Review of: "Membrane tension propagation couples axon growth and collateral branching"

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Review on manuscript entitled “Membrane tension propagation couples axon growth and collateral branching” by Zheng Shi, Sarah Innes-Gold and Adam E. Cohen (https://doi.org/10.1101/2022.01.09.475560)

The paper by Shi et al., extends a previous study by the authors (https://doi.org/10.1016/j.cell.2018.09.054), showing that plasma membrane tension can vary substantially over micron scale in cells. This is indeed an exciting and fundamental aspect of cellular membranes and relates to many diverse biological functions. In this paper, the authors have studied the specifics of membrane tension propagation in neurons. They show that membrane tension propagation differs hugely between neuronal dendrites and axons. They furthermore show that fast propagation of membrane tension along axons represents a mechanism by which mechanical cues at one part of the axon can affect growth dynamics remotely. Specifically, the observed opposing feature of axon elongation (by low tension at the growth cone) and branch induction with simultaneous growth cone stalling is very exciting. Such an inverse regulation of axonal elongation and axonal branching has been described in the literature (e.g. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6856728/); it is thought to depend on a constant growth supply allocated by spatiotemporally imposed neuron-intrinsic and/or extrinsic cues. In this context, the described mechanism of axonal membrane tension propagation in this paper may be such an intrinsic cue. One would envision such a mechanism contributing, for example, to terminal branching where branch induction by tension propagation along the axon shaft might serve to efficiently fill the target region with branches without overlap.

General Comments

1. The central questions addressed in this work are exciting and represent a fundamental aspect of neuron biology, specifically axonal biology. The manuscript is well-curated and the results are elegantly presented and discussed.

2. Comment on the developmental stage/day in vitro (DIV) age of the neurons analyzed

It is not always clear what was the DIV of neurons analyzed. The authors state that they generally analyzed DIV7-14 neurons, but this is a wide range spanning stages of neuronal maturation. This wide range might impact more on dendrite differentiation, morphology and physical properties (e.g. spines). Also, this wide range may impact on axons, for example, electrical maturation. Do the authors have any comment for age-dependency in the propagation of membrane tension in axons and/or dendrites? Also, how the axon/dendrite caliber might affect propagation of membrane tension? For example,

there seems to be a slight but visible variability in axonal process calibers in Fig, 3A vs 3C.

Specific Comments

1. On the molecular tools employed in this study. In their previous paper (Cell, 2018) the authors used a membrane bound GPI-GFP marker for fluorescent measurements of membrane tension, while in this study membrane tension was measured by using cytosolic GFP. It is unclear to us whether the use of a cytosolic sensor to measure membrane tension is indeed comparable to a membrane-bound sensor. Furthermore, given the considerable differences in cytosolic architecture between the thin tubular axon and wider dendrites proximal to nearly spherical somata, a cytosolic readout might be affected more than a membrane bound. Is cytosolic GFP possibly primarily a readout for cytosolic diffusion/tension and only an indirect readout of membrane tension? In addition, to measure growth cone size in response to pharmacologically or mechanically altering membrane tension in the current manuscript, the authors used a pHluorin-tagged neurexin construct instead of the cytosolic eGFP. This construct monitors neurexin abundance at the plasma membrane and is therefore potentially affected by additional mechanisms independent from membrane tension.

In conclusion, the variation in the use of fluorescent sensors is not explained clearly in the text. Does a cytosolic vs membrane construct only change the sensitivity of fluorescence/membrane tension measurements and to what extent? One plausible explanation might be the difficulty in sufficient labeling of thin axonal shafts by membrane- targeted constructs? If this is the case the authors could comment on that.

2. Figure 1. How close to soma is the location of these tethers on dendrites? Is there a possibility that in more distal parts tension propagation changes? Have the authors tried any secondary or tertiary dendrite? (This also relates to General Comment 2)

3. Figure 2. On the nature of the induced pearling on the axon. Axon pearling has been described as an early sign of axon degeneration. There also appears to be a link to mechanical properties of the cytoskeleton and membrane tension (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6731471/), where disruption of the cytoskeleton can induce pearling of the axon. Do the authors suspect that membrane tension is indeed the driving force for this observed pearling and that loss of cytoskeletal maintenance of axon-membrane tension underlies the behavior? Or could alternatively altered membrane tension regulate stability of the cytoskeletal core (microtubules) of the axon to induce pearling? Simultaneous visualization of membrane tension and the cytoskeleton could be very informative here.

4. On the terminology of “branches” used in Figure 3 & 4.We are not sure whether the term “branch” really applies to the small membrane protrusions observed along the axonal shaft and proximal to the growth cone upon altering membrane tension (Fig.3D, 3G, 4A, & 4C). Branches have been defined in literature to be protrusions > 10µm in length or as containing a microtubule-based core, while branch-precursors are usually shorter and contain F-actin. The size of the observed nascent "protrusions" fall well below what is considered to be a branch. It is furthermore unclear whether these formed "branches" contain any F-actin or even tubulin at this stage. We understand the author's reasoning since it is true

that these nascent protrusions can be filopodial/branch precursors, but the term branch is used rather in a generic way here. This is not trivial or simply a terminology issue, especially for the results on preexisting branches in figure 4. One can only imagine the vastly distinct implications for engagement of molecular mechanisms by membrane tension propagation if we are addressing small membrane-based protrusions vs actin-driven filopodia vs microtubule-driven branches.

5. Specific comments to experiments of Figure 3 and 4.

a) It would be very interesting to know, mechanistically, whether the observed protrusions are purely membrane based or whether they contain F-actin (or even microtubules), e.g. using the tools from the 2018 paper (mOrange2-KRAS and Lifeact-CFP). In case these protrusions contain F-actin, it would also be very interesting to measure whether membrane protrusions could precede (or even trigger entry of) F-actin filaments, or whether membrane-tension first feeds towards the cytoskeleton to induce a “pushing out” of the protrusion.

b) One important question regarding the implications for neuron morphology in Figure 3 concerns the lifetime of these nascent protrusions. For example, upon diffusion of the deoxycholate or mannitol solution do these protrusions persist? continue to grow? or do they revert?

c) Figure 4: The term retraction for the growth cone might be overemphasized. Stalling of the growth cone or simply size reduction would be more precise.

d) In Fig 4C it is not clear what the dark and white spots represent. Probably differences in fluorescence between T2-T1 and T3-T2? This should be stated clearly, with further indicating which color represents loss versus addition of a structure.

6. One obvious feature that might support fast propagation and the bidirectional regulation of protrusive activity along axons is the F-actin cytoskeleton and specifically the axonal F-actin rings which the authors discuss thoroughly (page 6). The authors could proceed in disruption of the F-actin rings by limited latrunculin treatments (as in ref 40) and then measure again membrane tension propagation. That should be, in principle, an easy experiment to perform and would impact significantly on their study.

Minor comments

1. Fig. S1. The breaking of the axon does indeed cause a large increase of Ca^{2+} signal but it is unclear whether the Ca^{2+} reporter has a dynamic range suitable to report localized and presumably not large Ca^{2+} signals along the axonal shaft upon tether pulling. Have the authors tried localized application of Ca^{2+} channel agonists as a positive control that they can indeed measure these small, localized signals? Measurements of membrane tension propagation/axonal pearling after blocking of the candidate Ca^{2+} channels or buffering of extracellular Ca^{2+} could be alternative experiments here.

2. Some figure legends and method descriptions would benefit from more detail:

- Figure 2: Do the panels (especially panel E) show example traces for one measurement or are these all measurements? There is no information on how often the experiments were repeated in the figure legend.
- Figure 3: At which time point after perfusion was the number of protrusions quantified?

- Figure 4: D&E; were the t-tests adjusted for multiple comparisons?

- Especially the image analysis methods descriptions are very underdeveloped: (a) How was the area of the growth cone quantified? As integrated fluorescence density with thresholding (how was the threshold chosen)? Or as manual area selections based on morphology? (Fluorescence based area quantification could be affected more by membrane-abundance of the pHluorin-sensor compared to morphology-based area selection); (b) What exactly is the relationship of tether fluorescence and diameter? Where was tether fluorescence measured, how was background corrected and fluorescence accumulated for the “background-corrected cumulative cross-sectional fluorescence of the tether vs. axon”?; (c) How and where were GCaMP signals quantified?