

Supporting Information

Complexin-1 enhances the on-rate of vesicle docking via simultaneous SNARE and membrane interactions

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Experimental Methods:

Protein expression and purification. Full-length rat syntaxin-1A, full-length SNAP-25A, full-length synaptobrevin-2, full-length synaptotagmin-1 were expressed, purified as described previously ¹ with modifications ². Briefly, his-tagged syntaxin-1A and synaptobrevin-2 were expressed in C43 (DE3) cells ³ and SNAP-25A was expressed in BL21 (DE3) cells (Novagen). The proteins were purified by Ni²⁺-nitrilotriacetic acid (NTA) sepharose (Qiagen) affinity chromatography and further purified by size exclusion chromatography using a Superdex 200 10/300 column (GE Healthcare) in buffer containing 20 mM HEPES (pH 7.5), 300 mM NaCl, 1 mM TCEP and 110 mM octyl- β -D-glucoside (OG, ANATRACE). No OG was used for SNAP-25. Synaptotagmin-1 was expressed in *Sf9* insect cells (Invitrogen) and purified by Ni²⁺-NTA

affinity chromatography (Qiagen), followed by size exclusion chromatography on a Superdex 200 16/600 column (GE Healthcare) and further purified by anion-exchange chromatography on a Mono-S 5/50 column (GE Healthcare). His-tags were removed from syntaxin-1A, synaptobrevin-2, and SNAP-25A with TEV protease, or from synaptotagmin-1 with PreScission protease (GE Healthcare, Uppsala, Sweden), and proteins further purified as previously described ².

Soluble rat synaptobrevin-2 (residues 1-96) was expressed in BL21 (DE3) with an N-terminal TEV cleavable hexa-His tag. The protein was purified by Ni-NTA affinity chromatography using standard procedures and buffers (Qiagen), digested overnight with TEV protease, and further purified by size exclusion chromatography using a HiLoad Superdex 200 16/600 column that was pre-equilibrated with 25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5 mM EDTA and 0.5 mM tris(2-carboxyethyl)phosphine hydrochloride (TCEP).

Full-length rat complexin-1 (referred to as Cpx), and the “4M” mutant (R48A, R59A, K69A, Y70A) of complexin-1 (referred to as Cpx_{4M}) were purified as previously described. The C-terminally truncated mutant of complexin-1 (residues 1-86, referred to as CPX₁₋₈₆) was expressed as a glutathione S-transferase (GST) fusion protein from pGEX-KT (GE Healthcare) in BL21 (DE3) and purified on a glutathione-agarose column (GE Healthcare). The protein was eluted from a glutathione column by digestion with Thrombin (HTI), concentrated, and further purified by size exclusion chromatography on a HiLoad Superdex 200 16/600 column that was pre-equilibrated with 20 mM HEPES-KOH pH 7.5, 100 mM NaCl, and 0.5 mM TCEP.

Vesicle reconstitution of syntaxin-1A, synaptobrevin-2, and synaptotagmin-1. Syntaxin-1A, synaptobrevin-2, and synaptotagmin-1 were reconstituted into vesicles as previously described² except that distinct lipid dyes were added to v- and t-vesicles: 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate, DiIC18(5) (DiD)/ 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate, DiIC18(5) (DiI) labels were added during the reconstitution of v- and t- SNARE vesicles, respectively⁴. The particular composition followed our previous work¹: t-vesicles consisted of Brain Total Lipid Extract, supplemented with 20 mol% cholesterol, 3.5 mol% phosphatidylinositol-4,5-bisphosphate (PIP2), 0.2 mol% biotinylated phosphatidylethanolamine (PE), and 2 mol% DiD (all lipids from Avanti polar lipids) and v-vesicles consisted of phosphocholine (PC):PE: phenylserine (PS):Cholesterol:DiI in a ratio of 46:20:12:20:2.

As previously described⁴, lipid films were dissolved in 110 mM OG buffer and tag free proteins (synaptobrevin-2/synaptotagmin-1 and syntaxin-1A for v- and t-vesicles, respectively) were added at a protein to lipid ratio of 1:200. For t-vesicles, a large excess of soluble SNAP-25A (five times the concentration of syntaxin) was added to the protein-lipid mixture in order to reduce the possibility of formation of dead-end 2:1 syntaxin/SNAP-25 complexes. Detergent free buffer (20 mM HEPES, pH 7.4, 90 mM NaCl, 1 % 2-mercaptoethanol) was added to the protein-lipid mixture until the detergent concentration was at its critical micelle concentration. The vesicles were purified with a CL4B desalting column and dialyzed overnight with Bio-beads SM2 (Bio-rad) in detergent-free Vesicle Buffer (20 mM HEPES, pH 7.4, 90 mM NaCl, 20 μ M

EGTA, 1 % 2-mercaptoethanol). For the reconstitution of v-vesicles with both full-length synaptobrevin-2 and synaptotagmin-1, a 4.6:1 protein ratio was used in order to mimic the observed protein concentration in synaptic vesicles⁵.

PEG surface preparation. Details of the preparation for PEGylation of surfaces are described in reference⁴. Briefly, cleaned or new quartz slides and glass coverslips were incubated with 150 ml acetone (Fisher) solution containing 5 ml Tris(hydroxymethyl)aminomethane (Fisher) for 30 min. After washing with acetone, air-dried quartz slides and glass coverslips were assembled into a sandwich structure with PEG solution (10 mg Biotin-PEG-SVA and 90 mg mPEG-SVA dissolved in 0.1 M sodium bicarbonate, pH 8.5) in between; note that we used mPEG-SVA instead of mPEG-SCM at variance with the protocol published in Ref.⁴ since mPEG-SVA produces a better surface coverage⁴. After incubation in the dark for 2-10 hours, the quartz slides and glass coverslips were disassembled and washed with deionized water, air dried, and stored at -20 °C. Quality controls included checking the homogeneity of each surface preparation (Figure S6) and tests for non-specific binding (Figure 2B).

Single vesicle-vesicle docking experiments. A saturated layer of DiD-labeled t-vesicles was immobilized on an imaging surface via biotin/neutravidin interactions. Specifically, the DiD-labeled t-vesicle solution (described above) was diluted 10 × with Vesicle Buffer. 100 μl of the diluted t-vesicle solution was injected into the sample chamber and incubated for 30 min, followed by buffer exchange (1 × 200 μl vesicle buffer) for 6 sec. Next, the DiI-labeled v-vesicle solution (described above) was diluted 50-100 ×. 100 μl of diluted free-floating DiD-labeled v-

vesicle solution was injected into the sample chamber in the presence or absence of wildtype or mutant complexin-1 (10 μM). After an incubation period, unbound v-vesicles were removed by buffer exchange ($2 \times 200 \mu\text{l}$ vesicle buffer for ~ 20 sec) (Figure S1). 20 μM EGTA was included in all solutions for elimination of free Ca^{2+} ions.

We determined the optimum incubation period (25 sec) by trial and error, i.e., providing a sufficiently large number of docked vesicles below the density limit (~ 1000 in a $50 \times 50 \mu\text{m}^2$ field of view) in order to optically resolve individual vesicles. In addition, we performed intensity distribution analyses (Figure S4 and Figure S5) that allowed us to conclude that mostly single v-vesicles are docked to the surface (as opposed to multiple v-vesicles docked to one surface-immobilized t-vesicle).

Sample slides with 5 channels were monitored in a wide-field TIR fluorescence microscope (Nikon) using an electron multiplying charge-coupled device (CCD) camera (iXon+ DV 897E, Andor Technology). A program (smCamera) written in C++ was used for data acquisition and analysis (available from Taekjip Ha, University of Illinois). 10 images were taken at random locations within each channel on the quartz slide. Details regarding software, slide assembly, and imaging protocols are described in reference ⁴.

We confirmed that the t-vesicle-covered surfaces were saturated and produced a homogeneous distribution for each surface preparation with red laser excitation (633 nm) of the DiD-labeled

immobilized vesicles, as observed in a separate DiD channel on particular slide (Figure S5). As previously reported, more than 1000 vesicles could be immobilized with this method ⁶. Our preparation of a reproducible, homogeneous, and saturated surface of immobilized t-vesicles ensures that the number of docked DiI labeled v-vesicles is directly related to the docking probability.

For each set of comparisons between different conditions and/or mutants (Figures 2B, 2C, and 3) the same protein preparations and surface preparations (quartz slide with immobilized vesicles), and incubation times were used, and the conditions were run in separate channels on the same slide. The relative differences and ratios were statistically similar for different protein preparations.

Membrane binding experiment. Protein-free vesicles were made using the method described above, containing PC, PE, PS lipids and cholesterol at a molar ratio of 48:20:12:20 (the same ratio as used to reconstitute the v-vesicles, except that the 2% ratio corresponding to the lipid-dyes was added to the PC ratio). The vesicles encapsulated 0.1 M sucrose in order to increase the density of the vesicles. 1 μ M Cpx or Cpx₁₋₈₆ were incubated along with these vesicles (0.8 mM total lipid) at 4 °C for 2 hours. The vesicles were separated from unbound complexin by centrifugation at 230,000 G for 45 min. Membrane pellets were suspended in 2/5 the volume of 1X Laemmli sample buffer containing DTT, and supernatant fractions were diluted with 1/2 volume of 3x sample buffer. 5 μ l aliquots were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on an AnyKD gel (Biorad) and were blotted to a nitrocellulose membrane which was then probed with a polyclonal antibody raised against

human complexin-1 (Abcam, product ab102761). The blot was developed using standard procedures with a horseradish peroxidase coupled anti-Rabbit IgG (Peirce, product #31460).

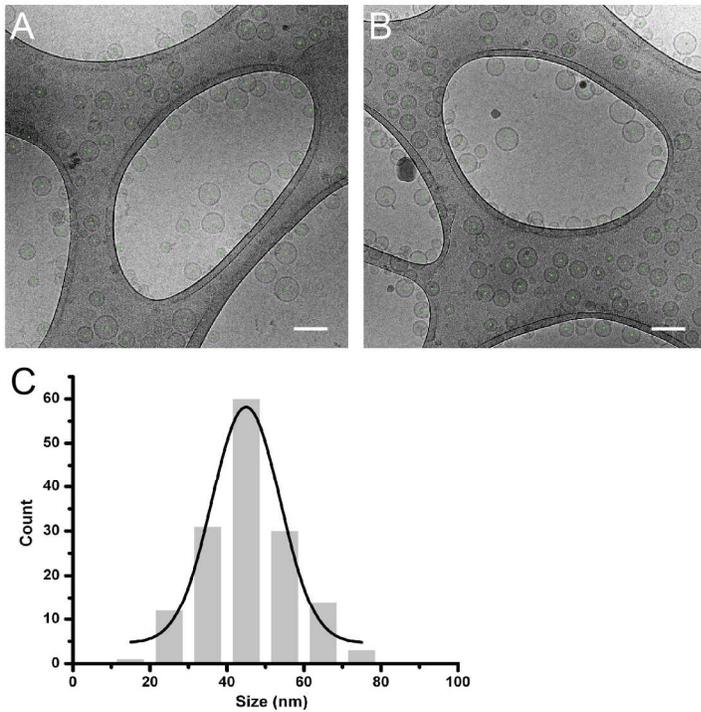


Figure S1. Vesicle diameter distribution. A and B: Cryo-EM images that were used for determining the vesicle diameter distribution. V- and t-vesicles were mixed for 30 minutes before flash freezing in liquid nitrogen using similar procedures as described in ref. ². Vesicles were picked by inspection of the cryo-EM images (indicated by a faint green dot in the center of a particular vesicle), and the diameter measured as an average over multiple center sections across the vesicle. Scale bars are 100 nm. C: A histogram showing the distribution of vesicle diameters of both t- and v-vesicles combined. The black line is a Gaussian fit to the observed diameter distribution; the mean diameter is 45 nm.

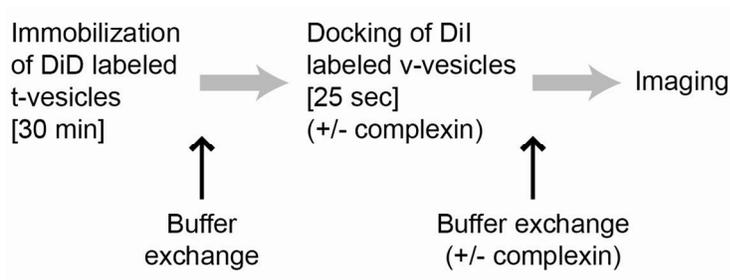


Figure S2. Experimental flow. Immobilization of DiD labeled t-vesicles, buffer exchange, incubation with DiI-labeled v-vesicles in presence of absence of complexin-1, and buffer exchange. The incubation period with DiI-labeled v-vesicles that we used in our experiments was in the range 7~25 sec. Significantly longer incubation periods resulted in too many docked vesicles that prevented optical separation of single vesicles (the theoretical optical separation limit for our setup is 237.5 nm, using the Airy disk approximation with a numerical aperture of 1.2 and a wavelength of 570 nm).

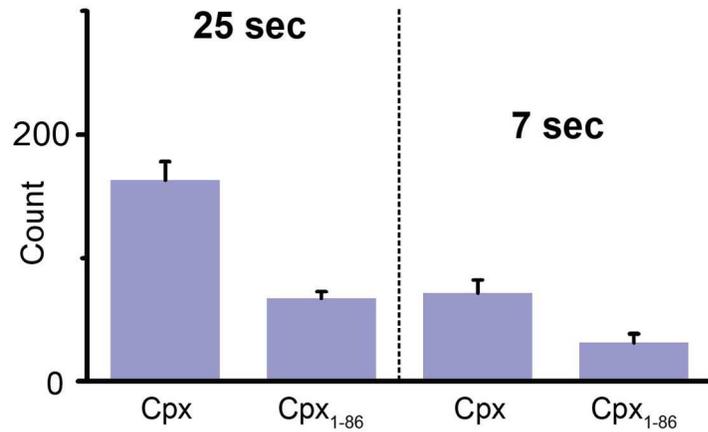


Figure S3. Dependence of the number of docked v-vesicles on the incubation time. A 100× dilution of DiI v-SNARE vesicles was used in this experiment. Because the injection method is a manual procedure, about 2~3 sec pass when switching to different buffers. Thus, there is some uncertainty in estimating the incubation time, affecting the total counts for the experiments. However, the ratio of the average counts between the two experimental conditions (SNAREs, synaptotagmin-1 & Cpx vs. SNAREs, synaptotagmin-1, & Cpx₁₋₈₆) should not be affected by this uncertainty. Indeed, the ratio of the two conditions (SNAREs, synaptotagmin-1 & Cpx vs. SNAREs, synaptotagmin-1 & Cpx₁₋₈₆) is 2.42 and 2.35 for incubation times of 25 and 7 sec, respectively. Error bars are SEM from 10 random imaging locations in the same sample channel.

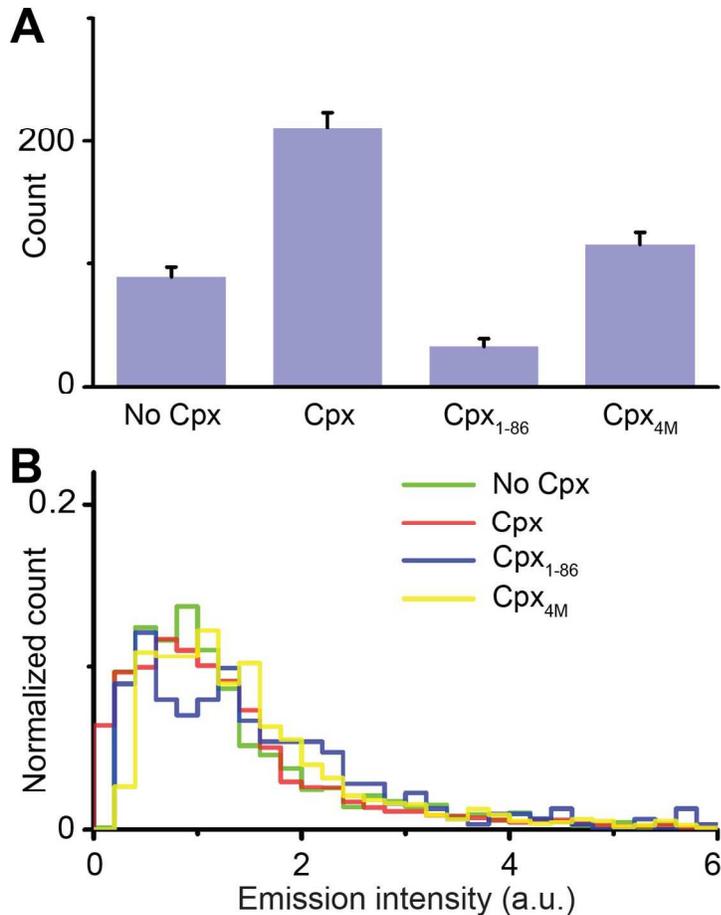


Figure S4. Docking experiments at lower v-vesicle concentration. (A) Number of docked v-vesicles for wildtype and mutants of complexin-1. The protocol described in Methods and Figure 1 was used. The v-vesicle concentration was half of that of the experiments shown in Figure 2. (A) The pattern of the docked vesicle counts is similar to that shown in Figure 2, i.e., the relative docking count is roughly independent of the v-vesicle concentration. (B) Distribution of the fluorescence intensity of all observed fluorescent spots for each of the four conditions, corresponding to the experiments shown in panel (A). The intensity distributions have maxima in the range of 0.4-1.2 (a.u.), suggesting that primarily single v-/t-vesicle pairs occur, i.e., there is a very low probability that two or more v-vesicles are docking to one t-vesicle.

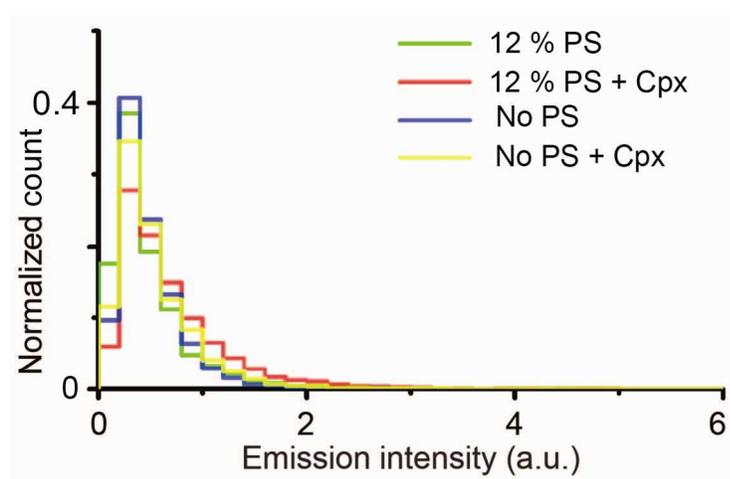


Figure S5. Fluorescence intensity distribution of fluorescent spots for the experiments shown in Figure 5. The intensity distributions have maxima in the range of 0.4-1.2 (a.u.), suggesting mostly single v-/t-vesicle pairs are observed.

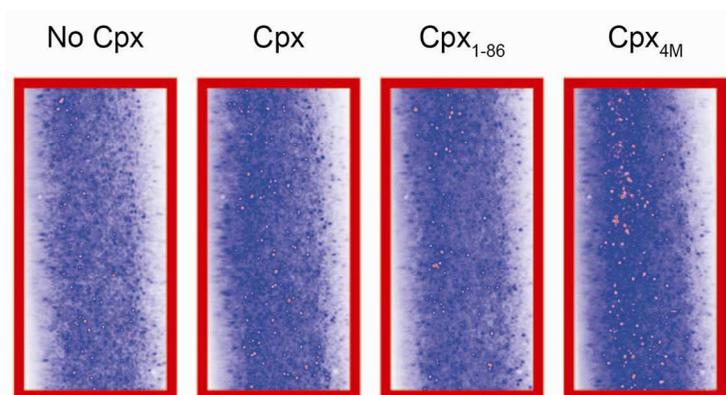


Figure S6. Fluorescent images of surface-immobilized DiD-labeled t-vesicles using red laser excitation. The images indicate that our Method generates saturated layers with more than 1000 immobilized t-vesicles, minimizing the possibility of non-specific surface interactions. Indeed, Figure 2B shows that there is only a low probability of non-specific binding.

References

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