

Clathrin-Mediated Endocytosis Is the Dominant Mechanism of Vesicle Retrieval at Hippocampal Synapses

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Properties of sypHy

SypHy was localized to active synapses

In the main paper, we used immunocytochemistry to demonstrate that sypHy was localized to synapses (Figure 2A). We also carried out a physiological test using the styryl dye FM4-64 to check whether these puncta of sypHy represented active synapses (Figure S1). First, we identified sites of sypHy fluorescence increase by applying a test train of 40 APs at 20 Hz. We then applied 15 μ M FM4-64 during a 600 AP train at 20 Hz and for one minute after, and then washed to remove the dye. Structures labeled with FM4-64 tended to co-localize with synapses identified using sypHy. We then stimulated the cultures using a 900 AP train at 20 Hz. Figure S1A shows that there was good co-localization between structures that lost FM4-64 on stimulation and sites of exocytosis identified with sypHy. These results confirm that sypHy was targeted to vesicles at active synapses.

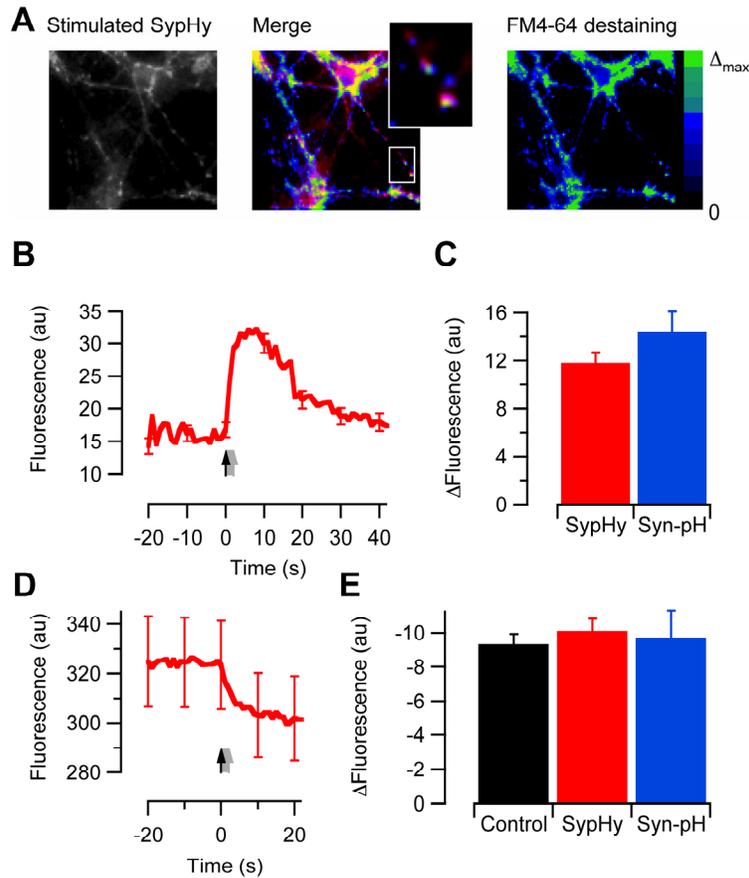


Figure S1. Exocytosis in synapses expressing sypHy characterized using FM4-64

(A) SypHy co-localizes with points of FM4-64 destaining. Snapshot of sypHy fluorescence after 40 stimulation pulses at 20 Hz (left), pseudocolor representation of the amount of FM4-64 destaining for a 900 AP train (right, $\Delta F = F_0 - F_{900AP}$). The middle panel is the sypHy image in red, merged with the pseudocolor destaining image. Yellow and purple spots highlight co-localization.

(B) Average sypHy response to a 40 AP train at 20 Hz (arrow, $n = 111$).

(C) Average amplitude of pHluorin response ($\Delta F = F_0 - F_{40AP}$) was similar for synapses expressing sypHy and synaptopHluorin ($n = 3-5$ cells, $p = 0.31$).

(D) Average FM4-64 trace for the same synapses when stimulated with a 40 AP train after dye-loading. ($n = 111$).

(E) Histogram to compare the amount of exocytosis, as judged from FM4-64 destaining for 40 AP trains at 20 Hz, at untransfected and sypHy- or synaptopHluorin-expressing synapses or synaptopHluorin ($n = 3-5$ cells, $p = 0.78$, one-way ANOVA).

Exocytosis was normal in synapses expressing sypHy

We next tested whether exocytosis was normal in synapses that expressed sypHy. First, the pHluorin responses of sypHy and synaptopHluorin were compared. The peak fluorescence change at the end of stimulation was not significantly different between the two reporters (Figure S1B and C; $p = 0.31$, Student's *t*-test). Next, we examined whether exocytosis was altered in synapses expressing sypHy by using FM4-64. The recycling pool of synaptic vesicles was loaded with the dye using a train of 600 APs at 20 Hz (Sankaranarayanan and Ryan, 2000), then dye release was monitored using a train of 40 AP at 20 Hz, which is sufficient to release the RRP (Rosenmund and Stevens, 1996). We found no significant difference in the amount of FM4-64 lost per synapse in untransfected neurons compared to those expressing synaptopHluorin or sypHy (Figure S1D and D).

SypHy was localized to synaptic vesicles better than synaptopHluorin

In the main paper we illustrate how sypHy allowed exocytosis to be detected with better signal-to-noise than synaptopHluorin (compare Figures 1B and 2B). This improvement was not due to significantly larger signals from sypHy (Figure S1C), but lower background due to much reduced surface staining (Figure 2A and Movie S1). To quantify this, we measured the surface fraction of the two constructs by performing NH_4Cl de-quenching experiments (Figure S2'; Sankaranarayanan et al., 2000). We found that the surface fraction of sypHy, 7.5 ± 1.1 %, was only a third of that for synaptopHluorin, 24.3 ± 3.9 %. Thus, sypHy was better localized to synaptic vesicles compared to synaptopHluorin. This is also evident in supplementary Movie 1 that provides a side-by-side comparison of synaptic responses imaged using sypHy and synaptopHluorin. Moreover, sypHy was less prone to escape from the site of exocytosis than synaptopHluorin (Figure 1, 2 and 8).

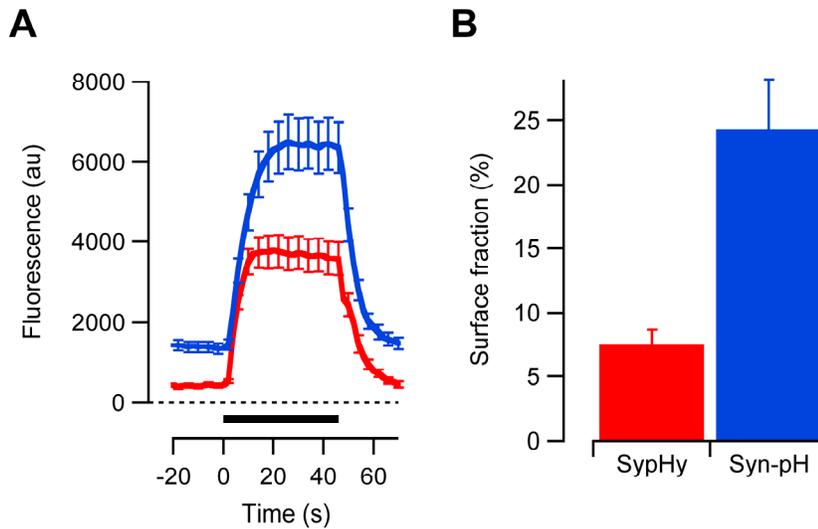


Figure S2. Estimates of surface expression for sypHy and synaptopHluorin

(A) Time course of pHluorin fluorescence changes during alkalinization using ammonium chloride. Average traces are shown for neurons expressing sypHy (red trace, $n = 24$) or synaptopHluorin (blue trace, $n = 23$). Baseline fluorescence (F_0) is the sum of highly fluorescent pHluorins non-specifically expressed on the cell surface (pH 7.4, F_{surf}) and the 20.7-fold lower fluorescence of pHluorins in acidic compartments (pH 5.6, F_{ves}) assuming a pK of 7.1 of superecliptic pHluorin (Miesenbock et al., 1998; Sankaranarayanan et al., 2000). Synapses were superfused with 50 mM NH_4Cl (black bar, $\Delta F = F_{\text{NH}_4\text{Cl}} - F_0$).

(B) Surface fraction of sypHy and synaptopHluorin. The relative contribution of surface expression to basal synaptic fluorescence is given as the surface fraction ($f = F_{\text{surf}} / F_{\text{NH}_4\text{Cl}} = (F_0 - F_{\text{ves}}) / F_{\text{NH}_4\text{Cl}}$, where $F_{\text{ves}} = \Delta F / 20.7$). For sypHy synapses f is $7.5 \pm 1.1\%$, significantly lower than $24.3 \pm 3.9\%$ for synaptopHluorin ($n = 4-6$ cells, $p = 0.0011$, Student's t -test).

Vesicle reacidification time measured with sypHy is similar to that measured with synaptopHluorin

The kinetics of fluorescence decay of intravesicular pHluorins after evoked release is often referred to as the time course of vesicle retrieval. This is not strictly true since the fluorescence decay is a two-step reaction with first endocytosis and then reacidification by means of the vesicular proton pump. If reacidification were much faster than the kinetics of endocytosis, direct measurement of the fluorescence recovery would serve as a convenient simplification. Direct measurements of

endocytosed vesicles in the process of reacidifying has provided time constants about 4 s (Atluri and Ryan, 2006). Similar experiments performed in neurons expressing sypHy gives a reacidification time constant of 3.4 s (Figure S3).

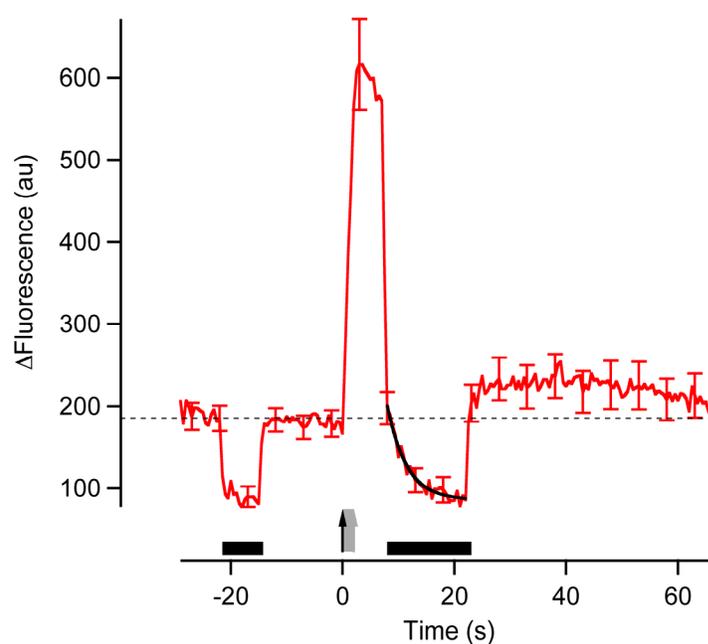


Figure S3. Vesicle reacidification time in synapses expressing sypHy

Average sypHy fluorescence trace (5 experiments, 51 synapses) with two periods of superfusion with pH 5.25 MES buffer (black bars). Endocytosed vesicles “trapped” during acidic buffer perfusion starting 5 s after 80 APs at 40 Hz (arrow), reacidified as described by a single exponential decay (black) with time constant 3.4 s. Error bars indicate s.e.m.

Fusion of individual vesicles could be resolved using sypHy

SypHy could be used to detect fusion of single vesicles. A representative experiment is shown in Figure S4, where the neuron was illuminated continuously and images captured at 5 Hz while stimulating with single pulses separated by 45 s. The upper trace in Figure S4A is the mean fluorescence over an ROI of $1.6 \times 1.6 \mu\text{m}$ centered over a single synapse. Events triggered by the stimulus can be seen superimposed on a baseline that is decaying due to photobleaching. This drift was corrected empirically, by fitting a double exponential function to “baseline periods” that excluded the 30 s following a stimulation pulse, and then subtracting this function from the original trace (see Supplementary Methods). The result is shown by the lower trace in Figure S4A. Amplitude histograms for the exocytic events in this

neuron were distributed with a quantal peak distinct from background noise (Figure S4B and C), indicating that it was possible to detect fusion of single vesicles.

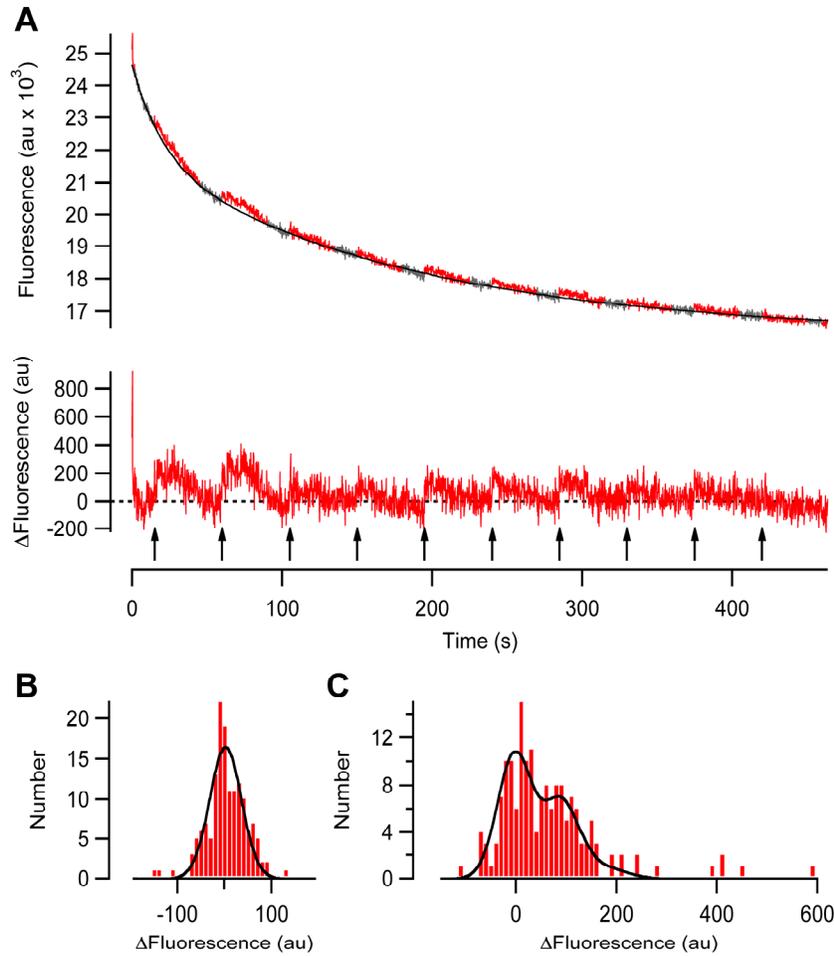


Figure S4. Single vesicle fusion events can be resolved with sypHy imaging

(A) Correction for photobleaching. Sample trace, 5 Hz acquisition rate, before (above) and after (below) bleach subtraction. A double exponential function (black) was fitted to the 15 s of baseline (grey) that preceded each AP (arrows). After subtraction, ΔF responses are readily visible, note the difference in scale.

(B and C) Frequency histograms to measure the background variability (B) and responses to APs (C). In C, ΔF histogram measured for the average response amplitude during 2 s after single stimuli compared to a 10 s baseline. Black line is a best-fit model with two equidistant quantal peaks with standard deviation (eq. 1, $\sigma_q = 3.1$, $q = 92$ au, 16 synapses in the same neuron). In B, ΔF variability measured as for responses in c but out-of-phase with the stimulation during grey periods in A. Black line is a normal distribution fit ($\sigma_0 = 34$).

eq. 1

$$\sigma_n = \sqrt{\sigma_0^2 + n\sigma_q^2}$$

An artifact introduced by selection of responses

In a previous study (Gandhi and Stevens, 2003), the kinetics of endocytosis after single action potentials was investigated by selecting release events before averaging. The criterion for selection was that the amplitude of the synaptopHluorin signal (ΔF) in an ROI of $2.7 \times 2.7 \mu\text{m}$, averaged for 0.3 s after the AP, be distributed in the range $q \pm \sigma$, where q is the amplitude of the first Gaussian used to fit the amplitude histogram and σ is the standard deviation of that Gaussian. We found that this method of analysis tends to introduce an “up-noise” bias that appears as a fast transient in the synaptopHluorin signal.

The nature of this problem is illustrated in Supplementary Figure S5, which shows an analysis from one neuron expressing synaptopHluorin. The ΔF histogram (Figure S5A) could be fit with a quantal model based on Gaussian distributions of baseline noise and quantal events. When *all* traces were averaged non-selectively, there was a hint of a fast phase of recovery in the synaptopHluorin signal within an ROI of $1.6 \times 1.6 \mu\text{m}$ (Figure S5B), but this component disappeared when an ROI of $4.8 \times 4.8 \mu\text{m}$ was used (Figure S5C), indicating that it was caused by lateral movement of synaptopHluorin rather than endocytosis. But, continuing the argument, a double exponential fit to the average trace in Figure S5B provided a “fast component” with $\tau = 770$ ms accounting for 31% of the biexponential decay. In contrast, when traces of amplitude $q \pm \sigma$ were selected before averaging, the “fast component” became more prominent (Figure S5D), accounting for 42% of the decay with a time constant of 1.1 s. As a control, we applied this selection procedure to a period of baseline noise collected from the same synapses without stimulation (Figure S5E and F). A similarly fast transient signal was obtained (Figure S5E), indicating that it was not related to exocytosis or endocytosis. The likely explanation is that this component in the averaged signal is generated by the “up-noise” events at the right extreme of the statistical noise distribution. In support of this interpretation, a fast negative deflection was observed after selecting events of amplitude $-q \pm \sigma$; the only likely explanation is that this negative deflection originates from selection of “down-noise”. “Up-noise” events could also be recreated in a fully simulated data set (Figure S5H and I).

Given these potential difficulties with an analysis based around the selection of events on statistical grounds, we decided the most reliable and unbiased analysis would be to simply use all traces to calculate the stimulus triggered average.

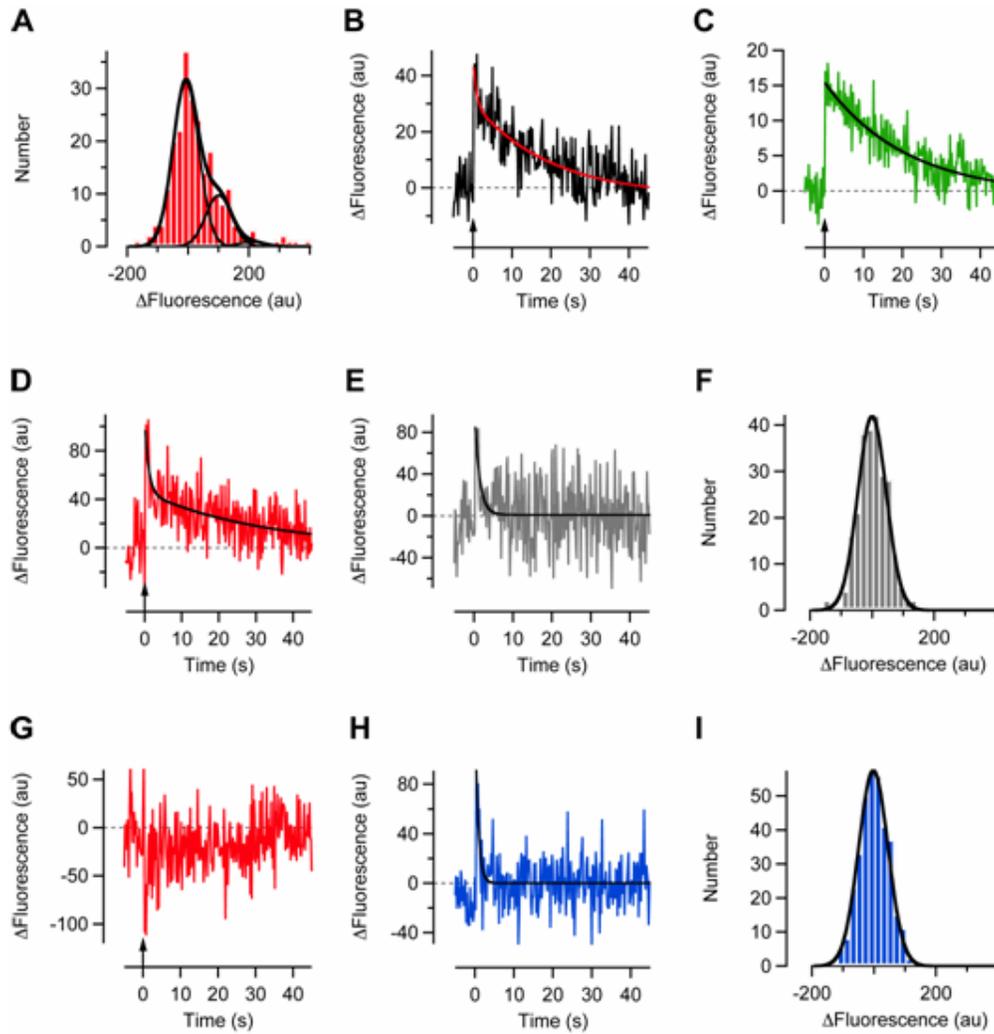


Figure S5. Selective averaging creates a false fast component

(A) Histogram of ΔF to single APs in one neuron expressing synaptopHluorin (12 synapses, 231 responses). Thin black lines are three evenly spaced gaussians in a quantal model as in Figure S4C eq. 1, with no release ($\sigma_0 = 41$) and single and double quanta ($\sigma_q = 11$, $q = 104$ au). Thick black line is the sum of the gaussians.

(B) All responses in A averaged. Red line is a double exponential curve fit with $\tau_{\text{fast}} = 769$ ms and $\tau_{\text{slow}} = 19.6$ s. The fast component accounts for 31% of the ΔF decay.

(C) As shown in the main paper, the fast component in B is a synaptotagmin movement artifact. The green trace is an average using a $4.8 \times 4.8 \mu\text{m}$ ROI instead of the $1.6 \times 1.6 \mu\text{m}$ normally used, and decays with a single exponential $\tau = 18.1 \text{ s}$.

(D) When the events in the first quantal peak ± 42 (from eq. 1) in A are singled out for averaging ($n = 51$) the fast component gets exaggerated. Black line is a double exponential with $\tau_{\text{fast}} = 1.09 \text{ s}$ and $\tau_{\text{slow}} = 32.1 \text{ s}$. Now the fast phase accounts for no less than 42%.

(E and F) The reason comes from selecting “up-noise” for averaging; E is an average of traces selected with the same parameters as in D ($n = 25$) but from the noise histogram in F where no APs were evoked ($n = 231$). The exponential curve fit to the average gives a $\tau = 1.28 \text{ s}$, the histogram is fit with a gaussian with $\sigma = 46$.

(G) When the selection parameters in D were used at the negative aspect of the data in A, a negative fast event can be seen ($n = 17$), most likely from selecting “down-noise” to be averaged.

(H and I) The problem with selective averaging can also be reproduced with a data set where traces are created from gaussian random noise. The average in H ($n = 30$) are from traces selected from the simulated data set illustrated in the histogram in I ($\sigma = 47$, $n = 340$). An artificial fast component with $\tau = 775 \text{ ms}$ is evident. The effect from “up noise” selection illustrated in this figure is expected to be a problem in particular to synapses with low release probability.

Supplemental Experimental Procedures

Primary cultures of rat hippocampal neurons

Hippocampi from E18 rat pups were dissected in cold Earl’s buffered salt solution with 10 mM HEPES and 100 U/ml penicillin-streptomycin (pen-strep). Each hippocampus was chopped in half and then incubated in Papain (10 U/ml in dissection solution) for 15 min at 37°C . Digested pieces were spun-down at 1500 rpm for 2 min, washed in 10 ml plating medium, spun again and resuspended in ~ 1 ml of plating medium. Pieces were dissociated with several passages through two flame-polished Pasteur pipettes of successively smaller diameter. Cells were plated on laminin/poly-D-lysine-coated cover slips at a density of 200,000-250,000 cells/ml, 150 μl per one 16 mm cover slip, two cover-slips per 35 mm well. Plating medium was modified eagle medium (MEM) supplemented with 100 U/ml pen-strep, 1% N2, 10% horse serum and 20 mM glucose, 1 mM sodium pyruvate, 25 mM HEPES. After 3-4 hr, 2 ml of plating medium was added per well (6 well format). Cells were fed the

following day (1 DIV) and every ~3 days by replacing 50% of the medium with plating medium. For some cultures, to improve transfection efficiency, the 4 DIV and subsequent feeds were done using Neurobasal medium supplemented with 2% B27, 1% pen-strep and 0.5 mM L-glutamine (Brewer et al., 1993). All medium was free of phenol red.

Molecular biology

For expression of synaptophysin in hippocampal neurons, synaptophysin from pCI Neo (kind gift from J.E. Rothman) was inserted into pEGFP-C1 at *Nhe* I and *Xba* I sites to substitute for EGFP. SypHy was made in two steps. First, rat synaptophysin1 cDNA (NM_012664, kind gift from H.T. McMahon) was amplified by PCR from the ATG to behind the third TM region introducing a Kozak region before the ATG and *Xho* I and *Age* I sites at the 5' and 3' ends respectively, using oligos 1 & 2 (see below). The product was then inserted into the synaptophysin vector via *Xho* I and *Age* I hereby connecting it to pHluorin and replacing the VAMP region of synaptophysin. Second, to restore the fourth TM region and the C-terminus of synaptophysin to sypHy we used PCR to introduce *Age* I sites at the 5' and 3' ends (oligos 3 & 4) for insertion into the previous plasmid (before the stop-codon) at *Age* I.

To express mRFP-tagged proteins, we made two vectors, pmRFP1-C1 and pmRFP1-N1, which were analogous to the Clontech Living Colours pEGFP-C1 and pEGFP-N1 plasmids. The cDNA for mRFP1 was amplified by PCR from mRFP1 (Campbell et al., 2002) in pHRSET-B using oligos 5 & 6 and inserted into pEGFP-C1 using *Age* I and *Not* I, or into pEGFP-N1 at *Age* I and *Bgl* II by using oligos 7 & 8. To make mRFP-synaptobrevin, mouse synaptobrevin was amplified from synaptophysin by PCR using oligos 9 & 10 to introduce a *Hind* III at the 5' end and a stop codon and an *Eco*R I sites at the 3' end. The product was cloned into pmRFP1-C1. To make mRFP-AP180-C, rat AP180 cDNA (kind gift from E. Ungewickell) corresponding to residues 530-896 was amplified by PCR to introduce *Hind* III and *Bam*H I sites at the 5' and 3' ends, respectively (using oligos 11 & 12). The product was inserted into pmRFP1-C1. To make LCa-mRFP, human non-neuronal clathrin light chain a (I.M.A.G.E. 3944942) was amplified using oligos 13 & 14 and subcloned into pmRFP1-N1 at *Bgl* II and *Eco*R I sites. To make Syp-mRFP, rat synaptophysin1 cDNA was amplified by PCR from the 5' ATG to the 3' stop codon introducing a

Xho I site and a Kozak region 5' as well as deleting the stop codon and introducing a *Bam*H I site 3' (oligos 15 & 16). The product was inserted into pmRFP1-N1. All constructs were verified by restriction digest or automated DNA sequencing (MRC Geneservice, UK).

CHC and scrambled siRNA were as previously described (Royle et al., 2005).

No.	Oligo name	Sequence (5'-3')
1	5' SypHy (1. step)	CGCTAGCTCGAGCCACCATGGACGTGGTGAATCAGCTGGTGGC
2	3' SypHy (1. step)	TCCACCGGTGTTCCCTGTCTGGCGGCACATGG
3	5' SypHy (2. step)	CCCACCGGTGCGCCAGACAGGGAACACATGCAAGG
4	3' SypHy (2. step)	GACACCGGTCTGCAGATTACATCTGATTGGAGAAGGAGG
5	5' pmRFP1-C1	TGCATACCGGTGCGCCACCATGGCCTCCTCCGAGGACGTC
6	3' pmRFP1-C1	GCTCGAGATCTGAGTCCGGAGGCGCCGGTGGAGTGGCGG
7	5' pmRFP1-N1	TGCATACCGGTGCGCCACCATGGCCTCCTCCGAGGACGTC
8	3' pmRFP1-N1	AGAGTCGCGGCCGCTTTAGGCGCCGGTGGAGTGG
9	5' VAMP2	GCTCAAGCTTCGATGTCGGCTACCGCTGCCAC
10	3' VAMP2	TGCAGAATTCTTAAGTGTGAAGTAAACGATGATGATGATG
11	5' AP180	GTGGCAGCCACTAAGCTTCTGCTGCCACCACCACCGCCGCCGCTG
12	3' AP180	TCCGGTGGATCCTTACAAGAAATCCTTGATGTTAAGATCC
13	5' LCa	GACTCAGATCTGCCACCATGGCTGAGCTGGATCCGTTC
14	3' LCa	CTGCAGAATTCCGGTGCACCAGCGGGGCTGC
15	5' Syp-mRFP	CGCTAGCTCGAGCCACCATGGACGTGGTGAATCAGCTGGTGGC
16	3' Syp-mRFP	GATGGATCCTGATTGGAGAAGGAGGTGGGCGC

Neuronal transfection

Hippocampal neurons were transfected using Lipofectamine2000 in MEM according to the manufacturers instructions. For synaptopHluorin, sypHy and protein diffusion experiments cells were double transfected at 8 DIV with mRFP and either synaptopHluorin or sypHy for pHluorin experiments; or sypHy and an mRFP fusion protein for protein movement experiments (4 µg total DNA per well). Both DNAs were in the same mixture. Cells were used for experiments from 14 to 21 DIV. For clathrin RNAi experiments, cells were transfected with sypHy and mRFP (4 µg total

DNA per well) and either CHC or scrambled siRNA (12 μ l of 40 mM per well) in a separate mixture. For dominant-negative experiments, cells were transfected with either sypHy and mRFP or sypHy and mRFP-AP180-C (4 μ g total DNA per well). For these endocytosis interference experiments, neurons were transfected at 11 DIV and were used 3 days post-transfection.

Neuronal labeling and quantification

For immunocytochemistry, cultures were fixed in 3% paraformaldehyde and 4% sucrose in PBS (in mM: 1.5 NaH_2PO_4 , 8 Na_2HPO_4 , and 145 NaCl , pH 7.3) for 10 min at 4°C. Cells were permeabilized using 0.1% Triton X-100 in PBS for 10 min at 4°C. Non-specific sites were blocked using PBS containing 4% normal goat serum and 3% bovine serum albumin (blocking buffer). Antibodies were diluted to their final concentration in blocking buffer. Primary antibodies (X22, 6.0 μ g/ml; synapsin I, 0.4 μ g/ml both from Affinity BioReagents) were applied for 2 hr at RT. Cells were rinsed once in blocking buffer and three times for 5 min with PBS, and then secondary antibodies (Oregon green 488- or Cy3-conjugated goat anti-mouse or -rabbit) were applied for 2 hr at RT. Finally, cells were washed five times for 5 min with PBS and mounted onto slides with ProLong anti-fade.

To measure CME as the uptake of transferrin-Alexa546 or -Alexa488 (Molecular Probes), cells were incubated in serum-free MEM for 15 min at 37°C, then kept in serum-free MEM containing 50 μ g/ml transferrin-conjugate for 15 min at 37°C. Cells were washed twice in PBS and then fixed and mounted for microscopy. Quantification of CHC immunoreactivity or transferrin uptake in neurons was carried out as described previously (Royle et al., 2005).

Live imaging

Cells were perfused at \sim 0.2 ml/min with normal extracellular solution at $23 \pm 2^\circ$ C (in mM: 136 NaCl , 2.5 KCl , 10 HEPES, 1.3 MgCl_2 , 10 glucose, 2 CaCl_2 , 0.01 CNQX and 0.05 DL-APV, pH 7.4) using a Gilson minipuls 3 peristaltic pump. In some experiments Bafilomycin A (Calbiochem) was added to the perfusing buffer to a final concentration of 2 μ M 1 min preceding the experiment. Fluorescence imaging was carried out on a Nikon Diaphot 200 microscope with a 40 \times (1.3 NA) oil immersion

objective and a Xenon 100 W unit. The filter set for pHluorin comprised a 475AF40 excitation filter, a 505DRLP dichroic mirror and a 535AF45 emission filter (Omega Filters). Using these filters, the illuminating light leaving the objective was 300 mW. For mRFP constructs, the filter set comprised a 560AF55 excitation filter, a 595DRLP dichroic mirror and a 645AF75 emission filter.

To minimize bleaching of the cells during time-lapse experiments we synchronised camera exposure times and illumination using a Uniblitz VMM-D3 shutter driver with an on- and offset of 10 ms. Images were captured at a depth of 16-bit using a Photometrics Cascade 512B camera controlled by IPLab (Scanalytics). Electrical field stimulation was delivered via two parallel platinum wires 5 mm apart. Action potentials were evoked by 20 mA square-pulses of 1 ms duration. We did not attempt to reduce background noise by photobleaching synaptopHluorin or sypHy on the surface before applying test stimuli (Gandhi and Stevens, 2003). In our hands, extensive pre-bleaching damages the cells. After 45 min of pre bleaching (80% light attenuation, 60× 1.40 NA objective) $52 \pm 10\%$ ($n = 5$) of the synapses present before bleaching failed to respond to a 40 AP train. This was significantly different ($p = 0.036$, Student's t-test) to controls where $20 \pm 5.8\%$ ($n = 5$) failed to respond after waiting 42 min without illumination. Eight out of 18 somata of non-transfected neurons retained Trypan Blue, indicating a non-viable cell, after the pre-bleaching protocol while this was not the case for any of 17 control neurons.

For experiments monitoring pHluorin responses to single APs, images of 128×128 pixels (binned 2×2) were acquired at 5 Hz with 195 ms of exposure at full illumination. For each cover slip we acquired two data sets, the first one consisting of 10 single APs at 45 s intervals (2325 images in total), the second consisting of the same 10 single APs followed by a final train of 40 APs at 20 Hz (3000 images in total). To avoid bleaching pHluorin, transfected cells were first located using co-transfected mRFP.

For acid quench experiments images of 512×512 pixels (binned 2×2) were acquired at 2 Hz with 195 ms shuttered exposure with a neutral density filter transmitting 25% of the incoming light. The field of view was superfused with a gravity-fed laminar flow of buffer through a single barrel glass micropipette fitted with two MicroFil needles (WPI) reaching to the very tip to minimize dead-volume. The superfusing buffer was switched using solenoid valves (Lee products Ltd.)

between the standard pH 7.4 HEPES buffer described above and a pH 5.25 buffer with the HEPES substituted with equi-molar MES. AP stimulation was time-locked to frame acquisition and buffer changes.

For experiments monitoring the effects of interfering with CME, time-lapse images of sypHy (512×512 pixels) were acquired at 0.5 Hz with 250 ms of exposure at 25% illumination. Four or 40 APs were delivered at 20 Hz. To avoid bleaching pHluorin, transfected cells were first located using co-transfected mRFP or mRFP-AP180-C.

For experiments monitoring the diffusion of clathrin, synaptobrevin and synaptophysin two sets of time-lapse images (150 images each, 512×512 pixels) were acquired at 0.5 Hz with 250 ms of exposure at 25% illumination intensity. The stimulus protocol comprised 4, 40 and 400 APs delivered at 20 Hz after 40, 100 and 160 s. The first data set was taken with the mRFP filter set and the second (sypHy) data set was taken after 5 min.

Experiments in which intravesicular pHluorin was dequenched were carried out in time-lapse (60 images, 0.5 Hz, 256×256 pixels, binned 2×2 , 195 ms exposure time, 25% illumination). After 30 s of imaging under normal conditions, the extracellular medium was changed for 60 s to the same buffer containing 50 mM NH_4Cl (with NaCl content adjusted to maintain osmolality, pH 7.4). The solution switch was carried out with a Warner 6 channel valve controller and gravity flow at ~ 1 ml/s. To minimise pre-bleaching of the surface fraction of pHluorin when searching for transfected neurons, these were co-transfected with mRFP to allow identification using the red filterset.

Experiments imaging uptake and release of FM4-64 were essentially the same as time-lapse experiments except a different microscope (Zeiss Axiovert S100TV, $40\times$ 1.3 NA Plan-NEOFLUAR oil objective and appropriate filtersets) and camera (Princeton Instruments PentaMax with II-100 Image Intensifier) were used. After imaging the sypHy or synaptopHluorin response to a test train of 40 APs at 20 Hz, synapses were labeled with 15 μM FM4-64 (Molecular Probes) applied during and 1 min after a stimulus of 600 AP at 20 Hz to load all vesicles in the recycling pool. After washing with normal extracellular buffer (10 min) the neurons were again stimulated with a train of 40 APs at 20 Hz, followed by destaining with a stimulus of 900 AP at 20 Hz.

Image analysis

We analyzed images using custom-written macros and procedures in IPLab and IgorPro. Synapses were identified using “difference images” highlighting the stimulus-dependent increase in sypHy fluorescence. These difference images were constructed by subtracting a 5-frame average obtained immediately before a test train of 20 or 40 APs from a 5-frame average obtained just after stimulation. Square regions of interest (ROIs) were then positioned at the center of exocytic hot-spots, revealing active synapses. The standard ROI size was $1.6 \times 1.6 \mu\text{m}$ and the precise location was selected to maximize the basal fluorescence within the ROI before stimulation. Larger ROIs ($4.8 \times 4.8 \mu\text{m}$) and perimeter zones (outer width of $4.8 \mu\text{m}$ and depth of $0.8 \mu\text{m}$) were then positioned to encompass smaller ROIs within their centres.

In acid quench experiments ROIs were $2.4 \times 2.4 \mu\text{m}$. To reduce buffer flow artifacts and signals originating from glia, background noise from a same size ROI displaced in x- or y-direction to a representative region without synapses was subtracted frame by frame. Traces were then normalized to baseline fluorescence and averaged.

The illuminating intensities necessary to obtain synaptopHlurin or sypHy signals in response to single APs also caused significant bleaching, especially in experiments which required periods of continuous illumination (Figure S4A upper trace). This drift was corrected empirically, by fitting a double exponential function to “baseline periods” that excluded the 30 s following a stimulation pulse, and then subtracting this function from the original trace. The result was a trace with a relatively flat baseline. Responses to single APs could be clearly observed using sypHy (Figure S4A), but they were much less clear using synaptopHluorin (Figure 1B).

Non-phasic release of synaptic vesicles after an action potential will affect the apparent time constant of ΔF decay. When signals were averaged, delayed transmitter release lead to ΔF shoulders or delay-periods that deviate from exponentially decaying models. Therefore each response to single APs had to be individually

examined and traces containing non-phasic events rejected from further analysis (e.g. the second stimulation in Figure 2B, main paper).

Although the ΔF increments with the fusion of single synaptic vesicles could be resolved, some release events overlapped with baseline noise level and made it difficult to resolve whether or not vesicle release occurred (Figure S4C). To get an unbiased estimate of release probability, the average ΔF response amplitude to single APs was compared to the RRP estimate (40 APs) for each synapse, providing the release probability of a vesicle in the readily-releasable pool (P_{rv}). Thus it was possible to make selective averages of high-release and low-release synapses from the whole data set. The P_{rv} distribution was fit with a Gamma function $f(x) = Ax^{\alpha-1}e^{-\beta x}$.

For clathrin interference experiments, synapses were identified and $1.6 \mu\text{m} \times 1.6 \mu\text{m}$ ROIs were placed on the sypHy movie as described above. The local background was subtracted for each synapse by displacing the ROI \pm (1 ROI length + $0.8 \mu\text{m}$) in both x- and y-directions and averaging the two background ROIs with mean intensities, discarding max and min ROIs to avoid artefacts from outliers. The traces were corrected for bleach by subtracting a double exponential function that was fitted to baseline traces or non-responding regions of the neuron. The baseline of corrected traces was set to zero and then all traces were averaged.

For experiments monitoring the diffusion of clathrin, synaptobrevin or synaptophysin, synapses were identified and ROIs ($1.2 \times 1.2 \mu\text{m}$) were placed on the sypHy movie as described above. These ROIs were then copied onto the respective mRFP movie to monitor mRFP fluorescence at the active synapses. The traces for each individual synapse were normalized to the average fluorescence (five frames) before the first stimulus. They were then individually examined to reject traces (less than 5%) which showed fast transients caused by trafficking of mRFP-positive endosomes. All remaining traces from five experiments for each construct were averaged and corrected for photo-bleach by fitting a line to the trace before the 40 AP stimulus and subtracting this function.

For intravesicular dequenching experiments ROIs ($1.6 \times 1.6 \mu\text{m}$) were placed on synapses identified by their response to a 2 s 20 Hz (40 AP) test-train. The surface fraction was calculated as $f = (F_0 - 20.7(F_{\text{NH}_4\text{Cl}} - F_0)) / F_{\text{NH}_4\text{Cl}}$ (Sankaranarayanan et al., 2000) also described in Figure S2.

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