SUPPORTING INFORMATION

SUPPORTING METHODS

Plasmids and protein purification. cDNA encoding rat syt I (*1*) was provided by T.C. Südhof (University of Texas Southwestern Medical Institute, Dallas, TX) and the D374 mutation was corrected by substitution with a glycine residue (*2*). The cytoplasmic domain of syt was sub-cloned into a pTrcHis vector (Invitrogen Life Technologies), expressed in *E. coli*, and purified as described (*3*). Protein was eluted in wash buffer with 500 mM imidazole and dialyzed overnight against 25 mM HEPES pH 7.4, 100 mM KCl, 10% (w/v) glycerol, 1 mM DTT (Buffer A). cDNA encoding full-length complexin I was provided by J.M. Edwardson (University of Cambridge, Cambridge, England), sub-cloned into a pTrcHis vector (Invitrogen Life Technologies) and expressed and purified as described above for syt. A plasmid encoding GST-complexin I (26-83) V61C (*4, 5*) was provided by J. Rizo (UT Southwestern Medical Center, Dallas, TX) and expressed in *E. coli* and purified as described (*6*) in HEPES buffer (25 mM HEPES, pH7.4, 150 mM NaCl). Soluble complexin I (26-83) was prepared by thrombin cleavage (*7*) in 25 mM HEPES, pH 7.4, 200 mM KCl, 10% glycerol. t-SNARE heterodimers were generated using either a plasmid provided by J. E. Rothman (Columbia University, New York, NY)(*8*) or the pRSFDuet-1 SNAP-25+syx 1 construct described in supplementary ref. (*9*). t-SNARE heterodimers were expressed and purified as described (*10*). Plasmids to generate recombinant full-length synaptobrevin 2 and the cytoplasmic domain of synaptobrevin (cd-syb; a.a. 1–94; pET-rsybCD), were provided by J. E. Rothman (Columbia University, New York, NY) and proteins were expressed and purified as described (*10*). All proteins were washed in buffer containing nucleases and high salt to remove bacterial contaminants. Protein concentration of all recombinant proteins was determined using SDS-PAGE and staining with Coomassie brilliant blue using bovine serum albumin as a standard. Optical Densities were calculated using a UVP BioImaging System and Labworks software. With the exception of complexin-1 (26-83), tags used for purification remained attached to proteins via short linkers.

Preparation of SNARE-bearing vesicles. All lipids were obtained from Avanti Polar Lipids. Reconstitution of v-SNARE and t-SNARE vesicles was carried out as previously described (*10*). Briefly, v-SNAREs were reconstituted using a lipid mix composed of 27% 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (PE), 55% 1-palmitoyl, 2-oleoyl phosphatidylcholine (PC), 15% 1,2-dioleoyl phosphatidylserine (PS), 1.5% N-(7-nitro-2-1,3-benzoxadiazol-4-yl)-1,2-dipalmitoyl phosphatidylethanolamine (NBD-PE, donor), and 1.5% N-(lissamine rhodamine B sulfonyl)-1,2-dipalmitoyl phosphatidylethanolamine (Rhodamine-PE, acceptor) (mol/mol). t-SNAREs were reconstituted in 30% PE, 55% PC and 15% PS (mol/mol). When PS was omitted, PC was increased to 70% for the t -SNARE vesicles. v-SNARE (syb) and t-SNARE (SNAP-25 + syx 1) vesicles were reconstituted to give a minimum of ~ 60 copies and ~ 95 copies (yielding ~ 45 and ~ 65 copies in the correct orientation) per vesicle, respectively, as described (*9, 10*).

Reconstituted membrane fusion assays. Fusion assays were carried out as described (*9*). Briefly, each 75 μl reaction consisted of 45 μl purified t-SNARE vesicles (55%

PC/30%PE/15%PS) and 5 µl of purified, NBD/Rhodamine-PE labeled v-SNARE vesicles plus 0.2 mM EGTA (and 1mM Ca^{2+} when included) in 25 mM HEPES pH 7.4, 100 mM KCl, 1 mM DTT. All components of the fusion reaction, except v-SNARE vesicles, were combined, and pre-warmed to 37ºC for 15 min. v-SNARE vesicles were pre-warmed separately and added to reactions to initiate fusion at t = 0 min. Fusion was monitored as an increase in NBD fluorescence using a Biotek SynergyHT plate reader and Gen5 software with data acquisition every 8-15 seconds during rapid Ca^{2+} induced fusion, and every 1.5–2 min during slower phases. After 2 hrs, 0.5% (w/v) n-dodecylmaltoside (Roche Applied Science) was added to maximally dequench the NBD fluorescence yielding a maximum fluorescence signal. Raw fluorescence was converted to percent of the maximum fluorescence and plotted. Ca^{2+} was added at t = 20 min using injectors controlled by Gen5 software.

Co-flotation assays. Co-flotation assays were carried out as described previously (*11*). Briefly, cd-syb, syt, and complexin were mixed individually or in combinations (concentrations and order of addition indicated in figures and legends) with 40 µl of purified t-SNARE vesicles harboring PS (55% PC/30%PE/15%PS) or lacking PS (30%PE/70% PC). All binding reactions were carried out in a total volume of 100 µl Buffer A with either 0.2 mM EGTA or 1 mM Ca^{2+}. Samples were incubated with shaking for 1 hr at room temperature and were then mixed with an equal volume of 80% Accudenz media, transferred to a Beckman ultra-centrifuge tube and layered with 150 µl each of 35% and 30% Accudenz media in Buffer A. Finally, samples were layered with 20 µl Buffer A lacking glycerol and centrifuged at 280,000 x g for 2.5 hours at 4°C. All buffers/media contained either 0.2 mM EGTA or 1 mM Ca^{2+}. 40 µl of vesicles from the 0%/30% interface were collected from each tube, and ~ one sixth of the collected sample was resolved by SDS-PAGE and stained with Coomassie blue. Standards indicate the electrophoretic mobility of proteins.

SUPPORTING FIGURES

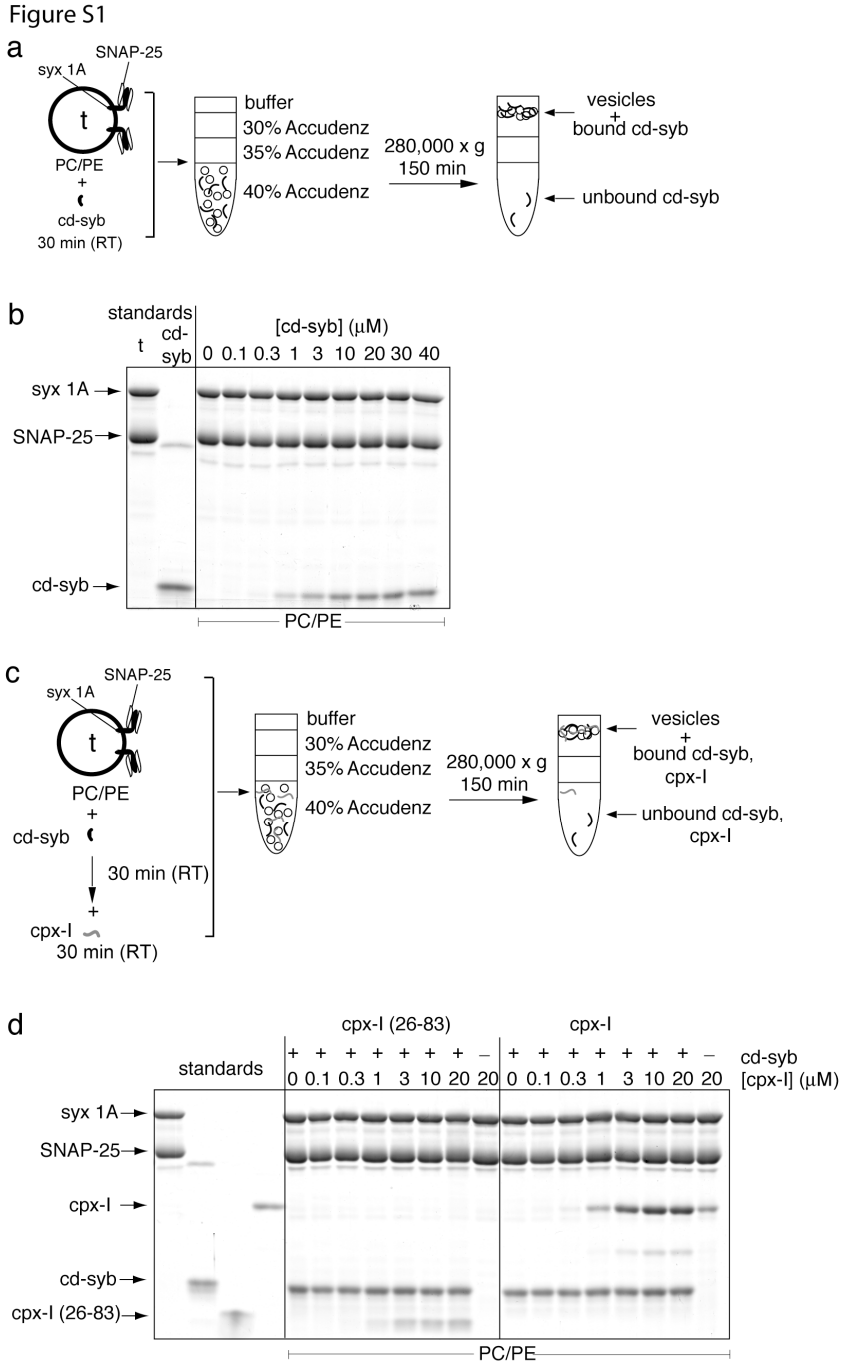

Figure S1: Saturation of membrane embedded ternary SNARE complexes with cpx-I, or cpx-I (26-83) (a) Illustration depicting ternary SNARE complex assembly. t-SNARE vesicles (t), containing syntaxin 1A (syx 1) and SNAP-25, were incubated with the cytosolic domain of synaptobrevin 2 (cd-syb) to form membrane embedded ternary SNARE complexes. t-SNARE vesicles harbored phosphatidylcholine and phosphatidylethanolamine (PC/PE; 70% and 30%, respectively). cd-syb will only float to

the top of the gradient if it forms complexes with t-SNAREs on the vesicles (cd-syb does not float with protein free-vesicles; data not shown). After centrifugation, samples were collected from the top of the gradient and analyzed by SDS-PAGE; proteins were visualized by staining with Coomassie blue. Standards indicate the electrophoretic mobility of proteins. (b) Increasing concentrations of cd-syb were added to t-SNARE vesicles and samples were subjected to the co-flotation assay. Saturation of t-SNARE heterodimers occurred at ~10 µM cd-syb. (c) Fully assembled ternary SNARE complexes were generated using 20 µM cd-syb. After a 30 min RT incubation, increasing concentrations of cpx-I or cpx-I (26-83) were added to the assembled ternary SNARE complexes. Reactions were then subjected to flotation as described in *a*. Duplicate samples containing 20 µM cpx-I or cpx-I (26-83) but lacking cd-syb were included. (d) Cpx-I saturated the SNARE complexes between 3-10 µM whereas 10-20 µM cpx-I (26-83) was required to reach saturation. All samples in *b* and *d* contained Ca^{2+} (1mM) and all gels are representative from $n \geq 3$.

Figure S2

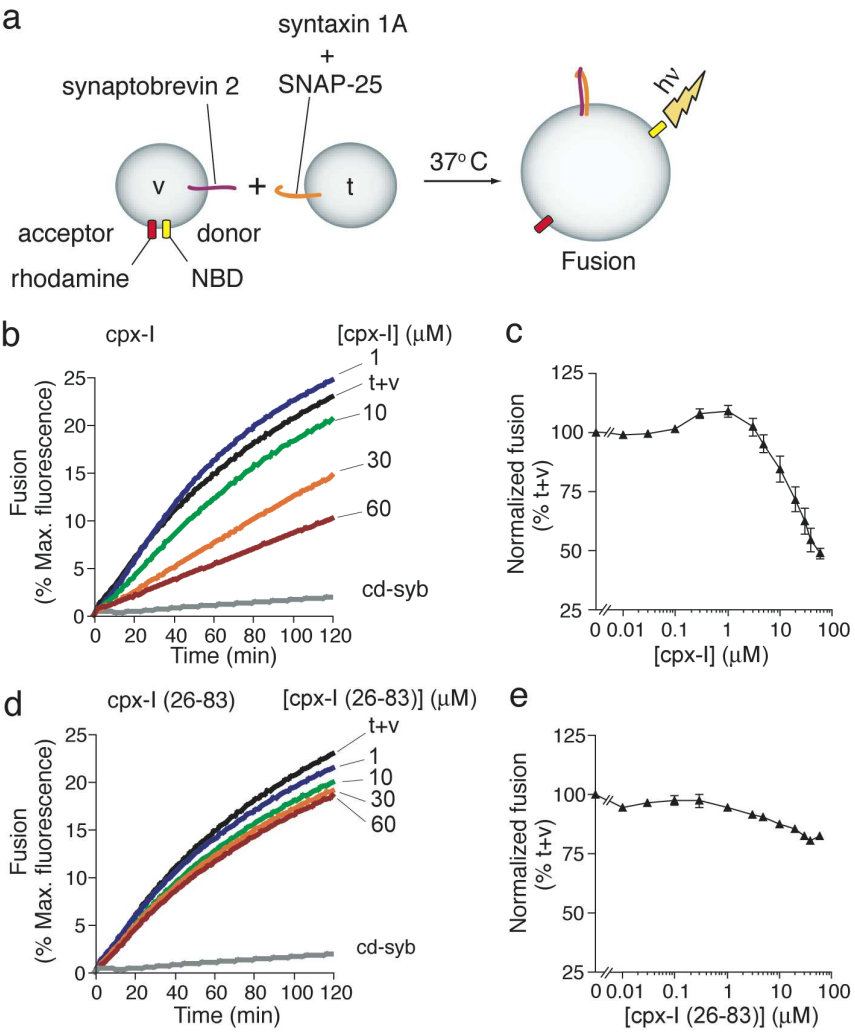

Figure S2: Effect of cpx-I on membrane fusion catalyzed by reconstituted neuronal SNARE proteins. (a) Illustration depicting the *in vitro* fusion assay used in this study. Fusion of v-SNARE (synaptobrevin 2; v) vesicles, containing a donor and acceptor FRET pair, with unlabeled t-SNARE vesicles (syntaxin 1A and SNAP-25; t), results in dilution of the FRET pair. Fusion is monitored as an increase in the donor fluorescence. (b) Increasing concentrations of cpx-I were added to fusion reactions. (t+v) denotes fusion reactions lacking cpx-I. As a control the cytosolic domain of synaptobrevin 2 (cd-syb, 10 μM) was added to (t+v) to inhibit SNARE-mediated fusion. Fusion was monitored for 120 min at 37°C, normalized to the maximum donor fluorescence signal (% Max. fluorescence) obtained by adding detergent to reactions at time =120 minutes, and plotted as a function of time. Reactions were carried out in 1 mM Ca^{2+}, but similar results were obtained for fusion reactions lacking Ca^{2+} (data not shown). (c) The final extent of fusion at each cpx-I concentration tested in *b* was normalized to the final extent of fusion obtained by (t+v) (% t+v). (d) Experiments were carried out as in *b* except cpx-I (26-83) was substituted for cpx-I. (e) Data from *d* were plotted as in *c*. All fusion traces are representative from n≥3. Data in *c* and *e* represent the mean +/- SEM from n≥3.

Figure S3

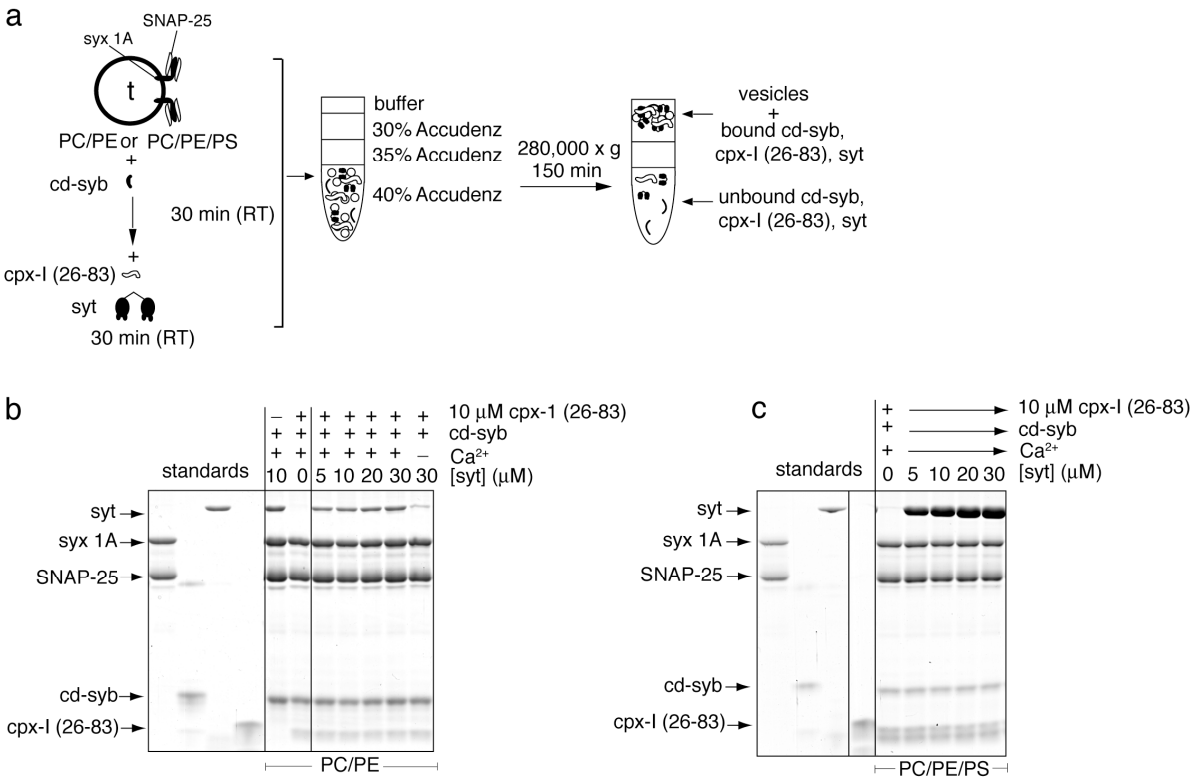

Figure S3: Binding of cpx-I (26-83) and syt to membrane embedded ternary SNARE complexes. (a) Diagram of the flotation assay used to monitor binding interactions (see also Figure S1a,b) (b) 10 μM cpx-I (26-83) and increasing concentrations of syt were added to ternary SNARE complexes individually or together, as indicated, then subjected to flotation. Vesicles harbored PC (70%), and PE (30%). Binding was monitored in the presence of Ca^{2+} (1 mM). A duplicate sample containing 30 μM syt and 10 μM cpx (26-83), but lacking Ca^{2+}, was also included. Proteins were visualized by staining with Coomassie blue. (c) Select experiments in *b* were repeated using vesicles that contained PE (30%), PC (55%) and PS (15%). All gels are representative from $n \geq 3$ and standards indicate the electrophoretic mobility of proteins. In this figure only, the line within the standards, and the line between the standards and experimental lanes, indicates lanes that were originally spaced further apart on the same gel and were combined for this figure.

SUPPORTING TEXT

Saturation of binding and fusion reactions with cpx-I

We note that different concentrations of cpx-I are required to attain saturation in the binding experiments shown in Figure S1 and the fusion experiments shown in Figure S2. Because of differences in the methodology used for the binding and fusion assays, we did not expect to see a perfect correlation between the co-flotation experiments used to monitor binding and the fusion reactions used to monitor function (we note that we did not observe any visible precipitate in our fusion reactions). We believe that this apparent discrepancy is likely due to the use of *cis*-SNARE complexes in the binding assay as compared to adding cpx-I to *trans*-SNARE complexes in the fusion assay. In the co-flotation assays, cpx-I was added to pre-formed *cis*-SNARE complexes and incubated for one hour before the vesicles were floated through a gradient and analyzed. In this case, cpx-I had a long time to equilibrate with the ternary SNARE complexes. In the fusion assays v-SNARE and t-SNARE vesicles attach and begin fusing immediately. Thus, at any given moment, the fusion reaction contains a mixed population of vesicles. This mixture includes attached/docked v-SNARE and t-SNARE vesicles that have not yet fused and are susceptible to inhibition by cpx-I. The mixture also contains v-SNARE and t-SNARE vesicles that have completely fused or have attached and progressed past the point at which cpx-I can inhibit fusion. In addition, the fusion mixture contains excess t-SNAREs that can bind cpx-I through the weak interactions demonstrated in Figure S1d.

Thus, higher concentrations of cpx-I may be needed for saturation in the fusion assay in comparison to the co-flotation assay. We see this same apparent "discrepancy" between the binding and fusion reactions with syt as well.

Inhibition by cpx-I is largely occluded by apo–syt

In contrast to what was observed when cpx-I was added to reactions containing SNAREs alone, addition of higher concentrations of cpx-I to reactions containing apo–syt only modestly inhibited fusion beyond what was already achieved by apo–syt alone (Figure 2a,b). Our data demonstrate that, before the Ca^{2+} signal, the inhibitory ability of cpx-I is largely abrogated by apo–syt. For example, 30 μM cpx-I inhibits fusion by ~ 37% in the absence of apo–syt (Figure S2b,c). However, addition of 30 μM cpx-I to reactions that contain apo–syt (which are already inhibited by ~25% in the absence of Ca^{2+}) only yields an additional ~10-15% more inhibition rather than an additional 37% inhibition (Figure 2a,b). Thus, the inhibitory function of cpx-I is largely occluded by apo–syt in fusion reactions prior to the addition of Ca^{2+}. This may be attributed to the fact that apo–syt binds t-SNAREs before, during and after ternary SNARE complex formation, whereas complexin preferentially binds to ternary SNARE complexes (see ref. (*9, 12*), and Figure S1). Please see main text for further discussion of this point.

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