Supporting Information

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SI Methods

Protein Constructs. The protein constructs were from *Rattus norvegicus*. They were cloned into the expression vector pET28a. Expression constructs of the full-length protein (amino acids 1–421) and of the soluble domain of synaptotagmin (amino acids 97–421), have been described before (1). In the same publications, the calcium mutants of the full-length protein have also been described (1): C2a*B (D178A, D230A, and D232A), C2Ab* (D309A, D363A, and D365A), C2a*b* (D178A, D230A, D232A, D309A, D363A, and D365A), and KAKA mutant (K326A and K327A). The constructs for the neuronal SNAREs were the SNARE motif of syntaxin 1A with its *trans*-membrane domain (amino acids 183–288), a cysteine-free variant of SNAP-25A (amino acids 1–206), and synaptobrevin 2 without its *trans*-membrane domain (amino acids 1–96). The synaptotagmin 1 (amino acids 97–421) single cysteine variant (S342C) was obtained after first removing the single native cysteine (C278S) and then introducing a point mutation at position 342 (1, 2).

Protein Purification and Labeling. All proteins were expressed in *Escherichia coli* strain BL21 (DE3) and purified using Ni^{2+}-nitrilotriacetic acid beads (GE Healthcare), followed by further purification using ion exchange chromatography as described (1) with a few modifications. The protein concentrations were determined by a Bradford assay or UV absorption (2). Labeling of the synaptotagmin-1 (amino acids 97–421) single cysteine variant (S342C) with Alexa Fluor 488 C_5 maleimide was done as follows. First the proteins were dialyzed against the labeling buffer (50 mm Hepes, pH 7.4, 500 mm NaCl, 100 μm Tris(2-carboxyethyl) phosphine). The dialyzed protein solution was incubated with the fluorophore for 2 h at room temperature. Thereafter, the labeled protein was separated from the unreacted dye using a Sephadex G50 superfine column. The labeling efficiency was ∼40%. Syntaxin 1A (183–288) and synaptobrevin 2 (1–96) were purified by ion-exchange chromatography (2) in the presence of 15 mm CHAPS. The binary complex containing syntaxin 1A (183–288) and SNAP-25A was assembled from purified monomers and subsequently purified by ion-exchange chromatography in the presence of 1% CHAPS (2). The ternary SNARE complex syntaxin 1A (183–288), SNAP-25A, and synaptobrevin 2 (1–96) was generated by incubation of the binary complex and synaptobrevin 2 (1–96) in a ratio of 1:2 overnight at 4 °C. The excess synaptobrevin 2 was removed with Sephadex G50 superfine column during liposome reconstitution. Full-length synaptotagmin was purified in the presence of 1% CHAPS using ion exchange chromatography (as described in ref. 2).

Liposome Reconstitution. All lipids were purchased from Avanti Polar Lipids except for the Texas Red-labeled phosphatidylethanolamine (TRPE) and Oregon Green-labeled phosphatidylethanolamine (OGPE), which were purchased from Invitrogen. Lipid mixtures according to Table S1 were first prepared by resolving lipid films in HP buffer (20 mM Hepes, 150 nM KCl, 2 mM DTT, pH 7.4) containing 5 % sodium cholate (mass fraction). The final concentration of the lipids was 27 mM. To 16.7 μL of the lipid mixtures protein was added to achieve a protein:lipid ratio of 1:1,000, expect the synaptotagmin-SNAREs experiments (here the synaptotagmin-to-lipid ratio was 1:750). The lipid protein mixtures were adjusted with HP buffer containing 1.5% sodium cholate (mass fraction) to a final volume of 50 μL. The liposomes were formed by detergent removal using a Sephadex G50 superfine column (Sigma; Bio-Rad). The running buffer for the column was HP150 buffer (20 mM Hepes, 150 nM KCl, 2 mM DTT, pH 7.4). The collected liposome volume was about 250 μL. The size of liposomes was about 50 nm. For all liposomes used in this study the average lipid number per liposome was ∼12,000. Also, in all experiments the liposome concentration was 10 nM liposomes corresponding to a lipid concentration of 0.09 mg/mL for each type of colored liposomes [based on liposome sizes (3) and space required for lipids (4)]. In Table S1, the composition of the liposomes for all data shown in Figs. 1–4 and Figs. S2 and S3 are shown.

Fluorescence Cross-Correlation Spectroscopy (FCCS) Setup. For simultaneous two-photon excitation of differently labelled liposomes we used a titanium-sapphire laser (800 nm, 87 MHz, Fig. S1*A*). The laser beam was expanded using a lens system and coupled with a dichroic mirror (715 DSCPXR; AHF) into a UPlanSApo 60×/1.2-W water immersion objective (Olympus). The emitted photons passed through the objective and the dichroic mirror. Scattered light from excitation beam was blocked by a short pass filter (E700SP2; AHF). The emission was collimated using a second lens system, separated by a second dichroic mirror (590 DCXR; AHF), filtered in each direction with a band pass filter (HQ 645/75 and HQ 535/50; AHF) and collected by separate avalanche photodiodes (APD) (SPCM-AQR-13; PerkinElmer). The transistor–transistor logic (TTL) signals from the APD were analyzed using a four-channel router (PRT 400; PicoQuant) and a time-correlated single photon counting (TCSPC) card (TimeHarp200; PicoQuant) and saved in time-tagged time-resolved (TTTR) format. The correlation was processed using a homemade program.

For the measurements without or with 100 μM Ca^{2+} either the buffer containing 20 mM Hepes, 150 mM potassium chloride, 1 mM EGTA or the buffer containing 20 mM Hepes, 150 mM potassium chloride, 1 mM EGTA, 1.1 mM calcium chloride was used. The reaction volume was 100 μL. The measurement was started by diluting the red and green liposome stock solutions into the corresponding reaction buffer and loading a droplet (20 μL) onto the coverslip after short vortexing. The final concentration of the liposomes was ∼10 nM for each color (corresponding to ∼0.09 mg/mL lipids). The signal traces for the TP-FCCS analysis were recorded six times for 12 seconds for each droplet resulting in a total measuring time of 72 seconds per droplet. This procedure was repeated several times with different droplets from the same solution. Each experiment using different liposome protein and lipid compositions as well as Ca^{2+} concentrations was repeated at least one time with fresh liposome and buffer preparations.

Tethering Assay and Binding of Labeled, Soluble C2AB-Fragments. The tethering assay has been described in detail (3). In general, the average number of particles in the focal detection volume that carry Oregon Green-labeled lipids, N_g, can be calculated from the inverse of the autocorrelation amplitude for the Oregon Green fluorescence $N_g = G_g(0)^{-1}$ at small lag times (green line in Fig. S1*B*). Here, a particle can be either a single liposome or a particle consisting of two or more tethered liposomes for which at least one liposome also contains Oregon Green-labeled lipids. Under our experimental conditions, the influence of different liposome/particle compositions on N_g can be neglected (3). In the same manner the average particle number for Texas Red-labeled particles, N_r, can be calculated from the inverse of the autocorrelation amplitude for the Texas

Red fluorescence N_r, = $G_r(0)^{-1}$ at small lag times (red line in Fig. S1*B*). The average particle number in the focal detection volume that carries both types of labeled lipids, N_{rg}, was calculated from the particle numbers N_g and N_r and the cross-correlation amplitude for the Texas Red and Oregon Green fluorescence (blue line in Fig. S1*B*) at small lag times: N_{rg} = $G_{rg}(0)\cdot N_g\cdot N_r$. By comparing this number of double-labeled particles, N_{rg}, with the total number of particles carrying green labels, N_g, the tethering percentage can be calculated: Tethering (%) = $N_{rg}/N_g \cdot 100$. Only in the case of Fig. 3*A* this percentage was calculated by $N_{rg}/N_r\cdot 100$ because here the number of green-labeled C2AB fragment was present in large excess in most cases in comparison with the number of red liposomes. Therefore, the tethering percentage $N_{rg}/N_r\cdot 100$ represents the percentage of liposomes carrying significant amounts of C2AB fragments in comparison with the total amount of liposomes. On the contrary, the $N_{rg}/N_g\cdot 100$ used for Fig. 3*B* represents the amount of C2AB fragment attached to liposomes. In this case only relative extents in C2AB fragments binding can be given at higher percentages of bound C2AB, because a liposome carrying many green C2AB fragments is a lot brighter than a single-labeled C2AB fragment. However, even though only relative bound fractions can be exactly concluded from the analysis shown in Fig. 3*B* it provides clear evidence that at 215 nM C2AB a significantly smaller fraction of C2AB is bound to the membranes than at 50 nM C2AB. This can only be explained by a saturation of the membranes. Because full tethering is still not observed at 860 nM soluble C2AB (Fig. 2), this provides evidence that clustering occurs at saturating concentrations.

1. Stein A, Radhakrishnan A, Riedel D, Fasshauer D, Jahn R (2007) Synaptotagmin activates membrane fusion through a Ca^{2+}-dependent *trans* interaction with phospholipids. *Nat Struct Mol Biol* 14:904–911.
2. Radhakrishnan A, Stein A, Jahn R, Fasshauer D (2009) The Ca^{2+} affinity of synaptotagmin 1 is markedly increased by a specific interaction of its C2B domain with phosphatidylinositol 4,5-bisphosphate. *J Biol Chem* 284:25749–25760.
3. Cypionka A, et al. (2009) Discrimination between docking and fusion of liposomes reconstituted with neuronal SNARE-proteins using FCS. *Proc Natl Acad Sci USA* 106: 18575–18580.
4. Nagle JF, Tristram-Nagle S (2000) Structure of lipid bilayers. *Biochim Biophys Acta* 1469: 159–195.

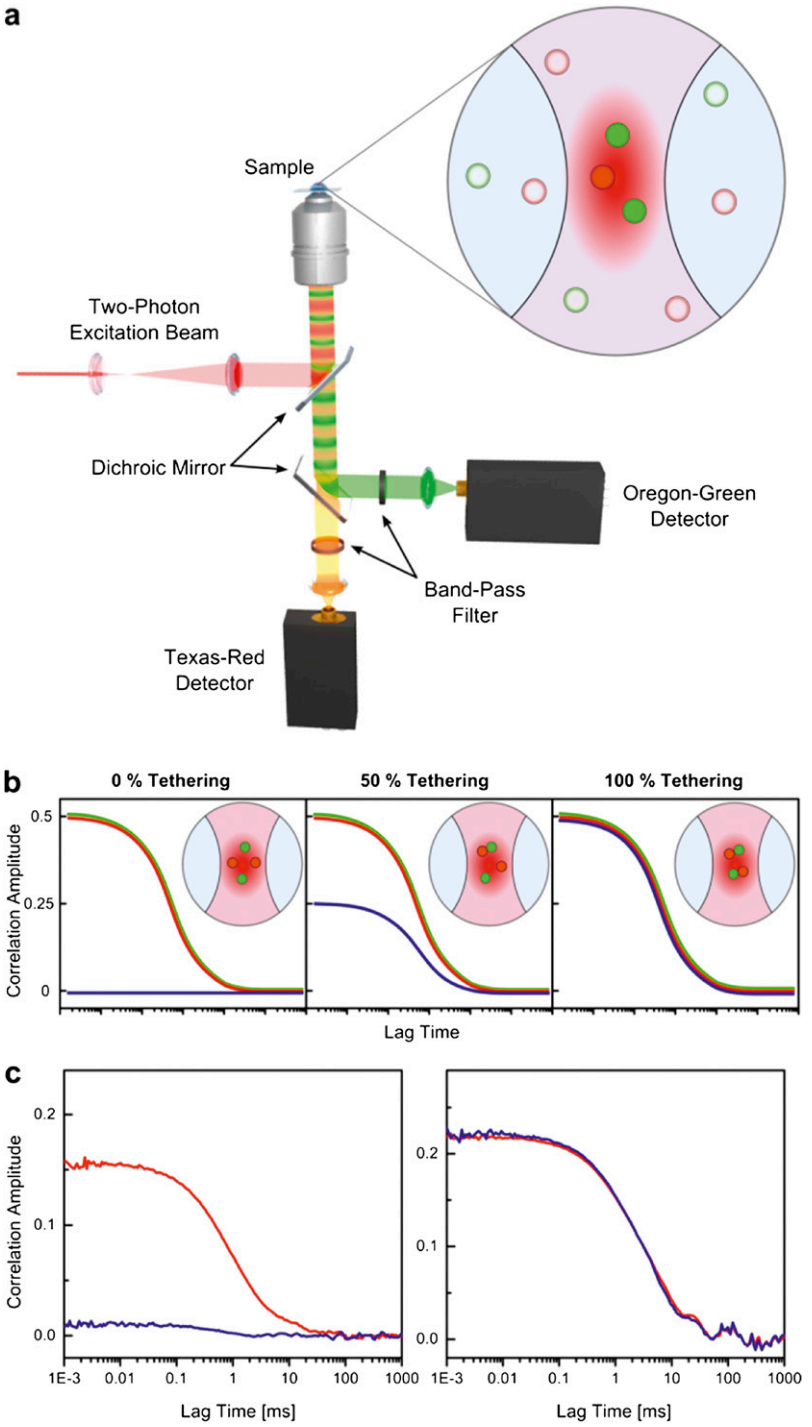

Fig. S1. TP-FCCS tethering assay. (*A*) In the TP-FCCS set-up fluorescence fluctuations caused by diffusion of labeled liposomes through the microscopic two-photon excitation volume (dimensions ~200–500 nm) are recorded and analyzed by correlation functions. (*B*) Schematic presentation of the correlation functions. Amplitudes of the red or green auto-correlation curves (correspondingly colored curves) are inversely proportional to the average number of red- or green-labeled liposomes in the detection volume, respectively. For example, the amplitudes of 0.5 of the red and green curves reflect approximately two red- or green-labeled particles that are on average in the excitation volume. Cross-correlation amplitude (blue) relative to the autocorrelation amplitudes is a direct measure for the proportion of tethered red-green liposomes in the total liposome population. (*C*) Exemplary measured autocorrelation curve of Texas-Red labeled, synaptotagmin-containing vesicles (red) and of the cross-correlation (blue) with Oregon-Green–labeled liposomes. *Left*, no or little tethering with an inactive mutant of synaptotagmin-1 (D178A D230A D232A D309A K325A K326A D363A D365A). *Right*, 100% tethering by wild-type synaptotagmin-1. For more details see ref. 1.

1. Cypionka A, et al. (2009) Discrimination between docking and fusion of liposomes reconstituted with neuronal SNARE-proteins using FCS. *Proc Natl Acad Sci USA* 106: 18575–18580.

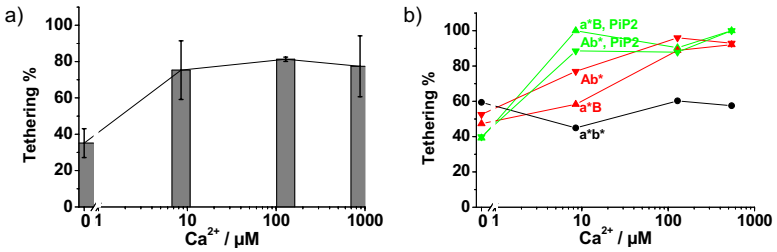

Fig. S2. Tethering of liposomes mediated by membrane-bound synaptotagmin-1. Dependence on the Ca^{2+} concentration and on the presence of PiP_2 in the target membrane. Tethering was measured as in Fig. 1. (*A*) Ca^{2+}-concentration dependence of membrane tethering by wild-type synaptotagmin in the absence of PiP_2 in the target membrane. (*B*) Ca^{2+}-titration curves of synaptotagmin mutants. At Ca^{2+} concentrations of about ~8.5 μM the tethering for both, C2A*b* and C2a*B*, is lower than at ~100 μM (red curves) but in the presence of 1 mol% PiP_2 in the target membrane full tethering is observed (green curves). In contrast to wild-type synaptotagmin, no significant increase of tethering with increasing Ca^{2+} concentrations can be observed for the double mutant a*b* (black curve). See Fig. 1 legend for an explanation of the synaptotagmin variants.

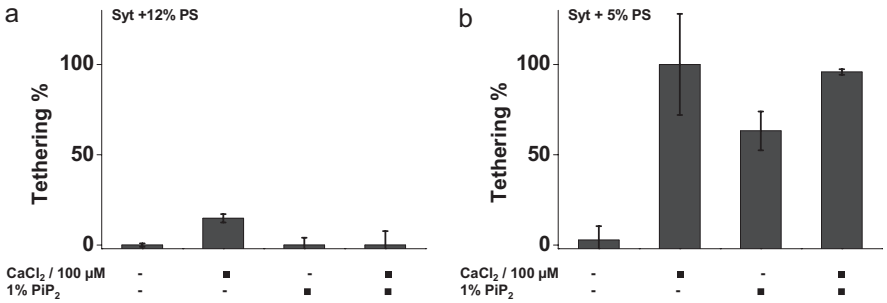

Fig. S3. Membrane tethering by synaptotagmin in the presence of 5 and 12% PS in the synaptotagmin-containing membrane. (*A*) Presence of 12% PS prevented membrane tethering in a very similar fashion as 20% PS (Fig. 1), regardless of whether the target membrane contains PS only or PS plus PiP_2. (*B*) A total of 5% PS is not sufficient anymore to inhibit the activity of synaptotagmin-1 by *cis*-binding if either 100 μM Ca^{2+} is present in the solution or 1% PiP_2 in the target membrane. Only the absence of both allows an inactivation of synaptotagmin tethering by the presence of 5% PS in the synaptotagmin-1–containing membranes.

Table S1. Percentages of lipid composition

	PC	PE	TRPE	OGPE	PS	Chol	PiP_2
Fig. 1*A*, red	70	19	1	0	0	10	0
Fig. 1*A*, green	50	18.5	0	1.5	20	10	0
Fig. 1*B*, red	70	19	1	0	0	10	0
Fig. 1*B*, green	49	18.5	0	1.5	20	10	1
Fig. 1*C*, red	50	19	1	0	20	10	0
Fig. 1*C*, green	50	18.5	0	1.5	20	10	0
Fig. 1*D*, red	50	19	1	0	20	10	0
Fig. 1*D*, green	49	18.5	0	1.5	20	10	1
Fig. 2, red	50	19	1	0	20	10	0
Fig. 2, green	50	18.5	0	1.5	20	10	0
Fig. 3 *A* and *B*, red	50	19	1	0	20	10	0
Fig. 4 *A*, *B*, and *D*, red	70	19	1	0	0	10	0
Fig. 4, all green	70	18.5	0	1.5	0	10	0
Fig. 4 *C* and *E*, red	50	19	1	0	20	10	0
Fig. S2*A*	70	19	1	0	0	10	0
Fig. S2*B*, black and red	70	19	1	0	0	10	0
Fig. S3, green	49	18.5	0	1.5	20	10	1
Fig. S3*A*, red	65	19	1	0	5	10	0
Fig. S3*B*, red	58	19	1	0	12	10	0
Fig. S3 *A* and *B*, green	50	18.5	0	1.5	20	10	0
Fig. S3 *A* and *B*, green	49	18.5	0	1.5	20	10	1

Chol, cholesterol; OGPE, Oregon green phosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP_2, phosphatidylinositol 4,5-bisphosphate; PS, phosphatidylserine; TRPE, Texas red phosphatidylethanolamine.