name: Eva Ruusuvuori
Journal Submitted to: EMBOreports
Manuscript Number: EMBOR-2020-50145V1

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

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

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

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Estimation of sample size, including rounds of transfections, is based on our previous work/publications on the field (TIRF assay/Gateva et al., Current Biology 2017; Latrunculin B assay/Hotulainen et al., Mol Biol Cell 2005 and as outlined in the Material and Methods section).
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Estimation of animal numbers is based on our previous work/publications on the field (IUE, mEPSCs and in vivo spine analysis/Fiumelli et al. 2012 CerebCortex; neuronal cultures and spine analysis in vitro/Puskarjov et al. 2014 EMBOreports)
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Pre-established inclusion/exclusion criteria are given in the materials and methods (mEPSC measurements) and in the source data legends (other experiments). The only experiment where an outlier is removed is the expression level quantification/Figure EV4. The exclusion is indicated in the Figure EV4 spreadsheet where one lane (mutant 2 on 18.1.2019) is excluded because of a mistake in the cell transfection. There is no EGFP band on the lane but the actin band is normal, so most likely the DNA or transfection reagent was forgotten from the transfection mix.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	When comparing mEPSCs and spine density/morphology in WT and CA7 KO animals, we used littermates when possible. For IUE, all pups/damn were transfected with the same constructs. Randomization was not used.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Masking was used during data collection and analysis as outlined in Matrerial and Methods (for mEPSC) and in the source data legends (for transfections/Pearson's coefficient and spine analysis in vivo).
4.b. For animal studies, include a statement about blinding even if no blinding was done	The person making and/or the person analysing the experiments was blind to the genotype (mEPSC and spine analysis in vivo).
5. For every figure, are statistical tests justified as appropriate?	Satistical test used and the exact p values (when > 0.001) are given in the figure legends and figures, respectively. The IUE experiments were performed as proof-of-concept to demonstrate that the effects observed in cultured cells are present in neurons in vivo as well. For technical reasons, the quantitative and mechanistic analysis focused on cultured NIH3T3 cells and cultured neurons and no statistical analysis were done for IUE data.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Student's t test or ANOVA were used when the data sets passsed the normality test.When data did not pass normality test we used appropriate statistical tests.

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Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Alexa Fluor 594 Phalloidin : Invitrogen cat no A12381;Alexa Fluor 488 Phalloidin: Invitrogen cat no A12379; Phalloidin- iFluor 488: Abcam cat no ab176753. Mouse anti-GFP (Clontech, cat nr: 632381); anti-mouse Starbright Blue 700 (Bio-Rad, cat:nro 12004158); anti-actin-rhodamine (Bio-Rad, cat:nro 12004164)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NIH3T3: Sigma (cat no 93061524), the line was not tested for mycoplasma contamination when used for the experiments.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Animals were housed and bread in a conventional animal house. Both mice (C57bl/6 and ICR) and rats (Wistar) were group housed (weaned littermates of same sex in groups of 2-6) under a 12:12h light:dark schedule (lights on at 0600h). Altromin food pellets and tap water were available ad libitum and animals had wood bedding/nesting material and wood blocks for enrichment. The CA7 KO mice (deletion of exons 5-7 of the Car7 gene) have been backcrossed to C57bl/6. Male P35-40 mice and were used for the mEPSC recordings and spine analysis. For IUE, time-pregnant ICR mice with E14.5 embryos were operated. All born pups were transcardially perfused at P40 to obtain histological sections.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All experiments involving animals were conducted in accordance with the European Directive 2010/63/EU, and were approved by the National Animal Ethics Committee of Finland or the Local Animal Ethics Committee, University of Helsinki
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E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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