

Peer Review File

Manuscript Title: Regulation of the mammalian-brain V-ATPase via ultra-slow mode-switching

Redactions – unpublished data

Parts of this Peer Review File have been redacted as indicated to maintain the confidentiality of unpublished data.

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

The manuscript, “Pump-Rest-Leak-Repeat: regulation of the mammalian-brain V-ATPase via ultra-slow mode-switching” by Kosmidis et al. seeks to characterize and quantify the activities of V-ATPases in native synaptic vesicles (SVs) isolated from rat brain at the single-molecule scale. SVs drive protons into the SV lumen and this pH gradient, in turn, drives the loading of neurotransmitters into the SV lumen. It has been previously reported by proteomics mass spectrometry and other methods that each individual SV contains approximately 1.2-1.4 V-ATPases in its membrane on average. Hence, V-ATPase activity and the underlying SV lifecycle is understood to be governed to a significant extent by the activities just a single V-ATPase.

Following foundations established by work from this laboratory (Veshaguri et al. Science 2016; reference 19), and others (Hernandez et al. Science 2012), the authors set out to directly measure V-ATPase activities in single SVs using a pH indicator, DOPE-pHrhodo. To achieve this goal, they employed a clever technique that their group harnessed, wherein isolated SVs are fused in situ, on the microscope with surface-immobilized large unilaminar vesicles (LUVs) that are first filled with DOPE-pHrhodo dyes and reconstituted SNAP/SNARE machinery. The details on how this was precisely achieved, and how the authors ensure that “hundreds” of fluorophores are encapsulated per surface-immobilized LUVs was difficult to follow based on the information given. How big are the LUVs precisely? How do they get an estimate of “hundreds” of fluorophores in each LUV lumen (this seems quite high if LUVs are 150-200 nm vesicles)? How much SNAP/SNARE fusion machinery was used to efficiently capture/fuse with the isolated SVs? Details on these points seem relevant (see comments below).

Prior efforts to measure pH changes within SVs were performed using genetically encoded pH sensitive protein, super-ecliptic pHluorin (ref. 11), which the authors indicate lacked the single-to-noise ratio needed to reveal what is claimed to be revealed by the present investigation. In this earlier study, the authors classified their SVs are either containing vesicular GABA or vesicular glutamate transporter 1 using “on-stage” antibody labeling. I infer from this report that the SVs used in the present investigation represent a mixture of SVs containing different neurotransmitter types. The authors should clarify this point. They should also clarify the ultimate size of their fused SV-LUV proteoliposomes and calculate by how much the protein concentration in the SVs drops after the fusion event to inform the reader on the extent of dilution. From Figure 1a, the message is that the change in dilution may be considered relatively minor, but if an SV is ~40 nm and an LUV is approximately 150 nm (per standard definitions), then the effective change in surface area of the proteoliposome is on the order of 30 fold, yes? The fold-dilution could indeed be much larger if the

authors definition of LUV is more liberal (eg. 500 nm) Clarity on this point touches on the matter of how the authors pack “hundreds” of DOPE-pHrhodo fluorophores into the LUV lumen to start with. If the LUVs were 150 nm, the original reconstitution must have been performed with hundreds of μM to mM dye concentrations. In this regime, do the dyes perform as advertised?

Using this unique set up, which has much higher dynamic range than previous efforts on similar systems, the authors investigate proton pumping into the lumen in response to ATP, resulting in a lowering of the luminal pH that increases the average brightness of the DOPE-pHrhodo dyes therein. The authors nicely show that the luminal dyes for an ensemble average of approximately 450 fused SVs becomes about 1.5-fold brighter upon the addition of ATP, where the range for individual vesicles measured was roughly 1.2-2 (Figure 1b). In ref. 11, the normalized fluorescence intensity change was reported to be about 1.3, no? Can the authors comment on whether the strategy employed indeed increases the signal to noise above and beyond ref. 11? Perhaps I am misinterpreting something. The authors show through control experiments that the pH change for the ensemble reflects a change in luminal pH from about 7.1 to about 6.2 (ED Fig.4), globally consistent with the results of glutamatergic and GABAergic vesicles isolated from mouse brain (ref. 11). Does the variance in range evidenced somehow reflect the overall sizes of the fused proteoliposomes? Do the authors make an effort to estimate the extant range of vesicle sizes measured? Can the authors derive an ATP hydrolysis rate or proton-pumping rate from their data to establish connections to prior literature on these points? Although this may be extremely difficult, as it must take into consideration the passive protein leak rate, it seems like it may be helpful to the reader. The authors calibration curve of normalized intensity and the known pH, which they convert to pH in the ED, would also be more helpful if moved up in the text and more clearly explained.

The main conclusion from this initial section is that their findings are consistent with approximately 1.2 V-ATPase proteins per fused SV on average, which is in line with expectation given previous studies.

A key differentiating aspect of the present study appears to be the extended time of imaging afforded by the hundreds of luminal fluorophores. The full imaging time scale appears to be multiple hours. Here the authors report that the V-ATPase does not pump continuously but rather switches stochastically between proton-pumping, inactive and proton leaky modes (of two distinct types – pump independent and pump dependent). The observed switching behavior takes place on the order of tens of minutes. As the authors conclude, this finding would suggest that pumping rate of a single V-ATPase may be about 170% higher than inferred from ensemble measurements. At this juncture in the story, it would seem relevant to comment on the physiological relevance of the hours-long time scale to make the case that the finding is important to SV function? Don't synapses release the majority of vesicles on the seconds time scale?

Figures 1C and d, directly show these switching behaviors and while clearly a departure from a saturating plateau as may be suggested from Figure 1b, it seems remarkable more complex than what could be expected from an on-off toggle switch in single molecule activity. For instance, the top panel of Figure 1c seems to fluctuate quite madly during the first 30-40 minutes. This single vesicle appears to exhibit the full range of fluorescence intensities exhibited by the ensemble (Fig. 1b). Is this a representative trace? Based on the fluorescence intensity/pH calibrations shown in the

ED, it would seem that this would suggest fluctuations in a full pH unit or more over the course of time. Is this true? What do the distribution of trace behaviors look like? Does this trace suffer from time averaging of the switching modes or is there something else going on like variable ATP hydrolysis, variable proton pumping rates, or fluorophore photophysics that are convolved in the signal as well?

Another key question is whether the behavior of V-ATPase with ATP and Bafilomycin (baf) added somewhere in the 1-3 hour range (similar to that is shown in 1b) shows a permanent decay of the pH gradient to the background pH value as it should? Robust suppression of the signal changes for individual V-ATPases would be very grounding as it would convey to the reader that the stochastic behaviors over these extended periods indeed stem from stochastic mode switching of the protein not the sensors or other variables associated with the measurements. The more temporally focused (0-30 minute long) traces shown in Fig.1d appear to show more clearly defined switching behaviors but even there, the fluctuations appear quite dramatic given that the signal arises from the collective brightness of hundreds of luminal fluorophores.

Analyzing data that has been reduced in complexity using Bayesian stochastic filtering (Chung-Kennedy filter) methods (see ED Fig.3), the authors go on to report that ATP concentration regulates the switching probability of the V-ATPase to be in the proton pumping mode, not the ATP hydrolysis and thus the proton pumping rates. On the other hand, electrochemical proton gradient regulates both pumping rate and the lifetime of the pumping and inactive mode. If I understand things correctly, a key assertion is that their theoretical modeling can explain the types of noise fluctuations evidenced in Fig. 1 and ED Fig.3. This would be confidence inspiring. Moreover, it is also inferred that ATP hydrolysis continues at steady state during the switching periods, such that proton pumping is somehow specifically uncoupled from the enzyme's ATPase activity. Have the authors verified this to be the case? Or is this inferred from prior literature? This seems important to delineate more clearly for the reader. The origins of the off-states are not at all clear and the authors do not attempt speculation on this front.

On the whole, the manuscript touches on a highly significant biological question and a potentially new perspective on SV biology and V-ATPase activity that has not been appreciated previously. The manuscript is organized in a logical fashion to convey the overall findings to the reader, but additional clarity on the technical details of how the work was achieved seems paramount to reproducibility of the findings. The concept of mode switching in biological systems, particular in the membrane, have been documented previously (eg. Ion channel literature and smFRET data on GlTPh). A perceived strength of the present finding of mode switching in the SV V-ATPase is further support to the argument that mode-switching behaviors in individual molecules may serve important biological functions.

In the present case, the manuscript's perceived significance would thus seem to hinge on the physiological relevance of the proposed switching events. Are SVs preserved for such extended periods of time (tens of minutes to hours) in the animal brain? What role could such behaviors serve? If tied to a biological function, one could more easily overlook the possible impacts of the non-native environments on the V-ATPase activity established in the present investigations— such as those arising from significant dilutions into lipids that may not be representative of the rat brain and

or the fluorophore and SNAP/SNARE components in the reconstituted fused, SVs. Have the authors examined whether there is a lipid dependency to the switching behavior?

Another potentially fascinating take away is that the V-ATPase must exhibit an intermediate state in which proton pumping is terminated but ATP turnover is not affected. Is this something that the authors could speculate on structurally given prior structural/functional work? More context to this finding and the potential mechanistic significance of the proposed switching behavior would also seem critical to convey to the reader to provide adequate context. Is it not also possible that the data may be explained by the reversible binding of an inhibitor to the V-ATPase? Further clarification on how such a model can be definitively excluded seems important to the message.

Referee #2 (Remarks to the Author):

Review comments for Author

The acidification of vesicles by V-ATPase is responsible for protein quality control, neurotransmitter accumulation, and autophagy, etc. The pH of acidic vesicles is different in different vesicles, therefore the proton pumping activity of V-ATPase should be regulated.

In this study, the authors measured pH changes in a single vesicle by fusing synaptic vesicles containing V-ATPase to single-membrane liposomes containing a pH probe immobilized on a glass surface. Although the method is not new, as it is almost identical to that used in previous studies by the author's group, there are some interesting points in the results obtained. In particular, the authors claim to have captured the fact that V-ATPase repeats the on-off mode of the proton pump. This is the first time that the on-off mode of the enzyme has been reported, and the single molecule measurement of the proton pumping activity of V-ATPase in synaptic vesicles is also interesting.

The novelty of the results and their physiological importance of V-ATPase regulation mechanism have attracted the interest of researchers in many fields, but on the other hand, there are some questions about the interpretation of the experimental data that need to be answered before this paper can be published. In particular, the relationship between the pHroad fluorescence value and the pH in the vesicle needs to be interpreted carefully, since the pHroad fluorescence varies with various factors.

major points:

1. Number of V-ATPase in a fused vesicle

Synaptic vesicles are fused to a large liposome (LUV) fixed to a glass surface. In this case, multiple synaptic vesicles could be fused to a single LUV. The authors claim that most of the observed liposomes have only one V-ATPase because of the magnitude of the pumping activity of the V-ATPase. In addition to pump activity, counting the number of V-ATPases in other ways would increase the reliability of the experiments. For example, a recent paper reports a method to count the number of V-ATPases in intracellular lysosomes using fluorescent labeled SidK (J Cell Biol (2022)

221 (3): e202107174). I strongly recommend that this experiment be performed.

2. Size of the fused liposomes

The authors confirmed the homogeneity of purified SVs by cryoEM. This is excellent. On the other hand, there is no mention of the size of LUVs. The size of reconstituted liposomes has a great impact on the measurement of proton pumping activity; it would be more convincing if the size of LUVs fused with SVs were also measured by cryoEM. This is a simple experiment and should be performed.

3. Stability of liposomes on a glass substrate

In this experiment, liposomes were exposed to the glass surface for a long time, and then the fluorescence of liposome was measured. In order to exclude the possibility of liposomes fusion each other or adsorbing on the glass substrate, it would be good to show that the liposomes maintain their shape stably on the glass surface for a long time. For example, it is possible to indicate the stability of liposome on the glass surface by observation of fluorescently labeled liposomes for a long time.

4. Stability of V-ATPase

I afraid human-derived V-ATPase is not stable for a long time incubation at room temperature. It is necessary to show by biochemical methods that the activity of the V-ATPase reconstituted in liposomes is stable even if it incubated at room temperature for one hour.

5. $\Delta\text{pH}_{\text{max}}$ values

It is interesting that the fluorescens of pHroad reaches a constant plateau after ATP is added (Fig.1c, d). The proton motive force formed by V-ATPase is determined by ΔG of ATP hydrolysis. Therefore, changing the ratio of ATP:ADP:Pi should change the plateau value of pHroad. This experiment will increase the reliability of the $\Delta\text{pH}_{\text{max}}$ value.

6. Effect of chrolide ion on $\Delta\text{pH}_{\text{max}}$ value

In Fig. 3, the authors show the results of an experiment in which the addition of chrolide ion increased the ΔpH value. If the chrolide ions were transported into the vesicle through the synaptic vesicle-derived transporter, this increase in ΔpH should be cancelled out by inhibitors to the transporter. The authors should preform the negative control experiments.

7. Effect of VNUTs on the experiments

Synaptic vesicles contain VNUTs, which are transporters of ATP, and they transport ATP into the vesicles by proton-driven force. The transported ATP may affect the pH in the vesicles. There is also a possibility that the ATP concentration in the measurement system may change as ATP is transported into the vesicles. To exclude this possibility, it would be better to have a control experiment that inhibits the activity of VNUT.

8. Relationship between ATP concentration and $\Delta\text{pH}_{\text{max}}$

As shown in Fig. 4, it is interesting to note that the plateau value of $\Delta\text{pH}_{\text{max}}$ remains constant even when the ATP concentration is changed. This indicates that for a single vesicle, there is no correlation between the number of protons transported per second and the measured ΔpH . A

discussion of the theoretical explanation for this in terms of vesicle volume, number of protons transported, and expected pH change in the vesicle will help many readers understand this.

minor points

1. p7, line 168

Fig.2c should be changed to Fig.2d.

2. p11, line 300

V/F-ATPase should be changed to V/A-ATPase.

Referee #3 (Remarks to the Author):

The present manuscript describes the characterization of proton pumping by mammalian V-ATPases in hybrid synaptic vesicles. These hybrid SVs (hSVs) are comprised of purified rat SVs fused to liposomes in which the lumen has been enriched with lipid conjugated pH sensitive fluorescent reporters. Further, the hSVs vesicles are tethered to glass slides making them amenable for imaging under TIRF illumination with single hSV resolution. The high copy number of fluorescent reporters dramatically increases the experimental photon budget and allows the authors to achieve higher signal resolution and longer total measurement duration of single SVs compared to previous work that relied on genetically encoded fluorescent reporters.

This work builds upon previous investigations by the same group into the proton pumping characteristics of the P-type ATPase. The key findings in the current manuscript, stochastic switching between proton-pumping, inactive and proton-leaky modes, are similar to the previous work. However, the current work does delve into deeper detail about the regulation of mode lifetimes and pumping rate. Overall, the quality of data is very impressive, the results are convincing and clearly support the interpretations that are made.

Minor points

- The authors should better support or temper the claim of three “novel” modes by more closely comparing this work to the findings in the P-type ATPase work.
- Similarly, phrasing such as “upending conventional wisdom” when describing mode-switching base regulation should be moderated, especially when later stating that “mode-switching... appears to be a general property of enzymes”.
- The authors infer that similar behaviour is likely to occur in SVs. Could the authors be more explicit in the physical differences between SVs and hSVs (luminal volume, buffering capacity, etc), and how these differences could potentially influence inference about V-ATPase activity in native SVs. (For example, in the averaged ensemble measurements (Fig 1b), acidification reaches steady state after about 15 minutes. In previous work in native SVs this occurs on the order of 10's of seconds.)

Author Rebuttals to Initial Comments:

Response to Reviewers' Comments:

Reviewer 1 (R1)

Comment 1.1

The manuscript, "Pump-Rest-Leak-Repeat: regulation of the mammalian-brain V-ATPase via ultra-slow mode-switching" by Kosmidis et al. seeks to characterize and quantify the activities of V-ATPases in native synaptic vesicles (SVs) isolated from rat brain at the single-molecule scale. SVs drive protons into the SV lumen and this pH gradient, in turn, drives the loading of neurotransmitters into the SV lumen. It has been previously reported by proteomics mass spectrometry and other methods that each individual SV contains approximately 1.2-1.4 V-ATPases in its membrane on average. Hence, V-ATPase activity and the underlying SV lifecycle is understood to be governed to a significant extent by the activities just a single V-ATPase.

Following foundations established by work from this laboratory (Veshaguri et al. Science 2016; reference 19), and others (Hernandez et al. Science 2012), the authors set out to directly measure V-ATPase activities in single SVs using a pH indicator, DOPE-pHrodo. To achieve this goal, they employed a clever technique that their group harnessed, wherein isolated SVs are fused in situ, on the microscope with surface-immobilized large unilamellar vesicles (LUVs) that are first filled with DOPE-pHrodo dyes and reconstituted SNAP/SNARE machinery.

Reply

We are truly grateful to R1 for the in depth reading of this MS and the many valuable, insightful and constructive comments. In the following we strived to address in full all your concerns with new experiments, as well as to clarify a few potential misunderstandings.

Motivated by your question on the physiological relevance of the hybrid/diluted SVs, we performed an entirely new set of experiments in intact native SVs. These new experiments validate mode-switching of the V-ATPase and stochastic vesicle acidification, in the physiologically relevant environment of intact native SVs.

As highlighted by reviewer #2, the activity of the V-ATPase has to be regulated. Here we document mode-switching of the V-ATPase for the first time, but most importantly we show that physiologically relevant parameters (electrochemical gradients and ATP) regulate activity predominantly by regulating mode-switching. This suggests that a broad physiologically important role of mode-switching is regulating the activity of the V-ATPase.

We sincerely hope the substantial body of new experiments, combined with the new kinetic modelling of acidification and the detail answers provided below, address all the reviewer's concerns and will prompt them to recommend publication of this manuscript.

Comment 1.2

The details on how this was precisely achieved, and how the authors ensure that "hundreds" of fluorophores are encapsulated per surface-immobilized LUVs was difficult to follow based on the information given.

Reply

R1 correctly remarks that very little technical information was provided in the main text. Especially for steps that have already been published we only added 1-2 relevant citations. The full information on sample preparation, as well as additional relevant literature, was in the section *Methods* but we did not make that clear in the main text. We apologize for this omission, and we are very thankful to R1 for bringing it to our attention. We have now insured that the *Methods* section is well cited in the main text.

Revised main text:

- "...instead isolated the endogenous V-ATPase in intact SVs purified from rat brain^{7,8} ([see Methods](#))"
- "...using a well-tested protocol of SNARE-mediated vesicle fusion⁹ ([see Methods](#))"
- "... we first tethered on a glass slide large unilamellar vesicles that contained DOPE-pHrodo and the SNARE acceptor complex, syntaxin 1 and SNAP 25 (LUVs) ([see Methods](#))".

Comment 1.3

How big are the LUVs precisely? How do they get an estimate of "hundreds" of fluorophores in each LUV lumen (this seems quite high if LUVs are 150-200 nm vesicles)?

Reply

New cryoEM experiments measure LUV diameter and allow us to calculate that the number of fluorophores/LUV ranges 16-340, as requested.

We are very thankful to R1 for these two very relevant questions. New CryoEM measurements (Extended data Fig. 2) demonstrate that LUVs are unilamellar and have an average diameter of 72 nm. The distribution of diameters had a standard deviation of ± 43 nm and extends up to ~ 180 nm.

Here the fluorescent pH indicator is conjugated to a lipid (DOPE). The lipid conjugate (DOPE-pHrodo) partitions exclusively to the membrane of the LUVs i.e. it is not water soluble and it is not encapsulated in the lumen of the LUV. This is now clarified in the main text and clearly illustrated in Fig. 1A.

Having measured the size of the LUVs with cryoEM, we calculated that the total number of fluorophores per LUV ranges between 16-340, see revised main text. The calculation is based on:

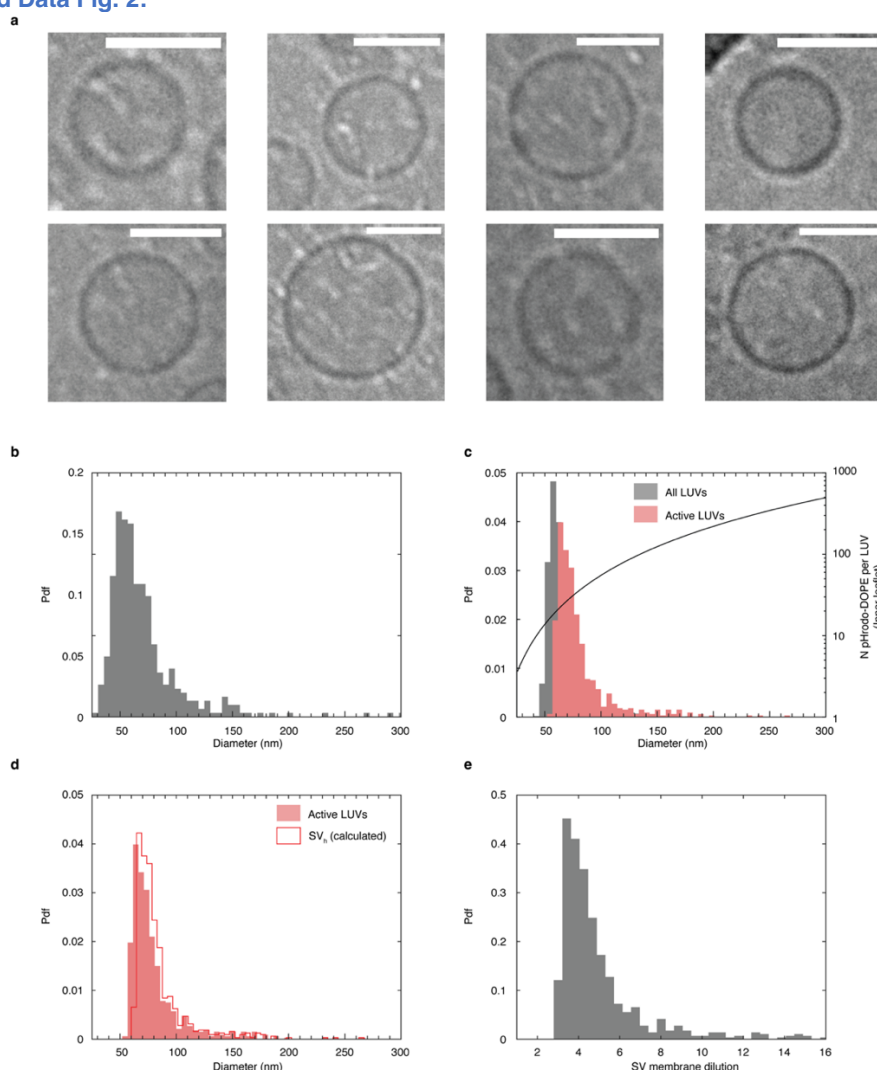
- 1) the measured LUV size,
- 2) the known molar ratio of DOPE-pHrodo in the LUV membrane (0.1 %)
- 3) the average area per lipid ($\sim 0,65 \text{ nm}^2$)^{10,11}

New Extended data Fig. 2c-black line (appended below) shows the total number of fluorophores per LUV. Note that the single vesicle-based pH calibration has taken into account the fact that only half of them are facing the vesicle lumen (See ED Fig. 7, 10).

Revised main text:

“Here, to observe the activity of the V-ATPase for periods of up to three hours we incorporated 16 - 340 fluorophores of photostable synthetic pH indicators (DOPE-pHrodo) in the membrane of SVs, using a well-tested protocol of SNARE-mediated vesicle fusion⁹ (see Methods and Extended Data Fig. 2, 4).”

New Extended Data Fig. 2:



Extended Data Fig. 2 | Morphology and diameters of LUVs; number of fluorophores in LUVs; SV fold-dilution upon fusion with a LUV.

a, Cryo-electron micrographs of LUVs reconstituted with the SNARE acceptor complex ΔN . Scale bars are 50 nm. **b**, Distribution of diameters of LUVs as measured by cryoEM. The mean and SD of the distribution is 72 ± 43 nm. **c**, The cryo-electron data can be used to calibrate the vesicle size in the fluorescence microscopy images using previously established methods^{1,12} (Supplementary discussion). Grey shaded histogram shows single LUV fluorescent intensities quantitatively converted to diameter in nanometres, 66 ± 18 nm. Red shaded histogram corresponds to the size distribution of the $\sim 5\%$ of LUVs that showed V-ATPase activity, 80 ± 25 nm. The average diameter of active LUVs is ~ 14 nm larger than the rest of the population, since larger LUVs fuse more efficiently via SNARE machinery¹³. Black line corresponds to the estimated number of pHrodo-DOPE lipids in LUVs and scales with the surface area of the vesicle. The number of pHrodo-DOPE lipids in the membrane of fused vesicles ranged between 16-340 and was ~ 40 on the average. To calculate the number of lipids per vesicle we used a surface area per lipid of ~ 0.65 nm² (^{10,11}). **d**, Red-shaded histogram is the same as in c, 80 ± 25 nm. Red-outlined histogram corresponds to the calculated diameters of SV_h produced by fusion of LUVs with 40 nm SVs, 84 ± 24 nm. **e**, Dilution factor of the SV membrane in SV_h calculated using a SV diameter of 40 nm. The mean SV membrane dilution is 5.4-fold.

Comment 1.4

How much SNAP/SNARE fusion machinery was used to efficiently capture/fuse with the isolated SVs?

Reply

We performed the requested calculation, the average number of SNARE complexes/LUV is ~ 130 . The SNARE/lipid molar ratio was 1:600, as mentioned in the section “Methods - Proteoliposome preparation and characterization”. Having measured LUV diameters, we can now calculate that on the average we expect 130 complexes/LUV that, as earlier controls have shown, are preferentially oriented towards the outside of the LUV¹⁴. This additional information is now specified in the revised text. Overall, we followed standard protocols of SNARE reconstruction from the Jahn lab that are extensively documented in the literature^{9,14,15}.

Revised Methods text:

“The protein:lipid ratio (mol:mol) was adjusted to 1:600 amounting to approx. 130 complexes/liposome that, as earlier controls have shown¹⁴, are preferentially oriented towards the outside of the liposome.”

Comment 1.5

Prior efforts to measure pH changes within SVs were performed using genetically encoded pH sensitive protein, super-ecliptic pHluorin (ref. 11), which the authors indicate lacked the single-to-noise ratio needed to reveal what is claimed to be revealed by the present investigation.

Reply

Farsi, Z. *et al.* do not show single SV kinetics at all. How severely limiting was the poor signal to noise in Farsi, Z. *et al.*¹⁶ is indicated by the fact that though the title was “Single-vesicle imaging reveals different transport mechanisms ...” in actuality none of the figures showed single SV kinetics. Due to poor signal to noise, the shown kinetics were actually averages of ~ 500 single SVs.

Comment 1.6

...In this earlier study, the authors classified their SVs are either containing vesicular GABA or vesicular glutamate transporter 1 using “on-stage” antibody labeling. I infer from this report that the SVs used in the present investigation represent a mixture of SVs containing different neurotransmitter types. The authors should clarify this point.

Reply

R1 is correct. Thank you for the comment, this is now clarified in the Methods section.

Revised Methods text:

The purified SV fraction contains a mixture of all SVs present in the cerebral cortex. Recently, the distribution of all known vesicular neurotransmitter transporters has been quantified in this preparation¹⁷. Accordingly, close to 80% of SVs are glutamatergic and 15 % are GABAergic, confirming an earlier study¹⁸. Vesicles containing other transporters (for dopamine, acetylcholine etc.) contribute only a few percent, with the exception of the Zn²⁺ transporter ZnT3, which colocalizes with about a third of the glutamatergic vesicles¹⁷.

Comment 1.7

They should also clarify the ultimate size of their fused SV-LUV proteoliposomes and calculate by how much the protein concentration in the SVs drops after the fusion event to inform the reader on the extent of dilution. From Figure 1a, the message is that the change in dilution may be considered relatively minor, but if an SV is ~40 nm and an LUV is approximately 150 nm (per standard definitions), then the effective change in surface area of the proteoliposome is on the order of 30 fold, yes? The fold-dilution could indeed be much larger if the authors definition of LUV is more liberal (eg. 500 nm).

Reply

We performed the requested calculation, the mean membrane dilution is 5-fold.

We thank R1 for these two excellent suggestions. As requested, in the new ED Fig. 2 we now show

- 1) the histogram of SV and LUV measured diameters (ED Fig. 1c,2b)
- 2) the histogram of SV_h diameters calculated as LUV+SV (ED Fig. 2d) and
- 3) the histogram of calculated single SV_h “dilution-factors” (ED Fig. 2e).
- 4) The mean membrane dilution is 5.4-fold.

Example calculation: given a SV diameter of 40 nm and an active LUV diameter of ~ 80 nm, the dilution is $(D_{LUV}^2 + D_{SV}^2) / D_{SV}^2 = (80^2 + 40^2) / 40^2 = 5\text{-fold}$.

Revised text:

The mean membrane dilution of SV membranes in SV_h was ~ 5.4-fold (Extended Data Fig. 2).

Comment 1.8

Clarity on this point touches on the matter of how the authors pack “hundreds” of DOPE-pHrhodo fluorophores into the LUV lumen to start with. If the LUVs were 150 nm, the original reconstitution must have been performed with hundreds of microM to mM dye concentrations. In this regime, do the dyes perform as advertised?

Reply

We used the membrane-bound DOPE-pHrodo at 10-fold below its self-quenching concentration.

Self-quenching is indeed an important issue that we took great care to avoid:

- 1) DOPE-pHrodo is a lipid conjugate that partitions exclusively to the membrane of the LUVs and not their lumen.
- 2) We purposefully chose the molar fraction of fluorophore in the membrane to be 0.1% i.e. a factor of ten below 1-2 %, the fraction at which lipids start to get on the average within energy transfer distance and self-quenching begins¹⁹.
- 3) We do not assume the dye to have a certain pH dependence. We validate experimentally the pH dependence of the dye by a state-of-the-art, in situ pH calibration performed at the single SV_h level, see ED Fig. 10.
- 4) Lastly, though not relevant for our experiment, we note that DOPE-pHrodo is a remarkably reliable pH reporter that retains its pH dependence even at concentrations which are 100-fold higher (10%) than the ones we used here (0.1%)²⁰.

Comment 1.9

Using this unique set up, which has much higher dynamic range than previous efforts on similar systems, the authors investigate proton pumping into the lumen in response to ATP, resulting in a lowering of the luminal pH that increases the average brightness of the DOPE-pHrodo dyes therein. The authors nicely show that the luminal dyes for an ensemble average of approximately 450 fused SVs becomes about 1.5-fold brighter upon the addition of ATP, where the range for individual vesicles measured was roughly 1.2-2 (Figure 1b). In ref. 11, the normalized fluorescence intensity change was reported to be about 1.3, no? Can the authors comment on whether the strategy employed indeed increases the signal to noise above and beyond ref. 11? Perhaps I am misinterpreting something.

Reply

Genetically encoded sensors could not resolve single vesicle kinetics at all.

It is crucial to highlight that our manuscript is based on the ability to resolve the acidification kinetics of **single V-ATPases in single vesicles**. In contrast, the kinetics of Farsi et al. (*Science* **351**, 981–984 (2016), Fig. 1A, C) are **ensemble averages** of >500 vesicles.

The signal/noise from the genetically encoded sensors is so low that Farsi et al. were not able to show or analyze single vesicle kinetics at all. Since Farsi et al. do not present single vesicle acidification kinetics at all, our experiments cannot be strictly compared. Thus, it is not feasible for us to strictly quantify the improvement of our signal/noise.

Regarding the **fold-response** of the sensors to pH changes: the maximal fold increase of DOPE-pHrodo is on the average ~ 9 (Fig. S10C), i.e. almost double the fold-increase of super ecliptic-pHluorin which is ~ 5 (Farsi, et al. Science (2016), Fig. S2A)¹⁶.

It is worth adding however that both for us and Farsi et al. the signal/noise (vesicle fluorescence / background-surface fluorescence) is limited primarily by the total **photon budget** (quantum yield and photostability), and less so by the **maximal fold-response** of the sensor or its **dynamic range** (range of pH that affects fluorescence). To maximize the total photon budget it is crucial to have more fluorophores and more photostable organic dyes. It is revealing that our total photon budget was so large that not only we did not have to average ~ 500 vesicles, but we were in addition able to record up to ~ 200 -fold more frames/images (c.f. our Fig. 1C with Fig. 1C of ref ¹⁶). Thus, our effective photon budget could be loosely approximated to be $500 \times 200 = 100,000$ -fold higher.

Revised main text:

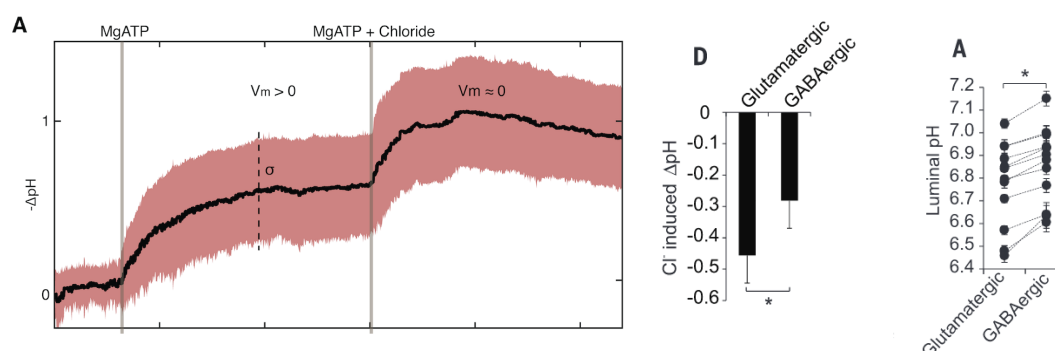
“Proton pumping of the V-ATPase could then in principle be observed by measuring the luminal pH of SVs using genetically encoded pH sensors^{16,21}. However, the expression levels, quantum yield and photostability of these systems^{16,21} have historically been limiting the signal/noise and duration of such recordings.”

Comment 1.10

The authors show through control experiments that the pH change for the ensemble reflects a change in luminal pH from about 7.1 to about 6.2 (ED Fig.4), globally consistent with the results of glutamatergic and GABAergic vesicles isolated from mouse brain (ref. 11).

Reply

R1 concludes correctly that the ATP induced acidification we observed is globally consistent with the data presented in ref.¹⁶. This is best seen by comparing Fig. 3A in our manuscript to Fig. 2A from ref. ¹⁶ (see figure below). In both experiments, in the absence of Cl⁻, ATP addition reduces the pH by ~ 0.5 (from 7.1 to 6.5). As shown below, c.f. Fig. 3A and 3D, further addition of Cl⁻ reduces further the pH by ~ 0.5 , to reach a total reduction of ~ 1 (from 7.1 to ~ 6.1).



Left: Fig. 3A from our manuscript (sequential addition of ATP and then Cl⁻). Middle & right: Fig. 3D (+ ATP, + Cl⁻) and Fig. 2A (+ ATP, - Cl⁻) from Farsi et al. Science (2016).

Minor clarification: ED Fig. 4 in our first submission (now renumbered as ED Fig. 7) was not describing ATP induced acidification but rather control experiments whereby pH gradients/jumps were applied by exchanging the buffer outside the surface tethered SV_h. The kinetics in these control experiments allowed us to determine the passive leakage/permeability of protons through the membrane.

Comment 1.11

Does the variance in [acidification] range evidenced somehow reflect the overall sizes of the fused proteoliposomes? Do the authors make an effort to estimate the extant range of vesicle sizes measured?

Reply

New analysis shows that acidification does not correlate with SV_h size

Motivated by this interesting comment we performed the requested analysis. First, we calibrated the diameters of single SV_h in situ, see new ED Fig. 2. We have described the size calibration methodology in detail in references^{1,12,22,23}. The size calibration allowed us to test directly the hypothesis proposed by R1. Our data demonstrate that the variance in ΔpH between single SV_h is not correlated with their diameter, see figure below.

The variance in ΔpH likely originates from stochastic differences in the pumping rates between single V-ATPase molecules (see also new ED Fig. 6). Like all other single enzymes studied to date^{1–3,24–26}, V-ATPases would not be expected to exhibit identical catalytic rates at the single molecule level (a phenomenon referred to as ‘static disorder’ in the literature).

[REDACTED]

Comment 1.12

Can the authors derive an ATP hydrolysis rate or proton-pumping rate from their data to establish connections to prior literature on these points? Although this may be extremely difficult, as it must take into consideration the passive protein leak rate, it seems like it may be helpful to the reader.

Reply

As requested, new kinetic modelling of pumping and leakage allowed to estimate the pumping rate: 7 ± 5 H⁺/s.

As R1 correctly points out extracting the absolute pumping rates is extremely difficult as it has to take into account the passive protein leak, buffering but also the mode-switching probability. However, motivated by your suggestion we added to the manuscript kinetic physical modelling of pumping, resting and leaky modes that we used to fit the single V-ATPase kinetics, see new ED Fig. 6.

We were thus able, for the first time to our knowledge, to use single-molecule V-ATPase activity data to explicitly correct for 1) mode-switching probability and 2) proton leakage. We thus calculate the intrinsic single-molecule V-ATPase proton-pumping rate to be 7 ± 5 H⁺/s, see new ED Fig. 6.

Briefly, we employed a physical kinetic model that we have previously validated for fitting single P-type ATPase acidification kinetics in Veshaguri et al., *Science* **351**, 1469–1473 (2016). The model accounts for several parameters that affect the acidification kinetics, including passive and active ionic fluxes across the membrane, proton buffering in the lumen and build-up of membrane potential. The stochastic switching of the pump between pumping and inactive modes was extracted directly from the traces and used as time-dependent input to the model. The model is constrained to fit the entire trace, and it provides a realistic description of the full electrochemical gradient and a direct estimation of the absolute numbers of pumped and leaked ions. For more details see also supplementary discussion.

Revised main text:

- “We next tracked the acidification of SV_h at the single particle level over periods of up to 3 hours comprising $\sim 10^5$ of proton turnovers.”
- “To calculate the absolute maximal proton pumping rate we employed a physical kinetic model that accounts for multiple parameters including passive and active ionic fluxes across the membrane, proton buffering in the lumen and build-up of membrane potential^{1,27,28}. The stochastic switching of the pump between pumping and inactive modes was extracted directly from the traces and used as time-dependent input to the model, see ED Fig. 6 and Supplementary Discussion. We can thus estimate the maximal average pumping rate of the V-ATPase: 7 ± 5 H⁺ s⁻¹.”

Revised supplementary discussion:

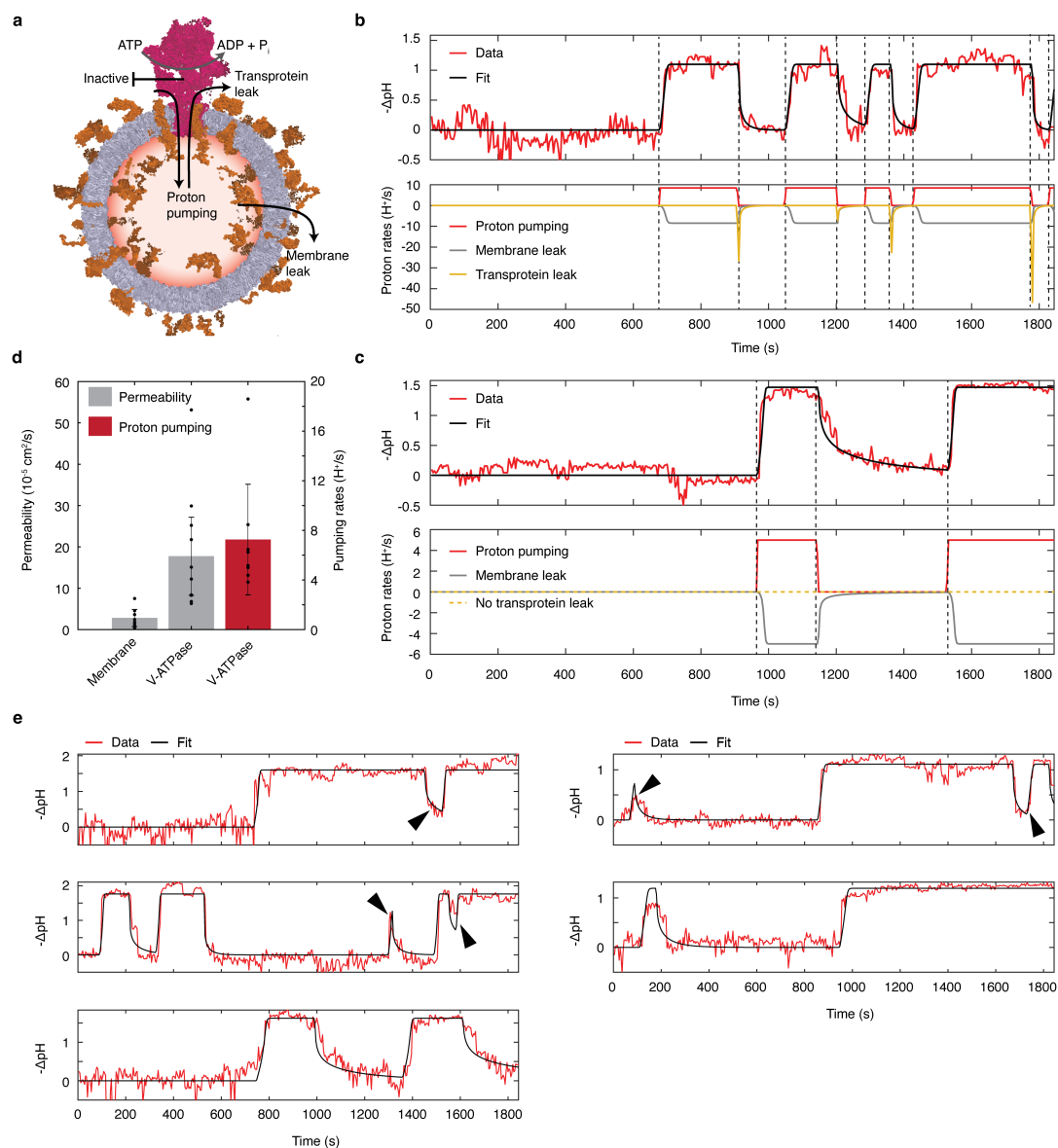
Physical model

We utilized the non-equilibrium model we developed in previous work to extract the pumping rates and permeabilities from single-molecule data^{1,28,29}. The acidification kinetics of single SV_h by the V-ATPase described by the following system of equations:

$$\begin{aligned}\frac{dpH_i}{dt} &= -\frac{1}{\beta(pH_i)} \frac{d[H^+]_T}{dt} \\ \frac{dH_i^+}{dt} &= I_p - (P_{leak}S + P_{V-ATPase}) \frac{U([H^+]_i - [H^+]_o e^{-U})}{1 - e^{-U}} \\ [H^+]_i &= 10^{-pH} \\ \frac{dCl_i^-}{dt} &= P_{Cl}S \frac{U([Cl^-]_i - [Cl^-]_o e^U)}{1 - e^U} \\ U = \frac{\psi}{k_B T} &= \frac{1}{k_B T} \frac{FV}{C_o S} \left(\left(-[Cl^-]_i + \int_{pH_o}^{pH_i} \beta(pH) dpH \right) - B \right)\end{aligned}$$

Quantities in brackets correspond to concentrations of ionic species. Subscripts *i* and *o* correspond to intra- and extraluminal values respectively. β corresponds to the luminal buffering capacity of the system in which protons are buffered by both MOPS and pHrodo-DOPE molecules. I_p is the proton-pumping rate established by the V-ATPase. P_{leak} is the passive proton permeability of the SV_h membrane. S is the surface area and V is the volume of SV_h. S and V are experimentally determined following as mentioned above and in ED Fig. 2. $P_{V-ATPase}$ is the transprotein permeability. U is the reduced membrane potential. In our model, I_p , P_{leak} , and $P_{V-ATPase}$ are free parameters to be determined by the model. P_{Cl} is the chloride permeability of the system. For the modelling performed in the present paper it was assumed that chloride can relatively freely permeate the mebrane as a result of a plethora of mechanisms that transport chloride ions (VGluTs, Cl⁻/H⁺ antiport, passive permeation). Therefore, P_{Cl} is set to a large value. ψ is the membrane potential. Single molecule traces fitted with the model are shown in E.D. Fig. 6. For more details regarding the model and the parameters involved we refer the reader to ^{1,28,29}.

New Extended Data Fig. 6:



Extended Data Fig. 6 | Non-equilibrium physical modelling of pumping, resting and leaky modes provides the single-molecule pumping and leaking rate.

a, Schematic illustration of the main parameters used in the model. Note: this illustration is also shown in Fig. 2a. **b**, **c**, Top: Representative single-molecule traces displaying mode-switching dynamics. Proton pumping was stochastically interrupted by inactive and proton-leaky modes. During active periods of the V-ATPase, a dynamic equilibrium between proton-pumping and passive leakage is established, therefore reaching a single acidification plateau. Leakage currents are a convolution of transprotein and passive membrane proton efflux. Transprotein leakage currents are temporally distinct from proton pumping and may activate directly after the enzyme switched off. Data was fitted with a non-equilibrium model as described in the supplementary information. Pumping rates and permeabilities (both membrane and transprotein) are calculated as free parameters by the model. Bottom: Pumping dynamics for the proton-pumping rate (red), the passive membrane efflux rate (grey) and the transprotein efflux rate (yellow). In **b** the transprotein leak was the primary efflux pathway while in **c** proton efflux manifested only passively through the membrane. **d**, Proton pumping rates and permeability estimates of the model for the data shown in this figure (**b**, **c**, and **e**). Pumping rates were found to be 7 ± 5 H⁺/s. Membrane and V-ATPase permeability were 3 ± 2 cm/s and 18 ± 9 cm/s. Transprotein permeability of the V-ATPase is nearly an order of magnitude larger than passive membrane permeability indicating the regulatory importance of the proton-leaky mode. **e**, Additional single-molecule traces fitted the model. Arrows point out the mode-switching events during which dynamic ($\Delta\text{pH} = \Delta\text{pH}_{\text{max}}$) or static (baseline, $\Delta\text{pH} = 0$) equilibrium was not reached.

Comment 1.13

The authors calibration curve of normalized intensity and the known pH, which they convert to pH in the ED, would also be more helpful if moved up in the text and more clearly explained.

Reply

As requested, we have now added a paragraph in the main text that briefly describes the pH calibration procedure. The full 1.5-page detailed description can still be found in the section 'Supplementary methods/Calibration of pH in vesicles' and in ED Fig. 10.

We would be happy to move the calibration description up in the text, however because Fig. 1 and 2 do not contain pH calibrated data we suggest it may facilitate the flow of the manuscript if the calibration is first described together with Fig. 3.

Revised main text:

The pH calibration procedure is described in detail in Extended Data Fig. 10 and in Supplementary Methods. Briefly, in order to convert intensity to pH at the end of each experiment we exchange the bulk pH from 7.1 to 2.85 and add the protonophore CCCP to equilibrate bulk and luminal pH. This gave us an accurate in-situ measure of the maximal intensity response per each single SV_h which we combined with ensemble average measurements of the pK_a and the rate to get a single SV_h-specific analytic function that we used to convert intensity to pH.

Comment 1.14

A key differentiating aspect of the present study appears to be the extended time of imaging afforded by the hundreds of luminal fluorophores. The full imaging time scale appears to be multiple hours. Here the authors report that the V-ATPase does not pump continuously but rather switches stochastically between proton-pumping, inactive and proton leaky modes (of two distinct types – pump independent and pump dependent). The observed switching behavior takes place on the order of tens of minutes. As the authors conclude, this finding would suggest that pumping rate of a single V-ATPase may be about 170% higher than inferred from ensemble measurements.

Reply

As R1 correctly points out, the ability of our assay to recording pumping by single-molecules for extended periods allowed us to observe mode-switching. Consequently, we were able to deconvolve the contribution of resting modes from pumping modes and therefore determine the true pH gradient ($\Delta\text{pH}_{\text{max}}$) established in SV_h. Indeed, Farsi et al., Science, (2016) found a maximal acidification $\Delta\text{pH} \sim 0.9$ (Fig. 2A). Even upon addition of chloride the additional enhancement of ΔpH found was ~ 0.4 . Comparatively we found $\Delta\text{pH}_{\text{max}} \sim 1.8$ in E.D. Fig. 11. This confirms that indeed the previous estimations of ΔpH in similar systems were skewed due to averaging of pumping and resting modes.

Comment 1.15

At this juncture in the story, it would seem relevant to comment on the physiological relevance of the hours-long time scale to make the case that the finding is important to SV function? Don't synapses release the majority of vesicles on the seconds time scale?

Reply

V-ATPases readily switch modes during the life-cycle of most single SVs

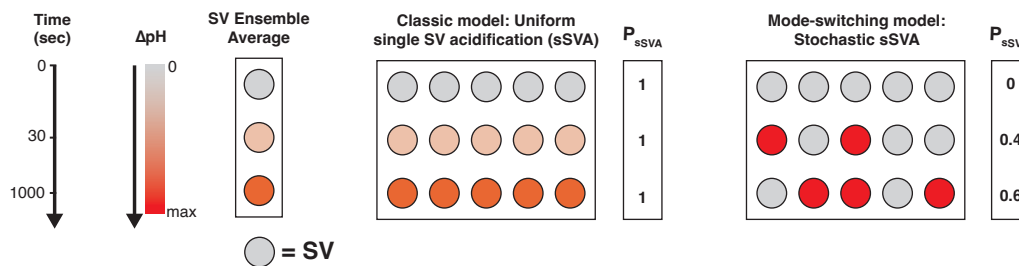
First, it may be important to add that the fastest SV recycling time is about 60 s, however, usually vesicles are stored for longer times. The actively recycling pool has a half-time in the 100 s – 1200 s range, and there are pools with a much longer half-life³⁰. In comparison, the average **lifetime** of the active/inactive modes is respectively 174 s and 122 s (see exponential histograms of lifetimes in Fig. 2). Thus, **SVs are not “trapped” in one mode**, on the contrary the V-ATPase should have enough time to **readily switch modes during the life-cycle of most single SVs**.

We agree fully with R1. The hours-long recording time is a **technical feature of the experiment that, by definition, has no physiological relevance**. Ultra-long recordings were however crucial to observe directly reversible mode-switching and extract mode-switching lifetime and probability.

Second, the physiologically relevant metric is the active probability P_{pumping} . Since $P_{\text{pumping}} = 0.6$, on the average V-ATPases spend $\sim 40\%$ of the time in an inactive mode. Because SVs contain ~ 1 V-ATPase, at any given moment $\sim 40\%$ of SVs contain an inactive V-ATPase. **Thus, at any given moment $\sim 40\%$ of SVs should not be able to sustain the proton-motive force necessary for neurotransmitter loading.**

P_{pumping} is a **property of the V-ATPase**, thus the fraction of non-acidified SVs is **independent** of recycling time. Stochastic single SVs acidification can be visualized as the blinking lights of a Christmas tree, where at any given moment only 60 % of them are bright/acidified (and 40% are not). A very short recycling time is equivalent to a very short movie or snapshot of the blinking lights. One may not be able to see the blinking anymore in the snapshot, but it is still the blinking that determines the fraction of dark/non-acidified SVs to be exactly 40%. **Thus mode-switching to inactive and leaky states emerges as a novel regulatory mechanism of controlling the fraction of the SVs that is acidified and is thus competent to drive neurotransmitter loading.**

We illustrate the important concept of **stochastic single SV-acidification** in new Fig. 5i.



Caption: Schematic illustration summarising the features of the classical ‘uniform single SV acidification’ model versus the ‘mode-switching’ model. The classical paradigm assumes that the probability of single SV acidification is equal to one ($P_{\text{sSVA}} = 1$), i.e. all individual SVs acidify simultaneously and continuously until they reach an equilibrium pH that is equal to the ensemble average pH. In contrast, our measurements revealed that single SV acidification is stochastic and P_{sSVA} ranges from 0 - 0.6 i.e. SV acidification is not synchronized and at any given movement at least 40 % of SVs are not acidified.

Comment 1.16

Figures 1C and d, directly show these switching behaviors and while clearly a departure from a saturating plateau as may be suggested from Figure 1b, it seems remarkable more complex than what could be expected from an on-off toggle switch in single molecule activity.

Reply

~ 30% of stochastic single molecule activity traces switch off before they reach a pH plateau due to the slow response time of the measurement.

Binary molecular activity (on/off switch) is not necessarily recorded as a binary temporal signal because it is invariably convoluted by the ‘response function’ of the measurement/instrument. This is a well-known problem e.g., single channel recordings of small currents (~ 1 pA) typically require temporal averaging/filtering that can distort both the lifetimes and the amplitude of fast events.

The single V-ATPase currents we are recording here are < 0,000001 pA. Because of these ultra-small currents, the **response time** of the measurement (defined as the average time it takes for pH-equilibration in a SV_h) is ~ 30 s, see new ED Fig. 13. *On*-events that are shorter than the response time will not be able to reach a pH plateau. Similarly, *off*-events that are shorter than the response time will not reach zero ΔpH .

Comparison of the response time (pH build-up/leakage lifetimes) with on/off-times suggests that ~30% of events will not reach dynamic equilibrium or zero ΔpH respectively (see new ED Fig. 13). This is now clearly stated in the main text and in new ED Fig. 13.

The binary on/off switch model is sufficient to explain our data.

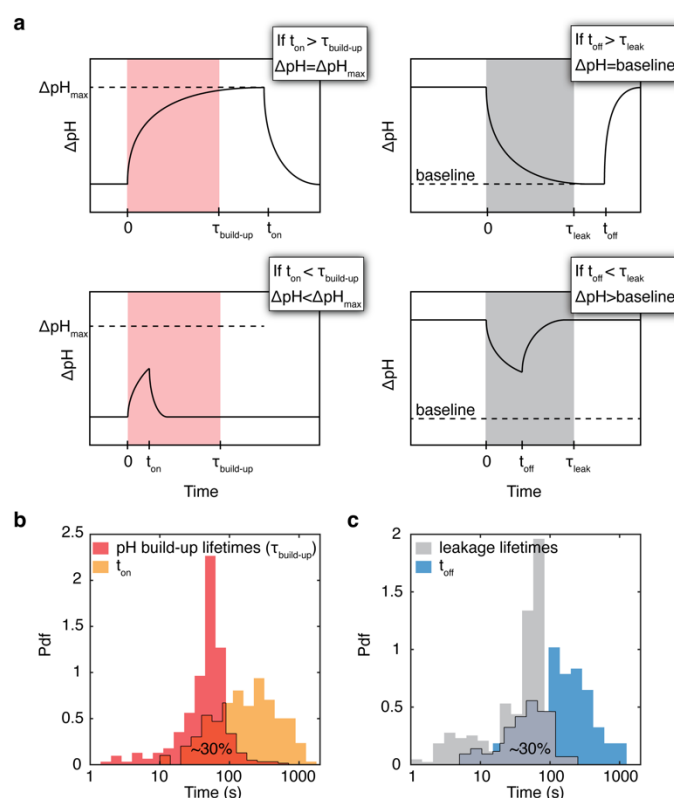
- The first observation supporting the binary model is that ~70% of single-molecule active traces do show transitions between stable plateaus and zero ΔpH .
- The second observation supporting the binary model is that all events, including ones which do not saturate, can be well fitted with kinetic modelling that assumes only on/off **switching of a fixed pumping rate**. Few examples of fits on events that do not saturate are indicated with black arrows in the new ED Fig. 6E.

To summarize, the hypothesis of **variable** pumping rate does not appear to be necessary to explain our data.

Revised main text:

As discussed in detail in Extended Data Fig. 13, ~ 30 % of mode-switching events are faster than the time it takes for pH equilibration.

Revised Extended Data Fig. 13:



Extended Data Figure 13 | ~30% of mode-switching events are too fast to reach a pH plateau or decay to zero ΔpH .

In approximately 30% of single-molecule data, dynamics of switching are faster than the dynamics of pumping or leakage. a, Schematic representation of different cases of mode-switching events, pH build-up and leakage lifetimes. Top left: the pumping dwell time (t_{on}) is longer than the pH build-up time ($\tau_{\text{build-up}}$), allowing for the acidification to reach a constant plateau ($\Delta\text{pH}_{\text{max}}$). Bottom left: t_{on} is shorter than $\tau_{\text{build-up}}$, and therefore $\Delta\text{pH}_{\text{max}}$ cannot be reached. Top right: the inactive dwell time (t_{off}) is longer than the leakage lifetime (τ_{leak}) allowing for the ΔpH to reach zero. Bottom right: t_{off} is shorter than τ_{leak} and therefore the pH will not be able to reach the baseline value. b, Populations of pH build-up lifetimes and proton-pumping mode dwell durations. pH build-up data (red) was produced by fitting single exponentials on kinetic traces during periods of rise of activity. Data for t_{on} (yellow) is taken from Extended Data Fig. 9b, second row and shown in a logarithmic scale. c, Populations of leakage lifetimes of periods of activity of SV_h and resting dwell times. Dataset for leakage is the same as that in Fig 2e while dataset for resting dwells is taken from Extended Data Fig. 9c, second row.

Comment 1.17

For instance, the top panel of Figure 1c seems to fluctuate quite madly during the first 30-40 minutes. This single vesicle appears to exhibit the full range of fluorescence intensities exhibited by the ensemble (Fig. 1b). Is this a representative trace?

Based on the fluorescence intensity/pH calibrations shown in the ED, it would seem that this would suggest fluctuations in a full pH unit or more over the course of time. Is this true? What do the distribution of trace behaviors look like?

Reply

As requested, we calculated the distribution of trace behaviors: 70% of all stochastic single molecule activity traces exhibit stable pH plateaus.

~ 10 % of all active traces subjected to lifetime analyses (Fig. 2) did not exhibit any stochastic pauses during observation. Of course, analysis of stochastic behavior could not be performed on this minority population.

~ 90 % of all active traces exhibited stochasticity. This is now clarified in the revised main text: “**Strikingly, ~ 90 % of single SV_h kinetics revealed reversible stochastic pauses**”. Analysis of stochasticity focused on this majority population. As we discussed in the main text and the figure caption, the trace in Fig. 1c is indeed representative of traces that exhibit stochastic pauses.

~ 20 % of all active traces that exhibit stochasticity had >1 plateaus indicating >1 molecules. This was discussed and quantified in Fig. 1d, e. As was also explicitly clarified in the main text “We restricted all subsequent analysis to SV_h containing a single active V-ATPase.”

~ 70% of all stochastic single molecule activity traces exhibit stable pH plateaus. As R1 very correctly points out, **the trace in Fig. 1c is not representative of traces that exhibit stable pH plateaus**. For that reason, this trace was never discussed as an example of the “stable-plateau phenotype”. Instead, representative examples of traces with plateaus are shown in Fig. 1d.

At the discretion of R1, we would suggest to keep the original trace in Fig. 1c because it showcases both events that do not reach a plateau as well as events that do each a plateau (zoom in Fig. 1c). However, if R1 feels this is likely to confuse the readers we would be happy to include another trace, see example in revised Fig. 1c.

Last but not least, we should stress the acidification is strictly specific to addition of ATP. We **never** observe acidification (stable or stochastic) in the absence of ATP. Second, we should stress that acidification is inhibited irreversibly and with 100 % efficiency by bafilomycin. We **never** observe acidification (stable or stochastic) after bafilomycin addition. These controls demonstrate unequivocally that DOPE-pHrodo reports V-ATPase-specific acidification of SVs.

Comment 1.18

...Does this trace suffer from time averaging of the switching modes or is there something else going on like variable ATP hydrolysis, variable proton pumping rates, or fluorophore photophysics that are convolved in the signal as well?

Reply

As we discuss in detail in Comments 1.16 and 1.17 and ED Fig. 13, the reason why 30 % of single molecule events do not reach a stable plateau is that mode-switching is faster than the **response-time** of the measurement (time required for pH-equilibration). Here it is important to reiterate that traces which do not reach equilibration are excluded from subsequent analysis of $\Delta\text{pH}_{\text{max}}$ values as shown e.g. in Fig. 3, 4.

Time averaging of mode-switching due to the **acquisition frame rate** is not a significant source of noise because acquisition is much faster than mode-switching. Average on/off switching times (174 s and 122 s) are ~2 orders of magnitude greater than frame acquisition (2 s).

Fluorophore photophysics contribute in general to background noise but these fluctuations are negligible compared to mode-switching. Importantly, under our imaging conditions (16-340 fluorophores per vesicle and very low excitation power) we cannot, and do not, ever, observe single fluorophore photobleaching or photo-blinking (see e.g. collection of 60 typical traces in new ED Fig. 5).

We have to emphasize that mode-switching events are explicitly and quantitatively discriminated from background noise by the Bayesian model we used for event-detection. The model is using noise analysis to calculate explicitly the probability (red peaks in lower part of Fig. 2b) that each detected event is distinct from photophysical noise. Events are accepted only when this probability is > 0.99.

Here we should clarify a crucial misunderstanding. The **Bayesian event-detection analysis** we implemented here is not related to **Chung-Kennedy filtering/smoothing**. We did not use the Bayesian event-detection analysis to filter/smooth the traces but to perform event-detection. The Bayesian event-detection analysis is not performed on filtered data but on the contrary relies on analyzing the raw noise of the traces to estimate the probability that an event is distinct from noise.

As discussed in detail in Comments 1.12 and 1.17 1) the observation of stable plateaus in 70% of the single molecule traces and 2) the new kinetic modelling, both support quantitatively the on/off mode-switching model.

Comment 1.19

Another key question is whether the behavior of V-ATPase with ATP and Bafilomycin (baf) added somewhere in the 1-3 hour range (similar to that is shown in 1b) shows a permanent decay of the pH gradient to the background pH value as it should? Robust suppression of the signal changes for individual V-ATPases would be very grounding as it would convey to the reader that the stochastic behaviors over these extended periods indeed stem from stochastic mode switching of the protein not the sensors or other variables associated with the measurements.

Reply

Bafilomycin addition induced a permanent/irreversible decay to background in 100 % of active SV, n=340 traces for n=15 independent experiments, Fig. 1b. We thank R1 for enquiring on this important point which is now explicitly stated in the revised main text.

Clarification note: The three hour-long experiments shown in Fig 1c and ED Fig. 4 did not include addition of Bafilomycin.

Revised main text:

Addition of the V-ATPase specific inhibitor bafilomycin (Fig. 1b, $t = 20$ min) blocked proton pumping and collapsed completely and irreversibly the proton gradient in 100 % of the single SV_h .

Comment 1.20

The more temporally focused (0-30 minute long) traces shown in Fig.1d appear to show more clearly defined switching behaviors but even there, the fluctuations appear quite dramatic given that the signal arises from the collective brightness of hundreds of luminal fluorophores.

Reply

Photon noise is large due to low excitation power and variations in fluorophores/ SV_h .

R1 correctly notices that photon noise in the traces is larger than what one might expect for many fluorophores,. There are two reasons for this apparent discrepancy:

- 1) First and foremost, we purposefully tuned down the excitation power to find a compromise between 1) moderate signal/background ratio in single frames and 2) maximizing the total number of frames we can record without significant photobleaching. Photon noise scales with the number of photons, and though we have many fluorophores we have purposefully kept the numbers of photons low (~ 100 per vesicle, per frame). A notable advantage of collecting photons from many fluorophores simultaneously is that stochastic single-fluorophore photophysics is averaged out.
- 2) Due to the variation in LUV size, the number of membrane-anchored DOPE-pHrodo fluorophores per LUV varies between ~ 16 -340. Consequently, the background noise can vary significantly between individual SV_h (c.f. the 4 traces in Fig. 1d).

However, we have to reiterate that previous attempts to image single SV acidification did not resolve single vesicle kinetics at all, please see discussion at *Comment 1.10*. Thus, our data represent a significant advancement of the state-of-art.

Finally, it is worth highlighting that **under our imaging conditions we cannot, and do not, ever, observe single fluorophore photobleaching or photo-blinking. We have highlighted this now to the readers by adding control traces of inactive vesicles (shown in black)** in Fig. 1b, 1c, ED Fig. 4, and in new ED Fig. 5c.

Comment 1.21

Analyzing data that has been reduced in complexity using Bayesian stochastic filtering (Chung-Kennedy filter) methods (see ED Fig.3), the authors go on to report that ATP concentration regulates the switching probability of the V-ATPase to be in the proton pumping mode, not the ATP hydrolysis and thus the proton pumping rates. On the other hand, electrochemical proton gradient regulates both pumping rate and the lifetime of the pumping and inactive mode. If I understand things correctly, a key assertion is that their theoretical modeling can explain the types of noise fluctuations evidenced in Fig. 1 and ED Fig.3. This would be confidence inspiring.

Reply

The Bayesian event-detection analysis indeed allowed us to distinguish with very high statistical certainty ($P > 0.99$) mode-switching events from background noise in the fluorescent signal.

It is also crucial to reiterate that the events we attributed to mode-switching:

- 1) were 100 % specific to addition of ATP and
- 2) were blocked by Bafilomycin with 100 % efficiency.

These two extremely “clean” controls, together with the Bayesian noise-based event detection, **unambiguously demonstrate the detected events represent V-ATPase-specific acidification and not stochastic noise in the fluorescent signal**. See black traces of control inactive vesicles in Fig. 1b, 1c, ED Fig. 4, and in new ED Fig. 5c

Clarification notes: 1) The Bayesian event-detection analysis we implemented here is not related to Chung-Kennedy filtering/smoothing. 2) **We did not use the Bayesian event-detection analysis to filter/smooth the traces but to perform ‘event-detection’.** 3) **The Bayesian event-detection analysis is not performed on filtered data but on the contrary relies on analysing the raw noise of the traces to estimate the probability that an event is distinct from noise.** 4) Bayesian event-detection was not a model that provided theoretical estimates of noise in the traces, it actually analysed the real/experimental noise to calculate statistical probabilities

of event detection. 5) The Chung-Kennedy filter has not been used as a tool for analysis but only as an aid to visualizing single-molecule traces. All of these points are now clarified in the revised Supplementary discussion.

Comment 1.22

... Moreover, it is also inferred that ATP hydrolysis continues at steady state during the switching periods, such that proton pumping is somehow specifically uncoupled from the enzyme's ATPase activity. Have the authors verified this to be the case? Or is this inferred from prior literature? This seems important to delineate more clearly for the reader. The origins of the off-states are not at all clear and the authors do not attempt speculation on this front.

Reply

We agree completely with R1, these are all salient points that out to be discussed.

Regarding the **inactive-mode**, we have now added to the revised main text the following discussion: "The structure(s) underlying the inactive mode we observed here are not known at present, it is thus currently unclear whether ATP hydrolysis continues while pumping is arrested^{27,31,32}. It is possible they are early, reversible, steps in the conformational trajectory that ultimately leads to the complete dissociation of V_1 from V_O ^{33,34}. However, they are likely not related to the ADP-bound autoinhibited structures reported in the literature^{35–37} because mode-switching is not affected by ADP (see Fig. 4e further down)."

Regarding the **leakage-mode**, the original main text included the following discussion: "Notably, the leakage mode is temporally distinct from the pumping mode. Thus, though the leakage mode reduces the ensemble average (and time average) pumping rates and ATP/net-proton-transport stoichiometry, it does not affect the pumping rates or the stoichiometry of the pumping mode. The leakage mode is thus conceptually and mechanistically distinct from substrate slippage hypothesised to occur stochastically in the thermodynamic pumping cycles^{27,32}."

Regarding the invariance of the **active-mode** pumping-rates on [ATP], the original main text included the following discussion: "These experiments suggest that coupling of shaft rotation to proton shuttling across the membrane becomes the dominant rate limiting step in proton pumping well below the K_m and suggest further increase in the concentration of ATP results in futile cycles of hydrolysis and rotation. The reduction of ΔpH_{max} at 10 μM ATP (Fig. 4c), suggests hydrolysis becomes rate limiting below 30 μM likely due to diffusion-limited binding of ATP to the V-ATPase³⁸."

Comment 1.23

On the whole, the manuscript touches on a highly significant biological question and a potentially new perspective on SV biology and V-ATPase activity that has not been appreciated previously. The manuscript is organized in a logical fashion to convey the overall findings to the reader, but additional clarity on the technical details of how the work was achieved seems paramount to reproducibility of the findings. The concept of mode switching in biological systems, particular in the membrane, have been documented previously (eg. Ion channel literature and smFRET data on GlTPh). A perceived strength of the present finding of mode switching in the SV V-ATPase is further support to the argument that mode-switching behaviors in individual molecules may serve important biological functions.

In the present case, the manuscript's perceived significance would thus seem to hinge on the physiological relevance of the proposed switching events. Are SVs preserved for such extended periods of time (tens of minutes to hours) in the animal brain? What role could such behaviors serve? If tied to a biological function, one could more easily overlook the possible impacts of the non-native environments on the V-ATPase activity established in the present investigations— such as those arising from significant dilutions into lipids that may not be representative of the rat brain and or the fluorophore and SNAP/SNARE components in the reconstituted fused, SVs. Have the authors examined whether there is a lipid dependency to the switching behavior

Reply

R1 questioned the physiological relevance of V-ATPase mode-switching for two different reasons:

- A. The first reason was the non-native environment of the hybrid SV that could possibly impact the activity of the V-ATPase.
- B. The second reason was the assumption that mode-switching lifetimes are much greater than SV-recycling lifetime, hence switching would be unlikely to occur during the lifetime of SV.

Below we try to address conclusively these two questions.

A. New experiments (Fig. 5) quantitatively validate mode-switching in intact SVs.

To exclude conclusively any possible impact of the non-native environment of the hybrid SV on our measurements, we performed new single V-ATPase pumping experiments in intact SVs. As we show in Fig. 5e,f, inactive/active lifetimes of the V-ATPase in intact SVs are identical to the ones recorded in hybrid SVs. Furthermore, the proton leaky mode is also present in intact SV (Fig. 5h). **These new experiments validate mode-switching of the V-ATPase and stochastic vesicle acidification, in the physiologically relevant environment of intact native SVs.**

Respectfully, we should mention that state-of-the-art single transporter experiments are typically performed with exogenously expressed bacterial homologues, that are purified and reconstituted either in detergent or at best in artificial liposomes devoid of any other proteins¹⁻⁶. The hybrid SV is not perfect, as R1 correctly remarks, but we would argue that it certainly represents a significant advance over the state-of-the-art. Indeed, circumventing altogether the traditional approach of purification and reconstitution, allowed us to perform for the first time, to the best of our knowledge, single transporter activity measurements on an **endogenous mammalian transporter in presence of the relevant proteome**, albeit $\times 5$ -diluted in lipids chosen to mimic the lipid composition of SVs.

B. Mode-switching lifetimes are actually comparable or smaller than the recycling lifetime of most SVs pools (see also Comment 1.15)

Regarding the physiological relevance of the mode-switching lifetime relative to the SV-recycling lifetime:

- First, mode-switching lifetimes (~ 100 s) are actually comparable or smaller than the recycling lifetime of most SVs pools (~ 60 s - 5000 s). **The V-ATPase will readily switch during the lifecycle of a typical SV supporting the physiological relevance of mode-switching.**
- Second, as we explain in detail in Comment 1.15, in our opinion, the relevant metric is not lifetime but probability. **The probability of single SV-acidification is independent of SV-recycling lifetime and is thus of physiological relevance for all SV pools.**

As highlighted by reviewer #2, the activity of the V-ATPase has to be regulated. Here we document mode-switching of the V-ATPase for the first time, but most importantly we show that physiologically relevant parameters (electrochemical gradients and ATP) regulate activity predominantly by regulating mode-switching. **This suggests that a broad physiologically important role of mode-switching is regulating the activity of the V-ATPase.**

Our data also suggest that **a new possible biological function of single V-ATPase mode-switching is regulating the fraction of SVs that is acidified and consequently, the fraction of SVs that is able to drive neurotransmitter loading.** To communicate these concepts better to the readers we included in new Fig. 5i, an illustration that juxtaposes the classical model of uniform single SV-acidification with the novel phenotype of stochastic single SV-acidification.

Speculation of further possible biological roles.

Energy saving mechanism: The V-ATPase appears to be responsible for the majority of the energy consumption of the brain (more than any of the other ion pumps such as the Na/K ATPase)^{39,40}. Making sure that it is off 40% of the time will have a major impact on economizing energy consumption. Indeed, previous findings at the Calyx of Held⁴¹ show that inhibition of the V-ATPase with Bafilomycin A1 resulted in only slow (10-20 minutes) glutamate efflux from SVs. This suggests that the V-ATPase does not have to keep acidifying after the SV is filled. In such a case, inactive modes that do not consume ATP would become a mechanism to minimizing energy expenditure post-loading. The electrochemical positive feedback also would work as **energy saving mechanism**, as it would reduce the time spend hydrolyzing ATP (by ~ 3 -fold) when acidification is not successful e.g. in compromised/leaky SVs or when the V-ATPase is at the PM.

SVs filled with neurotransmitters have been previously shown to have a higher release probability than empty ones^{42,43}. This suggests mode switching and stochastic SV acidification could act as a **regulatory factor in SV exocytosis**.

Revised main text:

“The observation that V-ATPase regulation by ATP and electrochemical gradients is based on mode-switching reveals a biological role of ultra-slow mode-switching/disorder in the regulation of protein function.”

“The V-ATPase appears to be responsible for the majority of the ATP consumption of the brain at basal conditions^{39,40}. If the inactive modes do not consume ATP, they would contribute significantly to economizing energy consumption.”

“Mode-switching introduces dramatic all/none fluctuations in the electrochemical gradient of SV_h . However, additional experiments motivated by anonymous reviewer comments, revealed identical mode-switching phenotypes in intact SVs (Fig. 5). Such all/none fluctuations in the electrochemical gradient of SVs will by definition regulate in a stochastic manner also proton-coupled secondary active neurotransmitters and neurotransmitter loading to SVs¹⁶”

Revised methods:

Lipid incorporation into intact synaptic vesicles

To image single intact SV acidification kinetics we established a protocol to incorporate lipid molecules into the native membranes of SVs. To do so, we first dissolved 10 μ L pHrodo-DOPE (1 mg/mL) and 5 μ L DSPE-PEG(2000)-biotin (10 mg/mL) in 85 μ L of chloroform. Then, we formed a lipid film by evaporation of chloroform under nitrogen flow. The lipid film then is stored in vacuum for at least 30 minutes. Subsequently, the lipid film was rehydrated in 20 μ L of synaptic vesicles diluted in 80 μ L of activity buffer and mixed initially with repeated pipetting. Once the lipid film is dissolved, we shake the sample for half an hour at 600 rpm and 37 °C. Finally, we spin down non-hydrated debris at 13,000 rpm for 1 minute and remove SVs without disturbing the pellet. The sample is then distributed into 10 μ L aliquots and freeze-thawed prior to use. To image synaptic vesicles, we immobilize them on a functionalized glass using the same protocols as with SV_h imaging. Once immobilized, the activity of the V-ATPase is triggered upon addition of ATP. Single-molecule proton-pumping data is shown in Fig. 5.

New Fig. 5:

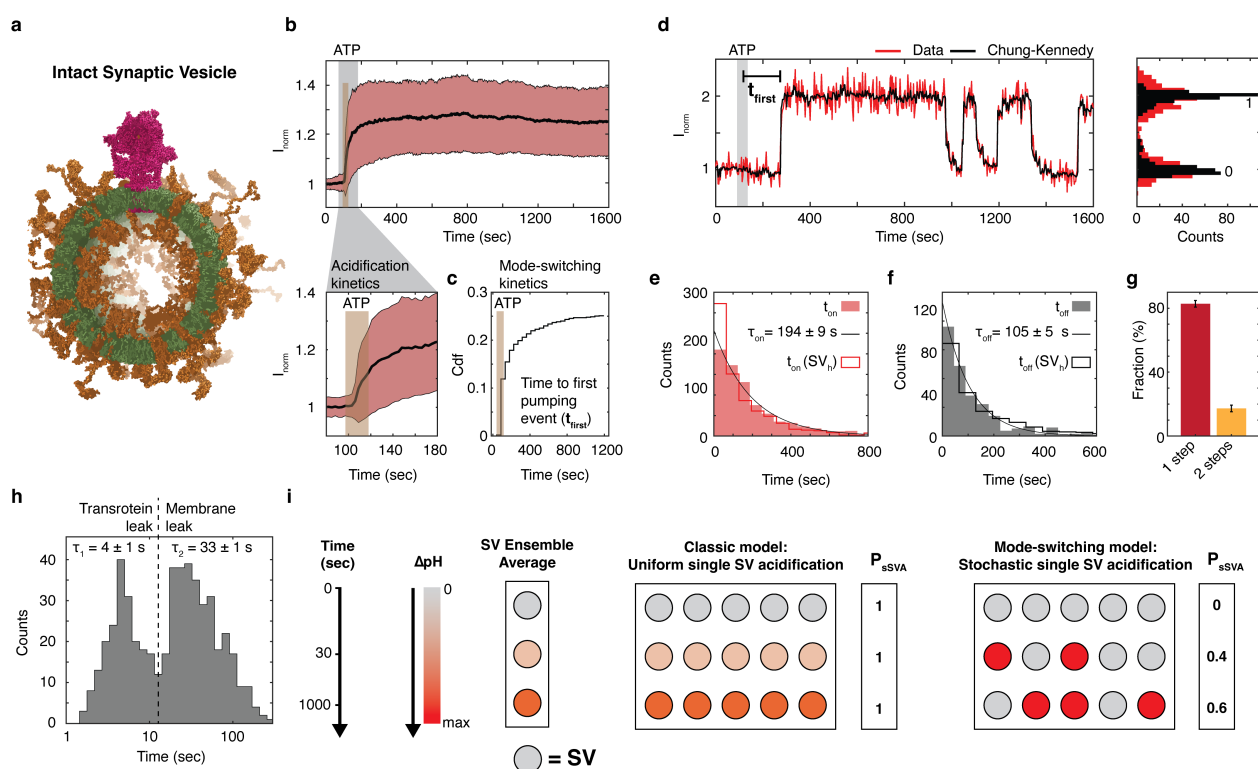


Figure 5 | Mode switching of the V-ATPase in intact SVs. a, Illustration of the membrane proteome of an intact SV. The V-ATPase is shown in magenta. The lipid conjugated pH sensor as well as biotinylated lipids were incorporated into the vesicle as described in supplementary information. **b**, SVs acidify in the order of tens of seconds. Top: ensemble average of ~ 950 single SVs. Activity was initiated by the addition of ATP. Data was corrected for photobleaching. Red shaded area depicts the SD of the single-SV traces. Bottom: zoom-in of the grey-shaded region corresponding to the time immediately after injection of ATP. Number of experiments $n = 3$. **c**, Cumulative histogram of the time elapsed until the initiation of the first pumping event (t_{first}) after the injection of ATP. The stochastic delay in activating single V-ATPases is convoluted in the ensemble average acidification kinetics seen in **b**. **d**, Representative single-vesicle acidification kinetics reveal mode-switching. Histograms of intensity (right) reveal acidification plateaus established by single V-ATPases. **e**, **f** Histograms of proton-pumping

and resting dwells fitted with a single exponential in intact SVs (shaded histograms). Error bars correspond to the error of the fit. Outlined histograms show the dwell time histograms of SV_h . The proton-pumping and inactive mode lifetimes of SVs are nearly identical to those of SV_h ($\tau_{on}(SV) = 194 \pm 9$ s; $\tau_{on}(SV_h) = 174 \pm 9$ s and $\tau_{off}(SV) = 105 \pm 5$ s; $\tau_{off}(SV_h) = 122 \pm 9$ s). **g**, Number of active V-ATPases in intact SVs as calculated by step analysis of acidification plateaus. The average synaptic vesicle contains $\sim 1.20 \pm 0.04$ active V-ATPases. Error bars correspond to the SD between $n=3$ experiments. **h**, Proton-leakage lifetimes reveal a transprotein leak at 4 ± 1 s, as in SV_h . **i**, Schematic illustration of the classical uniform single SV acidification model and the mode-switching model whereby single SV acidify stochastically. At equilibrium, the probability that a random SV is acidified equals the probability the V-ATPase is in the proton pumping mode, $P_{SVA} = P_{pumping} = 0.6$.

Comment 1.24

Another potentially fascinating take away is that the V-ATPase must exhibit an intermediate state in which proton pumping is terminated but ATP turnover is not affected. Is this something that the authors could speculate on structurally given prior structural/functional work? More context to this finding and the potential mechanistic significance of the proposed switching behavior would also seem critical to convey to the reader to provide adequate context.

Reply

We agree completely with R1, as already discussed in Comment 1.22, these are all salient points that out to be examined in the main text.

Regarding the invariance of the **active-mode** pumping-rates on [ATP], the original main text included the following discussion: “These experiments suggest that coupling of shaft rotation to proton shuttling across the membrane becomes the dominant rate limiting step in proton pumping well below the K_m and suggest further increase in the concentration of ATP results in futile cycles of hydrolysis and rotation. The reduction of ΔpH_{max} at $10 \mu M$ ATP (Fig. 4c), suggests hydrolysis becomes rate limiting below $30 \mu M$ likely due to diffusion-limited binding of ATP to the V-ATPase³⁸.”

Regarding the **inactive-mode**, we have now added to the revised main text the following discussion: “The structure(s) underlying the inactive mode we observed here are not known at present, it is thus currently unclear whether ATP hydrolysis continues while pumping is arrested^{27,31,32}. It is possible they are early, reversible, steps in the conformational trajectory that ultimately leads to the complete dissociation of V_1 from V_0 ^{33,34}. However, they are likely not related to the ADP-bound autoinhibited structures reported in the literature^{35–37} because mode-switching is not affected by ADP (see Fig. 4e further down).”

Regarding the **leakage-mode**, the original main text included the following discussion: “Notably, the leakage mode is temporally distinct from the pumping mode. Thus, though the leakage mode reduces the ensemble average (and time average) pumping rates and ATP/net-proton-transport stoichiometry, it does not affect the pumping rates or the stoichiometry of the pumping mode. The leakage mode is thus conceptually and mechanistically distinct from substrate slippage hypothesised to occur stochastically in the thermodynamic pumping cycles^{27,32}.”

Comment 1.25

Is it not also possible that the data may be explained by the reversible binding of an inhibitor to the V-ATPase? Further clarification on how such a model can be definitively excluded seems important to the message.

Reply

R1 puts forward a very important question: Could the V-ATPase inactive mode arise from the reversible binding of an pumping inhibitor that is hypothetically present in the membrane or lumen of SVs? Similarly, one could ask if the leaky mode could be induced by the reversible binding of an activator of proton-leakage? **Below we list a number of arguments that in our opinion suggest these two hypotheses are highly unlikely:**

- We are not aware of any mention in the existing literature that supports the hypothetical presence in SVs of a reversible V-ATPase inhibitor or activator.
- The systematic analysis of SV-content does not suggest any plausible candidates^{44,45}.
- In contrast, existing V-ATPase literature that we have cited, does discuss the existence of uncoupled structures^{33,46,47}.
- The inactive and leaky modes have also been observed in a purified and reconstituted P-type ATPase where the presence of a reversible inhibitor or activator can be reasonably excluded.
- If such molecules were hypothetically present in intact SVs, their initial concentration would be diluted in hybrid SV by $\times 5.4$ on the average, and up to $\sim \times 15$ in the largest LUVs. Such dilution would cause a significant shift in the equilibrium interactions and the active/inactive lifetimes. However, our new experiments demonstrate lifetimes are identical in intact and in hybrid SVs, refuting the hypothetical existence of reversible modulators.

These arguments combined are leading us to conclude with reasonable certainty that the modes we are observing are an intramolecular property of the V-ATPase. However, we made sure this issue is clearly discussed in the main text.

Revised text:

“We did consider the possibility mode-switching is due to the reversible association of the V-ATPase with a hypothetical pumping-inhibitor, or a leakage-activator, however the systematic analysis of SV content does not suggest any plausible candidates making this hypothesis unlikely.”

Reviewer 2

Comment 2.1

The acidification of vesicles by V-ATPase is responsible for protein quality control, neurotransmitter accumulation, and autophagy, etc. The pH of acidic vesicles is different in different vesicles, therefore the proton pumping activity of V-ATPase should be regulated.

In this study, the authors measured pH changes in a single vesicle by fusing synaptic vesicles containing V-ATPase to single-membrane liposomes containing a pH probe immobilized on a glass surface. Although the method is not new, as it is almost identical to that used in previous studies by the author's group, there are some interesting points in the results obtained. In particular, the authors claim to have captured the fact that V-ATPase repeats the on-off mode of the proton pump. This is the first time that the on-off mode of the enzyme has been reported, and the single molecule measurement of the proton pumping activity of V-ATPase in synaptic vesicles is also interesting.

The novelty of the results and their physiological importance of V-ATPase regulation mechanism have attracted the interest of researchers in many fields, but on the other hand, there are some questions about the interpretation of the experimental data that need to be answered before this paper can be published. In particular, the relationship between the pHroad fluorescence value and the pH in the vesicle needs to be interpreted carefully, since the pHroad fluorescence varies with various factors.

Reply

We are truly grateful to R2 for the in depth reading and the many insightful and constructive comments. In the following we strived to address in full all your concerns. Briefly:

2. New measurements count V-ATPases in intact SVs (to exclude the possible influence of multiple rounds of SV/LUV fusion)
3. New CryoEM measurements provide the requested diameters of LUVs, as requested
4. New measurements validate the stability of immobilized LUVs, as requested
5. New 1hr long acidification measurements validate the stability of the V-ATPase at RT, as requested
6. The requested influence of ADP on pumping rates is shown in Fig 4e
7. New physical modelling of acidification kinetics quantifies the relation between pumping and $\Delta\text{pH}_{\text{max}}$, as requested
8. We argued that the multiple Cl-flux pathways and the absence of good inhibitors make inhibition experiments an impractical avenue to explore
9. The bafilomycin control in Fig. 1b excludes the possible influence of VNUTs on acidification

We sincerely hope that the new experiments combined with the detailed discussion, address all your concerns and will thus prompt the reviewer to recommend publication.

Comment 2.2

Number of V-ATPase in a fused vesicle.

Synaptic vesicles are fused to a large liposome (LUV) fixed to a glass surface. In this case, multiple synaptic vesicles could be fused to a single LUV. The authors claim that most of the observed liposomes have only one V-ATPase because of the magnitude of the pumping activity of the V-ATPase. In addition to pump activity, counting the number of V-ATPases in other ways would increase the reliability of the experiments. For example, a recent paper reports a method to count the number of V-ATPases in intracellular lysosomes using fluorescent labeled SidK (J Cell Biol (2022) 221 (3): e202107174). I strongly recommend that this experiment be performed.

Reply

Counting V-ATPases in intact SVs conclusively excludes the possibility of “over counting” due to multiple fusion rounds.

We appreciate the reviewer's concern for multiple rounds of SV fusion per LUV. To resolve this concern, we carried out a new set of experiments in intact SVs (new Fig. 5). Counting V-ATPases in intact SVs conclusively excludes the possible influence of multiple fusion rounds. Additionally, the new experiments in intact SVs allowed us to conclusively exclude any putative consequences of fusion to the function of the V-ATPase.

Measurements in intact SVs (Fig. 5g) revealed the average number of active V-ATPases was 1.17 ± 0.05 . This is in excellent agreement with

literature values based on mass spectrometry^{44,45} (number ~ 1.4)

- literature values based on staining with fluorescent antibodies^{48,49} (1.3 ± 0.2) and

- our measurements on hybrid SVs 1.2 ± 0.1 , Fig. 1e.
- In addition, the proton leaky mode is also present in intact SV (Fig. 5h).

In summary, these new experiments validated counting of V-ATPases, as well as mode-switching between active, inactive and leaky modes in the native environment of intact SVs.

We feel it is important to mention that counting of multiple ionic current plateaus/steps to infer the presence of multiple single active molecules, is a robust criterion unanimously adopted in tens of thousands of papers on ion channels. As we have already demonstrated^{1,50}, this criterion applies also to transporters because ionic currents are additive in general. We would thus propose it is reasonable and justified to consider this is a well-proven method to count the number of active ion conducting/transporting molecules.

We are thankful to R2 for bringing to our attention the very relevant reference on SidK⁵¹. This is now clearly cited and discussed in the main text. Revised text: “Interestingly, recent work demonstrated that most lysosomes in HeLa cells carry only 1 - 4 V-ATPases suggesting they may also be subject to stochastic fluctuations in acidification⁵¹.”

Lastly, we should stress that we acknowledge SidK-staining would be a useful corroboration in the case of hybrid SVs. We sincerely hope the reviewer will condone our strategy to circumvent the problem altogether by using intact SVs.

Additional comments:

We can estimate that 98,5% of SV_h fused with only one SV. To prevent multiple fusion rounds, our experimental conditions were tuned so that the vast majority of LUVs (~ 97 %) did not fuse at all with a SV as judged by their inability to acidify. Assuming random fusion i.e. Poissonian statistics, we can calculate the probability of more than one fusion rounds to be vanishingly small (~ 10⁻⁴). From this probability we can also directly estimate that ~ 98,5 % of all SV/LUV fusion events are only one round.

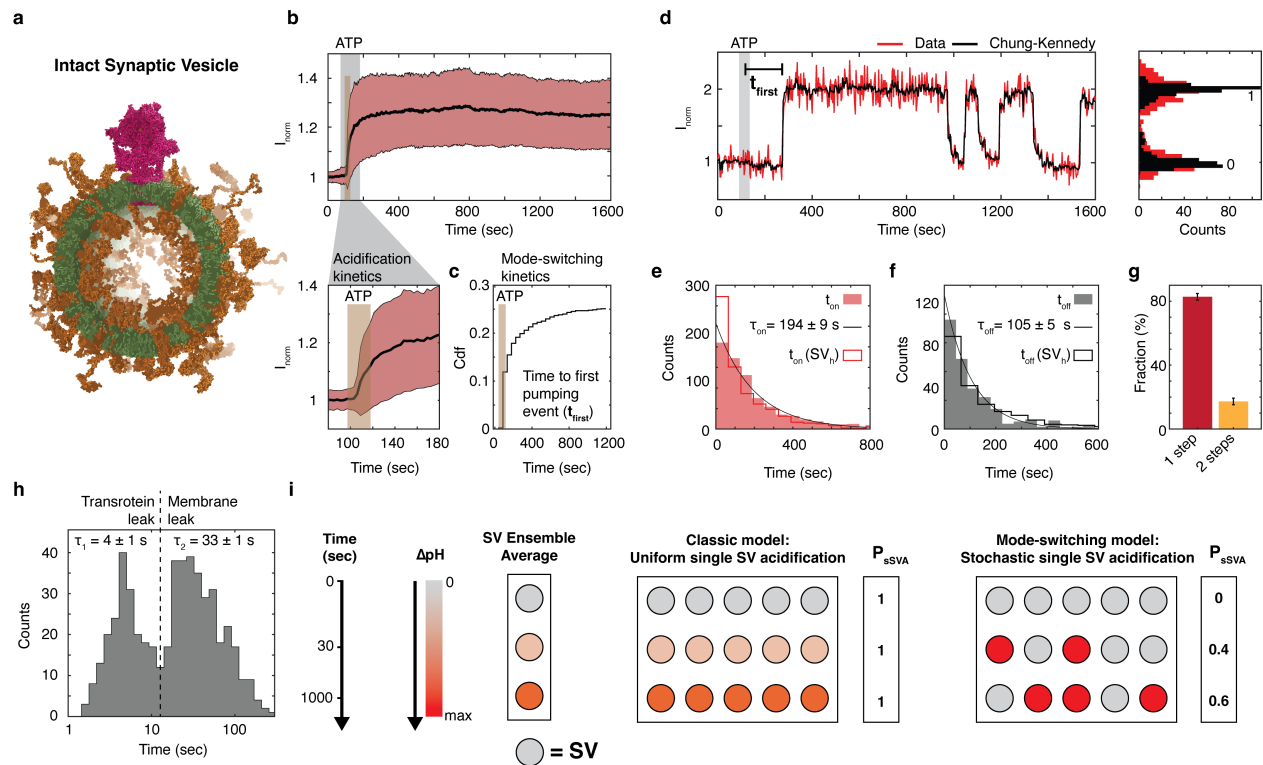
The mono-exponential shape of the lifetime histograms also indicates individual single molecules, thus validating the counting based on steps of acidification plateaus. Apart from counting steps in the acidification plateaus, another independent criterium that can discern the presence of multiple active single molecules per SV_h is the shape of the lifetime histograms. Briefly, apparent lifetimes are inversely proportional to the number of molecules, i.e. 2 or 3 V-ATPases/SV_h would appear to have 1/2 or 1/3 of the intrinsic lifetime, and such histograms would comprise more than one exponential component. The fact that the four different histograms of lifetimes we measured (Fig. 2c, 2d, 5e, 5f) are all fitted very well with monoexponentials strongly demonstrates that the stochasticity we observe originates predominantly from individual single molecules. Thus, validating the counting based on steps of acidification plateaus. This is now stated in the revised main text. “These two histograms [of lifetimes] were fitted well with mono-exponential functions further supporting the assertion we that observe the activity of single molecules.”

We have shown previously using DLS that small unilamellar vesicles (SUV) with a higher deltaN density (protein:lipid = 1:300) on average undergo only one fusion event/SV (ref ⁵², table 2). **The two-fold lower protein to lipid ratio used here (1:600) was chosen to increase even further the probability of only one round of fusion.** Mechanistically, the single-round of fusion can also partly be attributed to the fact that even though only few SNARE complexes are required for fusion, after fusion any unreacted complexes will diffuse, mix, react and deactivate each other.

Revised methods:

Lipid incorporation into intact synaptic vesicles

To image single intact SV acidification kinetics we established a protocol to incorporate lipid molecules into the native membranes of SVs. To do so, we first dissolved 10 µL pHrodo-DOPE (1 mg/mL) and 5 µL DSPE-PEG(2000)-biotin (10 mg/mL) in 85 µL of chloroform. Then, we formed a lipid film by evaporation of chloroform under nitrogen flow. The lipid film then is stored in vacuum for at least 30 minutes. Subsequently, the lipid film was rehydrated in 20 µL of synaptic vesicles diluted in 80 µL of activity buffer and mixed initially with repeated pipetting. Once the lipid film is dissolved, we shake the sample for half an hour at 600 rpm and 37 °C. Finally, we spin down non-hydrated debris at 13.000 rpm for 1 minute and remove SVs without disturbing the pellet. The sample is then distributed into 10 µL aliquots and freeze-thawed prior to use. To image synaptic vesicles, we immobilize them on a functionalized glass using the same protocols as with SV_h imaging. Once immobilized, the activity of the V-ATPase is triggered upon addition of ATP. Single-molecule proton-pumping data is shown in Fig. 5.



New Figure 5 | Mode switching of the V-ATPase in intact SVs. Mode-switching of the V-ATPase in SVs shows nearly identical kinetics as in SV_h. **a**, Illustration of the membrane proteome of the intact synaptic vesicle. The V-ATPase is shown in magenta. The lipid conjugated pH sensor as well as biotinylated lipids were incorporated into the vesicle as described in supplementary information. **b**, SVs acidify in the order of tens of seconds. Top: ensemble average of ~950 single SV acidification kinetics. Activity was initiated by the addition of ATP. Data was corrected for photobleaching. Red shaded area depicts the SD of the single-vesicle traces. Bottom: zoom-in of the grey-shaded region corresponding to the time immediately after injection of ATP. Acidification kinetics take place in the order of tens of seconds. Number of experiments is $n=3$. **c**, Cumulative histogram of the time elapsed until the initiation of the first pumping event (t_{first}) after the injection of ATP. The stochastic delay of single V-ATPase activity shapes the ensemble average kinetics seen in **b**. **d**, Representative single-vesicle acidification kinetics showcasing mode-switching. Unprocessed data is shown in red while in black is data processed by a Chung-Kennedy filter. Histograms correspond to acidification plateaus established by single V-ATPases. **e**, **f** Histograms of proton-pumping and resting dwells fitted with a single exponential in intact SVs (shaded histograms). Error bars correspond to the error of the fit. Outlined histograms show the dwell time histograms of SV_h. The proton-pumping and inactive mode lifetimes of SVs are nearly identical to those of SV_h ($\tau_{\text{on}}(\text{SV}) = 194 \pm 9$ s; $\tau_{\text{on}}(\text{SV}_h) = 174 \pm 9$ s and $\tau_{\text{off}}(\text{SV}) = 105 \pm 5$ s; $\tau_{\text{off}}(\text{SV}_h) = 122 \pm 9$ s). **g**, Number of active V-ATPases in intact SVs as calculated by step analysis of single-vesicle traces. The average synaptic vesicle contains ~1.2 active V-ATPases. Errorbars correspond to the SD between $n=3$ experiments. In the data analyzed, no vesicles with three or more active V-ATPases could be observed. **h**, Proton-leakage lifetimes reveal nearly identical leakage kinetics as in SV_h. The transprotein leak is 4 ± 1 s while the passive membrane leak is 33 ± 1 s. **i**, Schematic illustration of the classical acidification model and the mode-switching model. In the classical paradigm all vesicles simultaneously and continuously ($P_{\text{sSVA}} = 1$) acidify gradually to reach an equilibrium value that matches the experimentally determined ensemble average acidification kinetics. Here, we propose the mode-switching model, whereby each SV independently can stochastically switch on and reach the maximal pH gradient ($\Delta\text{pH}_{\text{max}}$). At equilibrium, the probability the V-ATPase is found in a pumping mode is $P_{\text{pumping}} = 60\%$. Therefore, at the steady state, the probability that a random synaptic vesicle is found in an acidified state corresponds to $P_{\text{sSVA}} = P_{\text{pumping}} = 60\%$.

Comment 2.3

Size of the fused liposomes.

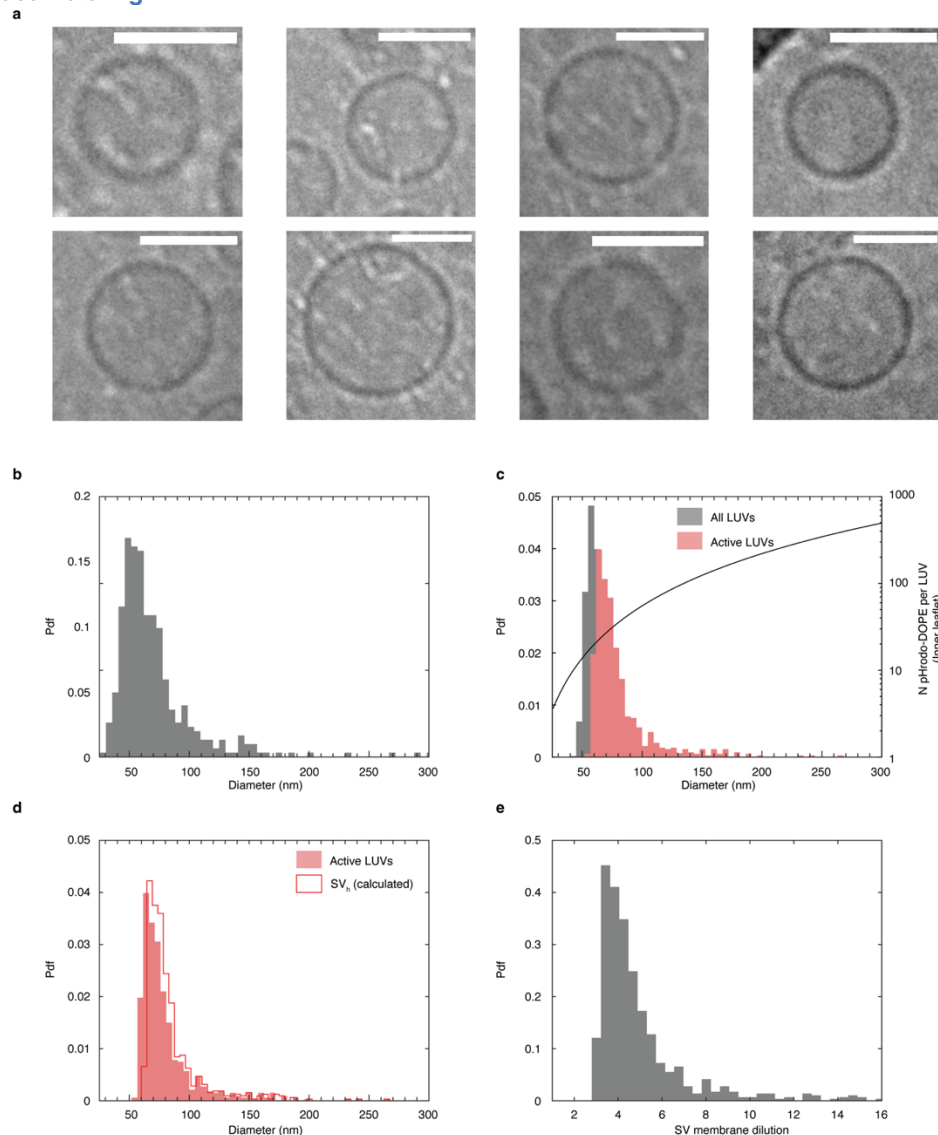
The authors confirmed the homogeneity of purified SVs by cryoEM. This is excellent. On the other hand, there is no mention of the size of LUVs. The size of reconstituted liposomes has a great impact on the measurement of proton pumping activity; it would be more convincing if the size of LUVs fused with SVs were also measured by cryoEM. This is a simple experiment and should be performed.

Reply

We performed the requested cryoEM experiment. We completely agree with R2 that knowing the LUV diameter is important. The new cryoEM experiments on LUVs containing the ΔN SNARE complex (new ED Fig. 2a, b) show that the mean LUV diameter is 72 ± 43 nm (s.d.).

Knowing the size distribution of LUVs from cryoEM allowed us to calibrate in situ the sizes of single immobilized LUVs using a well-established method we have validated in several publications^{1,12} (see also Supplementary discussion). We found that the diameter of LUVs that underwent fusion and could thus be acidified was 80 ± 25 nm (ED. Fig. 2c). Additionally, we calculated the number of lipid-conjugated pHrodo-DOPE molecules found in each vesicle. Finally, we calculated the final volume of SV_h (ED. Fig. 2d) and the final dilution of the SV proteome in hybrid vesicles, ~5-fold (ED. Fig. 2e).

New Extended Data Fig. 2:



Extended Data Fig. 2 | Morphology and diameters of LUVs; number of fluorophores in LUVs; SV fold-dilution upon fusion with a LUV.

a, Cryo-electron micrographs of LUVs reconstituted with the SNARE acceptor complex ΔN . Scale bars are 50 nm. **b**, Distribution of diameters of LUVs as measured by cryoEM. The mean and SD of the distribution is 72 ± 43 nm. **c**, The cryo-electron data can be used to calibrate the vesicle size in the fluorescence microscopy images using previously established methods^{1,12} (Supplementary discussion). Grey shaded histogram shows single LUV fluorescent intensities quantitatively converted to diameter in nanometres, 66 ± 18 nm. Red shaded histogram corresponds to the size distribution of the ~5% of LUVs that showed V-ATPase activity, 80 ± 25 nm. The average diameter of active LUVs is ~14 nm larger than the rest of the population, since larger LUVs fuse more efficiently via SNARE machinery¹³. Black line corresponds to the estimated number of pHrodo-DOPE lipids in LUVs and scales with the surface area of the vesicle. The number of pHrodo-DOPE lipids in the membrane of fused vesicles ranged between 16-340 and was ~40 on the average. To calculate the number of lipids per vesicle we used a

surface area per lipid of $\sim 0.65 \text{ nm}^2$ (^{10,11}). **d**, Red-shaded histogram is the same as in c, $80 \pm 25 \text{ nm}$. Red-outlined histogram corresponds to the calculated diameters of SV_h produced by fusion of LUVs with 40 nm SVs, $84 \pm 24 \text{ nm}$. **c**, Dilution factor of the SV membrane in SV_h calculated using a SV diameter of 40 nm . The mean SV membrane dilution is 5.4-fold.

Comment 2.4

Stability of liposomes on a glass substrate.

In this experiment, liposomes were exposed to the glass surface for a long time, and then the fluorescence of liposome was measured. In order to exclude the possibility of liposomes fusion each other or adsorbing on the glass substrate, it would be good to show that the liposomes maintain their shape stably on the glass surface for a long time. For example, it is possible to indicate the stability of liposome on the glass surface by observation of fluorescently labeled liposomes for a long time.

Reply

We performed the requested experiments. As requested, we performed new experiments shown in the new ED Fig. 5b and 5c-black-traces, where we observed DOPE-pHrodo labeled LUVs for a long time. These control experiments are indicative of liposome stability. They show that the intensity of individual LUVs does not exhibit any stochastic variation/ switching similar to the one observed after fusion with SVs (and strictly after ATP addition). To emphasize this clearly to the reader, we also added such typical traces of LUVs in Fig. 1b and c.

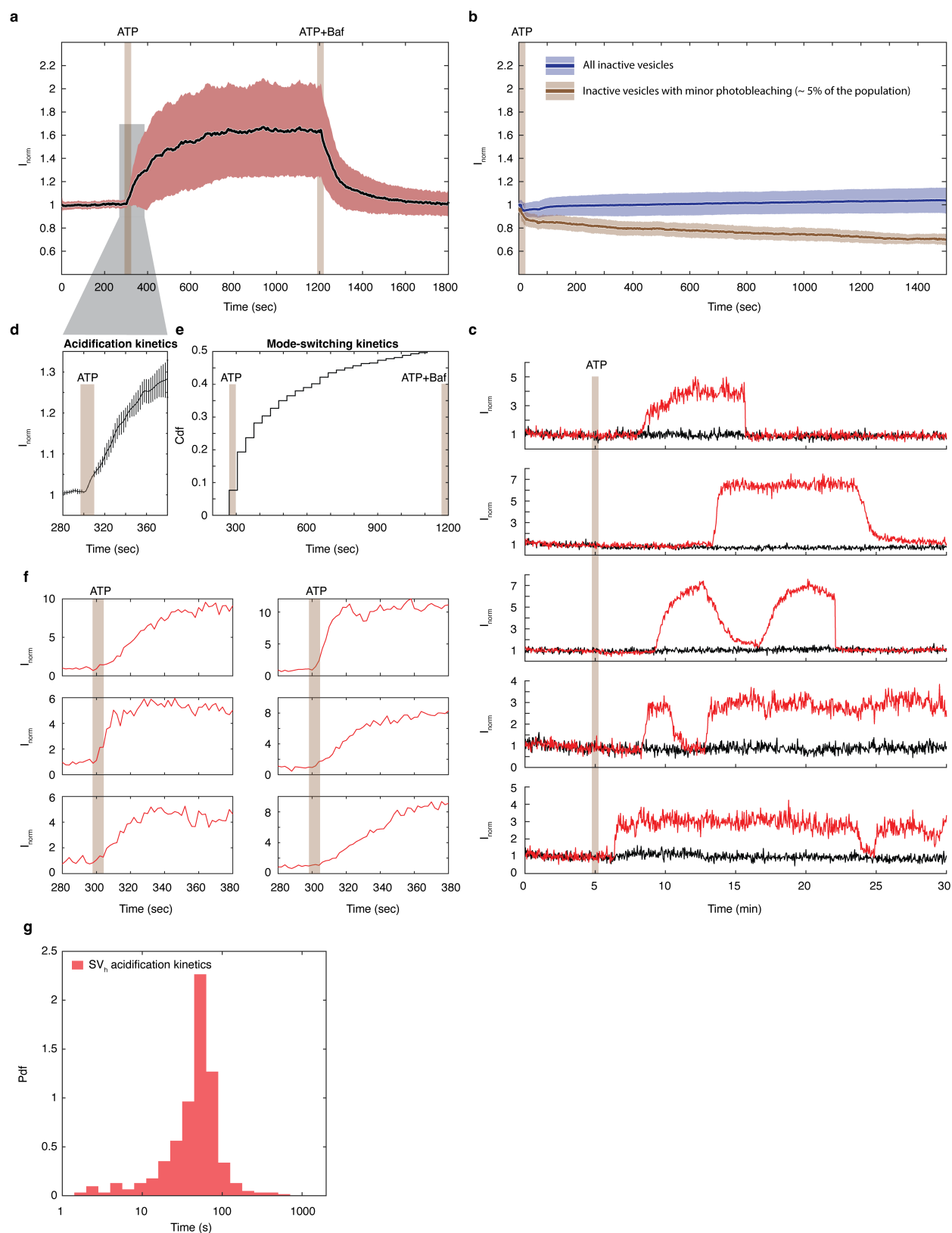
The concerns raised by R2 are very astute, however all of them have been addressed in a series of publications from our lab dealing with the surface immobilization of intact liposomes, their characterization at the single liposome level, and their use for high throughput single liposome assays. In particular, we have previously demonstrated that:

1. Single surface-immobilized liposomes are stable and maintain their shape.^{22,23,53–56}
2. Single surface-immobilized liposomes are stable and intact to soluble organic fluorophores^{23,50} and most importantly to ions.^{1,57}

The above state-of-the-art was already summarily described and cited in the main text in the following paragraph:

“As we and others have shown, the low surface density allowed observation of single vesicles^{1,16} while the controlled tethering conditions preserved membrane permeability to ions^{1,57}, vesicle sphericity²² and the function of transmembrane proteins^{1,2,23}.”

New Extended Data Fig. 5:



Extended Data Fig. 5 | Single-vesicle acidification kinetics are fast (~ 30 s), the slow apparent ensemble average acidification kinetics are governed by slow mode-switching (~ 1000 s). Under our imaging conditions we never observe single-molecule photo-blinking or photo-bleaching.

a, Ensemble average acidification kinetics of single SV_n . Red shaded area corresponds to one SD of all single vesicle traces. Note: this figure is also shown in Fig. 1b. **b**, The majority of inactive single vesicle traces (~ 95% of

the population) showed photostability with no indication of photobleaching or self-quenching. ~5% of the vesicles showed signs of minor photobleaching on the order of 30% after 25 minutes of recording. We never observed an increase in fluorescent intensity while imaging which would be indicative of self-quenching. **c**, Collections of active and inactive vesicles. Inactive vesicles retained stable baseline signals throughout the course of experiments. No photoblinking was observed for the totality of inactive vesicles **d**, Ensemble average acidification kinetics immediately after ATP injection. Error bars correspond to the SD between ensemble average traces of multiple replicates ($n = 4$). Acidification until the dynamic equilibrium of pumping and leakage is reached takes place in the order of tens of seconds, consistent with previous ensemble measurements of acidification¹⁶. **e**, Cumulative histogram of the time elapsed until the onset of the first proton-pumping event. Mode-switching dynamics are slow (~1000 seconds) and are responsible for the slow kinetics seen in the ensemble average traces in **a**. **f**, Zoom in regions of representative single-vesicle traces showcase that acidification occurs in the order of tens of seconds. **g**, Histogram of single-vesicle acidification kinetics shows that most vesicles acidify in 10-100 s.

Comment 2.5

Stability of V-ATPase.

I afraid human-derived V-ATPase is not stable for a long time incubation at room temperature. It is necessary to show by biochemical methods that the activity of the V-ATPase reconstituted in liposomes is stable even if it incubated at room temperature for one hour.

Reply

Stability of the V-ATPase in rat-brain hybrid or intact SVs was not a problem we had to contend with.

We performed the requested experiments (new ED Fig. 3a below) and monitored rat-brain-derived V-ATPase induced acidification for 1 hr at RT. The stable plateau in this control demonstrates conclusively that V-ATPase activity in hybrid SVs is stable at RT.

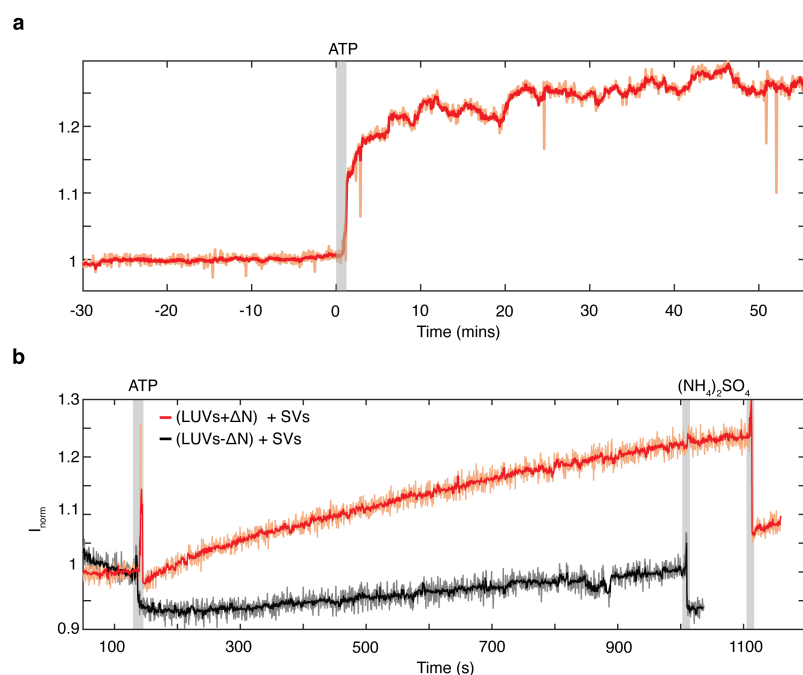
Supplementary comments:

It is absolutely true however that mammalian-derived transmembrane proteins are notoriously susceptible to denaturation. Indeed, this has been a major bottleneck to their biochemical and structural characterization, and even more so to their study at the single-molecule level. Consequently, single-molecule measurements of transporters have been predominantly restricted to ultra-stable (frequently hyperthermophilic) bacterial homologues^{2-6,24}.

However, it is crucial to highlight that such studies typically are performed on detergent solubilized protein, that has been purified and then either stabilized in micelles or reconstituted in pure synthetic liposomes. In contrast, **the V-ATPase used in this study has never been solubilized, it was neither ever purified, and it remains ‘accompanied’ by the entire native SV proteome and lipidome** (albeit diluted by ~5-fold during fusion). It is our current understanding that the comparative stability of our samples is due to these crucial differences in sample preparation. Thus, we had stated in the submitted main text that: *“To preserve the proton pumping activity of the mammalian brain V-ATPase, which is not as stable as its bacterial homologs⁶⁸, we avoided detergent solubilization protocols and instead isolated the endogenous V-ATPase in intact SVs purified from rat brain^{7,8} (see Methods).”*

We should also mention that **the submitted manuscript already contained several long-term activity measurements at RT**. E.g.: ED Fig. 2 in the submitted manuscript (now ED Fig. 3) depicted an experiment performed in bulk where the incubation of the V-ATPase for 45 min at RT was followed by a ~30 min measurement at 37 °C demonstrating that the V-ATPase retains stability over extended periods of time. In the single-vesicle experiments the V-ATPase was frequently incubated at RT for ~1 hour prior to the start of activity measurements to allow for SNARE-mediated fusion. Subsequently single-molecule activity measurements lasted at least 30 minutes and up to three hours (Fig. 1b, 3a, 4a, 5a, ED Fig. 12a, ED Fig. 4). In all of these experiments the V-ATPase was active for several hours of total incubation at RT. Overall, stability of the rat-derived V-ATPase in either hybrid or intact SVs was not an issue we had to contend with.

Revised ED Fig. 3a



Extended Data Fig. 3 | Long-term activity measurements in SVh that are surface-immobilized or suspended in solution.

a, Activity measurements for SV_h immobilized on the glass substrate at room temperature. Proton-pumping was initiated upon addition of ATP. Activity was retained for one hour demonstrating the biochemical stability of the V-ATPase. **b**, Activity measurements for SV_h suspended in solution. Proton pumping was initiated by addition of 2 mM ATP and stopped upon addition of 20 mM ammonium sulfate. Proteoliposomes reconstituted with the ΔN complex were able to fuse with SVs and produce SV_h that contain the V-ATPase (red trace). No fusion and therefore no activity was observed for liposomes lacking the ΔN acceptor complex (black trace). Raw data (orange and grey trace) was processed with a Chung-Kennedy filter (red and black trace)⁵⁹. All subsequent acidification traces shown in figures have been processed with a Chung-Kennedy filter, unless stated otherwise.

Comment 2.6

ΔpH_{max} values.

It is interesting that the fluorescence of pHroad reaches a constant plateau after ATP is added (Fig. 1c, d). The proton motive force formed by V-ATPase is determined by ΔG of ATP hydrolysis. Therefore, changing the ratio of ATP:ADP:Pi should change the plateau value of pHroad. This experiment will increase the reliability of the ΔpH_{max} value.

Reply

The requested experiment can be found in Fig. 4e. Indeed, as the reviewer suggests addition of ADP reduces ΔpH_{max} by ~20 % (from 1.6 to 1.3) in relatively good agreement with a new theoretical calculation (see below).

Theoretical calculation of the Thermodynamic limit. One can calculate the thermodynamic limit of acidification ΔpH_{th} using the formalism and reasonable assumptions described by Grabe et al. in ^{27,28}. Under our experimental conditions $-\Delta pH_{th}(-ADP) = 3.8$ in the absence of ADP and is predicted to drop to $-\Delta pH_{th}(+ADP) = 2.2$ when ADP is added. The theoretically expected reduction due to ADP addition is ~40%, is in relatively good agreement with the experimental reduction of ~20%. Note also that due to passive membrane leakage the vesicles never reach the thermodynamic limit, i.e. $\Delta pH_{max} = 1.6 - 1.3 < \Delta pH_{th} = 3.8 - 2.2$. See reply to Comment 2.9 and new ED Fig. 6 for further discussion on passive leakage.

Comment 2.7

Effect of chloride ion on ΔpH_{max} value.

In Fig. 3, the authors show the results of an experiment in which the addition of chloride ion increased the ΔpH value. If the chloride ions were transported into the vesicle through the synaptic vesicle-derived transporter, this increase in ΔpH should be cancelled out by inhibitors to the transporter. The authors should preform the negative control experiments.

Reply

This is in theory a very good suggestion unfortunately however, in practice, such experiments are complicated and unreliable to the extent we would feel very uncomfortable to interpret their outcome.

The three most important issues are:

1. based on our hands-on experience J Biol Chem. 1993 Nov 5;268(31):23122-7⁶⁰; Preobrashenski et al., 2014 and 2018^{61,62} and to the best of our knowledge, there are no good pharmacological tools available
2. To the best of our knowledge, all VGLUT inhibitors are fluorescent and cannot be used in conjunction with pHrodo^{61,63}.
3. there are at least three possible pathways through which chloride can enter SVs: VGLUTs^{61,64,65}, CIC3⁶⁶ and possibly VGAT^{66,67}. Inhibiting them all simultaneously is very complicated.

Comment 2.8

Effect of VNUTs on the experiments.

Synaptic vesicles contain VNUTs, which are transporters of ATP, and they transport ATP into the vesicles by proton-driven force. The transported ATP may affect the pH in the vesicles. There is also a possibility that the ATP concentration in the measurement system may change as ATP is transported into the vesicles. To exclude this possibility, it would be better to have a control experiment that inhibits the activity of VNUT.

Reply

The bafilomycin control proves the V-ATPase is solely responsible for acidification. The V-ATPase-specific inhibitor bafilomycin, inhibits acidification completely, in 100% of SV_h (Fig. 2b). This demonstrates conclusively that the only facilitator of acidification in our experiment is the V-ATPase. We can thus conclude that VNUT, whether it is present or not, does not contribute to acidification to a measurable degree.

Furthermore, proteomics studies did not identify VNUTs as a resident protein in SVs^{7,45}. Admittedly, not much is known about the distribution and function of VNUT, largely due to the fact that good antibodies are scarce. The Jahn laboratory is working on VNUT since several years, and it appears that its abundance on SVs is low as it is restricted in the CNS to aminergic and serotonergic neurons that make up less than 1% of all synaptic vesicles (data not shown). For these reasons, we did not expect any interference by VNUT in our experiments.

Comment 2.9

Relationship between ATP concentration and $\Delta\text{pH}_{\text{max}}$.

As shown in Fig. 4, it is interesting to note that the plateau value of $\Delta\text{pH}_{\text{max}}$ remains constant even when the ATP concentration is changed. This indicates that for a single vesicle, there is no correlation between the number of protons transported per second and the measured ΔpH . A discussion of the theoretical explanation for this in terms of vesicle volume, number of protons transported, and expected pH change in the vesicle will help many readers understand this.

Reply

$\Delta\text{pH}_{\text{max}}$ depends strictly on the pumping rates

We would like to draw the attention of the reviewer on this comment because it may reflect a crucial misunderstanding. As we discuss in revised the main text:

- The number of transported protons per second (proton pumping rates) does correlate with $\Delta\text{pH}_{\text{max}}$.
- To clarify this to the readers we added to the manuscript kinetic physical modelling of the dynamic equilibrium between active proton pumping and passive proton leakage, new ED Fig. 6 and revised supplementary discussion for more information.
- The fact the average $\Delta\text{pH}_{\text{max}}$ remains ~ 1.4 when increasing ATP above 30 μM , directly indicates that pumping rates also remain constant. Note, the dye can readily report $\Delta\text{pH}_{\text{max}}$ up to 3 (see single SV_h histograms in ED Fig. 11d, 15c, 15d).
- This reveals the existence of a **rate limiting step (reached at 10 μM – 30 μM [ATP]) above which faster hydrolysis and shaft rotation cannot be coupled to proton pumping** across the membrane. This is one of the major findings of the manuscript.

The acidification plateau reflects the dynamic equilibrium between active influx and passive efflux. It may be relevant to add that the acidification plateau we observe reflects the dynamic equilibrium between two opposing proton fluxes 1) proton influx/pumping in the SV lumen due to ATP hydrolysis and 2) proton efflux from the SV lumen due to passive proton leaking through the membrane, see figure below. See new ED Fig. 6 and revised supplementary discussion for more information.

Briefly, we employed a physical kinetic model that we have previously validated for fitting single P-type ATPase acidification kinetics in Veshaguri et al., *Science* **351**, 1469–1473 (2016). The model accounts for several parameters that affect the acidification kinetics, including passive and active ionic fluxes across the membrane, proton buffering in the lumen and build-up of membrane potential. The stochastic switching of the pump between pumping and inactive modes was extracted directly from the traces and used as time-dependent input to the model. The model is constrained to fit the entire trace, and it provides a realistic description of the full electrochemical gradient and a direct estimation of the absolute numbers of pumped and leaked ions.

Revised supplementary discussion:

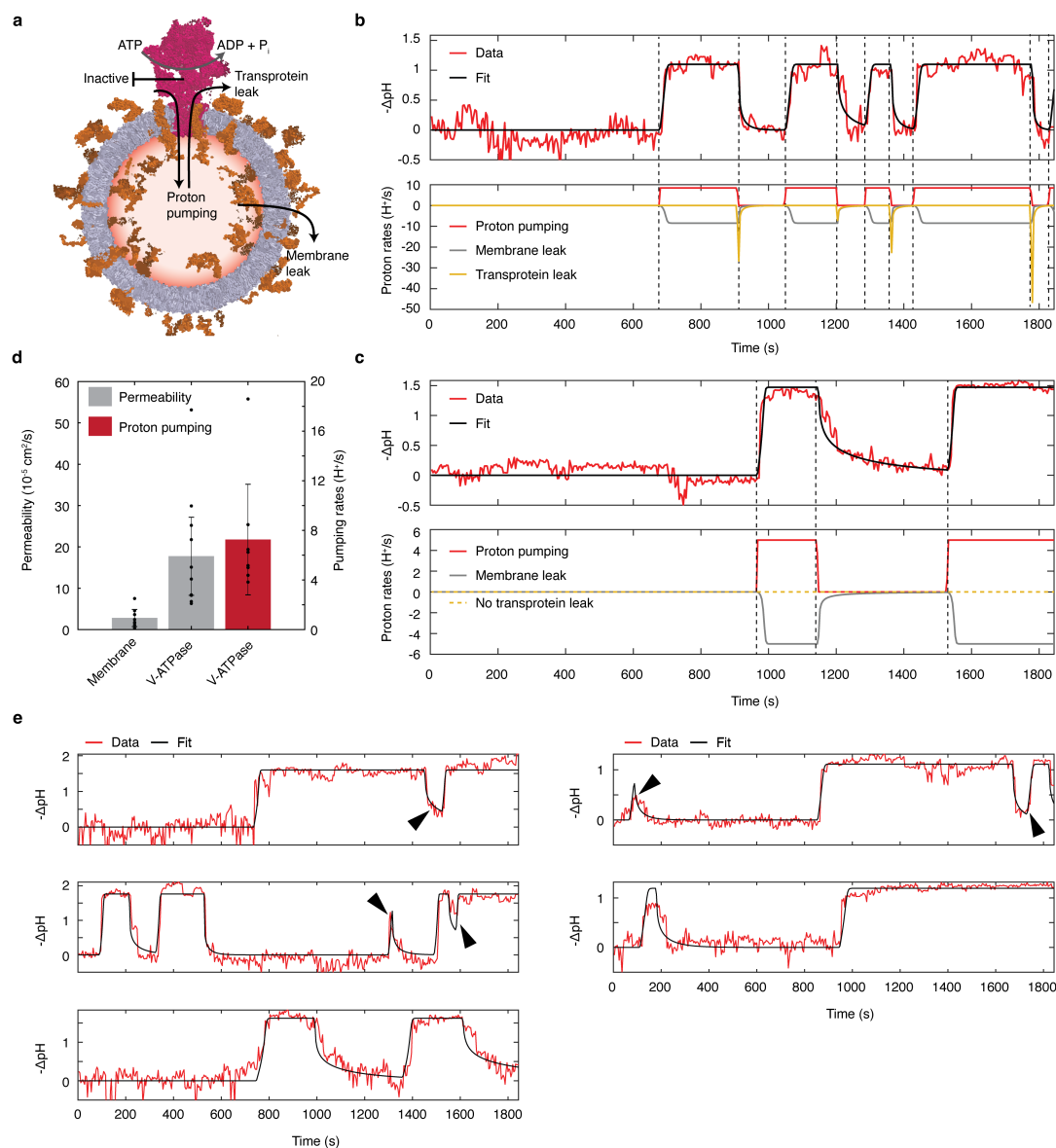
Physical model

We utilized the non-equilibrium model we developed in previous work to extract the pumping rates and permeabilities from single-molecule data^{1,28,29}. The acidification kinetics of single SV_h by the V-ATPase described by the following system of equations:

$$\begin{aligned}\frac{dpH_i}{dt} &= -\frac{1}{\beta(pH_i)} \frac{d[H^+]_T}{dt} \\ \frac{dH_i^+}{dt} &= I_p - (P_{leak}S + P_{V-ATPase}) \frac{U([H^+]_i - [H^+]_o e^{-U})}{1 - e^{-U}} \\ [H^+]_i &= 10^{-pH} \\ \frac{dCl_i^-}{dt} &= P_{Cl}S \frac{U([Cl^-]_i - [Cl^-]_o e^U)}{1 - e^U} \\ U = \frac{\psi}{k_B T} &= \frac{1}{k_B T} \frac{FV}{C_o S} \left(\left(-[Cl^-]_i + \int_{pH_o}^{pH_i} \beta(pH) dpH \right) - B \right)\end{aligned}$$

Quantities in brackets correspond to concentrations of ionic species. Subscripts *i* and *o* correspond to intra- and extraluminal values respectively. β corresponds to the luminal buffering capacity of the system in which protons are buffered by both MOPS and pHrodo-DOPE molecules. I_p is the proton-pumping rate established by the V-ATPase. P_{leak} is the passive proton permeability of the SV_h membrane. S is the surface area and V is the volume of SV_h. S and V are experimentally determined following as mentioned above and in ED Fig. 2. $P_{V-ATPase}$ is the transprotein permeability. U is the reduced membrane potential. In our model, I_p , P_{leak} , and $P_{V-ATPase}$ are free parameters to be determined by the model. P_{Cl} is the chloride permeability of the system. For the modelling performed in the present paper it was assumed that chloride can relatively freely permeate the membrane as a result of a plethora of mechanisms that transport chloride ions (VGluTs, Cl⁻/H⁺ antiport, passive permeation). Therefore, P_{Cl} is set to a large value. ψ is the membrane potential. Single molecule traces fitted with the model are shown in E.D. Fig. 6. For more details regarding the model and the parameters involved we refer the reader to ^{1,28,29}.

New Extended Data Fig. 6:



Extended Data Fig. 6 | Non-equilibrium physical modelling of pumping, resting and leaky modes provides the single-molecule pumping and leaking rate.

a, Schematic illustration of the main parameters used in the model. Note: this illustration is also shown in Fig. 2a. **b**, **c**, Top: Representative single-molecule traces displaying mode-switching dynamics. Proton pumping was stochastically interrupted by inactive and proton-leaky modes. During active periods of the V-ATPase, a dynamic equilibrium between proton-pumping and passive leakage is established, therefore reaching a single acidific plateau. Leakage currents are a convolution of transprotein and passive membrane proton efflux. Transprotein leakage currents are temporally distinct from proton pumping and may activate directly after the enzyme switched off. Data was fitted with a non-equilibrium model as described in the supplementary information. Pumping rates and permeabilities (both membrane and transprotein) are calculated as free parameters by the model. Bottom: Pumping dynamics for the proton-pumping rate (red), the passive membrane efflux rate (grey) and the transprotein efflux rate (yellow). In **b** the transprotein leak was the primary efflux pathway while in **c** proton efflux manifested only passively through the membrane. **d**, Proton pumping rates and permeability estimates of the model for the data shown in this figure (**b**, **c**, and **e**). Pumping rates were found to be 7 ± 5 H⁺/s. Membrane and V-ATPase permeability were 3 ± 2 cm/s and 18 ± 9 cm/s. Transprotein permeability of the V-ATPase is nearly an order of magnitude larger than passive membrane permeability indicating the regulatory importance of the proton-leaky mode. **e**, Additional single-molecule traces fitted the model. Arrows point out the mode-switching events during which dynamic ($\Delta\text{pH} = \Delta\text{pH}_{\text{max}}$) or static (baseline, $\Delta\text{pH} = 0$) equilibrium was not reached.

Comment 2.10

- p7, line 168. Fig.2c should be changes to Fig.2d.
- p11, line 300V/F-ATPase should be changed to V/A-ATPase.

Reply

We thank R2 for spotting these mistakes that have now been corrected.

Reviewer 3

Comment 3.1

The present manuscript describes the characterization of proton pumping by mammalian V-ATPases in hybrid synaptic vesicles. These hybrid SVs (hSVs) are comprised of purified rat SVs fused to liposomes in which the lumen has been enriched with lipid conjugated pH sensitive fluorescent reporters. Further, the hSVs vesicles are tethered to glass slides making them amenable for imaging under TIRF illumination with single hSV resolution. The high copy number of fluorescent reporters dramatically increases the experimental photon budget and allows the authors to achieve higher signal resolution and longer total measurement duration of single SVs compared to previous work that relied on genetically encoded fluorescent reporters.

This work builds upon previous investigations by the same group into the proton pumping characteristics of the P-type ATPase. The key findings in the current manuscript, stochastic switching between proton-pumping, inactive and proton-leaky modes, are similar to the previous work. However, the current work does delve into deeper detail about the regulation of mode lifetimes and pumping rate. Overall, the quality of data is very impressive, the results are convincing and clearly support the interpretations that are made.

Reply

We are grateful to R3 for acknowledging the quality of the data and the broad relevance of the findings. Below we strived to address in full all their concerns. We sincerely hope the substantial body of new experiments, combined with the new kinetic modelling of acidification and the detail answers provided below, address all the reviewer's concerns and will prompt them to recommend publication.

Comment 3.2

The authors should better support or temper the claim of three “novel” modes by more closely comparing this work to the findings in the P-type ATPase work. Similarly, phrasing such as “upending conventional wisdom” when describing mode-switching base regulation should be moderated, especially when later stating that “mode-switching... appears to be a general property of enzymes”.

Reply

Following the advice of R3 we tempered the requested claims in the main text. Below we also add a few brief comments to explain why we had felt these claims were relevant to the readers.

It was not our intention to claim the modes were novel in general. We just wanted to highlight there were novel for the V-ATPase specifically. We felt this is important because single molecule experiments that measured rotation (not pumping)³⁶ had hypothesised that V-ATPases pump continuously, which we show is not the case.

The phrase “upending conventional wisdom” referred specifically to the fact “physiologically relevant concentrations of ATP do not regulate the intrinsic pumping rate”. It did not refer to “the existence of mode-switching in general” nor to “the regulation of mode-switching lifetimes”. We felt this is particularly important to highlight because the field unanimously assumes pumping rates scale with ATP concentration, which we show is not the case.

Lastly, we want to mention that while the single-molecule community is in general familiar with fluctuations in enzymatic catalysis rate, the transporter community (including biochemists and structural biologists) is overall very unfamiliar and skeptical about the concept. For the transporter community, because P-type and V-Type ATPases are completely unrelated in sequence and structure, they should not a priori be assumed to have similar properties (including mode-switching). And of course, strictly speaking this is absolutely correct. We feel this manuscript contributes towards reconciling these two divergent points of view.

Revised text:

Instead, they stochastically switch between three ~~novel~~ ultra-long-lived proton-pumping, inactive, and proton-leaky modes. ~~Upending conventional wisdom~~ Surprisingly, direct observation of pumping revealed that physiologically relevant concentrations of ATP do not regulate the intrinsic pumping rate.

Comment 3.3

The authors infer that similar behaviour is likely to occur in SVs. Could the authors be more explicit in the physical differences between SVs and hSVs (luminal volume, buffering capacity, etc), and how these differences could potentially influence inference about V-ATPase activity in native SVs. (For example, in the averaged ensemble measurements (Fig 1b), acidification reaches steady state after about 15 minutes. In previous work in native SVs this occurs on the order of 10's of seconds.)

Reply

New experiments quantitatively validate mode-switching in intact SVs. The reviewer raises a very good question. To exclude conclusively any possible impact of the non-native environment of the hybrid SV on the properties of the V-ATPase, we performed new experiments in intact SVs. As we show in Fig. 5e,f, inactive/active lifetimes of the V-ATPase in intact SVs are identical to the ones recorded in hybrid SVs. Furthermore, the proton leaky mode is also present in intact SV (Fig. 5h). Taken together these results demonstrate that mode-switching is an intrinsic property of the V-ATPase and is unaffected by fusion with synthetic vesicles.

Ensemble acidification comprises two distinct kinetic processes, intrinsic acidification and mode-switching.

Motivated by the very astute observation of the reviewer we performed a new in-depth analysis of acidification kinetics which resulted in a number of novel, and in our opinion important, findings:

- If one examines our ensemble acidification kinetics in a time window comparable to the literature (~ 60 s) they would actually draw the conclusion that the time constant is of the order of ~ 30 s, see ED Fig. 5d, which is in apparent agreement to literature^{16,68}.
- Examining our ensemble acidification kinetics in a time window of ~ 1800 s (Fig. 1b, ED Fig. 5a) reveals an additional kinetic component which is nearly 10-fold slower (~ 300 s).
- The slow kinetic component arises from mode-switching. V-ATPase are not synchronized and thus not all of them start pumping at the same time, the time of ATP application. This is quantified in ED Fig. 5e that plots a cdf of the first activation events.
- Slow asynchronous activation of the V-ATPase takes place also in intact SVs, see Fig. 5b.
- Due to the convolution of acidification and mode-switching, the true/intrinsic acidification rates cannot be inferred from the ensemble average traces. They can however be extracted by fitting single vesicle kinetics.
- ED Fig. 5g shows the histogram of single SV_h acidification kinetics, it has a mean of ~ 30 s and spans a range of 10 s -100 s.

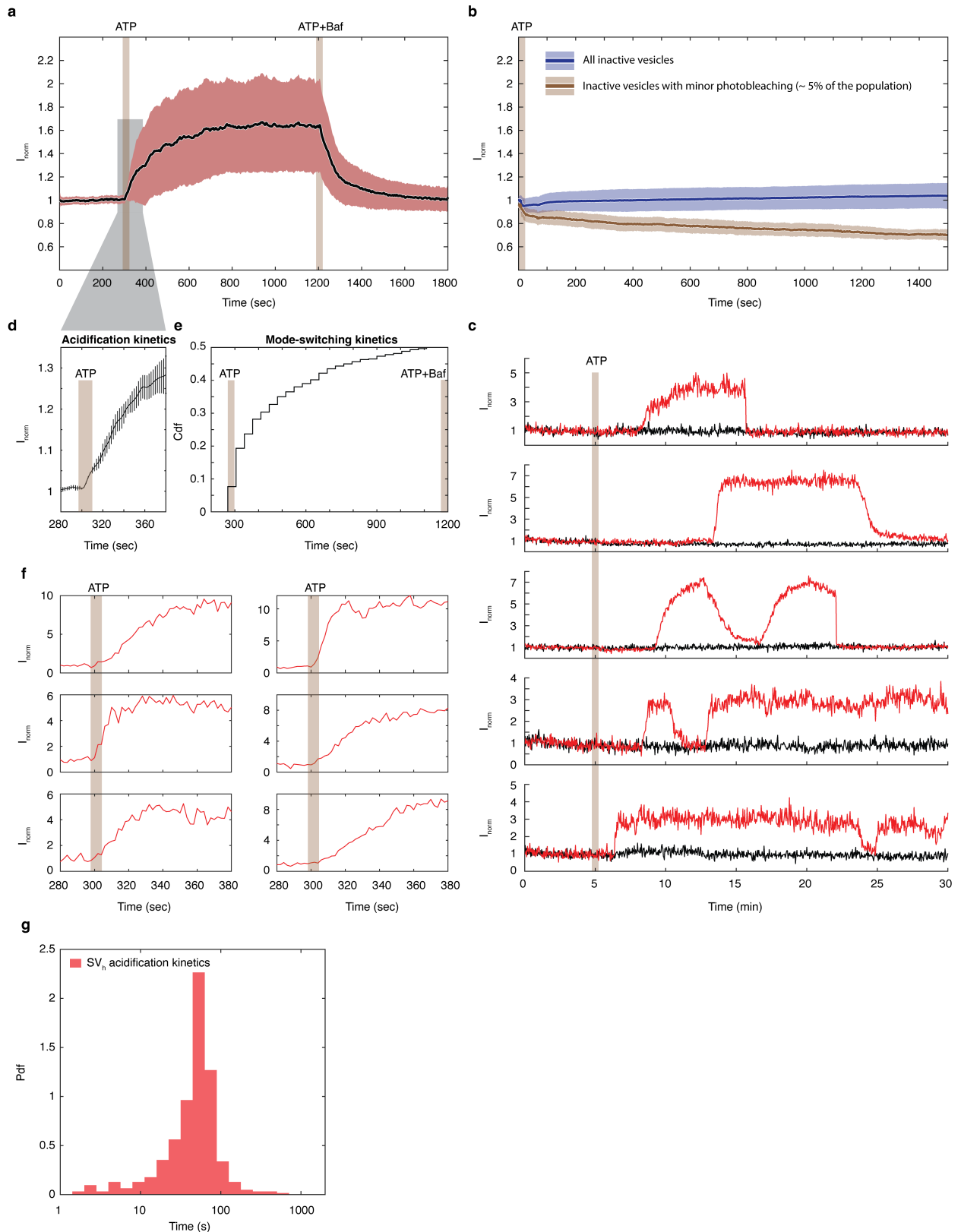
Influence of vesicle size on acidification rates

To answer this interesting question, we first measured the sizes of LUV using cryoEM (see new ED Fig. 2). Then we employed a physical kinetic model of acidification that accounts for several parameters including passive and active ionic fluxes across the membrane, proton buffering in the lumen and build-up of membrane potential, vesicle size etc. See new ED Fig. 6 and revised supplementary discussion for more information.

Increasing the vesicle diameter from 40 nm to 80 nm would be expected to slow down the rate of acidification by ~4-fold (from 10 s to 44 s).

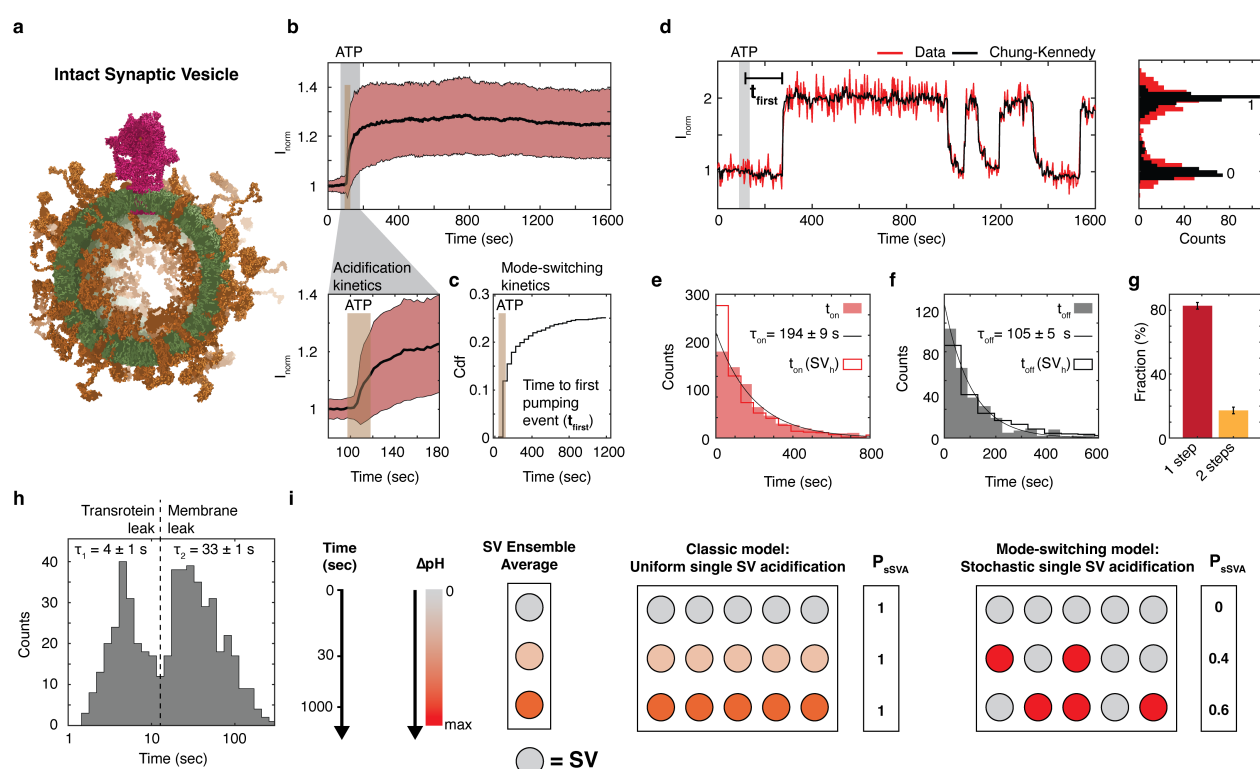
Revised text and new figures

- ED Fig. 5
- Fig. 5
- ED Fig. 2
- ED Fig. 6



- **Extended Data Fig. 5 | Single-vesicle acidification kinetics are fast (~30 s), the slow apparent ensemble average acidification kinetics are governed by slow mode-switching (~1000 s). Under our imaging conditions we never observe single-molecule photo-blinking or photo-bleaching.**
- **a**, Ensemble average acidification kinetics of single SV_n. Red shaded area corresponds to one SD of all single vesicle traces. Note: this figure is also shown in Fig. 1b. **b**, The majority of inactive single vesicle traces (~95% of the population) showed photostability with no indication of photobleaching or self-

quenching. ~ 5% of the vesicles showed signs of minor photobleaching on the order of 30% after 25 minutes of recording. We never observed an increase in fluorescent intensity while imaging which would be indicative of self-quenching. **c**, Collections of active and inactive vesicles. Inactive vesicles retained stable baseline signals throughout the course of experiments. No photoblinking was observed for the totality of inactive vesicles **d**, Ensemble average acidification kinetics immediately after ATP injection. Error bars correspond to the SD between ensemble average traces of multiple replicates ($n = 4$). Acidification until the dynamic equilibrium of pumping and leakage is reached takes place in the order of tens of seconds, consistent with previous ensemble measurements of acidification¹⁶. **e**, Cumulative histogram of the time elapsed until the onset of the first proton-pumping event. Mode-switching dynamics are slow (~ 1000 seconds) and are responsible for the slow kinetics seen in the ensemble average traces in **a**. **f**, Zoom in regions of representative single-vesicle traces showcase that acidification occurs in the order of tens of seconds. **g**, Histogram of single-vesicle acidification kinetics shows that most vesicles acidify in 10-100 s.

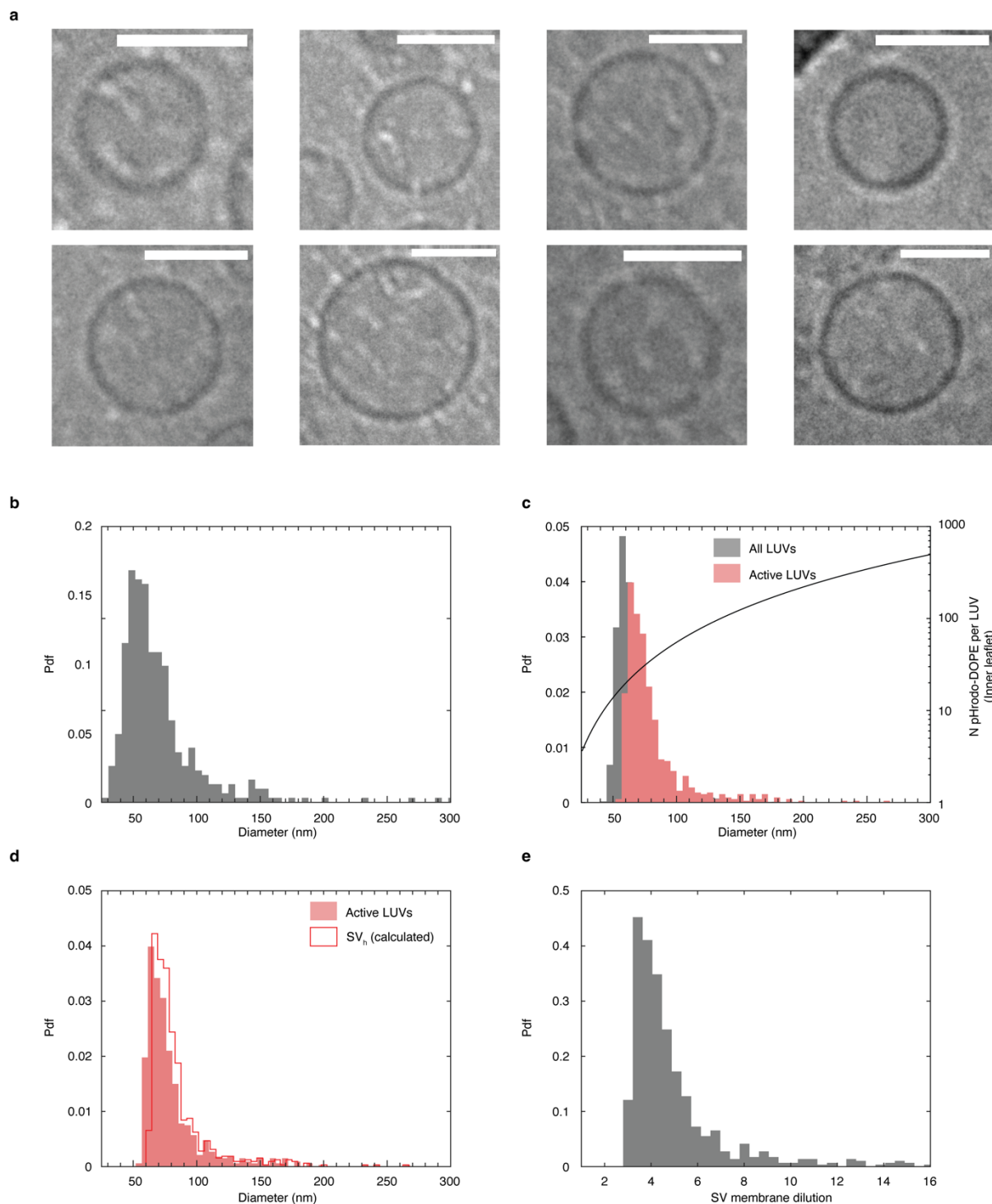


New Figure 5 | Mode switching of the V-ATPase in intact SVs. Mode-switching of the V-ATPase in SVs shows nearly identical kinetics as in SV_h. **a**, Illustration of the membrane proteome of the intact synaptic vesicle. The V-ATPase is shown in magenta. The lipid conjugated pH sensor as well as biotinylated lipids were incorporated into the vesicle as described in supplementary information. **b**, SVs acidify in the order of tens of seconds. Top: ensemble average of ~ 950 single SV acidification kinetics. Activity was initiated by the addition of ATP. Data was corrected for photobleaching. Red shaded area depicts the SD of the single-vesicle traces. Bottom: zoom-in of the grey-shaded region corresponding to the time immediately after injection of ATP. Acidification kinetics take place in the order of tens of seconds. Number of experiments is $n=3$. **c**, Cumulative histogram of the time elapsed until the initiation of the first pumping event (t_{first}) after the injection of ATP. The stochastic delay of single V-ATPase activity shapes the ensemble average kinetics seen in **b**. **d**, Representative single-vesicle acidification kinetics showcasing mode-switching. Unprocessed data is shown in red while in black is data processed by a Chung-Kennedy filter. Histograms correspond to acidification plateaus established by single V-ATPases. **e**, **f** Histograms of proton-pumping and resting dwells fitted with a single exponential in intact SVs (shaded histograms). Error bars correspond to the error of the fit. Outlined histograms show the dwell time histograms of SV_h. The proton-pumping and inactive mode lifetimes of SVs are nearly identical to those of SV_h ($\tau_{on}(SV) = 194 \pm 9$ s; $\tau_{on}(SV_h) = 174 \pm 9$ s and $\tau_{off}(SV) = 105 \pm 5$ s; $\tau_{off}(SV_h) = 122 \pm 9$ s). **g**, Number of active V-ATPases in intact SVs as calculated by step analysis of single-vesicle traces. The average synaptic vesicle contains ~1.2 active V-ATPases. Errorbars correspond to the SD between $n=3$ experiments. In the data analyzed, no vesicles with three or more active V-ATPases could be observed. **h**, Proton-leakage lifetimes reveal nearly identical leakage kinetics as in SV_h. The transprotein leak is 4 ± 1 s while the passive membrane leak is 33 ± 1 s. **i**, Schematic illustration of the classical acidification model and the mode-switching model. In the classical paradigm all vesicles simultaneously and continuously ($P_{sSVA} = 1$) acidify gradually to reach an equilibrium value that matches the experimentally determined ensemble average acidification

kinetics. Here, we propose the mode-switching model, whereby each SV independently can stochastically switch on and reach the maximal pH gradient ($\Delta\text{pH}_{\text{max}}$). At equilibrium, the probability the V-ATPase is found in a pumping mode is $P_{\text{pumping}} = 60\%$. Therefore, at the steady state, the probability that a random synaptic vesicle is found in an acidified state corresponds to $P_{\text{sSVA}} = P_{\text{pumping}} = 60\%$.

Lipid incorporation into intact synaptic vesicles

To image single intact SV acidification kinetics we established a protocol to incorporate lipid molecules into the native membranes of SVs. To do so, we first dissolved 10 μL pHrodo-DOPE (1 mg/mL) and 5 μL DSPE-PEG(2000)-biotin (10 mg/mL) in 85 μL of chloroform. Then, we formed a lipid film by evaporation of chloroform under nitrogen flow. The lipid film then is stored in vacuum for at least 30 minutes. Subsequently, the lipid film was rehydrated in 20 μL of synaptic vesicles diluted in 80 μL of activity buffer and mixed initially with repeated pipetting. Once the lipid film is dissolved, we shake the sample for half an hour at 600 rpm and 37 °C. Finally, we spin down non-hydrated debris at 13,000 rpm for 1 minute and remove SVs without disturbing the pellet. The sample is then distributed into 10 μL aliquots and freeze-thawed prior to use. To image synaptic vesicles, we immobilize them on a functionalized glass using the same protocols as with SV_h imaging. Once immobilized, the activity of the V-ATPase is triggered upon addition of ATP. Single-molecule proton-pumping data is shown in Fig. 5.



Extended Data Fig. 2 | Morphology and diameters of LUVs; number of fluorophores in LUVs; SV fold-dilution upon fusion with a LUV.

a, Cryo-electron micrographs of LUVs reconstituted with the SNARE acceptor complex ΔN . Scale bars are 50 nm.

b, Distribution of diameters of LUVs as measured by cryoEM. The mean and SD of the distribution is 72 ± 43 nm.

c, The cryo-electron data can be used to calibrate the vesicle size in the fluorescence microscopy images using previously established methods^{1,12} (Supplementary discussion). Grey shaded histogram shows single LUV fluorescent intensities quantitatively converted to diameter in nanometres, 66 ± 18 nm. Red shaded histogram corresponds to the size distribution of the $\sim 5\%$ of LUVs that showed V-ATPase activity, 80 ± 25 nm. The average diameter of active LUVs is ~ 14 nm larger than the rest of the population, since larger LUVs fuse more efficiently via SNARE machinery¹³. Black line corresponds to the estimated number of pHrodo-DOPE lipids in LUVs and scales with the surface area of the vesicle. The number of pHrodo-DOPE lipids in the membrane of fused vesicles ranged between 16-340 and was ~ 40 on the average. To calculate the number of lipids per vesicle we used a

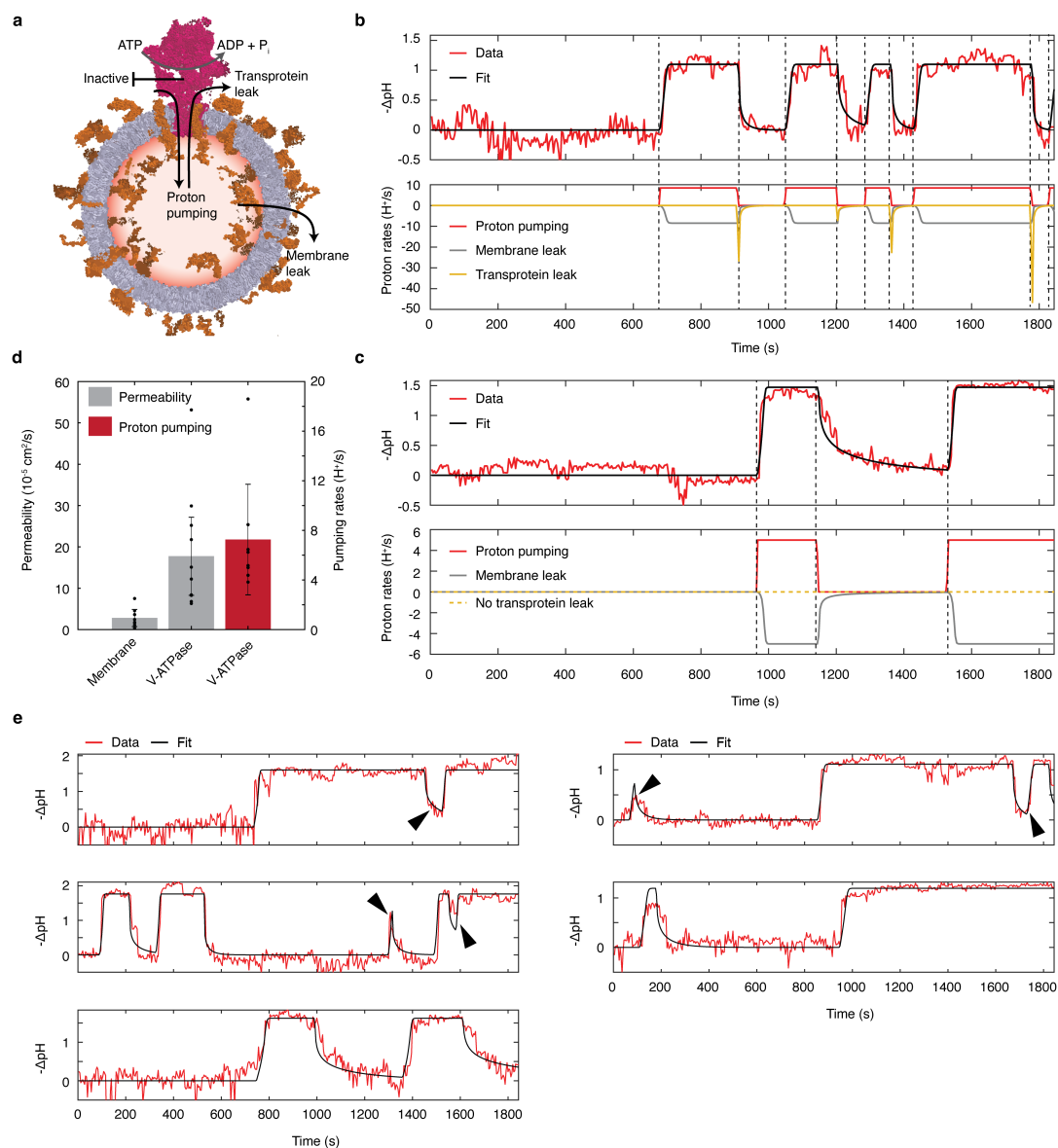
surface area per lipid of $\sim 0.65 \text{ nm}^2$ ^(10,11). **d**, Red-shaded histogram is the same as in c, $80 \pm 25 \text{ nm}$. Red-outlined histogram corresponds to the calculated diameters of SV_h produced by fusion of LUVs with 40 nm SVs, $84 \pm 24 \text{ nm}$. **c**, Dilution factor of the SV membrane in SV_h calculated using a SV diameter of 40 nm. The mean SV membrane dilution is 5.4-fold.

Physical model

We utilized the non-equilibrium model we developed in previous work to extract the pumping rates and permeabilities from single-molecule data^{1,28,29}. The acidification kinetics of single SV_h by the V-ATPase described by the following system of equations:

$$\begin{aligned}\frac{dpH_i}{dt} &= -\frac{1}{\beta(pH_i)} \frac{d[H^+]_T}{dt} \\ \frac{dH_i^+}{dt} &= I_p - (P_{leak}S + P_{V-ATPase}) \frac{U([H^+]_i - [H^+]_o e^{-U})}{1 - e^{-U}} \\ [H^+]_i &= 10^{-pH} \\ \frac{dCl_i^-}{dt} &= P_{Cl}S \frac{U([Cl^-]_i - [Cl^-]_o e^U)}{1 - e^U} \\ U = \frac{\psi}{k_B T} &= \frac{1}{k_B T} \frac{FV}{C_o S} \left(\left(-[Cl^-]_i + \int_{pH_o}^{pH_i} \beta(pH) dpH \right) - B \right)\end{aligned}$$

Quantities in brackets correspond to concentrations of ionic species. Subscripts *i* and *o* correspond to intra- and extraluminal values respectively. β corresponds to the luminal buffering capacity of the system in which protons are buffered by both MOPS and pHrodo-DOPE molecules. I_p is the proton-pumping rate established by the V-ATPase. P_{leak} is the passive proton permeability of the SV_h membrane. S is the surface area and V is the volume of SV_h . S and V are experimentally determined following as mentioned above and in ED Fig. 2. $P_{V-ATPase}$ is the transprotein permeability. U is the reduced membrane potential. In our model, I_p , P_{leak} , and $P_{V-ATPase}$ are free parameters to be determined by the model. P_{Cl} is the chloride permeability of the system. For the modelling performed in the present paper it was assumed that chloride can relatively freely permeate the membrane as a result of a plethora of mechanisms that transport chloride ions (VGluTs, Cl^-/H^+ antiport, passive permeation). Therefore, P_{Cl} is set to a large value. ψ is the membrane potential. Single molecule traces fitted with the model are shown in E.D. Fig. 6. For more details regarding the model and the parameters involved we refer the reader to ^{1,28,29}.



Extended Data Fig. 6 | Non-equilibrium physical modelling of pumping, resting and leaky modes provides the single-molecule pumping and leaking rate.

a, Schematic illustration of the main parameters used in the model. Note: this illustration is also shown in Fig. 2a. **b**, **c**, Top: Representative single-molecule traces displaying mode-switching dynamics. Proton pumping was stochastically interrupted by inactive and proton-leaky modes. During active periods of the V-ATPase, a dynamic equilibrium between proton-pumping and passive leakage is established, therefore reaching a single-molecule pumping plateau. Leakage currents are a convolution of transprotein and passive membrane proton efflux. Transprotein leakage currents are temporally distinct from proton pumping and may activate directly after the enzyme switched off. Data was fitted with a non-equilibrium model as described in the supplementary information. Pumping rates and permeabilities (both membrane and transprotein) are calculated as free parameters by the model. Bottom: Pumping dynamics for the proton-pumping rate (red), the passive membrane efflux rate (grey) and the transprotein efflux rate (yellow). In **b** the transprotein leak was the primary efflux pathway while in **c** proton efflux manifested only passively through the membrane. **d**, Proton pumping rates and permeability estimates of the model for the data shown in this figure (**b**, **c**, and **e**). Pumping rates were found to be 7 ± 5 H⁺/s. Membrane and V-ATPase permeability were 3 ± 2 cm/s and 18 ± 9 cm/s. Transprotein permeability of the V-ATPase is nearly an order of magnitude larger than passive membrane permeability indicating the regulatory importance of the proton-leaky mode. **e**, Additional single-molecule traces fitted the model. Arrows point out the mode-switching events during which dynamic ($\Delta\text{pH} = \Delta\text{pH}_{\text{max}}$) or static (baseline, $\Delta\text{pH} = 0$) equilibrium was not reached.

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Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have responded in full to the questions raised during initial review by revising aspects of the text, revising certain descriptions of their findings and performing new control experiments and additional calculations to clarify issues related to reader comprehension and technical concerns/reproducibility. Certain language is also toned down appropriately as requested by Referee 2. These changes make the manuscript more readable and easy to understand. The advance over prior research in this area is also made more evident as is the potential physiological relevance of 'mode-switching' behaviors uncovered by their single-molecule analyses. The main conclusion if I understand things correctly is that slow mode switching in the V-ATPase has the net effect of rendering a significant (up to half) of the total pool of synaptic vesicles inactive. Thus, the authors contend that variances in the mode switching rate can regulate the available pool of competent synaptic vesicles in the synapse. This is an interesting supposition to be sure. The authors present this model/hypothesis, but do not take it further. Hence the validity of the hypothesis remains to be tested.

Referee #2 (Remarks to the Author):

In the revised manuscript, the authors have demonstrated the regulatory mechanism (likely mechanical slipping) of the proton pump of V-ATPase by a sophisticated experimental system. Data obtained from sufficient number of experiments are carefully statistically processed, and the data are basically reliable.

As noted in the first comment, the number of V-ATPases per fused liposome is of great importance to the reliability of the results, and therefore the reviewer requested confirmation by another method. The authors have shown, through careful experimentation and interpretation, that the number of V-ATPases per liposome is approximately 1. The authors have provided answers to the problems of LUV size identification and unintended adsorption on glass substrates through additional experiments and careful interpretation. The stability of the V-ATPase affects on the reliability of the data obtained after several hours of observation. The authors have shown data that at least no inactivation occurs during the measurement. This is amazing, but it does guarantee the reliability of the results obtained in this study. The influence of other transporters in the SV is also a concern. Therefore, the control experiments with inhibitors were proposed. Instead, the authors suggest interpreted the experimental data to show that there is no effect of other transporters.

In summary, the reviewer believe that the revised manuscript addresses the issues pointed out and has been improved to be worthy of publication.

Referee #3 (Remarks to the Author):

The authors have done an outstanding job addressing all concerns expressed in my last review. The additional experiments and analysis included in the revised manuscript clarify many points and strengthen this fantastic work. I have no further concerns.