

Supporting Information

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SI Methods

Unless stated otherwise, all chemicals were purchased from Merck, Sigma, or VWR. FM 1-43 (SynptoGreen) was purchased from Biotrend.

Buffers. All concentrations are in mM.

Drosophila HL3 buffer [used for injection (1)]: NaCl 70, KCl 5, CaCl₂ 1.5, MgCl₂ 20, NaHCO₃ 10, trehalose 5, sucrose 115, and Hepes 5 (pH 7.2).

Caenorhabditis elegans buffer (2): NaCl 150, KCl 5, CaCl₂ 5, MgCl₂ 4, glucose 10, sucrose 5, and Hepes 15 (pH 7.3).

Locust standard saline (3) (also used for crickets): NaCl 140, KCl 10, NaH₂PO₄ 4, Na₂HPO₄ 6, CaCl₂ 2, and sucrose 90 (pH 6.8). For high-potassium saline, KCl concentration was increased to 50 mM with a corresponding reduction in NaCl concentration (100 mM).

Zebrafish Ringer (4): NaCl 116, KCl 2.9, CaCl₂ 1.8, and Hepes 5 (pH 7.2).

Frog Ringer (5): NaCl 115, KCl 2, CaCl₂ 1.8, and Hepes 5 (pH 7.2).

Mouse Ringer (6): NaCl 154, KCl 5, CaCl₂ 2, MgCl₂ 1, glucose 11, and Hepes 5 (pH 7.3).

Chicken Ringer: NaCl 150, KCl 3, CaCl₂ 2.5, MgCl₂ 1.2, glucose 17, and Hepes 10 (pH 7.2; modified from ref. 7).

For all animals injected with FM 1-43, dissections were performed in ice-cold Ca²⁺-free buffer (CaCl₂ was replaced with MgCl₂, and 1 mM EGTA was added).

For all *Drosophila* stimulation experiments (FM dye loading and pHluorin flies; see below), the following buffer was used (standard *Drosophila* buffer) (8, 9): NaCl 130, sucrose 36, KCl 5, CaCl₂ 2, MgCl₂ 2, and Hepes 5 (pH 7.3).

Animals. All animals were obtained either from laboratories specialized in their use or from commercial suppliers. All animals were treated in accordance with the regulations of the University of Göttingen and with the regulations of the State Niedersachsen (Landesamt für Verbraucherschutz, LAVES, Braunschweig, Germany). The wild-type fly stocks used here were w¹ and w¹¹¹⁸ and were kindly provided by Dr. Carolin Wichmann, Free University of Berlin, Germany. For the *shibire* experiments, *shibire*^{ts1} mutant flies were used (10), which were provided by Prof. Stephan Sigrist, Free University of Berlin, and contained a GFP and bar-eye balancer for recognition of homozygous mutants (*shibire*^{ts1}/FM7-Actin GFP). For synapsin null mutant experiments, we used “cantonized” homozygous Syn^{97CS} flies kindly provided by Prof. Erich Buchner, University of Würzburg, Germany (11). pHluorin flies expressing synaptobrevin-pHluorin in motoneurons were obtained by crossing males from the D42-GAL4 driver line (obtained from Prof. Andre Fiala, University of Göttingen, Germany) to UAS-synaptophluorin III females (obtained from Prof. Stephan Sigrist, Free University of Berlin); third instar larvae were used in all experiments. Flies were generally kept at 21 °C; pHluorin flies were grown at 25 °C with a 12 h day/night rhythm and used at 21 °C during the experiments. Wild-type Bristol N2 *C. elegans* worms were kindly provided by Ling Luo and Dr. Stefan Eimer, European Neuroscience Institute, Göttingen, Germany, and were maintained at 20 °C according to standard methods (12). Locusts were provided by Andrea Wirmer and Dr. Ralf Heinrich, University of Göttingen. They were maintained in a terrarium at room temperature and fed on special locust food (Nekton) and grass. Crickets were obtained from a commercial supplier and were maintained on commercial pellet food according to the

supplier's instructions. Zebrafish and frogs were obtained from commercial suppliers. Zebrafish were maintained in an aquarium at 24 °C. Frogs were kept at 16 °C and fed on live prey at least once per week. Wild-type (female CD-1, B6/N, and B6/J and male B6/N and B6/J) mice were obtained from the animal facility of the European Neuroscience Institute, Göttingen, Germany, and were maintained in standard cages. Chicken embryos (wild-type, LSL white eggs; Geflügelzucht Horstmann) were kindly provided by Alexandra Klusowski and Dr. Till Marquardt, European Neuroscience Institute, Göttingen, Germany. Fertilized eggs were kept at 37.8 °C until embryonic day (E) 11 to E12. Rats (Sprague-Dawley) were provided by Charles River Laboratories.

Injection of FM 1-43. Injections were performed to obtain a final concentration of approximately 10 μM FM 1-43 in the animal (see also ref. 13). FM 1-43 was diluted in the respective buffer and filtered (Millipore Ultrafree-MC Sterile centrifugal filter units, 0.22 μm). For *Drosophila* third instar larvae, microinjection was performed using a FemtoJet express microinjecting device (Eppendorf), using glass micropipettes prepared with a P-97 pipette puller (Sutter Instrument) from glass tubes (thin wall, 3 inches, 1-mm diameter; World Precision Instruments). Injection was monitored using a dissection microscope (Leica MZ6). Generally, approximately 20–50 nL were injected into the body cavity; volume was checked by subsequent injection with the same settings and tip into oil (Halocarbon oil 700; Sigma). Successful microinjection was monitored subsequently using a Leica MZ10F fluorescence stereomicroscope equipped with a GFP Plant filter set (Leica). For *C. elegans*, injections were performed into the pseudocoelom of young adult worms using Femtotips (Eppendorf) on a FemtoJet express microinjecting device (Eppendorf). Injection was monitored using a Zeiss Axiovert 200 microscope equipped with a 40× 0.5 N.A. objective from Olympus. For all larger animals, injection was performed using 1-mL syringes (Terumo) equipped with 0.3 × 20-mm needles (catalog no. 13.201-09, Unimed). For locusts, 10–20 μL were injected into the abdominal coelom. For zebrafish, approximately 20 μL were injected s.c. close to the caudal fin. In the case of frog, 20–100 μL (depending on frog size) were injected s.c. into the ventral part of the thorax. For mice, approximately 300 μL were injected s.c. into the neck area. For chicken embryos (E11 to E12; stages 37–38) (14), approximately 50 μL were injected s.c. into the upper region of the thigh, after cutting a 1.5 × 1.5-cm window in the egg shell. To prevent dehydration we sealed the window with Parafilm (VWR). Finally, in the case of crickets, 50–100 μL were injected into the thorax or the first segments of the abdomen.

Maintenance After Injection. During the time between injection and dissection, animals were generally kept in an environment that allowed them to move, eat, and sleep. *Drosophila* larvae were kept on standard cornmeal medium. *C. elegans* were rescued with M9 buffer (22 mM KH₂PO₄, 22 mM Na₂HPO₄, 85 mM NaCl, and 1 mM MgSO₄) after injection and maintained on nematode growth medium (16 g Agar, 3 g NaCl, 3 g peptone, and 1 L ddH₂O; autoclaved). Locusts and crickets were maintained on fresh grass; zebrafish were maintained in normal aquarium water; frogs were kept in a small terrarium with sufficient water, and occasionally fed with live prey; mice were kept in a standard cage provided with food and water. Eggs were maintained in a waterbath at ≈38 °C. All animals survived injection and behaved after injection (moved, ate, slept, or communicated/interacted with other animals) in ways that were indistinguishable from control (uninjected) animals.

Dissections. At the specified time after injection, dissections of the respective muscles (Fig. 1) were performed rapidly in a mixture of ice-cold/frozen Ca^{2+} -free buffer, in sylgard-coated culture dishes. *Drosophila* larva dissection was performed according to ref. 8. For adult flies, the thorax was cut longitudinally, leaving the ventral side with legs and coxa intact; the femur was removed. *C. elegans* were cut horizontally into three pieces. For locusts, animals were killed by decapitation, the third pair of legs was removed, the femurs were opened, and the *tibial flexor* muscle was spread out. For zebrafish, animals were killed, and skin and superficial muscles were removed from the tail to expose the *adductor caudalis ventralis* and *flexor caudalis ventralis inferior* and *superior* muscles. Dissection of the frog *cutaneous pectoris* muscle was done as previously described (15). Preparation of the mouse *levator auris longus* muscle was done as previously described (6). For the chicken embryo (14), animals were killed by decapitation, legs were removed, and the *gastrocnemius pars externa* and *interna* muscles were exposed. Crickets were killed by decapitation, and the eye and optic lobe with lamina and medulla were removed (see refs. 16 and 17).

Fixation and Photooxidation. After dissection preparations were fixed for 30 min in 2.5% glutaraldehyde (in PBS) on ice, followed by further fixation for 30 min to 1 h in 2.5% glutaraldehyde at room temperature. All remaining steps (washing and quenching) were performed at 4 °C. Preparations were washed in PBS and quenched with 100 mM ammonium chloride (in PBS). After further PBS washing, samples were incubated in 1.5 mg/mL di-amino-benzidine (DAB) for 30–45 min. Photooxidation was performed as previously described (18). Briefly, a Zeiss Axioskop 2 FS plus microscope equipped with a 20× 0.5 N.A. objective from Olympus was used, and preparations were illuminated until the appearance of a dark precipitate. We used an EGFP Long Pass filter set (AHF; 470/40-nm excitation filter, 495-nm dichroic mirror, 500-nm long pass emission filter).

Electron Microscopy Procedures. After photooxidation and subsequent PBS washing, preparations were processed as previously described (18). Briefly, they were treated with 1% osmium tetroxide (Fluka, Sigma) for approximately 1 h at room temperature and dehydrated with an ascending series of ethanol and propylene oxide (PO; Electron Microscopy Sciences). Samples were then incubated in 50% Epon resin (Plano) in PO for 12–18 h under continuous agitation, followed by incubation for at least 8 h in 100% Epon in open vials, to allow for PO evaporation. Finally, preparations were incubated for 36 h at 60 °C. For *C. elegans*, the procedure was slightly modified to prevent poor penetration of resin/osmium. Incubation in 2% osmium tetroxide was performed for 1 h at room temperature and for 1 h at 4 °C. Furthermore, two additional steps of incubation with 100% resin for 6 h under continuous agitation were added before evaporation of PO. Resin polymerization was increased to 48 h. For adult flies (*shibire* experiment) and crickets, treatment with 1% osmium tetroxide at room temperature was increased to 1.5 h. Cross-sections (90–100 nm thick) were collected from the blocks. For the *shibire* samples (larvae and adults) and for the investigation of frog Schwann cells, sections were poststained with 2% uranyl acetate (Plano) in 50% ethanol for 2 min. Electron micrographs were acquired using a Zeiss EM 902A microscope, equipped with a 1,024 × 1,024 CCD detector (Proscan CCD HSS 512/1024; Proscan Electronic Systems).

The 3D reconstructions were made from serial sectioning (90- to 100-nm sections), with several micrometer-long reconstructions obtained from individual synapses (and thus with reconstruction volumes of up to several μm^3).

Electrical Stimulation. Electrical stimulation was performed as previously described (18). Briefly, preparations were mounted in

a chamber equipped with a platinum plate field stimulator (8-mm distance between the plates, custom-made in the workshop of the Max Planck Institute for Biophysical Chemistry, Göttingen, Germany). Generally, 100-mA shocks were delivered at a frequency of 30 Hz for 5 min (10 s for the fluorescence recovery after photobleaching experiment; Fig. 4 A and B), using an A385 stimulus isolator and an A310 Accupulser stimulator (both from World Precision Instruments).

For dye-loading experiments, samples were immersed in 10 μM FM 1-43 in the respective (Ca^{2+} -containing) buffer during stimulation. To allow for complete vesicle recycling, preparations were allowed to recover in the presence of the dye for 5–10 min. For locust and cricket, stimulation was done by depolarizing the preparations in high-potassium saline (50 mM KCl, with 10 μM FM 1-43) for 5 min to avoid mounting these fragile preparations in the stimulator chamber. Stimulation protocols were typically followed by brief washing in Ca^{2+} -free medium (at 4 °C), before fixation and photooxidation.

Testing Vesicle Use In Vivo by HRP Injection. We used postnatal day 12 (P12) Sprague-Dawley rats. A solution of 10 mg/mL HRP was injected into the brainstem of the anesthetized rat, followed by recovery from anesthesia within 2 min. The rat was allowed to behave normally for 30 min (i.e., under sound conditions commonly found in a laboratory environment), before transcatheterial perfusion with 4% paraformaldehyde. Brain slices were then incubated with DAB, resulting in strong oxidation and labeling of recycling organelles. Note that HRP can be cross-linked by the fixative and is therefore retained in the space between the cells (unlike the FM dyes). Importantly, very strong HRP labeling is visible in the synaptic cleft (black arrowhead in Fig. 1B, calyx of Held panel), demonstrating that HRP did penetrate into the synapses and was fully available for labeling of endocytic compartments.

Electron Microscopy Data Analysis. Image analysis was performed using software custom-written in MATLAB (Mathworks) as previously described (5). Active zones, vacuoles (labeled and unlabeled), plasma membrane, and vesicles (labeled and unlabeled) were drawn manually, using a Wacom PL 720 LCD tablet monitor.

Testing Dye Availability in Body Fluids After Injection. Eleven crickets were injected in the abdomen with 50–100 μL of 180 μM FM 1-43 in standard *Drosophila* buffer, resulting in a final concentration in the animals of $\approx 10 \mu\text{M}$. *Drosophila* buffer was used because the body fluids collected afterward were used to label *Drosophila* larvae in vitro. Body fluids were then retrieved by compressing the skull, at 2 h after injection. Fluids were pooled and centrifuged for 10 min at maximal speed (25,000 × g) in an Eppendorf 5417R cooled centrifuge. The supernatant was collected and diluted 1:1 in standard *Drosophila* buffer. Four *Drosophila* third instar larvae were dissected and stimulated (30 Hz, 5 min; same stimulation device as described above) in the diluted cricket body fluids. Preparations were then washed in standard *Drosophila* buffer for 5 min at 4 °C and imaged using a Zeiss Axio Examiner.Z1 microscope equipped with a 63× 1.0 N.A. objective and a 100-W Hg lamp (Osram). Images were acquired using a 470/40 excitation filter, a 495 beamsplitter, and a 525/50 emission filter (Zeiss). Image acquisition was done using a QuantEM:512 SC camera (Photometrics) and Axiovision software (Zeiss). As a control, we diluted a solution of 10 μM FM1-43 (equal to the volume of cricket body fluids) as we did for the body fluids and used this solution to stimulate four additional *Drosophila* larvae, as described for the body fluids. Images were analyzed by calculating the average intensity of the nerve terminals (signal), in a manually determined region of interest. The

background intensity was similarly measured in the neighboring muscle area and was subtracted from the signal intensity.

Fluorescence Spectrophotometry. Crickets were injected in the abdomen as above. Body fluids were retrieved after 2 h by compressing the skull. Samples were snap-frozen with liquid nitrogen and stored overnight at -20°C . Samples were then cleared by centrifugation for 60 min at maximal speed ($25,000 \times g$) in an Eppendorf 5417R cooled centrifuge, and supernatants were collected. A dilution series of FM 1-43 was prepared in standard *Drosophila* buffer and diluted 1:1 in 20% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (19). Dilutions (1:1) in 20% CHAPS were also prepared for the cricket supernatants. All solutions were measured in quartz cuvettes (1.5-mm width) in a Fluoromax-2 fluorescence spectrophotometer (Jobin Yvon, Horiba Scientific Instruments) at an excitation of 488 nm (2-nm bandwidth) and an emission of 538 nm (3-nm bandwidth). Duplicate readings were obtained for the FM 1-43 dilution series and averaged; the small volume of the experimental samples allowed for only one reading per sample. The concentration of FM in the cricket fluid was obtained by linear interpolation from the FM 1-43 dilution series.

Drosophila larvae were injected with FM dye as described above (in HL3 buffer) and after 2 h were cut in segments of less than ≈ 0.3 mm. The material from three larvae was collected in one tube, diluted with 7 μL of buffer, mixed 1:1 with 20% CHAPS, and sonified for 20 min in an ice-water bath (Bandelin Sonorex, Bandelin Electronic) before clearing by centrifugation (15 min, same conditions as above). The fluorescence of the resulting solution was measured using a Fluoromax-2 fluorescence spectrophotometer, as above.

pHluorin Experiments. Flies expressing pHluorin in motoneurons were obtained as described above. Imaging was performed with a Zeiss Axioskop 2 FS plus microscope equipped with a 63×1.0 N.A. objective from Zeiss, using the same filter set as above. Bafilomycin A1 was used at a final concentration of $\approx 1 \mu\text{M}$ (0.5 μM for the stimulation experiments). Data analysis was performed using software custom-written in MATLAB; regions of interest encompassing the synapses were selected manually; the average fluorescence in the synapses was then calculated, subtracting the average background fluorescence of the regions immediately adjacent to the synapses.

The values presented in Fig. 2B were corrected for the fluorescence of the surface pool of pHluorin (which represents $\approx 70\%$ of the fluorescence of control terminals, four independent experiments). Surface fluorescence was determined by subtracting the fluorescence of synapses imaged at pH 5.5 from that at pH 7.2, as performed in the past (20). To obtain a more accurate estimate for the recycling vesicle pool, the values were further corrected for the fluorescence of the quenched vesicles (which is easily derived from the increase we obtained upon NH_4Cl treatment; a quenched vesicle is 9.9-fold less fluorescent than a dequenched one; four independent experiments).

shibire Paralysis Experiment. Third instar *shibire* or control larvae (with at least one wild-type copy of dynamin) were placed in standard *Drosophila* buffer in a water bath at 32°C , mounted on a stereomicroscope (MZ6, Leica). Adult *shibire* or control flies were placed in a tube inside a waterbath at 32°C , which allowed monitoring of paralysis and “dropping” to the bottom of the tube. The animals were dissected in ice-cold Ca^{2+} -free buffer either immediately after the onset of paralysis (≈ 15 seconds) or after 10 min at nonpermissive temperature. They were then fixed as described above and processed for electron microscopy.

Control larvae and flies displayed no loss of vesicles after 10 min at nonpermissive temperature.

Predator/Prey Experiment. Locusts were injected with FM 1-43 as described above. After 2 h one locust was placed into a terrarium ($29.5 \times 32 \times 19$ cm) with three frogs. After the locust had been caught and ingested, the respective frog was immediately killed and the locust retrieved from its stomach, dissected in ice-cold Ca^{2+} -free buffer, fixed, photooxidized, and processed for electron microscopy.

Comparing Vesicle Release and Recycling. We recorded electrophysiologically synaptic release in *Drosophila* larvae in vitro, while simultaneously labeling the recycling vesicles with FM 1-43. After ≈ 10 min of recording they were fixed, photooxidized, and processed for electron microscopy. On average $\approx 8\%$ of the synapse volume was reconstructed [1–4 neuromuscular junction (NMJ) segments were reconstructed for each synapse; the total volume of the synapses was estimated from fluorescence imaging in the live preparations; see below]. The number of labeled (recycled) vesicles found in the reconstructions was scaled to the total volume of the synapses and compared with the number of vesicles released (recorded electrophysiologically).

Electrophysiology recordings were performed as previously described (8), using third instar *Drosophila* larvae. The nerves connecting the muscles of interest to the upstream ventral ganglia were severed before the measurements. Glass micropipettes (25–30 M Ω resistance) were prepared using a P-97 pipette puller (Sutter Instrument) from glass tubes (thin wall, 3 inches, 1.5-mm diameter; World Precision Instruments). Filling solution was 3 M potassium acetate. An ELC-03 XS amplifier (npi electronic) was used and digitized with an INT-20 \times interface (npi electronic) using CellWorks software (version 6.0b1; npi electronic). Spontaneous miniature end-plate potentials (mEPPs) were recorded for ≈ 10 min. The number of spontaneous events was determined manually using a routine written in MATLAB (5). No measurements could be obtained during the brief time frame (≈ 1 min) during which the preparation was handled before fixation; we assumed the same release rate for this period as for the rest of the recorded trace.

During electrophysiological recordings, FM 1-43 (10 μM) was present in the buffer to label recycling vesicles. The fluorescence of the *Drosophila* subsynaptic reticulum was acquired on the unfixed preparation by using a Zeiss Axio Examiner.Z1 microscope equipped with a 20×1.0 N.A. objective (Zeiss) and a 100-W Hg lamp (Osram). Images were acquired using a 470/40 excitation filter, a 495 beamsplitter, a 525/50 emission filter (Zeiss), a QuantEM:512 SC camera (Photometrics), and Axiovision software (Zeiss). To allow for visualization of the complete terminal, z-stacks were performed (400-nm interval between the images) and were later deconvolved using AxioVision 4 deconvolution software (Zeiss). We used two methods to investigate the volume of the fluorescently labeled preparations. First, we drew the outline of the preparations in each deconvolved stack section and calculated the volume corresponding to each individual outline. In a second approach, we summed all stack frames and filled ellipses into each bouton area in the summed image. The two methods resulted in almost identical volume estimates (the second method estimates the volume to $97 \pm 8\%$ of the first method values; $n = 7$ synaptic volume reconstructions).

Preparations were washed with ice-cold PBS, fixed and photooxidized, and processed for electron microscopy as described above. For each preparation, the quantity of labeled vesicles was determined for one to four nerve terminal reconstructions. The volume of the surrounding subsynaptic reticulum was determined by manually drawing its surface in each electron microscopy section and adjusting for the corresponding section thickness. The volume of the entire reconstructed reticulum was corrected for the shrinking in volume produced by glutaraldehyde fixation and plastic-embedding, as determined previously (21). The number of labeled vesicles was then scaled up to the total volume of the synapse (determined by fluorescence microscopy).

Fluorescence Recovery After Photobleaching. Imaging was performed using a Leica SP5 STED laser-scanning confocal microscope. Excitation was provided by an Argon laser (488 nm line, at 20% of the current). We used 10% of the laser light transmission for image acquisition and 100% for bleaching. A 63 \times 1.4 N.A. oil-immersion objective was used (Leica). After FM dye loading by 30 Hz, 10-s stimulation, *Drosophila* larvae were mounted on glass coverslips, ventral side down, to allow for optimal imaging using oil immersion. Images (512 \times 512 pixels in size, with a pixel width of 32 nm) were collected every 5.24 s, with a laser dwell time of 20 μ s per pixel. For bleaching, points of interest were selected in nerve terminals, and the laser was applied to the particular point for 150 ms. Images were collected at different detector gains, to allow different terminals to be imaged at similar dynamic range; detector gain does not influence the recovery (21).

Data analysis was performed using self-written routines in MATLAB. Fluorescence was measured within the bleached spot,

in the bouton containing the bleached spot, in the neighboring boutons, and in the nonterminal background area. The background was subtracted from all other values. The fluorescence in the neighboring boutons was then quantified and used to correct the fluorescence of the bleached spot for the photobleaching induced by image acquisition. The fluorescence in the bleached spot was expressed as percentage of the loss during bleaching. We assumed that vesicles could only come into the bleached spot from the bouton containing it (21). Therefore, we calculated the fraction of the total fluorescence lost by this bouton during bleaching and used this value as the maximal possible recovery (100%; see details also in ref. 21).

Statistics. Values indicate mean \pm SEM unless otherwise noted. Numbers indicate the number of independent preparations unless otherwise noted. *P* values refer to Student *t* tests unless otherwise noted.

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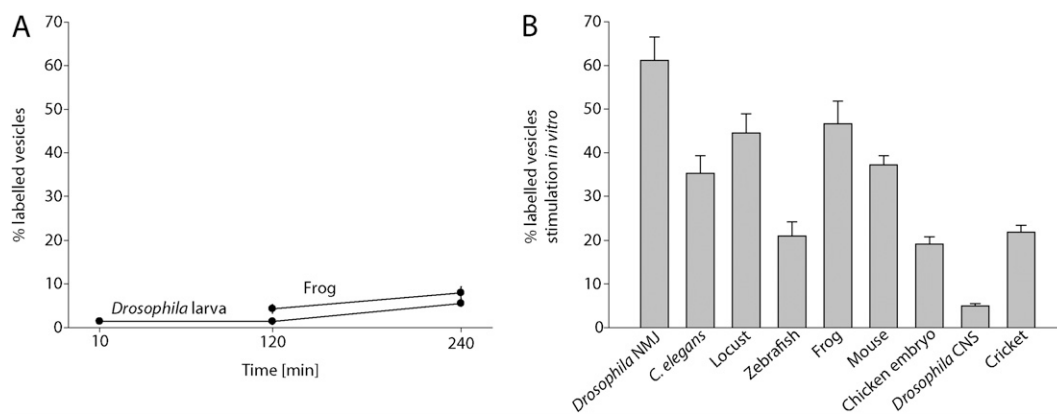


Fig. S1. Vesicle labeling in vivo compared with stimulation in vitro. (A) Percentage of labeled vesicles at different times after injection in the *Drosophila* larval NMJ or the frog NMJ (three to six independent preparations \pm SEM; note that the error bars are occasionally smaller than the symbol size). Note the slow increase, indicative of slow intermixing between the active and inactive vesicles. (B) Percentage of labeled vesicles after strong in vitro stimulation (generally 30 Hz, 5 min; locust and cricket: 50 mM high potassium, 5 min). At least 10 synapses from 2 to 9 independent preparations were analyzed; bars show means \pm SEM.

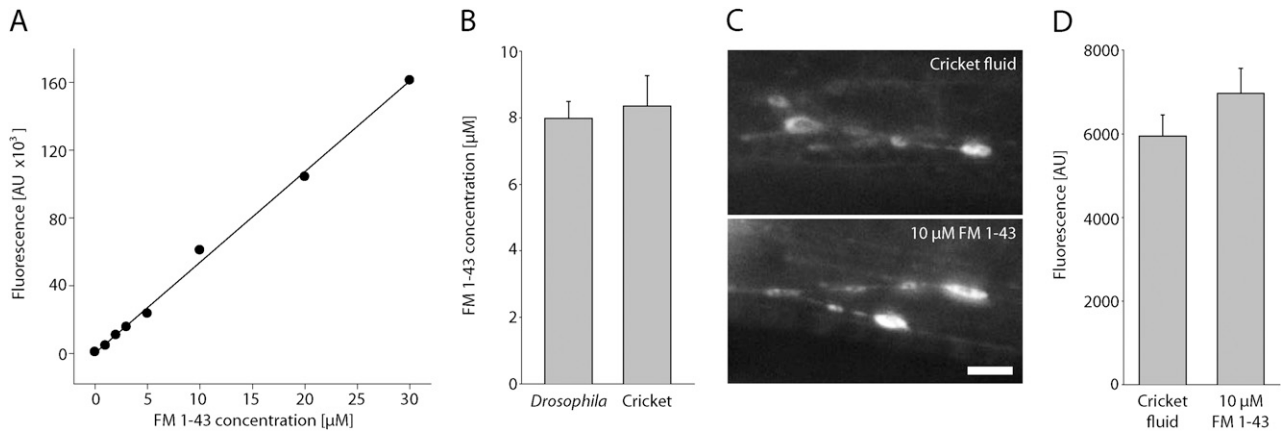


Fig. 52. FM dye persists in the animal body fluids for hours. It was conceivable that the dye was not available in the body fluids bathing the synapses. However, body fluids collected at hours after injection contained the dye, as verified by both fluorescence spectrophotometry (A and B) and imaging (C and D). (A) Dilutions of FM 1-43 in 10% CHAPS were measured using a Fluoromax-2 fluorescence spectrophotometer. The line shows a linear fit to the data. (B) The FM concentration in body fluids collected from animals 2 h after FM dye injection was determined by linear interpolation from graphs such as the one indicated in A. Bars show mean \pm SEM from 13 crickets and from three measurements of *Drosophila* larvae (note that each measurement in *Drosophila* pooled the fluids from three larvae, because the small volume obtained from only one larva could not be measured). Dye concentration was $\approx 8 \mu\text{M}$ on average and above $4.5 \mu\text{M}$ for all preparations measured. Note that we obtained optimal photooxidation in the past with FM 1-43 concentrations as low as $1.2 \mu\text{M}$ (1). Note that the amount of dye injected was calculated to result in a final concentration of $\approx 10 \mu\text{M}$ in the animal's body. (C) *Upper*: Exemplary fluorescence image of *Drosophila* nerve terminals stimulated for 5 min at 30 Hz in body fluids obtained from crickets that had been injected with FM 1-43 2 h earlier (body fluids were diluted 1:1 in normal *Drosophila* buffer). *Lower*: Exemplary fluorescence image of *Drosophila* nerve terminals stimulated for 5 min at 30 Hz in conventional *Drosophila* buffer (10 μM FM 1-43, diluted 1:1 in normal *Drosophila* buffer). (Scale bar, 10 μm .) (D) Comparison of fluorescence intensity of *Drosophila* terminals stimulated in body fluids of injected crickets and in conventional *Drosophila* buffer. No significant difference in fluorescence intensity is observed ($P > 0.19$, t test). Graph shows averages from at least 29 different synapses from four larvae (\pm SEM).

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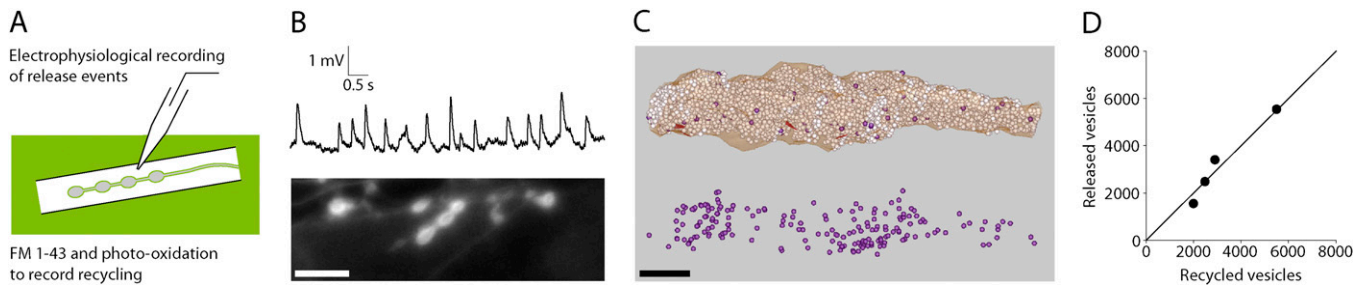


Fig. 53. Correlation between the number of vesicles released and the number of photooxidized vesicles. It seemed possible that the technique misses some of the vesicles that release neurotransmitter. To test this, we quantified all synaptic vesicles released over a few minutes in the *Drosophila* NMJ by electrophysiology (in vitro), while labeling recycling vesicles by FM photooxidation. The numbers of vesicles released and recycled (found labeled in electron microscopy) were virtually identical. (A) Experimental schematic: comparison of vesicle release and recycling. Spontaneous neurotransmitter release is recorded for approximately 10 min. Simultaneously, vesicle recycling is recorded by FM dye uptake, followed by photooxidation. (B) *Upper*: Exemplary spontaneous end-plate potential recordings. Each peak corresponds to a single released vesicle. *Lower*: Fluorescence image of a nerve terminal bathed in FM 1-43. The image indicated is a projection of a stack of fluorescence frames captured at different heights, spanning the entire volume of the synapse. (Scale bar, 20 μm .) (C) 3D reconstruction of a nerve terminal labeled as in A and B. *Lower*: Labeled vesicles only. (Scale bar, 500 nm.) (D) Quantification of the number of vesicles released/recycled. The number of labeled vesicles from the 3D reconstructions was scaled to the total volume of the preparation (obtained from the fluorescence images as depicted in B) and correlated to the number of vesicles recorded electrophysiologically. The identity line is shown, for comparison.

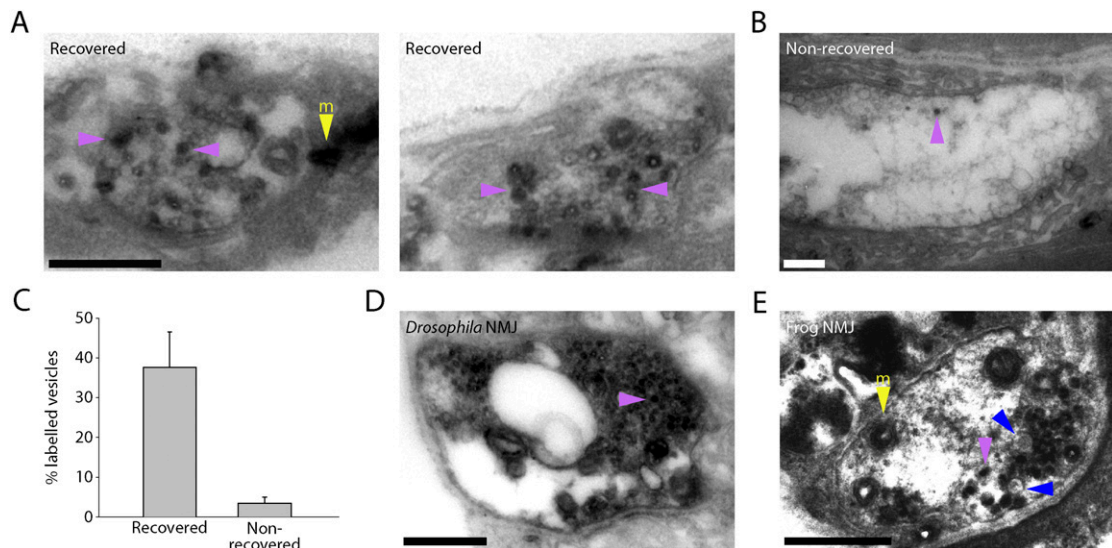


Fig. 54. Strong FM dye uptake in vivo after recycling inhibition. Despite the results of the previous experiments, it was still possible that the vesicles cannot take up the dye in vivo, for unknown reasons. To test this we used a *Drosophila* temperature-sensitive dynamin mutant (*shibire*) in which vesicle reformation is blocked at temperatures above 29 °C (1). This treatment results in vesicle depletion (due to the physiological activity of the synapses), with numerous vesicles remaining fused into the plasma membrane. Returning to room temperature forces the retrieval of all of these vesicles (recovery). Performing this protocol after FM dye injection into a *shibire* larva should therefore result in substantial vesicle labeling. This was indeed the case, indicating that the dye reaches the synapses efficiently (A). Importantly, synapses fixed without a recovery period contained few or no FM-labeled vesicles (B). Purple arrowheads indicate labeled vesicles. Yellow arrowheads indicate oxidized mitochondria. (A) We injected the FM dye in *shibire* larvae and placed them for 5 min at nonpermissive temperature. The animals were then placed at the permissive temperature for 10 min, resulting in a complete recovery of motion behavior. Note the high number of labeled vesicles. (Scale bar, 300 nm.) (B) A *shibire* larva injected with FM 1-43 was placed for 10 min at nonpermissive temperature and immediately dissected. Note the low number of labeled vesicles. (Scale bar, 300 nm.) (C) Quantification of vesicle labeling in injected *shibire* larvae allowed to recover (Left) or dissected immediately after the nonpermissive temperature treatment (Right). As indicated in A, numerous vesicles were labeled in recovered animals. Which percentage of all vesicles should have been labeled? As indicated below (Fig. S6), during 10 min at the nonpermissive temperature approximately 60% of the vesicles are depleted. Assuming that the vesicle release and depletion proceed at a more or less constant rate, approximately 30% of the vesicles should have been released over 5 min at the nonpermissive temperature in the experiment from A. Thus, approximately 30% of the vesicles should have been labeled in these preparations after switching to the permissive temperature. This was confirmed by FM photooxidation: on average approximately 37% of the vesicles were labeled in the animals allowed to recover. Note that we placed the larvae at the nonpermissive temperature for only 5 min in this experiment because their recovery from the 10-min treatment seemed longer and more difficult (at the level of larva behavior). Hardly any labeled vesicles were found in the nonrecovered animals. Graph shows means from 3 to 15 different synapses from four independent preparations (\pm SEM). (D and E) Examples of heavily labeled preparations from *Drosophila* (D) or frog (E) NMJs subjected to stimulation at 30 Hz, for 300 s or 60 s, respectively. Note the abundance of labeled vesicles, indicating that photooxidation can indeed label a large fraction of the vesicles, at least under strong stimulation conditions in these relatively robust preparations. Blue arrowheads in E point to two dense-core (nonacetylcholine) vesicles; note that they are unlabeled, as expected, because they do not readily release and recycle upon stimulation. (Scale bars, 500 nm.)

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