Supporting Information

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SI Materials and Methods

Immunostaining and Western Analysis. Immunostaining was performed on third instar larvae at wandering stage after rearing at 25 °C as described previously (1). Anti-myc (Clontech), anti-Drosophila melanogaster Complexin (DmCpx; 1:5,000), and anti-HRP (1:10,000, Jackson ImmunoResearch) were used for immunostaining (1). Immunoreactive proteins were visualized on a Zeiss Pascal Confocal with PASCAL software (Carl Zeiss MicroImaging, Inc.) using fluorescent secondary antibodies (Molecular Probes). Western blotting of whole adult head lysates was performed using standard laboratory procedures with anti-myc (1:1,000, 1:500; GencTex) and anti-DmCpx (1:5,000 (1). The equivalent of one head was loaded per lane. Equal loading was assayed using anti-tubulin clone B-5-1-2 at 1:60,000 (T5168; Sigma-Aldrich). Western blots were visualized on a Li-Cor Odyssey infrared imaging system.

Protein Constructs, Expression, and Purification. SNARE motif of Drosophila Syntaxin and SNAP25. The SNARE domains of Drosophila Syntaxin and SNAP25 were cloned into a pCDFDuet-1 vector. The resulting plasmids were GST-PreScission-DmSyntaxin (containing Drosophila Syntaxin residues 194–265), GST-PreScission-dmSNAP25N (containing Drosophila SNAP25 residues 18–89), and GST-PreScission-dmSNAP25C (containing Drosophila SNAP25 residues 149–211). The details of these plasmids, and protein expression and purification were similar to that previously described (2, 3). Briefly, these plasmids were expressed in Rosetta2 (DE3; Novagen) Escherichia coli bacterial strain. Cells were pelleted, resuspended, and passed through a cell disruptor. The lysate was centrifuged, and the supernatant was incubated with glutathione agarose (Pierce, Thermo Fisher Scientific Inc). The glutathione beads and the supernatant was incubated with glutathione agarose (Superdex 75; GE Healthcare) with a buffer containing 25 mM Hepes (pH 7.4), 400 mM KCl, 10% (vol/vol) glycerol, and 1 mM DTT. Each individual protein was eluted and further purified by gel filtration chromatography (Superdex 75; GE Healthcare) with a buffer containing 25 mM Hepes (pH 7.4), 400 mM KCl, 10% (vol/vol) glycerol, and 1 mM DTT.

N-terminal domain and entire cytosolic domain of Drosophila neuronal VAMP. The N-terminal domain (residues 48–79) and entire cytosolic domain (residues 1–115) of Drosophila neuronal VAMP (n-syb) were cloned into a pET28a vector that contains N-terminal His15-SUMO tag. The details of these plasmids, and protein expression and purification were similar to that previously described (3–5). Briefly, these plasmids were expressed in Rosetta2 (DE3) E. coli bacterial strain. Cells were pelleted, resuspended, and passed through a cell disruptor. The lysate was centrifuged, and the supernatant was incubated with Nickel-NTA agarose (Thermo Fisher Scientific Inc). The Nickel-NTA beads were collected and washed. The His15-SUMO tag was cleaved by incubating the protein (attached to Nickel-NTA beads) with SUMO protease. Each individual protein was eluted and further purified by gel filtration chromatography (Superdex 75; GE Healthcare) with a buffer containing 25 mM Hepes (pH 7.4), 400 mM KCl, 10% glycerol, and 1 mM DTT.

mCpx variants. Full-length WT human Complexin 1 (mCpx) and its mutant forms were cloned into a pET15b vector containing an N-terminal His tag and a Thrombin cleavable site. These mCpx variants contained wild-type mCpx (residues 1–134), switchbreaker (SB) mCpx mutant (residues 1–134, 4R8D Y52A), nonclamp (NC) 2x mCpx mutant (residues 1–134, L41E A44E), and NC 4x mCpx mutant (residues 1–134, A30E A31E L41E A44E). The C-terminal domain mCpx was created by cloned residues 48–134 of mCpx into a pET28a vector that contains N-terminal His15-SUMO tag. The details of these plasmids, and protein expression and purification were similar to that described above and previously (3, 5). The N-terminal His6 or His6-SUMO tag was cleaved by incubating the protein (attached to Nickel-NTA beads) with thrombin (from bovine plasma; Sigma-Aldrich) or SUMO protease, respectively.

Isothermal Titration Calorimetry Analysis. To study the interactions between partially zippered SNARE and NC mCpx mutants, DmSyntaxin (residues 194–265), DmSNAP25N (residues 18–89), DmSNAP25C (residues 149–211) and the N-terminal SNARE motif from Drosophila VAMP (residues 48–79) were mixed together at a 1:1:2:1:2 molar ratio and incubated at 4 °C overnight to form the pDmSNARE79 partial complex. Before isothermal titration calorimetry (ITC) experiments, pDmSNARE79 and mCpx variants (mCpx 48–134, WT mCpx, NC 2x mCpx, and NC 4x mCpx) were purified by gel filtration using a regular or HiLoad Superdex 75 column (GE Healthcare Life Sciences) and PBS (pH 7.4; 137 mM NaCl, 3 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic) with 0.25 mM tris-(2-carboxy-ethyl)phosphine hydrochloride (TCEP) (Thermo Scientific Pierce) as the running buffer, respectively. Peak fractions were pooled and concentrated. To ensure that all of the central helix binding sites of the partial SNARE complex were occupied, excess mCpx 48–134 was added into pDmSNARE79 with a molar ratio of at least 2:1, and incubated overnight at 4 °C or 1 h at room temperature to form blocked pDmSNARE79. mCpx variants and blocked pDmSNARE79 were then dialyzed in the same flask against 4 L of PBS buffer with 0.25 mM TCEP for 4 h at 4 °C and then dialyzed against another 4 L of fresh PBS buffer with 0.25 mM TCEP overnight at 4 °C. The concentrations of dialyzed proteins were determined using the Thermo Scientific Pierce bichinonic acid protein assay kit with BSA as the standard and/or Bradford assay.

To study the interactions between postfusion, full-zippered DmSNARE complex and SB mCpx mutant (R48D, Y52A), DmSyntaxin (residues 194–265), DmSNAP25N (residues 18–89), DmSNAP25C (residues 149–211), and the complete cytosolic domain from Drosophila VAMP (residues 1–115) were mixed together at a 1:1:2:1:2 molar ratio and incubated at 4 °C overnight to form the full-zippered DmSNARE complex. Before ITC experiments, full DmSNARE complex and mCpx variants (WT mCpx and SB mCpx) were purified by gel filtration using a regular or HiLoad Superdex 75 column and PBS buffer, respectively. mCpx variants and full DmSNARE complex were then dialyzed in the same flask against 4 L of PBS buffer with 0.25 mM TCEP for 4 h at 4 °C and then dialyzed against another 4 L of fresh PBS buffer with 0.25 mM TCEP overnight at 4 °C.

ITC experiments were performed with a MicroCal ITC200 instrument similarly to that described previously (3, 6). Typically, ~200 μL of SNARE solution was loaded into the sample cell, and ~60 μL of mCpx solution was loaded into the syringe. An initial 0.1-μL injection was followed by several injections of constant volume. A 180-s equilibration time was used after each injection to ensure complete binding. The heat change from each injection was integrated and then normalized by the moles of mCpx in the injection. All ITC experiments were carried out at 23 °C to match the temperature (T) at which all of the physiological experiments with Drosophila were performed.

MicroCal Origin ITC200 software package was used to analyze the titration calorimetric data and obtain the stoichiometric
number \( (N) \), the molar binding enthalpy \( (\Delta H) \), and the association constant \( (K_a) \). A nonlinear least-squares fit assuming a simple one site chemical reaction was used. The equilibrium dissociation constant \( (K_D) \), the binding free energy \( (\Delta G) \), and the binding entropy \( (\Delta S) \) were calculated using the thermodynamic equations

\[
K_D = \frac{1}{K_a}
\]

\[
\Delta G = \Delta H - T\Delta S = -RT\ln(K_a)
\]

Cell–Cell Fusion Assays. Cell–cell fusion assays were performed as previously described (7–9). Stable cell lines expressing flipped WT VAMP2 and ΔRed2-nuclear export signal (v-cells) were transfected with GPI-anchored Cpx [WT mCpx, DmCpx, or SB mCpx (R48D, Y52A)], YFP-nuclear localization signal (NLS), and flipped GPI-anchored Synaptotagmin I where indicated. Afterward, v-cells were seeded on top of stable cell lines expressing flipped Syntaxin 1, flipped SNAP 25, and CFP-NLS (v-cells). Cells were incubated overnight and then treated with 0.5 U/mL phosphatidylinositol-specific phospholipase C (MP Biomedicals) for 30 min at 37°C to cleave the GPI anchor. Cells were fixed with 4% (vol/vol) paraformaldehyde and mounted using Prolong Gold Antifade (Molecular Probes). Images were acquired using a Zeiss 510 confocal microscope and analyzed with Zeiss LSM imaging software. The \( n \) indicates independent experiments analyzed separately, where \( >30 \) fields were imaged for each. Statistical significance was determined using one-way ANOVA with post hoc Tukey analysis (n.s., \( P > 0.05 \), *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \)).

Molecular Cloning for Drosophila Genetics. WT and mutant mCpx genes subcloned into a modified pValum construct (N-terminal myc tag), which uses of the Gal4/UAS expression system (10). The constructs were injected into \( y; \) atT third chromosome docking strains by Genetic Services Inc. Homozygous third chromosome UAS lines were then recombined into the cpx\textsuperscript{SH} null mutant (1). The C155 elav-GAL4 driver was used for neuronal expression of transgenes.

Superclamp (SC) mCpx and mCpx (L41E, A44E) mutant genes were synthesized using custom gene synthesis services (IDT). For the remaining mutants, QuickChange (Stratagene) was used for site-directed mutagenesis on the existing cloned mouse mCpx I (11). The following primers (all 5′→3′) were used to generate point mutations.


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Fig. S1. Transgenically expressed mutant mCpxs are expressed and localized similarly to WT mCpx at *Drosophila* synapses. (A) Overall protein expression of transgenically expressed myc-tagged WT or mutant mCpxs driven by the neuronal C155*elav-Gal4* driver in the indicated WT or *cpx*SH1 backgrounds was analyzed by Western blot analysis using adult transgenic head lysates. Blots were probed using anti-myc, anti-DmCpx, and anti-tubulin. Expression of individual mutant mCpxs was similar to WT mCpx expression with the exception of reduced expression of mCpx 51–134. Similar results were obtained in at least two independent experiments. (B) Third instar wandering larvae of the indicated genotypes were dissected, fixed, and costained with anti-HRP and anti-myc antisera. Representative images demonstrate that transgenically expressed myc-tagged WT and mutant mCpxs, with the exception of mCpx 1-50, are localized and enriched similarly at *Drosophila* muscle 6/7 neuromuscular junctions. (Scale bar: 20 μm.)
**Fig. S2.** Interaction of mCpx central helix with prefusion, partially assembled pDmSNARE79. ~330 μM mCpx 48-134 was titrated into ~27 μM pDmSNARE79. (Upper) Raw data in power vs. time during the injection after subtracting the baseline. (Lower) Integrated heat of each injection normalized by the moles of injectant vs. the molar ratio between mCpx and SNARE in the sample cell. The solid lines represented the best fit to the black squares obtained from a nonlinear least-squares fit assuming a simple one-site chemical reaction. The result gave the thermodynamic parameters for the binding reaction: affinity constant $K_D = 667 \pm 90$ nM, $\Delta H = -13.3 \pm 0.2$ kcal·mol$^{-1}$, and $\Delta S = -16.6 \pm 0.8$ kcal·mol$^{-1}$·°C$^{-1}$.

**Fig. S3.** Interaction of mCpx accessory helix with prefusion, partially assembled pDmSNARE79. (A) ~310 μM WT mCpx was titrated into ~15.5 μM blocked pDmSNARE79, (B) ~360 μM double-mutant mCpx L41E A44E was titrated into ~25 μM blocked pDmSNARE79, and (C) ~603 μM quadruple-mutant mCpx A30E A31E L41E A44E was titrated into ~30 μM blocked pDmSNARE79. (Upper) In each reaction, raw data in power vs. time during the injection after subtracting the baseline. (Lower) Integrated heat of each injection normalized by the moles of injectant vs. the molar ratio between mCpx and SNARE in the sample cell. The solid lines represented the best fit to the black squares obtained from a nonlinear least-squares fit assuming a simple one-site chemical reaction. The results gave the thermodynamic parameters for each binding reaction, which are listed in Table S1.
Table S1. Summary of mCpx mutants expressed in cpx null mutants

<table>
<thead>
<tr>
<th>mCpx mutant (N-terminal myc tag)</th>
<th>Mutations</th>
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<tbody>
<tr>
<td>Superclamp (SC)</td>
<td>D27L, E34F, R37A</td>
</tr>
<tr>
<td>Nonclamp (NC)</td>
<td>A30E, A31E, L41E, A44E</td>
</tr>
<tr>
<td>Helixbreaker (HB)</td>
<td>GGG insertion between residues A50 and K51</td>
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<tr>
<td>mCpx 51–134</td>
<td>Deletion of N terminus 1–50 up to the central helix</td>
</tr>
<tr>
<td>Switchbreaker (SB)</td>
<td>R48D/E, Y52A</td>
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Table S2. Thermodynamic parameters of various mCpx accessory helix (WT mCpx, mCpx L41E A44E, and mCpx A30E A31E L41E A44E) binding to blocked pDmSNARE79

<table>
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<tr>
<th>Interaction</th>
<th>Stoichiometric coefficient, N</th>
<th>$K_D$, μM</th>
<th>$\Delta H$, kcal·mol$^{-1}$</th>
<th>$\Delta S$, cal·mol$^{-1}$·°C$^{-1}$</th>
<th>$\Delta G$, kcal·mol$^{-1}$</th>
<th>$\Delta G$, $k_B T$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT mCpx/prefusion dmSNARE79</td>
<td>1.00 ± 0.05</td>
<td>11 ± 1</td>
<td>−5.7 ± 0.4</td>
<td>3.6 ± 1.5</td>
<td>−6.7 ± 0.1</td>
<td>11.5 ± 0.1</td>
</tr>
<tr>
<td>Double mutant/prefusion dmSNARE79</td>
<td>1.04 ± 0.15</td>
<td>45 ± 8</td>
<td>−3.9 ± 0.7</td>
<td>6.9 ± 2.8</td>
<td>−5.9 ± 0.1</td>
<td>10.0 ± 0.1</td>
</tr>
<tr>
<td>Quadruple mutant/prefusion dmSNARE79</td>
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<td>Not detected</td>
<td>Not detected</td>
<td>Not detected</td>
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