

Reporting Summary

Nature Portfolio wishes to improve the reproducibility of the work that we publish. This form provides structure for consistency and transparency in reporting. For further information on Nature Portfolio policies, see our [Editorial Policies](#) and the [Editorial Policy Checklist](#).

Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

n/a Confirmed

- | | | |
|-------------------------------------|-------------------------------------|--|
| ☐ | ☒ | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement |
| ☐ | ☒ | A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☐ | ☒ | The statistical test(s) used AND whether they are one- or two-sided
<i>Only common tests should be described solely by name; describe more complex techniques in the Methods section.</i> |
| ☒ | ☐ | A description of all covariates tested |
| ☐ | ☒ | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ☐ | ☒ | A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| ☐ | ☒ | For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
<i>Give P values as exact values whenever suitable.</i> |
| ☐ | ☒ | 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 |

Our web collection on [statistics for biologists](#) contains articles on many of the points above.

Software and code

Policy information about [availability of computer code](#)

Data collection	Microscopy data was collected using the commercially available software: - xcellence rt (version 2.0, Olympus, now discontinued) - cellSens Dimension (version 3.2, Olympus)
Data analysis	All data analysis and modelling was performed in custom software written in MATLAB 2018a and newer versions up to 2020b. Bayesian filtering algorithm was written in C++11.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio [guidelines for submitting code & software](#) for further information.

Data

Policy information about [availability of data](#)

All manuscripts must include a [data availability statement](#). This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

The data that support the findings of this study are available from the corresponding authors upon request.

Human research participants

Policy information about [studies involving human research participants and Sex and Gender in Research](#).

Reporting on sex and gender	Not applicable
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.

Field-specific reporting

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☒ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see [nature.com/documents/nr-reporting-summary-flat.pdf](https://www.nature.com/documents/nr-reporting-summary-flat.pdf)

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 $n \geq 3$ with the only exception of data shown in Figure S7 where $n = 2$. n is defined as the number of independent runs of recordings per condition. 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 1000 liposomes in each FOV per repeat as stated in the manuscript. Liposomes were detected via a particle tracking algorithm and selected via various thresholding techniques. Due to each experimental run containing ~1000 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 15 independent experiments (typically $n = 3-15$). Only exception is the data in Fig. S7 ($n = 2$) and the cryo-electron micrographs in Fig. S1 and S2 ($n = 1$, 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.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

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

Methods

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.

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