Supplementary Information for

Molecular mechanism of the synaptotagmin–SNARE interaction in Ca\(^{2+}\)-triggered vesicle fusion

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Supplementary Figure 1 Ensemble binding of Syt3 to SNARE complex. (a-b) Shown are Coomassie stained SDS-PAGE gels. (a) Unlabeled and dye-labeled Syt3 bind SNARE complex. Pull-down of SNARE complex by unlabeled (top gel) and dye-labeled Q410C N554C Syt3 (bottom gel), in the presence of 50 mM KCl. Legend on top of the gels (+ or – signs) indicates whether Syt3, SNARE, EGTA and/or Ca\(^{2+}\) were incubated with streptavidin beads. Boiling refers to whether sample was boiled (+) or not (-) prior to gel loading. In the absence of boiling the neuronal SNARE complex is stable in SDS-PAGE gel loading buffer, while after boiling it dissociates into its four components. For reference, the proteins used for the pull-down assay are shown in lanes labeled as “input”. (b-c) Syt3 binding to SNARE complex is dependent on ionic strength and enhanced by Ca\(^{2+}\). (b) Pull down of SNARE complex using immobilized Syt3 at different ionic strengths (the KCl concentration is indicated on top of the lanes). As a control, we incubated the SNARE complex with streptavidin beads in the absence of Syt3 (labeled as “No Syt3”). We did not boil eluted fractions prior to gel loading, thus the size of eluted protein corresponds to SNARE complex. To verify the presence of Syt3 on beads, we boiled beads in SDS-PAGE loading buffer after elution. (c) Effect of ionic strength on SNARE complex binding to immobilized Syt3 (the black line is the amount of SNARE complex bound in the presence of EGTA, and the red line in the presence of Ca\(^{2+}\)). We estimated the amount of SNARE complex bound to the beads by measuring the intensity (Matlab, The Mathworks, MA) of eluted SNARE complex bands from the Coomassie stained SDS-PAGE gel image shown in panel (b).
**Supplementary Figure 2** Ensemble binding of labeled Syt3 to liposomes. (a) Binding of the labeled Syt3 to liposomes occurs in a Ca\(^{2+}\)-dependent manner. Shown are the dot blots of top to bottom fractions of a sucrose density gradient obtained from a liposome co-floatation assay. We detected fractions containing fluorescently labeled Syt3 by a fluorescence scanner (Typhoon, Amersham Pharmacia). (b) smFRET efficiency histograms of Syt3 molecules labeled with Cy3/Cy5 in the presence of 100 µM Ca\(^{2+}\) (43 molecules), and liposomes (composition as described in panel (a); 86 molecules). The experimental conditions are as in Fig. 3 except that we used different dyes (Cy3/Cy5 instead of Alexa555/Alexa647). The corresponding histograms (black line in panel (b) and red line in Figure 3b) are slightly different. However, the areas of the high FRET peak in both distributions are similar: we fitted the smFRET histograms to the sum of two Gaussians as shown in panels (c) (Alexa555/Alexa647 dyes) and (d) (Cy3/Cy5 dyes). We determined the fraction of the high FRET peak by calculating the respective areas underneath Gaussian distributions (red lines). The area of the high FRET peak in the presence of Ca\(^{2+}\) is 22% for the Alexa555/Alexa647 dyes and 19% for the Cy3/Cy5 dyes (the sum is shown as a green line, the individual distributions are shown as blue and red lines, while the black lines are the smFRET distributions). The higher overlap between high and low FRET peaks for the Cy3/Cy5 smFRET distribution stems from a lower signal to noise ratio for Cy3/Cy5 dyes relative to Alexa555/Alexa647 dyes when attached to Syt3. The lower FRET peak is also slightly right-shifted by ~0.17 for Cy3/Cy5 compared to Alexa555/Alexa647, as expected due to the difference in Förster radii between the two dye pairs.
Supplementary Figure 3 C2AB linker, metal and Ca\(^{2+}\) binding sites in Syt3. (a) Simulated annealing omit map of the linker connecting the C2 domains of the Syt3 C2AB fragment. Linker residues are shown as sticks with electron density shown as a blue mesh. (b) The interface of C2A and C2B domains contains a metal binding site that is coordinated by Cys298, Asp419, His543, and Glu546 (balls and sticks). Superimposed is an anomalous difference electron density map (red mesh) contoured at 5\(\sigma\), clearly showing a binding site of an anomalously scattering element (“Me” label stands for metal). (c) Simulated annealing omit maps of the Ca\(^{2+}\) binding regions in the C2A and C2B domains. The Ca\(^{2+}\)-binding loops are shown in ribbon representation, Ca\(^{2+}\) ions as spheres, and the electron density contoured at 7\(\sigma\) (blue) and 9.5\(\sigma\) (red) is shown in mesh representation. The C2A domain contains three fully occupied Ca\(^{2+}\) (at 10-11\(\sigma\) electron density level) that are at the Ca1, Ca2, and Ca3 sites. The Ca4 position is not occupied in the Ca\(^{2+}\)-bound C2AB Syt3 structure even at 25 mM Ca\(^{2+}\) concentration used during crystallization, similar to what is observed for isolated C2A Syt1\(^{70}\). The Syt3 C2B domain contains two fully-occupied Ca\(^{2+}\) in the Ca1, Ca2 positions (at 12\(\sigma\) electron density level). These Ca\(^{2+}\) positions are similar to the structure of the isolated C2B domain of Syt1\(^{66,67}\). There is also weak electron density (at 9\(\sigma\)) for Ca\(^{2+}\) in the Ca4 position indicating a lower affinity binding site. (d) Schematic representation of the most probable Ca\(^{2+}\) coordination by the Ca\(^{2+}\)-binding loops of the C2A and C2B domains.
Supplementary Figure 4 Crystal packing of the SNARE-induced Ca\(^{2+}\)-bound crystal structure of Syt3. The three independent C2AB molecules in the asymmetric unit are shown in red, yellow, and blue, respectively. Symmetry related C2AB molecules (black) are shown up to a 100 Å radius around the primary molecules. Two different orientations are shown depicting relatively small crystal contacts and large water filled channels throughout the crystal, resulting in a 84% solvent content of the crystals.
Supplementary Figure 5 Biotinylated-Syt3 binding to the streptavidin surface is specific, the majority of observed spots are single molecules, and the observed Syt3 behavior is not surface dependent. We prepared the surface as described in Supplementary Methods. (a) Left panels: Shown are representative images of N-terminally biotin tagged Syt3 bound to the biotin-BSA glass surface in the presence and absence of streptavidin. The fluorescence intensity is scaled from low (black) to high (white). Bright spots (white) are fluorescently labeled Syt3 molecules. Right panel: The graph shows the average number of diffraction limited Cy5 or Alexa647 spots in a 40x80 µm area using 20 different samples (6 areas per sample). (b) Left panels: We used colocalized fluorescence intensity (arbitrary units) traces of Cy3/Alexa555 and Cy5/Alexa647 dyes to determine the number of dyes per single fluorescent spot (donor shown in green and acceptor in red). The laser illumination sequence is indicated on top of panels (red for 635nm and green for 532nm illumination). Right panel: The graph shows the average number of dyes (either acceptor or donor) per diffraction limited spot for approximately 100 spots per sample using 3 samples. Note that a single Q410C, N554C-labeled Syt3 molecule has at most two dyes attached. (c) Comparison of Syt3 conformational dynamics on BSA (black line) and PEG (grey line) pacified surfaces. Apart from the surface preparation the experimental conditions are the same as in Fig. 3. Shown are smFRET efficiency histograms of C2AB molecules in the absence of Ca^{2+} and absence of SNARE complex (left panel), absence of Ca^{2+} and presence of SNARE complex (middle panel), and presence of Ca^{2+} and SNARE complex (right panel).
**Supplementary Figure 6** Syt3 C2AB conformational dynamics at higher ionic strength. The experimental set-up is the same as in Figs. 3 and 5 except that we imaged the samples in 150 mM KCl, 100 mM Hepes-Na, pH 7.2 buffer. Shown are C2AB smFRET efficiency histograms (a) without (black) and with (red) Ca$^{2+}$, (b) without Ca$^{2+}$, and absence (black) and presence (green) of 6.0 µM SNARE complex, (c) with Ca$^{2+}$, and absence (red) and presence (blue) of 6.0 µM SNARE complex.
Supplementary Figure 7 Syt3 C2AB conformational dynamics in the presence of different concentrations of SNARE complex. The experimental set-up is the same as in Figs. 3 and 5. (a) smFRET efficiency histograms of C2AB molecules without Ca$^{2+}$, and absence (black) and presence of 0.6 µM (green) and 6.0 µM (red) SNARE complex. These SNARE complex concentrations correspond to a molar ratio of surface bound Syt3 to SNARE complex in solution of approximately 1:1x10$^{-6}$ and 1:1x10$^{-7}$ respectively. Thus, they represent saturating conditions. (b) smFRET efficiency histograms of C2AB molecules with Ca$^{2+}$, and absence (black) and presence of 0.6 µM (green) and 6.0 µM (red) SNARE complex. Samples in (a) and (b) were kept in 50 mM KCL, 100 mM Hepes-Na, pH 7.2 buffer. The distance between X-axis ticks corresponds to histograms’ bin width (0.025).
Supplementary Figure 8 Biosensor characterization of the Syt3-SNARE interaction. Sensorgrams of minimal SNARE complex (10 µM to 612 pM as a two-fold serial dilution with many concentrations in duplicate) binding to a Syt3-coated biosensor in 50 mM KCl, 10 mM Hepes-Na, pH 7.2 buffer. (a) In the presence of 100 µM Ca\(^{2+}\). (b) In the absence of Ca\(^{2+}\) and presence of 100 µM EGTA. (c) At higher ionic strength (150 mM KCl) in the presence of 100 µM Ca\(^{2+}\). We normalized the binding responses by the level of biotin-Syt3 captured via streptavidin tips. In the presence of Ca\(^{2+}\) the SNARE binding response is higher than in the absence of Ca\(^{2+}\), and follows different binding kinetics. The biosensor data are consistent with the pull-down experiments\(^{17}\) (Supplementary Fig. 1), which show an enhancement of the Syt3-SNARE interaction in the presence of Ca\(^{2+}\). (b), (d), and (f) represent the equilibrium binding analysis of the data shown in (a), (c), and (e) respectively. We plotted the (near) equilibrium binding responses recorded at the end of the association phase as a function of SNARE concentration and fitted them to a two-site binding model (red curves). All binding data were too heterogeneous to fit to a single-site binding model (blue curves). The high-affinity site had a \(K_D\) of approximately 6, 7, and 50nM while the low-affinity site had a \(K_D\) of \(~\)500, 1100, and 2500 nM for panels (b), (d), and (a).
Supplementary Figure 9  The SNARE-induced Ca^{2+}-bound Syt3 crystal structure matches the observed high FRET efficiency state. Shown are the results of restrained molecular dynamics simulation of Syt3 with a distance restraint of 44.1 Å between pseudo-atoms at positions 410 and 554 (dye labeling sites used for smFRET). We kept the C2 domains as rigid bodies connected by the linker with variable torsion angles. We represented the dyes center positions as pseudoatoms connected to the rigid C2 domains (see Online and Supplementary Methods). The imposed distance constraint between the pseudoatom positions of 44.1 Å corresponds to FRET of 0.8 using R_0=55.5 Å where R_0 is based on measurements and calculations described in the accompanying paper^{18}. We sampled the trajectory every 100 molecular dynamics steps and the corresponding structures from the simulation are shown in a Cα-backbone representation (gray thin lines). For comparison, the crystal structure of Syt3 in the SNARE-induced Ca^{2+}-bound state (Fig. 1) is shown as a red ribbon diagram along with the bound Ca^{2+} ions (yellow spheres).
Supplementary Figure 10 Similarities between the SNARE-induced Ca\(^{2+}\)-bound Syt3 crystal structure and the smFRET-derived model of the Syt1–SNARE complex\(^\text{18}\). Panels a and b show a superposition of the SNARE-induced Ca\(^{2+}\)-bound Syt3 crystal structure (cyan) and of the smFRET-derived model of the Syt1 structure (yellow) in complex with SNARE\(^\text{18}\). The SNARE complex is shown as a ribbon with synaptobrevin colored in blue, syntaxin in red, and SNAP-25 in green. Ca\(^{2+}\) ions bound to Syt3 are shown as gray spheres, while the Ca\(^{2+}\)-binding loops of Syt1 are colored red.
Supplementary Figure 11 Chemical structures of fluorescent dyes. (a) Cy3-maleimide, (b) Cy5-maleimide, (c) AlexaFluor 555 maleimide linker, and (d) AlexaFluor 647-maleimide. Structures of Cy3 and Cy5 are shown as publicly available from GE Healthcare, NJ (www.gehealthcare.com), AlexaFluor 647 and AlexaFluor 555 linker from Invitrogen, CA (www.invitrogen.com; personal communication).
Supplementary Table 1. Comparison of the interface of the SNARE-induced Ca\textsuperscript{2+}-bound Syt3 C2AB structure with other protein–protein interfaces

### Dimer interface statistics

|                        | ASA (Å\textsuperscript{2}) | Hydrogen Bonds per 100 Å\textsuperscript{2} |
|------------------------|-----------------------------|---------------------------------------------|
|                        | Mean σ                      | Mean σ                                      |
| **Syt3 SNARE-induced Ca\textsuperscript{2+}-bound** |                             |                                             |
| Syt3 Ca\textsuperscript{2+}-free                  | 371                         | 0.52 (1.03*)                                 |
| Syt1                   | 863                         | 0.46                                        |
| Antibody–Protein       | 777 135                     | 1.06 0.51                                   |
| Enzyme–Inhibitors      | 785 74                      | 1.37 0.37                                   |
| Hetero complexes       | 983 582                     | 1.13 0.47                                   |
| Hetero complexes2      | 848 243                     | 0.85 0.37                                   |
| Homodimers             | 1685 1101                   | 0.70 0.46                                   |

### Shape correlation

|                        | PDB code     | shape correlation |
|------------------------|--------------|-------------------|
| **Syt3 SNARE-induced Ca\textsuperscript{2+}-bound** | (3HN8)       | 0.63              |
| Syt3 Ca\textsuperscript{2+}-free                  | (1DQV)       | 0.49              |
| Syt1                   | (2R83)       | 0.70              |
| Lysozyme/HYHEL-5 FAB   | (2HFL)       | 0.65              |
| neuraminidase/NC41 FAB | (1NCA)       | 0.66              |
| neuraminidase subunit interface | (1NCA) | 0.71              |
| insulin dimer          | (9INS)       | 0.72              |
| trypsin/trypsin inhibitor | (2PTC)     | 0.76              |
| subtilisin/cymotrypsin inhibitor | (2SNI) | 0.72              |
| V\textsubscript{H}/V\textsubscript{L} of FABs   |              | 0.58-0.78         |
| C\textsubscript{H1}/C\textsubscript{L} of FABs   |              | 0.49-0.73         |

ASA is the accessible surface area per monomer calculated with SURFACE\textsuperscript{56}. Shape correlation was calculated with program SC\textsuperscript{57}. Hydrogen bonds were analyzed with HBPLUS\textsuperscript{58}. *including metal coordination residues.
Supplementary Methods

Protein expression and purification. For crystallization, we cloned the C2AB fragment of Syt3 from *R. norvegicus* (residues 292-587) into a pET28 vector and expressed it in BL21(DE3) cells by induction with 1 mM IPTG and growth at 37 °C for 3 hr. We lysed cells in 50 mM sodium phosphate, 300 mM NaCl, 10 (v/v) % ethylene glycol, 10 mM imidazol, 2 (v/v) % Triton-X-100, 0.1 (v/v) % β-mercaptoethanol, 1 mM PMSF, 10 µg ml⁻¹ DNase I, 1 µg ml⁻¹ leupetin, 1 µg ml⁻¹ pepstatin, 1 µg ml⁻¹ benzamidine. We bound the protein to Ni-NTA resin, washed it in 50 mM and eluted in 250 mM imidazole. We cut the hexa-histidine tag with TEV protease, and further purified Syt3 by ion-exchange chromatography and exchange into buffer containing 20 mM Hepes pH 7.8, 75 mM NaCl, 5 mM DTT, 1 mM EDTA. The final yield was 0.2 mg L⁻¹ of cells.

For single-molecule FRET experiments, we cloned a mutant of the cytoplasmic region of Syt3 from *R. norvegicus* (residues 232-588, C298H Q410C C438S C523S C533V N554C) into a GST fusion vector where we inserted an Avi-tag (Avidity, CO) between the GST tag and the N-terminus of Syt3 in the pGEX-6P1 vector (GE Healthcare). In this mutant construct we substituted the four endogenous cysteine residues with other residues, and introduced cysteine residues at residues 410 and 554 for labeling (see below for choice of these particular sites). The replacement of C533 with serine resulted in aggregation. We therefore replaced C533 by valine based on sequence alignment with other members of synaptotagmin family. We biotinylated the mutant Syt3 construct *in vivo* by expression in AVB101 cells (Avidity, CO) and induced it with 40 µM IPTG overnight at 18 °C in the presence of 50 µM biotin. We lysed cells in 50 mM Hepes, pH 7.5, 0.5 M NaCl, 5 (v/v) % glycerol, 10 mM β-mercaptoethanol, DNAase, PMSF, and protease inhibitors (Roche). We bound the protein to glutathione resin, washed it with 2 mM CaCl₂ followed by 3 mM EGTA, and eluted it in 25 mM Hepes pH 7.5, 0.2 M NaCl, 5 (v/v) % glycerol, 0.5 mM TCEP, 10 mM glutathione. We cleaved the GST tag off using Precission protease (GE Healthcare), and further purified Syt3 using ion-exchange and size exclusion chromatography in 0.25 M NaCl, 25 mM Hepes pH 7.5, 0.5 mM TCEP. The final yield was approximately 1 mg of cleaved protein per 1L of cells. The Syt3 mutant is properly folded since it binds to SNARE complex (Supplementary Fig. 1), binds liposomes containing negatively charged lipids in the Ca²⁺-dependent manner using a liposome co-flotation assay (Supplementary Fig. 2a), and it has a nearly identical circular dichroism spectra to that of wild type Syt3 (data not shown).

We used the following constructs for the SNARE complexes: the “minimal” SNARE core complex (synaptobrevin[28-89], syntaxin[191-256], SNAP-25[7-83] and SNAP25[141-204]) and the more “extended” SNARE complex (synaptobrevin[25-96] syntaxin[28-262], SNAP-25[1-83] and SNAP-25[120-206]). Proteins were expressed in *E. coli*. Purification, assembly and isolation of the ternary SNARE complex followed previously published protocols. Minimal SNARE complex is monomeric as assessed by multi angle light scattering and dynamic light scattering (data not shown).

Syt3 fluorophore labeling. We mixed the mutant Syt3 construct (C298H Q410C C438S C523S C533V N554C) with Cy3 and Cy5 or Alexa555 and Alexa647 maleimide dyes (Syt3 : Cy3/Alexa555 : Cy5/Alexa647 = 1:20:20) in 0.25 M NaCl, 25 mM Hepes pH
7.5, 0.1 mM TCEP and incubated the mixture overnight at 4 °C. We separated free dye and protein using a desalting column followed by size-exclusion chromatography. Based on the absorption measurements at the respective wavelengths of the dyes the labeling efficiency was near 100%.

We chose the labeling sites, Q410C (located on the C2A domain) and N554C (located on the C2B domain) based on several criteria. First, the sites are distant from the Ca\(^{2+}\)-binding loops so that they would not interfere with Ca\(^{2+}\) binding. Second, the sites produce very distinct FRET efficiencies for the two conformations observed in the crystal structures of Syt3 (Fig. 3a). Third, the sites are surface exposed in both crystal structures and should not affect folding of the C2 domains. Fourth, the presence of labels at these positions does not affect SNARE complex and membrane binding (Supplementary Figs. 1 and 2, respectively). For the two chosen sites (Q410 and N554) the Cα-Cα distance in the SNARE induced Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free conformations is 2.5 nm and 7.3 nm, respectively. Based on molecular dynamics simulations (see below), the average distance between the centers of the fluorophores attached at Q410C and N554C in the SNARE-induced Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free conformations is 35 and 85 Å, respectively, corresponding to FRET efficiencies of 0.9 for the SNARE-induced Ca\(^{2+}\)-bound and 0.05 for the Ca\(^{2+}\)-free conformation, respectively.

**Single-molecule FRET setup.** We constructed sample chambers from quartz slides and borosilicate glass coverslips. We cleaned slides by boiling in detergent, following with 30 min sequential sonication in acetone, ethanol and 1 M potassium hydroxide. Prior to use we cleaned slides and coverslips with 75 (v/v) % sulfuric acid : 25 (v/v) % (30 (v/v) % hydrogen peroxide) solution for 2 hr and extensively rinsed with water. We tethered labeled mutant C2AB fragment of Syt3 (referred to as just Syt3) to the surface as follows. (1) We pacified surfaces with biotinylated bovine albumin (bBSA) (Sigma-Aldrich and Pierce) at 1 mg ml\(^{-1}\), 10 min, 20 °C, in 100 mM Hepes pH 7.2, 150 mM KCl, 0.1 mM TCEP buffer. (2) We added streptavidin (Sigma-Aldrich) at 20 µg ml\(^{-1}\), 5 min, 20 °C. (3) We added N-terminal biotin-tagged Syt3 at 2–4 pM, 5 min (100 mM Hepes pH 7.2, 150 mM KCl, 5 (v/v) % glycerol, 0.1 mM TCEP). Syt3 binding to the surfaces is specific (i.e., there is little non-specific binding to the BSA surface in the absence of streptavidin) and the majority of the Syt3 molecules are single (Supplementary Fig. 5 a,b). We obtained similar binding specificity and smFRET distribution histograms on polyethyleneglycol (PEG) pacified surfaces (Microsurfaces, Inc, TX) for a variety of different conditions (Supplementary Fig. 5c). We additionally pacified the PEG surface with 1 mg ml\(^{-1}\) of BSA. We added streptavidin and Syt3 as described for the BSA-pacified surface. On the PEG surface, in the presence of streptavidin, the average number of diffraction limited Alexa647 spots in a 40x80 µm area was 80 ± 25, and in the presence of streptavidin saturated with biotin prior to addition of Syt3 was 5 ± 3. The smFRET histograms on the PEG surface (Supplementary Fig. 5c) are somewhat nosier compared to ones on the BSA surface due to the smaller number of molecules in the histograms (28 molecules for the PEG surface and 170 for the BSA surface in the Supplementary Fig. 5c left panel, 42 for the PEG surface and 93 for BSA surface in the Supplementary Fig. 5c middle panel, and 23 for the PEG surface and 123 for the BSA in the Supplementary Fig. 5c right panel). Thus, the observed conformational dynamics of Syt3 follows the same trend for both BSA and PEG pacified surfaces. We conclude that
the observed smFRET distributions are intrinsic to Syt3 rather than being dependent on the particular surface preparation. (4) We performed experiments in the presence of 100 µM Ca^{2+} or 100 µM EGTA and oxygen scavengers 1.5x10^{-3} U ml^{-1} protocatechuate 3,4-dioxygenase, 2.5 mM protocatechuic acid, Trolox 0.5 mM, 6 mM b-mercaptopetoethanol, 100 µM cyclooctatetraene or 1 mM ascorbic acid, 1 mM Parquat (all from Sigma-Aldrich, MO). (5) When specified, we added SNARE complex in either 50 mM or 150 mM KCl, 100 mM Hepes-Na, pH 7.2. For the experiments shown in Fig. 5, Supplementary Fig. 5c and Supplementary Fig. 6 we have added 6 µM of core SNARE complex.

We collected the single-molecule FRET (smFRET) data with a prism-based total internal reflection (TIR) fluorescence microscope. We excited fluorophores with 532 nm diode-pumped solid-state (Melles Griot, CA) and 635 nm solid-state (Cube, Coherent, CA) lasers. We collected fluorescence with a 1.2 N.A. × 60 water immersion objective (PlanApo; Nikon, Japan) on a commercial inverted microscope (Eclipse TE2000; Nikon). We recorded fluorescence intensities from Cy3/Alexa555 and Cy5/Alexa647 simultaneously using a 550 nm long-pass filter (Chroma, VT), a 650 nm dichroic mirror (Chroma), and bandpass filters HQ700/75 (Chroma) and HQ580/60 (Chroma) with an electron multiplying charge-coupled device (CCD) camera (Cascade 512B; Roper Scientific, AZ) at 100 msec integration time. We collected images using an alternating laser illumination sequence (acceptor, 635 nm, for approximately 2 sec; donor, 532 nm for approximately 100 sec; acceptor, 635 nm for approximately 30 sec).

Fluorescence intensity data analysis. We used co-localized Alexa555/Cy3 and Alexa647/Cy5 emission spots to calculate the FRET efficiency. We used the alternating laser illumination sequence to determine the number of acceptors per single spot and if photobleaching occurred. We analyzed only traces containing single acceptor and single donor, as judged by the fluorescence intensity (examples are in Figs. 3a and 4). We corrected collected images for background fluorescence by a rank leveling approach. First, we scanned the entire image (512x512 pixels) with a 16x16 pixel window to determine the median intensity value in each 16x16 pixel neighborhood. Second, we defined median values of each window as the background level for each local neighborhood and combined them into a two-dimensional array. Third, we constructed the background image by resizing the background array to the original image size using bilinear interpolation.

We calculated FRET efficiencies from single fluorescence intensity traces during donor illumination for co-localized spots, \( E = I_a / (I_a + I_d) \), where \( E \) = FRET efficiency, \( I_a \) = background and donor-acceptor crosstalk (8%) corrected acceptor intensity and \( I_d \) = background corrected donor intensity. We selected FRET sections of acceptor and donor fluorescence intensity traces during donor illumination as follows. For zero FRET, we included sections of the intensity traces as long as acceptor presence was confirmed. We judged this by anticorrelated occurrence of non-zero FRET following zero FRET and/or detection of acceptor using direct acceptor excitation at the end of the observation period. For non-zero FRET, we selected sections of donor and acceptor intensity traces until spontaneous loss of donor and/or acceptor fluorescence occurred due to the photobleaching or blinking of the fluorophores. In the case of blinking (i.e., a reversible non-fluorescent state of the dye that lasts a few hundred milliseconds; an example is
shown in Fig. 4a, right panel) we selected the section of the intensity trace until the occurrence of the first blinking event as a representative FRET instance for that molecule.

The fractional occurrence shown in Figs. 3 and 5 and Supplementary Figs. 2b-d, 5c, 6 and 7 represent normalized summation of individual FRET efficiency histograms of ~100–200 molecules. We generated individual FRET efficiency histograms by normalizing each single molecule FRET efficiency histogram (bin width 0.025) by the total number of its FRET instances. We performed analysis of fluorescence intensity trajectories using MATLAB (The Mathworks, MA). The majority of FRET efficiency values are in a stable intermediate range. The distribution is rather broad indicating an ensemble of many different conformational states and it exhibits a long tail towards high FRET states (Fig. 3b). We observed occasional transitions between FRET states (Fig. 4b) (by ~ 10–15% of molecules), although the limited data collection time for each molecule due to photobleaching and low fraction of those traces precluded kinetic analysis of these transitions.

**Syt3-SNARE interaction studies by pull-down.** We immobilized 20 µg Syt3 (232-588, C298H Q410C C438S C523S C533V N554C) onto 10 µL streptavidin beads via Syt3’s biotin-tag and incubated immobilized Syt3 with 4 µg SNARE complex in either 100 µM EGTA or 100 µM Ca²⁺ and 100 mM Hepes-Na, pH 7.2 buffer and different amount of KCl (as indicated in Supplementary Figure 1). We washed the beads in the same buffer, and eluted bound “minimal” SNARE complex with SDS gel running buffer. After SNARE complex elution, we boiled the beads to verify the presence of Syt3. As a control, we incubated SNARE complex with the streptavidin beads in the absence of Syt3.

**Syt3-liposome interaction studies.** We mixed Cy3/Cy5 labeled Syt3 (232-588, C298H Q410C C438S C523S C533V N554C) with liposomes for 30 min at 20°C before loading the mixture to a 40/30/0 sucrose density gradient and spinning it for 4 hr at 220 000 g. We composed liposomes of phospholipid:dihydrocholesterol = 60:40. The headgroup composition of phospholipids mimicked the ratio reported for total brain extracts, that is, (POPC: (DOPE:POPE=1:1): brain SM: brain PS: brain PI(4,5)P2 = 37:32:9:18:3. Dihydrocholesterol was from Sigma-Aldrich, MO, all other lipids from Avanti Polar Lipids, AL: 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (POPC), Dioleoyl Phosphatidylethanolamine(DOPE), 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphoethanolamine (POPE), brain phosphatidylinositol-4,5-bisphosphate (PI(4,5)P2), brain phosphatidylserine (PS), brain sphingomyelin (SM). We dried the chlorofom solution of lipids under argon, further dried lipids under vacuum, rehydrated and then extruded the lipids through a 50 nm pore filter (Avestin, Inc. Canada).

**Syt3–SNARE interactions studies by bio-layer interferometry.** We used an Octet Red (ForteBio, Inc.) equipped with a streptavidin SA biosensors (ForteBio, Inc.) to study the SNARE–Syt3 interaction in the absence and presence of Ca²⁺. This instrument uses biolayer interferometry to detect and quantify molecular interactions using disposable fiber-optic biosensors. We performed the experiments at 25 °C in 10 mM Hapes pH 7.2, 50 mM or 150 mM KCl, 100 µM EGTA or 100 µM Ca²⁺ and 0.05 (v/v) % Tween-20 as running buffer. We bound site-specifically biotinylated Syt3 to the streptavidin biosensor
and incubated it with minimal SNARE complex (or control buffer) at different concentrations. We added Tween-20 in order to remove nonspecific binding of SNARE to streptavidin coated tips. Under these conditions, nonspecific binding to the naked streptavidin tips at the highest SNARE concentration (10 µM) was ~ 0.1 nm. We observed further decrease in nonspecific binding upon protein capture during control experiments with biotinylated BSA. We agitated Syt3 samples at 1000 rpm and bound to the biosensor at 2.5 µg ml⁻¹ for 10 min, which typically resulted in capture levels of 3.5 nm within a row of eight tips. We incubated minimal SNARE complex with the sensor at two-fold serial dilution starting at 10 µM (Supplementary Fig. 8). We monitored association for 15 min and followed dissociation into buffer for 15 minutes. We blank-subtracted the resulting data using an in-line buffer control and processed the data using Scrubber (version 2.0a, BioLogic Software, Center for biomolecular interaction analysis, University of Utah).

Dynamic light scattering experiments (Wyatt Technology, CA) (not shown) confirmed that the increased signal in presence of Ca²⁺ is not due to aggregation of the minimal SNARE complex, as the radius of hydration (Rₜₚ) of minimal SNARE complex is similar in the presence or absence of Ca²⁺ (3.3 nm and 3.6 nm).

**Crystallization, data collection, and refinement.** We grew crystals of the C2AB fragment of Syt3 by the hanging drop method at 4 °C using C2AB with the extended SNARE complex. Despite the presence of SNARE complex in the crystallization condition, we found no residual electron density that could account for stably bound SNARE complexes. Control crystallization experiments using the extended SNARE complex alone produced no crystals. In addition, Syt3 demonstrated limited solubility in the buffer condition used for SNARE complex formation prior to crystallization (water pH 8.0). We used micro-crystal seeding with crystals grown under identical conditions to improve the consistency of crystal nucleation.

We solved the structure by molecular replacement using Ca²⁺-free structure of the C2AB fragment of Syt3 as the search model (PDB ID 1dqv). Initially we found no solutions using this search model. We therefore separated the search model into individual C2A and C2B domains, which were searched for independently by molecular replacement with PHASER⁶⁴. After we located two C2A and two C2B domains, we refined their positions and orientations by rigid body and positional minimization in CNS. We subsequently located the positions and orientation of a third C2A domain with PHASER using the four refined and fixed C2 domains. We manually modeled the last C2B domain into an electron density map computed with phases from molecular replacement solutions of five C2 domains (3 C2A domains, and two C2B domains).

After alternate cycles of refinement and manual building, the structure of C2B has total of 74.1 % of the residues in the most favored regions of the Ramachandran plot, 21.2 % in the additional allowed regions as calculated with MOLPROBITY⁶⁵. The final model contains 6672 atoms (6651 protein, and 21 ions).

**Metal binding sites in Syt3.** The Ca²⁺ coordination is shown in Supplementary Fig. 3c,d. While the accuracy of the Ca²⁺ coordination might be limited by the resolution of the structure, it appears nearly identical to the Ca²⁺ coordination from crystal structures of the individual C2A and C2B domains of Syt1. The Ca²⁺ coordination sphere in the C2A
domain is completed by Glu476 (Supplementary Fig. 3d, shown in blue) from a symmetry (NCS and/or crystallographic) related molecule for positions Ca2 and Ca3 for all three NCS molecules. These residues might mimic completion of the Ca$^{2+}$ coordination sphere by interaction with negatively charged lipid headgroups. The Syt3 C2B domain contains two fully-occupied Ca$^{2+}$ in the Ca1, Ca2 positions (at 12 $\sigma$ electron density level). These Ca$^{2+}$ positions are similar to structure of the isolated C2B domain of synaptotagmin $^{1,66,67}$. There is also weak electron density (at 9 $\sigma$) for Ca$^{2+}$ in the Ca4 position indicating a lower affinity binding site.

At the interface between the two C2 domains there is a metal site (Supplementary Fig. 3b). Based on the coordinating ligands, the metal is most likely zinc, copper, nickel, iron, or cobalt. We could not determine the exact identity of the metal since all of the likely metals have an anomalous signal at the wavelength that we collected the data at (0.98 Å). Since we did not supplement any of these elements in the crystallization condition, they must have been carried from either the expression or purification steps.

Simulation of dye positions and expected FRET efficiencies. We calculated the distance between fluorophore centers (defined as the positions of the CAO atoms, see Supplementary Fig. 11) in each model and then we averaged these distances to yield the average fluorophore distance. To obtain good sampling of all possible conformations, we employed a high simulation temperature (T=300,000 K, with a time step of 0.00075 ps for 5000 molecular dynamics steps).

Model of synaptotagmin-SNARE interaction. In Figure 6, SNARE proteins and accessory proteins, synaptotagmin and complexin, are shown as ribbon diagrams for protein fragments for which crystal structures are available (SNARE and complexin PDB ID 1KIL$^{68}$; SNARE induced Ca$^{2+}$-bound structure of Syt3 (Fig. 1), also corresponding to the SNARE-induced conformation of Syt3 in the absence of Ca$^{2+}$, Fig 5) and thin lines for protein fragments for which structures are not available. Transmembrane domains of syntaxin and synaptobrevin are shown as helical ribbons$^{69}$. We docked Syt3 and the SNARE complex based on the best model of the Syt1 - SNARE complex that was obtained by docking calculations using 34 smFRET-derived distances between Syt1 and SNARE complex. We generated the model by superposition of the C2B domain of the SNARE induced Ca$^{2+}$-bound Syt3 crystallographic structure onto the C2B domain of Syt1 in the smFRET derived Syt1-SNARE complex model (Supplementary Fig. 10). We generated conformations of uncomplexed Syt3 molecules in the plasma membrane from unrestrained molecular dynamics simulations. We drew the ratio of phospholipids and cholesterol, and ratio of different phospholipid headgroups to correspond to the composition of synaptic vesicles$^{61,62}$. 
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