

Supplementary Information

Orthosteric and allosteric inhibition of the brain V-ATPase outside the proton transport cycle

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Supplementary Methods

Image analysis

Data from light microscopy experiments were analyzed using a custom software suite and MATLAB scripts. A single particle localization algorithm¹ identified vesicles and their positions, with a drift correction algorithm² addressing potential XY drifts caused by thermal fluctuations. Following image stabilization and particle identification, 3x3 pixel regions of interest (ROIs) were defined around each particle, and integrated intensity readouts were obtained by summing the pixel values.

Single-molecule normalized intensity, defined as raw intensity divided by the mean baseline raw intensity ($I_{SM} = I_{raw}/I_{base}$), and standardized intensity, defined as raw intensity reduced by the mean baseline intensity and divided by the standard deviation of the signal at the baseline (Standardized Intensity = $(I_{raw}-I_{base})/\sigma_{base}$), were employed. Normalized intensities were used for data analysis and visualization, as well as to convert single-molecule traces to pH units and classify traces based on their number of intensity steps (Supplementary Fig. 2 and Supplementary Fig. 3). Standardized intensities were used in the stochastic filtering algorithm.

Measuring mode-occupancies through single-molecule local referencing for compound screening

To screen compounds, we first acquire a stack of images of immobilized vesicles in activity buffer (300 mM glycine, 2 mM MOPS, 2 mM MgSO₄, pH 7.1–7.15, adjusted with Tris). This defines the baseline fluorescence of synaptic vesicles (SVs). Next, 1 mM MgATP is added to initiate proton pumping, along with 30 mM choline chloride to counteract positive charge accumulation in the SV lumen^{3–5}. This condition reveals a vesicle population that responded to ATP. This is referred to as the *apo* condition. Note that at the *apo* condition we sample a random snapshot that captures the active vesicles at the time of the recording. Subsequently, increasing concentrations of V-ATPase inhibitors are sequentially introduced at 6.5-minute intervals, which allowed incubation and action of inhibitors. After each interval, a 20-frame stack is acquired.

Active vesicles were identified for each treatment by thresholding based on the intensity change relative to the baseline. A trace was classified as active if $\geq 50\%$ of frames exceeded the threshold. For each treatment, the number of active traces and mean fluorescence intensity per vesicle were extracted from the recording. Mean intensities were converted to Δ pH values and used to estimate acidification and proton pumping rates. At the end of the imaging sequence, a 20-frame stack was acquired under saturating pH conditions to calibrate fluorescence-to-pH conversion (Supplementary Fig. 3, Supplementary methods).

For contour plots and population histograms (Fig. 2 and Supplementary Fig. 9, Supplementary Fig. 10), intensities were normalized per vesicle, such that the baseline/inactive mode was set to 0 and the active mode was set to 1. This normalization preserves intra-vesicle variability across

conditions. Mode-occupancy distributions were fitted with double Gaussians via maximum likelihood estimation with grid search for ensuring global maxima. When active-mode occupancy was too low for robust fitting, a lower bound was imposed on the mean of that population.

Note: All snapshot experiments used 1 mM ATP supplemented with 30 mM choline chloride unless stated otherwise.

Single-vesicle kinetic measurements

Acidification kinetics were initiated by recording baseline fluorescence of immobilized vesicles in activity buffer (300 mM glycine, 2 mM MOPS, 2 mM MgSO₄, pH 7.1–7.15, adjusted with Tris) for 5 minutes. Activity was then triggered by adding 1 mM MgATP and 30 mM choline chloride to counteract the buildup of membrane potential^{3–5}. After a 10-minute equilibration (15 minutes for SidK), compounds were introduced, and vesicle responses were monitored over the subsequent 15 minutes. At the end of the recording, a 20-frame image stack was acquired under saturating pH conditions to enable fluorescence-to-pH conversion via calibration curves (Supplementary Fig. 3, Supplementary methods).

Active V-ATPase transport kinetics were identified using signal-to-noise ratio (SNR)–based thresholding. SNR for each vesicle was defined as

$$\text{SNR} = (I_{\text{SM,max}} - I) / \sigma_{\text{SM,base}},$$

where $I_{\text{SM,max}}$ is the peak normalized intensity, and $\sigma_{\text{SM,base}}$ is the baseline standard deviation. To reduce spike-related outliers in $I_{\text{SM,max}}$, traces were smoothed with a Chung–Kennedy filter⁶.

Active transport traces were manually classified based on the number of discrete intensity steps (plateaus; Supplementary Fig. 3). Only vesicles exhibiting a single step—indicative of individual V-ATPase activity—were included in the single-molecule analysis. Traces lacking characteristic stochastic behaviour were excluded.

Note: All kinetic experiments included 1 mM ATP and 30 mM choline chloride unless otherwise specified.

Single-molecule local referencing vs kinetics: efficiency and throughput improvement

The single-molecule local referencing assay provides a major improvement in experimental and data-analysis throughput compared to the previous kinetic-based approach⁷. Briefly, single-molecule local referencing offers a ~100-fold improvement in experimental data acquisition over a range of concentrations of ligands because it shortens the acquisition time from ~1800s down to ~10s per condition (~20 frames), effectively taking a snapshot of the binary normalised single synaptic vesicle intensities. In exposing the sample to only ~20 frames per condition, in between 6min long incubation steps, it also minimizes phototoxicity that could potentially arise from uninterrupted sequential exposure (kinetic recordings comprise ~600–800 sequential frames), thus enhancing temporal stability of the sample. Moreover, due to the short period recordings per

condition, we were able to record ~10 fields-of-view simultaneously per condition increasing the data throughout by 10-fold.

Lastly, data analysis is accelerated by roughly two orders of magnitude compared with our previous kinetic approach, because the single-molecule local referencing assay avoids the computationally and labour-intensive identification and fitting of individual events in long single-molecule traces. Instead, the binary values of normalised single synaptic vesicle intensities yield population mode occupancies for each condition, which is intrinsically much faster while still yielding robust statistics. A custom, fully automated MATLAB pipeline processes a complete titration dataset and outputs all distributions within ~10 minutes.

Taken together, single-molecule local referencing provides a $\sim 10^5$ -fold improvement in effective combined throughput relative to our kinetic-based method.

Calibration of pH in vesicles

A local calibration approach was employed using DOPE-pHrodo labeled liposomes with the specified lipid composition (DOPC:DOPS:Chol:(18:1 Biotinyl Cap PE):DOPE-pHrodo 64.4:10:25:0.5:0.1). Immobilized liposomes were subjected to calibration buffer (150 mM K-Gluconate - 2 mM MOPS - 2 mM MgSO_4) with 60 nM valinomycin and 5 μM CCCP at nine pH values. The custom software identified vesicles, and raw intensities were extracted and fitted with a sigmoidal function. Normalization to pH 7.1 baseline intensity yielded mean values for pKa and rates. I_{base} and I_{max} , the defining fluorophore dynamic range, were locally defined for each vesicle after activity acquisition.

Following single vesicle acidification kinetics at pH 7.10-7.15, we incubated SVs in saturation buffer (300 mM Glycine - 2 mM MOPS – 2 mM MgSO_4) at pH 2.85-3.15 (adjusted with citric acid) with 5 μM CCCP and 30 mM choline chloride. Calibration involved normalizing raw traces, accounting for pHrodo-DOPE bilayer contribution, and calculating pH as a function of intensity. The assumption of equal distribution of pHrodo-DOPE lipids between bilayer leaflets was maintained throughout the analysis. For a more detailed description of the calibration procedure, we refer the reader to previous work ⁷. See also supplementary Fig. 3.

Single SV pH gradients

To extract single SV acidification equilibria ($\Delta\text{pH}_{\text{SM}}$) we selected regions of the kinetic traces during which proton pumping and proton leakage are in equilibrium. In these regions, the pH gradient established by the V-ATPase is the maximal possible given that the permeability of the membrane counteracts the proton pumping. For a region of a trace to qualify to extract the $\Delta\text{pH}_{\text{SM}}$, it must display a stable plateau for at least 5 frames (typically 15 seconds of real-time imaging). The value of $\Delta\text{pH}_{\text{SM}}$ is the mean value of the frames in the plateau selected.

Intensity plateaus and step counting

We have shown previously that the presence of multiple transporters in a single vesicle would appear as multiple steps in the acidification plateaus⁷. Indeed, performing a step analysis we found that $85\% \pm 3\%$ of the single vesicle data showed a single step, $13\% \pm 2\%$ showed 2 steps and $2\% \pm 1\%$ showed 3 steps or more (Supplementary Fig. 2). Note that purified SVs from different batches yielded slightly different step counts, this was accounted for throughout the present paper. As a result, our data suggests that, on average, the SV contains 1.2 ± 0.1 copies of active V-ATPases which is in excellent agreement with proteomic studies^{8–10}. These findings confirm the intrinsic single-molecule nature of the SV. All single-molecule analysis was restricted to single-step data.

pH gradients at the thermodynamic limit

To estimate the maximum pH gradient that a V-ATPase can establish, we considered the free energy change of ATP hydrolysis under various intracellular conditions. The V-ATPase is assumed to translocate 10 protons per 3 ATP molecules hydrolyzed.

The Gibbs free energy change for ATP hydrolysis is given by:

$$\Delta G = \Delta G^0 + RT \ln \frac{[\text{ADP}][\text{P}_i]}{[\text{ATP}]}$$

where $\Delta G^0 = -30 \text{ kJ} \cdot \text{mol}^{-1}$, $R = 8.314 \times 10^{-3} \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, and $T = 298 \text{ K}$. The free energy available per proton translocated is:

$$\Delta G_{H^+} = (3/10) \cdot \Delta G$$

This energy drives the formation of a transmembrane pH gradient, where:

$$\Delta G_{H^+} = -2.303RT \cdot \Delta \text{pH}$$

Solving for the pH gradient gives:

$$\Delta \text{pH} = -\frac{\Delta G_{H^+}}{2.303RT}$$

Using a fixed ATP concentration of 1 mM and 1 μM ADP and 1 μM Phosphate (in our experiments, the concentrations of P_i and ADP are likely much lower), we computed the theoretical maximum to be $\Delta \text{pH}_{\text{max}} = 4.28$. These calculations define the thermodynamic upper limit of proton motive force generation by V-ATPases and demonstrate that in our single-molecule experiments, the gradient generated by the V-ATPase is substantially below the thermodynamic limit ($\Delta \text{pH}_{\text{SM}} \sim 1-1.5$)

Kinetic assay: dwell time analysis

Employing the stochastic filtering algorithm, mode-switching events in single-molecule data were identified, and manual fine-tuning of events was performed on approximately 30-40% of the detected events.

Dwell time calculations considered only single-molecule data exhibiting at least one stochastic event. Active dwell times (t_{ON}) were determined from the onset of acidification to the point at which the signal decreased, signifying V-ATPase deactivation. Inactive dwell times (t_{OFF}) were computed from the start of signal decrease until the subsequent active event. Analysis of dwell times commenced from the initiation of the first proton-pumping event, uninterrupted by the recording's conclusion.

Kinetic assay: probability estimates

In kinetic experiments, the probability that the V-ATPase is found in an active mode is estimated as, $P_{ON} = \sum t_{ON} / t_{treatment}$, where $t_{treatment}$ is the total duration of the recording under a specific condition (e.g., in the presence of an inhibitor).

Snapshot assay: probability estimates and ensemble average acidification

The number of active vesicles for each treatment (i) is normalized to the total number of active vesicles identified under all conditions. We calculate the uncorrected probability of the active mode per treatment to be

$$P_{ON,i}^* = \frac{N_i}{N_{Total}}$$

$P_{ON,i}^*$ includes SVs that contain more than one V-ATPase and therefore skew the total probability towards higher active mode probability. To correct the probability for the number of active V-ATPases, we perform the following correction:

The probability that a SV with n V-ATPases, ${}^n P_{OFF}$, is found in an inactive mode is given by

$${}^n P_{OFF} = [P_{OFF}]^n$$

Where, P_{OFF} is the inactive mode probability ($P_{OFF} = 1 - P_{ON}$) of a SV containing a single V-ATPase. For the entire population of SVs then the total probability to be found in an inactive mode becomes

$$P_{OFF}^* = \sum_{n=1}^3 w_n \cdot [P_{OFF}]^n$$

Where w_n is the corresponding fraction of SVs containing $n = 1, 2$ or 3 (or more) V-ATPases as calculated by the step analysis (Supplementary discussion and Supplementary Fig. 2). Therefore, the corrected active mode probability, P_{ON} , is numerically extracted using

$$P_{ON,i}^* = \frac{N_i}{N_{Total}} = \sum_{n=1}^3 w_n \cdot (1 - P_{ON})^n$$

The average acidification per treatment i , ($\Delta pH_{AV,i}$) is a convolution of the single-molecule acidification of that treatment ($\Delta pH_{SM,i}$) reduced by the probability to be found in an active mode (P_{ON}) and is expressed as

$$\Delta pH_{AV,i} = \Delta pH_{SM,i} \cdot P_{ON,i}$$

Physical model

We employed the previously developed non-equilibrium model to extract pumping rates and permeabilities from single-molecule data ^{7,11,12}. The following system of equations describes the acidification kinetics of single synaptic vesicles by the V-ATPase:

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

Concentrations of ionic species are represented as quantities in brackets. Subscripts i , o indicate intraluminal and extraluminal values of variables, respectively. Luminal buffering capacity is defined as β . In the lumen of SVs, protons are buffered by MOPS and pHrodo-DOPE molecules (the buffering capacity of the SV proteome has not been considered). Single-molecule proton pumping rates, passive proton permeability, and transprotein permeability are represented by I_p , P_{leak} , and $P_{V-ATPase}$ respectively and are the free parameters in the model. S and V are the surface area and volume of SVs, respectively. P_{Cl} is the permeability of SVs to Cl^- and is chosen such that chloride ions can traverse freely across the membrane. Ψ is the membrane. Single-molecule traces fitted with the model are shown in Supplementary Fig 4.

In a previous publication, we demonstrated the existence of a transprotein leakage mode of the V-ATPase. To fit the single-molecule data with the physical model, it was necessary to include transprotein permeability as a free parameter. However, in the present manuscript, the leaky mode was not further investigated.

Calculation of the transition energy barrier heights for on-to-off and off-to-on cycle transitions.

Transition rates were calculated using transition state theory¹³, where the pumping and inactive modes are considered ground states separated by an activation barrier (the transition state). According to transition state theory, the energy required to achieve the transition state (and cross the barrier) is:

$$\Delta G^\ddagger = -RT \ln \frac{h k_{ij}}{k_B T}$$

where R is the gas constant, T is the absolute temperature, k_B is Boltzmann's constant, h is Planck's constant, k_{ij} is the measured transition rate from active to inactive or from inactive to active modes. Under the assumption that we have a two-mode model, active and inactive mode rate constants can be calculated using equations

$$k_{on,off} = \frac{1}{\langle t_{on} \rangle}$$
$$k_{off,on} = \frac{1}{\langle t_{off} \rangle}$$

Where, $\langle t_{on} \rangle$ and $\langle t_{off} \rangle$ are the average dwell times in active and inactive modes for the V-ATPase. Free energy barriers for mode transitions in the presence of inhibitors are found in Supplementary Table 2 and Fig. 5.

Calculation of free energy difference between active and inactive modes.

We calculated the Gibbs free energy difference between active and inactive modes of the V-ATPase for the *apo* and in the presence of inhibitors (see Fig. 5 and Supplementary Table 2) using the equation,

$$\Delta G = -RT \ln \frac{P_{ON}}{1-P_{ON}}$$

where P_{ON} and $1-P_{ON}$ represent the probabilities of the pump being in active or inactive modes, respectively.

Stochastic model description

Here, we provide a brief summary of the Bayesian event detection methodology developed in a previous work. For a more detailed description of the method, we refer the reader to earlier publications^{7,14}.

The signal in the experiment refers to variations in light intensity caused by the activity of V-ATPase. This signal depends on change-point times $\mathbf{T} = \{T_1, T_2, \dots, T_n\}$ representing mode-switching between active and inactive modes of the proton pump. The proton-pumping mode leads to increased fluorescence modelled as an exponential increase to an equilibrium level (α) with rate (β), while the inactive mode results in exponential decay back to baseline with rate (γ). The overall signal model, $\mu(t)$, switches between these modes at change-points.

Exponential rates for proton-pumping and inactive events are denoted as β_i and γ_i , respectively. Equilibrium level α remains constant. Parameters are grouped as $\mathbf{v} = \{\alpha, \beta_1, \gamma_1, \dots, \beta_n, \gamma_n\}$. Time between mode-switching events is denoted as $\boldsymbol{\theta} = \{\theta_1, \theta_2, \dots, \theta_n\} = \{T_1, T_2 - T_1, \dots, T_n - T_{n-1}\}$, which replaces $\mathbf{T}$ in the model. Unknowns, including change-point times, are treated as random variables with unbiased prior distributions in a Bayesian setting.

Stochastic filtering, based on Bayesian techniques, is employed to estimate the signal, considering prior opinions and updating with observed data. Maximum a posteriori probability (MAP) estimates for change-points and parameters are obtained from the joint distribution. The likelihood function L is defined based on discrete observed data, approximating continuous-time likelihood. Numerical constraints are applied for parameter ranges.

A sliding method of Bayesian stochastic filtering is introduced to address the computational complexity associated with a large number of change-points. This method segments data into regions with one mode-switch event, reducing complexity. The Bayesian event detection algorithm is novel, focusing on raw data to identify mode-switching events without smoothing.

Model selection via simulated Bayesian Information Criterion (BIC) differences

To determine whether lifetime distributions are better described by a single or double-exponential model, we performed fitting and simulation-based inference. First, lifetimes were fit using both a single- and double-exponential model for maximum likelihood estimation (MLE) with grid search for ensuring global maxima to obtain parameter estimates. The Bayesian Information Criterion (BIC) is defined as

$$\text{BIC} = k * \ln(n) - 2 * \ln(\hat{L})$$

and the change in BIC as

$$\Delta\text{BIC} = \text{BIC}_{\text{Single}} - \text{BIC}_{\text{Double}}$$

where indices indicate BIC values for a single- and double-exponential model respectively. k describes the number of fit parameters for the respective model, n the number of events and $\hat{L}$ the maximized likelihood value, which in our case is extracted from the best fit.

Next, we generated 500 synthetic datasets under each model given the fit results and data size of the experiment. We then performed the same MLE on each synthetic dataset with both a single- and double-exponential model and determined their ΔBIC .

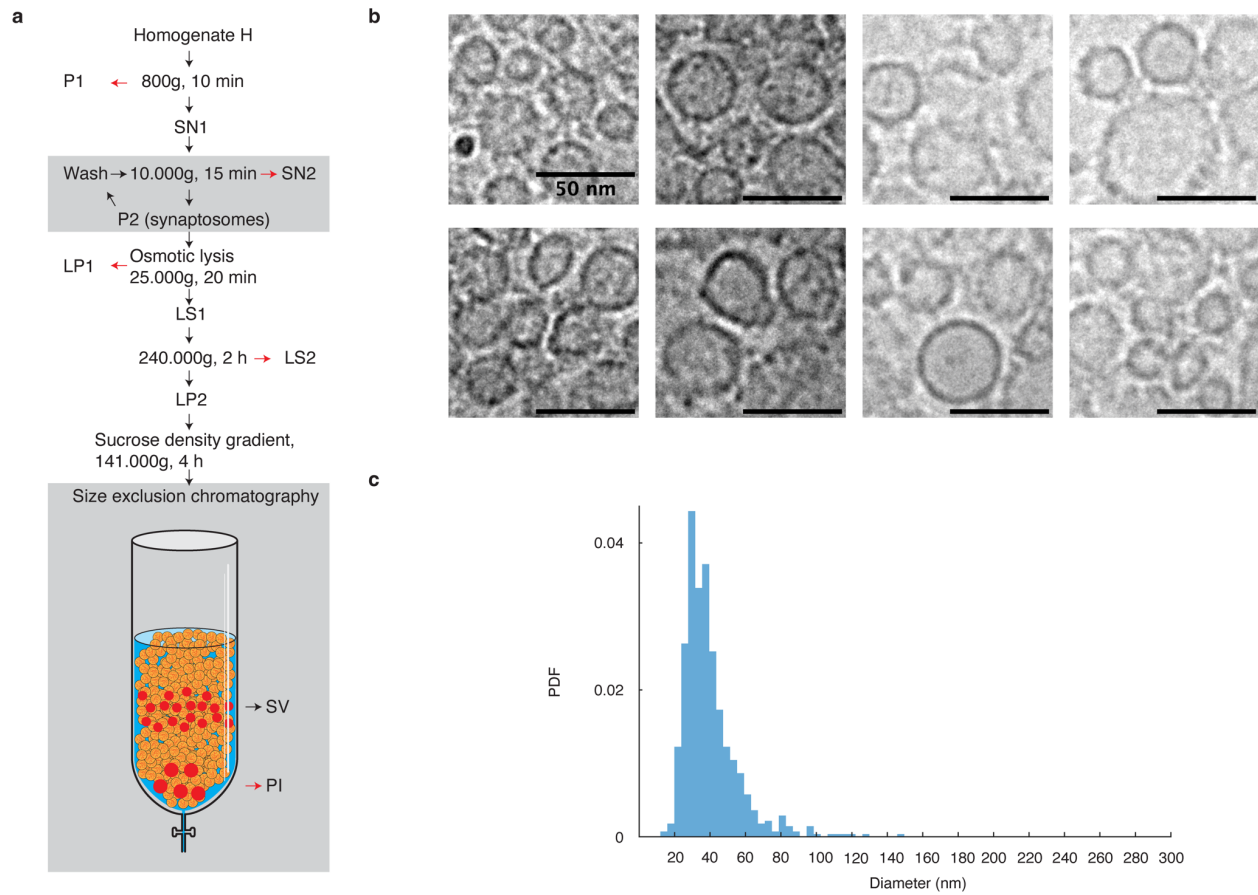
We then estimated the probability density functions (pdf) of observing a given ΔBIC value under each model, $p_{\text{Single}}(\Delta\text{BIC})$ and $p_{\text{Double}}(\Delta\text{BIC})$ respectively using Kernel Density Estimation (KDE) in Python ¹⁵.

The posterior probability of a double-exponential distribution producing the experimental ΔBIC value (termed ΔBIC^*) was then calculated using Bayes theorem via

$$P_{\text{Double}}(\Delta\text{BIC}^*) = \frac{p_{\text{Double}}(\Delta\text{BIC}^*)}{p_{\text{Single}}(\Delta\text{BIC}^*) + p_{\text{Double}}(\Delta\text{BIC}^*)}$$

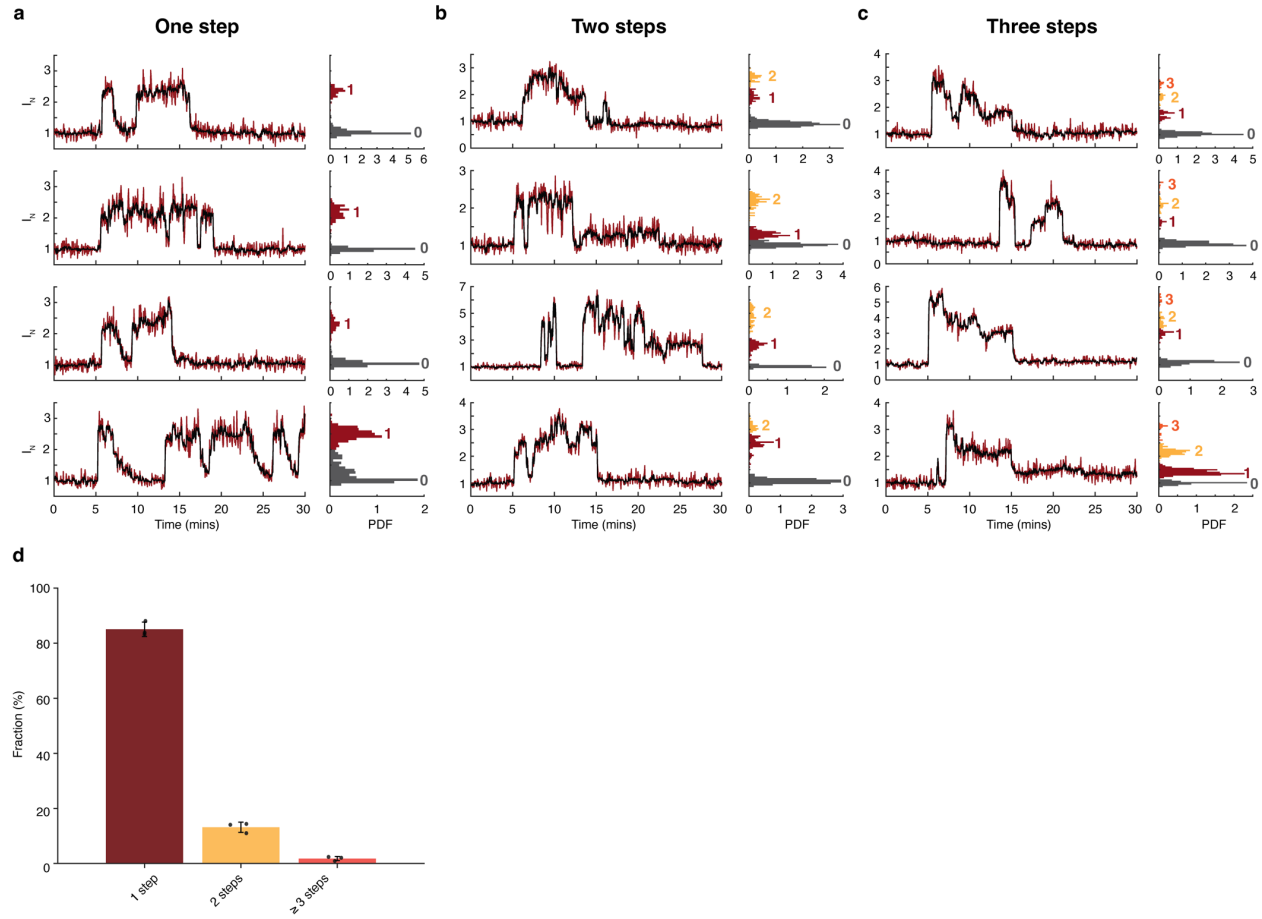
assuming equal priors since no prior knowledge is available to favour either of the two models. Results of this analysis are found in Supplementary Table 1.

Supplementary Figures



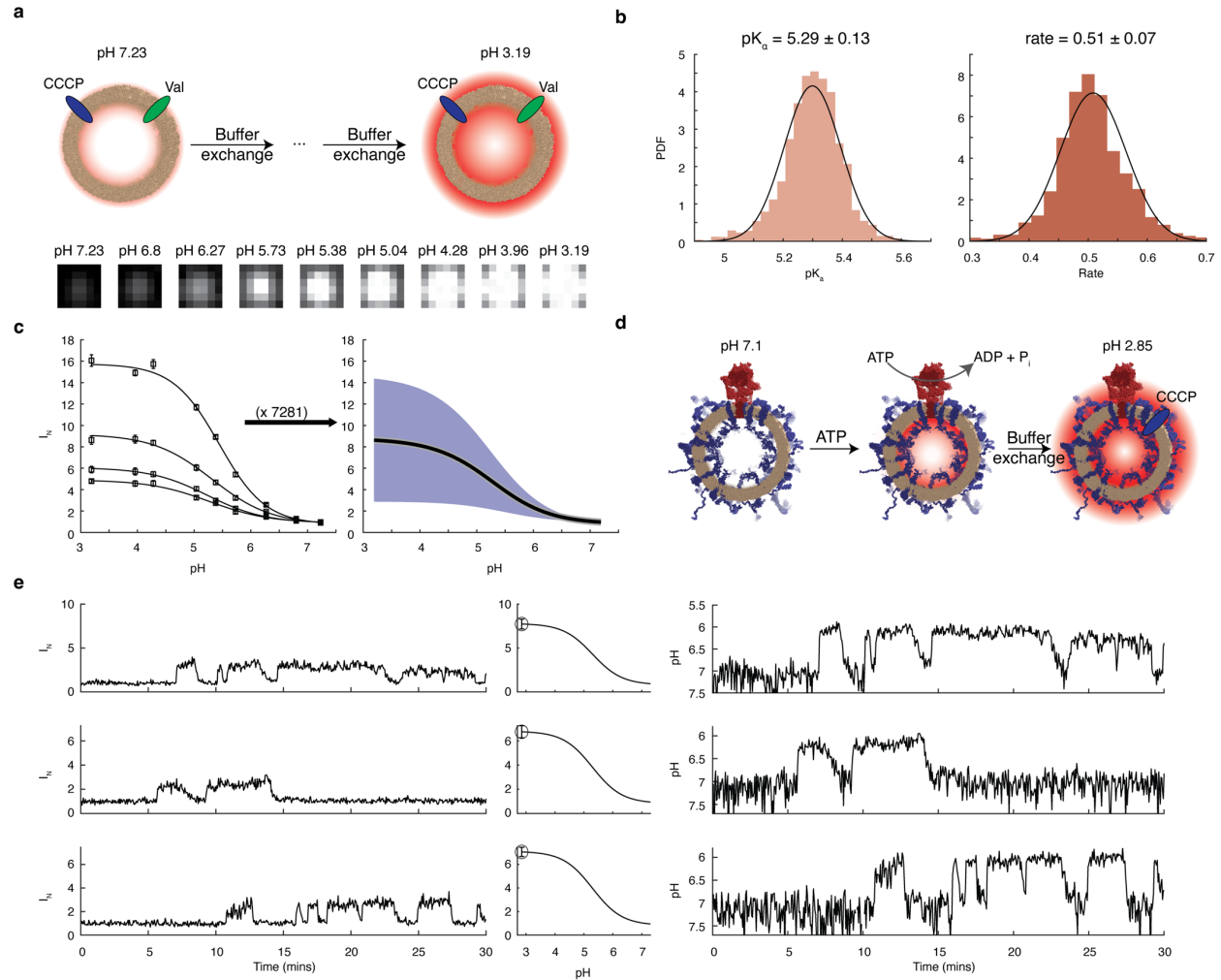
Supplementary Fig. 1. Purification and morphology of SVs.

a, Schematic illustration of the purification protocol for synaptic vesicles (SVs). **b**, Cryo-electron micrographs depicting the high purity of the SV fraction. Larger vesicles were infrequently observed in the images. The data is derived from numerous micrographs obtained during a single experimental session. The scale bar corresponds to 50 nm. **c**, Size distribution of SVs as observed in cryo-electron micrographs. The SV population displayed homogeneous morphology and a uniform distribution, with a consistent mean vesicle diameter of 38 ± 1 nm based on the S.E. of the fit.



Supplementary Fig. 2. Synaptic vesicles contain predominantly single V-ATPases.

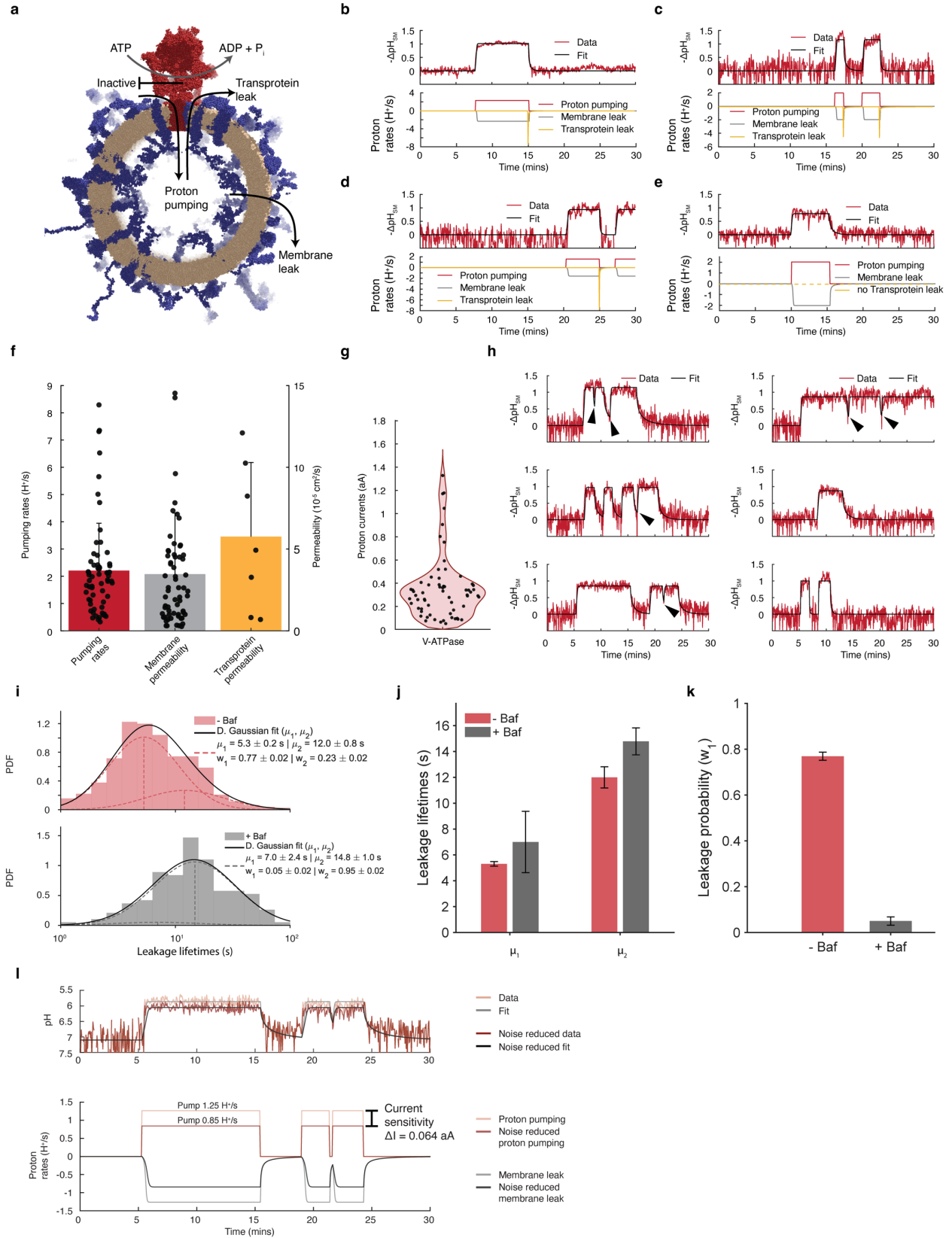
a-c, Left: Single vesicle acidification kinetics showcasing mode-switching dynamics with different acidification plateaus (steps). Each acidification plateau corresponds to a different dynamic equilibrium between pumping and leakage. Red traces correspond to normalized intensities, while black traces correspond to data treated with a Chung-Kennedy filter. Right: Histograms of accumulated intensities for single-vesicle traces. Distinct populations of steps can be identified. **a**, Representative traces showcasing a single acidification plateau. A single plateau corresponds to a single V-ATPase switching to an active mode. **b**, Representative traces that contain two plateaus correspond to two V-ATPases stochastically switching on. **c**, Similarly, traces that contain three or more plateaus correspond to multiple V-ATPases switching to an active mode in a single SV. **d**, Bar chart of the fraction of vesicles that showed single (dark red), double (yellow), or multiple plateaus (orange). Taken together, on average, the synaptic vesicle contains 1.2 ± 0.1 V-ATPases; $n = 3$. n denotes independent experimental replicates.



Supplementary Fig. 3. Single-vesicle-specific pH calibration.

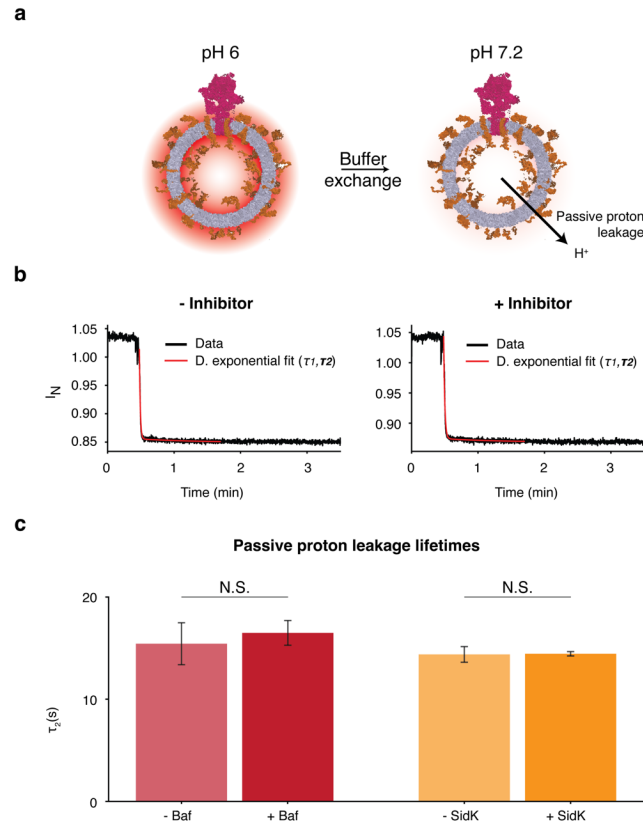
a, Illustration of the pH titration experiments to determine the calibration parameters pK_a and growth rate. Briefly, large unilamellar vesicles (LUVs) are immobilized on the glass surface and incubated in a calibration buffer containing K-Gluconate-MOPS at pH 7.23. Then buffers of the same composition with decreasing pH are sequentially exchanged in the presence of CCCP and valinomycin to maintain ionic balance and negate the membrane potential across vesicular membranes. **b**, Left: example data from single-vesicle pH titrations that are fitted with a sigmoidal function. Data points and error bars correspond to the mean and s.d. of stacks of 20 images per data point. Right: ensemble average (mean) and spread (s.d.) of the sigmoidal fits to the total population of LUVs. **c**, Distributions of the dissociation constant, pK_a , and growth rate from single LUV fits. **d**, Schematic of a typical single-molecule activity measurement. The synaptic vesicle illustration is adapted from *Cell*, Vol. 127, Takamori, S. *et al.*, Molecular Anatomy of a Trafficking Organelle, Pages 831–846, Copyright (2006), with permission from Elsevier. SVs are immobilized on glass, and subsequently, ATP is injected to commence activity. At the end of the measurement, a saturation buffer containing glycine-MOPS at a pH of ~ 3 is injected, along with CCCP and choline chloride, to neutralize the membrane potential. A stack of 20 images is then captured. This stack is used to calibrate intensities to pH as described briefly in the supplementary text and in ⁷. **e**, Example traces before (left) and after (right) calibration along with their corresponding calibration curve (mid). Each calibration curve is created using the dissociation constant and the growth

rate determined in (c) as well as the saturation and baseline intensity determined in situ from activity and saturation measurements, which are distinct for each LUV.



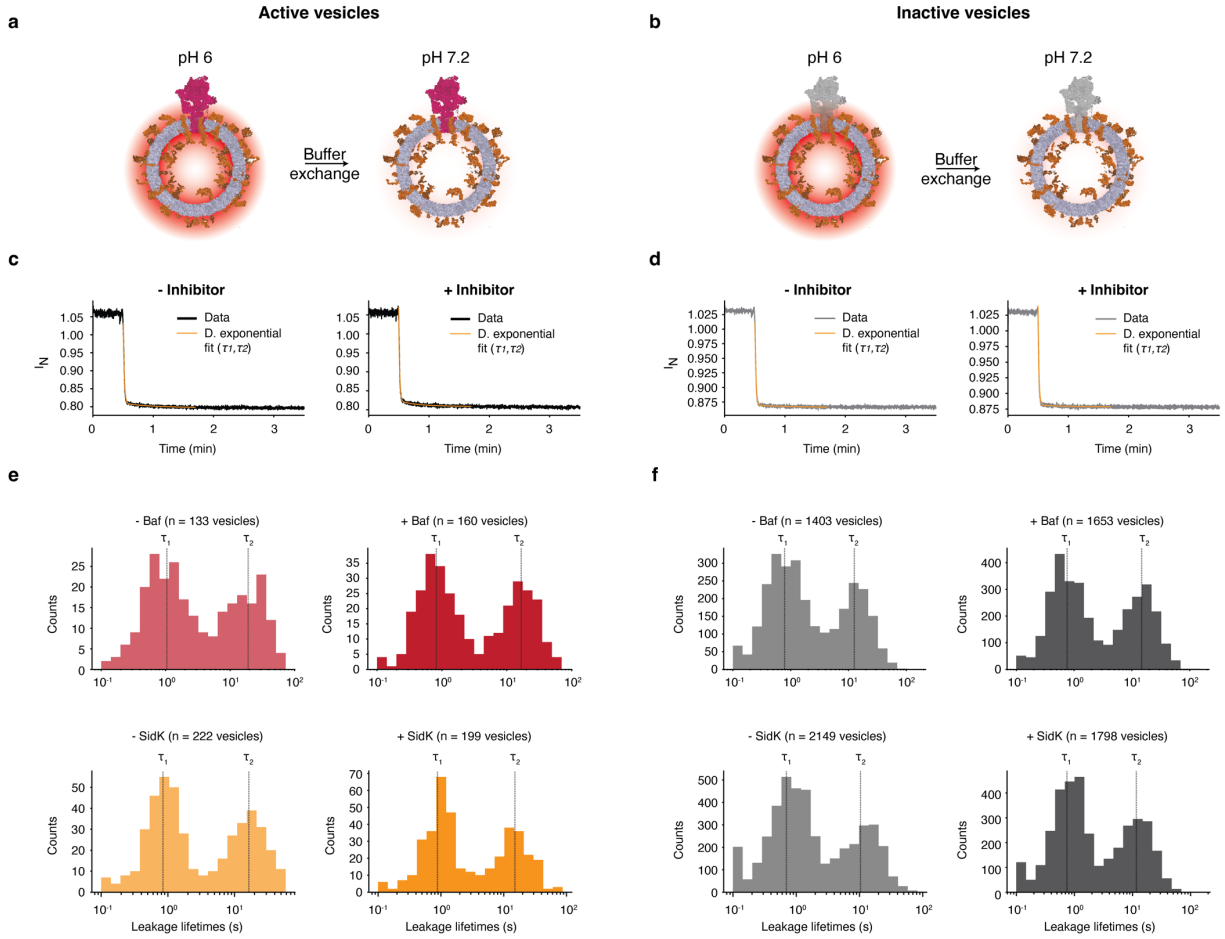
Supplementary Fig. 4. Non-equilibrium modelling reveals single-molecule proton transport kinetics and leakage pathways.

a, Schematic of model parameters. The synaptic vesicle illustration is adapted from *Cell*, Vol. 127, Takamori, S. *et al.*, Molecular Anatomy of a Trafficking Organelle, Pages 831–846, Copyright (2006), with permission from Elsevier. **b-e**, Top: Representative single-molecule traces showing stochastic mode-switching dynamics. Active proton pumping is intermittently interrupted by inactive and proton-leaky modes. During active phases, a dynamic equilibrium is established between pumping and leakage, resulting in a single acidification plateau. During an active mode, which can last up to hours, a V-ATPase may complete thousands of conformational cycles and ATP/H⁺ turnover events before randomly switching to an inactive mode. Leakage arises from both membrane diffusion and transprotein efflux. Transprotein leakage initiates immediately upon V-ATPase deactivation and displays distinct temporal kinetics. Traces were fitted using a non-equilibrium model (see Supplementary discussion), allowing for the independent estimation of pumping rates and permeabilities. Bottom: Modelled rates for proton pumping (red), membrane leak (grey), and transprotein leak (yellow). In (b-d), efflux is dominated by transprotein leakage, while in (e), by membrane diffusion. **f**, Model-derived proton transport rates. Pumping rates (red) average $2.2 \pm 1.1 \text{ H}^+ \text{ s}^{-1}$. Membrane (grey) and transprotein (yellow) permeabilities are estimated at $\sim 3 \pm 2 \times 10^{-5} \text{ cm s}^{-1}$ and $6 \pm 5 \times 10^{-5} \text{ cm s}^{-1}$, respectively. Error bars, s.d.; $n = 24$ (pumping), $n = 24$ (membrane), $n = 8$ (transprotein). n denotes independent fits on single vesicles. **g**, Violin plot of atto-Ampere proton currents generated by single V-ATPases. **h**, Additional single-molecule traces with model fits. Arrows indicate mode-switching events where equilibrium was not reached ($\Delta\text{pH}_{\text{SM}} \approx \text{max or } 0$). Transprotein leakage modes were previously reported^{7,11} but are not further analysed here. **i**, Histogram of proton leakage lifetimes in active SVs $\pm$ Baf. The fast leakage component (μ_1) corresponds to a transprotein leakage pathway through the V-ATPase, while the slow component (μ_2) corresponds to membrane leakage. **j**, Characteristic leakage lifetimes extracted from double Gaussian fits of the leakage populations in (i). **k**, Leakage probability of active V-ATPase $\pm$ Baf shows that bafilomycin dramatically reduces the probability of the transprotein leakage pathway. **l**, Quantitative estimation of the detectable changes in the steady-state currents. The steady-state plateaus are reduced by one S.D. of the noise observed during these plateaus. Subsequently both the original trace and the noise-reduced trace are fitted with the model (top) and the corresponding proton rates are generated (bottom).



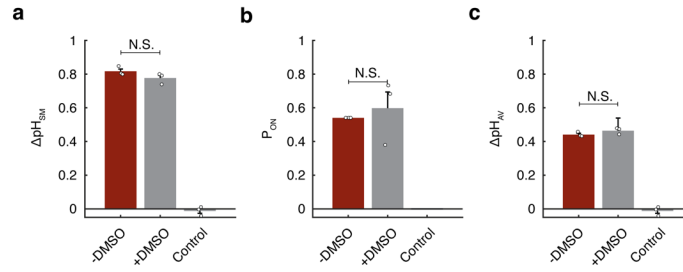
Supplementary Fig. 5. Bafilomycin and SidK do not affect the intrinsic membrane permeability to protons.

a, Illustration of pH jump experiments. The synaptic vesicle illustration is adapted from *Cell*, Vol. 127, Takamori, S. *et al.*, Molecular Anatomy of a Trafficking Organelle, Pages 831–846, Copyright (2006), with permission from Elsevier. SVs are equilibrated at pH 6 and subsequently are subjected to an external pH of 7.2 such that luminal protons passively permeate across the lipid bilayer inducing a drop in fluorescence intensity. pHrodo lipids are found in both leaflets of the bilayer. Fluorescence response of the outer monolayer has a much faster response time that depends on the flow rate of the microfluidic setup. Contrary, the fluorescence response of pHrodo lipids located in the inner monolayer solely depends on the leakage rate of luminal protons across the membrane. **b**, Ensemble average fluorescence (black and grey traces) of pH jump experiments $\pm$ inhibitor fitted with a double exponential function (red line). **c**, Characteristic lifetimes of the fluorescence response of the inner monolayer (τ_2) $\pm$ Baf or $\pm$ Sidk. Mean $\pm$ S.E.M. $n = 3$ independent experiments; Two-sided paired t-test. N.S. ($P = 0.81$, τ_2 , $\pm$ Baf); N.S. ($P = 0.96$, τ_2 , $\pm$ SidK).



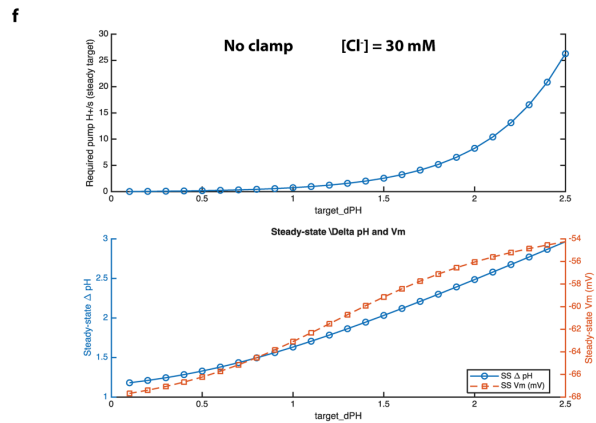
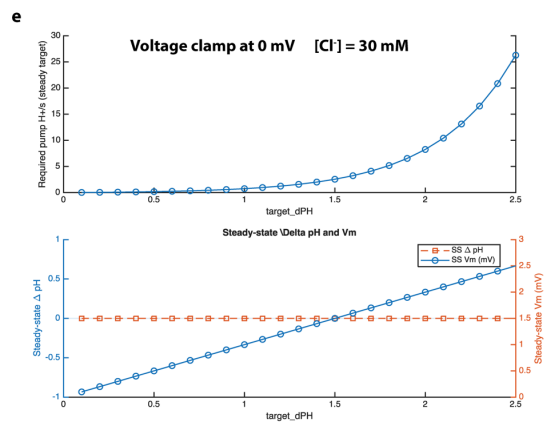
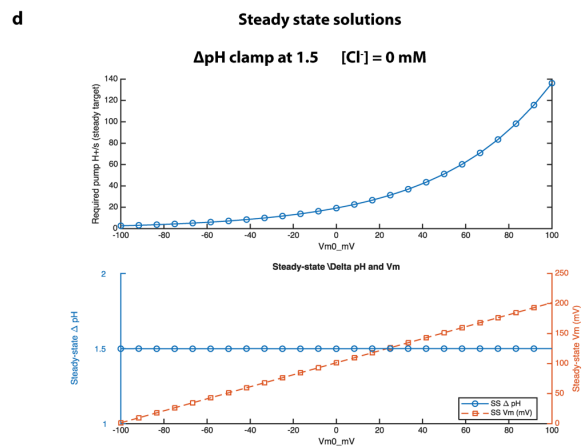
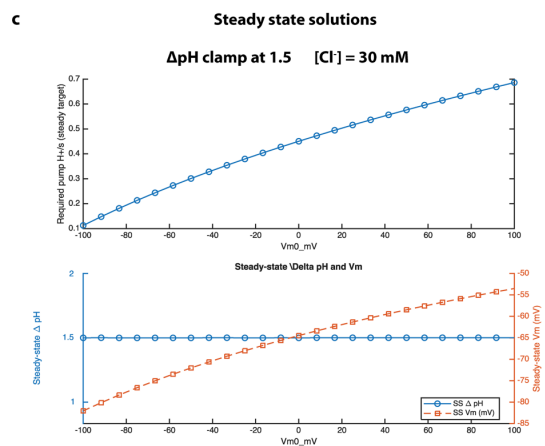
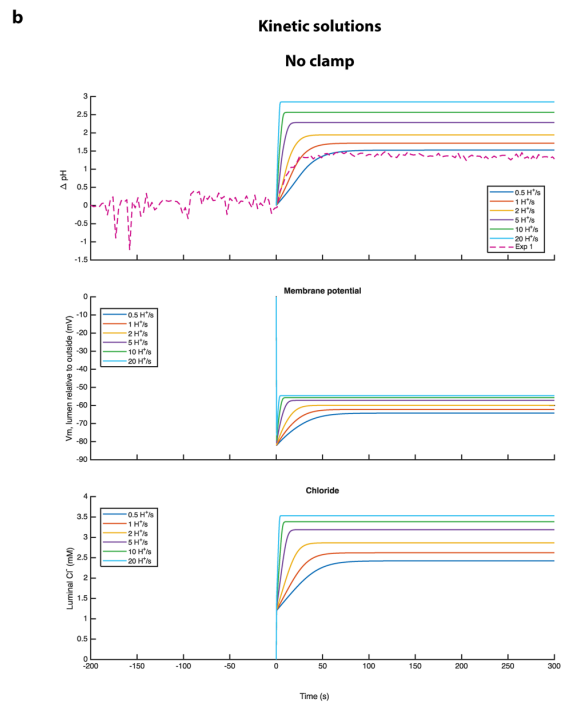
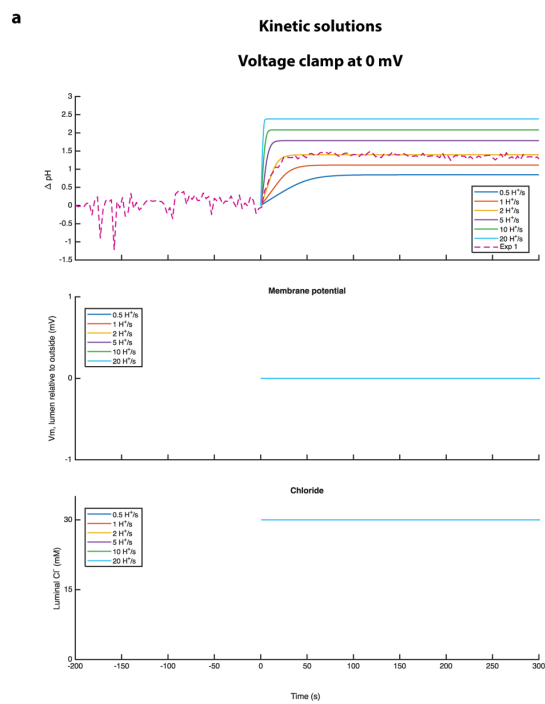
Supplementary Fig. 6. Additional single-vesicle proton leakage experiments confirm that Baf and SidK do not alter the intrinsic passive permeability of the membrane.

a, b, Illustration of pH jump experiments for vesicles that contained active (a) and inactive (b) V-ATPases. The synaptic vesicle illustration is adapted from *Cell*, Vol. 127, Takamori, S. *et al.*, Molecular Anatomy of a Trafficking Organelle, Pages 831–846, Copyright (2006), with permission from Elsevier. **c, d**, Example traces of fluorescence response (black and grey traces) in pH jump experiments $\pm$ inhibitor for SVs containing active or inactive V-ATPases fitted with a double exponential function (yellow line). **e, f**, Histograms of single-vesicle lifetimes of proton leakage. All single-vesicle pH jump traces were fit with a double exponential function that generated two distinct lifetime populations corresponding to the outer and inner monolayer. The outer monolayer had a characteristic response time, $\tau_1 = 0.8\text{--}1$ s across all conditions and is rate-limited by the microfluidic setup. The inner monolayer generated a characteristic response time, $\tau_2 = 11\text{--}19$ s across all conditions and represents the lifetimes of proton leakage across the membrane. τ_2 is at least one order of magnitude longer than τ_1 which is consistent with previous findings⁷.



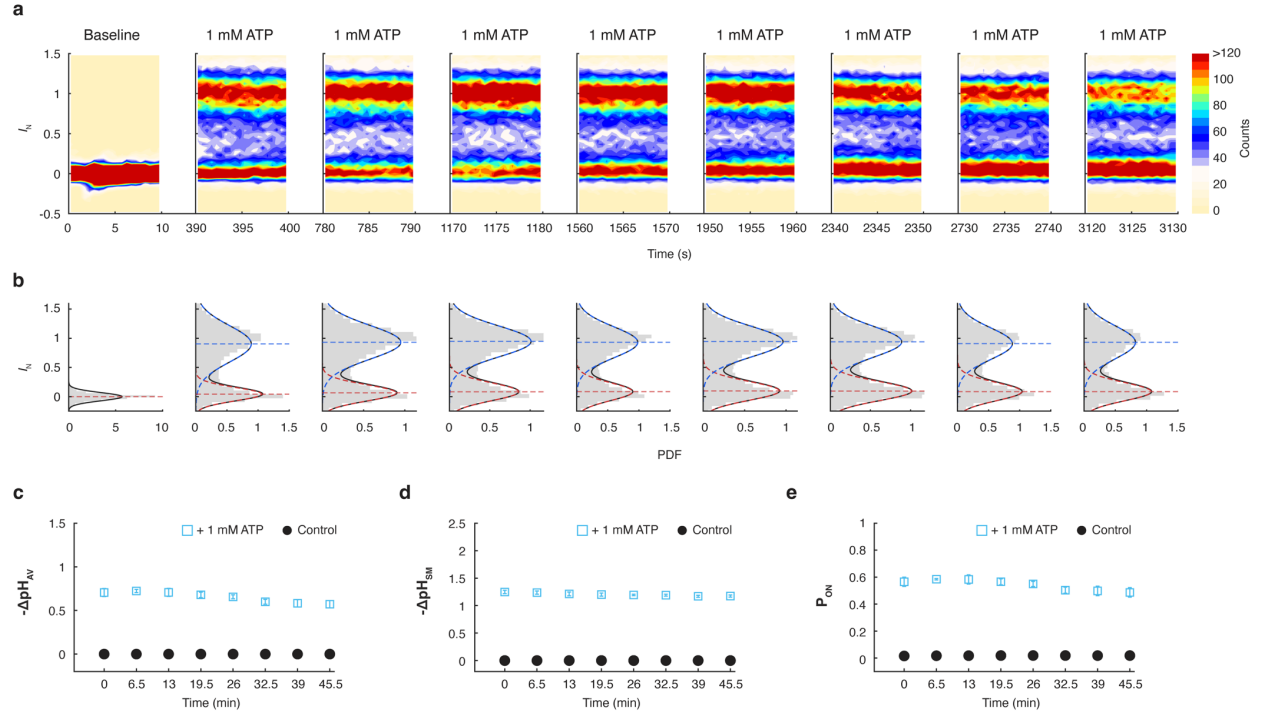
Supplementary Fig. 7. DMSO does not affect single-molecule and ensemble average pH gradients and active mode probabilities.

a, Single-molecule pH gradients under apo conditions (+ATP only) with and without DMSO show no significant difference. Error bars, s.e.m.; $n = 4$; n.s. ($P = 0.18$); two-sided Student's t-test. Control refers to SVs that remained inactive (stable at the baseline) throughout the experiment. **b**, Active mode probabilities remain unchanged in the presence of DMSO under apo conditions. Error bars, s.e.m.; $n = 4$; n.s. ($P = 0.63$); two-sided Student's t-test. **c**, Ensemble average acidification is also unaffected by DMSO. Error bars, s.e.m.; $n = 4$; n.s. ($P = 0.18$); two-sided Student's t-test. n denotes independent experimental replicates.



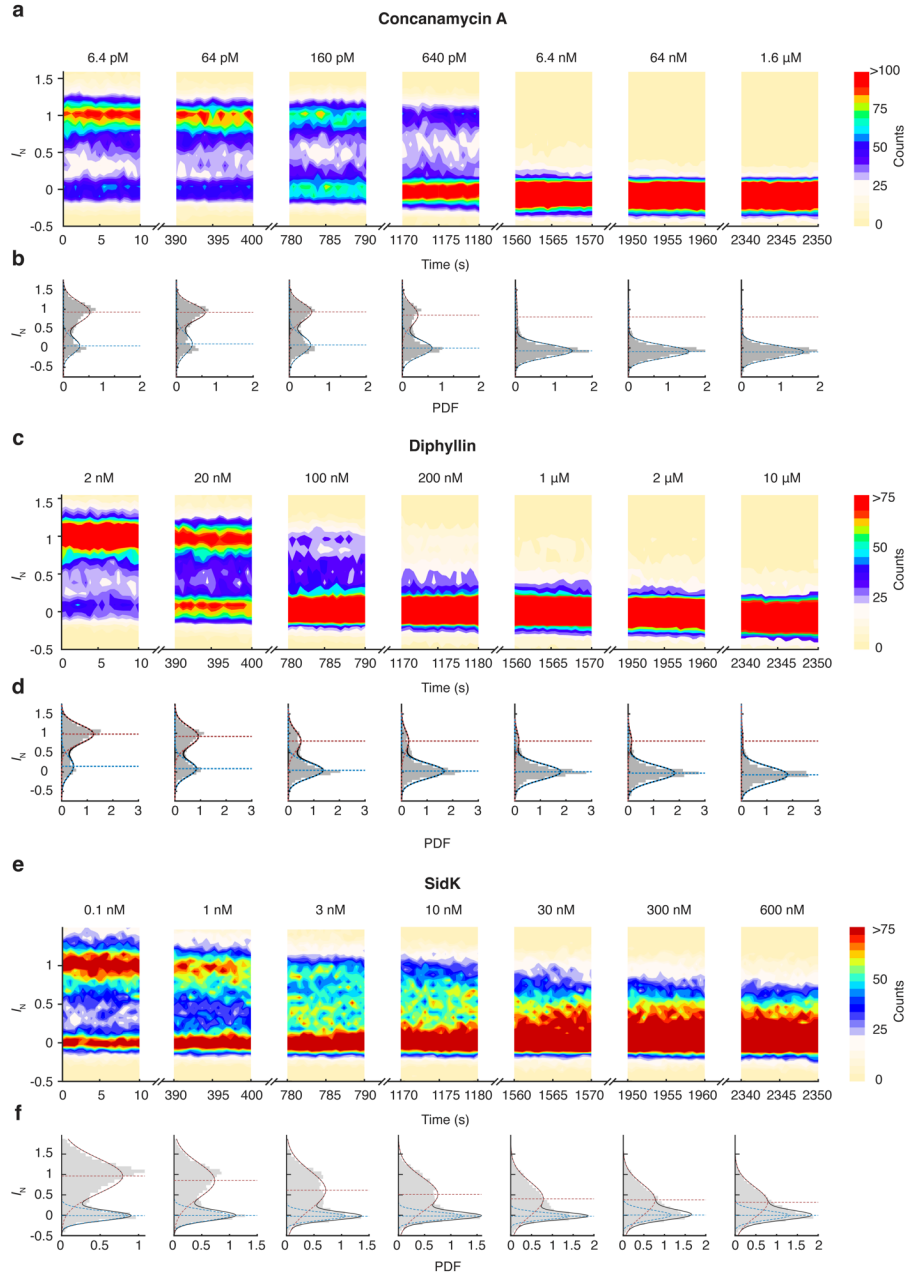
Supplementary Fig. 8. Non-equilibrium simulations show that low active-state proton pumping rates are sufficient to generate experimentally observed synaptic-vesicle pH gradients.

a–b, Kinetic simulations of single-vesicle acidification after ATP addition. The model solves the coupled time evolution of luminal pH, membrane potential, and luminal chloride concentration for a single synaptic vesicle with experimentally relevant volume, passive proton permeability, buffer capacity, membrane capacitance, and chloride permeability. Proton pumping was simulated at the indicated active-state rates, while passive proton leak, chloride flux, and voltage buildup were calculated dynamically. The magenta trace shows an example experimental single-vesicle trace aligned to ATP addition. With voltage clamped at 0 mV (**a**), corresponding to ideal charge compensation, increasing pump rate primarily increases the ΔpH plateau. Without voltage clamp (**b**), proton pumping drives membrane-potential changes and chloride influx, but low H^+ s^{-1} pumping rates still reproduce the experimentally observed acidification range. **c–d**, Required pumping rate at fixed target ΔpH . Steady-state calculations determined the proton-pumping rate required to maintain $\Delta\text{pH} = 1.5$ while varying membrane potential. With external chloride present, $\text{Cl}^-_{\text{out}} = 30$ mM (**c**), the required pump rate can remain at low levels across a broad voltage range. In contrast, without external chloride (**d**), the required rate increases steeply at positive membrane potentials, illustrating that poor charge compensation can strongly penalize proton accumulation. **e–f**, Required pumping rate as a function of target ΔpH . Under voltage clamp at 0 mV with, $\text{Cl}^-_{\text{out}} = 30$ mM (**e**), low proton-pumping rates are sufficient to maintain ΔpH values within the experimentally observed range. The required rate increases nonlinearly only at larger gradients, where passive proton leak becomes stronger. Similar behavior is observed without voltage clamp when chloride is present (**f**), indicating that chloride permeability efficiently compensates electrogenic proton influx under the simulated experimental conditions. Together, these simulations provide a system-specific quantitative justification for the low active-state pumping rates inferred from single-vesicle measurements. Because synaptic vesicles have very small luminal volumes, only a limited number of free protons is required to generate substantial acidification, and under chloride-compensated conditions the pump mainly needs to balance passive proton leakage. Thus, rates of a few H^+ s^{-1} are sufficient to establish and maintain the observed ΔpH , supporting the interpretation that the measured low rate reflects active-state proton flux rather than a time average diluted by inactive periods. Note that while the model for the simulations shown in this figure was developed in-house based on the system of differential equations described in Supplementary text, others have developed similar software for synaptic vesicle acidification¹⁶.



Supplementary Fig. 9. Single-molecule local referencing control confirms the stability of ΔpH_{SM} over periods of 45 minutes.

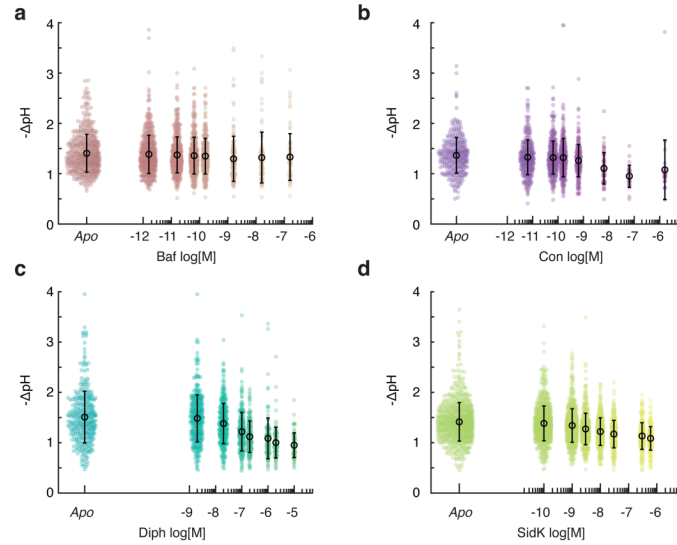
a, Contour plots of normalized fluorescence intensity over time reveal discrete transitions between inactive ($I_N \approx 0$) and active ($I_N \approx 1$) modes at a constant ATP concentration (1 mM ATP). **b**, Histograms of accumulated intensities fit to a double Gaussian distribution; mode occupancies of active and inactive modes remain constant over time. **c**, Ensemble-average acidification (ΔpH_{AV}) remains constant over time; $n = 4$, mean $\pm$ s.e.m. **d**, Single-molecule acidification (ΔpH_{SM}) remains unchanged across inhibitor concentrations; $n = 4$, mean $\pm$ s.e.m., one-way ANOVA, n.s. ($P = 0.57$). **e**, Active-mode probability (P_{ON}) remains constant over time; mean $\pm$ s.e.m. from independent experiments; $n = 4$.



Supplementary Fig. 10. Single-molecule local referencing inhibition dose-response reveals regulation of mode probabilities by Con while Diph and SidK regulate both mode-switching and proton gradients.

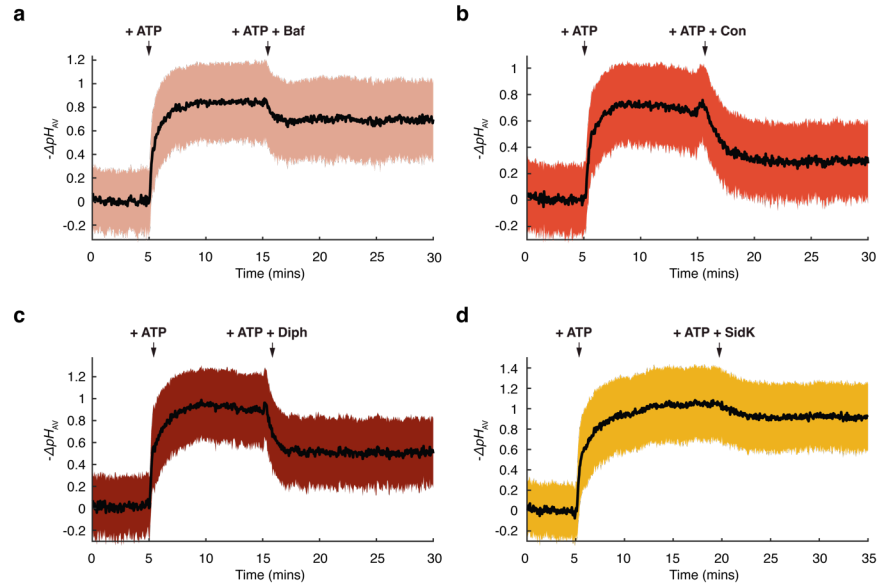
a, Contour plots of normalized fluorescence intensity over time reveal discrete transitions between inactive ($I_N \approx 0$) and active ($I_N \approx 1$) modes across Con concentrations. **b**, Histograms of accumulated intensities fit to a double Gaussian distribution, increasing Con shifts occupancy toward the inactive mode without altering active mode intensity. **c**, Contour plots of normalized fluorescence intensity over time reveal discrete transitions between inactive and active modes across Diph concentrations with a slight decrease of active mode intensities. **d**, Histograms of accumulated intensities fit to a double Gaussian distribution; increasing Diph shifts occupancy toward the inactive mode slightly altering active mode intensity. **e**, Contour plots of normalized fluorescence intensity over time and SidK concentrations. V-ATPases exhibit gradual and concentration dependent decrease in intensity (I_N) simultaneously with

a decrease of the active-mode population. **f**, Histograms of accumulated intensities fit to a double Gaussian distribution; increasing SidK shifts the population toward the inactive state and gradually alters the I_N value of the active state.



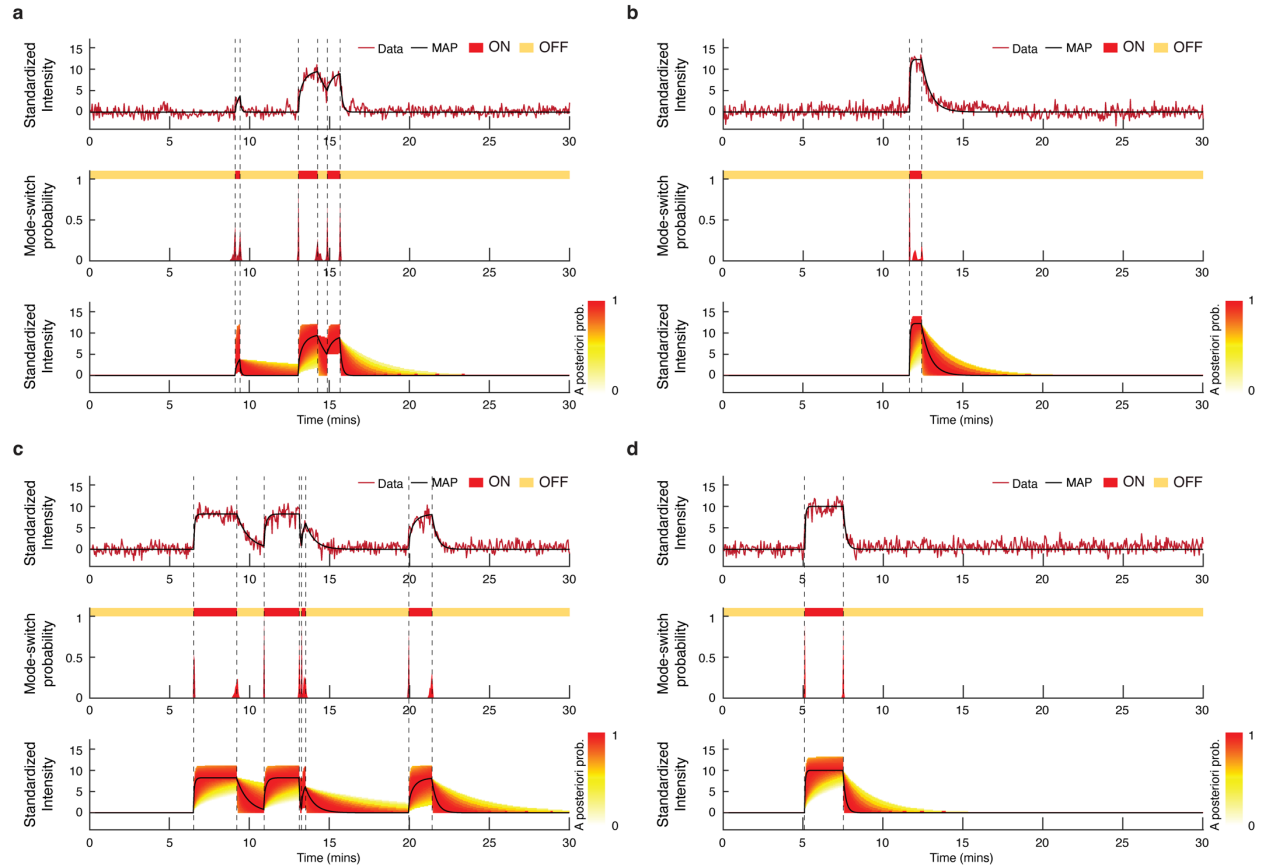
Supplementary Fig. 11. Orthosteric and allosteric inhibitors predominantly regulate the fraction of V-ATPases in an active mode and can regulate proton gradients.

a-d, Beeswarm plots of single-molecule acidification as a function of inhibitor concentration bafilomycin, concanamycin, diphyllin, and SidK. Coloured data points correspond to $\Delta p H_{SM}$ by individual SVs, while black data points and error bars correspond to the mean and one s.d. of the population of SVs. $\Delta p H_{SM}$. Remarkably, the number of active SVs is markedly reduced in the presence of increasing concentrations of inhibitors. Diphyllin and SidK additionally regulate proton gradients. Taken together, all inhibitors predominantly regulate the probability that the V-ATPase enters inactive modes. Data has not been corrected for the control.



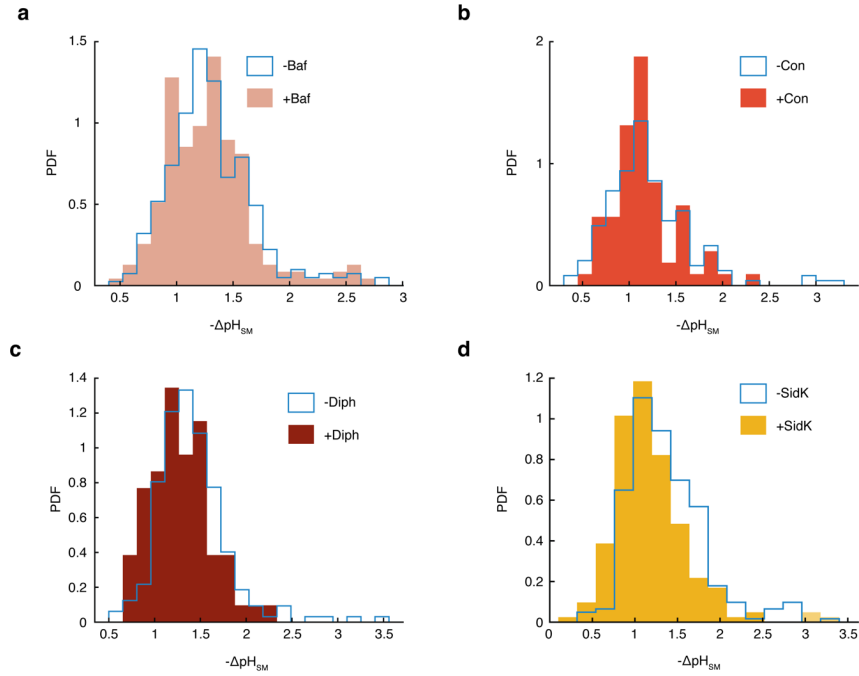
Supplementary Fig. 12. Partial reduction of ensemble average acidification by the V-ATPase at intermediate concentration of inhibitors.

a-d, Ensemble average acidification kinetics of 834, 519, 452, and 824 single, active SVs in the presence of Baf, Con, Diph, and SidK respectively. Activity is initiated upon the addition of ATP after 5 minutes of baseline recording. After 10 minutes, inhibitors are injected (after 15 minutes for SidK), and net acidification is notably reduced. Black line and coloured areas reflect the mean and one s.d. between SVs. The ensemble average trace for Baf and SidK have been corrected for bleaching as necessary.



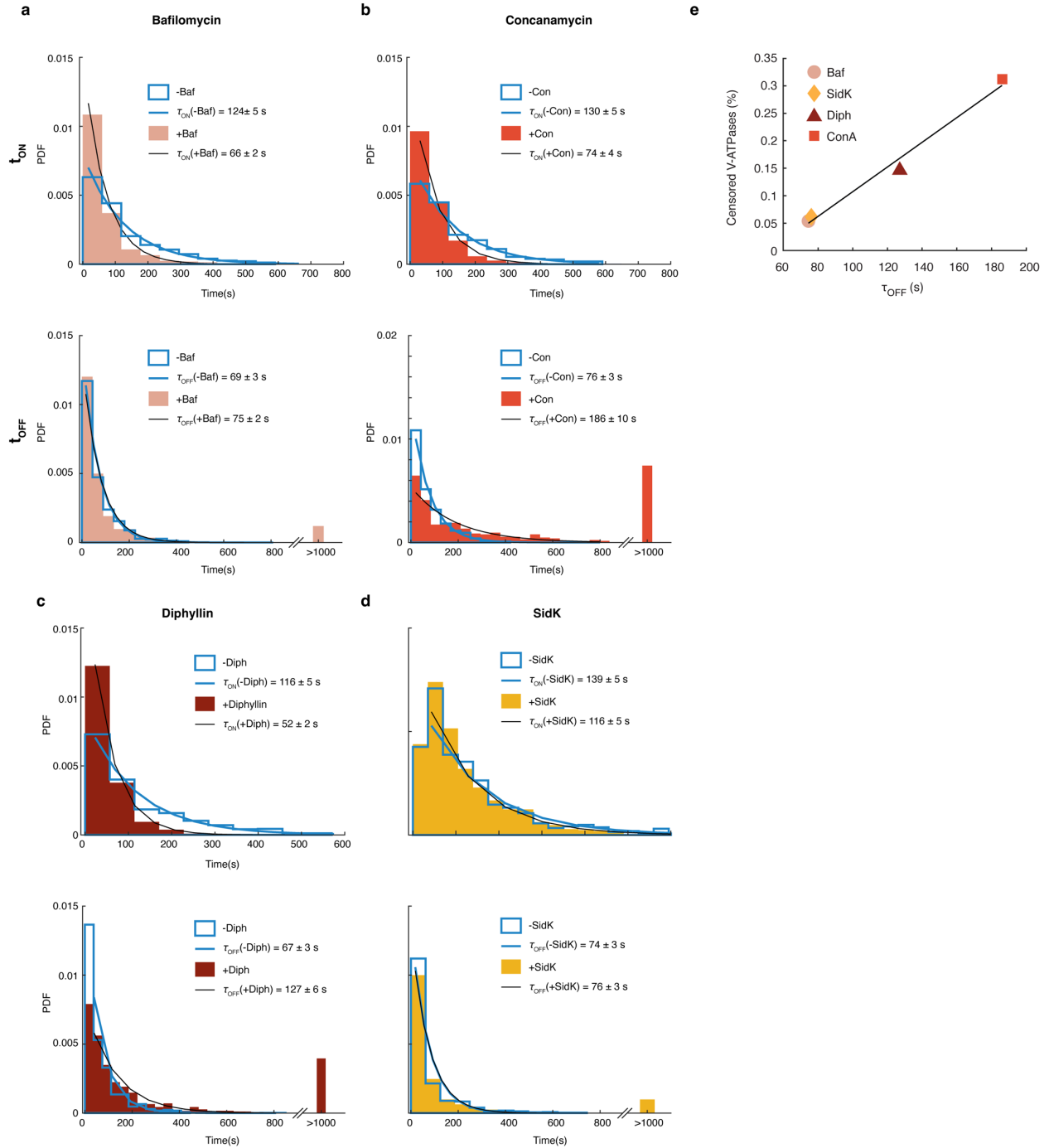
Supplementary Fig. 13. MAP-based detection of mode-switching events using a stochastic exponential model.

a-d, Single-molecule fluorescence traces were analysed using Bayesian stochastic filtering based on a piecewise exponential model of V-ATPase activity. The signal reflects alternating proton-pumping and inactive states, modelled by exponential increases or decays in fluorescence intensity. Change-points (mode-switching events) were identified from the posterior distribution of the signal, with events marked at time points reaching $\geq 99\%$ confidence (dashed lines). MAP (maximum a posteriori) estimates were derived by optimizing over the joint posterior distribution of model parameters and change-point times (see Supplementary text and reference ^{7,14}). Red curves in the probability panels indicate the marginal posterior distribution for switching events. Heatmaps show the posterior probability of signal location; MAP estimates are overlaid in black.



Supplementary Fig. 14. Inhibitors do not affect the levels of acidification established by single-V-ATPases.

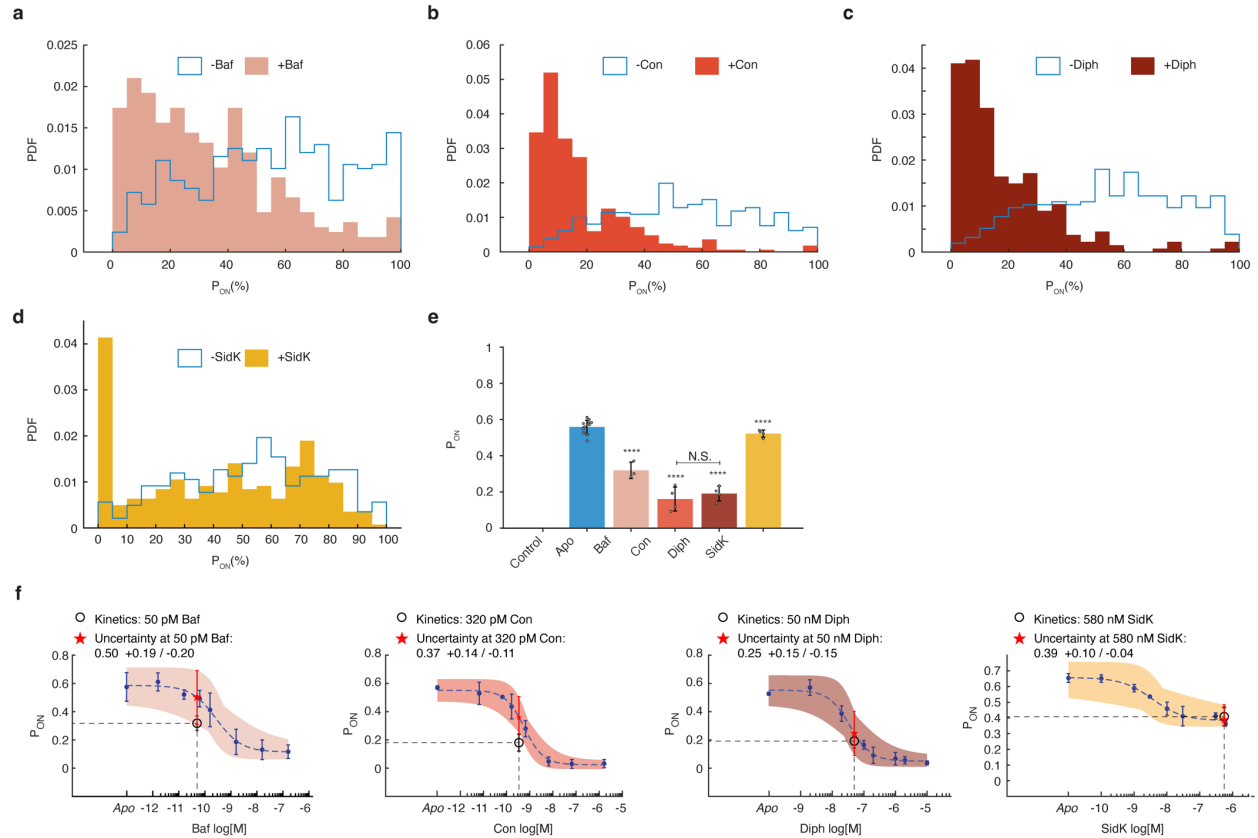
a-d, Distributions of single-molecule acidification levels in SVs by the V-ATPase in the presence and absence of inhibitors estimated from acidification plateaus of single-molecule kinetic measurements. Concentrations of inhibitors used were: 50 pM Baf; 320 pM Con; 50 nM Diph. n.s. ($P = 0.36$, $\pm$ Baf); n.s. ($P = 0.63$, $\pm$ Con); n.s. ($P = 0.71$, $\pm$ Diph); **** $P = 8 \cdot 10^{-5}$ ($\pm$ SidK); two-sided two-sample Students t-test.



Supplementary Fig. 15. Bafilomycin and SidK regulate active mode dwell times but leave the inactive mode unaffected; Concanamycin and Diphyllin both simultaneously downregulate active mode dwell times while upregulating inactive mode dwell times.

a-d, Active and inactive mode dwell times in the presence and absence of inhibitors. Colored histograms correspond to dwell time populations in the presence of inhibitory molecules, while outlined histograms correspond to dwell times in the presence of only ATP. Characteristic dwell times (τ_{ON} and τ_{OFF}) were extracted by fitting histograms with single exponentials. Errors correspond to the standard error of the fit. The concentrations of the inhibitors were 50 pM (Baf),

320 pM (Con), 50 nM (Diph) and 580 nM (SidK). **e**, Fraction of truncated/censored vesicles as a function of inactive-mode lifetimes for each inhibitor. There is a linear correlation of inactive-mode lifetimes and the fraction of SVs that contain V-ATPases that remained inactive for the duration of the experiment. In other words, the larger the effect of inhibitors was on the inactive-mode, the larger the fraction of V-ATPases that were not captured in an active-mode for the duration of the experiment.



Supplementary Fig. 16. Kinetic experiments demonstrate that all inhibitors regulate the single-molecule active mode probability.

a-d, Distributions of single-molecule active mode probability in the presence and absence of inhibitors. All inhibitors reduced the probability that the V-ATPase is found in an active mode. Concentrations of the inhibitors used were: 50 pM Baf, 320 pM Con, 50 nM Diph, and 580 nM SidK. **** $P < 10^{-10}$ ($\pm$ Baf); **** $P < 10^{-10}$ ($\pm$ Con); **** $P < 10^{-10}$ ($\pm$ Diph); **** $P = 2 \cdot 10^{-5}$ ($\pm$ SidK); two-sided two-sample Kolmogorov-Smirnov tests. **e**, Bar charts summarizing the regulation of active mode probability (P_{ON}) by the inhibitors used in single-molecule kinetic experiments. All inhibitors investigated significantly reduced the probability of the V-ATPase being found in an active mode. mean $\pm$ s.d. from independent experiments; n.s. ($P = 0.20$, Con-Diph). **f**, Comparison of kinetic and single-molecule local referencing experiments reveals similar estimates of P_{ON} within error estimates. Titration curves of P_{ON} were supplemented by uncertainty envelopes based on the sigmoidal fit parameter uncertainties that minimize or maximize the curve. Red data corresponds to P_{ON} of the curve at the concentration used in kinetic experiments. Black data corresponds to P_{ON} measured in kinetic experiments as seen in (e).

	<i>Apo</i>	Baf	ConA	Diph	SidK
P_{Double}^{ON}	0 (n.s.)	>0.99 (**)	0 [‡] (n.s.)	0 [‡] (n.s.)	0 [‡] (n.s.)
P_{Double}^{OFF}	0.69 (n.s.)	>0.99 (**)	0.99 (**)	0.98 (*)	0.92 (n.s.)

Supplementary Table 1. Bayesian analysis reveals that bafilomycin introduces a second exponential lifetime for the active mode while concanamycin, diphyllylin and SidK modulate the single exponential lifetime of the *apo* condition. Baf, Con, and Diph stabilized a secondary inactive mode, while SidK statistically confirmed modulation of a single inactive mode.

As described in supplementary text, modelling of the difference of the Bayesian Information Criterion (ΔBIC) distributions for single and double exponential fits of lifetime distributions determined the probability that the lifetimes are expressed by a double exponential for the active, P_{Double}^{ON} , and the inactive, P_{Double}^{OFF} , mode. Active mode: the *apo* condition is best represented by a single exponential distribution as the double exponential Maximum Likelihood Estimation (MLE) based fit generated two monoexponentials with identical lifetimes. Bafilomycin, unambiguously introduced a second population of ON lifetimes. Concanamycin, diphyllylin and SidK are best represented by a single exponential. Inactive mode: we cannot confirm whether the *apo* condition is best represented by a single or a double exponential as P_{Double}^{OFF} is not significant. However, three inhibitors unambiguously stabilized a secondary exponential with high statistical significance while for SidK we could not confirm a double exponential. The significance was produced via parametric-bootstrap Bayesian model comparison. [‡]We fitted ON lifetimes of concanamycin, diphyllylin and SidK with a double exponential model that generated exponential terms with overwhelming relative weight of one term over the other (98% for Con, >99% for Diph) or two identical double exponentials for SidK. Therefore, these datasets are represented by single exponentials.

	$\Delta G^{\ddagger}_{ON}(\text{kJ} \cdot \text{mol}^{-1})$	$\Delta G^{\ddagger}_{OFF}(\text{kJ} \cdot \text{mol}^{-1})$	$\Delta G(\text{kJ} \cdot \text{mol}^{-1})$
<i>Apo</i>	85.0 ± 0.05	84.2 ± 0.1	-0.4 ± 0.1
Bafilomycin	83.4 ± 0.1	83.7 ± 0.1	1.9 ± 0.6
Concanamycin	83.6 ± 0.1	85.9 ± 0.1	4.1 ± 1.2
Diphyllin	82.7 ± 0.1	85.0 ± 0.1	3.6 ± 0.7
SidK	84.8 ± 0.1	83.7 ± 0.1	0.9 ± 0.9

Supplementary Table 2. Calculations of forward, backward transition activation barriers and free energy differences between active and inactive modes in the absence (*apo*) and presence of inhibitors.

We employed transition state theory to derive the forward ($\Delta G^{\ddagger}_{ON}$) and backward ($\Delta G^{\ddagger}_{OFF}$) activation barriers, as well as the free energy difference (ΔG). For more details, we refer the reader to the corresponding section in the Supplementary text. The concentrations of the inhibitors were 50 pM (Baf), 320 pM (Con), 50 nM (Diph) and 580 nM (SidK).

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Give P values as exact values whenever suitable.
- ☐ ☒ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- ☒ ☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- ☒ ☐ Estimates of effect sizes (e.g. Cohen's d , Pearson's r), indicating how they were calculated

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Software and code

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Data collection Microscopy data was collected using the commercially available software:
- cellSens Dimension (version 3.2, Olympus)

Data analysis All data analysis and modelling was performed in custom software written in MATLAB 2023b and newer versions up to 2025b. Bayesian filtering algorithm was written in C++11. The Bayesian filtering algorithm was previously published in Kosmidis et al., Nature (2022) and the source code is publicly available in https://github.com/pete906/Bayesian_Filtering_Proton_Pump.git.

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The data that support the findings of this study are available from the corresponding author upon request. Source data are provided with this paper. The data

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Reporting on sex and gender	Not applicable
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Population characteristics	Not applicable
Recruitment	Not applicable
Ethics oversight	Not applicable

Note that full information on the approval of the study protocol must also be provided in the manuscript.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	All sample sizes used in the present manuscript are $n \geq 3$. n is defined as the number of independent experimental replicates. More specifically each independent experiment included a freshly thawed aliquot and a new microfluidic flow chamber was used. The sample for each independent experimental run contained a total of approximately 2000 synaptic vesicles in each FOV per repeat as stated in the manuscript. Fluorescent synaptic vesicles were detected via a particle tracking algorithm and selected via various thresholding techniques as described in the supplementary material and in Kosmidis et al. (2022). Due to each experimental run containing ~2000 we could extract large number for reliable statistical analysis.
Data exclusions	Single vesicle data were identified via single particle detection algorithms. SNR thresholding was used to separate active vesicles. Step-counting on single-vesicle traces was used to determine the number of molecules per vesicle. Single molecule data used for dwell time analysis was used only if stochastic behavior was observed. Details on data exclusion can be found in the manuscript.
Replication	Reproducibility was reliably verified by performing experiments on separate days on frozen and aliquoted samples and freshly made buffers. All data included has been replicated. Data was replicated by up to 5 independent experiments. Only exception is the data in the cryo-electron micrographs in Fig. S1 ($n = 1$, multiple micrographs).
Randomization	Randomization is not applicable. Single-molecule data is intrinsically randomized as their properties are unknown prior to investigation.
Blinding	Blinding is not applicable. No a priori knowledge was and could be assumed prior to the investigation of the switching dynamics of the V-ATPase. All data analyzed via sets of strict rules and/or automated processes as discussed in the manuscript.

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Materials & experimental systems

Methods

n/a	Involved in the study
☒	☐ Antibodies
☒	☐ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Animals and other research organisms

Policy information about [studies involving animals](#); [ARRIVE guidelines](#) recommended for reporting animal research, and [Sex and Gender in Research](#)

Laboratory animals	Adult Wistar rats were purchased from Charles River Laboratories, or Janvier, and were kept until 5 to 6 weeks of age at a 12:12 hr light/dark cycle with food and water ad libitum.
Wild animals	The study did not involve wild animals.
Reporting on sex	Not applicable. The molecular function of the V-ATPase in synaptic vesicles, which was analysed in this study, is not affected by sex.
Field-collected samples	The study did not contain samples collected from the field.
Ethics oversight	Certificate of approval for using animals was issued by the Landkreis Goettingen Office of Veterinary Affairs and Consumer Protection. Extraction of brain material from animals does not require an ethics committee oversight according to the law issued by the state of Lower Saxony. The state itself in this case should act as the ethics committee. Adult Wistar rats were procured from Charles River Laboratories or Janvier and were housed until they reached 5 to 6 weeks of age under a 12:12-hour light/dark cycle, with access to food and water ad libitum. TierSchG (Tierschutzgesetz der Bundesrepublik Deutschland, Animal Welfare Law of the Federal Republic of Germany) as documented by 33.23-42508-066-§11, dated Nov 16th, 2023 ("Erlaubnis zum Halten von Wirbeltieren zur Versuchszwecken", "Permission to keep vertebrates for experimental purposes") by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (Lower Saxony State Office for Consumer Protection and Food Safety).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Plants

Seed stocks	No plants were used
Novel plant genotypes	No plants were used
Authentication	No plants were used

Orthosteric and allosteric inhibition of the brain V-ATPase outside the alternating-access cycle

Corresponding Author: Professor Dimitrios Stamou

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Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This manuscript builds on the authors' previous observations that the V-ATPase can enter an ultra-long-lived inactive mode and proposes a novel pharmacological concept: targeting this inactive mode as a mechanism of inhibition. The idea of "two orthogonal axes" of pharmacological control is mechanistically interesting and potentially impactful. The study elegantly characterizes four known V-ATPase inhibitors – bafilomycin, concanamycin A, diphyllin, and SidK. In brief, bafilomycin and concanamycin bind the V0 subunit and block proton translocation, SidK binds the V1 subunit and blocks ATP hydrolysis, while the mechanism of diphyllin remains unknown. Despite previously resolved high-resolution structures supporting steric inhibition of proton translocation, the manuscript argues that bafilomycin does not act via this classical mechanism but instead switches active molecules into an ultra-long inactive mode. There is no doubt that this work will stimulate new discussions in transporter pharmacology and may open avenues for more precise drug design.

Major

1. Definition of "single-molecule proton transport rate."

Throughout the manuscript, changes in ΔpHSM appear to be the primary indicator of the single-molecule proton transport rate. However, ΔpHSM is not the pumping rate itself; it is a steady-state gradient shaped by proton leak, buffering capacity, calibration, and possibly counter-ion conductance. Although the authors mention deconvolving pumping and leak via a physical model, much of the language reads as if the transport rate is measured directly. The main evidence for "unchanged pumping" is largely based on ΔpHSM , which does not uniquely report on catalytic turnover.

Relatedly, the claim that inhibitors cause "no slowing of the alternating-access cycle" is a strong mechanistic leap. Even if the fitted parameter is unchanged during ON events, this does not strictly prove that the canonical transport cycle is unaffected. The assay reports net H^+ flux, which could be buffered by other steps. When referring to the "cycle," the authors should explicitly acknowledge that they measure proton flux, not mechanical rotation or conformational transitions directly.

2. Interpretation of mode switching versus classical inhibition.

The work elegantly demonstrates dose-dependent responses of single V-ATPases to inhibitors. However, it is unclear how molecules that were already inactive prior to inhibitor application were treated. In Fig. 2 and Fig. S5, a substantial fraction of vesicles appear OFF even in the presence of ATP and the lowest concentration of inhibitors or the absence of inhibitors. Can these molecules re-enter the active state? How are they distinguished from defective or damaged transporters? Are the initial ON/OFF fractions consistent across vesicle preparations?

Moreover, it is difficult to reconcile the observation that neither bafilomycin nor concanamycin affects single-molecule acidification (Fig. 3C) with their known steric interference with proton translocation. One possible explanation is that molecules that bind inhibitors immediately enter the inactive mode, while those that remain active are simply unbound. In that case, however, "being inhibited" and "entering the ultra-long inactive mode" become operationally indistinguishable. This conceptual issue deserves more explicit discussion.

For orthosteric blockers such as bafilomycin and concanamycin, a plausible alternative model is rapid binding and unbinding that produces intermittent long pauses, although hard to imagine for inhibitors binding at pmol affinities. This is still inhibition, but could be interpreted as acting on-cycle and generating OFF-like periods rather than promoting a distinct off-cycle state. It is therefore unclear whether "direct blockage" is considered on-cycle or off-cycle, and the conceptual framework would benefit from clarification.

More generally, it remains unclear how, in a raw single-molecule trace, the authors distinguish between (i) a molecule that is bound to an inhibitor and directly inhibited and (ii) a molecule that has been switched by the inhibitor into the ultra-long-lived

inactive mode. These two scenarios appear experimentally equivalent in the current readout.

I found Fig S12 could be possibly helpful for this matter. If I understand correctly, t_{off} should be controlled by two factors: mode switching and inhibition. Given that the inhibitors bind with pM affinity, t_{off} for inhibition should be quite long, likely longer than the inactive mode t , which was about 1-2 min as reported in Fig 4. As the concentration of inhibitors increases, the population controlled by inhibition (transporters bound to inhibitors) should increase if the timescales of the two components are distinguishable.

3. Assumptions underlying estimation of active-mode probability.

The probability of being in the active mode appears to be inferred entirely from ensemble acidification and single-molecule ΔpHSM , assuming that only pumping capacity and mode occupancy determine the ensemble signal. This seems speculative. Could other factors contribute to maintaining a constant ΔpHSM , like changes in membrane permeability, counter-ion conductance, or pHrodo response properties? It would strengthen the manuscript to explicitly address whether these components play a role and to provide specific controls. Likewise, are there intermediate or partially active states that might be forced into a binary ON/OFF classification by the analysis? Finally, was $P(\text{ON})$ in Fig 3D derived from values from Fig 3B and C, or independently measured from the $I(\text{N})$ shifts in Fig S6?

4. Heterogeneity of inactive states.

The OFF dwell times appear highly heterogeneous, ranging from seconds to many minutes. Especially, some are almost always inactive (Fig. S12). Do the authors believe that all these OFF periods correspond to the same inactive state and the same molecular mechanism, or that multiple distinct inactive states exist? This distinction is important for interpreting the concept of a single “ultra-long inactive mode” as a pharmacological target.

Minor

Pages 12–13 and 14–15 of the Methods section appear to be duplicated. The Methods may be incomplete or incorrectly compiled.

When first claiming that most vesicles contain a single V-ATPase, the text should explicitly refer to Fig. S8, where the step analysis supporting this conclusion is presented.

The scales in panels of Figure 3 are inconsistent, making it difficult to compare data across inhibitors.

The inhibition fits should report IC_{50} and Hill coefficients.

Y-axis labeling in S6F seems off. The decrease in the maximum of the upper $I(\text{N})$ peak appears to be much greater than ~25%, as shown in Figure 3 and stated in the text.

It is also unclear how SidK has almost no effect on either ON to OFF or OFF to ON lifetimes (Fig 4F,G), and yet decreases the fraction of ON mode by ~50% (Fig S6).

There is an inconsistency between the results in Figures 3 and 4. For example, for Baf, the IC_{50} is 200 pM, based on Fig. 3. However, in Fig. 4, 50 pM decreases the ON time by 50%, which would define this concentration as the IC_{50} . Similarly, the IC_{50} for Con is ~600 pM (Fig. 3), while 320 pM results in a nearly 2-fold decrease in ON time and an over 2-fold increase in OFF time, yielding at least 75% inhibition. Results seem more consistent for Diph is 32 nM.

What is the scenario behind the different doses chosen for each inhibitor in Figure 4? It seems odd to use everything differently for comparison. Is it possible to use IC_{50} for all?

How is n determined in these experiments? Are they the same preparation but different molecules (movies), or different vesicle preparations? This should be stated in the figure legends or the methods section.

Reviewer #2

(Remarks to the Author)

Overall, the authors present a well-designed single-vesicle assay to dissect how various inhibitors of the V-ATPase act at the single-molecule level, specifically by prolonging the OFF-mode dwell time. The manuscript provides important insights that cannot be obtained from bulk phase measurements. However, several methodological and conceptual issues need to be addressed.

General Assessment

The central contribution of this manuscript is the demonstration that the inhibitory effects of multiple V-ATPase inhibitors are mediated primarily by increasing the dwell time spent in the OFF mode, rather than decreasing the catalytic turnover rate of individual enzyme molecules. The authors successfully established a single-synaptic-vesicle assay by loading purified SVs with pHrodo, enabling fluorescence-based monitoring of the proton-pumping activity of individual native V-ATPases. The study convincingly shows that the OFF-mode occupancy increases in a dose-dependent manner upon addition of various inhibitors, and similar behavior is observed across different inhibitor classes. This finding fundamentally challenges traditional interpretations derived from bulk kinetic measurements, in which inhibition is typically attributed to reduced turnover of continuously cycling enzymes. Thus, the manuscript provides a new conceptual framework for understanding inhibitor action on active transporters.

It should also be noted that the same group previously published a single-molecule proton-pumping assay for V-ATPase in *Nature* (2022), and the present work is effectively an extension of that platform toward pharmacological applications.

While the concept of mode-dependent regulation is novel in the transporter field, the underlying idea—that the ensemble activity of an enzyme population is determined by the fraction of molecules in an OFF state—has been previously demonstrated in the single-molecule rotation experiments of Kinoshita and colleagues on $\text{F}_1\text{-ATPase}$ (*Science* 2001, et al). For example, the ADP-inhibited state represents an OFF mode, and the ensemble ATPase activity is reduced simply because a subpopulation is trapped in this inactive state, even though the catalytic turnover of active molecules remains unchanged (Hirono-Hara, *PNAS* 2001). Likewise, in the case of V-ATPase, the same group has already reported

single-molecule evidence of mode switching. Therefore, although the experimental system is sophisticated and the data interpretation is valid, the conceptual novelty may be limited.

Nevertheless, the present manuscript provides a significant contribution by applying single-vesicle measurements to multiple inhibitors and demonstrating consistent OFF-mode regulation across different chemical classes.

Major Points Requiring Clarification

1. Assumption of One V-ATPase per SV

The analysis is performed under the assumption that each SV contains only a single V-ATPase. However, electron microscopy analysis for SVs (Science 2024) has shown that a considerable fraction of SVs contains two or more V-ATPase complexes. The authors' previous work also reported SVs harboring multiple pumps. In the current manuscript, such multi-pump SVs appear not to be present, or not discussed. The authors must clarify why these differences arise between datasets and how multi-pump SVs were excluded or handled. This is essential because multiple pumps would alter the interpretation of dwell-time analysis and ON/OFF classification.

2. Lack of Discussion of the Leaky Mode

In the previous single-molecule study by the authors, a distinct "leaky mode" was identified. In the present manuscript, the analysis focuses exclusively on ON and OFF modes, and the leaky mode is not discussed, although Supplemental Figures suggest its presence.

Were leaky events observed in this dataset?

If so, how frequently?

Were these leaky events affected by inhibitors, or do they represent a separate regulatory pathway?

The omission of this mode needs justification.

In addition, the manuscript should clarify whether inhibitor-independent OFF modes were also observed, and how such intrinsic OFF events can be distinguished from inhibitor-induced OFF modes.

3. Stability of V_1 Association During ATP Addition

It is reported in the literature that, upon acidification during neurotransmitter loading, the V_1 domain can detach from V_0 , leading to irreversible loss of proton-pumping activity.

If ATP triggers V_1 dissociation under the current experimental conditions, such events should manifest as irreversible OFF modes.

Were such dissociation events observed?

How can the authors distinguish inhibitor-induced OFF states from OFF states caused by V_1 dissociation?

4. Proton Transport Rate Appears Too Low

The estimated proton transport rate of $\sim 2 \text{ H}^+/\text{s}$ is surprisingly low.

If V-ATPase hydrolyzes ATP at rates comparable to F-ATPase, this value seems inconsistent.

The authors must discuss why the apparent pumping rate is so low in their experimental setup.

Potential factors include:

limited buffering and leakage characteristics of SV membranes,
suboptimal ATP concentrations,
rate limitations imposed by pHrodo response kinetics,
mechanical or conformational constraints of the native complex.

Furthermore, did the authors measure the ATPase activity of the SV preparation in bulk?

Measuring bulk ATPase rates (with and without inhibitors) would help validate whether the single-molecule rate estimates are physiologically reasonable.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I appreciate the authors' thorough responses to my concerns. I have no further comments.

Reviewer #2

(Remarks to the Author)

In this revision, several of the previously raised concerns have been addressed appropriately, and the manuscript has been substantially improved. However, the responses to the following points are still not entirely sufficient and require further clarification and improvement. If these issues are adequately addressed, the manuscript is likely to meet the requirements for publication.

1. Comments 2–7: Stability of V_1 Association During ATP Addition

The authors have provided a thoughtful response to the concern regarding the potential dissociation of the V_1 domain upon ATP addition. The clarification that no clearly irreversible OFF events are observed is helpful, and the additional analysis

showing a correlation between bounded events and dwell time (Fig. S12) strengthens the argument.

However, the current response does not fully resolve the central issue. The explanation that long-lived OFF events arise from finite observation windows is plausible, but not sufficient to distinguish these events from potential structural dissociation followed by slow reassociation. The observed scaling behavior supports a kinetic interpretation, but does not uniquely rule out alternative mechanisms.

Given that V_1 dissociation under acidification has been reported in the literature, it is important to more clearly address why such behavior is not observed under the present experimental conditions. Differences in membrane environment, ATP concentrations, or luminal pH conditions should be discussed explicitly.

To more definitively resolve this issue, the most straightforward approach would be to biochemically verify that V_1 dissociation does not occur upon ATP addition under the experimental conditions used in this study, for example using the prepared synaptic vesicles.

2. Comments 2–8: Proton transport rate remains insufficiently explained

The authors attempt to address the concern regarding the unexpectedly low proton transport rate ($\sim 2 \text{ H}^+/\text{s}$).

While the authors cite variability in coupling ratios and literature values, the discussion remains largely generic and does not directly explain why such a low rate is observed in the present experimental system. In particular, the explanation focuses on variability across conditions and prior studies, rather than providing a quantitative, system-specific justification.

Furthermore, the argument based on intermittent activity (i.e., long inactive periods) may account for a reduced time-averaged flux, but it does not clarify whether the intrinsic pumping rate during active periods is itself reduced. This distinction is critical, and the current response conflates these two possibilities.

In addition, the authors have not addressed the suggestion to directly measure ATPase activity in the prepared vesicles. It would be important to measure both proton pumping activity and ATPase activity in bulk assays for the same synaptic vesicle preparation, and to include these raw data in the Supplementary Information. A quantitative discussion of the turnover rate in the main text would substantially strengthen the reliability and interpretability of the study.

minor point

The chemical structure of concanamycin appears to be incorrect. Please revise it using the correct structure.

Reviewer #3

(Remarks to the Author)

Kosmidis et al. investigated the orthosteric and allosteric inhibition of V-ATPase using the single-vesicle assay they previously developed. In this work, they newly identified an inhibitor-induced off-cycle mode. Although the mechanistic basis by which the inhibitors affect both the on/off state transitions and the intrinsic pumping rate remains unclear, the observations are sufficiently novel and interesting to the field. All of my concerns have been adequately addressed in the point-by-point response, and I therefore support publication of this manuscript. I only have one minor comment.

minor comment

1. In Figure 1F, the authors used a single y-axis. However, the y-axis for P_{on} is unitless and should not be presented together with ΔpH on the same scale. At first glance, this was somewhat confusing.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)

The revised manuscript has been significantly improved. The authors have responded carefully to the reviewers' comments and have incorporated substantial new analyses, data, and discussion that strengthen the study. While some mechanistic questions remain unresolved, these limitations are appropriately acknowledged and do not detract from the main conclusions of the work. Overall, the authors' responses are satisfactory, and I believe that the manuscript is now suitable for publication.

Reviewer #4

(Remarks to the Author)

I am very impressed with the manuscript that provides a quantitative approach to an important physiological system - synaptic vesicle acidification - that has been historically difficult to measure under normal conditions and against pharmacological challenges.

I found the article and the response to the reviewers letter to be clear and surgical in the treatment of the received feedback: overall the quality of the claim language has improved and I found it matches the strength of the evidence while making spaces for potential field-wide limitations.

I was specifically asked to comment on the authors' use of the computational modeling approach during the revision, and I can say that it leverages established frameworks with appropriate citations to the literature. These models have several open parameters that may complicate their interpretation (<https://pubmed.ncbi.nlm.nih.gov/40471464/> - no need to add the

reference to the ms!!), but I think the authors do an excellent job of acknowledging this limitation. In fact, the technique developed in this manuscript can help the field better fit parameters for future computational model. Depending on the editorial policy, it might be a good idea to share the code together with the manuscript (eg, GitHub, Zenodo, etc)

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Orthosteric and allosteric inhibition of the brain V-ATPase outside the alternating-access cycle

Authors: Eleftherios Kosmidis^{1§}, Maria Fokaeos^{1§}, Christopher G. Shuttle¹, Michael Isselstein¹, Alessandra Narducci¹, Aleksander Cvjetkovic¹, Peter J. Johnson², Jesper L. Pedersen³, Reinhard Jahn⁴, Julia Preobraschenski^{4,5,6}, Dimitrios Stamou^{1,7*}

Point-by-point response to reviewers

We thank the reviewers for their thoughtful and constructive evaluation of the manuscript. Below we respond point-by-point and, for each comment, also indicate the specific changes that we recommend incorporating into the revised manuscript.

Reviewer #1

Comment 1.1

Reviewer comment

This manuscript builds on the authors' previous observations that the V-ATPase can enter an ultra-long-lived inactive mode and proposes a novel pharmacological concept: targeting this inactive mode as a mechanism of inhibition. The idea of "two orthogonal axes" of pharmacological control is mechanistically interesting and potentially impactful. The study elegantly characterizes four known V-ATPase inhibitors – bafilomycin, concanamycin A, diphyllin, and SidK. In brief, bafilomycin and concanamycin bind the V0 subunit and block proton translocation, SidK binds the V1 subunit and blocks ATP hydrolysis, while the mechanism of diphyllin remains unknown. Despite previously resolved high-resolution structures supporting steric inhibition of proton translocation, the manuscript argues that bafilomycin does not act via this classical mechanism but instead switches active molecules into an ultra-long inactive mode. There is no doubt that this work will stimulate new discussions in transporter pharmacology and may open avenues for more precise drug design.

Response

We thank the reviewer for the careful reading of our manuscript and for the very positive assessment. We are encouraged that the reviewer found the concept of two orthogonal axes of pharmacological control to be mechanistically interesting and potentially impactful, and that the study may stimulate broader discussion in transporter pharmacology and drug design. We particularly appreciate the reviewer's recognition that the central advance of the work is the identification of long-lived inactive modes as a distinct pharmacological route for inhibition.

Comment 1.2

Reviewer comment

Definition of "single-molecule proton transport rate."

Throughout the manuscript, changes in $\Delta pHSM$ appear to be the primary indicator of the single-molecule proton transport rate. However, $\Delta pHSM$ is not the pumping rate itself; it is a steady-state gradient shaped by proton leak, buffering capacity, calibration, and possibly counter-ion conductance. Although the authors mention deconvolving pumping and leak via a physical model, much of the language reads as if the transport rate is measured directly. The main evidence for "unchanged pumping" is largely based on $\Delta pHSM$, which does not uniquely report on catalytic turnover.

Relatedly, the claim that inhibitors cause "no slowing of the alternating-access cycle" is a strong mechanistic leap. Even if the fitted parameter is unchanged during ON events, this does not strictly prove that the canonical transport cycle is unaffected. The assay reports net H^+ flux, which could be buffered by other steps. When referring to the "cycle," the authors should explicitly acknowledge that they measure proton flux, not mechanical rotation or conformational transitions directly.

Response

We thank the reviewer for emphasizing the important distinction between the alternating-access cycle of conformations and substrate transport. We fully agree that this distinction should be made clear to the readers and revised the manuscript accordingly. As requested, we now explicitly state that the assay measures net proton flux and not mechanical rotation or conformational transitions. We have introduced this clarification prominently both in the Introduction, and in the Conclusions.

In the **Introduction**, we now state: "We should highlight that, by definition, transport assays do not directly resolve conformational transitions but instead report the rate of productive substrate turnovers, which we refer to here as the 'transport cycle rate'. In the **Conclusions**, we now state: "Because we do not directly measure mechanical rotation, we cannot exclude that the rate of uncoupled conformational transitions is modulated by inhibitors."

At the same time, we propose that measurements of substrate flux are important **functional** readouts of the rate of **productive** alternating-access cycles i.e. of the conformational cycles which are coupled to substrate transport. In comparison, mechanical rotation or conformational dynamics assays report on all conformational changes whether they are productively coupled to substrate transfer or not. We therefore propose to sharpen the terminology in the revised

manuscript and define more clearly that, in this study, “*transport cycle rate refers to the rate of productive substrate turnovers*”.

We also absolutely agree that ΔpH_{SM} is not itself the proton pumping rate. We had explicitly stated this distinction in the first paragraph of page 4, lines 100-106, in Figs. S2 and S3, and in the Supplementary text (page 30, section “Physical model”) where we describe that ΔpH_{SM} depends on the pumping rate together with passive membrane leakage, transprotein leakage, luminal buffering, chloride/counter-ion conductance, membrane potential, vesicle geometry and fluorescence calibration.

Now, we also make this clear in the results section: “A non-equilibrium physical model (Fig. 1D black line) allowed us to deconvolve these two opposing proton kinetics, as well as the influence of luminal buffering, chloride/counter-ion conductance, membrane potential, vesicle geometry and fluorescence calibration...” and “Single-molecule measurements of proton pumping thus reveal that bafilomycin does not induce a detectable reduction in ΔpH_{SM} , suggesting it does not slow down the proton-transport cycle of the V-ATPase”.

At the same time, while ΔpH_{SM} is indeed not the pumping rate itself, it is a **quantitatively constrained observable** from which changes in pumping rate can be inferred with high sensitivity, as shown in references (1, 2). Inspired by the reviewer’s feedback, we now provide a quantification of the assay sensitivity: under our specific conditions (including buffering, photon counts, etc.) we can readily resolve ~ 0.2 atto-ampere proton currents generated by single V-ATPases (Fig. S3F, G) and **changes** in steady state currents of ~ 0.06 atto-ampere (see Fig. S3K).

Lastly, we would like to comment on a possible terminological ambiguity. In the single-molecule field, the term “**direct** observation” is used to distinguish **single-molecule** measurements from **ensemble-average** measurements, not to imply that a molecular property is observed without inference. For example, single-molecule conformational changes are typically inferred from changes in fluorescence resonance energy transfer between fluorophores, and ionic movement through single ion channels is inferred from electrical signals recorded by a patch-clamp circuit via an Ag/AgCl electrode.

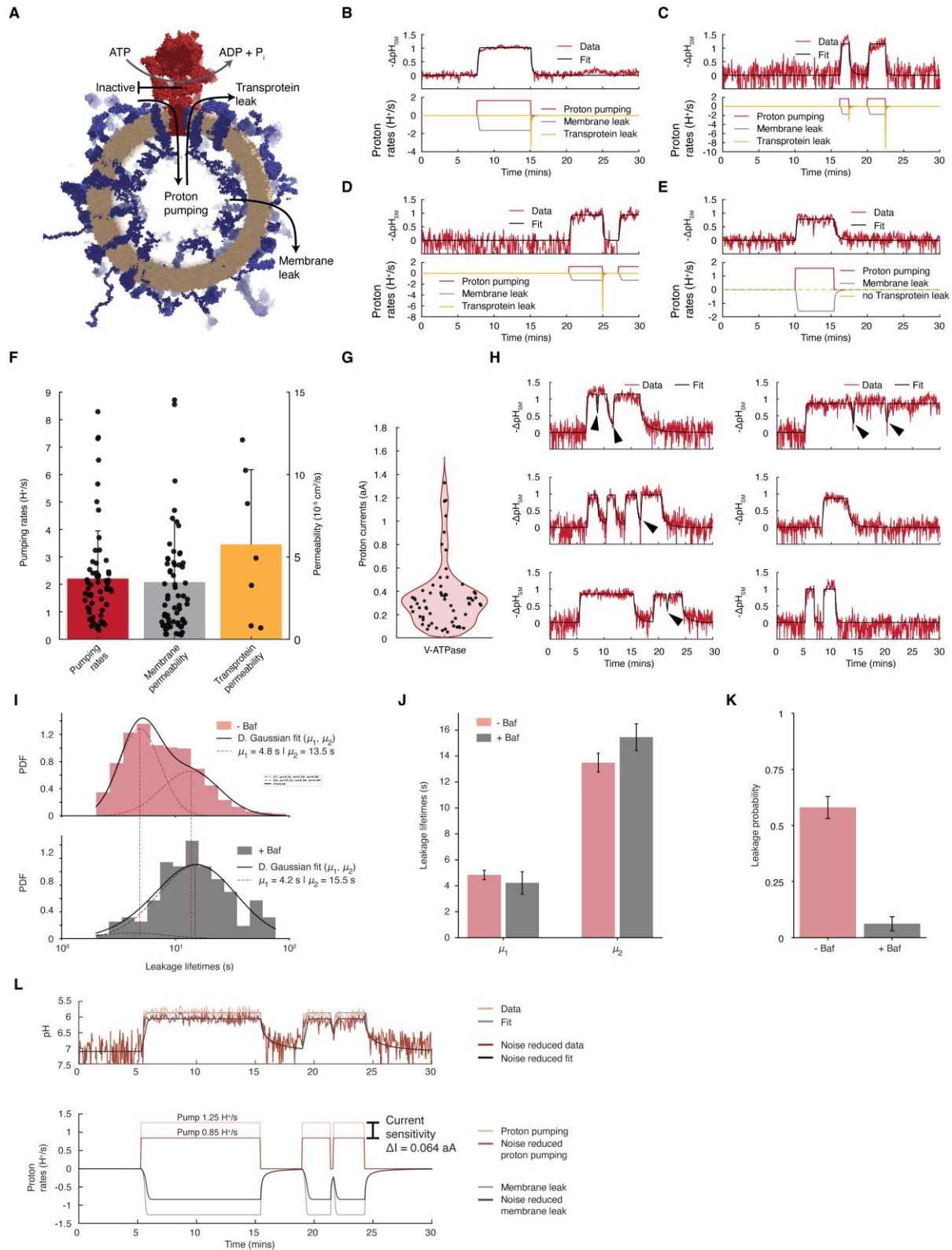


Fig. S3. Non-equilibrium modelling reveals single-molecule proton transport kinetics and leakage pathways.

(A) Schematic of model parameters. The synaptic vesicle illustration is adapted from *Cell*, Vol. 127, Takamori, S. *et al.*, Molecular Anatomy of a Trafficking Organelle, Pages 831–846, Copyright (2006), with permission from Elsevier. (B)–(E), Top: Representative single-molecule traces showing stochastic mode-switching dynamics. Active proton pumping is intermittently interrupted by inactive and proton-leaky modes. During active phases, a dynamic equilibrium is established between pumping and leakage, resulting in a single acidification plateau. During an active mode, which can last up to hours, a V-ATPase may complete thousands of conformational cycles and ATP/H⁺ turnover events before randomly switching to an inactive mode. Leakage arises from both membrane diffusion and transprotein efflux. Transprotein leakage initiates immediately upon V-ATPase deactivation and displays distinct

temporal kinetics. Traces were fitted using a non-equilibrium model (see Supplementary discussion), allowing for the independent estimation of pumping rates and permeabilities. Bottom: Modelled rates for proton pumping (red), membrane leak (grey), and transprotein leak (yellow). In (B)–(D), efflux is dominated by transprotein leakage, while in (E), by membrane diffusion. (F) Model-derived proton transport rates. Pumping rates (red) average $2.2 \pm 1.1 \text{ H}^+ \text{ s}^{-1}$. Membrane (grey) and transprotein (yellow) permeabilities are estimated at $\sim 3 \pm 2 \times 10^{-5} \text{ cm s}^{-1}$ and $6 \pm 5 \times 10^{-5} \text{ cm s}^{-1}$, respectively. Error bars, s.d.; $n = 24$ (pumping), $n = 24$ (membrane), $n = 8$ (transprotein). n denotes independent fits on single vesicles. (G) Violin plot of atto-Ampere proton currents generated by single V-ATPases. (H) Additional single-molecule traces with model fits. Arrows indicate mode-switching events where equilibrium was not reached ($\Delta\text{pH}_{\text{SM}} \approx \text{max or } 0$). Transprotein leakage modes were previously reported^{8,9} but are not further analysed here. (I) Histogram of proton leakage lifetimes in active SVs $\pm$ Baf. The fast leakage component (μ_1) corresponds to a transprotein leakage pathway through the V-ATPase, while the slow component (μ_2) corresponds to membrane leakage. (J) Characteristic leakage lifetimes extracted from double Gaussian fits of the leakage populations in (I). (K) Leakage probability of active V-ATPase $\pm$ Baf shows that bafilomycin dramatically reduces the probability of the transprotein leakage pathway. (L) Quantitative estimation of the detectable changes in the steady-state currents. The steady-state plateaus are reduced by one S.D. of the noise observed during these plateaus. Subsequently both the original trace and the noise-reduced trace are fitted with the model (top) and the corresponding proton rates are generated (bottom).

Comment 1.3

Reviewer comment

Interpretation of mode switching versus classical inhibition.

The work elegantly demonstrates dose-dependent responses of single V-ATPases to inhibitors. However, it is unclear how molecules that were already inactive prior to inhibitor application were treated. In Fig. 2 and Fig. S5, a substantial fraction of vesicles appear OFF even in the presence of ATP and the lowest concentration of inhibitors or the absence of inhibitors. Can these molecules re-enter the active state? How are they distinguished from defective or damaged transporters? Are the initial ON/OFF fractions consistent across vesicle preparations?

Response

We thank the reviewer for raising this point. The OFF fraction in Fig. 2 and Fig. S5 does not represent **permanently** inactive, defective or damaged molecules. Rather, these are molecules that are capable of activation but, like the rest of the population, stochastically and **reversibly switch between active and inactive modes** in the presence of ATP. Thus, the fraction that appears OFF at any given time reflects the occupancy of the inactive mode, i.e. the activation probability, not a distinct population of permanently inactive enzymes.

This behavior, termed mode-switching, is an intrinsic feature of the native brain V-ATPase and was established in our 2022 single-molecule study, where all active single vesicles exhibited reversible stochastic pauses over recordings that spanned hours (2). Herein, reversible mode-switching was discussed in pg. 4 (L98-109) and shown in figures 1d-f, 4a-d, and S2, S3, S8, S10.

P_{ON} is consistent between brain preparations and technical replicates $P_{\text{ON}} \sim 0.6 \pm 0.1$, c.f. with (2).

Motivated by the comment, we revised the manuscript to explicitly state that: “the V-ATPase is not active continuously over time; instead, it **stochastically switches** between ultralong-lived but **reversible** active and inactive modes” and “the inactive mode does not represent permanently inactive molecules.”.

Comment 1.4

Reviewer comment

Moreover, it is difficult to reconcile the observation that neither bafilomycin nor concanamycin affects single-molecule acidification (Fig. 3C) with their known steric interference with proton translocation. One possible explanation is that molecules that bind inhibitors immediately enter the inactive mode, while those that remain active are simply unbound. In that case, however, “being inhibited” and “entering the ultra-long inactive mode” become operationally indistinguishable. This conceptual issue deserves more explicit discussion.

For orthosteric blockers such as bafilomycin and concanamycin, a plausible alternative model is rapid binding and unbinding that produces intermittent long pauses, although hard to imagine for inhibitors binding at pmol affinities. This is still inhibition, but could be interpreted as acting on-cycle and generating OFF-like periods

rather than promoting a distinct off-cycle state. It is therefore unclear whether “direct blockage” is considered on-cycle or off-cycle, and the conceptual framework would benefit from clarification.

More generally, it remains unclear how, in a raw single-molecule trace, the authors distinguish between (i) a molecule that is bound to an inhibitor and directly inhibited and (ii) a molecule that has been switched by the inhibitor into the ultra-long-lived inactive mode. These two scenarios appear experimentally equivalent in the current readout.

I found Fig S12 could be possibly helpful for this matter. If I understand correctly, $t(\text{off})$ should be controlled by two factors: mode switching and inhibition. Given that the inhibitors bind with pM affinity, $t(\text{off})$ for inhibition should be quite long, likely longer than the inactive mode t , which was about 1-2 min as reported in Fig 4. As the concentration of inhibitors increases, the population controlled by inhibition (transporters bound to inhibitors) should increase if the timescales of the two components are distinguishable.

Response

We thank the reviewer for this important comment and agree that the conceptual distinction should be made more explicit. Functionally, a **pre-existing** inactive mode stabilized by inhibitor binding and a **new inhibitor-induced** non-transporting (‘locked’) mode can indeed appear equivalent in a single proton-flux trace; neither can be distinguished event-by-event from loss of acidification alone.

Our aim was therefore not to claim that these scenarios are directly separable from a raw trace, but to distinguish them statistically through the lifetime distributions (just as the reviewer suggested). This is precisely the purpose of the discussion and analysis in the second-to-last Results paragraph (Pg10, L278-290), Fig. S12 and Table S1. There, we ask whether the active- and inactive-mode dwell-time distributions are better described by single- or double-exponential models. The data indicate that both mechanistic regimes are possible: some inhibitors are more consistent with modulation/stabilization of a pre-existing OFF mode, whereas others are more consistent with stabilization of an additional/new inhibitor-specific long-lived non-transporting mode.

We also agree that, even in the latter case, this mechanism remains fundamentally different from continuous slowing of a normally cycling enzyme. A bind-and-lock mechanism is digital and does not progressively reduce the proton flux of molecules that remain active. Furthermore, we would argue that both pre-existing and inhibitor-induced OFF modes can be distinguished from canonical cycle intermediates because they have lifetimes which are several orders of magnitude larger than any cycle intermediate.

We will therefore clarify in the revised manuscript (Pg10) that the relevant mechanistic alternatives are: (i) stabilization of a pre-existing OFF mode, (ii) induction of an additional inhibitor-bound long-lived inactive mode, and (iii) slowing of the productive transport cycle. Our data argue strongly against mechanism (iii) for bafilomycin, concanamycin and diphyllin, while allowing a mixture of (i) and/or (ii), depending on the inhibitor. On the contrary, SidK appears to recruit both modulation of active/inactive mode lifetimes, mechanism (ii), and slowing of the productive transport cycle, mechanism (iii.)

Comment 1.5

Reviewer comment

Assumptions underlying estimation of active-mode probability.

The probability of being in the active mode appears to be inferred entirely from ensemble acidification and single-molecule $\Delta\text{pH}_{\text{SM}}$, assuming that only pumping capacity and mode occupancy determine the ensemble signal. This seems speculative. Could other factors contribute to maintaining a constant $\Delta\text{pH}_{\text{SM}}$, like changes in membrane permeability, counter-ion conductance, or pHrodo response properties? It would strengthen the manuscript to explicitly address whether these components play a role and to provide specific controls.

Response

We thank the reviewer for this comment. P_{ON} is not inferred from ensemble acidification and single-molecule $\Delta\text{pH}_{\text{SM}}$. Rather, all three quantities are measured independently in our assay: P_{ON} is obtained directly from the single-vesicle intensity distributions in the local-referencing assay (Fig. 2c and Fig. S6), or independently assessed from the single-molecule kinetic recordings (Fig.

4, S13). $\Delta\text{pH}_{\text{SM}}$ is measured directly at the single-vesicle level. $\Delta\text{pH}_{\text{AV}}$ is also measured directly as the ensemble average over all SVs.

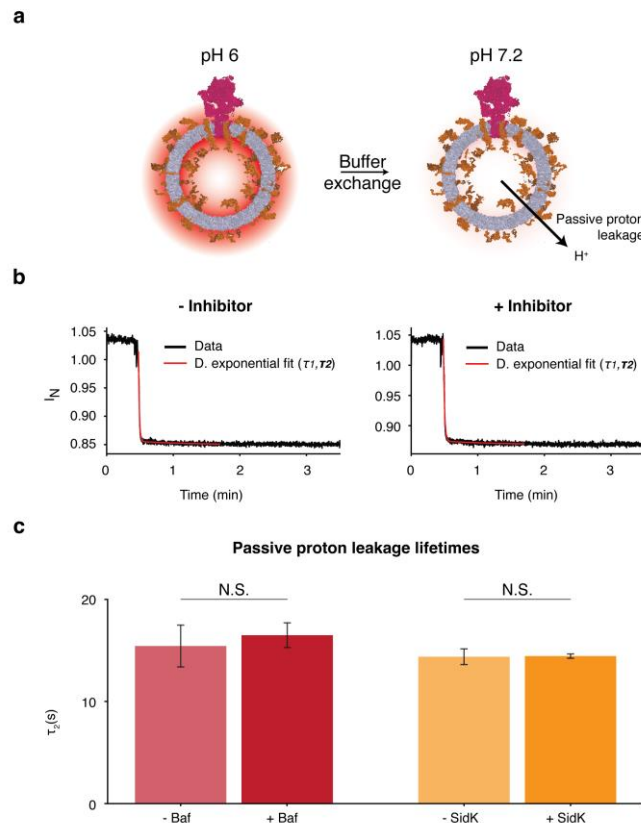
The relation $\Delta\text{pH}_{\text{AV}} = \Delta\text{pH}_{\text{SM}} \times P_{\text{ON}}$ is therefore not used to derive P_{ON} ; it is used to conceptually summarize how the independently measured observables combine to yield the ensemble signal. This is now clearly stated in the revised text: “*Note that all three quantities are independently determined in the experiment.*”

We completely agree that $\Delta\text{pH}_{\text{SM}}$ is directly influenced by many factors other than pumping alone, and our modelling quantitatively captures these influences. If we understood correctly, the reviewer asks whether it is conceivable that one of these factors changes to the exact same magnitude but with the opposite sign, i.e., in a manner that exactly cancels out the inhibition of the pumping rates. Thank you for raising this hypothesis that we address below:

Membrane permeability: Inspired by the reviewer comments, we have now performed a comprehensive series of time-consuming control experiments with Baf and SidK (new Fig. S14, S15) to demonstrate that inhibitor addition does not affect passive membrane leakage. By performing pH jumps $\pm$ inhibitor we can test for any variation in passive membrane permeability to protons. The new data shown in Fig. S14 and S15 demonstrate that passive proton leakage is not altered by addition of neither Baf nor SidK. This provides convincing control for the variable effect these two inhibitors had on $\Delta\text{pH}_{\text{SM}}$. Note that protons have orders of magnitude higher passive permeability than any other ion; thus, this experiment is a particularly stringent test for passive ion permeation.

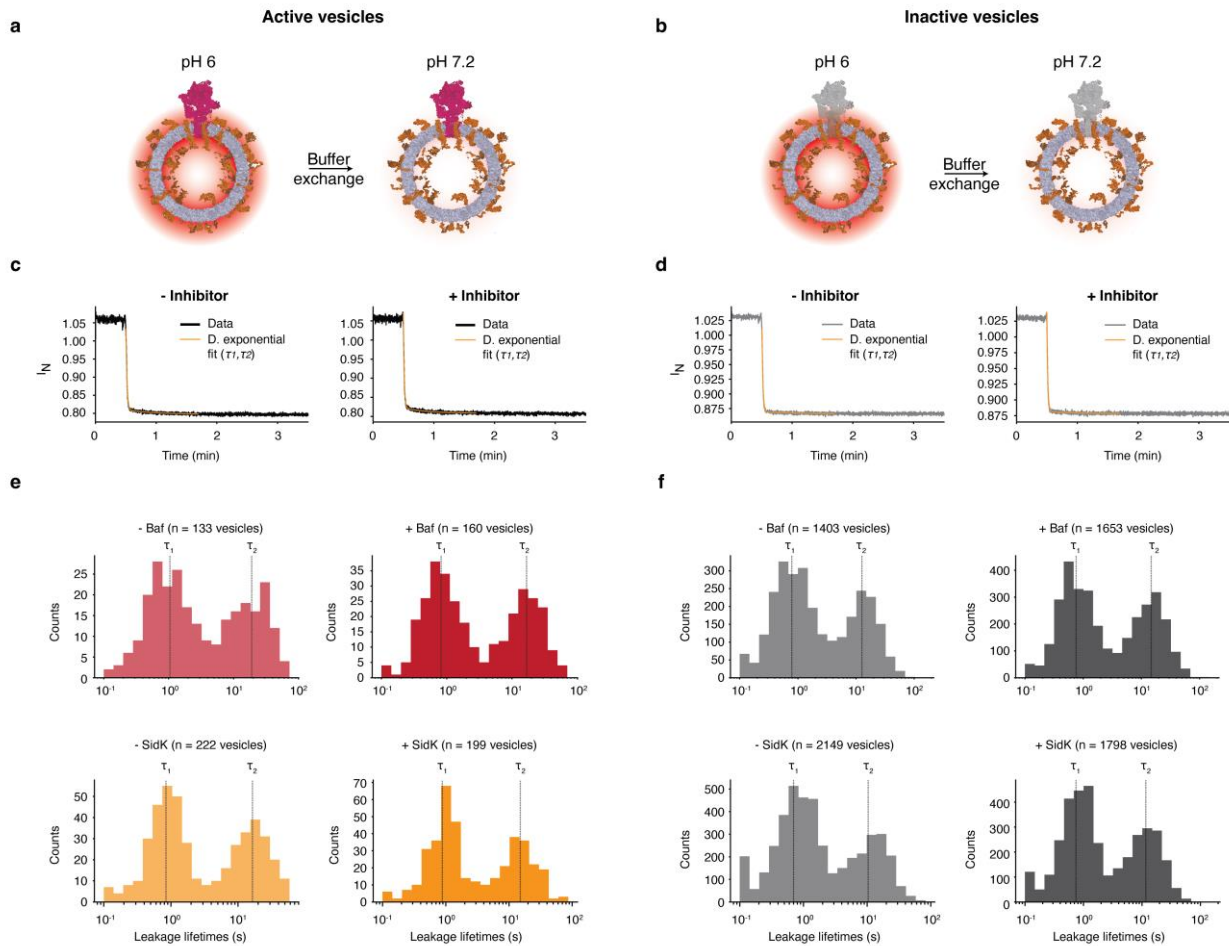
Counter-ion conductance: the only small counter-ion present in our experiments is Cl^- (See Methods). As discussed in reference (2) (see also references therein and Fig. 3), Cl^- flux mediated by synaptic Cl^- channels and/or transporters releases the membrane potential built by proton pumping. We have no rational reason to expect that the V-ATPase-specific inhibitors tested here affect Cl^- flux.

pHrodo response: the manuscript already contains control experiments showing that addition of vehicle (DMSO) and that addition of DMSO + inhibitor, do not affect the fluorescent properties of pHrodo (please see Fig. S4, and black circles/control in Fig. 3).



New Fig. S14. Bafilomycin and SidK do not affect the intrinsic membrane permeability to protons.

a, Illustration of pH jump experiments. The synaptic vesicle illustration is adapted from *Cell*, Vol. 127, Takamori, S. *et al.*, Molecular Anatomy of a Trafficking Organelle, Pages 831–846, Copyright (2006), with permission from Elsevier. SVs are equilibrated at pH 6 and subsequently are subjected to an external pH of 7.2 such that luminal protons passively permeate across the lipid bilayer inducing a drop in fluorescence intensity. pHrodo lipids are found in both leaflets of the bilayer. Fluorescence response of the outer monolayer has a much faster response time that depends on the flow rate of the microfluidic setup. Contrary, the fluorescence response of pHrodo lipids located in the inner monolayer solely depends on the leakage rate of luminal protons across the membrane. **b**, Ensemble average fluorescence (black and grey traces) of pH jump experiments $\pm$ inhibitor fitted with a double exponential function (red line). **c**, Characteristic lifetimes of the fluorescence response of the inner monolayer (τ_2) $\pm$ Baf or $\pm$ Sidk. Mean $\pm$ S.E.M. $n = 3$ independent experiments; Two-sided paired t-test. N.S. ($P = 0.81$, τ_2 , $\pm$ Baf); N.S. ($P = 0.96$, τ_2 , $\pm$ SidK).



New Fig. S15. Additional single-vesicle proton leakage experiments confirm that Baf and SidK do not alter the intrinsic passive permeability of the membrane.

a, b, Illustration of pH jump experiments for vesicles that contained active (a) and inactive (b) V-ATPases. The synaptic vesicle illustration is adapted from *Cell*, Vol. 127, Takamori, S. *et al.*, Molecular Anatomy of a Trafficking Organelle, Pages 831–846, Copyright (2006), with permission from Elsevier. **c, d,** Example traces of fluorescence response (black and grey traces) in pH jump experiments $\pm$ inhibitor for SVs containing active or inactive V-ATPases fitted with a double exponential function (yellow line). **e, f,** Histograms of single-vesicle lifetimes of proton leakage. All single-vesicle pH jump traces were fit with a double exponential function that generated two distinct lifetime populations corresponding to the outer and inner monolayer. The outer monolayer had a characteristic response time, $\tau_1 = 0.8\text{--}1$ s across all conditions and is rate-limited by the microfluidic setup. The inner monolayer generated a characteristic response time, $\tau_2 = 11\text{--}19$ s across all conditions and represents the lifetimes of proton leakage across the membrane. τ_2 is at least one order of magnitude longer than τ_1 which is consistent with previous findings⁸.

Comment 1.6

Reviewer comment

Likewise, are there intermediate or partially active states that might be forced into a binary ON/OFF classification by the analysis? Finally, was $P(\text{ON})$ in Fig 3D derived from values from Fig 3B and C, or independently measured from the $I(N)$ shifts in Fig 3E?

Response

We thank the reviewer for these related questions. We do not find evidence for statistically significant intermediate or partially active single-molecule states in the kinetic analysis. In both the apo condition and the inhibitor conditions, the event-level acidification amplitudes of **single active V-ATPases** are described by a single distribution for each condition rather than by discrete intermediate subpopulations (see histograms in 1d, 4a-d, S11).

Similarly, the ‘single molecule local referencing’ histograms are overall well described by two Gaussians (Fig. 2, S5, S6). Note, that intermediate intensity levels can appear in these histograms for two reasons that, however, do not imply partially active single molecules: (i) a minority of vesicles (~15%) contain more than one active V-ATPase, and (ii) a finite fraction of vesicles are sampled during pH build-up or decay rather than at steady state (see detailed analysis in ED Fig. 5 of reference (2)). For these two reasons, histograms in Fig. 2 and S6 contain marginal deviations from perfect double Gaussian behavior.

Regarding P_{ON} , the value in Fig. 3D was not inferred but measured independently from the snapshot intensity distributions shown in Fig. S6. We revised the figure caption to state this explicitly: “*The relative area under the two gaussians provides a direct estimation of P_{ON}* ”.

In addition, P_{ON} was independently assessed from the single-molecule kinetic recordings in Fig. 4 / Fig. S13, where the dwell times of the ON and OFF modes are resolved directly.

Comment 1.7

Reviewer comment

Heterogeneity of inactive states.

The OFF dwell times appear highly heterogeneous, ranging from seconds to many minutes. Especially, some are almost always inactive (Fig. S12). Do the authors believe that all these OFF periods correspond to the same inactive state and the same molecular mechanism, or that multiple distinct inactive states exist? This distinction is important for interpreting the concept of a single “ultra-long inactive mode” as a pharmacological target.

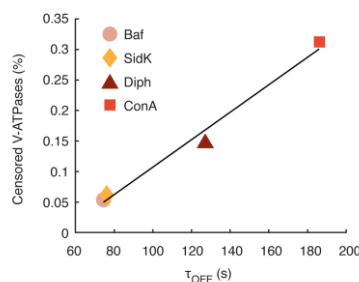
Response

We thank the reviewer for this important point. The broad span of OFF dwell times from seconds to minutes does not by itself imply strong heterogeneity in the statistical sense. In the apo condition, the inactive-mode lifetime distribution is well described by a single exponential, i.e. by a single characteristic timescale. A wide range of observed dwell times is expected for any exponential process **when the mean lifetime itself is long**; this is distinct from a genuinely ‘stretched’ distribution.

We agree, however, that some inhibitor conditions do reveal additional complexity. As summarized by the Bayesian comparison in Table S1, several inhibitors stabilize an additional OFF component, whereas the apo condition is consistent with one kinetically defined ultra-long inactive mode. We therefore do not claim that every inhibitor-induced inactive period corresponds to one identical microscopic state. Rather, our conclusion is that the native enzyme exhibits a pre-existing long-lived inactive mode, and some inhibitors can indeed additionally stabilize secondary inactive subpopulations. Please see discussion Pg10/L280-292 and also reply to Comment 1.4.

Finally, once the mean OFF lifetime becomes comparable to the finite recording window, a small number of traces will inevitably appear inactive for most or all of the observation period. This is expected censoring of long-lived reversible events, not evidence for a separate permanently inactive population. Note that out of the 16 histograms shown in Fig. S12, only two of them show significant percent (>10%) of molecules exceeding the recording duration after inhibition ($\tau_{off} + \text{Con}$, and $+\text{Diph}$).

Inspired by the comment we now provide additional supporting data to demonstrate that the percent of ‘censored’ events scales with the average lifetime, as expected. This is now in Fig. S12.



Longer inactive-mode lifetimes result in a larger fraction of truncated/censored lifetimes within the finite recording window. Fraction of truncated/censored vesicles as a function of inactive-mode lifetimes for each inhibitor. There is a linear correlation of inactive-mode lifetimes and the fraction of SVs that contain V-ATPases that remained inactive for the duration of the experiment. In other words, the larger the effect of inhibitors was on the inactive-mode, the larger the fraction of V-ATPases that were not captured in an active-mode for the duration of the experiment.

Comment 1.8

Reviewer comment

- Pages 12–13 and 14–15 of the Methods section appear to be duplicated. The Methods may be incomplete or incorrectly compiled.
- When first claiming that most vesicles contain a single V-ATPase, the text should explicitly refer to Fig. S8, where the step analysis supporting this conclusion is presented.
- The scales in panels of Figure 3 are inconsistent, making it difficult to compare data across inhibitors. The inhibition fits should report IC50 and Hill coefficients.
- Y-axis labeling in S6F seems off. The decrease in the maximum of the upper I(N) peak appears to be much greater than ~25%, as shown in Figure 3 and stated in the text.
- It is also unclear how SidK has almost no effect on either ON to OFF or OFF to ON lifetimes (Fig 4F,G), and yet decreases the fraction of ON mode by ~50% (Fig S6).
- There is an inconsistency between the results in Figures 3 and 4. For example, for Baf, the IC50 is 200 pM, based on Fig. 3. However, in Fig. 4, 50 pM decreases the ON time by 50%, which would define this concentration as the IC50.
- Similarly, the IC50 for Con is ~600 pM (Fig. 3), while 320 pM results in a nearly 2-fold decrease in ON time and an over 2-fold increase in OFF time, yielding at least 75% inhibition. Results seem more consistent for Diph is 32 nM.
- What is the scenario behind the different doses chosen for each inhibitor in Figure 4? It seems odd to use everything differently for comparison. Is it possible to use IC50 for all?
- How is n determined in these experiments? Are they the same preparation but different molecules (movies), or different vesicle preparations? This should be stated in the figure legends or the methods section.

Response

We thank the reviewer for these careful technical comments. We will address them as follows.

- Thank you for pointing out the page duplication error, it has now been corrected.
- We added an explicit first reference to Fig. S8 when stating that most vesicles contain a single V-ATPase.
- We harmonized the axes across the panels of Fig. 3 to facilitate comparison. We also report IC50 and Hill coefficients for the dose-response fits in the figure legends. Moreover, data has been color coded based on inhibitor for additional clarity.
- We corrected the labeling/presentation of Fig. S6F.
- , f), g) The two independent estimations of P_{ON} by the two different assays (local referencing vs kinetics) are comparable within uncertainties for all compounds. Especially considering these experiments spanned three years and multiple different preparations of SVs and batches of inhibitors. For Baf (0.50 ± 0.20 vs 0.32 ± 0.05), Con (0.37 ± 0.14 vs 0.16 ± 0.07), Diph (0.25 ± 0.15 vs 0.19 ± 0.04), SidK (0.39 ± 0.08 vs 0.41 ± 0.09). We have made a new figure panel (Fig. S13F) to precisely illustrate this. Note that the lifetimes shown in Fig 3. do not include censored events, and cannot thus be used to

accurately estimate P_{ON} . The correct P_{ON} values extracted from kinetics that include censored lifetimes are shown in Fig. S13E.

- h) We performed these measurements guided by literature values before we knew the exact IC_{50} of all compounds under our conditions. In general, we tried to inhibit ~50% of events, in the case of SidK, this meant we had to exceed by far the IC_{50} because of its low efficacy. Importantly, the absolute concentrations used in Fig. 4 have no bearing on any of the conclusions.
- i) We have clarified, wherever applicable, in the figure legends and Methods how n is defined, distinguishing independent biological preparations from the total number of single-vesicle traces / vesicles analyzed.

Changes to the manuscript

- **Methods section:** Corrected the duplicated pages / compilation issue and ensured the final compiled Methods are complete.
- **Main text and Fig. S8 cross-referencing:** Added an explicit cross-reference to Fig. S8 at the first statement that most vesicles contain a single V-ATPase.
- **Figure 3 and accompanying legend:** Harmonized the axis scaling across inhibitor panels and add the IC_{50} and Hill coefficients to the legend and/or to a new supplementary fit-parameter table.
- **Figure S6F and Results text:** Corrected the y-axis / labeling and clarify in the text that SidK lowers P_{ON} primarily through shortening τ_{ON} while having little effect on τ_{OFF} .
- **Figure 4 legend / Methods:** Added a sentence explaining the rationale for the representative inhibitor concentrations used for kinetic analysis and define n explicitly as independent experiments and total traces / vesicles analyzed.
- **Updated Figure S13:** The figure now contains a comparison of kinetic and local referencing experiments, showing that P_{ON} estimates between the two methods are within error margins for all inhibitors (Fig. S13F).

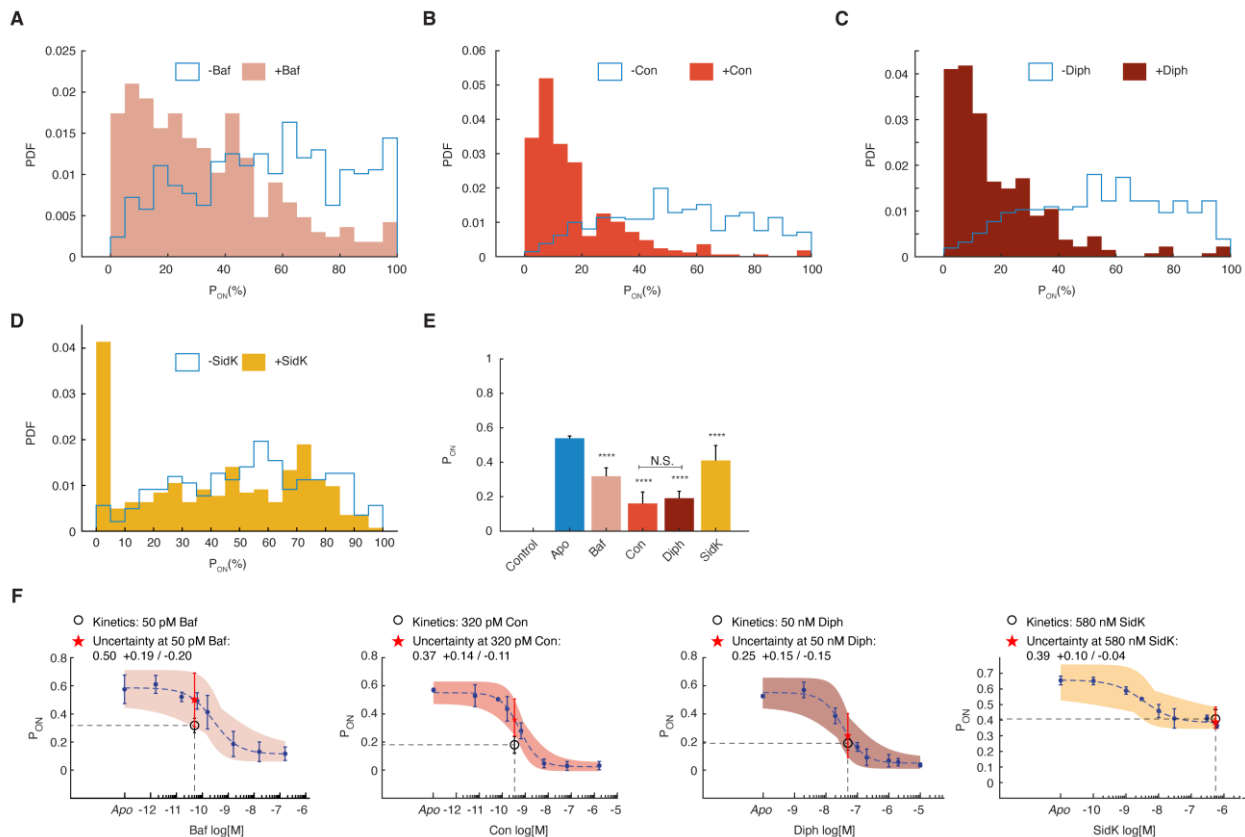


Fig. S13. Kinetic experiments demonstrate that all inhibitors regulate the single-molecule active mode probability.

(A)-(D), Distributions of single-molecule active mode probability in the presence and absence of inhibitors. All inhibitors reduced the probability that the V-ATPase is found in an active mode. Concentrations of the inhibitors used were: 50 pM Baf, 320 pM Con, 50 nM Diph, and 580 nM SidK. **** $P < 10^{-10}$ ($\pm$ Baf); **** $P < 10^{-10}$ ($\pm$ Con); **** $P < 10^{-10}$ ($\pm$ Diph); **** $P = 2 \cdot 10^{-5}$ ($\pm$ SidK); two-sided two-sample Kolmogorov-Smirnov tests. **(E)** Bar charts summarizing the regulation of active mode probability (P_{ON}) by the inhibitors used in single-molecule kinetic experiments. All inhibitors investigated significantly reduced the probability of the V-ATPase being found in an active mode. mean $\pm$ s.d. from independent experiments; n.s. ($P = 0.20$, Con-Diph). **(F)** Comparison of kinetic and single-molecule local referencing experiments reveals similar estimates of P_{ON} within error estimates. Titration curves of P_{ON} were supplemented by uncertainty envelopes based on the sigmoidal fit parameter uncertainties that minimize or maximize the curve. Red data corresponds to P_{ON} of the curve at the concentration used in kinetic experiments. Black data corresponds to P_{ON} measured in kinetic experiments as seen in (E).

Reviewer #2

Comment 2.1

Reviewer comment

Overall, the authors present a well-designed single-vesicle assay to dissect how various inhibitors of the V-ATPase act at the single-molecule level, specifically by prolonging the OFF-mode dwell time. The manuscript provides important insights that cannot be obtained from bulk phase measurements. However, several methodological and conceptual issues need to be addressed.

Response

We thank the reviewer for the positive and constructive overall assessment. We are pleased that the reviewer recognizes the value of the single-vesicle functional/transport assay for dissecting inhibitor action beyond what can be learned from bulk measurements. We appreciate the methodological and conceptual points raised below and have addressed them point-by-point; several of them have prompted clarifications that will strengthen the revised manuscript.

Comment 2.2

Reviewer comment

General Assessment

The central contribution of this manuscript is the demonstration that the inhibitory effects of multiple V-ATPase inhibitors are mediated primarily by increasing the dwell time spent in the OFF mode, rather than decreasing the catalytic turnover rate of individual enzyme molecules. The authors successfully established a single-synaptic-vesicle assay by loading purified SVs with pHrodo, enabling fluorescence-based monitoring of the proton-pumping activity of individual native V-ATPases.

The study convincingly shows that the OFF-mode occupancy increases in a dose-dependent manner upon addition of various inhibitors, and similar behavior is observed across different inhibitor classes. This finding fundamentally challenges traditional interpretations derived from bulk kinetic measurements, in which inhibition is typically attributed to reduced turnover of continuously cycling enzymes. Thus, the manuscript provides a new conceptual framework for understanding inhibitor action on active transporters.

It should also be noted that the same group previously published a single-molecule proton-pumping assay for V-ATPase in Nature (2022), and the present work is effectively an extension of that platform toward pharmacological applications.

Response

We thank the reviewer for this balanced assessment. We agree with the reviewer's summary of the central contribution: the key result is that chemically distinct V-ATPase inhibitors suppress ensemble acidification predominantly by increasing OFF-mode occupancy and/or dwell time, rather than by uniformly slowing the active-state proton flux of all molecules. We also agree that the present work extends the single-molecule platform established in our 2022 Nature study into the pharmacological domain (2).

Comment 2.3

Reviewer comment

While the concept of mode-dependent regulation is novel in the transporter field, the underlying idea—that the ensemble activity of an enzyme population is determined by the fraction of molecules in an OFF state—has been previously demonstrated in the single-molecule rotation experiments of Kinosita and colleagues on F_1 -ATPase (Science 2001, et al). For example, the ADP-inhibited state represents an OFF mode, and the ensemble ATPase activity is reduced simply because a subpopulation is trapped in this inactive state, even though the catalytic turnover of active molecules remains unchanged (Hirono-Hara, PNAS 2001). Likewise, in the case of V-ATPase, the same group has already reported single-molecule evidence of mode switching.

Therefore, although the experimental system is sophisticated and the data interpretation is valid, the conceptual novelty may be limited. Nevertheless, the present manuscript provides a significant contribution by applying single-vesicle measurements to multiple inhibitors and demonstrating consistent OFF-mode regulation across different chemical classes.

Response

We thank the reviewer for this thoughtful perspective. We agree completely that the novelty of this manuscript does not lie in reporting the existence of OFF modes per se. Indeed, as the Reviewer noted, the key novelty and impact lies in "*providing a new conceptual framework for understanding inhibitor action on active transporters.*" Identifying a novel pharmacological mechanism which does not rely on slowing the canonical transport cycle is the central conceptual novelty of the present manuscript.

Comment 2.4

Reviewer comment

Assumption of One V-ATPase per SV

The analysis is performed under the assumption that each SV contains only a single V-ATPase. However, electron microscopy analysis for SVs (Science 2024) has shown that a considerable fraction of SVs contains two or more V-ATPase complexes. The authors' previous work also reported SVs harboring multiple pumps. In the current manuscript, such multi-pump SVs appear not to be present, or not discussed. The authors must clarify why these differences arise between datasets and how multi-pump SVs were excluded or handled. This is essential because multiple pumps would alter the interpretation of dwell-time analysis and ON/OFF classification.

Response

We thank the reviewer for raising this important point. Multi-pump vesicles are present in all of our preparations and are explicitly quantified rather than ignored (see Fig. S8, and Pg29, L779-788).

Step analysis in Fig. S8 shows that $85 \pm 3\%$ of active vesicles exhibit a single activation step, $13 \pm 2\%$ exhibit two steps, and $2 \pm 1\%$ exhibit three or more, corresponding to an average of approximately 1.2 ± 0.1 active V-ATPases per vesicle. This is consistent with our 2022 study and with multiple previous estimates of 1.2 ± 0.1 V-ATPase per native synaptic vesicle (2,5,6,7).

As discussed in Fig. S8 and in Supplementary Text (Pg30, L808-817), all kinetic analyses are restricted to single-step traces only; therefore, multi-pump vesicles do not contribute to the lifetime measurements or the ON/OFF classification in Fig. 4.

Inspired by the reviewers comment we now state this explicitly also in the main text Pg8, L235: "*This kinetic dwell-time analysis was restricted to the 85% of SVs carrying only one copy of the V-ATPase, see Fig. S8*".

As described in Supplementary Text (Pg30, L822-843), for the snapshot analysis, we explicitly correct P_{ON} for the 15% of vesicles containing two or more active pumps using the stoichiometric weights obtained from the step-counting analysis. We will make this handling clearer in the main text and the figure caption: "*Here, we corrected P_{ON} for the minority of vesicles containing two or more active pumps*".

Lastly, we wanted to point out that the reference suggested by the reviewer, follows a SV purification method that inherently is biased towards isolation of SVs that contain a larger number of V-ATPases. Specifically, SidK was used as a handle to selectively pull-down V-ATPase-containing native vesicles. As the authors acknowledge, this inevitably biased their SV samples, towards higher copy numbers of the V-ATPase.

Comment 2.5

Reviewer comment

Lack of Discussion of the Leaky Mode

In the previous single-molecule study by the authors, a distinct "leaky mode" was identified. In the present manuscript, the analysis focuses exclusively on ON and OFF modes, and the leaky mode is not discussed, although Supplemental Figures suggest its presence. Were leaky events observed in this dataset? If so, how frequently? Were these leaky events affected by inhibitors, or do they represent a separate regulatory pathway? The omission of this mode needs justification.

Response

We thank the reviewer for pointing out this omission. Yes, proton-leaky events were observed in this dataset, as expected from our previous work, and their presence is already discussed in Fig. S3 and in the underlying non-equilibrium analysis. Leakage probability, just like active probability is quite stable across preparations and technical replicates. To mend the omission of a statistical analysis of the transprotein leakage pathway we have revised Fig. S3 to include the histogram of single-molecule leakage lifetimes ($\pm$ Baf), Fig. S3, as well as bar charts of membrane and transprotein leakage lifetimes and transprotein leakage probability ($\pm$ Baf), Fig. S3J, K.

We did not focus on the leaky mode in the present manuscript because the explicit purpose of this study was to understand whether inhibitors slow-down the canonical transport cycle/rates, or instead regulate P_{ON} . Specifically, the manuscript addresses how inhibition redistributes the enzyme between transport-active and transport-inactive modes, thereby altering P_{ON} , τ_{ON} and τ_{OFF} . A detailed study focused on the regulation of leaky modes by small molecules would be outside the scope of the current submission, but is something that we plan to follow up on in a future manuscript. Thank you for the suggestion.

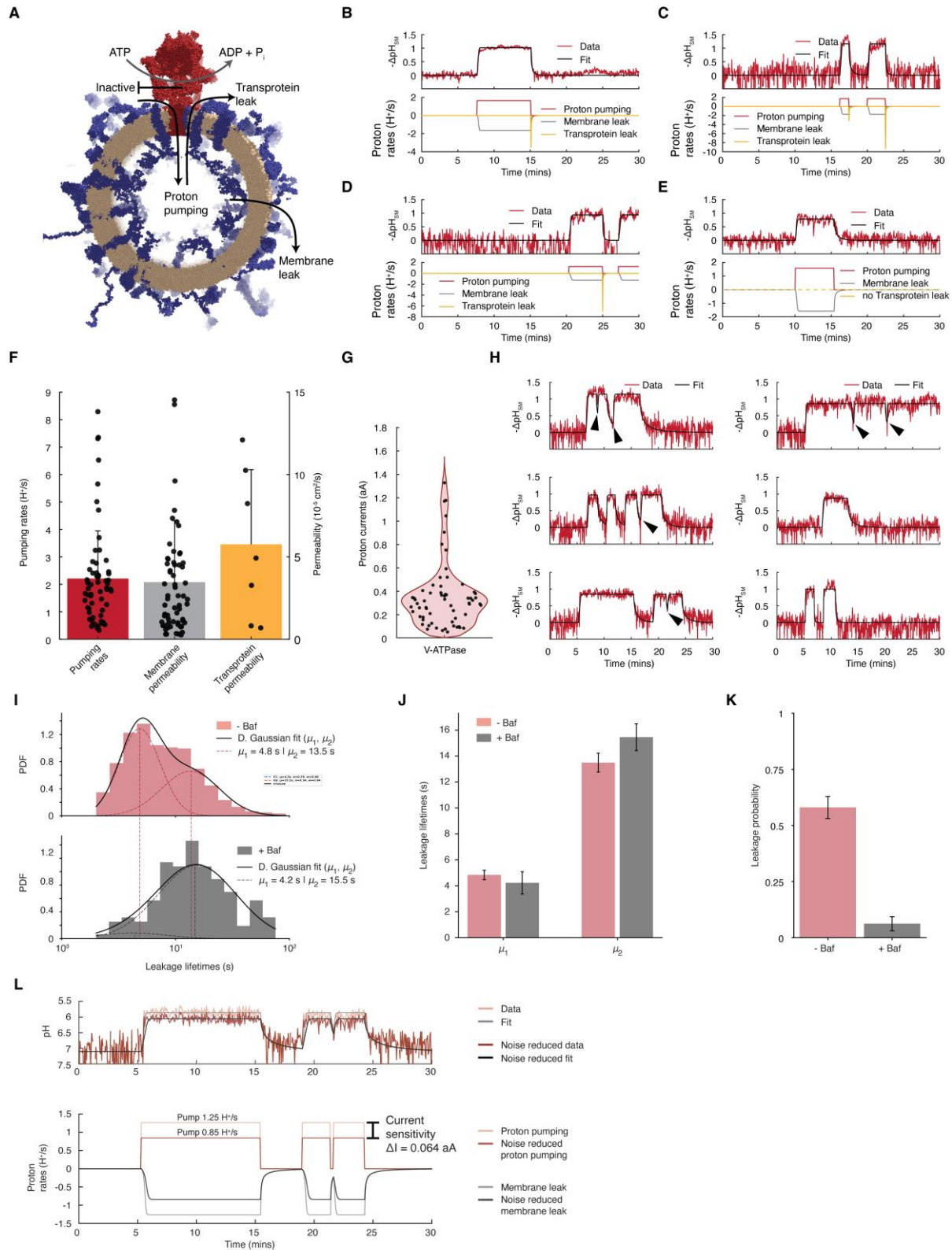


Fig. S3. Non-equilibrium modelling reveals single-molecule proton transport kinetics and leakage pathways.

(A) Schematic of model parameters. The synaptic vesicle illustration is adapted from *Cell*, Vol. 127, Takamori, S. *et al.*, Molecular Anatomy of a Trafficking Organelle, Pages 831–846, Copyright (2006), with permission from Elsevier. (B)–(E), Top: Representative single-molecule traces showing stochastic mode-switching dynamics. Active proton pumping is intermittently interrupted by inactive and proton-leaky modes. During active phases, a dynamic equilibrium is established between pumping and leakage, resulting in a single acidification plateau. During an active mode, which can last up to hours, a V-ATPase may complete thousands of conformational cycles and ATP/H⁺ turnover events before randomly switching to an inactive mode. Leakage arises from both membrane diffusion and transprotein efflux. Transprotein leakage initiates immediately upon V-ATPase deactivation and displays distinct

temporal kinetics. Traces were fitted using a non-equilibrium model (see Supplementary discussion), allowing for the independent estimation of pumping rates and permeabilities. Bottom: Modelled rates for proton pumping (red), membrane leak (grey), and transprotein leak (yellow). In (B)–(D), efflux is dominated by transprotein leakage, while in (E), by membrane diffusion. (F) Model-derived proton transport rates. Pumping rates (red) average $2.2 \pm 1.1 \text{ H}^+ \text{ s}^{-1}$. Membrane (grey) and transprotein (yellow) permeabilities are estimated at $\sim 3 \pm 2 \times 10^{-5} \text{ cm s}^{-1}$ and $6 \pm 5 \times 10^{-5} \text{ cm s}^{-1}$, respectively. Error bars, s.d.; $n = 24$ (pumping), $n = 24$ (membrane), $n = 8$ (transprotein). n denotes independent fits on single vesicles. (G) Violin plot of atto-Ampere proton currents generated by single V-ATPases. (H) Additional single-molecule traces with model fits. Arrows indicate mode-switching events where equilibrium was not reached ($\Delta\text{pH}_{\text{SM}} \approx \text{max or } 0$). Transprotein leakage modes were previously reported^{8,9} but are not further analysed here. (I) Histogram of proton leakage lifetimes in active SVs $\pm$ Baf. The fast leakage component (μ_1) corresponds to a transprotein leakage pathway through the V-ATPase, while the slow component (μ_2) corresponds to membrane leakage. (J) Characteristic leakage lifetimes extracted from double Gaussian fits of the leakage populations in (I). (K) Leakage probability of active V-ATPase $\pm$ Baf shows that bafilomycin dramatically reduces the probability of the transprotein leakage pathway. (L) Quantitative estimation of the detectable changes in the steady-state currents. The steady-state plateaus are reduced by one S.D. of the noise observed during these plateaus. Subsequently both the original trace and the noise-reduced trace are fitted with the model (top) and the corresponding proton rates are generated (bottom).

Comment 2.6

Reviewer comment

In addition, the manuscript should clarify whether inhibitor-independent OFF modes were also observed, and how such intrinsic OFF events can be distinguished from inhibitor-induced OFF modes.

Response

We thank the reviewer for raising this point. Yes, inhibitor-independent OFF modes are clearly observed in the absence of inhibitor. OFF-modes are a **pre-existing intrinsic property** of the brain V-ATPase and were the central finding of our 2022 study; the current manuscript likewise shows reversible apo switching between active and inactive modes before any inhibitor is added, see Fig. 1d, 4a-d, S2, S3, S8, S10 (2).

We completely agree that intrinsic OFF events and inhibitor-induced ‘bind-lock’ OFF-like states are not distinguishable event-by-event in a raw proton-flux trace, because both appear as loss of acidification. Our distinction is therefore statistical rather than phenomenological. Specifically, in the second-to-last Results paragraph, Fig. S12 and Table S1, we tested whether inhibitors simply redistribute occupancy within a pre-existing mode landscape or whether they stabilize an additional kinetically distinct inactive state. The data indicate that different inhibitors behave differently: some are consistent with modulation of the intrinsic OFF mode, whereas others stabilize an additional long-lived reversible inactive population.

Importantly, both OFF modes reflect a digital (all-or-none) mechanism fundamentally different from continuous slowing of a normally cycling enzyme. Furthermore, we would argue that both pre-existing and inhibitor-induced OFF modes can be kinetically/energetically distinguished from canonical cycle intermediates because they have lifetimes which are several orders of magnitude larger than any cycle intermediate.

These clarifications are now clearly stated in the Results section (Pg11, L283-295).

Comment 2.7

Reviewer comment

Stability of V_1 Association During ATP Addition

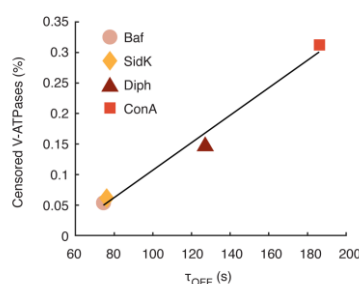
It is reported in the literature that, upon acidification during neurotransmitter loading, the V_1 domain can detach from V_o , leading to irreversible loss of proton-pumping activity. If ATP triggers V_1 dissociation under the current experimental conditions, such events should manifest as irreversible OFF modes. Were such dissociation events observed? How can the authors distinguish inhibitor-induced OFF states from OFF states caused by V_1 dissociation?

Response

We thank the reviewer for this important question. We do not observe irreversible OFF events upon ATP addition in the apo condition. On the contrary, the OFF events resolved in our assay are reversible, consistent with repeated stochastic switching between active and inactive modes

rather than irreversible loss of function. This is consistent with our 2022 study, where single active vesicles exhibited recurrent ON/OFF switching but could be followed over periods of hours without any loss of function (2).

The same conclusion applies in the presence of inhibitors: the dominant behavior is still reversible switching. In a small minority of traces, and only under a minority of conditions in which OFF lifetimes become comparable to the finite recording window, a vesicle can remain OFF for the remainder of the movie. We do not interpret these events as evidence for irreversible V1 dissociation; rather, they are the expected consequence of observing very long-lived reversible states within a bounded time window. Inspired by the comment we now provide additional supporting data to demonstrate that the percent of ‘bounded events scales with the average lifetime, as expected. This figure is now in Fig. S12.



Longer inactive-mode lifetimes result in a larger fraction of truncated/censored lifetimes within the finite recording window. Fraction of truncated/censored vesicles as a function of inactive-mode lifetimes for each inhibitor. There is a linear correlation of inactive-mode lifetimes and the fraction of SVs that contain V-ATPases that remained inactive for the duration of the experiment. In other words, the larger the effect of inhibitors was on the inactive-mode, the larger the fraction of V-ATPases that were not captured in an active-mode for the duration of the experiment.

We agree that the V1-dissociation literature should be acknowledged more explicitly. However, as discussed in reference (2), if ATP-triggered V1 dissociation were the dominant process under our assay conditions, we would expect irreversible loss of activity rather than recurrent reversible switching. We will therefore clarify in the revised manuscript that: *“The reversible inactive mode does not represent permanently inactive molecules and is thus not consistent with the ATP-triggered irreversible V1 dissociation⁸”*.

Comment 2.8

Reviewer comment

Proton Transport Rate Appears Too Low

The estimated proton transport rate of $\sim 2 \text{ H}^+/\text{s}$ is surprisingly low. If V-ATPase hydrolyzes ATP at rates comparable to F-ATPase, this value seems inconsistent. The authors must discuss why the apparent pumping rate is so low in their experimental setup. Potential factors include: limited buffering and leakage characteristics of SV membranes, suboptimal ATP concentrations, rate limitations imposed by pHrodo response kinetics, mechanical or conformational constraints of the native complex.

Response

Thank you for this crucial comment. As we discuss below, our estimates of proton pumping rates are in general consistent with V-ATPase literature.

An important distinction we should make is that ATP hydrolysis rates and proton pumping rates are not always proportional because the coupling ratio is variable. As discussed extensively in (10) proton rates can vary between 120 /s to -60 /s (i.e. inverse direction!) depending on changes in the electrochemical potential alone. Furthermore, the actual rates also depend on the species and the reconstitution conditions. Specifically, there are electrochemical conditions described in Fig. 7 in (10) that encourage similar pumping rates to those we found in the current paper as well as in Kosmidis et al. (2). However, things are further complicated by the finding that at the single

molecule level, pumping is interrupted by long inactive periods that would change the apparent ensemble average coupling ratio on which most literature data is based.

To our knowledge, the most accurate estimation of pumping rates is our previous Kosmidis et al. paper. There, we deconvolve P_{ON} from pumping rates for the first time, and we also use a model that accounts explicitly for buffering, passive proton leakage, transprotein leakage, counter-ion conductance and membrane potential which allowed us an accurate estimation of proton pumping rates.

One of the most significant insights reached in Kosmidis et al. is that increased [ATP] increases P_{ON} but not the single-molecule pumping rates. As discussed in this paper, a direct consequence of this discovery is that the maximum coupling ratio is reached well below the K_M for [ATP] hydrolysis, and thus, further increase in hydrolysis rates is not coupled to proton pumping. The proton pumping rate we measured in hybrid synaptic vesicles ($7 \pm 5 \text{ H}^+/\text{s}$) (2) is very similar to the one we measure here in intact synaptic vesicles ($2.2 \pm 1.9 \text{ H}^+/\text{s}$).

Changes to the manuscript

- **Results / figures / supplementary information:** We discovered a minor error in the pH calibration which is not corrected. This minor correction increases the absolute pumping-rates from 1.7 to 2.2. Moreover new Fig. S3 contains a panel that shows single-molecule currents in units of atto-Ampere (Fig. S3G). Transprotein leakage rates and probability $\pm$ Baf are now analyzed in new panels in Fig. S3I-K.
- **Discussion:** Added one explicit sentence that F1 rotation / hydrolysis rates are not directly comparable to net V-ATPase proton flux measured in synaptic vesicles.
- **Supplementary Methods / model description:** Clarified again that the full kinetic model includes buffering, leakage, counter-ion conductance and membrane potential.
- **Discussion / benchmarking paragraph:** Cited Egashira et al. (9) and Grabe et al. (10) as an external benchmark showing that synaptic-vesicle proton influx is of the same order of magnitude as the corrected estimates.
- **Additional experiments:** We performed additional experiments that show that passive leakage is not affected by the presence of inhibitors at the ensemble average and single-vesicle level (new Fig. S14 and S15).

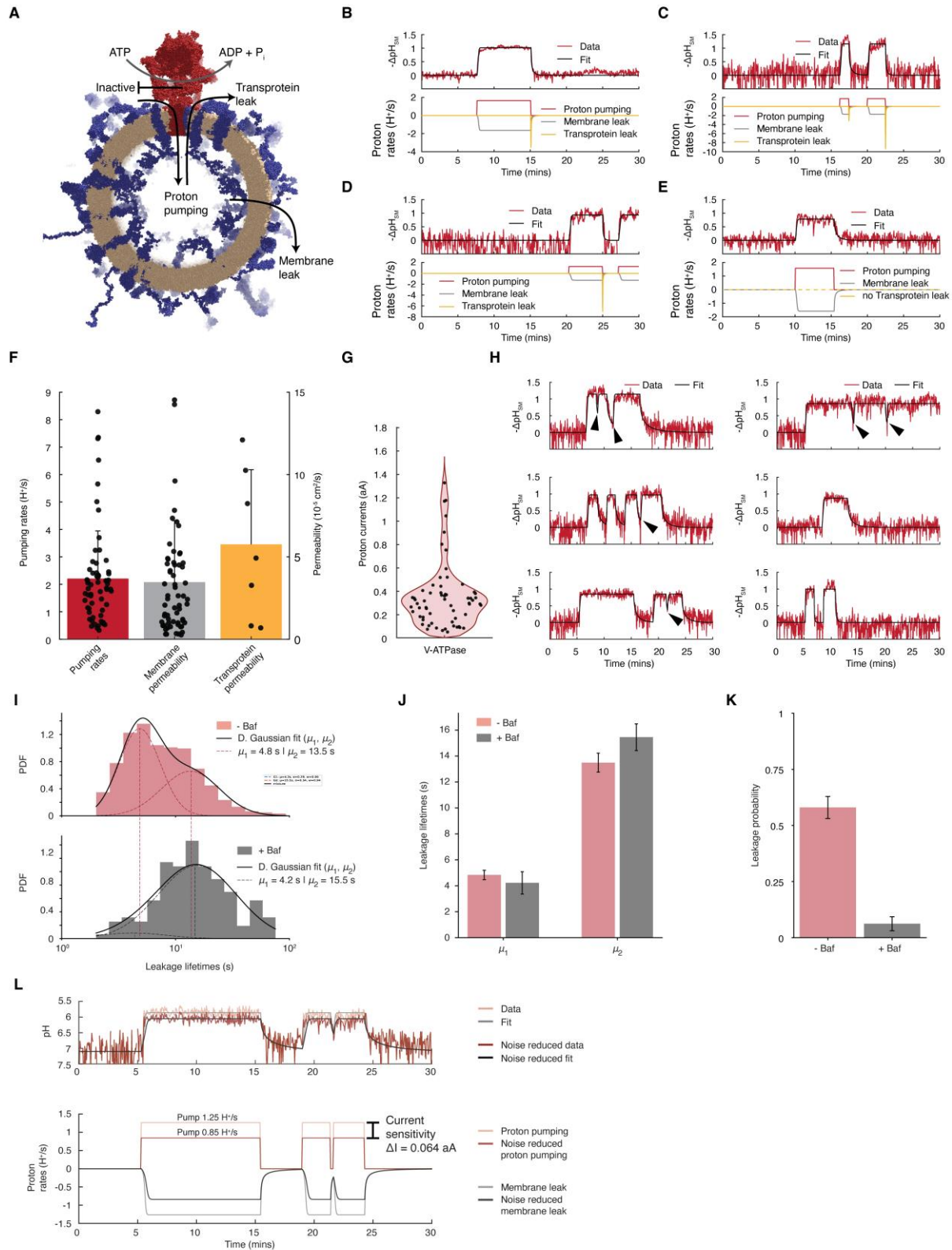


Fig. S3. Non-equilibrium modelling reveals single-molecule proton transport kinetics and leakage pathways.

(A) Schematic of model parameters. The synaptic vesicle illustration is adapted from *Cell*, Vol. 127, Takamori, S. *et al.*, Molecular Anatomy of a Trafficking Organelle, Pages 831–846, Copyright (2006), with permission from Elsevier. **(B)–(E)**, Top: Representative single-molecule traces showing stochastic mode-switching dynamics. Active proton pumping is intermittently interrupted by inactive and proton-leaky modes. During active phases, a dynamic equilibrium is established between pumping and leakage, resulting in a single acidification plateau. During an active mode, which can last up to hours, a V-ATPase may complete thousands of conformational cycles and ATP/H⁺ turnover events before randomly switching to an inactive mode. Leakage arises from both membrane diffusion and transprotein efflux. Transprotein leakage initiates immediately upon V-ATPase deactivation and displays distinct

temporal kinetics. Traces were fitted using a non-equilibrium model (see Supplementary discussion), allowing for the independent estimation of pumping rates and permeabilities. Bottom: Modelled rates for proton pumping (red), membrane leak (grey), and transprotein leak (yellow). In (B)–(D), efflux is dominated by transprotein leakage, while in (E), by membrane diffusion. **(F)** Model-derived proton transport rates. Pumping rates (red) average $2.2 \pm 1.1 \text{ H}^+ \text{ s}^{-1}$. Membrane (grey) and transprotein (yellow) permeabilities are estimated at $\sim 3 \pm 2 \times 10^{-5} \text{ cm s}^{-1}$ and $6 \pm 5 \times 10^{-5} \text{ cm s}^{-1}$, respectively. Error bars, s.d.; $n = 24$ (pumping), $n = 24$ (membrane), $n = 8$ (transprotein). n denotes independent fits on single vesicles. **(G)** Violin plot of atto-Ampere proton currents generated by single V-ATPases. **(H)** Additional single-molecule traces with model fits. Arrows indicate mode-switching events where equilibrium was not reached ($\Delta\text{pH}_{\text{SM}} \approx \text{max or } 0$). Transprotein leakage modes were previously reported^{8,9} but are not further analysed here. **(I)** Histogram of proton leakage lifetimes in active SVs $\pm$ Baf. The fast leakage component (μ_1) corresponds to a transprotein leakage pathway through the V-ATPase, while the slow component (μ_2) corresponds to membrane leakage. **(J)** Characteristic leakage lifetimes extracted from double Gaussian fits of the leakage populations in (I). **(K)** Leakage probability of active V-ATPase $\pm$ Baf shows that bafilomycin dramatically reduces the probability of the transprotein leakage pathway. **(L)** Quantitative estimation of the detectable changes in the steady-state currents. The steady-state plateaus are reduced by one S.D. of the noise observed during these plateaus. Subsequently both the original trace and the noise-reduced trace are fitted with the model (top) and the corresponding proton rates are generated (bottom).

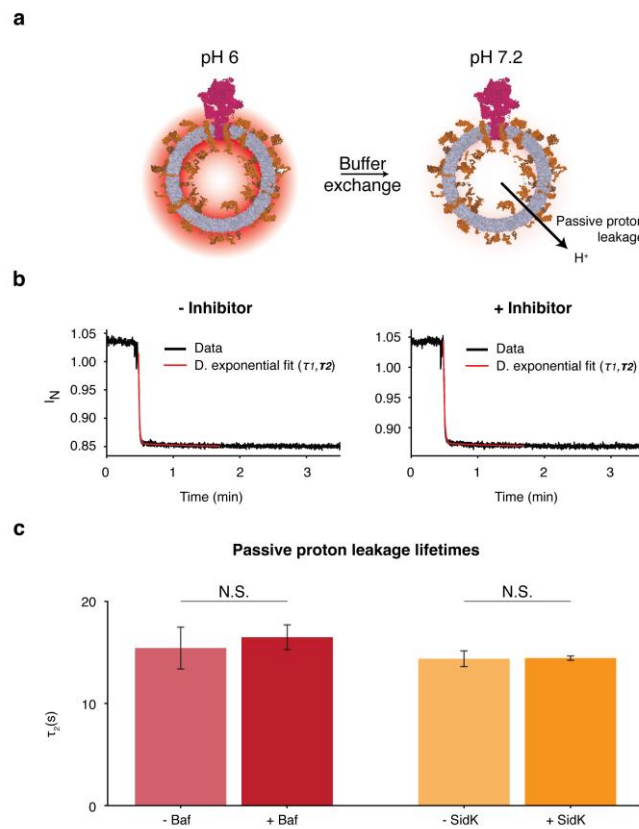


Fig. S14. Bafilomycin and SidK do not affect the intrinsic membrane permeability to protons.

a, Illustration of pH jump experiments. The synaptic vesicle illustration is adapted from *Cell*, Vol. 127, Takamori, S. *et al.*, Molecular Anatomy of a Trafficking Organelle, Pages 831–846, Copyright (2006), with permission from Elsevier. SVs are equilibrated at pH 6 and subsequently are subjected to an external pH of 7.2 such that luminal protons passively permeate across the lipid bilayer inducing a drop in fluorescence intensity. pHrodo lipids are found in both leaflets of the bilayer. Fluorescence response of the outer monolayer has a much faster response time that depends on the flow rate of the microfluidic setup. Contrary, the fluorescence response of pHrodo lipids located in the inner monolayer solely depends on the leakage rate of luminal protons across the membrane. **b**, Ensemble average fluorescence (black and grey traces) of pH jump experiments $\pm$ inhibitor fitted with a double exponential function (red line). **c**, Characteristic lifetimes of the fluorescence response of the inner monolayer (τ_2) $\pm$ Baf or $\pm$ SidK. Mean $\pm$ S.E.M. $n = 3$ independent experiments; Two-sided paired t-test. N.S. ($P = 0.81$, τ_2 , $\pm$ Baf); N.S. ($P = 0.96$, τ_2 , $\pm$ SidK).

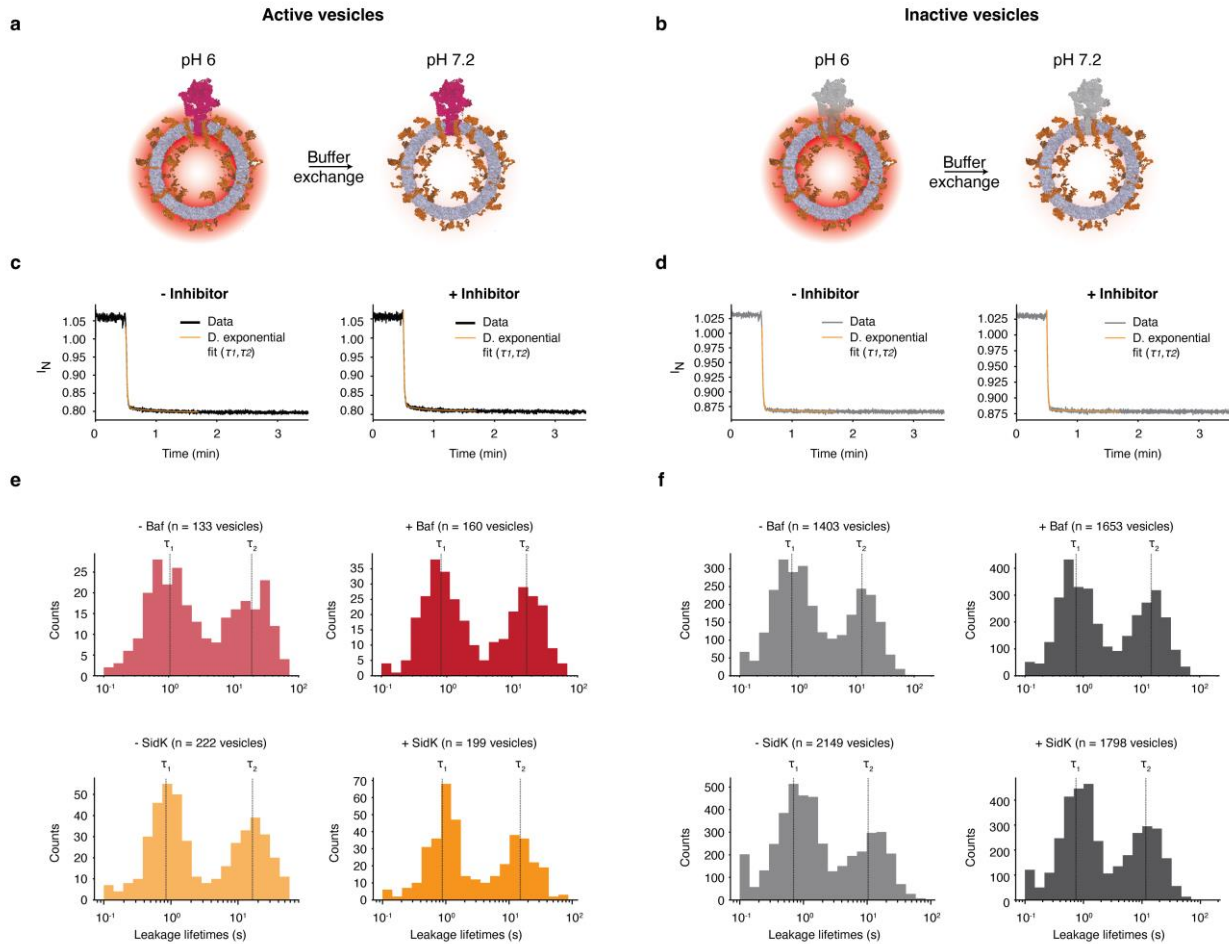


Fig. S15. Additional single-vesicle proton leakage experiments confirm that Baf and SidK do not alter the intrinsic passive permeability of the membrane.

a, b, Illustration of pH jump experiments for vesicles that contained active (a) and inactive (b) V-ATPases. The synaptic vesicle illustration is adapted from *Cell*, Vol. 127, Takamori, S. *et al.*, Molecular Anatomy of a Trafficking Organelle, Pages 831–846, Copyright (2006), with permission from Elsevier. **c, d**, Example traces of fluorescence response (black and grey traces) in pH jump experiments $\pm$ inhibitor for SVs containing active or inactive V-ATPases fitted with a double exponential function (yellow line). **e, f**, Histograms of single-vesicle lifetimes of proton leakage. All single-vesicle pH jump traces were fit with a double exponential function that generated two distinct lifetime populations corresponding to the outer and inner monolayer. The outer monolayer had a characteristic response time, $\tau_1 = 0.8\text{--}1$ s across all conditions and is rate-limited by the microfluidic setup. The inner monolayer generated a mean response time, $\tau_2 = 11\text{--}19$ s across all conditions and represents the lifetimes of proton leakage across the membrane. τ_2 is at least one order of magnitude longer than τ_1 which is consistent with previous findings⁸.

Comment 2.9

Reviewer comment

Furthermore, did the authors measure the ATPase activity of the SV preparation in bulk?

Measuring bulk ATPase rates (with and without inhibitors) would help validate whether the single-molecule rate estimates are physiologically reasonable.

Response

Please see reply to comment 2.8 above.

Suggested changes to the manuscript

In PG4 L107 we have added the sentence: “... consistent with previous literature⁸ that determined pumping rates in hybrid synaptic vesicles to be $\sim 7\text{ H}^+\text{s}^{-1}$...”.

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Orthosteric and allosteric inhibition of the brain V-ATPase outside the alternating-access cycle

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Point-by-point response to reviewers v2

We thank the reviewers for their thoughtful and constructive evaluation of the manuscript. Below we respond point-by-point and, for each comment, we also indicate the specific changes that we incorporated into the revised manuscript.

Reviewer #1

Comment 1.1

I appreciate the authors' thorough responses to my concerns. I have no further comments.

Response

We thank the reviewer for acknowledging the value our work and our responses to their concerns.

Reviewer #2

Comment 2.1

In this revision, several of the previously raised concerns have been addressed appropriately, and the manuscript has been substantially improved. However, the responses to the following points are still not entirely sufficient and require further clarification and improvement. If these issues are adequately addressed, the manuscript is likely to meet the requirements for publication.

Response

We thank the reviewer for acknowledging the value our work and our responses to most of their concerns. Below we address the remaining concerns in a concise manner and to a full extend.

Comment 2.2

Related to previous comment 2–7.

Stability of V_1 Association During ATP Addition

The authors have provided a thoughtful response to the concern regarding the potential dissociation of the V_1 domain upon ATP addition. The clarification that no clearly irreversible OFF events are observed is helpful, and the additional analysis showing a correlation between bounded events and dwell time (Fig. S12) strengthens the argument.

However, the current response does not fully resolve the central issue. The explanation that long-lived OFF events arise from finite observation windows is plausible, but not sufficient to distinguish these events from potential structural dissociation followed by slow reassociation. The observed scaling behavior supports a kinetic interpretation, but does not uniquely rule out alternative mechanisms.

Given that V_1 dissociation under acidification has been reported in the literature, it is important to more clearly address why such behavior is not observed under the present experimental conditions. Differences in membrane environment, ATP concentrations, or luminal pH conditions should be discussed explicitly.

To more definitively resolve this issue, the most straightforward approach would be to biochemically verify that V_1 dissociation does not occur upon ATP addition under the experimental conditions used in this study, for example using the prepared synaptic vesicles.

Response

We thank the reviewer for the careful follow-up. If we understood correctly the central questions are a) whether ATP addition and subsequent vesicle acidification generate *irreversible* OFF events/modes and b) whether such events could be confused with *irreversible* inhibitor-induced OFF events/modes. Below we address these two questions in detail and also outline several related revisions to the manuscript.

Briefly, we never observed *acidification*-induced irreversible OFF events, therefore distinguishing them from *inhibitor*-induced irreversible OFF events is a hypothetical problem that never arose in practice.

1. Since *acidification*-induced irreversible OFF events were never observed, distinguishing them from *inhibitor*-induced irreversible OFF events is a hypothetical problem that never arose in practice.

We agree with the reviewer that complete V_1 dissociation should produce irreversible loss of ATP-driven proton pumping. Indeed, given that the concentration of SVs in our experiments is <0.1 pM, V_1 reassociation time constants would be ~ 10 -60 hrs. However, we never observed irreversible OFF events under apo + ATP conditions. Instead, the apo OFF modes are always reversible and are always followed by renewed proton pumping. Since *acidification*-induced irreversible OFF events were never observed, distinguishing them from *inhibitor*-induced irreversible OFF events is a hypothetical problem that never arose in practice.

Because the apo OFF modes are reversible they do not appear to arise from complete V_1 dissociation. This conclusion is further supported by our observation of OFF modes in a P-type ATPase which does not have a subunit that can dissociate.

More broadly, as already emphasized in the discussion “*long-lived off-cycle conformations (often referred to in the literature as locked or autoinhibited states) are a recurrent feature across diverse families of primary^{1,2} and secondary³⁻⁶ active transporters, as well as in most enzymes studied at the single molecule-level⁷.*”

2. Reversible OFF modes are compatible with reversible V_1 – V_0 rearrangements

The reversibility of OFF modes is compatible with structural rearrangements within the V_1V_0 holoenzyme. As discussed in Kosmidis et al. 2022, “inactive modes could in principle represent early reversible conformational steps along a trajectory that, under other conditions, might lead to V_1 dissociation”. This is now highlighted in the main text.

Revised text: “Because OFF modes are readily reversible, they are likely not due to complete/irreversible dissociation of V_1 , however they could be associated with early reversible conformational steps along a trajectory that, under other conditions, might lead to V_1 dissociation⁸. Overall, the structural mechanism(s) that underpin reversible and irreversible OFF modes remain unclear”.

3. Pre-dissociated or rapidly dissociating complexes may well exist in the sample, but, by definition, they are outside the functional population that we can detect and analyze here upon inhibitor addition.

We further clarify that our experiments do not determine whether dissociated V_0 -only complexes are present in the SV preparation. Such complexes would not acidify upon ATP addition and would therefore be outside the functional single-vesicle population analyzed here.

Similarly, complexes that dissociate upon initiation of ATP hydrolysis, before significant acidification, would also not be detectable and would not contribute to the active trajectories. The suggested classical/bulk biochemical analysis averages over temporal trajectories and thus could not distinguish these populations.

Thus, our conclusion is not that dissociated complexes are absent from the preparation. We have no information, and make no claims, in that regard. Our data only suggest that complete V_1 dissociation likely does not explain the reversible ON/OFF/ON modes observed in active single-vesicle traces and which are regulated by inhibitors in a mostly reversible manner.

Revised text: “Note that by definition our readout does not detect and analyze non-functional complexes, e.g. pre-dissociated V_0 -only complexes or V_0 / V_1 complexes that may dissociate upon ATP hydrolysis and before significant acidification is established”.

4. Our current data do not allow us to elucidate differences among prior studies; such endeavor would be beyond the scope of this manuscript.

We are aware of one study that has reported acidification-dependent V_1/V_0 disassembly in purified SVs which we have cited and acknowledged explicitly⁹. We are also aware of other studies that have not reported this effect^{10,11}. Unfortunately, these studies differ between them in so many experimental aspects (including purification strategy, vesicle handling, specific biochemical conditions used before analysis, and the endpoint structural or biochemical readout) that it is not possible for us to identify plausible causal differences. Most crucially, in our opinion, elucidating differences among prior studies is beyond the scope of this single-molecule pharmacology manuscript.

Though our current data unfortunately do not allow us to determine why different structural or biochemical preparations report different extents of V_1 association or dissociation, we have noted the concern and do hope to provide additional insights in future work.

We have revised the Discussion to acknowledge more explicitly the importance of V_1/V_0 disassembly in the regulation of synaptic-vesicle and neurosecretory systems.

Revised text (Pg12 L321-343): “Several studies have reported pH-dependent V_1/V_0 assembly or disassembly in neurosecretory and synaptic-vesicle systems. These studies were performed in cellular recycling systems^{12,13} or endpoint structural/biochemical^{9,14} and therefore address a different experimental regime from the isolated single-vesicle functional assay used here. In our assay, complete acidification-induced V_1 dissociation would be expected to produce irreversible loss of ATP-driven proton pumping in one-pump vesicles, because no cytosolic V_1 pool or reassembly machinery is present. Because ATP addition alone produces recurrent reversible ON/OFF switching rather than irreversible loss of activity, the apo

OFF mode measured here is not consistent with complete V_1 dissociation under our experimental conditions.

To assess whether long inhibitor-associated OFF periods reflect irreversible loss or finite observation of reversible states, we quantified the fraction of censored OFF events in Fig. S12. The fraction of censored events scales with the mean inactive-mode lifetime, as expected for long-lived reversible states recorded within a bounded observation window. This observation is consistent with reversible events censored by the finite observation window but does not conclusive prove it. Furthermore, it does not rule out reversible rearrangements at or near the V_1/V_0 interface. As discussed in our previous work, reversible OFF states may reflect early, reversible steps along a conformational pathway that, under different biological or biochemical conditions, could progress to full V_1/V_0 dissociation⁸. We furthermore note that our assay reports the functional acidifying population. Pre-dissociated V_0 -only complexes or V_0 / V_1 complexes that may dissociate upon ATP hydrolysis and before significant acidification is established, if present in the preparation, would not be expected to contribute to ATP-dependent acidification traces and would therefore fall outside the analyzed single-molecule population. The data therefore do not exclude the existence of dissociated complexes in the preparation; rather, they suggest that complete V_1 dissociation is not the mechanism underlying the reversible apo OFF modes quantified here.”

5. This is the second ever manuscript on single-molecule V-ATPase function, the very first on pharmacology. We feel structural elucidation of the functional single-molecules phenotypes is beyond the scope of this manuscript.

OFF modes in the *absence* of inhibitors (+ATP, apo) can be distinguished from complete V_1 dissociation because they are always reversible.

In the *presence* of inhibitors, the dominant behavior remains reversible. In Figure S12 at ~EC50 of the four inhibitors, respectively 95%, 95%, 85% and 70% of all events are reversible. We cannot formally exclude that inhibitors induce complete V_1 dissociation in a small subset of molecules, therefore **we remain purposefully agnostic as the structural mechanism(s) underlying OFF modes and their regulation by inhibitors.**

Revised text: “The observation that the occurrence of irreversible OFF events scales with their lifetime, is consistent with reversible events censored by the finite observation window **but does not conclusive prove it.**”

We note that if inhibitors were producing irreversible dissociation of V_1 , our ensemble average activity would be expected to progressively decline to zero (realkalinization), this however is not the case. As can be seen in Fig S9, even though addition of inhibitors leads to rapid reduction in acidification (~1 min) indicating potent inhibition, subsequent activity remains stable for periods of one order of magnitude larger (>10min) consistent with reversible deactivation.

Comment 2.3

Related to previous comment 2–8.

Proton transport rate remains insufficiently explained

The authors attempt to address the concern regarding the unexpectedly low proton transport rate (~2 H^+/s). While the authors cite variability in coupling ratios and literature values, the discussion remains largely generic and does not directly explain why such a low rate is observed in the present experimental system. In particular, the explanation focuses on variability across conditions and prior studies, rather than providing a quantitative, system-specific justification.

Furthermore, the argument based on intermittent activity (i.e., long inactive periods) may account for a reduced time-averaged flux, but it does not clarify whether the intrinsic pumping rate during active periods is itself reduced. This distinction is critical, and the current response conflates these two possibilities. In addition, the authors have not addressed the suggestion to directly measure ATPase activity in the prepared vesicles. It would be important to measure both proton pumping activity and ATPase activity in bulk assays for the same synaptic vesicle preparation, and to include these raw data in the Supplementary Information. A quantitative discussion of the turnover rate in the main text would substantially strengthen the reliability and interpretability of the study.

Response

We thank the reviewer for the careful follow-up. If we understood correctly the central questions are:

1. Can we include in SI measurements of ATP hydrolysis for our SV samples.

Yes indeed, ATP hydrolysis has been measured for our samples, and we can therefore now add in SI an estimate of the perfect theoretical ensemble average coupling ratio. See revised SI (Pg 33, L939) and Table S3 (Pg 57, L 1194).

We note that the literature does not treat this perfect-coupling value as a “fixed expectation”^{15–17}, on the contrary experimentally determined coupling is widely reported to vary.

Item	Quantity	Value / calculation
Input 1	Total ATPase activity in isolated SVs	~75 pmol P _i min ⁻¹ per 1.3 micrograms SV protein
Input 2	NEM-sensitive V-ATPase-specific fraction	~20%, corresponding to ~15 pmol P _i min ⁻¹ per 1.3 micrograms SV protein
Step 1	SV number in 1.3 micrograms protein	Using ~42 MDa protein mass per SV: 1.3 micrograms / 6.98 x 10 ⁻¹¹ micrograms per SV = 1.86 x 10 ¹⁰ SVs
Step 2	ATP hydrolysis per SV	15 x 10 ⁻¹² mol min ⁻¹ / 1.86 x 10 ¹⁰ SVs = 8.06 x 10 ⁻²² mol min ⁻¹ per SV = ~485 ATP min ⁻¹ per SV = ~8.1 ATP s ⁻¹ per SV
Step 3	ATP hydrolysis per V-ATPase	Using 1.4 V-ATPases per SV: 8.1 / 1.4 = ~5.8 ATP s ⁻¹ per V-ATPase
Step 4	Perfect structural coupling	10 H ⁺ per 3 ATP gives 5.8 x 3.33 = ~19 H ⁺ s ⁻¹ per V-ATPase

2. Have we been reporting the intrinsic pumping rate of active periods/modes?

Yes indeed, throughout the text, SI and reply-to-reviewers we have been consistently reporting the pumping rate of ON modes (active periods).

3. Is the intrinsic pumping rate smaller than what would be theoretically expected from ATP hydrolysis assuming the structural stoichiometry and perfect coupling?

Yes indeed, the intrinsic pumping rates we reported here for intact SVs and in Kosmidis et al. for hybrid SVs² (~2.2 and 7 H⁺/s respectively), are 3-fold to 8-fold smaller than what would be expected from ATP hydrolysis measurements assuming the structural stoichiometry and perfect coupling.

4. Are we the first to report deviations from the theoretical perfect coupling ratio?

No, we are not the first to report deviations from perfect theoretical coupling, such observations are widespread. There is broad consensus in the field that V-ATPase ATP hydrolysis rates and proton pumping rates are not always proportional because the coupling ratio is not fixed, see e.g.^{15–17} and references therein.

Importantly, there is no “expectation” in the field that the experimentally measured coupling ratio should match the perfect theoretical one, on the contrary.

5. Why are the intrinsic pumping rates different than what theoretically expected from the structural stoichiometry assuming perfect coupling?

Neither we, nor the field in general, know exactly why the intrinsic pumping rates are consistently reported to be different than what theoretically expected from the structural stoichiometry assuming perfect coupling.

The deviation of measured coupling-ratios from the structural predictions is one of the main issues under debate in the field and resolving it is beyond the scope of the current manuscript which does not focus on the *absolute* value of the proton pumping rates (nor on the absolute value of the coupling stoichiometry) but in *relative changes* in proton pumping upon inhibition.

Prior V-ATPase literature shows that ATP hydrolysis and net proton transport cannot generally be described by a single/fixed coupling ratio. Mechanochemical treatments predict strong dependence of apparent proton flux and coupling on electrochemical conditions, and electrophysiological measurements in yeast vacuoles have reported variable apparent coupling ratios and proton shunt behavior^{15–17}. Additional biochemical and inhibitor studies show that V-ATPase proton transport and ATPase activity can be partially uncoupled¹⁸. Thus, **the structural stoichiometry provides an upper-bound mechanical reference, not a universal productive net flux expectation.**

6. Are there obvious reasons why we our measurements may systematically underestimate the proton pumping rates?

To our knowledge, there no obvious reasons why we our measurements may systematically underestimate the proton pumping rates.

To our knowledge, here and in Kosmidis et al., we provide the most accurate estimation of pumping rates ever reported because **for the first time we explicitly deconvolve pumping rates** from 1) P_{ON} , 2) buffering, 3) passive proton leakage, 4) transprotein leakage, 5) counter-ion conductance and 6) membrane potential.

7. Detection limit and sensitivity

We have revised the manuscript to add a system-specific simulation analysis that supports our ability to accurately measure rates of few protons per second and changes therein, Fig. S16. There we solve the same pump-leak-buffer-voltage framework under the experimental conditions of the single-vesicle assay, including vesicle geometry, passive proton permeability, luminal buffering, chloride permeability, membrane potential, and chloride-dependent charge compensation.

These simulations show that active-state proton fluxes of only a few $H^+ s^{-1}$ are sufficient to generate and maintain the experimentally observed ΔpH range in nanoscale SVs. The reason is physical rather than ad hoc. The synaptic-vesicle lumen is extremely small, so only a limited number of free protons is needed to establish substantial acidification. Once a steady-state gradient is reached (~ 20 s), the pump does not continuously acidify a large reservoir; it primarily balances passive proton leakage under the prevailing electrochemical load. With chloride-mediated charge compensation, the electrical penalty for electrogenic proton influx is minimized, so low active-state fluxes can readily maintain the observed gradients.

The same simulations also provide a quantitative description into how ΔpH would be adjusted under poor charge-compensation conditions, such as absence of external chloride (see SI Fig. 7 in Kosmidis et al.), low chloride permeability, or unfavorable positive membrane potentials, whereby proton accumulation is increasingly opposed by the electrochemical gradient as described in the literature^{15–17}.

Revised text (Pg33-34 L914-940):

“Physical model-based simulations

To test whether low active-state proton pumping rates are sufficient to generate experimentally observed synaptic-vesicle pH gradients under our experimental conditions, we solved the non-equilibrium pump-leak-buffer-voltage model for single synaptic vesicles. These simulations show that active-state pumping rates of only a few $H^+ s^{-1}$ are sufficient to generate and maintain the observed ΔpH range when chloride conductance compensates electrogenic proton influx. Changes as small as 1 $H^+ s^{-1}$ are readily detected. This follows from the very small luminal volume of synaptic vesicles and from the fact that, at steady state, the pump primarily balances passive proton leakage rather than acidifying a large reservoir. The same simulations show that substantially higher rates would be required under poor charge-compensation conditions or unfavorable positive membrane potentials due to very low chloride permeability or complete absence of external chloride.

Bulk ATPase Benchmark for Single-Vesicle Proton Flux

Our isolated SV preparations have a total ATPase activity of $\sim 75 \text{ pmol Pi min}^{-1}$ per 1.3 micrograms SV protein, a NEM-sensitive V-ATPase-specific fraction of $\sim 20\%$, and a total protein mass of $\sim 42 \text{ MDa}$ per SV, 1.4 V-ATPases per SV. Assuming perfect coupling of 10 H^{+} per 3 ATP, the corresponding upper-bound estimate of proton pumping is $\sim 19 \text{ H}^{+} \text{ s}^{-1}$ per V-ATPase. This value is higher than the $\sim 2.2 \text{ H}^{+} \text{ s}^{-1}$ active-state net flux measured here, confirming that the fitted rate is below a perfect-coupling estimate. However, this comparison assumes fixed productive coupling and cannot account for electrochemical load, or uncoupled ATP hydrolysis. Thus, the perfect-coupling estimate should be interpreted as a useful reference point rather than as a model-independent expectation for net proton flux in intact SVs. See also table S3."

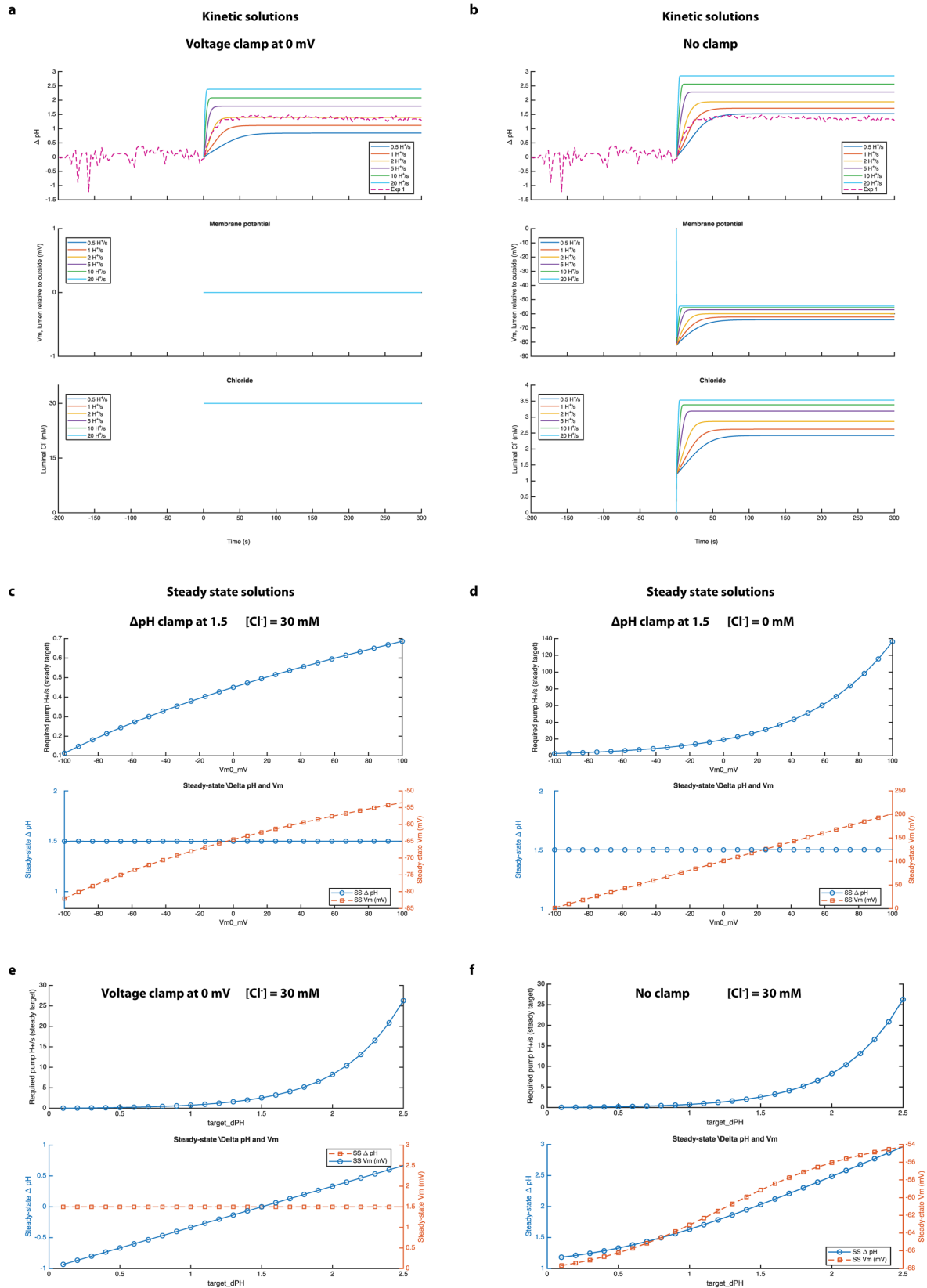


Fig. S16. Non-equilibrium simulations show that low active-state proton pumping rates are sufficient to generate experimentally observed synaptic-vesicle pH gradients.

a–b, Kinetic simulations of single-vesicle acidification after ATP addition. The model solves the coupled time evolution of luminal pH, membrane potential, and luminal chloride concentration for a single synaptic vesicle with experimentally relevant volume, passive proton permeability, buffer capacity, membrane capacitance, and chloride

permeability. Proton pumping was simulated at the indicated active-state rates, while passive proton leak, chloride flux, and voltage buildup were calculated dynamically. The magenta trace shows an example experimental single-vesicle trace aligned to ATP addition. With voltage clamped at 0 mV (**a**), corresponding to ideal charge compensation, increasing pump rate primarily increases the ΔpH plateau. Without voltage clamp (**b**), proton pumping drives membrane-potential changes and chloride influx, but low H^+ s^{-1} pumping rates still reproduce the experimentally observed acidification range. **c–d**, Required pumping rate at fixed target ΔpH . Steady-state calculations determined the proton-pumping rate required to maintain $\Delta\text{pH} = 1.5$ while varying membrane potential. With external chloride present, $\text{Cl}^-_{\text{out}} = 30 \text{ mM}$ (**c**), the required pump rate can remain at low levels across a broad voltage range. In contrast, without external chloride (**d**), the required rate increases steeply at positive membrane potentials, illustrating that poor charge compensation can strongly penalize proton accumulation. **e–f**, Required pumping rate as a function of target ΔpH . Under voltage clamp at 0 mV with, $\text{Cl}^-_{\text{out}} = 30 \text{ mM}$ (**e**), low proton-pumping rates are sufficient to maintain ΔpH values within the experimentally observed range. The required rate increases nonlinearly only at larger gradients, where passive proton leak becomes stronger. Similar behavior is observed without voltage clamp when chloride is present (**f**), indicating that chloride permeability efficiently compensates electrogenic proton influx under the simulated experimental conditions. Together, these simulations provide a system-specific quantitative justification for the low active-state pumping rates inferred from single-vesicle measurements. Because synaptic vesicles have very small luminal volumes, only a limited number of free protons is required to generate substantial acidification, and under chloride-compensated conditions the pump mainly needs to balance passive proton leakage. Thus, rates of a few H^+ s^{-1} are sufficient to establish and maintain the observed ΔpH , supporting the interpretation that the measured low rate reflects active-state proton flux rather than a time average diluted by inactive periods.

Comment 2.4

Reviewer comment

minor point

The chemical structure of concanamycin appears to be incorrect. Please revise it using the correct structure.

Response

We thank the reviewer for this observation. We have now added the correct structure of Concanamycin A in Figure 3.

Reviewer #3

Comment 3.1

Reviewer comment

Kosmidis et al. investigated the orthosteric and allosteric inhibition of V-ATPase using the single-vesicle assay they previously developed. In this work, they newly identified an inhibitor-induced off-cycle mode. Although the mechanistic basis by which the inhibitors affect both the on/off state transitions and the intrinsic pumping rate remains unclear, the observations are sufficiently novel and interesting to the field. All of my concerns have been adequately addressed in the point-by-point response, and I therefore support publication of this manuscript. I only have one minor comment.

minor comment

1. In Figure 1F, the authors used a single y-axis. However, the y-axis for P_{ON} is unitless and should not be presented together with ΔpH on the same scale. At first glance, this was somewhat confusing.

Response

We thank the reviewer for the response and the acknowledgement of the quality of our work. We have now corrected Figure 1F by separating the axes for P_{ON} and ΔpH .

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