

Supplementary Information

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

Authors: Eleftherios Kosmidis^{1,2§}, Maria Fokaeos^{1§}, Christopher G. Shuttle^{1,2}, Michael Isselstein^{1##}, Alessandra Narducci¹, Aleksander Cvjetkovic^{1#}, Peter J. Johnson³, Jesper L. Pedersen⁴, Reinhard Jahn⁵, Julia Preobraschenski^{6, 7, 8, 9}, Dimitrios Stamou^{1,2*}

Corresponding author: stamou@chem.ku.dk

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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 ($\Delta \text{pH}_{\text{SM}} \sim 1\text{-}1.5$)

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 .

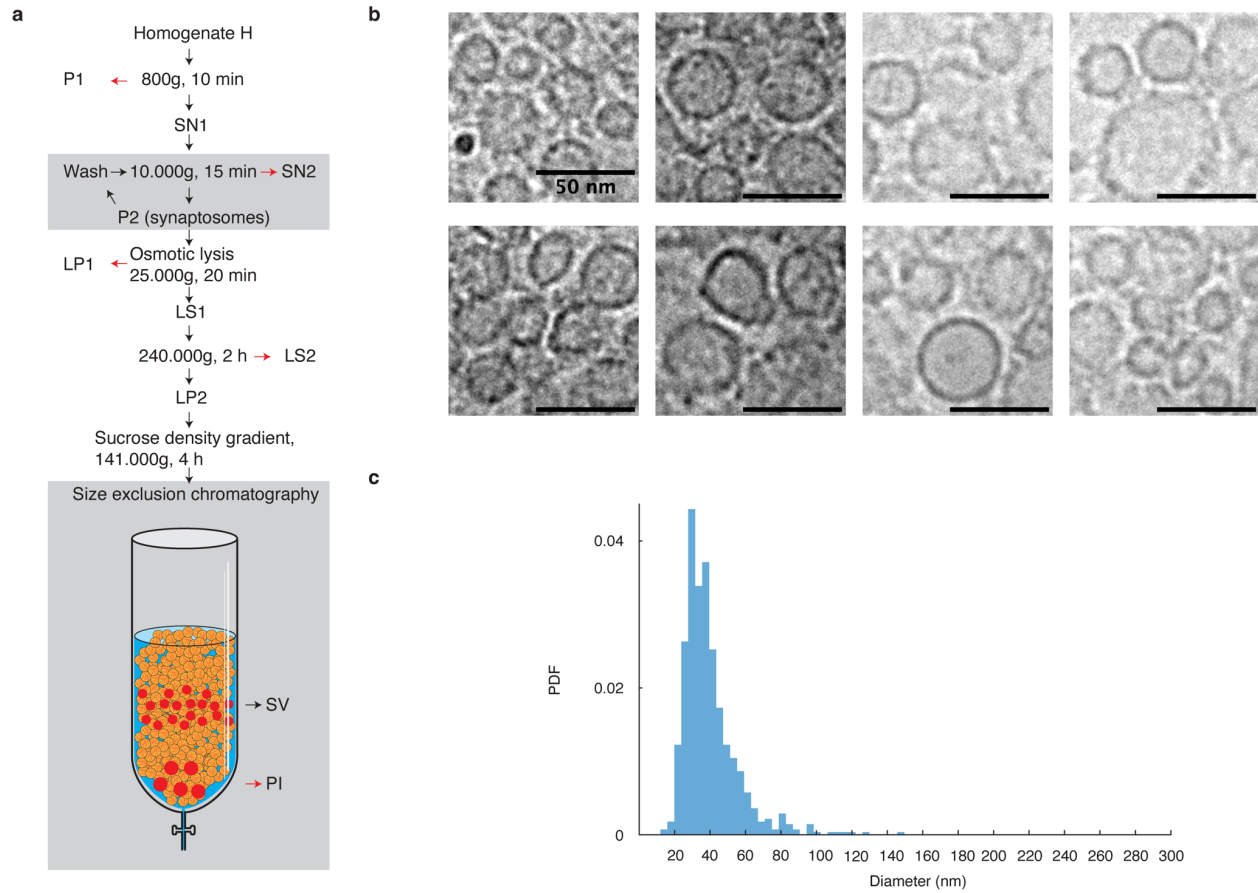
We then estimated the probability density functions (pdf) of observing a given ΔBIC value under each model, $p_{\text{Single}}(\Delta\text{BIC})$ and $p_{\text{Double}}(\Delta\text{BIC})$ respectively using Kernel Density Estimation (KDE) in Python ¹⁵.

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

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

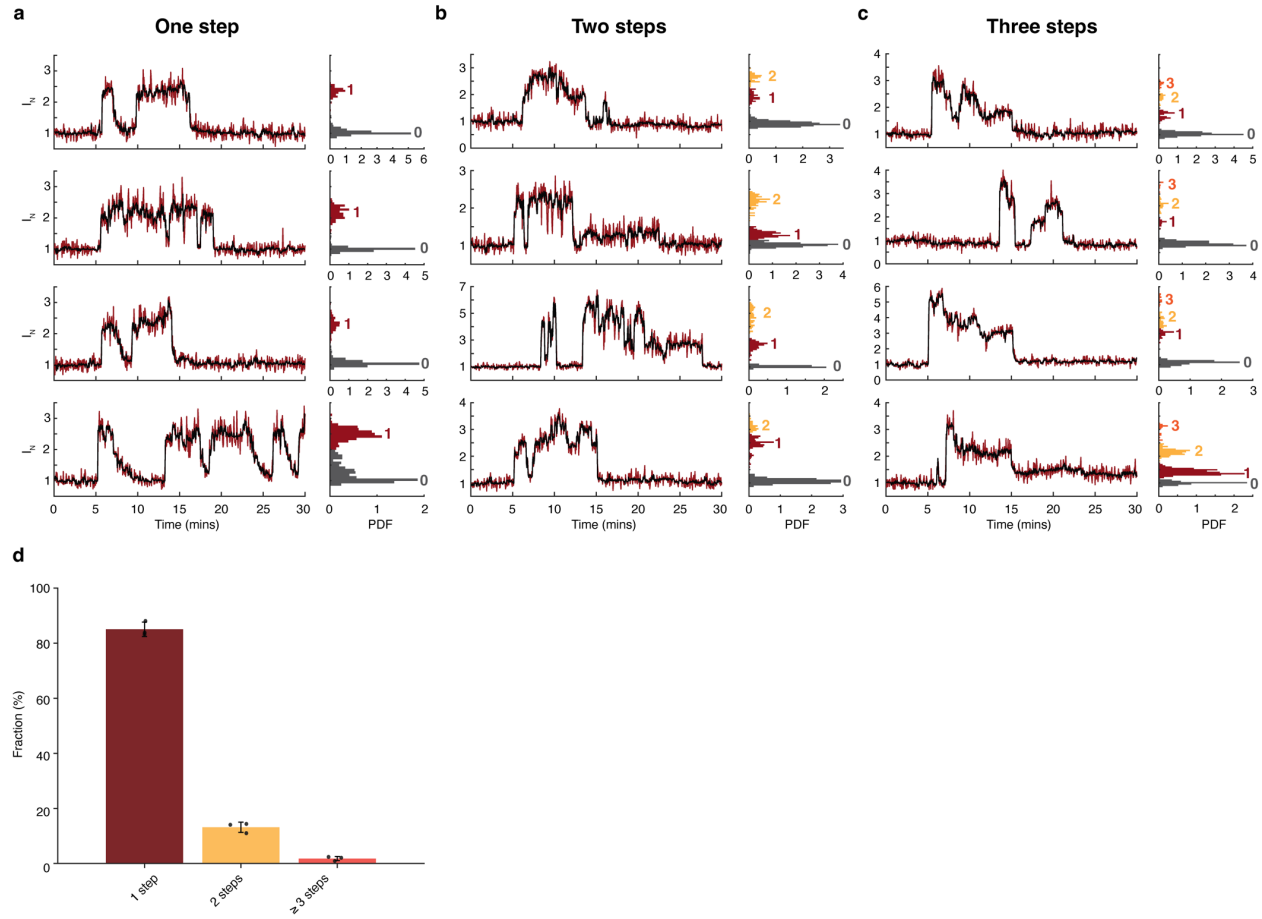
assuming equal priors since no prior knowledge is available to favour either of the two models. Results of this analysis are found in Supplementary Table 1.

Supplementary Figures



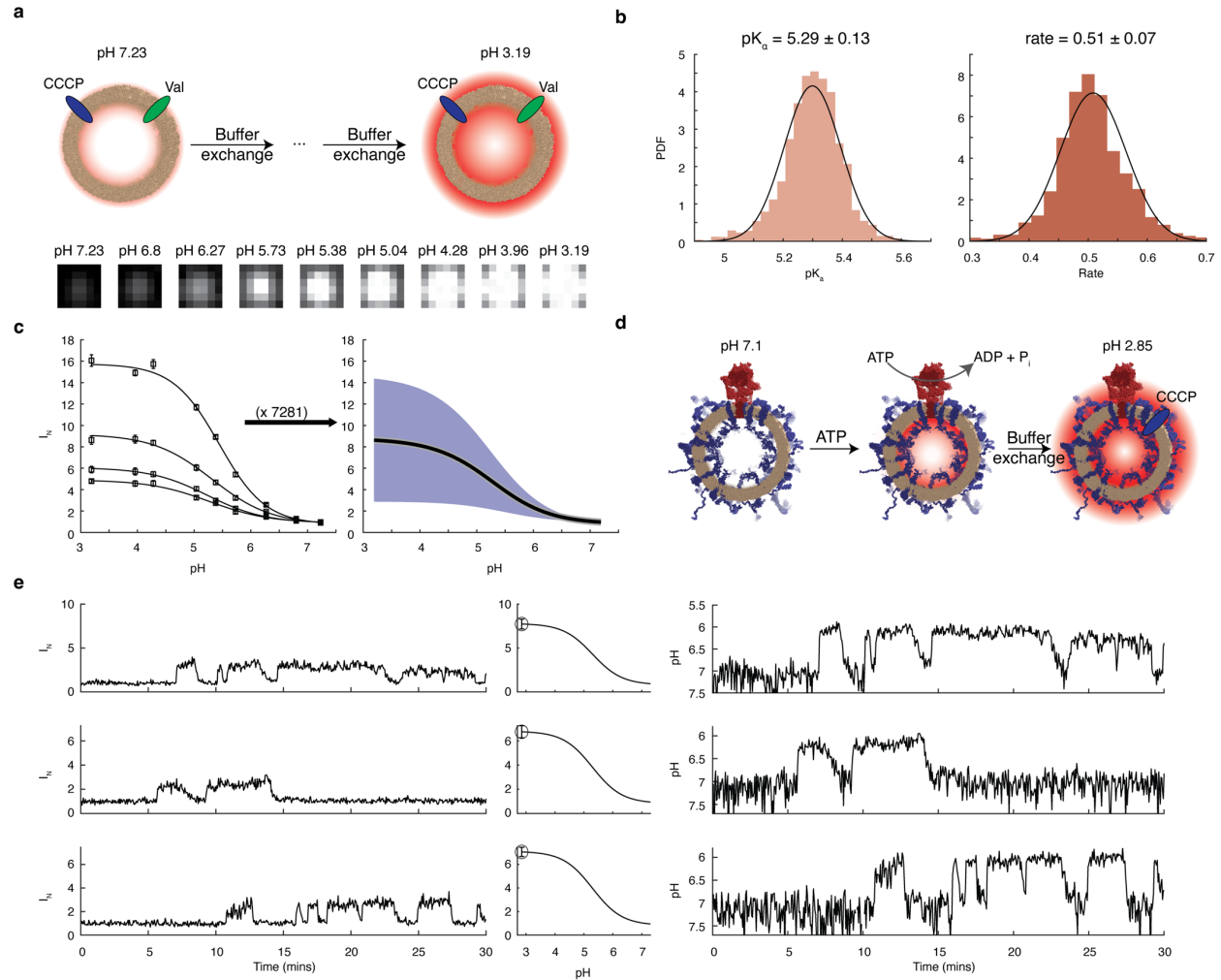
Supplementary Fig. 1. Purification and morphology of SVs.

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.

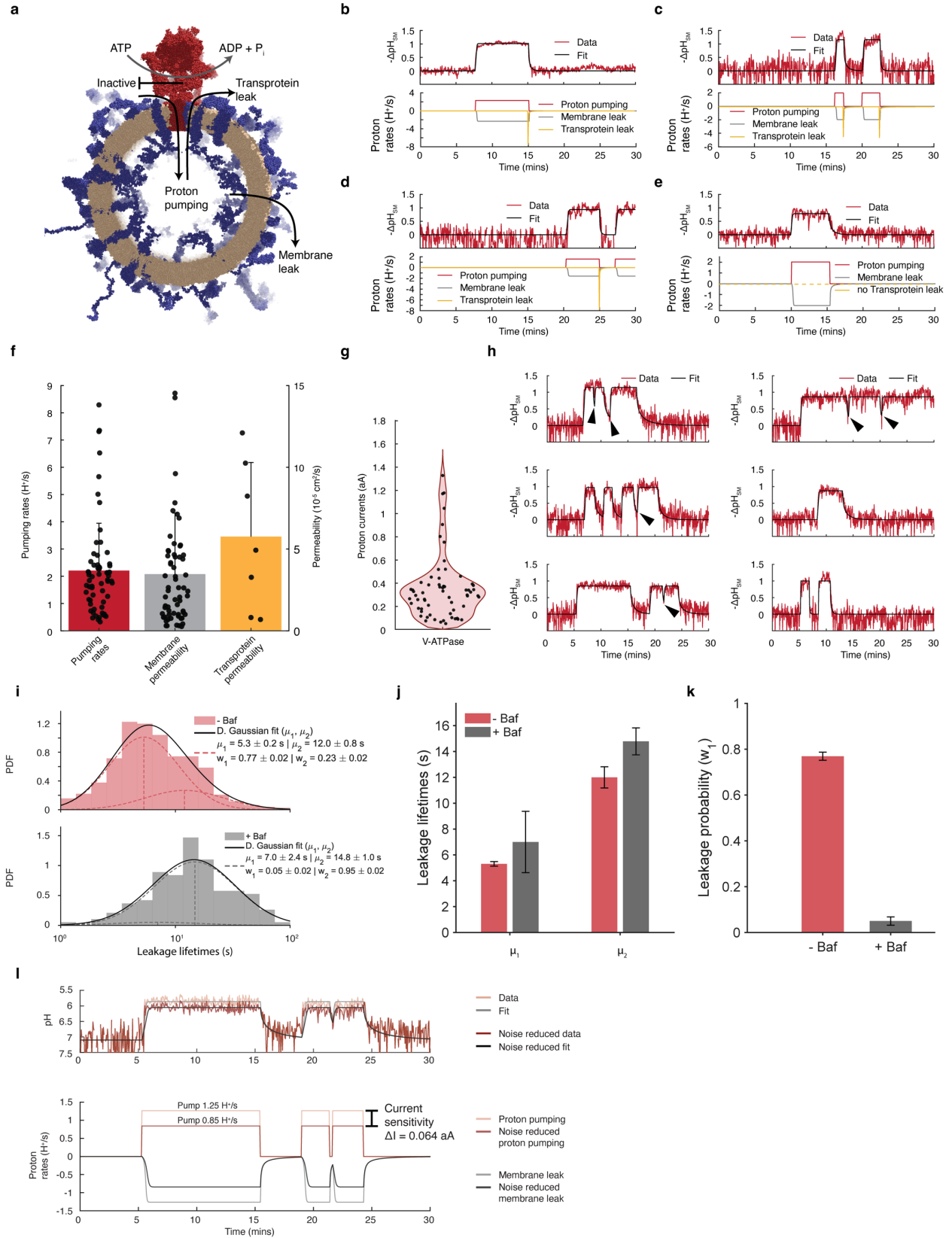
a-c, Left: Single vesicle acidification kinetics showcasing mode-switching dynamics with different acidification plateaus (steps). Each acidification plateau corresponds to a different dynamic equilibrium between pumping and leakage. Red traces correspond to normalized intensities, while black traces 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.

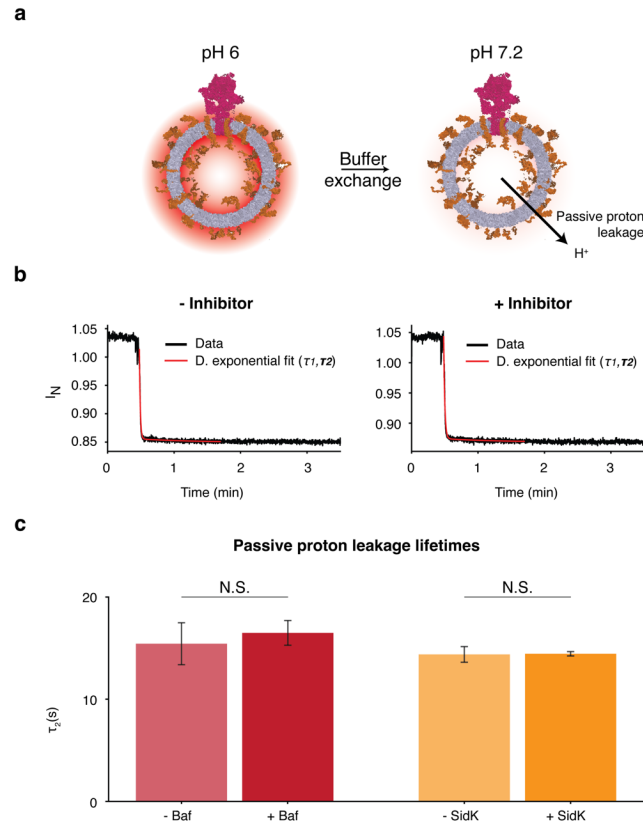
a, Illustration of the pH titration experiments to determine the calibration parameters pK_a and growth rate. Briefly, large unilamellar vesicles (LUVs) are immobilized on the glass surface and incubated in a calibration buffer containing K-Gluconate-MOPS at pH 7.23. Then buffers of the same composition 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.



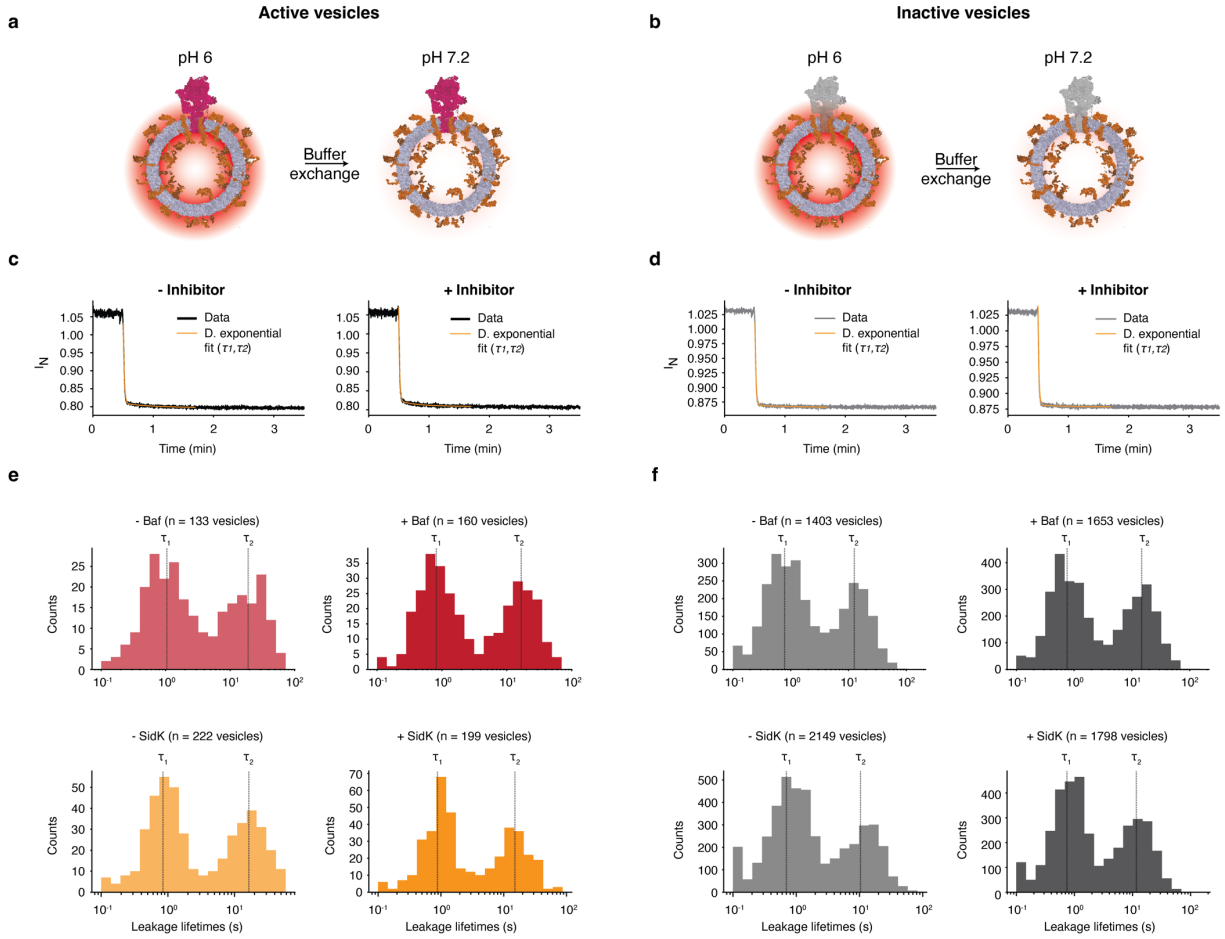
Supplementary Fig. 4. Non-equilibrium modelling reveals single-molecule proton transport kinetics and leakage pathways.

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).



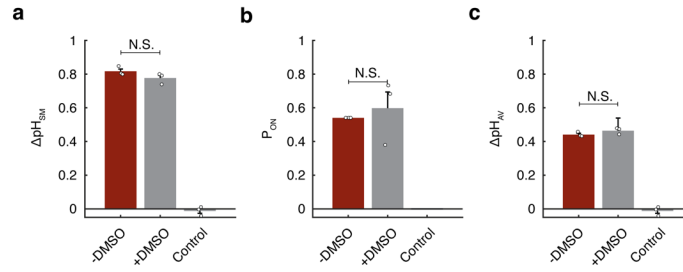
Supplementary Fig. 5. Bafilomycin and SidK do not affect the intrinsic membrane permeability to protons.

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).



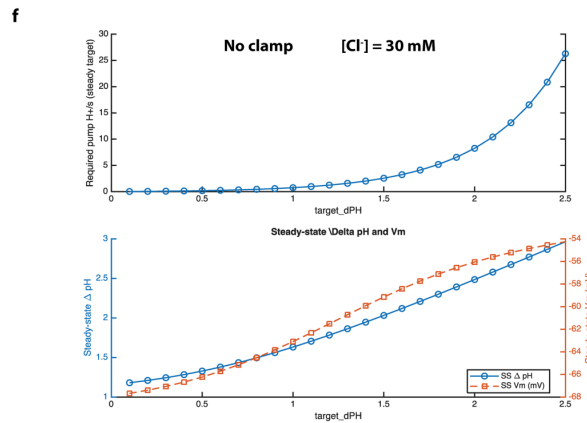
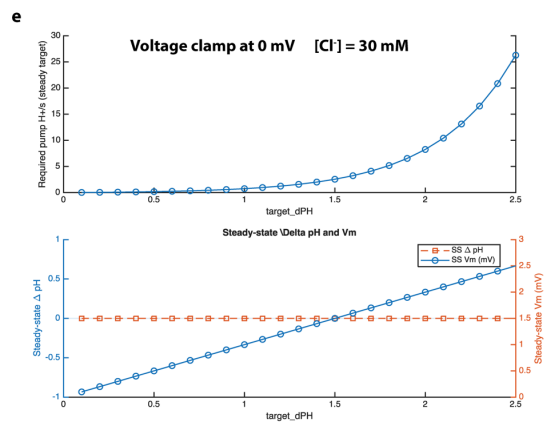
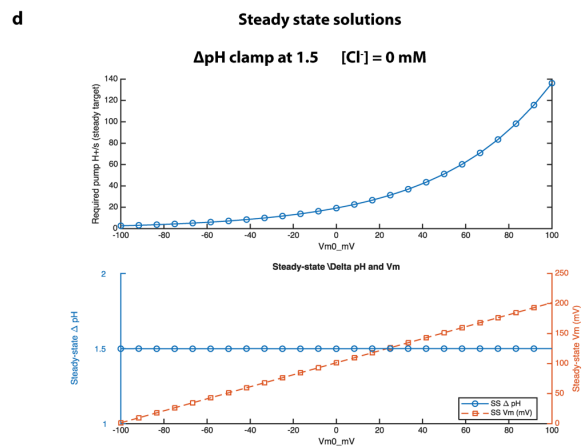
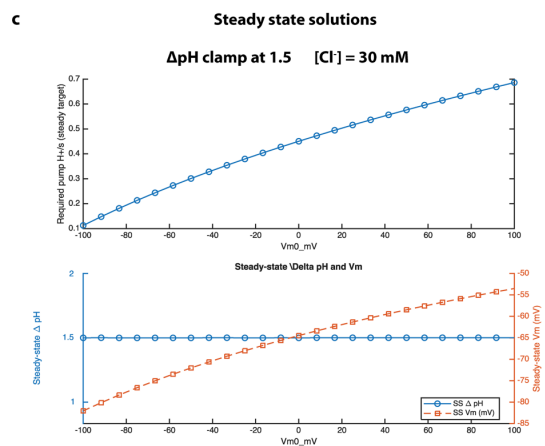
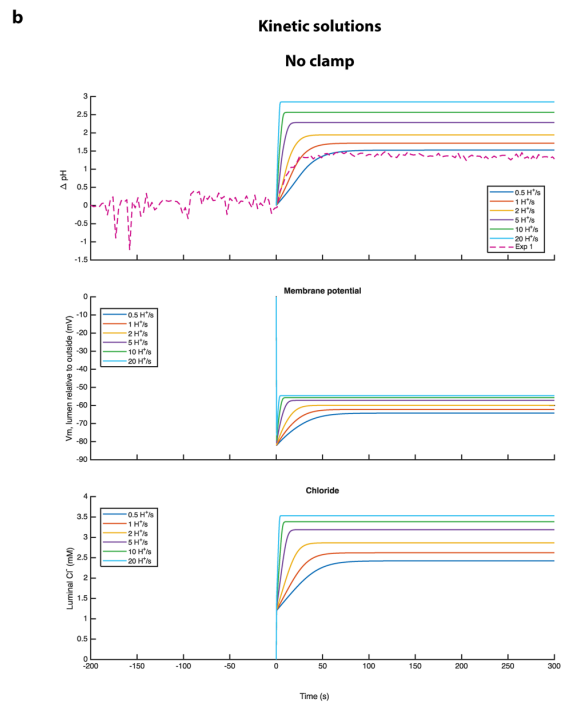
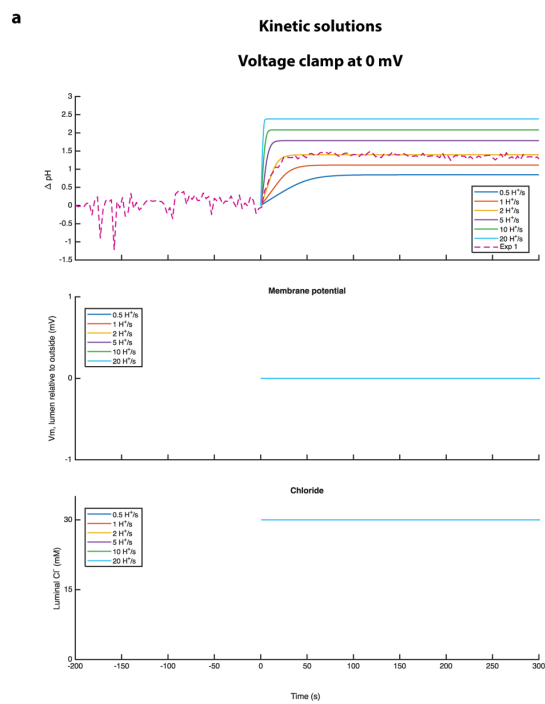
Supplementary Fig. 6. Additional single-vesicle proton leakage experiments confirm that Baf and SidK do not alter the intrinsic passive permeability of the membrane.

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.



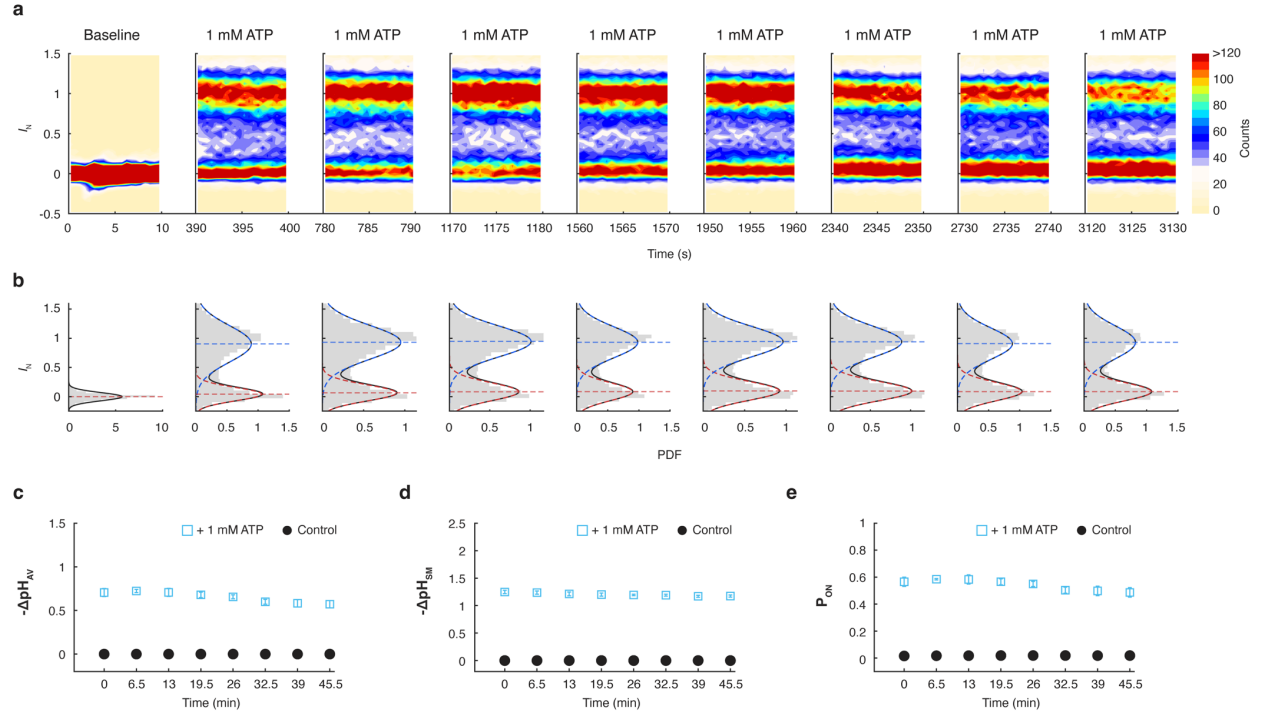
Supplementary Fig. 7. DMSO does not affect single-molecule and ensemble average pH gradients and active mode probabilities.

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.



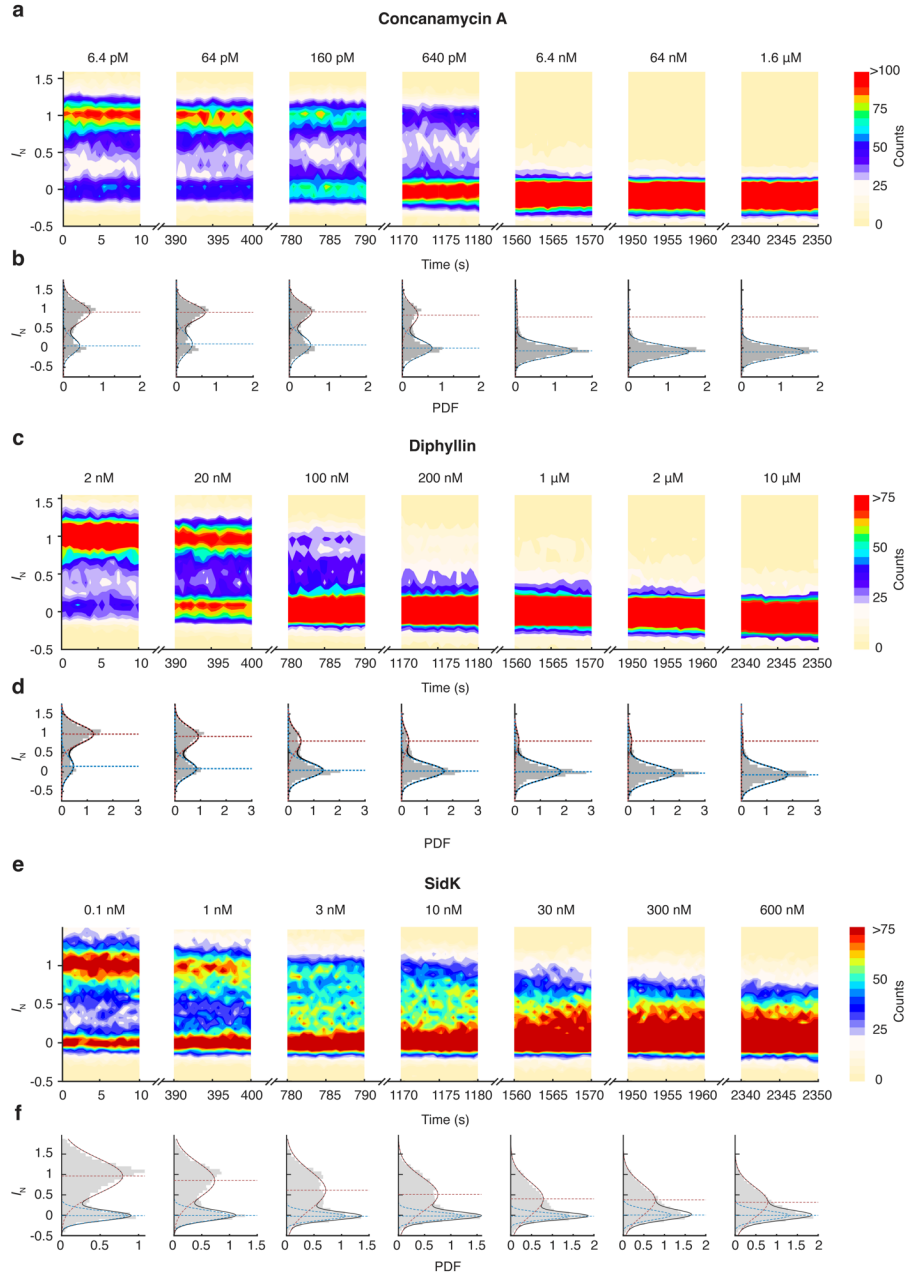
Supplementary Fig. 8. Non-equilibrium simulations show that low active-state proton pumping rates are sufficient to generate experimentally observed synaptic-vesicle pH gradients.

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.

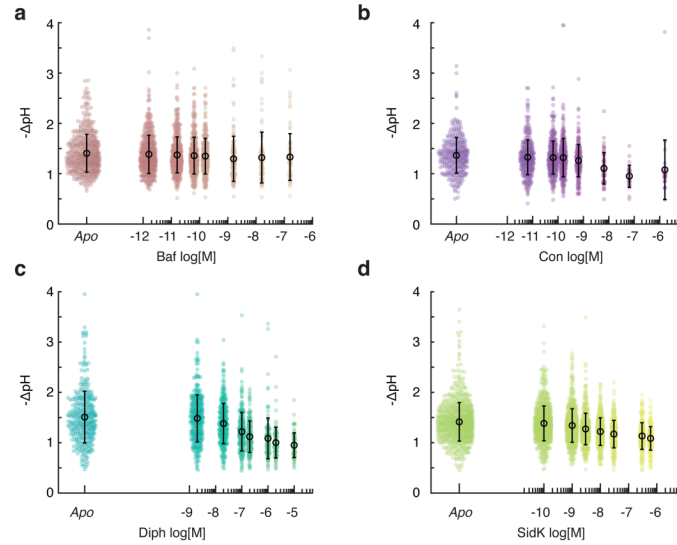
a, Contour plots of normalized fluorescence intensity over time reveal discrete transitions between inactive ($I_N \approx 0$) and active ($I_N \approx 1$) modes at a constant ATP concentration (1 mM ATP). **b**, Histograms of accumulated intensities fit to a double Gaussian distribution; mode occupancies 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.

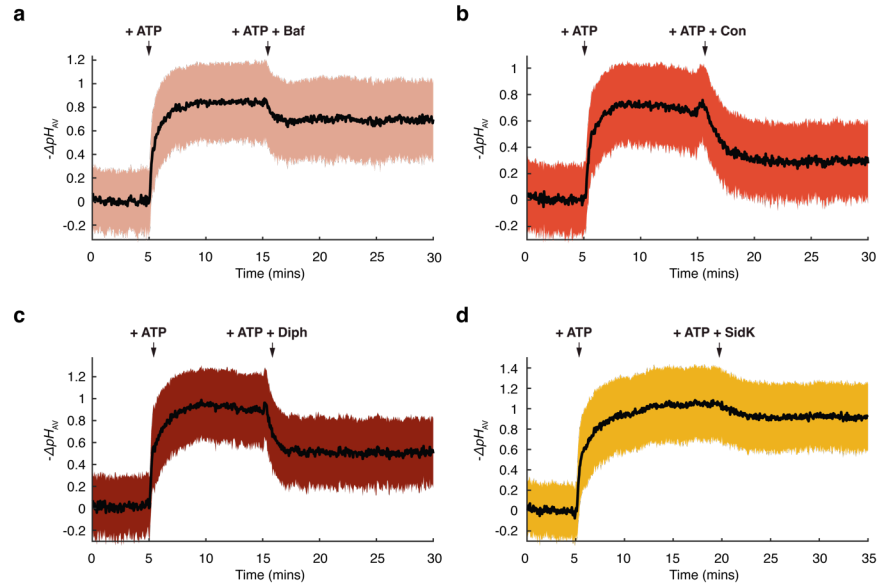
a, Contour plots of normalized fluorescence intensity over time reveal discrete transitions between inactive ($I_N \approx 0$) and active ($I_N \approx 1$) modes across Con concentrations. **b**, Histograms of accumulated intensities fit to a double Gaussian distribution, increasing Con shifts occupancy 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.



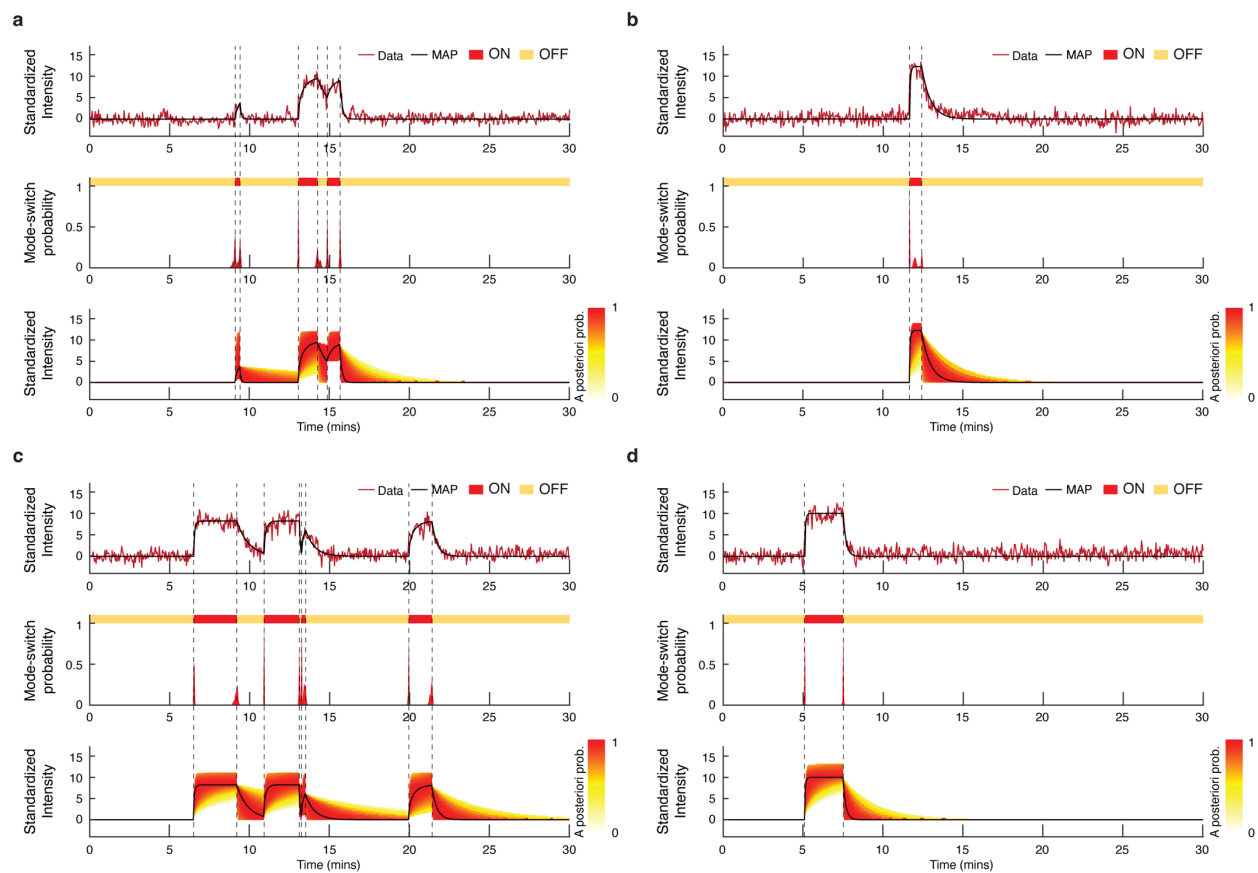
Supplementary Fig. 11. Orthosteric and allosteric inhibitors predominantly regulate the fraction of V-ATPases in an active mode and can regulate proton gradients.

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.



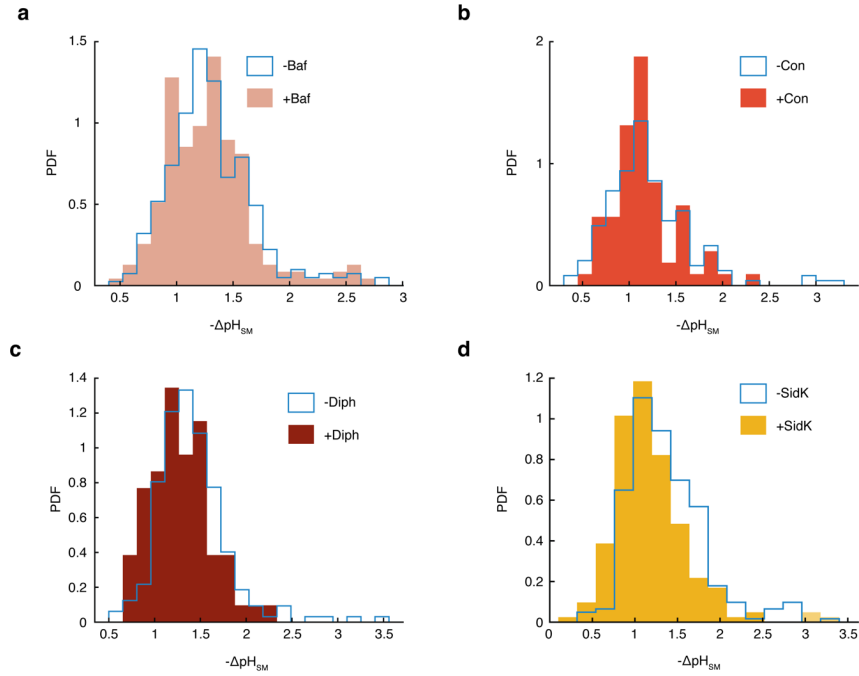
Supplementary Fig. 12. Partial reduction of ensemble average acidification by the V-ATPase at intermediate concentration of inhibitors.

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.



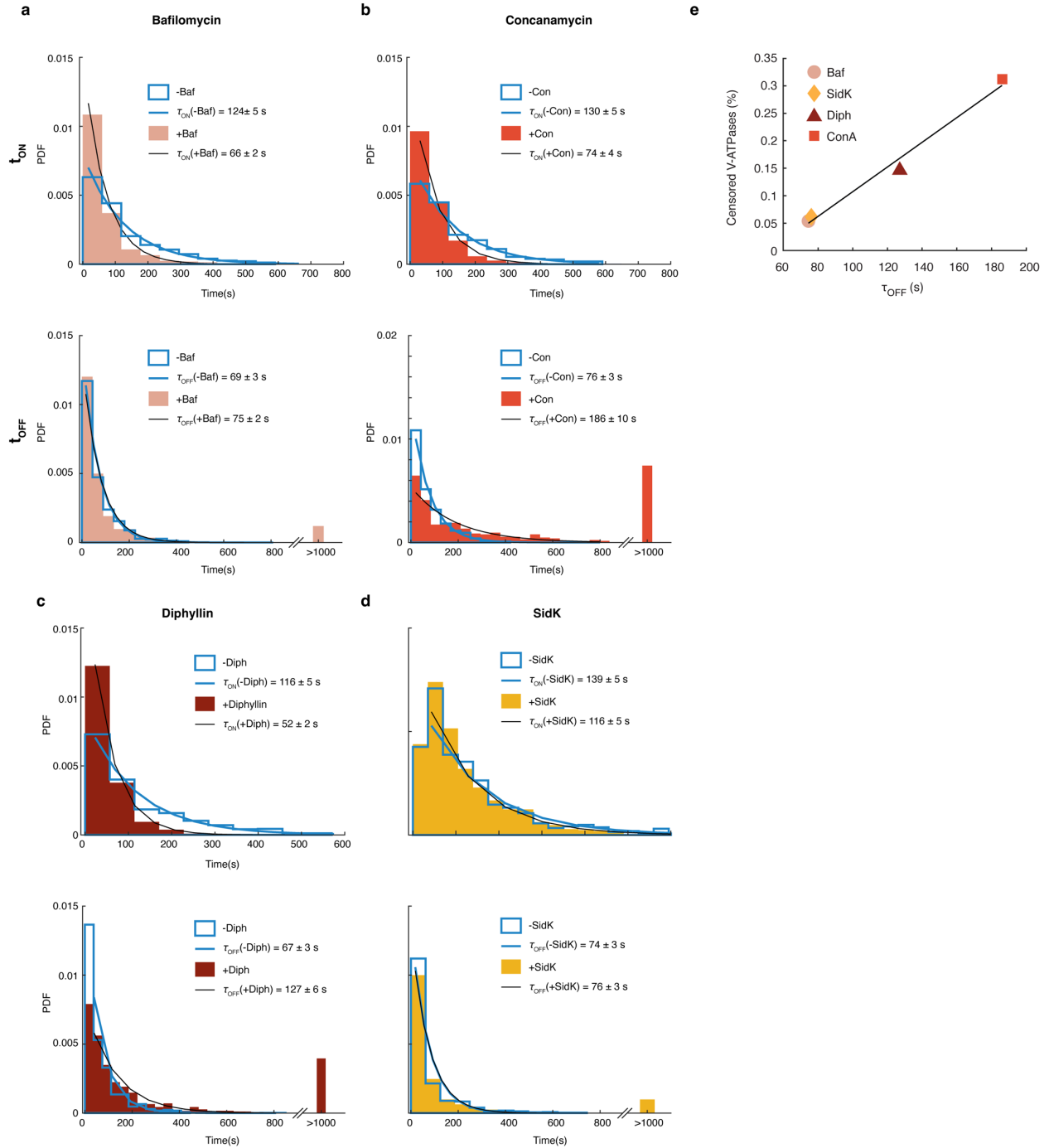
Supplementary Fig. 13. MAP-based detection of mode-switching events using a stochastic exponential model.

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.

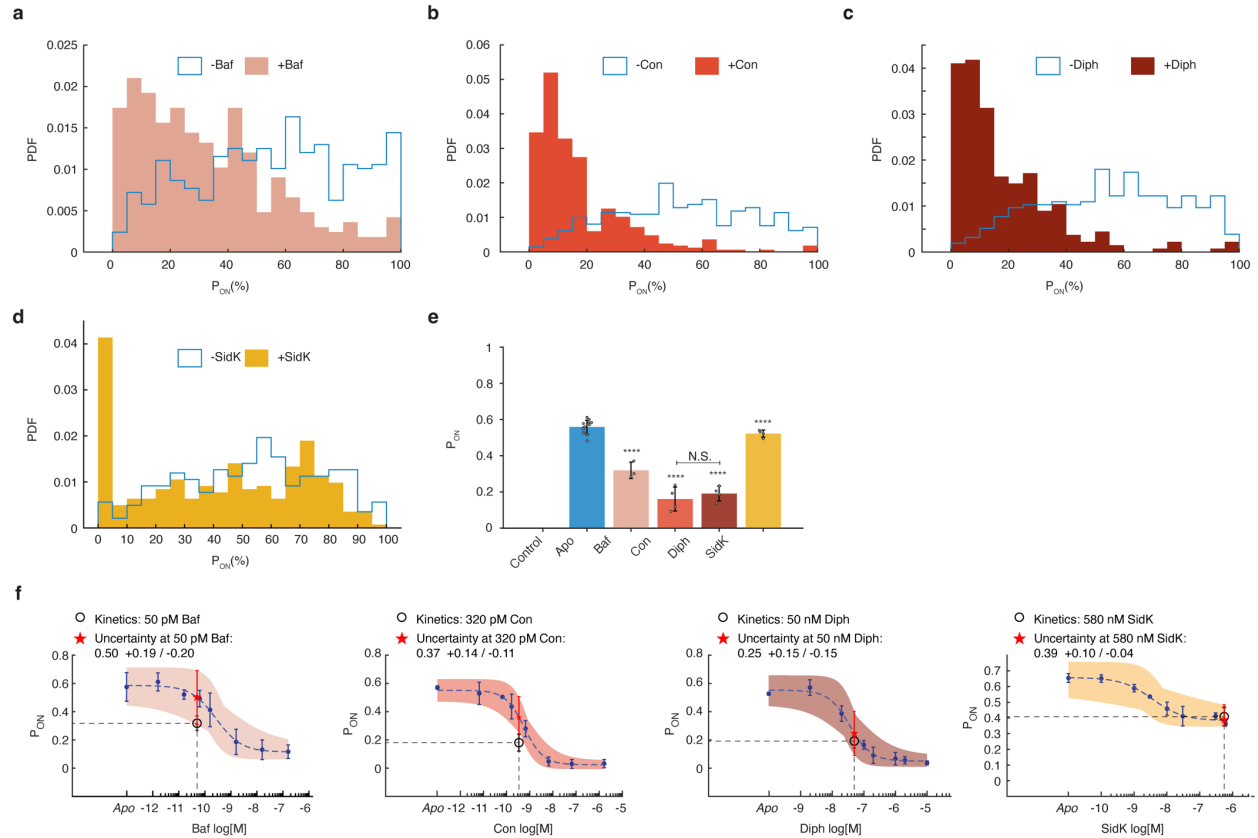
a-d, Distributions of single-molecule acidification levels in SVs by the V-ATPase in the presence and absence of inhibitors estimated from acidification plateaus of single-molecule kinetic measurements. Concentrations of inhibitors used were: 50 pM Baf; 320 pM Con; 50 nM Diph. n.s. ($P = 0.36$, $\pm$ 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, diphyllin 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, diphyllin 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, diphyllin 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).

Supplementary References

1. Furst, E. M. Particle tracking with Matlab. (2015).
2. Guizar, M. Efficient subpixel image registration by cross-correlation (<https://www.mathworks.com/matlabcentral/fileexchange/18401-efficient-subpixel-image-registration-by-cross-correlation>), MATLAB Central File Exchange. Retrieved September 28, 2021. (2021).
3. Preobraschenski, J., Zander, J.-F., Suzuki, T., Ahnert-Hilger, G. & Jahn, R. Vesicular Glutamate Transporters Use Flexible Anion and Cation Binding Sites for Efficient Accumulation of Neurotransmitter. *Neuron* **84**, 1287–1301 (2014).
4. Preobraschenski, J. *et al.* Dual and Direction-Selective Mechanisms of Phosphate Transport by the Vesicular Glutamate Transporter. *Cell Rep.* **23**, 535–545 (2018).
5. Farsi, Z. *et al.* Single-vesicle imaging reveals different transport mechanisms between glutamatergic and GABAergic vesicles. *Science* **351**, 981–984 (2016).
6. Chung, S. H. & Kennedy, R. A. Forward-backward non-linear filtering technique for extracting small biological signals from noise. *J. Neurosci. Methods* **40**, 71–86 (1991).
7. Kosmidis, E. *et al.* Regulation of the mammalian-brain V-ATPase through ultraslow mode-switching. *Nature* **611**, 827–834 (2022).
8. Wang, C. *et al.* Structure and topography of the synaptic V-ATPase–synaptophysin complex. *Nature* 1–6 (2024) doi:10.1038/s41586-024-07610-x.
9. Takamori, S. *et al.* Molecular Anatomy of a Trafficking Organelle. *Cell* **127**, 831–846 (2006).

10. Mutch, S. A. *et al.* Protein Quantification at the Single Vesicle Level Reveals That a Subset of Synaptic Vesicle Proteins Are Trafficked with High Precision. *J. Neurosci.* **31**, 1461–1470 (2011).
11. Veshaguri, S. *et al.* Direct observation of proton pumping by a eukaryotic P-type ATPase. *Science* **351**, 1469–1473 (2016).
12. Ishida, Y., Nayak, S., Mindell, J. A. & Grabe, M. A model of lysosomal pH regulation. *J. Gen. Physiol.* **141**, 705–720 (2013).
13. Laidler, K. J. & King, M. C. Development of transition-state theory. *J. Phys. Chem.* **87**, 2657–2664 (1983).
14. Johnson, P. & Pedersen, J. L. Bayesian changepoint detection for epidemic models. *Sci. Rep.* **15**, 20545 (2025).
15. Virtanen, P. *et al.* SciPy 1.0: fundamental algorithms for scientific computing in Python. *Nat. Methods* **17**, 261–272 (2020).
16. Baraglia, E. C., Fattorini, G., Chattaraj, S., Pasqualini, F. & Conti, F. An Open-Science Computational Model of Organelle Acidification to Integrate Putative Mechanisms of Synaptic Vesicle Acidification and Filling. *Neurochem. Res.* **50**, 181 (2025).