Supporting Information

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SI Text

Our studies address a number of issues that have been raised concerning whether the SNARE-dependent liposome fusion assay faithfully reflects a physiological process of membrane fusion (1–3). Although previous studies (2) expressed reservations about whether SNARE-dependent fusion could be observed in proteoliposomes formed by co-micellization versus direct incorporation, we found similar kinetic effects of CAPS when SNAREs were incorporated at low densities in either type of liposome (e.g., Fig. 1*A* and Fig. S3). However, as found in previous work (3), attempts to entrap small molecule probes in liposomes formed by the direct method were unsuccessful if SNARE surface densities exceeded 7,500 molecules/μm^2. At these higher SNARE densities, it was not feasible to conduct a contents mixing assay with low molecular weight Tb^{3+} and DPA probes (Fig. 2*B*). Although contents mixing was reported for SNARE-dependent liposome fusion (at high SNARE densities) with macromolecular content probes (4), the studies of Fig. 2*B* provide a more stringent test of whether fusion occurs without content leakage. It should be noted that at the SNARE densities required for small molecule probe entrapment, no fusion was observed in the absence of CAPS (Fig. 2*B* and Fig. S3). Thus, SNAREs as a minimal essential fusion machinery (5) may require additional factors such as Munc18–1 (6) or CAPS to promote fusion at physiological membrane densities. The surface SNARE densities used in our studies correspond well to what is found on synaptic vesicles (7) and on the PC12 cell plasma membrane (see *Materials and Methods* in the main text and Table S1).

SI Materials and Methods

Materials. PC (1-palmitoyl, 2-oleoyl phosphatidylcholine), 1,2-dioleoyl phosphatidylcholine (DOPC), 1,2-dipalmitoyl phosphatidylcholine (DPPC), 1,2-dioleoyl phosphatidylserine (DOPS), 1,2-dipalmitoyl phosphatidylethanolamine-(*N*-7-nitro-2,1,3-benzoxadiazol-4-yl) (NBD-PE), N-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl phosphatidylethanolamine (rhodamine-PE), 1,2-dioleoyl-*sn*-glycero-phospho-L-serine-(*N*-7-nitro-2,1,3-benzoxadiazol-4-yl) (NBD-PS), and L-α-phosphatidylinositol-4,5-bisphosphate (PI 4,5-P_2) were purchased from Avanti Polar Lipids. Lysophosphatidylcholine was purchased from Sigma-Aldrich.

Plasmids and Recombinant Protein Expression and Purification. Plasmid constructs pTW34 to co-express rat syntaxin-1A with an N-terminally His-tagged mouse SNAP-25B and pTW2 to express C-terminal His-tagged mouse VAMP2 were provided by J.E. Rothman (New Haven, CT) (5). Plasmids to express GST-rat syntaxin1A (1–265), GST-mouse SNAP-25B, and GST-synaptotagmin-1 C2AB were gifts from R. H. Scheller (San Francisco) and E. R Chapman (Madison, WI). A pGEX4T1 plasmid to express C-terminally tagged GST-rat syntaxin1A 1–288 was prepared by K. Boswell (Madison, WI). GST-tagged proteins were purified and eluted using standard methods by thrombin cleavage for 4 h at room temperature in presence of 1% OG followed by neutralization with 2 mM PMSF. CAPS-1 fragments corresponding to residues 859 to 1,073 and 655 to 1,289 were produced with an N-terminal 6xHis-maltose binding protein purification tag using FlexiVector cloning system (Promega) as previously described (8). All plasmids were transformed into *Escherichia coli* BL-21(DE3) cells for protein expression and purification. C-terminally myc-His-tagged rat CAPS-1 protein was produced in Sf9 insect cells or by COS-1 cell expression as previously described (9, 10). Purification of SNARE proteins was performed following published procedures (5).

Determination of Syntaxin Cell Surface Density. PC12 cells were cultured as described in DMEM supplemented with 5% horse serum and 5% calf serum (10). Cells were removed from 10-cm dishes using Hanks EDTA buffer, and cell number determined with a hemocytometer. Protein expression levels were determined from total cell lysates prepared in SDS sample buffer. Samples corresponding to 10^4 to 10^5 cells along with a standard curve of purified full-length syntaxin1A protein were resolved by SDS/PAGE and immunoblotted (Table S1). PC12 cells were stained with 5 nM FM4–64 and imaged on a Nikon C1 laser scanning confocal microscope with a ×60 oil immersion objective with an N.A. of 1.4. Z-series images were obtained with 100 nm sectioning with oversampling. Z-stacks were de-convolved with Autodeblur/Autovisualize software (AutoQuant Imaging) and surface area was quantified in ImageJ (National Institutes of Health) using an implementation of the marching cubes algorithm in the ImageJ_3D_viewer plug-in written by Benjamin Schmid. A plug-in updated by Albert Cardona to extract surface area measurements from rendered 3D mesh was used (A. Cardona, personal communication).

Proteoliposome Preparation. Proteoliposomes were formed by co-micellization in the presence of VAMP2 or co-expressed syntaxin-1A and SNAP-25B as previously described (5). To produce liposomes by co-micellization, a lipid film containing 1.5 μmol DOPC:DOPS in an 85:15 mole ratio was resuspended with 500 μL of 4.17 to 417.5 μg syntaxin-1/SNAP-25B in elution buffer (25 mM Hepes-KOH, pH 7.4, 100 mM KCl, 50 mM imidazole-OAc, pH 7.4, 1.0% β-octyl glucoside). Proteoliposomes made under these conditions had t-SNARE densities in the range of approximately 127 to 12,800 copies/μm^2 (≈1–100 copies of each SNARE/liposome). For liposomes that contained either 5 or 10 mol% PI 4,5-P_2, we used DOPC:DOPS mol ratios of 95:0 or 90:0. For fluorescent donor liposomes, we incorporated approximately 100 copies of VAMP2 per liposome [to correspond to VAMP2 densities on a synaptic vesicle (7)]. The dried lipid film containing a DOPC:DOPS:Rh-DOPE:NBD-DOPE lipid mix in an 82:15:1.5:1.5 mol ratio was resuspended with 500 μL of 280 μg/mL VAMP2 protein diluted in elution buffer. For fusion experiments to assess hemi-fusion, we substituted NBD-DOPS for NBD-DOPE lipids because PS undergoes less trans-bilayer flip/flop than PE (11). In experiments in which DOPC:PI 4,5-P_2 (95:5 mole ratio) liposomes were reconstituted with different syntaxin:SNAP-25 mol ratios, tagless syntaxin-1A and SNAP-25B proteins diluted in elution buffer were mixed together to re-suspend a lipid film containing 1.5 μmoles lipid mix for liposome generation by co-micellization. All lipid mixtures were spiked with 2 μCi of [^{3}H]-1,2-dipalmitoyl phosphatidylcholine (NEN) to enable determination of lipid recoveries and standardization of fusion reactions. Proteoliposomes were dialyzed overnight at 4 °C in reconstitution buffer [25 mM Hepes-KOH, pH 7.4, 100 mM KCl, 10% (wt/vol) glycerol, 1 mM DTT], mixed 1:1 with 80% Nycodenz (Accurate Chemical and Scientific), overlaid with 30% and 0% Nycodenz in reconstitution buffer without glycerol, and centrifuged at 45,000 rpm for 4 h at 4 °C using an SW60 rotor (Beckman). Proteoliposomes (≈400 μL) at the 0% to 30% Nycodenz interface were collected,

divided into aliquots, and flash-frozen. The liposome SNARE content was assessed by SDS/PAGE and visualized by Coomassie staining for SNAP-25/syntaxin-1A and amido-black staining for VAMP2 using BSA standards for comparison.

Mixing of the non-fluorescent content markers Tb and DPA results in marked increases in fluorescence at 4 distinct wavelengths when excited at 276 nm (Fig. S2*C*) and has previously been used to monitor the process of membrane fusion (12). We encapsulated Tb^{3+} and DPA in 100-nm-diameter liposomes using established methods (13). Briefly, 7.5 μmol lipid mixes, DOPC:PI 4,5-P_2 in a 95:5 mole ratio for t-SNARE liposomes, and DOPC:DOPS:RhPE-DOPE:NBD-DOPE in an 82:15:1.5:1.5 mol ratio for v-SNARE liposomes were dissolved in chloroform, and organic solvent was removed by evaporation under a stream of N_2 gas followed by vacuum for 30 min. The dried lipid film was re-hydrated with 200 μL reconstitution buffer for 30 min. The reconstitution buffer was substituted with buffer containing DPA (20 mM Hepes, pH 7.5, 150 mM KDAPA, 1 mM DTT) or Tb (20 mM Hepes, pH 7.5, 150 mM Na citrate, 15 mM $TbCl_3$, 1 mM DTT). The hydrated lipid film was vigorously vortexed for 5 min, subjected to 5 freeze–thaw cycles, and extruded with an Avanti mini-extruder using 38 passes through 100-nm track-etch polycarbonate membranes (Nucleopore). One hundred microliters of 50 μg t-SNAREs (syntaxin-1A:SNAP-25B 1:1) in reconstitution buffer or DPA buffer supplemented with 1.98% wt/vol β-octyl glucoside was added to 200 μL liposomes to give a concentration of 0.66% wt/vol. One hundred microliters of 48 μg VAMP2 in reconstitution buffer or Tb buffer was similarly supplemented with β-octyl glucoside and added to liposomes. This was gently mixed for 30 min at room temperature and diluted fivefold in reconstitution buffers lacking detergent but containing DPA or Tb. Proteoliposomes were dialyzed for 3 h at room temperature using 0.5 L reconstitution buffer without glycerol with 0.5 g Biobeads, then changed into 1 L of fresh buffer and further dialyzed overnight at 4 °C. Dialyzed proteoliposomes in which β-octyl glucoside was removed were further purified by sucrose density gradient flotation. Proteoliposomes were mixed 1:1 with 80% sucrose, overlaid with 30% and 0% sucrose in reconstitution buffer without glycerol, and centrifuged at 45,000 rpm for 4 h at 4 °C using an SW60 rotor (Beckman). Proteoliposomes (≈400 μL) at the 0%–30% sucrose interface were collected, divided into aliquots, and stored at 4 °C for a maximum of 5 d. Leakage studies of Tb or DPA revealed that liposomes did not retain any contents on freeze-thaw cycles or after extended storage at 4 °C. The liposome SNARE content was assessed by SDS/PAGE and visualized by Coomassie staining for SNAP-25/syntaxin-1 and amido-black staining for VAMP2 using BSA standards for comparison. These conditions produced 100 nm proteoliposomes with 318 syntaxin-1/SNAP-25 1:1 complexes/μm^2 and 640 VAMP2 complexes/μm^2. At these SNARE densities, leakage of aqueous content was minimal (<1.5%).

Lipid Mixing Fusion Studies. The lipid mixing assay between fluorescent donor liposomes and non-fluorescent acceptor liposomes was performed as described (5). Lipid mixing was reported by the loss of FRET between fluorescent lipids (NBD-PE and Rh-PE) in the VAMP2 liposomes that occurs upon fusion with the non-fluorescent SNAP-25/syntaxin-1 liposomes. The standard assay used 0.45 mM acceptor and 0.225 mM donor liposomes in a total volume of 75 μL reconstitution buffer without glycerol. CAPS protein was added at concentrations indicated in figure legends. Ninety-degree light scattering studies did not detect any aggregation of liposomes during the fusion reactions (Fig. S9). Fusion reactions were conducted in the absence of Ca^{2+} except where indicated. Blank reactions were prepared for all conditions by substituting SNAP-25/syntaxin-1 acceptor liposomes with protein-free (P_f) liposomes to detect non-SNARE-mediated lipid mixing. Reactions were assembled on ice and flick-mixed before addition to 96-well FluoroNunc plates (Nunc). Fusion was detected by measuring de-quenching of NBD fluorescence (excited at 460 nm emission at 538 nm) every 90 sec (shaken before each reading) for 2 h at 32 °C in a SpectraMax Gemini XS microplate spectrofluorometer (Molecular Devices). At the end of 2 h, fusion reactions were solubilized with 0.5% wt/vol dodecyl-d-maltoside and fluorescence readings were recorded for an additional 10 min. Lipid mixing results were quantified as previously described (14).

Measuring Inner Monolayer Mixing by Dithionite Treatment of v-SNARE Liposomes. Oxidation of outer monolayer NBD-PS of v-SNARE liposomes by addition of dithionite was performed as previously described (11). Dithionite treatment (10 mM) in 100 mM Tris pH10 efficiently oxidized NBD on outer leaflets of purified v-SNARE liposomes at approximately 1.5 mM lipid (Fig. S2*A*). A 50% to 60% decrease in NBD fluorescence occurred within 2 min at room temperature with no further decrease over a period of 30 min. NBD fluorescence was completely eliminated by treatment after detergent addition. Parallel control liposomes were prepared with 100 mM Tris pH10 lacking dithionite. All liposomes were re-purified on Accudenz gradients. Dithionite does not modify unlabeled lipids or affect rhodamine fluorescence (11), and this method produced liposomes labeled with fluorescent NBD-PS on the inner monolayer. Fusion assays were conducted with dithionite-treated and Tris-treated v-SNARE liposomes in parallel to compare inner monolayer lipid mixing with inner and outer leaflet mixing.

Measuring Aqueous Contents Mixing. Tb^{3+}-loaded v-SNARE liposomes and DPA-loaded t-SNARE liposomes were mixed at a 1:2 ratio to give a final lipid concentration of 0.75 mM in a total volume of 75 μL reconstitution buffer without glycerol. CAPS (1 μM) and 5 μM GST-syntaxin (1–265) were added to reactions as indicated. Parallel incubations were set up in reconstitution buffer without glycerol and with 150 mM DPA. Fusion was detected by measuring the Tb-DPA fluorescence (excited at 276 nm; emission at 495, 540, 585, and 624 nm) every minute (shaken before each reading) for 2 h at 32 °C in a SpectraMax Gemini XS microplate spectrofluorometer. Empty liposomes gave a significant fluorescence signal with a maximum at 545 nm (Fig. S2*C*) that overlapped with the strongest of the Tb-DPA complex emissions. Thus, we used readings at 585 and 624 nm for the analysis of fusion. Leakage, assessed at 1.5% ± 0.1% ($n = 6$), was measured during the fusion reactions as the difference between reactions assembled in reconstitution buffer with 150 μM DPA and parallel incubations in the absence of external DPA. Zero fusion was taken as the fluorescence level of the reaction mixture at t = 0. One hundred percent fusion was calibrated by addition of dodecyl-d-maltoside to fusion reactions assembled in reconstitution buffer with 150 μM DPA after 2 h.

Second-Dimension Heat Denaturation of SDS-Resistant SNARE Complexes Formed at 4 °C and Quantification of SNARE Complexes. v-SNARE and t-SNARE liposomes were incubated separately or together on ice for 16 h in the presence or absence of 1 μM CAPS. Reactions were assembled as outlined in *Materials and Methods* in the main text for SNARE complex formation. Reactions were resolved on 12% SDS/PAGE gels without boiling to maintain heat-sensitive SDS-resistant SNARE complexes. Molecular weight standards were run in lanes with reactions. Lanes were cut out and soaked in carbonate buffer (66 mM sodium carbonate, 2% SDS, and 0.67% β-mercaptoethanol (pH10) for 10 min. Gel slices were microwaved in carbonate buffer at highest setting for 5 × 10-sec bursts, removed, and washed in polymerizing stacking gel mix and overlaid horizon-

tally onto 12% SDS PAGE resolving gel with a 2-cm stacker. The gel slice was sealed in place with stacking gel. Molecular weight standards and protein standards were loaded in lanes adjacent to the gel slice (Fig. S7*E*). Gels were run at 89 V until the smallest molecular weight standard was run off. Gels were transferred and immunoblotted with syntaxin monoclonal antibody (HPC1; Sigma) or VAMP2 polyclonal antibody (Synaptic Systems). CAPS specifically promoted high molecular weight SNARE complexes migrating between 150 and 250 kDa in incubations of v- and t-SNARE liposomes (Fig. 3 *A–D* and Fig. S7 *A–D*). To calculate the specific immunoreactivity for high molecular weight SNARE complexes formed between 150 and 250 kDa, we subtracted the immunoreactivity that existed in this range for v- or t-SNARE liposomes in the absence or presence of 1 μM CAPS (not shown). The remaining signal was a consequence of pairing between v- and t-SNARE liposomes. We expressed the SNARE immunoreactivity between 150 and 250 kDa as a percentage of the total signal. For incubations with v- and t-SNARE liposomes, we found that 0.38% of the total VAMP2 migrated between 150 and 250 kDa as a consequence of incubation with t-SNARE liposomes. We observed no net increase in syntaxin-1 migrating between 150 and 250 kDa as a consequence of v-SNARE liposome incubation. For incubations with CAPS, we found that 1.13% of the total VAMP2 migrated in the range of 150 to 250 kDa as a consequence of t-SNARE liposome incubation, whereas 1.65% of the syntaxin migrated in the 150- to 250-kDa range as a consequence of v-SNARE liposome incubation. Therefore, the fractional amounts of VAMP2 and syntaxin1A migrating between the 150 and 250 kDa that can be attributed to CAPS stimulation are 0.75% and 1.65% of the total VAMP2 or syntaxin1A content, respectively. The SNARE and lipid content of our reactions were 40 pmol of VAMP2/4.2 nmol lipid (12%PS:85%PC:1.5%RhPE:1.5%NBDPE) and 17.2 pmol of syntaxin/4.1 nmol lipid (5%PIP2/95%PC). We resolved one quarter of this reaction in our gel electrophoresis separation of SNARE complexes. Using the estimates that 20% of the liposomes (50 nm diameter; 27,000 lipids/liposome) fuse at 32 °C with 1 μM CAPS, we calculate that approximately 9.7 (i.e., $\approx$9–10) *trans*-SNARE complexes per fusion event form in incubations with CAPS at 0 °C to 4 °C.

Immunoprecipitation of SNARE Complexes. v-SNARE and t-SNARE liposomes were incubated at 4 °C for 90 min with or without 1 μM CAPS. Reactions (20 μL) were incubated with 1.5 μM HPC-1 syntaxin antibody for a further 10 min on ice before addition of protein G beads (20 μL of washed packed beads). The liposome-antibody-bead reaction was nutated on an orbital shaker for 10 min at 4 °C. Beads were washed 3 times with 240 μL of reconstitution buffer containing 1% Triton X-100. Beads were boiled for 10 min in 35 μL 4× Laemmli sample buffer supplemented with 16% SDS. Boiled beads were spun out at 2,000 × *g* for 5 min, and supernatants were resolved by SDS/PAGE gels and immunoblotted for syntaxin-1 and VAMP2.

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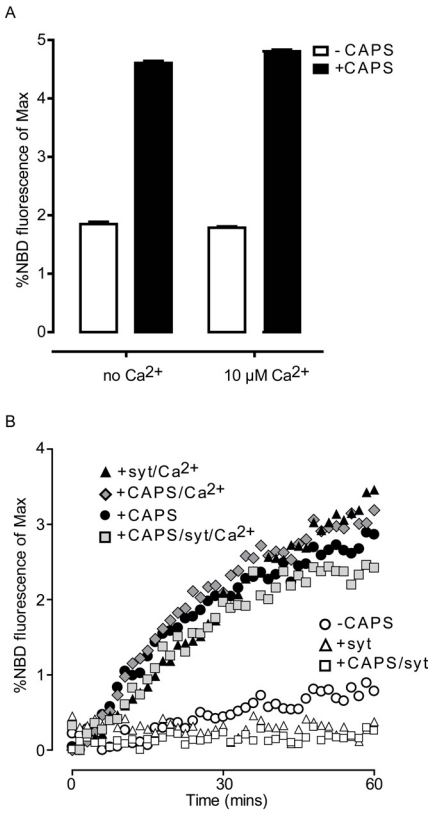

Fig. S1. (*A*) CAPS stimulation of fusion is not dependent on calcium. The final extent of lipid mixing was compared for incubations conducted with or without 10 μM Ca^{2+} in the absence (open bars) or presence of 1 μM CAPS (closed bars). (*B*) Synaptotagmin-1 C2AB confers calcium sensitivity to CAPS stimulation of SNARE-dependent fusion. Synaptotagmin-1 C2AB inhibited CAPS stimulation of lipid mixing and rendered it Ca^{2+}-dependent. Incubations (as in *A*) were conducted without additions (open circles), with 1 μM CAPS (filled circles),1 μM C2AB (Δ), or CAPS plus 1 μM C2AB (open squares); or in the presence of 100 μM Ca^{2+} with 1 μM CAPS (filled diamonds),1 μM C2AB (filled triangles), or CAPS plus 1 μM C2AB (filled squares).

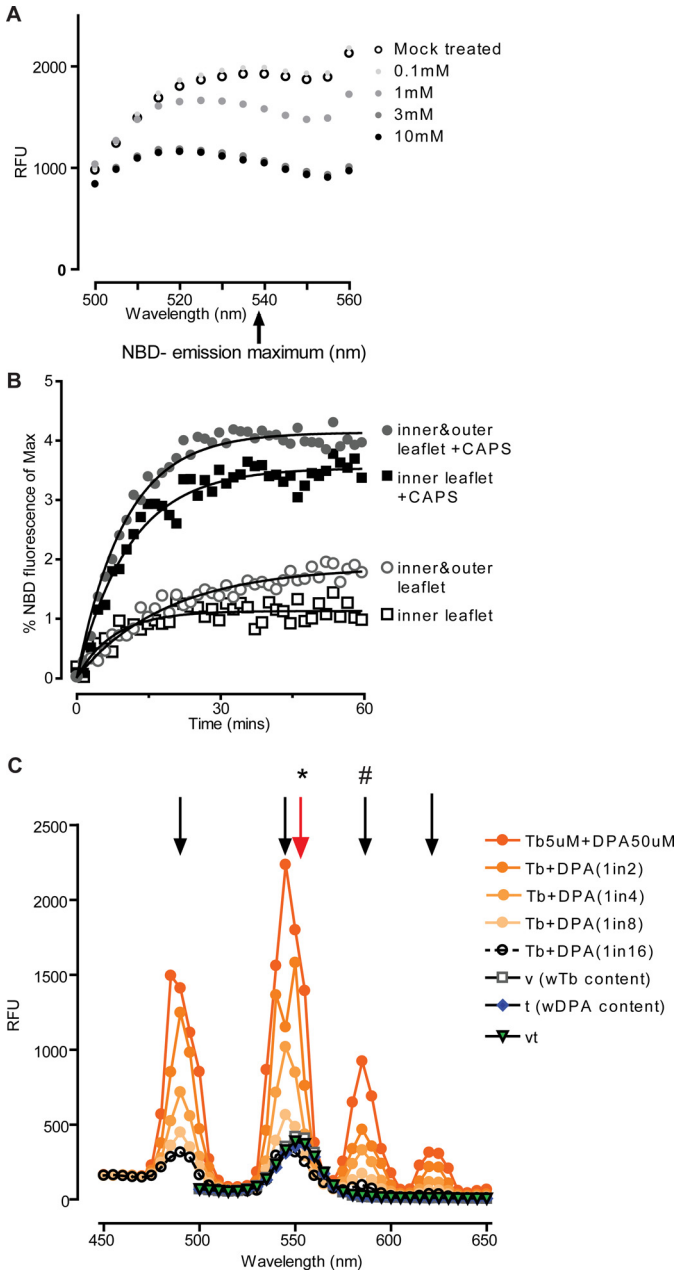

Fig. S2. (*A*) Dithionite quenching of liposome NBD-PS fluorescence. Dithionite at indicated concentrations was used to treat v-SNARE liposomes for fluorescence emission determinations with excitation at 438 nm. Dithionite (3 mM) reduced donor NBD emission at 538 nm and decreased rhodamine acceptor fluorescence at 595 nm by 50%. (*B*) CAPS accelerates both inner- and outer-lipid leaflet mixing. Dithionite-treated (open squares, filled squares) or untreated v-SNARE liposomes (open circles, filled circles) were incubated with t-SNARE/10% PIP_2 liposomes in the absence (open circles, open squares) or presence (filled circles, filled squares) of 1 μM CAPS (mean $\pm$ SD, $n = 3$). (*C*) Fluorescence emission of Tb-DPA complexes: 5 μM Tb^{3+} and 50 μM DPA were diluted as indicated and fluorescence spectra (450–650 nm) were monitored with 236 nm excitation. Peak emissions corresponded to 485, 540, 585, and 624 nm. Liposomes exhibited a peak emission corresponding to 550 nm (asterisk). For quantification of liposome content mixing, 585 nm emission (#) was used.

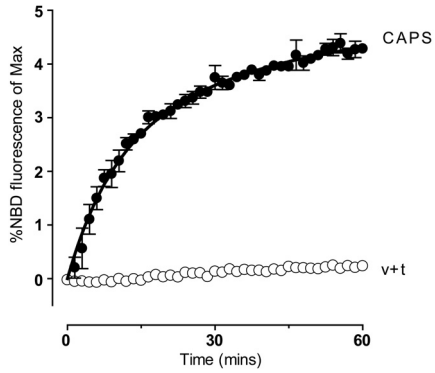

Fig. S3. CAPS stimulates SNARE-dependent fusion at low SNARE densities. Lipid mixing reactions using v- and t-SNARE liposomes with the same SNARE densities as in Fig. 2*B* were conducted without (open circles) or with (filled circles) 1 μM CAPS (mean $\pm$ SE, $n = 3$). The final NBD fluorescence of approximately 4.5% is equivalent to 0.2 rounds of fusion.

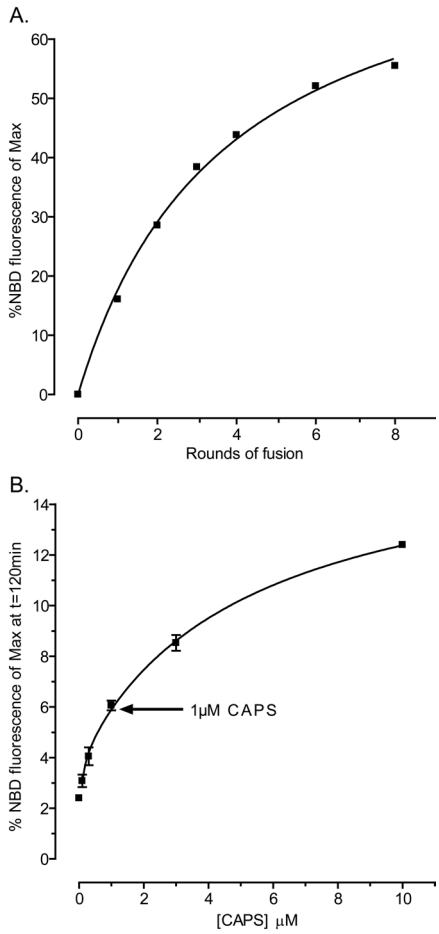

Fig. S4. (*A*) Calibration of NBD fluorescence as a measure of the number of rounds of fusion. To calibrate our fusion assay, we generated liposomes that mimicked the lipid dilution expected for the FRET pair over several rounds of fusion. NBD fluorescence was measured for each liposome set and expressed as a percent NBD of maximum observed after detergent solubilization. Using this calibration curve, we estimate that 50% of the v-SNARE liposomes (≈90 VAMP2 molecules/liposome) fuse with 2 t-SNARE liposomes (≈40 syntaxin/SNAP25 1:1/liposome) at the highest CAPS concentration used (10 μM). Overall, these results indicate that CAPS promotes efficient fusion. (*B*) CAPS promotes a dose-dependent increase in SNARE-dependent liposome fusion. Lipid mixing reactions using v- and t-SNARE liposomes were conducted without or with 0.1 to 10 μM CAPS (mean ± SE, $n = 3$). Plotted are the final extents of fusion at 120 min as percentage of maximal NBD fluorescence. The concentration of CAPS (1 μM) used in our experiments promotes approximately 6% of maximal NBD fluorescence at t = 120 min (arrow). This fluorescence corresponds to approximately 20% of v-SNARE liposomes undergoing 2 rounds of fusion with t-SNARE liposomes.

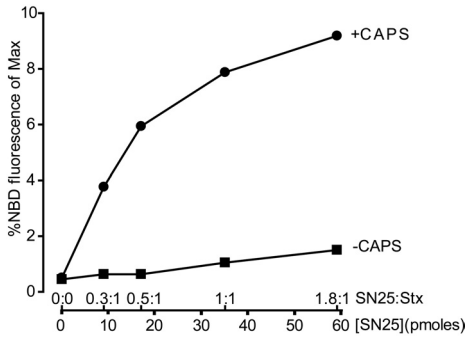

Fig. S5. CAPS stimulation of SNARE-dependent fusion requires SNAP-25 and operates optimally at a 1:1 SNAP-25:syntaxin molar ratios. Acceptor liposomes with 5% PIP_2 were generated with indicated SNAP-25:syntaxin mol ratios (see SI Materials and Methods) and incubated with v-SNARE liposomes in the absence (filled squares) or presence (filled circles) of 1 μM CAPS. Plotted are the final extents of fusion at 120 min as percentage of maximal NBD fluorescence.

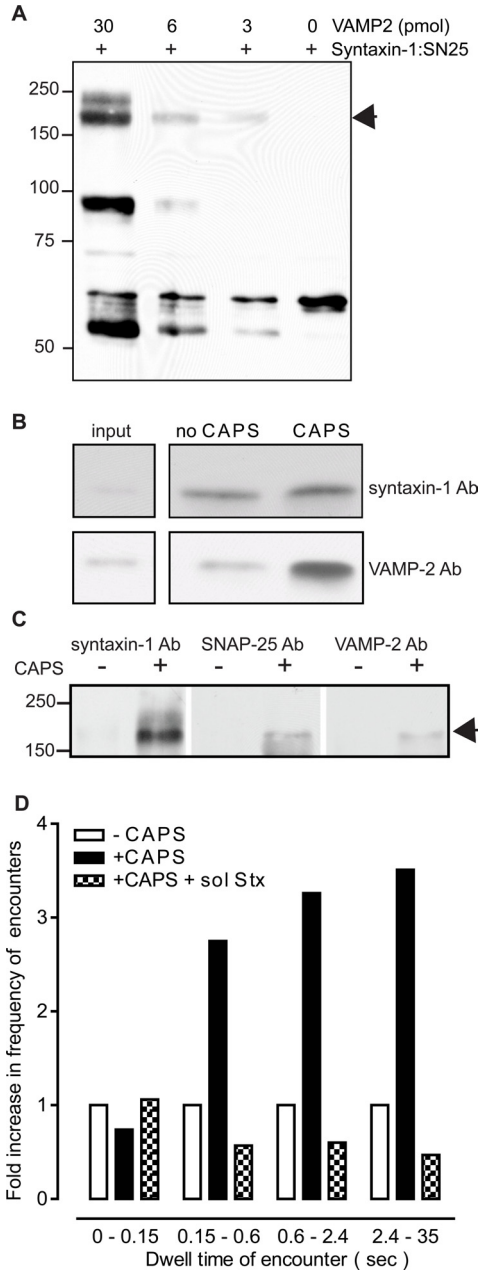

Fig. S6. A. SNARE complex formation with full-length SNARE proteins. Detergent-soluble full-length SNAREs readily form ≈85 kDa and ≈165 kDa complexes. Syntaxin1A:SNAP25 (25:64 pmol) in 0.8% octyl glucoside were incubated on ice for 30 min in the absence or presence of 30, 6, and 3 pmol of VAMP-2 protein. Samples were quenched by addition of one quarter volume of 4× Laemmli buffer. SNARE complexes were resolved by SDS/PAGE without heating. Arrowhead indicates ≈165 kDa complex. (*B*) CAPS increases the entry of VAMP2 into t-SNARE complexes. Co-immunoprecipitations using HPC-1 syntaxin1A antibody were performed as described in *SI Materials and Methods*. CAPS incubation with v- and t-SNARE liposomes increased the co-immunoprecipitation of VAMP2 with syntaxin1A by fourfold. Input (0.5%) and immunoprecipitate (50%) were resolved by SDS/PAGE. (*C*). CAPS stimulates SNARE complex formation. CAPS stimulated the formation of high molecular weight SDS-resistant SNARE complexes that contain syntaxin-1, VAMP2, and SNAP-25. Incubations were conducted with v-SNARE and t-SNARE (5% PIP_2) liposomes at 32 °C for 30 min in the absence or presence of 1 μM CAPS. SNARE complexes were resolved by SDS/PAGE for blotting with the indicated antibodies. Arrowhead indicates ≈165 kDa SNARE complex. (*D*) CAPS increases v-SNARE liposome dwell time on t-SNARE bilayers. Movies of Rh-PE v-SNARE liposome encounters with t-SNARE bilayers incubated with buffer (open bar), 1 μM CAPS (solid bar), or CAPS plus 5 μM soluble syntaxin (hatched bar) were recorded and analyzed as in SI Materials and Methods. All liposomes in a 10 μm^2 area entering or leaving the evanescent field were tracked over a period of 37 s, and dwell times were grouped into 4 bins.

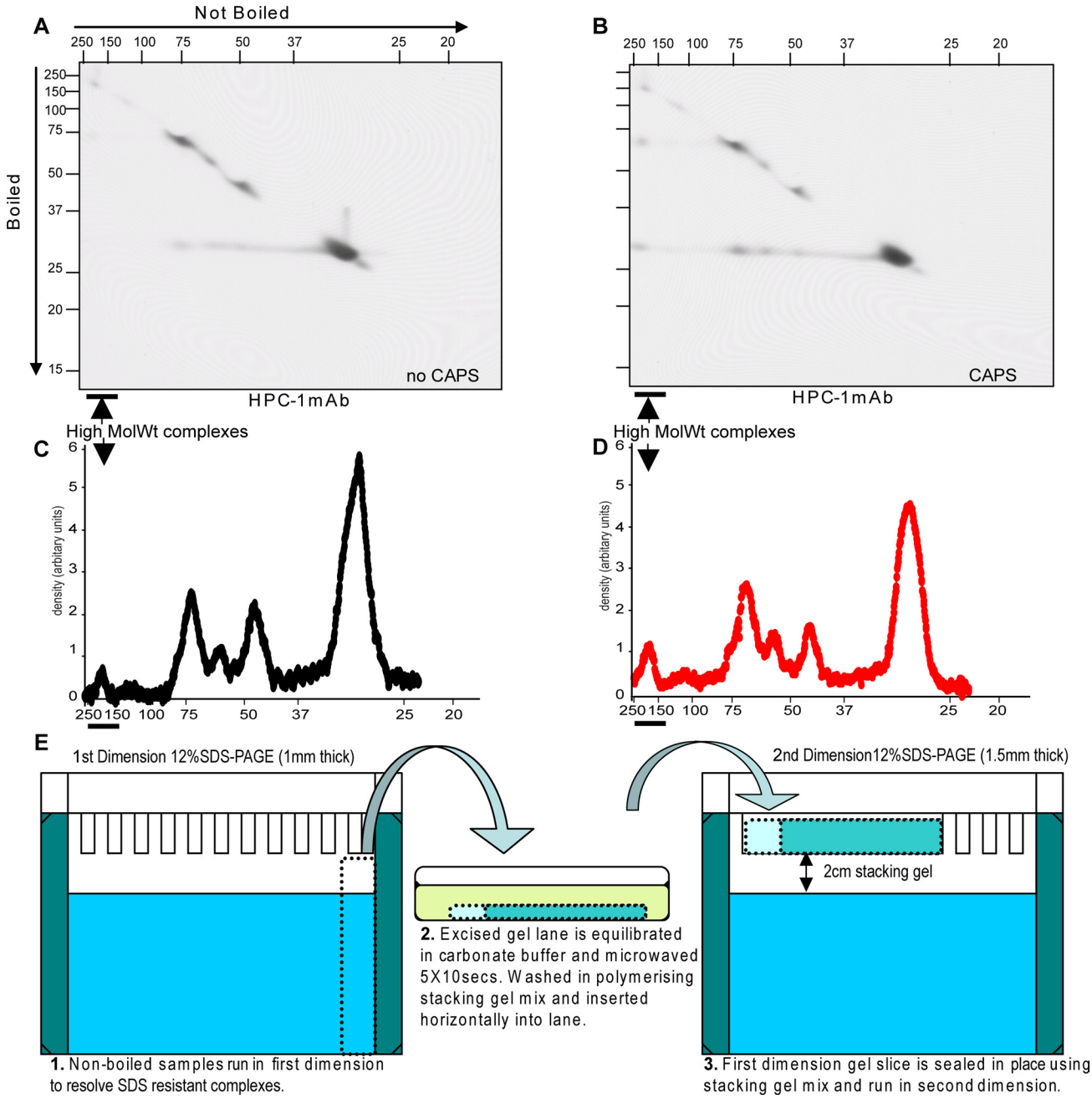

Fig. S7. Resolving syntaxin-1A and VAMP-2 content in high molecular weight SDS-resistant SNARE complexes. SDS-resistant SNARE complexes were formed during 16-h incubations at 4 °C. Reactions containing v-SNARE and t-SNARE(5% PIP_2) liposomes without (*A*) or with (*B*) 1 μM CAPS (1 μM) were resolved by non-boiled SDS/PAGE. Gel strips of resolved SDS-resistant SNARE complexes were excised, heated, and resolved in the second dimension. Nitrocellulose gel transfers were immunoblotted for syntaxin-1A (*A* and *B*) or VAMP-2 (not shown). Black bar with arrow indicates the molecular weight range of SDS-resistant complexes that are formed in incubations with CAPS. Scans of the immunoblots along the first dimension (*A* and *B*) are shown in *C* and *D*, respectively. Note that an increased density peak occurs at approximately 165 kDa. A schematic of the overall experimental method is indicated (*E*) and described in *SI Materials and Methods*.

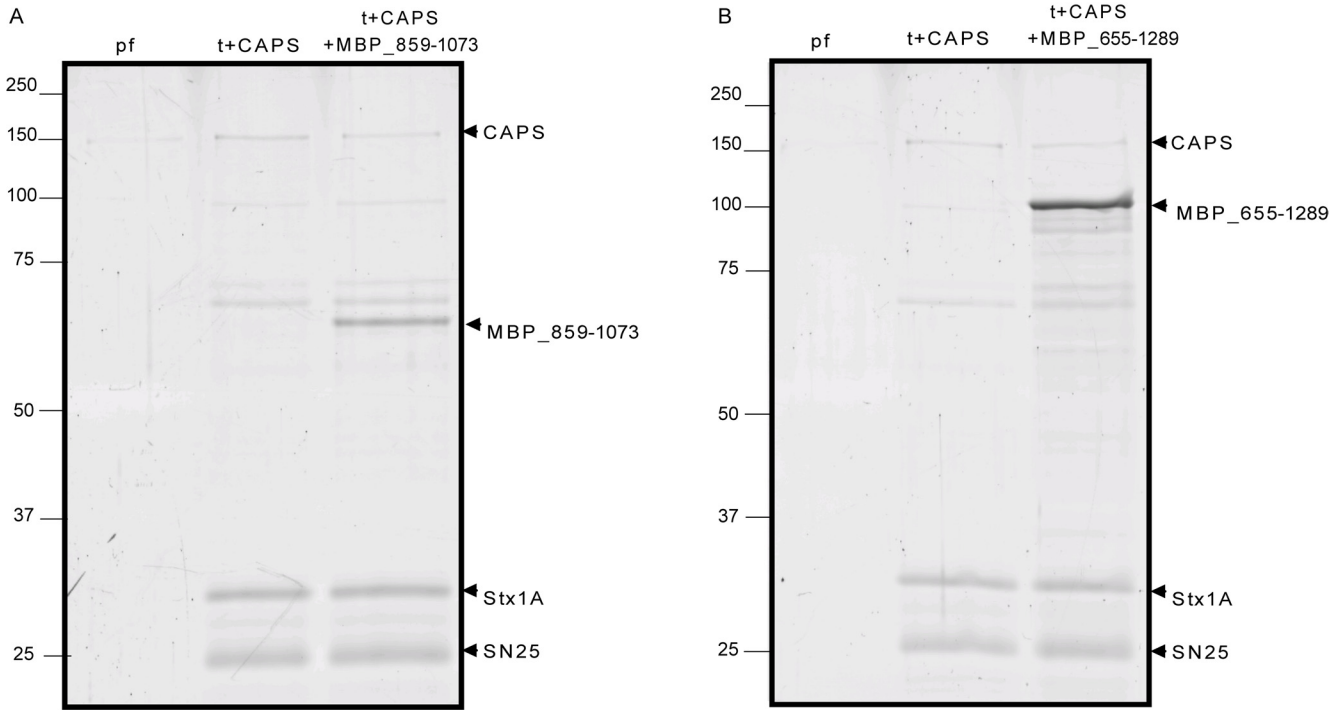

Fig. S8. CAPS fragments encompassing the MH domain inhibit CAPS binding to t-SNARE liposomes. CAPS binding to t-SNARE liposomes and competition by MH domain-containing fusion proteins were assessed as described in *Materials and Methods* in the main text. Liposomal binding was assessed by Accudenz gradient flotation to determine bound protein. Floated liposome fractions were resolved by SDS/PAGE and stained with SYPRO Ruby (Peribio). (*A* and *B*) Similar experiments for maltose binding protein CAPS(859–1073) and CAPS(655–1289) fusion proteins, respectively. Lane 1 corresponds to binding to protein-free liposomes, lane 2 to CAPS bound to t-SNARE liposomes, and lane 3 to reduced levels of CAPS bound to t-SNAREs upon co-incubation with corresponding CAPS fusion proteins. CAPS(859–1073) and CAPS(655–1289) at 1 μM significantly inhibited binding of 300 nM CAPS to t-SNARE liposomes, as summarized in Fig. 5*G*.

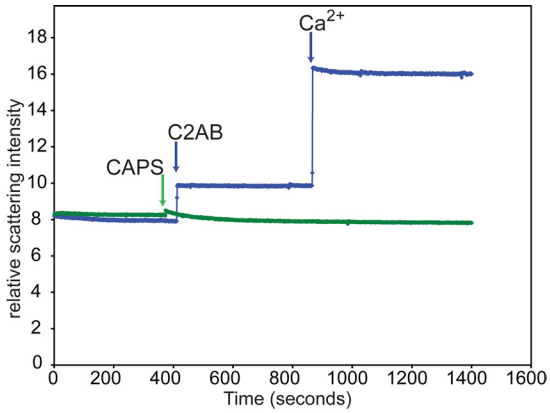

Fig. S9. CAPS did not aggregate liposomes. v- and t-SNARE/5% PIP2 liposomes were incubated as detailed in ''Lipid Mixing Fusion Studies'' in *SI Materials and Methods*. Reactions were scaled up to 200 μL, and v- and t-SNARE/5% PIP2 liposomes were mixed in a quartz cuvette. Ninety-degree light scatter intensity was monitored at excitation/emission wavelengths of 400 nm/400 nm using a QuantaMaster spectrofluorometer (Photon Technologies International). Synaptotagmin C2AB (10 μM; blue line) increased light scattering, and this was further increased by 1 mM Ca^{2+} addition. Addition of CAPS to 1 μM (green line) did not increase light scattering.

Table S1. Neuronal SNARE densities used in the in vitro liposome fusion assay with comparison versus determinations of physiological SNARE density

Source	vSNARE copies/ liposome	L:P(v) molar ratio	vSNARE copies/μm^2	tSNARE copies stx:sn25 1:1/ liposome	L:P(t) molar ratio	tSNARE copies/ μm^2	v [1]:t liposome ratio	v [1]:t protein	%NBD mixing at t = 120 min (%)	Diameter (nm)	Surface area (μm^2)	Notable differences
Weber *et al* (5)	750	22	117,952	75	230	11,795	15	1.5	20–30	45	0.0064	45 nm liposome PC[85]:PS[15], 65 A^2 lipid surface area
Schuette *et al*. (15)	64	100	22,647	64	100	22,647	1	1	22	30	0.0028	≈30 nm liposome PC[5]:PE[2]:PS[1]:PI[1]:Chol[1] reconstituted H3 and SN25 construct
Tucker *et al.* (16)	48–860	563–31	6,115–109,554	16–134	1,687–201	2,038–17,070	15	0.8–41.9	8–44	50	0.0079	50 nm liposome PC[85]:PS[15], 65 A^2 lipid surface area, 27,000 lipids/liposome
Present study	90	225	11,465	40	506	5,096	2	0.9	2	50	0.0079	≈50 nm liposomes PC[90–95]:PIP2[5–10]
Present study	20	4,469	637	10	8,938	318	2	1.0	0	100	0.0314	≈100 nm extruded liposomes PC[90]:PIP2[10]
Measurement of organelle SNARE density												
Takamori *et al* (7)	69.8	100.2	12,845	—	—	—	—	—	—	42	0.0054	Synaptic vesicle ≈42 nm diameter
Sieber *et al*. (17)	—	—	—	14	1,446	1,804	—	—	—	—	—	These syntaxin densities do not account for clustering effects which are demonstrated to occur
Present study	—	—	—	18	1,125	2,280 ± 60	—	—	—	—	—	