Neuron, Volume *70*

Supplemental Information

Synaptophysin Regulates the Kinetics of Synaptic Vesicle Endocytosis in Central Neurons

Sung E. Kwon and Edwin R. Chapman

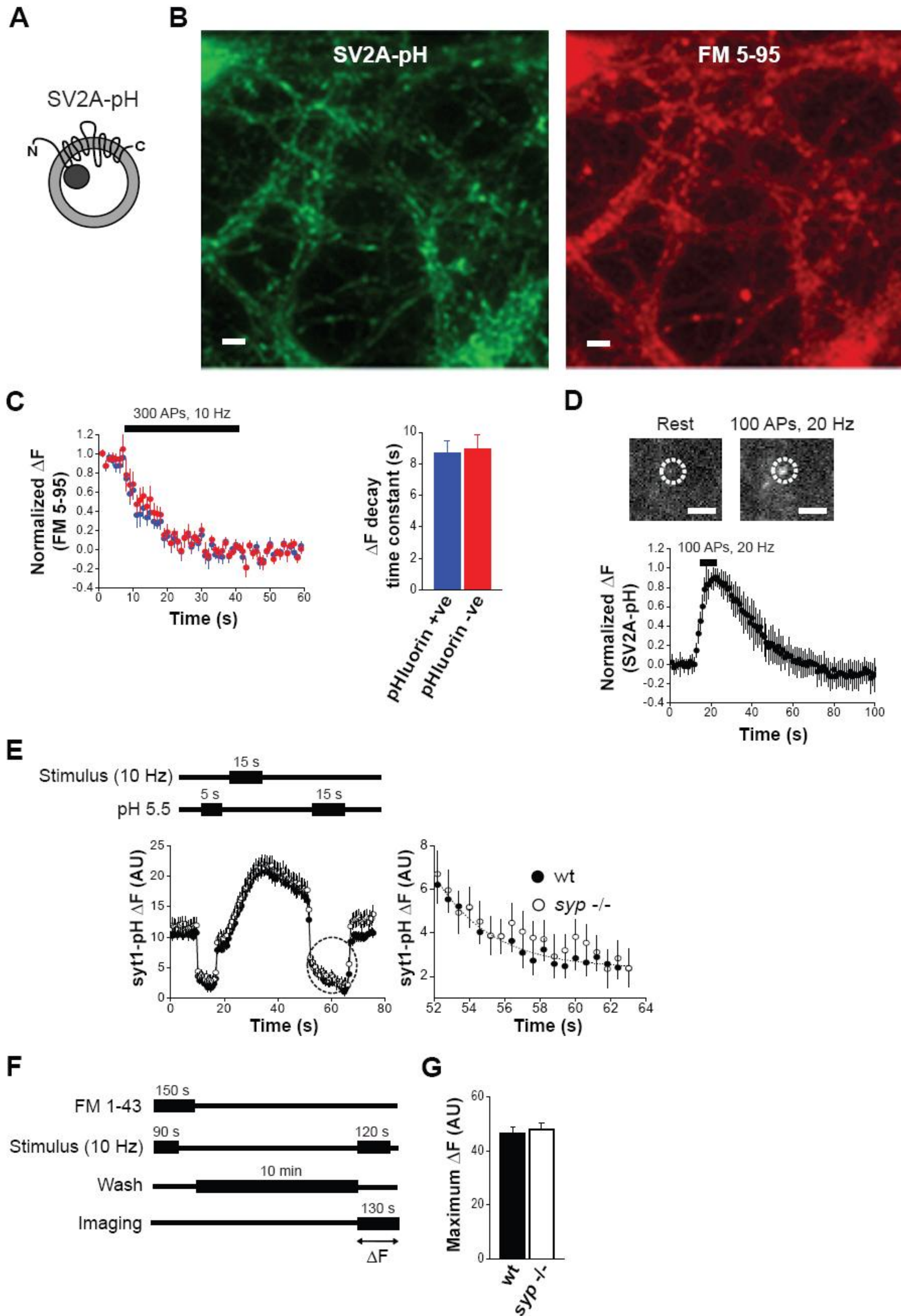

Fig. S1 (Related to Fig. 1).

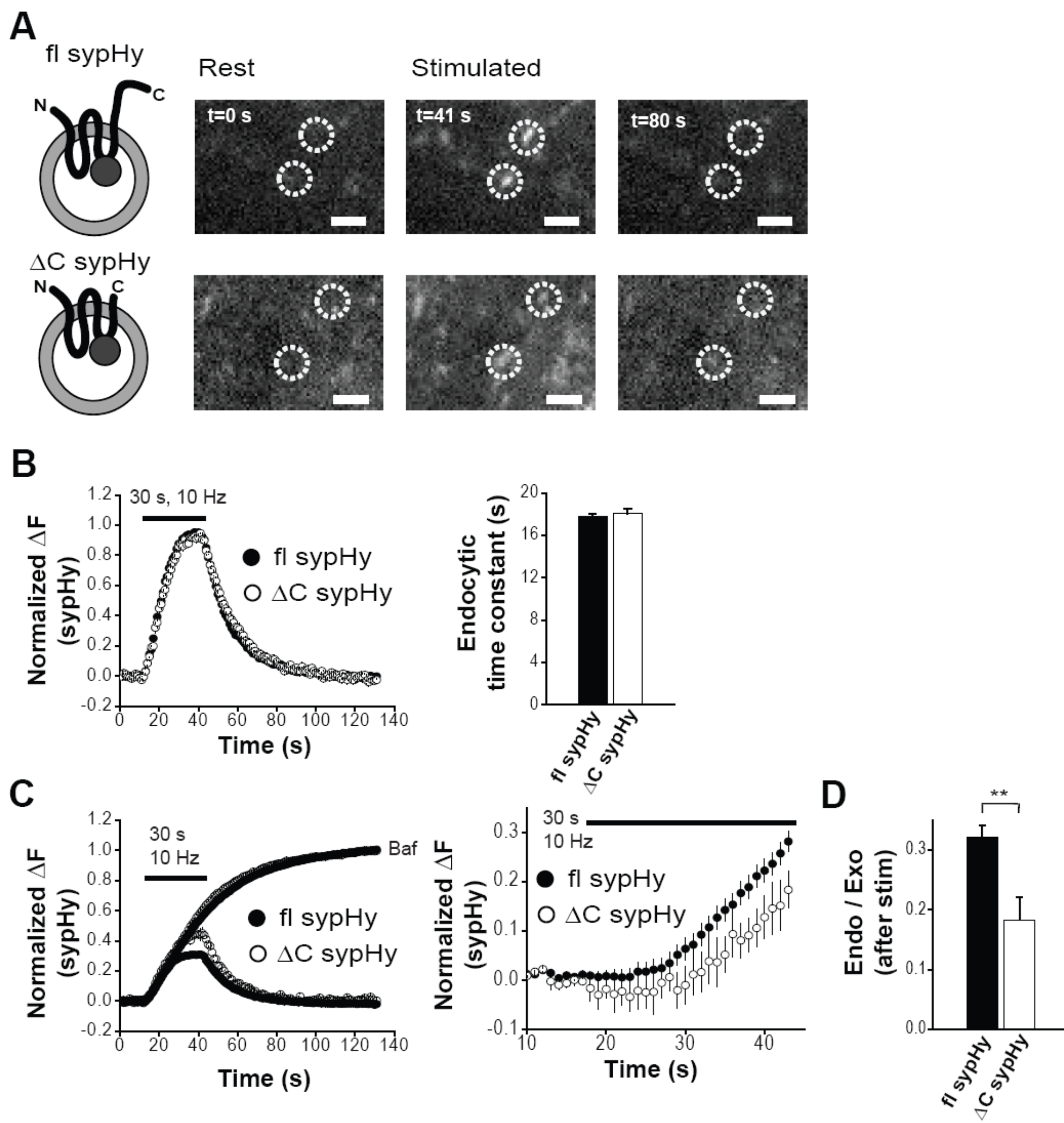

Fig. S2 (Related to Fig. 3).

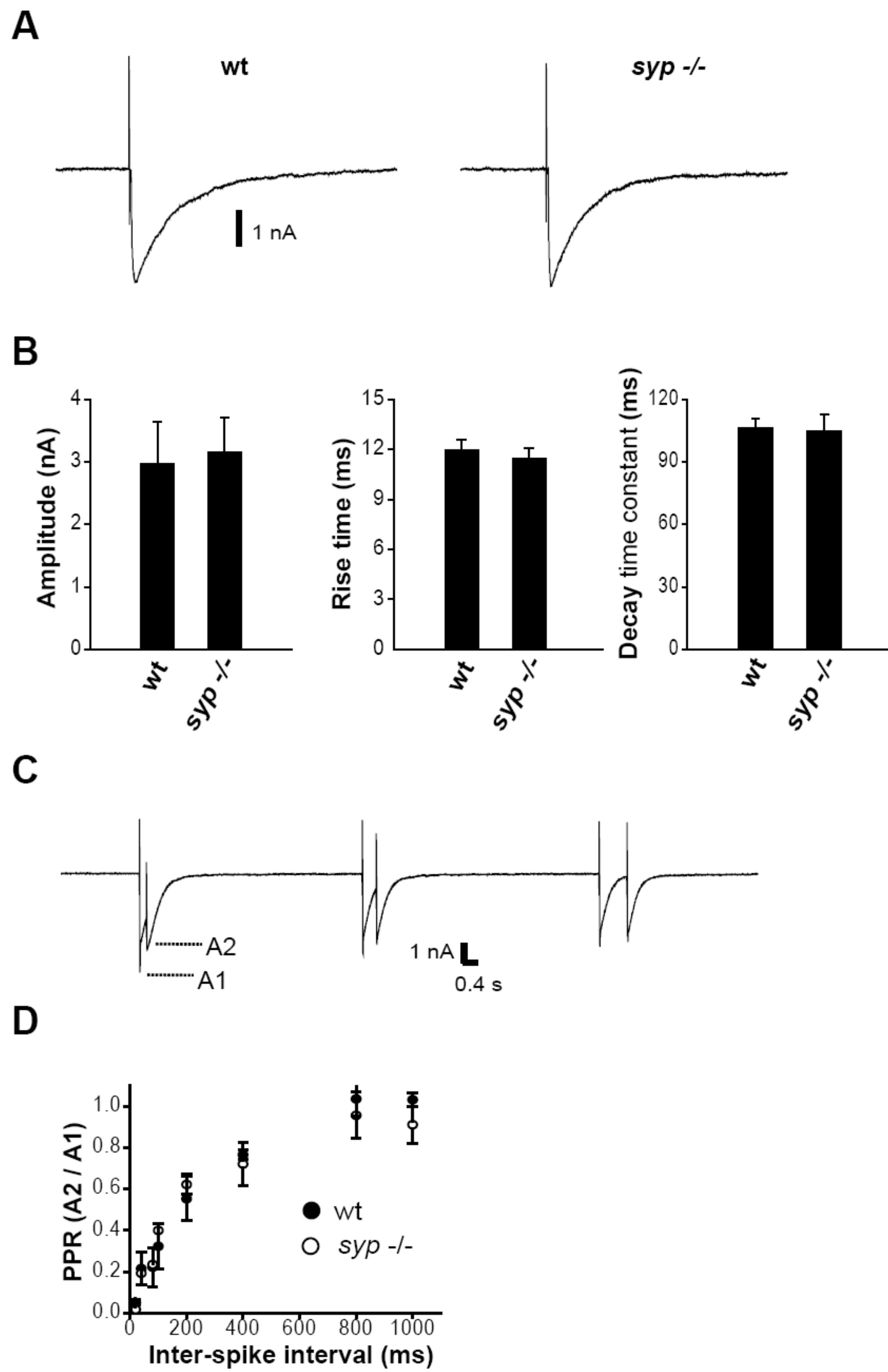

Fig. S3 (Related to Fig. 4)

Supplemental Figure Legends

Figure S1. **Validation of SV2A-pHluorin, measurements of re-acidification time course and total recycling pool in wt and syp -/- neurons.**
(A) Schematic diagram of SV2A-pHluorin. The pHluorin was inserted to the first intra-luminal loop of SV2A.
(B) SV2A-pH is efficiently targeted to recycling SVs labeled by FM 5-95. Cultured hippocampal neurons expressing SV2A-pH were subjected to FM 5-95 loading experiments using the same protocol as in *(F)*. The SV2A-pH image (Left) was acquired at rest in a bath solution containing ammonium chloride that alkalinizes the vesicle lumen thereby dequenching all pHluorin. Scale bars are 6 μm.
(C) (Left) Average traces of FM 5-95 destaining in neurons expressing SV2A-pH (blue) or in control neurons (red). Values were normalized to the maximal fluorescence change. Traces represent averages from 2 coverslips, 20 boutons each. (Right) Average decay time constants of FM 5-95 destaining were invariant ($\tau = 8.65 \pm 0.8$ s in 'pHluorin +ve', $\tau = 8.93 \pm 0.9$ in 'pHluorin –ve'). $p > 0.01$ (two tailed, unpaired *t*-test throughout). This shows that pHluorin expression does not interfere with the normal SV recycling pathway. Error bars are SEM.
(D) Dashed circles indicate boutons expressing SV2A-pH at rest (Top left) and after applying 100 stimuli at 20 Hz (Top right). Scale bars are 2 μm. (Bottom) Averaged fluorescence changes of SV2A-pH expressed in cultured mouse hippocampal neurons, in response to 100 stimuli at 20 Hz.
(E) (Top left) Protocol used to measure vesicle re-acidification following stimulation. Neurons expressing syt1-pH were briefly perfused with an acidic bath solution (pH 5.5) to quench all surface-stranded syt1-pH and then stimulated at 10 Hz for 15 s. During the decay phase that represents vesicle endocytosis and re-acidification, the neurons were again perfused with the acidic solution (pH 5.5) for 15 s. This latter perfusion unveiled a fraction of vesicles that are initially resistant but eventually become quenched (Bottom left). The time-course of fluorescence change (dashed circle) from the quenching resistant to the fully quenched state, ie., vesicle reacidification, was not different between wt and *syp* -/- ($\tau = 3.13 \pm 1.2$ s in wt, $\tau = 3.31 \pm 1.2$ s in *syp* -/-, $p > 0.01$) (Bottom right). Traces are averages from 2 coverslips, 10 boutons each, for WT and *syp* -/-. Error bars are SEM.
(F) Protocol for measuring the total recycling pool using a styryl dye, FM 1-43. Neurons were stimulated (900 APs, 10 Hz) in the presence of FM 1-43. The dye remained in the bath for an additional 1 min after cessation of the stimulation to allow maximal loading. After a 10 min wash in Ca^{2+}-free media, the neurons were stimulated again (1200 APs, 10 Hz), in the absence of FM 1-43 to unload the dye from recycling vesicles. The change in fluorescence intensity (in arbitrary units, AU) during the unloading step is an empirical measurement of the size of the total recycling SV pool.
(G) Average total recycling pool sizes of wild-type (black, 3 coverslips, 25 boutons each) or *syp* -/- neurons (white, 3 coverslips, 20 boutons each) were not significantly different (46.3 ± 2.5 AU in wt, 47.8 ± 2.4 AU in *syp* -/-, $p>0.01$). All error bars are SEM.

Figure S2. **The C-terminal cytoplasmic tail of synaptophysin regulates trafficking of syp during persistent neuronal activity, but is not important for trafficking following the cessation of stimulation.**

(A) (Left panels) Schematic diagrams of full-length synaptophysin-pHluorin (fl sypHy) and C-terminal truncation mutant synaptophysin-pHluorin (ΔC sypHy) lacking amino acids 244-307. (Right panels) Representative images of boutons (dashed circles) containing fl sypHy or ΔC sypHy at rest (t = 0 s) and 1 s (t = 41 s) or 40 s (t = 80 s) after 300 stimuli. Scale bars are 2 μm.
(B) (Left) Average fluorescence changes of fl sypHy (black) and ΔC sypHy (white) expressed in *syp* -/- hippocampal neurons, in response to 300 stimuli at 10 Hz. Values were normalized to the peak fluorescence change. Average from 5 coverslips, 30 boutons each. (Right) Comparison of post-stimulus time constants between fl sypHy and ΔC syp. The decay phase of ΔF traces were fitted with single exponential functions before normalization, and the time constants were calculated from the fits.
(C) (Left) Trafficking of fl sypHy (black) or ΔC sypHy (white) during stimulation was assayed in *syp* -/- neurons using the protocol in Fig 2(*A*). Average from 5 coverslips, 30 boutons each. (Right) Time-course of retrieval, during the 30 s stimulation, was calculated by taking the difference between ΔF traces before and after Baf application as in Fig. *2(E)*.
(D) Average magnitudes of internalization at the end of the 30 s stimulation.
All error bars are SEM. **, $p < 0.001$.

Figure S3. **Lack of syp does not affect the amplitude, the kinetics or the paired-pulse ratio of IPSCs.**
(A) Representative single IPSCs from wt or *syp* -/- neurons in 3 mM Ca^{2+}.
(B) (Left) Mean amplitude of IPSCs was not significantly different between wt (2.98 ± 0.7 nA, 10 cells) and *syp* -/- neurons (3.16 ± 0.5 nA, 10 cells). (Middle) Mean rise time was measured as the time difference between the base and the peak of each IPSC. There was no significant difference in the rise time between wt and *syp* -/- neurons (12.0 ± 0.6 ms in wt, 11.5 ± 0.6 in *syp* -/- neurons, 10 cells each, $p > 0.05$). (Right) Mean decay time constants were estimated by fitting the decay phase of each IPSC trace with a single exponential function. There was no significant difference in the decay time between wt and *syp* -/- neurons (106 ± 4.2 ms in wt, 105 ± 7.8 ms in *syp* -/-, 10 cells each, $p > 0.05$). Error bars are SD.
(C) Representative IPSC traces in response to paired pulses with inter-spike intervals were varied from 20 ms to 1000 ms. Paired-pulse ratio (PPR) was determined by dividing amplitude of the second peak (A2) by amplitude of the first peak (A1).
(D) PPRs, at different inter-spike intervals, were not significantly different between wt and *syp* -/- neurons, 5 cells each.

Supplemental Experimental Procedures

Molecular Biology

The C-terminus of sypHy was removed by amplifying the full-length sypHy with PCR primers that exclude the C-terminal tail (amino acids 244-307 of synaptophysin) and subcloning the product into BamHI and NotI sites of pLox vector. SV2A-pH was generated by inserting super-ecliptic pHluorin into the first intra-vesicular loop of rat SV2A (between amino acid 197 and 198)

using PCR. For lenti-viral delivery into neurons, syt1-pH, sypHy and SV2A-pH were sub-cloned into a bicistronic lentiviral vector, pLox Syn-DsRed-Syn-GFP lenti-viral vector (pLox) (kindly provided by Dr. Francisco Gomez-Scholl (Serville, Spain)) digested with BamHI and NotI. GFP in the pLox vector was replaced by mCherry to avoid spectral overlap with pHluorin.

Neuronal Cultures and Viruses

Hippocampi or cortices from 0-1 day old *syp* -/- or wt littermates were dissociated in 0.25 % trypsin, washed twice in Hank's Buffered Salt Solution (HBSS) medium containing 10 mM HEPES and plated onto poly-D-lysine coated 15 mm coverslips. All constructs used in this study were based on pLox lenti-viral vector system that has two synapsin promoters. Neurons were infected with viruses on day-in-vitro (DIV) 5 and subjected to imaging between DIV14-20 or electrophysiology between DIV14-17. Infection efficiency estimated by mCherry expression was ~95%.

Live-cell imaging

For acid perfusion experiments (Fig. S1E), acidic solution (pH 5.5) was prepared by replacing HEPES in the bath solution (140 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM HEPES, 10 mM Glucose, 50 μM D-AP5, 10 μM CNQX adjusted to 310 mOsm with glucose, pH 7.4) with 2-[N-morpholino] ethane sulphonic acid (pK_a=6.1). The solution exchange was driven by a MPS-2 multi-channel perfusion system (World Precision Instruments). For time-lapse imaging, a typical exposure time was 400 ms for pHluorin, and 100 ms for FM1-43. The following band pass filters (± 15~20 nm) were used: pHluorin (excitation 484 nm, emission 517), FM1-43 (excitation 484 nm, emission 605 nm), mCherry, FM5-95 (excitation 555 nm, emission 645 nm). A neutral density filter (ND 0.3, Chroma) was used for FM dye experiments to minimize photobleaching. In all experiments, images were acquired for 10 s prior to stimulation to establish a stable baseline. Fluorescence intensity values during the first 10 s were averaged and used for normalization in subsequent analysis. For analysis of image stacks, regions that show fluorescence changes (ΔF) larger than 5 AU during field-stimulation were initially selected and marked with 7-pixel diameter (corresponding to ~1.5 μm) circular selection areas. This threshold was determined as previously described (Richards et al., 2005). Those regions that showed abrupt ΔF larger than 5 AU in reverse direction over any 3 consecutive time-points during the base-line acquisition (~10 s prior to stimulation), or that exhibited any movement during stimulation, were discarded. Bleaching was less than 1% for 120 s~180 s in all imaging experiments. For FM dye imaging, the criteria used in (Richards et al., 2005) were applied with modifications. The cut-off for selection was 5 AU instead of 3.5 AU, due to use of different camera and light source. Data normalization and curve-fitting were carried out using Origin 6.0 software.

Supplemental References

Richards, D. A., Bai, J., and Chapman, E. R. (2005). Two modes of exocytosis at hippocampal synapses revealed by rate of FM1-43 efflux from individual vesicles. J Cell Biol *168*, 929-939.