Peer Review File

Manuscript Title: Mechanical actions of dendritic-spine enlargement on presynaptic exocytosis

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referee #1 (Remarks to the Author):

In this paper, Ucar et al. examined an interesting hypothesis that spine expansion may cause presynaptic functional changes by mechanical force from the dendritic spine. When they "pushed" presynaptic varicosity with a microneedle, they observed an increase in the probability of glutamate release, associated with the enhanced assembly of SNARE complex, measured with FRET. They also observed similar effects with mild osmolarity shock. Finally, they observed these presynaptic changes in a bouton near an expanding spine. Interestingly, these presynaptic changes need the spine to "push" the target bouton when it is expanding. Overall, the idea of mechanical coupling between post- and pre-synapses is novel and innovative, and the experiments are well constructed to prove their hypothesis.

However, there are several concerns in their manuscript:

1. Mechanical force perhaps is applied to multiple axons as well as astrocyte near the expanding spine. Also, before arriving at the bouton, it pushes away a big portion of the slice, causing the processes and somata on the whole track activated. So, this does not provide evidence that presynaptic force is causing the change in presynaptic terminals.

2. The dramatic change in the release probability with only 20 mM Sucrose (osmolarity +20) is surprising, as this kind of fluctuation in CSF would occur in normal conditions. Some neurons in the hypothalamus appear to be able to sense small osmolarity changes. However, in CA1, although there only some old reports, the synaptic transmission seems to be osmolarity-independent (e.g. Ballyk et al., 1991, J Neurophys). I wonder if this also occurs in in vivo situation or specific to organotypic slices measured at low temperatures (21-23 degrees) the authors use.

3. The cell shrinkage by hypertonic solution occurs in spines, axons, and astrocytes, increasing extracellular space. This would cause a "pull" force to each other. Overall it is not clear what kind of "force" the author is thinking here. Measuring the degree of shrinkage in spines and axons may help us understand the process and the direction of the force.

4. The only evidence that mechanical force may play a role in the post-to-pre synaptic coupling is the difference between "pushing" and "non-pushing". I think the number of samples for non-pushing is too small (N = 3).

5. Fig. 4: There is no evidence that the observed-bouton pairs are synaptically connected. For the mechanical coupling, do they need to be synaptically connected?

6. Figure 4f and 4h, Extended data 8: Why "before STDP" has samples much more than "pushing" + "non-pushing"? Are there spines other than "pushing" and "non-pushing"? Are some of the samples removed from the analysis?

7. Is the data for Figure 4 and Extended data 8 the same (guessed from the number of cells)? If so, please state so, and explain why D-FRET value in Fig. 4i is different from that n Extended Fig. 8c.

8. Extended figure 8: Please indicate the sample number for panels e and f.

9. All experiments are performed at a rather low temperature (21-23 degrees). Considering the membrane properties would be temperature-dependent, some of the key experiments for pushing, osmolarity, and spine enlargement would need to be performed at physiological temperature.

Referee #2 (Remarks to the Author):

This is an interesting manuscript from the Kasai lab. Here, the authors use a clever approach to mechanically stimulate individual presynaptic boutons, by pressing against them with a pipette, to try to mimic the hypothesized action of dendritic spines as they become enlarged and potentially press against an opposed bouton. During mechano-stimulation, trans-SNARE complex formation was observed via FRET; surprisingly, this was dependent on the polymerization of actin. The key finding was that the mechanical force that was applied served to potentiate transmission by increasing synaptic vesicle exocytosis. Spine enlargement has been reported during short- and long-term synaptic plasticity and is thought to be related to cognition and memory (and disruption of enlargement might be associated with psychiatric and neurodevelopmental disorders), so this is an important topic. In this study, the authors propose a provocative and novel model in which spine growth serves to press against boutons to enhance neurotransmitter release, thus contributing to synaptic strength. This is an exciting idea. Below is a list of strengths of this study, as well as list of a number of issues that need to be addressed.

Specific strengths:

1. The techniques used here are sophisticated and elegant. The approach of measuring putative trans-SNARE assembly by FRET-FLIM, and later comparing that to putative cis-SNARE assembly, is a substantial technical achievement.
2. Likewise, the data in Fig. 2 showing the dependence of SNARE assembly on pushing location are quite elegant.
3. The idea in this study – that growing spines push on boutons to enhance release - is extremely novel and interesting.

Major weaknesses:

1. A major concern is the weakness of the data supporting a link between spine enlargement and enhanced glutamate release. Extended data Fig. 9 contains no statistical analysis, nor does it demonstrate a dependence of enhanced glutamate release on bouton pushing (i.e. spines that grow in in length) vs non-pushing (i.e. spines that grow only in width and not in length) spine enlargement effects. Collecting more data on how spine enlargement drives glutamate release is critical; enough data must be collected to determine whether spine enlargement must actually push the bouton to enhance glutamate release.

Restated: Is there any glutamate release enhancement in spines that enlarge only in width? This would appear to be a crucial control that would help get at the idea that mechano-stimulation, and pressing against the bouton, is needed to enhance glutamate release, rather than just spine enlargement per se.

2. Related to point (1) above: spines enlarge relatively slowly, as compared to acutely pushing boutons with a pipette. So, there is a major concern that if the mechanical force of the pipette was applied as slowly and gradually as a growing spine, there would be no effect, as the bouton might adapt or accommodate, and the mechanical force would have no effect on release. The key

questions are: what is the time course of spine growth (from the literature it seems to be minutes), and can that time course be mimicked with the mechanical force from a pipette? And, if that is done, is there still an effect? This would seem to be an essential experiment.

3. In general, another major concern is a small sample size and a lack of clarity regarding the replicates for each experiment in the study. In many experiments, the data come from only a few boutons, and it is not clear at any point how many preparations or animals were used for each experiment. It seems that the authors should consider at least 15 boutons from at least 2-3 independent preparations to be sufficient for most experiments.

Minor issues (in no specific order):

1. Regarding the increased Pr (from pushing to 31.5 min) in Figure 1h, is this detected from the same bouton at a different time point or different boutons at different time points? It would be constructive to show a few examples of release from the same bouton at different time points.

2. The model in Figure 1A represents a hypothesis; it is not clear whether spine enlargement will mechanically stimulate a bouton or not, so this should be presented as an idea rather than a known phenomenon.

3. It looks like the glutamate increases before stimulation in Figure 1h,j. This apparent pre-stimulation increase does not occur in the sucrose experiments. How do the authors explain this pre-stimulation increase?

4. The authors provide only show 4 negative controls from strong pushing; clearly a higher sample number is needed. If the model is true, why does strong pushing have no effect on release? Perhaps the boutons were damaged? In this context, perhaps it is a bit premature to exclude mechanosensitive channels? Is there an increase in SNARE FRET during strong pushing? There is no effect on release, so it begs the questions as to whether SNAREs are or are not affected.

5. Is it possible to get enough spatial resolution to discern whether the 'SNARE assembly side', in each bouton, has more glutamate release?

6. There is a concern about potential presynaptic effects of glutamate uncaging. Can presynaptic effects be ruled out, perhaps by using pharmacological approaches?

Referee #3 (Remarks to the Author):

Ucar et al have produced an intriguing and potentially exciting paper. The idea has been around for some time that spine morphology, and in particular the changing morphology that accompanies plasticity, might directly and mechanically alter neurotransmitter release. However, I know of no direct evidence that such an effect existed. The authors here develop or apply a series of new methods and measures to discern such an effect, and demonstrate a surprisingly large and long-lasting influence on presynaptic function resulting from pressure directed on the bouton. The results suggest the revelation of an important factor in how we think about synaptic plasticity. The findings are unique, the experiments are apparently well-controlled and thoughtfully carried out, and the paper is generally understandably written despite its highly concise format. I do have several points of concern, however.

First, the strong implication of the results is that in the abundant literature of plasticity involving spine size changes, physiological recordings of synaptic transmission should prevalently show strong and long-lasting changes in presynaptic release probability. However, in general terms,

strong, long-lasting changes in Pr have not been much reported with classical LTP since the discovery of AMPAR insertion. The use of glutamate uncaging does obscure this critical question, but spine size increases are of course observed in numerous paradigms of LTP induction. Can the authors provide previous papers that reported a large change in Pr with STDP in the synapses used here? Importantly, a more convincing test is that according to the authors' model, increases in Pr (and decreases in PPR) should occur reliably and just as often in conditions where spine size is transiently increased but LTP is not produced. For instance, KN62 treatment to block CaMKII activation preserves a large but transient spine volume increase yet prevents both LTP and sustained spine size changes. In the context of this paper, KN62 given during synaptically induced LTP or chemLTP should not block presynaptic FRET changes consistent with the mechanotransduction effect, and a sustained increase in Pr should be seen that is insensitive to KN62. Do the authors find in the literature any evidence in such experiments of increases in Pr consistent with their model?

Second, the mechanism here is very loosely considered. One might argue that the overall novelty of the observations, the technological development that was necessary to carry them out, and the rigor with which they were executed obviate the need to demonstrate a clear potential mechanism. Nevertheless, it remains quite vague how this phenomenon arises, and the authors to my mind overstate the conclusions they can draw at this point. Other than the requirement of actin, as demonstrated with latrunculin, there is little to go on. Since of course the bath-applied latrunculin affects all cells, it is not even possible to determine in which cell the actin is acting (e.g. rigidity or tension in surrounding glia might be required for the FRET effect in boutons when they are pushed upon). Though the authors perfuse postsynaptic CK-666 via the patch pipette, that drug is also cell permeable and may affect Arp2/3 in other nearby cells such as the bouton. (The authors further weaken this part of the paper by citing an Arp2/3 knockout study when referencing the mechanism of action of CK-666.) The authors also give a strange and undefined name (actinARE) to the process(es) involved here, which seems unwarranted without further examination of the mechanisms. An abundance of potentially related mechanisms might be considered to deepen and solidify the model here, including integrins or other components of mechanotransduction in other cells.

Third, from a phenomenological standpoint, I am somewhat confused by what the "pipette pushing" is doing to the synapse. The authors indicate in Fig 2 that pushing onto the bouton in the area of highest FRET produces the largest effect. This area should be the active zone, and thus one might assume the pipette is in fact just directly pushing through the spine into the terminal. This sounds potentially disruptive to both cells. Could the authors instead simply push on the spine or even nearby glia to produce presynaptic changes?

A minor complaint is that the FRET experiments should report less processed results, potentially the raw lifetimes rather than just the rather heavily normalized "A1%." In addition, the N's for the critical experiments in Fig 4 (4f-I particularly) and Ext Data Fig 8 are quite low, and it is not clear what criteria were used for including data in those experiments.

Author Rebuttals to Initial Comments:

Referee #1 (Remarks to the Author):

Overall, the idea of mechanical coupling between post- and pre-synapses is novel and innovative, and the experiments are well constructed to prove their hypothesis.

Thank you for your careful reading and commenting on our manuscript. We have addressed all of your comments, performed many additional experiments and

revised the text accordingly. Added/corrected parts of the text were marked by red. I would be very glad, if you could appreciate how sincerely we have worked to address your concerns over a year.

1. Mechanical force perhaps is applied to multiple axons as well as astrocyte near the expanding spine. Also, before arriving at the bouton, it pushes away a big portion of the slice, causing the processes and somata on the whole track activated. So, this does not provide evidence that presynaptic force is causing the change in presynaptic terminals.

Thank you for pointing out an important issue, about which our description was insufficient. We have now presented the figure (Fig. 2e, Pushing[-]), where we found no increases in the FRET during insertion of the pipette into the slice and placing it close (< 2 μm) to the boutons. The FRET increases required the pipette to apparently touch and push the boutons. In fact, the pushing effects were very local (Fig. 2b,c; EFig. 2e). We limited the pipette insertion to 8 μm from the surface as now have described in the Methods. Furthermore, just the enlargement of spines which do not affect the surrounding tissues unlike pipette insertion could augment the FRET and Pr (Fig. 4, EFig. 10), similarly as pipette pushing (Figs. 1 and 2). The text has now modified as below.

(page 4, line 94-96) : "The FRET did not increase when the pipette was inserted into the slice tissue and positioned within 2 μm from the bouton without pushing (Fig. 2e, Pushing[-])."

(Page 11, line 242-243): " Pushing was performed within 8 μm from the surface of the slice."

2. The dramatic change in the release probability with only 20 mM Sucrose (osmolarity +20) is surprising, as this kind of fluctuation in CSF would occur in normal conditions. Some neurons in the hypothalamus appear to be able to sense small osmolarity changes. However, in CA1, although there only some old reports, the synaptic transmission seems to be osmolarity-independent (e.g. Ballyk et al., 1991, J Neurophys).

Ballyk et al. (J. Neurophys, 1991) reported 20 mM mannitol but not D-glucose affected the amplitudes of field spikes due to the change in the excitability, using electrical stimulation and extracellular recording. In contrast, we studied the effect of 20 mM sucrose using optogenetic stimulation and whole-cell recording. Although 20 mM sucrose was far smaller than the frequently used 300 mM sucrose, 20 mM sucrose has an osmotic pressure of 0.5 atm (5 meters of water, or 0.5kg/cm^2 or 50 nN/mm^2) by the Van't Hoff equation. Such force (0.5 kg/cm^2) is in a range comparable to muscle construction, and is sufficient to distort the subcellular structures, as we actually found (EFig. 4c). We have emphasized this point in the sentence below:

(Page 7, line 151-153) : "The degree of ΔFRET (~20%) was similar to that of 20 mM sucrose (Fig. 3f) with an osmotic pressure of 0.5 atm ≒ 0.5 kg/cm^2 (comparable to that of muscle contraction) ≒ 50 nN/μm^2,"

I wonder if this also occurs in in vivo situation or specific to organotypic slices measured at low temperatures (21-23 degrees) the authors use.

We purchased a heat controller for this purpose and we have performed 90% of the revision experiments at 31℃ (in total, *n* = 67), which reproduced the key results at 21-23℃. The data obtained at 21-23℃ and 31℃ were not different, and their compositions and actual values will be listed in the Source Data.

(Methods: Page 9, line 198-201): "Most imaging and electrophysiological experiments were performed at room temperature (21℃ to 23℃), while the key observations, for example, ΔPr (or ΔA1%) for pushing (pipette or spine enlargement or sucrose) were reproduced at 31℃ ."

3. The cell shrinkage by hypertonic solution occurs in spines, axons, and astrocytes, increasing extracellular space. This would cause a "pull" force to each other. Overall it is not clear what kind of "force" the author is thinking here. Measuring the degree of shrinkage in spines and axons may help us understand the process and the direction of the force.

You are right that both types of force might come into play. We have therefore directly measured the changes in the widths of boutons by 20 mM sucrose, and found the shrinkage of ~0.06 μm in the bouton diameters (EFig. 4c), suggesting that

the osmotic shrinkage was the major action of 20 mM sucrose. This is now described as follows:

(Page 5, line 119-120) ". The boutons were recessed by ~0.06 μm when 20 mM sucrose was applied (Extended Data Fig. 4c)."

4. The only evidence that mechanical force may play a role in the post-to-pre synaptic coupling is the difference between "pushing" and "non-pushing". I think the number of samples for non-pushing is too small (N = 3).

We have performed additional experiments so that now *n* is 14 for pushing and 9 for non-pushing spines (Fig. 4h). We have also performed addition experiments for Pr vs spine enlargement, *n* = 6 for pushing and *n* = 5 for non-pushing spines (EFig. 10).

5. Fig. 4: There is no evidence that the observed-bouton pairs are synaptically connected. For the mechanical coupling, do they need to be synaptically connected?

We have now added a series of new experiments to verify the synaptic connection of the bouton-spine pairs we used. We selected the pairs which were aligned in a horizontal plane, and there was a significant overlap between the boutons and spines (for the criteria see below) in 2P imaging where precise alignment of multicolor images is possible, unlike 1P imaging. To prove the synaptic connection, we have performed the experiments, where Ca-indicator dye (Cal520) was whole-cell perfused into postsynaptic cells to detect the synaptic transmission from the axonal boutons which was expressed with mTurquoise and CsChrimsonR and were optogentically stimulated. The presynaptic stimulation triggered spine Ca increases in18 out of 20 pairs. In addition, if the spine bouton pairs were close (< 2 μm) but without overlap, A1% increases were undetected (*n* = 5). Thus, we have added the description below.

(Page 6, line 139-142): "We selected the bouton-spine pairs in which multi-colour 2P imaging detected a precise horizontal alignment and a substantial overlap (Methods, Extended Data Fig. 8a–f). Presynaptic stimulation elicited Ca^{2+} transients in 18 out of 20 spines in such pairs (Extended Data Fig. 8g–i)."

(Page 7, line 156-160): “The A1% increases never occurred …by the enlargement of the spines which markedly violated the selection criteria although they were within 2 mm from the bouton (Methods, Extended Data Fig. 8d–f) (n = 5).”

(Methods, Page 15, line 327-354): “To find the bouton-spine pairs which were functionally connected, we labelled the boutons either with mTq2-Syntaxin1A (Fig. 4, Extended Data Fig. 8g) or iGluSnFR (Extended Data Fig. 8a,d, 10). Postsynaptic CA1 neurons were whole-cell perfused with 50 µM Alexa594-hydrazide (Invitrogen). These pre-and postsynaptic fluorescent probes (mTq2, iGluSnFR and Alexa594) have well-separated emission spectra. At the same time, they are excited simultaneously at the wavelength of 900 nm in 2P imaging due to the blue-shifted broad 2P excitation spectra of the fluorescent probes[36], which permits the precise alignment of multi-colour images[37], unlike 1P imaging. We acquired XY-images of the bouton-spine pairs at multiple Z-planes (13 to 15) with an interval of 0.5 mm. To show vertical and horizontal alignment, we redefined the X-axis parallel with the bouton-spine axis and derived the maximum intensity XY- and XZ-projections (Extended Data Fig. 8a,d). The fluorescence profiles along X- and Z-axis of the bouton-spine pairs (Extended Data Fig. 8b,c,e,f) were then obtained averaging across the entire bouton-spine width (yellow and blue boxes in Extended Data 8a,d,g). We defined the maximal overlap fractions, Fz and Fx, by the crossing of the peak-normalized bouton and spine fluorescence profiles (Extended Data Fig. 8b,c,e,f). Our criteria for selecting the bouton-spine pair were Fz > 0.8 for the precise horizontal alignment and Fx > 0.6 for the substantial overlap in the XY plane, which were stringently set to avoid potential non-synaptic bouton-spine pairs. The horizontal alignment (Fz > 0.8) enabled more precise measurements of the widths of the bouton and spine. The functional connection was confirmed by expressing the boutons with CsChrimsonR and mTq2-Syntaxin1A and whole-cell loading the dendritic spines with Alexa594 and a Ca^{2+} indicator dye, Cal520 (AAT-Bioquest, 50 mM). The three probes (mTq2, Alexa594 and Cal520) could be 2P excited simultaneously at 900 nm (Extended Data Fig. 8g,h). We optically stimulated the CA3 region at least ten times, and the fluorescence of Cal520 was measured at 490-540 nm. The spine Ca^{2+} responses were elicited in 18 out of 20 pairs (Extended Data Fig. 8h,i). In the remaining two pairs, optogenetic stimulation would have failed or Pr was less than 0.1. ”

6. Figure 4f and 4h, Extended data 8: Why “before STDP” has samples much more than “pushing” + “non-pushing”? Are there spines other than “pushing” and “non-pushing”? Are some of the samples removed from the analysis?

Sorry for the confusion. Those many data points were used to reflect the baseline fluctuations. We have now changed the policy in the presentation after Fig. 4g so

that the “Before STDP” bar has been removed consistently (Fig.,4g,h, EFig.9a, 10k-n).

7. Is the data for Figure 4 and Extended data 8 the same (guessed from the number of cells)? If so, please state so, and explain why D-FRET value in Fig. 4i is different from that n Extended Fig. 8c.

This was a mistake. We have now added many new experiments, and the data source and numbers of Fig. 4h and EFig. 9a-d are same (*n* = 23, see legends).

8. Extended figure 8: Please indicate the sample number for panels e and f.

We have decided to remove the CK666 experiments as it is not conclusive, as pointed out by the other reviewer. We have performed additional uncaging only experiments in total of *n* = 9 for EFig. 9e and f.

(Page 45, line 861-863): ” **e**, **f**, Absence of spine enlargement (**e**) and FRET changes (**f**) when uncaging was applied without postsynaptic spikes (green, *n* = 7 boutons, 7 slices, 5 rats). The control STDP traces are the same as Fig. 4d,g “

9. All experiments are performed at a rather low temperature (21-23 degrees). Considering the membrane properties would be temperature-dependent, some of the key experiments for pushing, osmolarity, and spine enlargement would need to be performed at physiological temperature.

As described above, we purchased a heat controller for this purpose and we have performed most additional experiments after revision (*n* = 67), and reproduced most key experiments at 31℃.

(Methods, Page 9, line 198-201):“Most imaging and electrophysiological experiments were performed at room temperature (21℃ to 23℃), while the key observations, for example, ΔPr (or ΔA1%) vs. pushing (pipette or spine enlargement or sucrose) were reproduced at 31℃.”

Referee #2 (Remarks to the Author):

This is an exciting idea. Below is a list of strengths of this study, as well as list of a number of issues that need to be addressed.
Specific strengths:
1. The techniques used here are sophisticated and elegant. The approach of measuring putative trans-SNARE assembly by FRET-FLIM, and later comparing that to putative cis-SNARE assembly, is a substantial technical achievement.
2. Likewise, the data in Fig. 2 showing the dependence of SNARE assembly on pushing location are quite elegant.
3. The idea in this study – that growing spines push on boutons to enhance release - is extremely novel and interesting.

Thank you so much for your deep appreciations of our work. Newly added or corrected parts of the text were marked by red. I would be more than happy, if you could appreciate how hard we have performed the additional experiments and analysis to address your concerns over a year, as we describe in more detail below.

Major weaknesses:
1. A major concern is the weakness of the data supporting a link between spine enlargement and enhanced glutamate release. Extended data Fig. 9 contains no statistical analysis, nor does it demonstrate a dependence of enhanced glutamate release on bouton pushing (i.e. spines that grow in in length) vs non-pushing (i.e. spines that grow only in width and not in length) spine enlargement effects. Collecting more data on how spine enlargement drives glutamate release is critical; enough data must be collected to determine whether spine enlargement must actually push the bouton to enhance glutamate release.

We have succeeded in additional STDP-FRET experiments for 14 pushing and 9 non-pushing spines (Fig. 4), and STDP-Pr experiments for 6 pushing and 5 non-pushing spines (Extended Data Fig. 10). The increases in SNARE/FRET and glutamate release could be only seen when spines were elongated. It has been particularly difficult to increase the number of experiments for STDP-Pr (and also STDP-FRET). 1) The proper quality and level of expressions must be maintained for combinations of virus vectors (iGluSnFR, CsChrimsonR, iSLIM, mScarlet). They were OK for two weeks, but non-ideal in the next three weeks, and we needed to readjust or remake the AAVs, and screened the optimal samples. 2) It is not easy to maintain the stable repetitive fluorescent imaging (iGluSnFR or iSLIM) for an hour when the same synapse was challenged by induction of plasticity under whole-cell

perfusion. In case of iGluSnFR for Pr measurement, we applied optogenetic stimulation 10 times for each time point and repeat 6 times altogether before and after STDP induction, and we could fail at any time. 3) The difficulty in finding out a good synaptic contact located relatively at the surface, which was required for good imaging and uncaging. On average, only 3-4 pairs were found in one pyramidal neuron where only 1or 2 of them had proper orientation, and it was rarely located at the superficial layer 20 μm from the surface, where uncaging can be performed in a well-controlled manner. 4) The slice preparations were often not good enough to achieve 1)-3). Once all these criteria were met, however, the results were always consistent. The credibility of our experiments was assured by the *P* values of non-parametric tests, and by the consistency of the results with the three different methods of pressure application (pipette, sucrose and spine enlargement for FRET, Pr and average release).

(Page 7, line 160-162): "Finally, we confirmed that the evoked presynaptic release was augmented by the spine enlargement (Extended Data Fig. 10a–c) only when the spines elongated and pushed the presynaptic boutons (Extended Data Fig. 10d–r)."

Restated: Is there any glutamate release enhancement in spines that enlarge only in width? This would appear to be a crucial control that would help get at the idea that mechano-stimulation, and pressing against the bouton, is needed to enhance glutamate release, rather than just spine enlargement per se.

This is the reason why we compared pushing and non-pushing spines that enlarged (EFig. 10). Enlarged spines always broadened their widths (Matsuzaki et al. Nature 2004; Okamoto et al., Nature Neurosci. 2004). The increases in glutamate release were uncorrelated with spine enlargement (EFig. 10o,p), but correlated with spine elongation (EFig. 10q,r). The results fully conform to the increases in the FRET of SNAREs (Fig. 4i, EFig. 9d).

2. Related to point (1) above: spines enlarge relatively slowly, as compared to acutely pushing boutons with a pipette. So, there is a major concern that if the mechanical force of the pipette was applied as slowly and gradually as a growing spine, there would be no effect, as the bouton might adapt or accommodate, and the mechanical force would have no effect on release. The key questions are: what is the time course of spine growth (from the literature it seems to be minutes), and can that time course be mimicked with the mechanical force from a pipette? And, if that is done, is there still an effect? This would seem to be an essential experiment.

The time course of spine enlargement was dependent on the duration of stimulation, and since our stimulation protocol is 1 Hz 80 time, it took 80 s to reach the peak (Matsuzaki et al. *Nature* 2004). It was difficult to push the bouton slowly in a well-controlled manner, since the position of pipette and boutons were slowly drifting. On the other hand, a mild sucrose treatment (20 mM) was applied with a time course of approximately 1 min, and it reproduced the effects of spine enlargement. This was already described in the previous version:

(Methods, Page 15, line 363-366): “For all experiments, the sucrose solutions were bath applied for 5 minutes and then washed-out with ACSF. After switching the solutions, buffer exchange was completed within 1 minute, which was confirmed by measuring the osmolarity of the solution in the recording chamber.”

3. In general, another major concern is a small sample size and a lack of clarity regarding the replicates for each experiment in the study. In many experiments, the data come from only a few boutons, and it is not clear at any point how many preparations or animals were used for each experiment. It seems that the authors should consider at least 15 boutons from at least 2-3 independent preparations to be sufficient for most experiments.

We have also desired to have large data numbers and tried our best to make additional experiments over one year of struggle. The number of slices and rats are now described in the legends. In most cases, only one data could be obtained from one slice or one rat, as only one bouton could be targeted for each experiment. The difficulty of our experiments has been described for your comments #1. The total number of our experiments was combinatorically huge. The credibility of our conclusion was assured also by the consistency of the results with the three different methods of pressure application (pipette, sucrose and spine enlargement [FRET and Pr]). We hope that you also feel convinced with our data in the revised version.

Minor issues (in no specific order):
1. Regarding the increased Pr (from pushing to 31.5 min) in Figure 1h, is this detected from the same bouton at a different time point or different boutons at different time points? It would be constructive to show a few examples of release from the same bouton at different time points.

The Fig. 1h data were obtained from the same bouton at different time points. Probably, you misunderstood, because of our mistake in the legend, “from optogenetically stimulated bouton**s**.”

(Page 23, line 543): This has been corrected to "from **an** optogenetically stimulated bouton."

2. The model in Figure 1A represents a hypothesis; it is not clear whether spine enlargement will mechanically stimulate a bouton or not, so this should be presented as an idea rather than a known phenomenon.

As you pointed out, Fig. 1a presents the initial idea for our study, and we have added "Putative" to the mechanical actions in our legend as follows.

(Page 22, line 525): "Putative mechanical actions of the spine enlargement on a presynaptic terminal."

3. It looks like the glutamate increases before stimulation in Figure 1h,j. This apparent pre-stimulation increase does not occur in the sucrose experiments. How do the authors explain this pre-stimulation increase?

The apparent pre-stimulation increases (Fig. 1h,j Pushing and 11.5 min) reflected the baseline noise as they are well within the range of 3 SD (the shade). The apparent increase may also be partly due to the binning to reduce the noise level. Fig. 1h,j are the raw data from single bouton and noisy, while Fig. 1j, k are averaged trace, where there is no pre-stimulation increases.

4. The authors provide only show 4 negative controls from strong pushing; clearly a higher sample number is needed. If the model is true, why does strong pushing have no effect on release? Perhaps the boutons were damaged? In this context, perhaps it is a bit premature to exclude mechanosensitive channels? Is there an increase in SNARE FRET during strong pushing? There is no effect on release, so it begs the questions as to whether SNAREs are or are not affected.

We have now performed altogether 12 strong pushing experiments where a pipette pushed the bouton over the middle line (EFig. 1a). In the new series of experiments, we pushed more gently than the earlier experiments. Consequently, the inhibitory effect of pushing was partial on average (EFig. 1e,f), and reversible in a few cases. We detected FRET increases for the strong pushing as in the fine pushing and no $[Ca^{2+}]_i$ increases in these cases as well (data not shown). We thus speculate that the

bouton was not damaged, but molecular machinery was vulnerable, as we already described in the previous version.

(Page 4, line 81-84): "If the pipette pushed the bouton over the axial line of the axon (strong pushing), the evoked release was partly suppressed (Extended Data Fig. 1a–e), and the diameters of boutons were recessed by ~0.14 μm (Extended Data Fig. 1f,g)."

(Page 7, line 169-173): "The stronger pushing suppressed the evoked release (Extended Data Fig. 1), suggesting that the proper alterations in the conformations of cytosolic molecules, their binding, and structures of the membranes, facilitated the SNARE assembly and vesicle replenishment in an actin polymerisation-dependent manner[22]."

5. Is it possible to get enough spatial resolution to discern whether the 'SNARE assembly side', in each bouton, has more glutamate release?

The diffusion of glutamate is so rapid that we cannot estimate the release site with sub-bouton resolution in our imaging with the time resolution of at most 100 ms. Assuming the free diffusion constant of iGluSnFR as 100 $\mu m^2/s$, MSD in 2D space is ~6 μm/100 ms. Thus, it is not possible to find the release site in a bouton with widths of 1-2 μm.

6. There is a concern about potential presynaptic effects of glutamate uncaging. Can presynaptic effects be ruled out, perhaps by using pharmacological approaches?

In EFig. 9f and g, uncaging only without postsynaptic spike did not cause spine enlargement and increase in SNARE/FRET (n = 9), indicating that the presynaptic effects were not explained by uncaging itself. We have rephrased the text.

(Page 7, line 156-158): "The A1% increases never occurred by uncaging alone without postsynaptic spikes, which did not induce spine enlargement (Extended Data Fig. 9e,f, green traces)."

Referee #3 (Remarks to the Author):

Ucar et al have produced an intriguing and potentially exciting paper. The findings are unique, the experiments are apparently well-controlled and thoughtfully carried out, and the paper is generally understandably written despite its highly concise format. I do have several points of concern, however.

Thank you so much for your appreciation and constructive comments. We have addressed all of your concerns as below. Added or corrected parts of the text were marked with red.

First, the strong implication of the results is that in the abundant literature of plasticity involving spine size changes, physiological recordings of synaptic transmission should prevalently show strong and long-lasting changes in presynaptic release probability. However, in general terms, strong, long-lasting changes in Pr have not been much reported with classical LTP since the discovery of AMPAR insertion. The use of glutamate uncaging does obscure this critical question, but spine size increases are of course observed in numerous paradigms of LTP induction. Can the authors provide previous papers that reported a large change in Pr with STDP in the synapses used here? Do the authors find in the literature any evidence in such experiments of increases in Pr consistent with their model?

Nigel Emptage and Alan Fine laboratories have been reporting the Pr increases after LTP induction using the optical quantal analysis:

1. Padamsey, Z., Tong, R. & Emptage, N. Glutamate is required for depression but not potentiation of long-term presynaptic function. *eLife* **6**, (2017).

2. Padamsey, Z. & Emptage, N. Two sides to long-term potentiation: a view towards reconciliation. *Philos Trans R Soc Lond B Biol Sci.* 369, 20130154 (2014).

3.Enoki, R., Hu, Y. L., Hamilton, D. & Fine, A. Expression of long-term plasticity at individual synapses in hippocampus is graded, bidirectional, and mainly presynaptic: optical quantal analysis. *Neuron.* 62, 242-253 (2009).

4. Ward, B. *et al.* State-dependent mechanisms of LTP expression revealed by optical quantal analysis. *Neuron.* 52, 649-661 (2006).

5. Emptage, N. J., Reid, C. A., Fine, A. & Bliss, T. V. Optical quantal analysis reveals a presynaptic component of LTP at hippocampal Schaffer-associational synapses. *Neuron.* 38, 797-804 (2003).

We'd like to refrain from making any comparisons of their results with our study at this stage, since their induction protocols, preparations and the methods of measurement were quite different from ours.

Importantly, a more convincing test is that according to the authors' model, increases in Pr (and decreases in PPR) should occur reliably and just as often in conditions where spine size is transiently increased but LTP is not produced. For instance, KN62 treatment to block CaMKII activation preserves a large but transient spine volume increase yet prevents both LTP and sustained spine size changes. In the context of this paper, KN62 given during synaptically induced LTP or chemLTP should not block presynaptic FRET changes consistent with the mechanotransduction effect, and a sustained increase in Pr should be seen that is insensitive to KN62.

KN62 markedly reduced the enlargement induced with STDP in our laboratory (unpublished) unlike in the 0 Mg uncaging experiments, and we could not meaningfully perform the experiments you suggested. In the spine enlarge experiments, FRET and Pr were already enhanced within 4 and 10 min, respectively (Fig. 4g, EFig. 10i), indicating that the transient phase of enlargement was enough to induce the pushing effects. Consistently, the time course of the pushing effects was similarly fast and sustained in the transient pipette pushing experiments (Figs. 1,2) and sucrose experiments (Fig.3), as in the spine enlargement experiments (Fig. 4g, EFig. 10i,j).

Second, the mechanism here is very loosely considered. One might argue that the overall novelty of the observations, the technological development that was necessary to carry them out, and the rigor with which they were executed obviate the need to demonstrate a clear potential mechanism. Nevertheless, it remains quite vague how this phenomenon arises, and the authors to my mind overstate the conclusions they can draw at this point. Other than the requirement of actin, as demonstrated with latrunculin, there is little to go on. Since of course the bath-applied latrunculin affects all cells, it is not even possible to determine in which cell the actin is acting (e.g. rigidity or tension in surrounding glia might be required for the FRET effect in boutons when they are pushed upon). Though the authors perfuse postsynaptic CK-666 via the patch pipette, that drug is also cell permeable and may affect Arp2/3 in other nearby cells such as the bouton. (The authors further weaken this part of the

paper by citing an Arp2/3 knockout study when referencing the mechanism of action of CK-666). The authors also give a strange and undefined name (actinARE) to the process(es) involved here, which seems unwarranted without further examination of the mechanisms. An abundance of potentially related mechanisms might be considered to deepen and solidify the model here, including integrins or other components of mechanotransduction in other cells.

We appreciate your thoughtful comments. According to your suggestion, we have decided to remove the CK-666 data for clarity. We have also replaced the specific name actinARE with more general one PREST(PREssure Sensation and Transduction mechanism) to faithfully represent the state of understanding.

(Page 8, line 173-176): "Thus, we found a new mechano-sensation[14] and transduction (PREST: PREssure Sensation and Transduction) mechanism which senses the focal pressure and responds by facilitation of evoked release at least for 20 min in the specific presynaptic terminals (Figs. 1, 3, 4).."

Third, from a phenomenological standpoint, I am somewhat confused by what the "pipette pushing" is doing to the synapse. The authors indicate in Fig 2 that pushing onto the bouton in the area of highest FRET produces the largest effect. This area should be the active zone, and thus one might assume the pipette is in fact just directly pushing through the spine into the terminal. This sounds potentially disruptive to both cells. Could the authors instead simply push on the spine or even nearby glia to produce presynaptic changes?

In Fig. 2b, we pushed the one side of the bouton possibly with or without AZ, but did not aim at the invisible small AZ with a dimeter of 0.02 – 0.2 μm. Thus, pushing must be effective even though the pipette was not located precisely on the AZ. We have tried for a year, but unable to find the putative synaptic contact where the bouton and spine were precisely aligned coincidentally along the axis of the pipette direction so that bouton could be pushed by the spine without its escaping. For pipette manipulation, these pairs need to be at the surface (20 μm) of slice. Interestingly, approximately in 5% of the pushing experiments with pipette, the boutons displaced before the pipette apparently touch the boutons, and in these cases pushing must be applied indirectly by an intervening tissue with unknown nature. FRET could be increased in such cases. Although it may relate to the condition you suggested, we have decided not to include such data, because we are unable to specify the type of cells and tissues which were responsible for pushing

(Methods, Page 11, line 243-245): "We excluded those data where the boutons escaped from the pipette within 1 min or where a pipette apparently pushed a bouton indirectly via intervening tissues."

A minor complaint is that the FRET experiments should report less processed results, potentially the raw lifetimes rather than just the rather heavily normalized "A1%."

We have made images for the averaged lifetime, as shown in EFig. 2g,h.

(page 14, line 317-319) "The average lifetimes[12,28] were also obtained and sample images were presented in Extended Data Fig. 2g,h for comparison with A1% (Extended Data Fig. 2e,f)."

In addition, the N's for the critical experiments in Fig 4 (4f-I particularly) and Ext Data Fig 8 are quite low, and it is not clear what criteria were used for including data in those experiments.

The initial version was submitted when we needed to halt our experiments by the outbreak of COVID19. After that, we have performed many additional experiments for spine enlargement and SNARE/FRET measurements shown in Fig. 4 and EFig. 9 (n =14 pushing and n = 9 non-pushing cases), and also Pr experiments in Fig. 10 (n = 6 pushing and n = 5 non-pushing cases). To succeed in these experiments, many conditions need to be realized. 1) The proper quality and level of expressions of virus vectors for multiple plasmids (CsChrimsonR, iSLIM, iGluSnFR, mScarlet). For example, they were OK for two weeks, but not ideal in the next three weeks, and we needed to readjust or remake the AAVs, and screened the optimal samples from time to time. 2) Stable repetitive fluorescent imaging (iSLIM/FLIM, iGluSnFR) for an hour when the same synapse was challenged by induction of plasticity under whole-cell clamping is a high-wire act. 3) The difficulty in finding out a good synaptic contact located relatively close to the surface, which was required for good uncaging and pipette manipulation. On average 4-5 pairs were found for on pyramidal neurons where only 1or 2 of them had proper orientation, and it was rare that such pair could be found in 20 μm from the surface so that uncaging and pipette pushing was better controlled. 4) The slice preparations were often not good enough to achieve 1)-3). Once, all these criteria were met, the results were always consistent. The credibility of our experiments was assured by the P values with non-parametric tests, and by the consistency of the results with the three different methods of pressure application (pipette, sucrose and spine enlargement).

Reviewer Reports on the First Revision:

Referee #1 (Remarks to the Author):

The authors addressed all concerns from the reviewers. It is an innovative idea, and their elegant experiments provide strong evidence that local pressure may play a role in synaptic plasticity.

Referee #2 (Remarks to the Author):

This is a thoroughly revised manuscript, and all of the concerns thatI raised during the review process have been fully addressed. This is a fascinating and remarkable study. I have no further suggestions. To the authors: well done.

Edwin Chapman

Referee #3 (Remarks to the Author):

- I appreciate the more conservative approach to the mechanisms at work here. It remains quite mysterious to me how this effect might come about, but this understanding can come later.
- the authors understate the size of the active zone and thus under-estimate their odds of directly pushing on it. (AZ's are rarely < 100 nm and frequently 200 to 500 nm in diameter, occupying 50% or more of the spine face of the bouton). Nevertheless, the data in their answer to this concern is adequate about my concern.

However, I remain somewhat unsure about the physiological relevance of the observations:
- The author's reticence to relate their findings to LTP literature is surprising, since it is a major motivation for the work. They provide in their response no evidence as to whether STDP as they have applied it is accompanied by increases in glutamate release, which weakens the impact of the work. Transient spine enlargement produced by other mechanisms (e.g. short-term enlargement after ineffectual LTP induction stimulus) is not generally seen as a mechanism of "working memory."

- From the response to Reviewer 1's concern: Note that Ballyk et al in fact concludes: "The osmotic effect on field potential amplitudes appeared to be independent of synaptic transmission because the inverse relationship with osmolality held for the antidromically evoked PS. Moreover, as recorded with respect to ground, the intracellular EPSP-inhibitory postsynaptic potential (IPSP) sequence (evoked from CA3 stratum radiatum) was not altered by osmolality." The work here is certainly internally consistent, and armed with numerous controls, but its relevance does depend on hoping for effects that are not strongly supported by other work.

Author Rebuttals to First Revision:

Referee #1 (Remarks to the Author):

The authors addressed all concerns from the reviewers. It is an innovative idea, and their elegant experiments provide

strong evidence that local pressure may play a role in synaptic plasticity.

Thank you so much for carefully reading our manuscript again. We have treasured your previous constructive comments to improve our manuscript.

Referee #2 (Remarks to the Author):

This is a thoroughly revised manuscript, and all of the concerns that I raised during the review process have been fully addressed. This is a fascinating and remarkable study. I have no further suggestions. To the authors: well done.

Edwin Chapman

Thank you so much for carefully reading our manuscript again. Your constructive comments have been a major driving force to pursuit the additional experiments presented now.

Referee #3 (Remarks to the Author):

Thank you so much for carefully reading our manuscript again. We have deeply appreciated your thoughtful comments for revision.

- I appreciate the more conservative approach to the mechanisms at work here. It remains quite mysterious to me how this effect might come about, but this understanding can come later.

We are glad to know that you like the new name (PREST) better.

- the authors understate the size of the active zone and thus under-estimate their odds of directly pushing on it. (AZ's

are rarely < 100 nm and frequently 200 to 500 nm in diameter, occupying 50% or more of the spine face of the

bouton). Nevertheless, the data in their answer to this concern is adequate about my concern. However, I remain somewhat unsure about the physiological relevance of the observations:

We appreciate that you agree with our answer.

- The author's reticence to relate their findings to LTP literature is surprising, since it is a major motivation for the work. They provide in their response no evidence as to whether STDP as they have applied it is accompanied by increases in glutamate release, which weakens the impact of the work. Transient spine enlargement produced by other mechanisms (e.g. short-term enlargement after ineffectual LTP induction stimulus) is not generally seen as a mechanism of "working memory."

The effects of the pushing on the increases in glutamate release are now provided in EFig. 10.

- From the response to Reviewer 1's concern: Note that Ballyk et al in fact concludes: "The osmotic effect on field potential amplitudes appeared to be independent of synaptic transmission because the inverse relationship with osmolality held for the antidromically evoked PS. Moreover, as recorded with respect to ground, the intracellular EPSP-inhibitory postsynaptic potential (IPSP) sequence (evoked from CA3 stratum radiatum) was not altered by osmolality." The work here is certainly internally consistent, and armed with numerous controls, but its relevance does depend on hoping for effects that are not strongly supported by other work.

We think the difference from the old study was due to the usage of new direct techniques in our study.