Supporting Information

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SI Materials and Methods

Slice Preparation and Electrophysiology. Transverse slices of 200 μm thickness from 7- to 9-d-old (for dual patch) or 13- to 15-d-old (for long-term stimulus) C57/BL6 mice were prepared with a vibratome (Leica VT 1200s) (1). All electrophysiological experiments were performed at room temperature (22–24 °C) with artificial cerebrospinal fluid (ACSF) containing the following (in millimolar) 125 NaCl, 2.5 KCl, 25 $NaHCO_3$, 3 *myo*-inostol, 2 Na-pyruvate, 1.25 NaH_2PO_4, 0.4 ascorbic acid, 25 D-glucose, 1 $MgCl_2$, and 2 $CaCl_2$ at a pH of 7.4 when oxygenated (95% O_2 and 5% CO_2). The presynaptic pipette (3–4 MΩ) solution contained (in millimolar) 125 Cs-gluconate, 20 CsCl, 4 MgATP, 10 Na_2-phosphocreatine, 0.3 GTP, 10 Hepes, and 0.05 BAPTA (pH 7.2 adjusted with CsOH). The postsynaptic pipette (2–3 MΩ) solution contained (in millimolar) 125 K-gluconate, 20 KCl, 4 MgATP, 10 Na_2-phosphocreatine, 0.3 GTP, 10 Hepes, and 0.5 EGTA (pH 7.2 adjusted with KOH). Presynaptic and postsynaptic whole-cell recording were obtained with an EPC-10 amplifier (HEKA) and an Axopatch 200B amplifier (Axon Instruments), with series resistances <15 MΩ and 10 MΩ compensated by 60% and 95% (lag 10 μs), respectively. The holding potential of −80 mV was corrected for a liquid junction of −11 mV between the extracellular and pipette solution (2, 3). For dual-patch experiments, tetrodotoxin (TTX; 1 μM) and tetraethylammonium (TEA; 20 mM, replacing 20 mM NaCl) were added to block Na^+ and K^+ channels, and cyclothiazide (CTZ; 0.1 mM) and kynurenic acid (KYN; 1 mM) were applied to prevent postsynaptic EPSC saturation and desensitization. Afferent stimuli were delivered by electrical stimulation (0.1 ms, 3–30 V) via a bipolar electrode positioned at the midline of the trapezoid body and EPSCs were recorded in the presence of CTZ (0.1 mM, all experiments) and KYN (0.1 mM, in Fig. 3 *D* and *E*). Folimycin (Calbiochem) was first dissolved in DMSO and then in ACSF. Stock solution (57.7 μM) was prepared and stored at −80 °C. The drug was diluted in ACSF to a final concentration of 2 μM on the day of use (4). The final DMSO concentration was <0.3%. The drug was applied to the patched cells by puffing at a distance of less than one cell diameter via a Picospritzer III microinjector.

Data were acquired using PatchMaster software at a sampling frequency of 20 kHz and filtered at 5 kHz. All data were analyzed with Igor Pro-6.2 (WaveMetrics).

FM Labeling and Fluorescence Imaging. Slices were incubated in 12 μM FM1-43FX-containing bath solution for 10 min and challenged with 15 min high K^+ (57.5 mM), followed with another 20–30 min for the styrl dye to load into recycling vesicles. Without washing, labeled slices were fixed with 2.5% (vol/vol) glutaraldehyde and cut into ∼200-μm parasagittal sections on a vibratome. The fluorescence images were collected on a two-photon microscope (FV1000 MPE Olympus BX61WI microscope with a 40 × 1 N.A. dipping objective) at 810-nm excitation.

Photoconversion and TEM. FM1-43FX–labeled slices were fixed with 2.5% (vol/vol) glutaraldehyde and photoconverted following a 10-min preincubation in diaminobenzidine (1.5 mg/mL). Blue light illumination from a mercury lamp was applied for ∼30 min. Slices were rinsed in PBS, and only tissue containing the medial nucleus of the trapezoid body (MNTB) was dissected under a stereoscope. The MNTB sections were postfixed in 1% osmium tetroxide for 1.5 h, dehydrated in an ascending series of ethanols [30, 50, 70, 85, 95, and 100% (vol/vol)], and stored in epoxy (EPON 812) overnight. The section was then embedded in epoxy resin at 60 °C for 48-h polymerization. Ultrathin sections (∼70 nm thick) were cut by Leica EM UC6 μLtramicrotome and mounted on Formvar-coated slot grids. After poststaining with 2% (wt/vol) uranyl acetate and 8% (wt/vol) lead citrate, ultrathin sections were examined on a Tecnai Spirit TEM (FEI) at 120 kV.

The quantification of vesicles as photoconverted (PC+) or nonphotoconverted (PC−) was carried out based on methods described previously (5).

FIB/SEM. C57/BL6 mice 13–15 d old were deeply anesthetized with a ketamine (100 mg/kg)/xylazine (20 mg/kg) mixture and fixed by transcardial perfusion of 2% (vol/vol) paraformaldehyde and 2% (vol/vol) glutaraldehyde. The brain was removed, fixed overnight at 4 °C, and then cut into ∼100-μm-thick sections. Only tissue containing the MNTB was dissected out and incubated in a mixture of 1.5% (wt/vol) potassium ferricyanide and 1% osmium tetroxide for 2 h, followed by 1% osmium tetroxide alone for another 1 h. After rinsing in distilled water, the samples were stained *en bloc* with 2% (wt/vol) uranyl acetate overnight, dehydrated in an ascending series of ethanols, and finally infiltrated with EPON 812 resin overnight. The sections were embedded in freshly made resin and polymerized at 60 °C for 48 h.

Semithin sections were obtained from the surface of the block and stained with toluidine blue to ensure that the calyx of Held was easily seen during subsequent observation using an SEM. The block was then glued onto a 45° inclined SEM aluminum sample stub using conductive silver paint and all of the surface of the block was coated with a layer of carbon to prevent charging artifacts. The ultrastructural 3D study was carried out using a Helios Nanolab 600i dual-beam SEM (FEI), which combines high-resolution field-emission SEM with a focused beam of gallium ions. A 1-μm-thick layer of platinum was deposited on the surface of the block above the region of interest to reduce FIB milling artifacts. The sequential automated use of FIB milling and SEM imaging was as described previously (6).

For the reconstruction of compartments of the calyx terminal, the block face was photographed at a total image size of 2,048 × 1,768 pixels and a field size of 4 × 3.45 μm. An acceleration voltage of 2 keV, a current of 0.17 or 0.34 nA, and a dwell time of 30 μs per pixel was used in serial imaging by the electron beam. The 20-nm-thick layer of the block was milled by FIB at an acceleration voltage of 30 keV and a current of 0.79 nA. For the reconstruction of the whole calyx of Held synapse, the block face was photographed at a total image size of 4,096 × 4,096 pixels and a field size of 45 × 45 μm, using an acceleration voltage of 3 keV, a current of 2.7 nA, and a dwell time of 10 μs per pixel. The milling depth was 100 nm completed by FIB at an acceleration voltage of 30 keV and a current of 2.5 nA.

All datasets were analyzed using TrakEM2 in the Fiji software package (fiji.sc/wiki/index.php/Fiji). After Z-stacks were aligned, synapses of interest were manually traced and reconstructed in 3D. Active zones were justified and distinguished from puncta adherentia based on a previous study (7).

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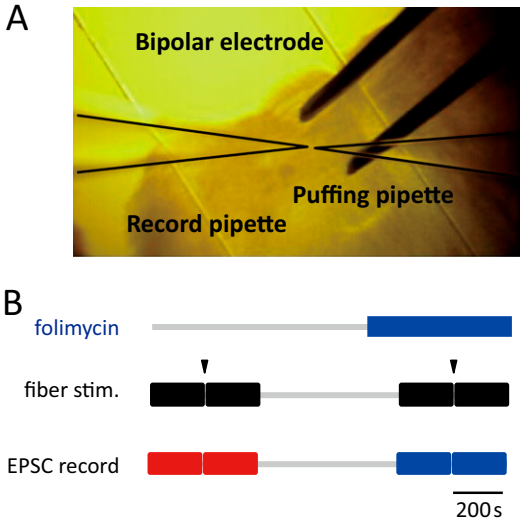


Fig. S1. Experimental protocol of EPSC recordings during sustained stimulation. (*A*) Differential interference contrast image (5×) of a brain slice containing the MNTB region. A bipolar electrode was used to stimulate afferent fibers, a patch pipette was used to make postsynaptic whole-cell recordings, and a puffing pipette was placed upstream of the circulation flow to apply 2 mM folimycin-containing bath solution. (*B*) Schematic of the experimental protocol. EPSCs were recorded during fiber stimulation trains (black) with 10-s Rs compensation breaks (arrowheads). After control recording (red) and ~17 min of rest for completion of endocytosis, folimycin was applied 3 min before folimycin recording (blue).

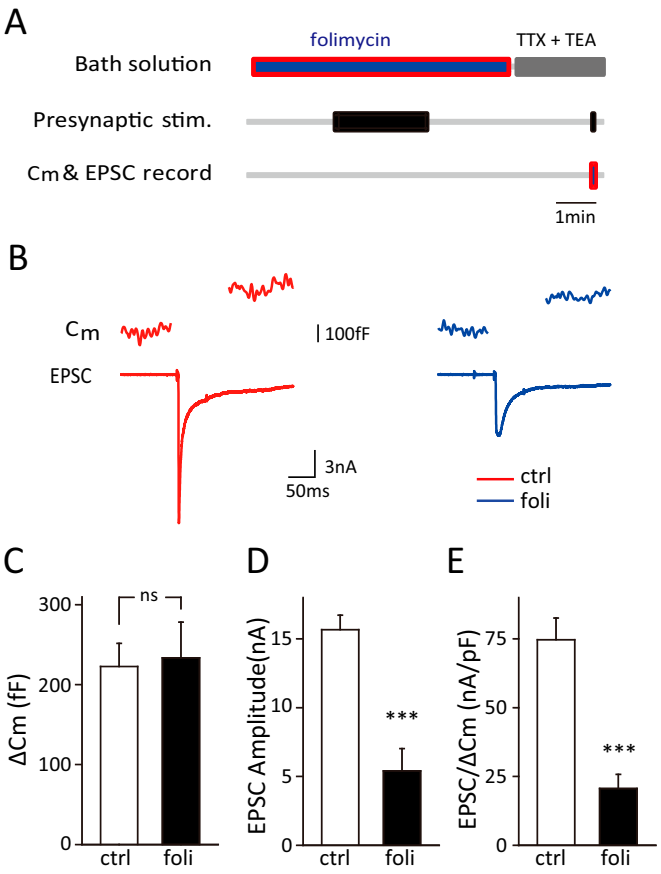


Fig. S2. Folimycin does not impair vesicle exocytosis. (*A*) Schematic of the experimental protocol. Presynaptic cell-attached stimulation (20 Hz for 82–142 s, black) was applied during normal or folimycin-containing bath solution (applied 2 min before stimulation and stopped at the completion of endocytosis). Whole-cell patch recorded presynaptic capacitance and EPSCs in response to 50-ms depolarization from −80 mV to 0 mV in TTX- and TEA-containing bath solution (dark gray). Blue bars indicate the folimycin condition. Red boxes indicate control. (*B*) Representative presynaptic capacitance and EPSC traces with (blue) and without (red) folimycin. (*C–E*) Summary of ΔCm (*C*, 222.8 ± 28.9 fF for control and 233.5 ± 44.7 fF for folimycin), EPSC amplitude (*D*, 15.6 ± 1.1 nA for control and 5.4 ± 1.6 nA for folimycin), and ratio of EPSC to ΔCm (*E*, 74.7 ± 7.9 nA/pF for control and 20.6 ± 5.1 nA/pF for folimycin) in response to 50-ms depolarization with ($n = 4$) and without ($n = 6$) folimycin. ***$P < 0.001$, *t* test.

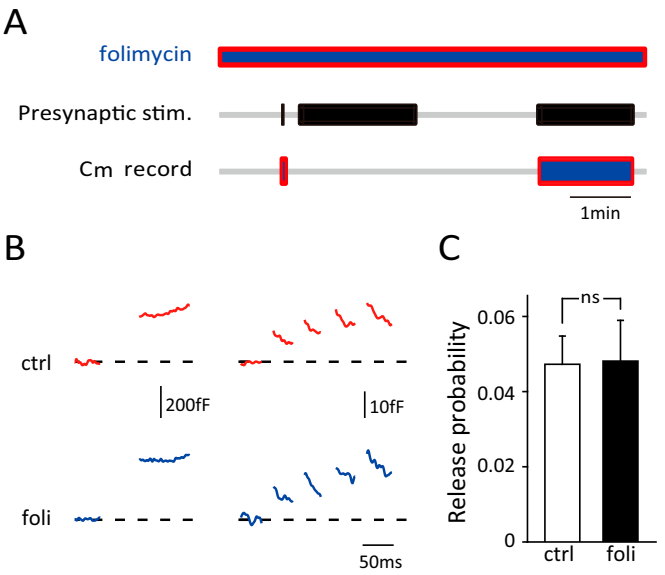


Fig. S3. Folimycin does not change vesicle release probability. (*A*) Schematic of the experimental protocol. All experiments were performed in normal or folimycin bath solution containing TTX and TEA. Presynaptic capacitance recording during 50-ms depolarization from −80 mV to +10 mV (RRP depletion, black) after 1-min folimycin application was followed by presynaptic action potential-like stimulation (1-ms depolarization from −80 mV to +40 mV at 20 Hz for 72–174 s). After 2-min recovery period, four action potential-like test pulses at 20 Hz and presynaptic capacitance recordings were repeated at 0.1 Hz. Blue bars indicate folimycin condition. Red boxes indicate control. (*B*) Representative presynaptic capacitance change (ΔCm) in response to RRP-depleting stimulation (*Left*) and 4 action potential-like stimuli (averaged trials, *Right*) with (blue) and without folimycin (red). (*C*) Statistics of release probability estimated as the ratio of the first action potential-like stimulus-induced ΔCm (*Right* in *B*) to the RRP depletion-induced ΔCm (*Left* in *B*) with (0.048 ± 0.011, $n = 8$) and without folimycin (0.047 ± 0.008, $n = 7$).

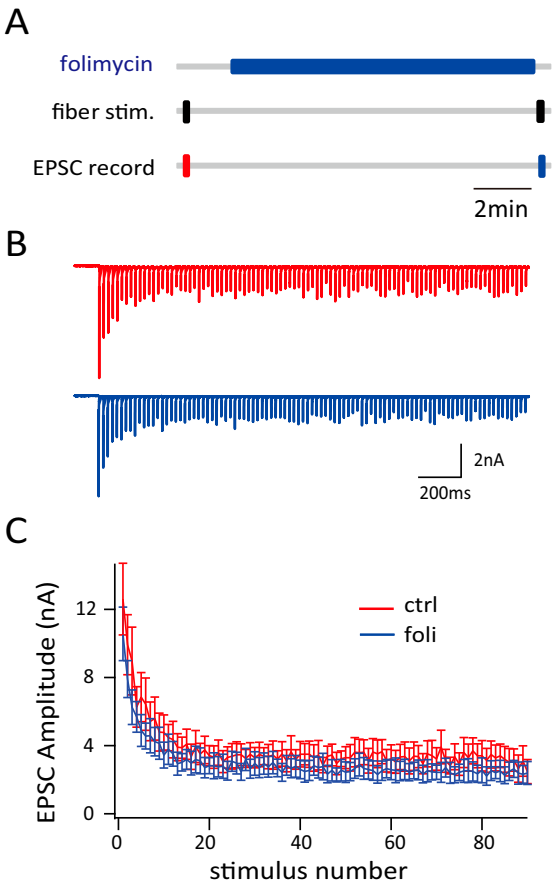


Fig. S4. Folimycin does not affect EPSCs by permeabilizing into the plasma membrane. (*A*) Schematic of the experimental protocol. EPSCs were recorded in response to afferent stimulation at 50 Hz for 2 s as a control recording (red). Two minutes after the control recording, folimycin was applied for 10 min before the test recording (blue). (*B*) Representative EPSCs and (*C*) statistics of EPSC amplitudes corresponding to the first (red) and second (blue) stimulus train ($n = 7$). There was no significant difference between the EPSC amplitudes in the control and folimycin groups.

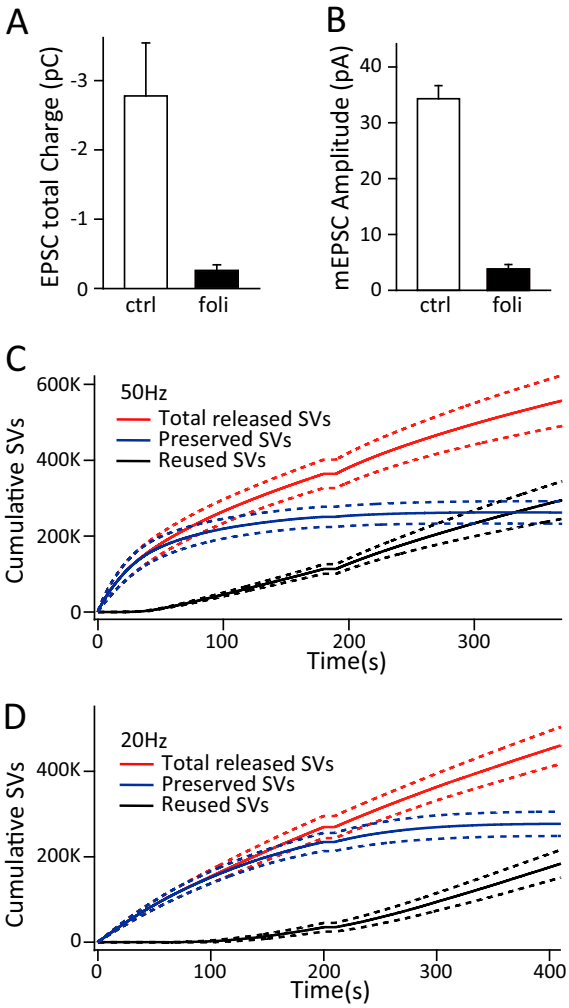


Fig. S5. Kinetics of preserved and reused vesicle release estimation based on EPSC electrical charge. (*A*) Statistical comparison of the average electrical charge from the last 100 EPSCs before and after folimycin application (−2.78 ± 0.76 pC for control and −0.26 ± 0.08 pC for folimycin, $n = 8$). (*B*) Summary of mEPSC amplitude measured in control and estimated in folimycin (34.31 ± 2.34 pA for control and 3.81 ± 0.80 pA for folimycin, $n = 8$). (*C* and *D*) Estimated cumulative number of total released vesicles (red), preserved vesicles (blue), and reused vesicles (black) under 50-Hz (*C*, $n = 4$) and 20-Hz stimulation (*D*, $n = 4$). The dashed lines denote mean ± SEM in *C* and *D*.

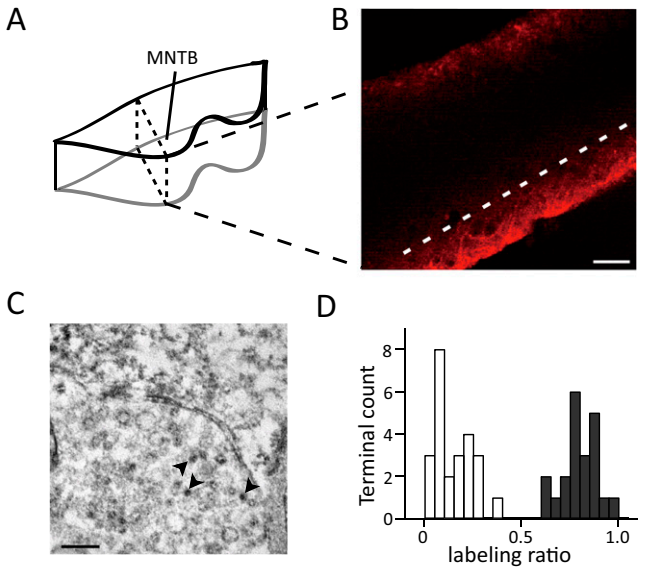


Fig. S6. Fraction of FM dye labeling and the location of tissue sampling. (*A*) Schematic of a brain slice. A slice containing the FM1-43FX–labeled MNTB was fixed and cut into parasagittal sections (dashed rectangle) under a two-photon microscope. (*B*) Representative parasagittal section with FM fluorescence under a two-photon microscope. Tissue within 50 μm of the slice surface (dashed line) was chosen for EM observation. (Scale bar, 40 μm.) (*C*) Representative EM image from the depth of the slice. Arrowheads indicate FM dye-labeled vesicles. (Scale bar, 200 nm.) (*D*) Summary data for the number of terminals with different ratios of FM-labeled vesicles to total vesicles. Images containing labeled terminals were collected from the interior (depth >50 μm, open bars, $n = 24$ terminals, 11 mice) and surface of the slice (depth <50 μm, filled bars, $n = 21$ terminals, 5 mice).

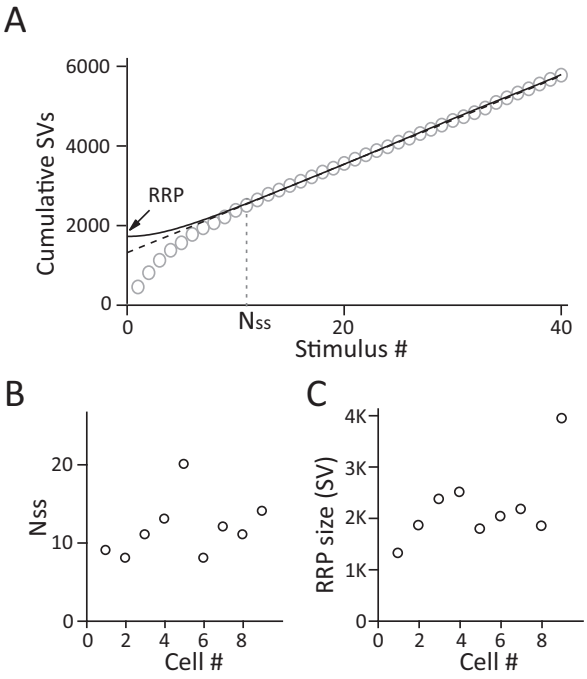


Fig. S7. Estimation of effective readily releasable pool size. (*A*) Representative plot of cumulative vesicle number versus stimulus number at 50 Hz. The arrow indicates the effective RRP size obtained according to the corrected estimation (1). The vertical dashed line indicates the starting point of the steady state (N_{ss}) for the best linear regression. (*B* and *C*) Plots of N_{ss} (*B*) and RRP size (*C*) for individual cells.

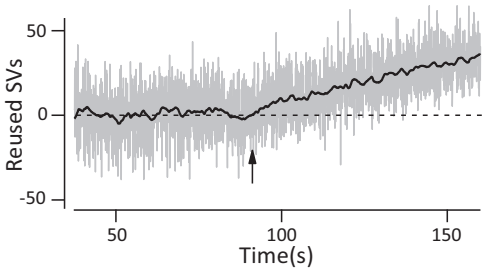


Fig. S8. Starting time point of vesicle reuse. Representative differentiated cumulative reused vesicle number (gray) and filtered with a low-pass filter at a cutoff frequency of 0.01. The starting time of reuse was determined as the last time point that deviated from the baseline in the reuse trace (arrowhead).

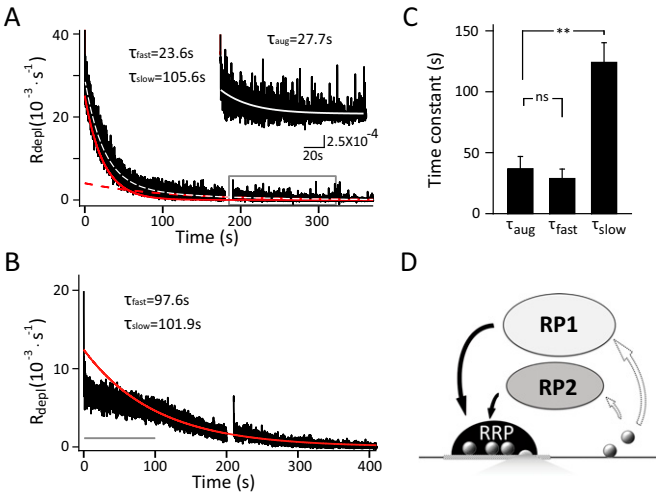


Fig. S9. Kinetics of the recycling pool depletion rate excludes two parallel pathways of vesicle recycling. (*A*) Representative normalized RP depletion rate (R_{depl}) after RRP depletion at 50 Hz. Fast (red solid line) and slow components (red dashed line) of the double-exponential fit (white dashed line). (*Inset*) Augmented trace by subtracting R_{depl} (in the gray box) by double-exponential fitting and then averaging every five sampling points as a low-pass filter. This trace is well fitted by monoexponential function (white line) with τ_{aug} after the initial period for RRP depletion. (*B*) Representative R_{depl} at 20 Hz with double-exponential fitting (fitting started from 100 s to the end, red curve). The R_{depl} in the initial 100 s (horizontal bar) showed a slow declining phase that cannot be exponentially fitted. (*C*) Statistics of time constants of the augmented component (τ_{aug}, 36.85 ± 9.99 s), fast component (τ_{fast}, 28.83 ± 7.76 s), and slow component (τ_{slow}, 124.17 ± 16.11 s) obtained by R_{depl} at 50 Hz ($n = 3$). $^{**}P < 0.01$, *t* test. (*D*) Two parallel-recycling pathway scheme.

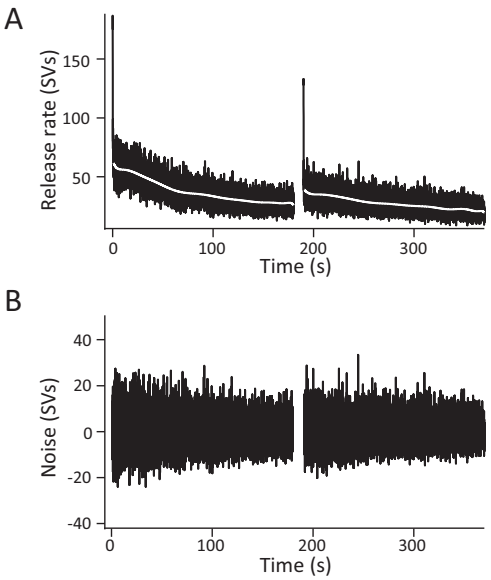


Fig. S10. Signal and temporal resolution of the proposed approach. (*A*) Representative vesicle release rate under 50-Hz stimulation with polynomial fitting (white curve). (*B*) Noise signal isolated by subtraction of the fitting curve from the rate curve. The noise has a root mean square of ~7.3 ± 0.5 vesicles and the temporal resolution of 20 ms as interstimulus interval.

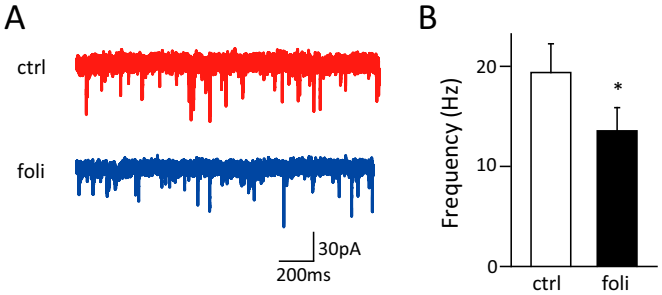
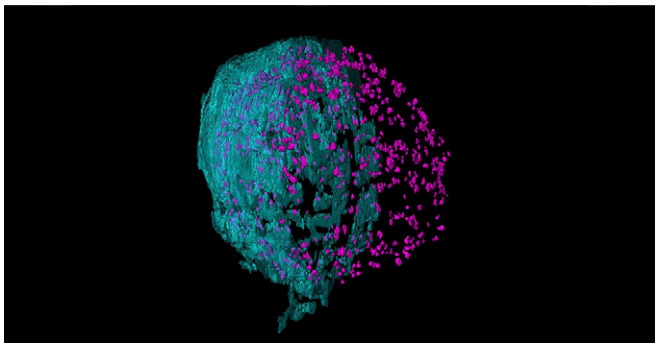
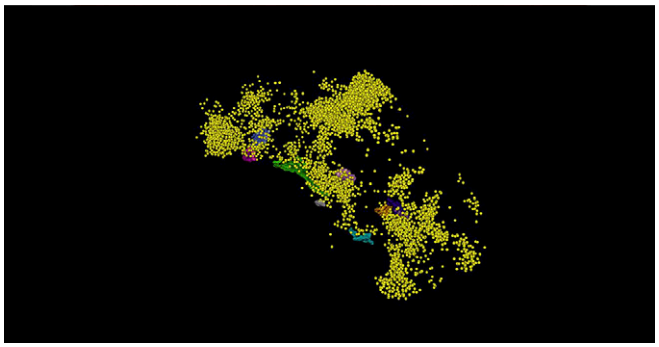


Fig. S11. Persistence of spontaneous release after recycling pool depletion. *(A)* Representative mEPSC traces after prolonged stimulation with (blue) and without (red) folimycin. (*B*) Statistics of mEPSC frequency recorded 5 min after prolonged stimulation (19.37 ± 2.88 Hz in the control and 13.55 ± 2.33 Hz after folimycin application, $n = 16$). *$P < 0.05$, *t* test.



Movie S1. Representative reconstruction of a calyceal terminal with AZs. One-half of the calyceal terminal outline is displayed in green and is rotated toward the postsynaptic part (principle cell not shown). Pink represents a single AZ within the presynaptic terminal, whereas purple shows the overlay between the calyceal terminal outline and the presynaptic AZs.

Movie S1



Movie S2. Representative reconstruction of a compartment of calyceal terminal. Presynaptic vesicles are shown in yellow, and individual AZs are outlined in red, pink, green, orange, cyan, blue, purple, and gray (a part of an AZ).

Movie S2