APP Maintains Structural Plasticity of Dendritic Spines by Regulating Extracellular D-Serine levels

Chengyu Zou, Sophie Crux, Stephane Marinesco, Elena Montagna, Shi Yuan, Song Shi, Kaichuan Zhua, Mario Dorostkar, Ulrike Müller and Jochen Herms

Corresponding author: Jochen Herms, DZNE - German Center for Neurodegenerative Diseases, Munich

Review timeline:

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<td>Revision received</td>
<td>20 July 2016</td>
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Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 8 July 2015

Thank you much for submitting your manuscript to The EMBO Journal. I am sorry for the delay in getting back to you with a decision, but as it is a busy time it unfortunately took a bit longer than anticipated to get the full set of reports back.

I have now received the reports on your manuscript and they are provided below. The referees do appreciate the interest of the topic. However they also raise concerns that I am afraid preclude publication here at this stage. Referee #2 is positive and support publication as is. However, both referees #1 and 3 also find that the analysis has to be extended for consideration here. While referee #1 requests more mechanistic insight into the link between APP and NMDAR, referee #3 finds that further insight into the effects on learning and memory is needed. Referee #1 also points out issues regarding the electrophysiology.

Given these concerns, I am afraid that I can't offer to invite a revision at this stage as it is too unclear if the issues raised can be addressed. However, should you be able to extend the analysis along the lines as suggested, I am open to look at a resubmission. I should add that we don't require the full mechanism for how APP affects NMDAR, but some further molecular insight is needed. I should point out that for resubmissions that we do consider novelty at time of resubmission and if needed might involve new referee(s).
REFEREE COMMENTS

Referee #1:

Zou et al. investigated the effect of APP KO in the spine turnover in living animal using in vivo two-photon microscopy. They found that APP KO has reduced spine turnover rate. In enriched environment, the WT gradually increases the spine density while KO did not have such effect. The KO has reduced proportion of thin spines and reduced contribution of NMDAR in mEPSC. The spine turnover phenotype was corrected by application of D-serine.

Overall, I feel this work lacks mechanistic insight into the role of APP. How does it link to reduction in NMDAR? The same group previously published an opposite result in APP KO on NMDAR (JNS, 2006). They demonstrated "that hippocampal neurons lacking APP show significantly enhanced amplitudes of evoked AMPA- and NMDA-receptor-mediated EPSCs" (cited from their abstract). The authors might argue that the preparation is very different here (hippocampus vs cortex, culture vs in vivo or acute slice). But this contradiction indicates that the action of APP is not that simple. The analyses remain rather superficial and do not reach criteria required for publication in EMBO J.

In addition, there are several technical concerns as listed below.

1. Figure 1. The authors should perform the same experiment without environment enrichment to demonstrate the stability of their recording.

2. How did the authors draw regression line in Figure 1G? There is a clear trend of reduction (hence the line should have negative slope) but it is not reflected in regression line.

3. Electrophysiological assay is rather rough. The author's method to use 0.5 mM Mg and Vh of -45 mV to monitor NMDAR is fine but not standard. They should show the amplitude of AMPAR mEPSC. Also, they should test CNQX and AP5 (or CPP) to see the contribution of AMPAR and NMDAR. They should measure evoked EPSC at negative and positive potentials. Averaged mEPSC event (with and without drugs) and EPSC traces must be shown.

4. Figure 6. The same experiment must be done with WT. The rescue effect of D-serine is not contradictory to the author's idea but still does not fully support, given various biological effect implicated for APP.

Minor comments.

Abstract. APPsalpha-KI. Define.

P5 Section title. Structural plasticity of spines... Consider "APP ectodomain is not sufficient to rescue the loss of APP..."


Referee #2:

This study provides interesting, exciting and potentially important insights into the pathogenesis of Alzheimer's disease. The experiments are well-designed, the data are exquisite, and the interpretations are appropriately cautious. I have no important advice for edits for revisions or corrections. The language is clear and well-crafted.
Referee #3:

Zou et al show that there is a functional link between APP and NMDA receptor induced spine morphology. The authors used an APP KO mouse model and looked at spine turnover rate (TOR), spine density and morphology. No difference was found in spine density between APP KO and WT mice, however, there were differences in spine elimination and formation with correlates to a significant reduction in TOR when APP is knocked out. Usually environmental enrichment increases spine density, however, this increase is not seen in the absence of APP suggesting APP plays a necessary role in enrichment induced spine formation. The author's next look at the relationship between APP and NMDA induced spine formation. The APP KO mice have a lower contribution of NMDA mediated currents in the generation of mEPSC. To verify the role of the NMDA receptor in this electrophysiological response they compared the expression levels of the NMDA receptor subunits in APP KO and WT mice and found they were significantly reduced in the absence of APP. Finally, they were able to recover the APP KO phenotype by treating mice with D-serine, an NMDA agonist. This data suggests that APP contributes to NMDA mediated synaptic dynamics. The findings linking APP to NMDA mediated spine morphology are novel and would be an excellent article in the EMBO Journal if authors connect this data to learning and memory deficits. The authors suggest in their discussion that APP's modulation of spine morphology may have a function in AD pathology and some experimental data could substantiate this claim. For example, the authors could look at changes in learning and memory in the APP KO mouse model when treated with D-serine using simple behavioral paradigms such as the Morris water maze, the Y maze and fear conditioning.

1st Revision – authors’ response 10 February 2016

We thank all the referees for comments with constructive suggestions. In the revised text, we have made necessary clarifications and provide additional experimental data requested along with the rebuttal of specific points made.

During the course of the revision we made the exciting finding that APP loss leads to a reduction of extracellular D-serine levels, which in turn cause the observed effects on dendritic spines. Therefore, we restructured the manuscript to focus on these mechanistic findings.

We removed the section on electrophysiology, as we found it technically too challenging to asses synaptic NMDA receptor function under adequately controlled conditions. The detailed explanations for these decisions are given in the answers to the reviewers’ questions, below.

Similarly, we realized that using sAPPα knock-in mice to determine which APP cleavage products may mediate the described effects was based on a too simplistic model: Cleavage of the sAPPα fragment from the holoprotein may determine the specific extracellular location of that fragment, which is not necessarily recapitulated by the knock-in mouse model. Therefore, we opted to remove those data as well.

Referee #1:

1. I feel this work lacks mechanistic insight in to the role of APP. How does it link to reduction in NMDAR?

Response: As exogenous D-serine treatment has successfully rescued the deficits of spine plasticity in APP-KO mice, the mechanism of APP in maintaining spine plasticity may be directly related with D-serine. To test this hypothesis, we measured cortical extracellular and total D-serine concentrations by microelectrode biosensor recordings and HPLC, respectively. These results confirmed that extracellular D-serine levels are dysregulated in APP-KO mice (Fig. 4). Combined with D-serine rescue experiments (Fig. 5), our data clearly demonstrate that decreased extracellular D-serine is the mechanistic reason for impaired spine dynamics and adaptive plasticity in APP-KO mice.
2. The same group previously published an opposite result in APP KO on NMDAR (JNS, 2006).

Response: We would like to point out that our previous results were obtained from cultured neurons, which are hardly comparable to the data from adult brain. In this previous study, it is noteworthy that enhanced NMDA receptor currents are probably due to increased synapse numbers in neuronal cultures, which are not observed in adult brain of APP-KO mice.

3. Figure 1. The authors should perform the same experiment without environment enrichment to demonstrate the stability of their recording.

Response: As requested, the experiments without enriched environment have been performed in WT and APP-KO mice (Fig. 1 in the revised manuscript).

4. How did the authors draw regression line in Figure 1G? There is a clear trend of reduction (hence the line should have negative slope) but it is not reflected in regression line.

Response: We used an extra sum-of-squares F test to determine whether the slopes significantly differed from the hypothetical value of 0 (i.e., no dynamic change). If the null hypothesis (no dynamic change) could not be rejected with a p value <0.05, then a slope of 0 is shown. This has now been clarified in the methods section.

5. Electrophysiological assay is rather rough. The author's method to use 0.5 mM Mg and Vh of -45 mV to monitor NMDAR is fine but not standard. They should show the amplitude of AMPAR mEPSC. Also, they should test CNQX and AP5 (or CPP) to see the contribution of AMPAR and NMDAR. They should measure evoked EPSC at negative and positive potentials. Averaged mEPSC event (with and without drugs) and EPSC traces must be shown.

Response: We attempted to pharmacologically isolate AMPAR and NMDAR currents, as suggested by the reviewer. We found that the amplitudes of miniature NMDAR-mediated currents were too small to discern over the noise. Although the addition of extracellular glycine or d-serine may have increased the amplitudes, we were afraid that these substances would mask the effects of endogenous d-serine which we discovered to play a critical downstream role in mediating the effects of APP knockout. Furthermore, we attempted to record evoked EPSCs. However in the cortex, unlike in the hippocampus, no distinct fiber bundles can be stimulated to evoke EPSCs in specific cell populations. Rather, stimulation electrodes are placed in the cortex and the response from nearby neurons is recorded. The resultant EPSC amplitudes, however, are strongly affected by the distance of the recorded neuron from the stimulation electrode and possibly other factors, thus causing large variance in the recorded currents. In practice, this is typically controlled for by quantifying the NMDA/AMPA current ratio (cf. e.g. Chaelon et al. J Neurophysiol 90: 771–779, 2003 and Figure 1, below). Using that approach, we found no difference in NMDAR/AMPA ratios between genotypes, suggesting that AMPAR function may be altered as well. Therefore, this approach is unsuitable to assess electrophysiological changes in NMDAR function.

As we lack the tools to investigate NMDAR function in these models in adequate detail, we preferred to leave out the electrophysiological data from this manuscript. Several previous studies showed effects of d-serine on NMDAR function, which we cite in the manuscript.
Figure 1: A: example traces of extracellular stimulation 50 – 100 µM apical of the patched cell. EPSC were recorded in the presence of 50 µM picrotoxin and low extracellular Mg²⁺ (0.5 mM). The cell was clamped at -90 mV for AMPAR currents and at +40 mV for NMDAR currents and stimulated via a nearby extracellular electrode. AMPAR and NMDAR current amplitudes were measured 10 and 40 ms after the stimulus artifact, respectively. B: NMDAR/AMPAR ratios.

6. Figure 6. The same experiment must be done with WT. The rescue effect of D-serine is not contradictory to the author's idea but still does not fully support, given various biological effect implicated for APP.

Response: As requested, WT mice have been treated with D-serine as APP-KO mice (Supp. Fig. 1). Exogenous D-serine administration does not change spine dynamics and adaptive spine plasticity in WT mice.

7. Abstract. APPsalpha-KI. Define

8. P5 Section title. Structural plasticity of spines... Consider "APP ectodomain is not sufficient to rescue the loss of APP..." 

Response: We realized that using sAPPα knock-in mice to determine which APP cleavage products may mediate the described effects was based on a too simplistic model: Cleavage of the sAPPα fragment from the holoprotein may determine the specific extracellular location of that fragment, which is not necessarily recapitulated by the knock-in mouse model. Therefore, we opted to remove those data


Response: This section has been deleted in the revised manuscript

Referee #2:

Response: We thank this referee for his/her highly encouraging and positive remarks and greatly appreciate his/her interest in our study

Referee #3:

1. The findings linking APP to NMDA mediated spine morphology are novel and would be an excellent article in the EMBO Journal if authors connect this data to learning and memory deficits.
The authors suggest in their discussion that APP's modulation of spine morphology may have a function in AD pathology and some experimental data could substantiate this claim.

Response: As suggested, we have performed novel objective recognition test in adult APP-KO mice (Fig. 6) and found that they do display memory deficits. Cognitive decline in APP-KO mice has been rescued after D-serine treatment over 5 weeks, illustrated by the fact that APP-KO mice treated with D-serine has explored the novel object significantly more. These results indicate impaired spine plasticity in APP knockout is associated with cognitive deficits.

2nd Editorial Decision 24 March 2016

Thanks for submitting your manuscript to The EMBO journal. This is an invited resubmission of manuscript 92035.

Your manuscript has now been re-reviewed by referees #1 and 3. As you can see from their comments below the response is a bit mixed. Referee #3 raises issues regarding novelty and advance, while referee #1 is now more positive. Having evaluated all the comments carefully and discussed them further with referee #1 and colleagues, I have decided to invite a final revision. I have made comments below regarding the specific comments raised by referees:

Referee points:

Ref#1: If there is actually reduction in NMDA current
Karin: Do you have further data on hand to support this?

Ref#1: What is the mechanism leading to the reduction in extracellular but not total D-serine
Karin: Any additional insight into this would be great - do you have data on hand to address this? If not lets discuss this issue further

Ref#1: If there is recovery of extracellular D-serine concentration by oral administration, and whether the reduction is specific to D-serine or not
Karin: This is an important control that needs to be done.

Ref#3: Novelty issues
Karin: I don't see a major issue here. Maybe good to cite the papers and discuss.

Ref#3: removal of the electrophysiology data (also related to point above raised by ref#1).
Karin: Has anything changed regarding this? and is this something that you might be able to sort out? If not let's discuss further

Ref#3: Identify the APP fragments that promote spine density.
Karin: Interesting point, but beyond the scope of this manuscript.

Why don't you send me a point-by-point response and then we can see the best way to move forward.
REFEREE COMMENTS

Referee #1:

The manuscript by Zou et al. is now improved. There are still number of weakness such as whether there is actually reduction in NMDA current, what is the mechanism leading to the reduction in extracellular but not total D-serine, whether there is indeed recovery of extracellular D-serine concentration by oral administration, and whether the reduction is specific to D-serine or not. I am not totally satisfied by the lack of mechanistic linkage of each component of this work. But this manuscript may stand as it is in EMBO J.

Minor comment.

P2 line 6. However, upon environment enrichment... Consider rephrasing. It is not clear what actually happened.

Referee #3:

In this revised manuscript, the authors concentrated on D-serine as a mediator for the connection of APP and NMDA receptors. They found that APP plays an important role in the regulation of D-serine level. Moreover, they reported that cognition impairment resulted from APP knockout can be rescued with treatment of D-serine. This revised manuscript partially answered questions raised by the reviewers. However, it has been reported that Ab and secreted APP can induce the release of D-serine (J Neuroinflammation. 2004;1(1):2; Curr Alzheimer Res. 2007;4(3):243-51), raising an issue of the novelty of this study. Another concern is that the authors chose to eliminate data in which the reviewer has asked about in this revised manuscript, rather than to experimentally address them. In addition, this lab has investigated the effect of g-secretase and b-secretase inhibitors on spine density and dynamics (J Neurosci. 2009;29(33):10405-9; Biol Psychiatry. 2015;77(8):729-39). It would be interesting if the authors identify the APP fragment(s) or other proteins responsible for modulation of spine density and dynamics.

Response to referees

Referee #1:

1. **Whether there is actually reduction in NMDA current.**

The results in the previous version of this work were obtained by measuring spontaneous AMPA mEPSC at a holding potential of -65 mV and combined AMPA + NMDA mEPSC at -45 mV. This provided only indirect evidence of altered NMDA receptor (NMDA Rs) function. During the course of the revision, we tested evoked NMDA Rs and AMPA Rs currents at positive and negative potentials, as the reviewers had suggested. By employing this more direct method, we did not observe any change in the NMDA Rs /AMPAs Rs current ratios (see figure below). We acknowledge that this result contrasts with previous observation describing altered NMDA Rs current in in vitro model of APP-deficiency (as mentioned in the previous discussion of this paper, referring to Hoe et al., 2009 [1]). However, we suspect NMDA Rs function critically change between in vivo and in vitro conditions. More importantly, we unveiled the new findings that disrupted D-serine homeostasis is the underlying mechanism of impaired structural spine plasticity observed in APP-KO brain. Therefore, we opted to leave out these contradictory NMDA Rs current data from this manuscript, keeping our work focused on the study of parameters measured from the intact brain.
2. What is the mechanism leading to the reduction in extracellular but not total D-Serine.

We thank the referee for the opportunity to clarify our interpretation of the mechanism. The cause of reduced extracellular levels but increased total D-serine in our data is most likely related to a defective release of D-Serine containing vesicles in APP-deficient astrocytes. It has been shown that loss of APP modulates cytosolic calcium concentration and reduces ER calcium filling in astrocytes [2, 3]. Meanwhile, it is well documented that calcium signaling is critical for D-serine release from astrocytes [4-7]. Together with our data, these evidences suggest that dysregulated calcium signaling in APP-KO astrocytes might decrease their ability to release D-serine in the extracellular space. At the same time, disrupted exocytosis in astrocytes likely triggers D-serine accumulation in intracellular vesicular pools, explaining why the total D-serine does not decrease in the brain, but actually increase compared to control mice. Note that the total D-serine concentration measured by HPLC is actually more than 99% intracellular [8]. The only D-serine storage mechanism presently described in literature is storage in intracellular vesicles of astrocytes (for a review [2]). In contrast, D-serine synthesized in neurons is not stored, but released through Asc1 transporters and eliminated through the blood or taken up by astrocytes. Therefore, D-serine accumulation in astrocytic vesicles would result in increased total brain D-serine levels as measured by HPLC. We have included these considerations in the modified discussion.

3. Whether there is indeed recovery of extracellular D-serine concentration by oral administration.

To figure out if oral administration of D-serine indeed recovers extracellular D-serine concentration in APP-KO mice, we have treated APP-KO mice with exogenous D-serine for five weeks and assessed cognition by the novel object paradigm and spine dynamics by two-photon imaging. We have found that after chronic D-serine treatment, the extracellular D-serine concentration in APP-KO mice is not significantly different as compared with WT mice (Fig.4). Surprisingly, the total D-serine level in APP-KO mice is also normalized after exogenous D-serine treatment. Thus, these data suggest that oral administration of D-serine not only successfully recovers extracellular D-serine concentration in APP-KO mice, but also restores D-serine homeostasis. Unfortunately, we have no experimental evidence that might explain why providing APP-KO mice chronically with exogenous D-serine restores normal total amounts of D-serine in the brain. However, our observations clearly link APP depletion to physiological alteration of production, maintenance and/or release of D-serine. To dissect further each single component responsible of this unbalanced homeostasis, it might need to perform in vitro approaches. This may, however, in turn compromise the pathophysiological condition where this phenomenon is detectable (an intact parenchymal environment). As we pointed this out in the manuscript, any further in vitro investigations about the role of D-serine in APP-deficient model need to consider these limitations.

4. Whether the reduction is specific to D-serine or not

In our new version of the paper, we included the quantification of L-serine by HPLC, the precursor of D-serine. The total amount of L-Serine changed following the same pattern of D-serine:
compared to controls, it is higher in APP-KO mice, and normalized after chronic D-serine treatment in the drinking water. Both of them, D-serine and L-serine, accumulate probably because the system detects the deficiency of extracellular D-serine and tries to compensate it by increasing its production. In our prospective, this is another indication that points the compromised D-serine release machinery to be the cause of serine accumulation, but not to its production. At the end, the restoration of normal extracellular D-serine we described in the intact brain of APP-KO mice allows D-serine homeostasis to be reverted into physiological conditions. Unfortunately, our in vivo approaches still cannot explain the entire mechanism and further investigations are needed to dissect the specific components resulting in this restoration process, which may also involve in vitro studies. It is also noteworthy that in vitro condition may not guarantee the physiological balance between intracellular and extracellular amino acids concentrations, important for a functional tonic and phasic D-serine release.

5. **P2 line 6. However, upon environment enrichment...** Consider rephrasing. It is not clear what actually happened.

As suggested, this sentence has been changed to “However, when housed under environmental enrichments, APP-KO mice...”

Referee #3:

1. **However, it has been reported that Ab and secreted APP can induce the release of D-serine** (J Neuroinflammation. 2004;1(1):23; Curr Alzheimer Res. 2007;4(3):243-51), raising an issue of the novelty of this study.

Indeed, these papers have investigated the relationship between APP fragments and D-serine release. Interestingly, these results have confirmed that APP fragments increases D-serine release, which seem to be in line with our findings that loss of APP decreases D-serine release. We have updated our manuscript with this information (Discussion, paragraph 4). To our knowledge, this study is not only the first to demonstrate that D-serine release is down-regulated in the absence of APP in vivo, but also shows the functional consequences associated with this. Here we provide direct evidence that decreased spine dynamics in APP-KO mice lead to impaired cognition. We thank the referee for this comment that prompted us examine cognitive behavior, which lead to this exciting finding

2. **Another concern is that the authors chose to eliminate data in which the reviewer has asked about in this revised manuscript, rather than to experimentally address them.**

Actually, we performed the experiments in the way they had been suggested by the referees. However, the results do not support our previous conclusion that NMDA Rs currents are reduced in APP-KO. In light of the new data, we admit that our previous data on mEPSC are not supportive enough to prove altered NMDA Rs function. Since the novel results do not add any insight into the mechanism of abnormal spine plasticity in APP knockout, we decided to remove the data from the revised manuscript and present these data only to the reviewers (see also reply to referee’ 1, point 1).

3. **In addition, this lab has investigated the effect of g-secretase and b-secretase inhibitors on spine density and dynamics** (J Neurosci. 2009;29(33):10405-9; Biol Psychiatry. 2015;77(8):729-39). It would be interesting if the authors identify the APP fragment(s) or other proteins responsible for modulation of spine density and dynamics.

We completely agree with the referee that APP fragments and their relative proteins need to be investigated for their roles in modulating spine density and dynamics. We have been working on this topic several years and we will definitely continue this work. We really appreciate the interest of the referee on our work.
Reference


3rd Editorial Decision 08 August 2016

Thanks for sending us your revised manuscript. Your revision has now been seen by referee #1 and I am happy to let you know that the referee appreciate the introduced revisions and support publication here. I am therefor very pleased to accept the manuscript for publication.

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REFEREE REPORT

Referee #1:

The authors addressed all my concerns. The manuscript should be published.
### Reporting Checklist for Life Sciences Articles (Rev. July 2015)

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.
   - Figures include only data points, measurements or observations that can be compared to each other in a scientific meaningful way.
   - Graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
   - If n<5, the individual data points from each experiment should be plotted and any statistical test employed should be justified.
   - Source data should be included to report the data underlying graphs. Please follow the guidelines set out in the authorship 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 or unpaired), simple x2 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 tests, e.g., if values are not parametric or normal, are described.
       - Definition of “center values” as median or average.
       - Definitions of error bars as s.d. or s.e.m.

   Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, models, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non-applicable).

#### B- Statistics and general methods

1. **How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?**

   - Based on our previous studies (Huang et al., Biochemistry 2016; Masuda et al., Nat Commun 2015; Ohs et al., Am J Physiol Cell Physiol 2015), the sample size of around five mice per group in long term in vivo imaging on dendritic spines is adequate. Also, the sample size in the publications from other research groups is also around 5 mice per group for in vivo imaging (Song et al., Science 2014; Li et al., Nat Neurosci 2015).

2. **What was the variance similar between the groups that are being statistically compared?**

   - False positives are not estimated.

#### C- Reagents

- Each figure and table should contain an explicit and unambiguous identification of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- 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.
  - Figures include only data points, measurements or observations that can be compared to each other in a scientific meaningful way.
  - Graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
  - If n<5, the individual data points from each experiment should be plotted and any statistical test employed should be justified.
  - Source data should be included to report the data underlying graphs. Please follow the guidelines set out in the authorship guidelines on Data Presentation.

- Each figure caption should contain the following information, for each panel where they are relevant:
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  - The assay(s) and method(s) used to carry out the reported observations and measurements.
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  - 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 or unpaired), simple x2 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 tests, e.g., if values are not parametric or normal, are described.
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Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, models, animal models and human subjects.

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D. Animal Models

- Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.
- For all experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

E. Human Subjects

- Identify the committee(s) approving the study protocol.
- Include a statement confirming that informed consent was obtained from all subjects and that the experiments were conducted in the spirit of the Declaration of Helsinki and the Department of Health and Human Services Belmont Report.
- For publication of patient photos, include a statement confirming that consent to publish was obtained.
- Report any restrictions on the availability (and/or use) of human data or samples.
- Report the clinical trial registration number (e.g., ClinicalTrials.gov or equivalent), where applicable.
- For phase 1 and 2 clinical controlled trials, 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.
- For future 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.

F. Data Accessibility

- Provide accession codes for deposited data. See author guidelines, under Data Availability.

- Data deposition in a public repository is mandatory for:
  - Protein, DNA and RNA sequences
  - Microarray data for small molecules
  - Crystallographic data
  - Functional genomics data
  - Proteomics and molecular interactions

- Access to human and genetic data should be provided to as few restrictions as possible while respecting legal obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access controlled repositories such as DBTIR (see link list at top right) or EGA (see link list at top right).

G. General

- For all articles, please list table at the top right of the document.

- Identify species, strain, gender, age of animals and genotype modification status where applicable. Please detail husbanding and husbandry conditions and the source of animals.

- For experiments involving live invertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

- We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 08, e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under Reporting Guidelines. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.

- For any study involving high throughput screening, please list table at the top right of the document.

- To show that antibodies were profiled for use in the system under study (antibody number and/or clone number), supplementary information or reference to an antibody validation profile, e.g., Antibodypedia (see link list at top right), Deregard (see link list at top right).

- For all antibodies, please list table at the top right of the document.

- Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.

- In rabbit anti-rat antibodies (tagged with Alexa 555) purchased from Invitrogen (see link list at top right), the rabbit anti-rat antibodies (tagged with Alexa 555) were purchased from Invitrogen (see link list at top right).

- For all experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.

- We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 08, e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under Reporting Guidelines. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.

- For any study involving high throughput screening, please list table at the top right of the document.