Supplemental Experimental Procedures

Immunostaining

Immunostaining was performed on wandering 3rd instar larvae reared at 25°C, unless otherwise indicated, as previously described (Huntwork and Littleton, 2007). Anti-myc (1:500, Clontech, Mountain View, CA), anti-DmCpx (1:5000, (Huntwork and Littleton, 2007)), anti-GluRIII (1:1000, (Marrus et al., 2004)), anti-BRP (1:250, obtained from Developmental Studies Hybridoma Bank, The Univ. of Iowa, Iowa, USA), and anti-HRP (1:10000, Jackson ImmunoResearch, West Grove, PA) were used for immunostaining. Samples were mounted in 70% glycerol and imaged with a 40x NA 1.3 Pan Neofluar oil immersion lens (Carl Zeiss, MicroImaging, Inc.). Immunoreactive proteins were visualized on a Zeiss Pascal Confocal with PASCAL software (Carl Zeiss MicroImaging, Inc.) using fluorescent secondary antibodies (Molecular Probes, Carlsbad, CA). Bouton number (as determined by morphology and enrichment of postsynaptic GluRIII staining) was quantified from confocal stacks taken of the entire NMJ at muscle 6/7 of segments A3 and A4. Bouton number was normalized to muscle area where indicated (bouton #/muscle 6/7 area μm^2 x $1x10^5$) (Lnenicka and Keshishian, 2000). To measure activity-dependent structural plasticity, experiments were performed as previously described (Sigrist et al., 2003; Zhong and Wu, 2004). Briefly, duplicate sets of 25 male and 25 female flies of indicated genotypes were allowed to lay eggs at 25°C for ~10 hours. Parent flies were then cleared, and laid eggs were reared at 25°C or 29°C in humidified chambers. Wandering 3rd instar larvae are subsequently collected, dissected, and assayed for synaptic bouton number as described.

Recombinant protein purification

WT Cpx, Cpx^{S126A}, Cpx^{S126D}, $Cpx^{K74A, Y75A}$, and $Cpx^{CTD\ (1-87)}$ were subcloned into pGEX-5X-1 GST-recombinant expression vector. Bl-21 host bacteria were transformed with GST constructs and grown in LB media containing ampicillin (100 μg/ml) to an optical density of 0.5. Expression of protein was induced by adding IPTG (0.5 mM final concentration). After 3 hours of growth at 37°C, bacteria were collected and resuspended in buffer (10 mM Tris-HCl, 100 mM KCl, 0.1 mM EDTA, and 0.05% Triton X100 for the SNARE pull-down experiments and 50 mM Tris-HCl, 300 mM NaCl, 5mM EDTA for the liposomal assays). Proteins were purified using glutathione sepharose beads (GE Healthcare) and bound proteins were washed using respective reconstitution buffers. For the liposomal flotation assay, proteins were eluted using 15 mM reduced glutathione (Sigma-Aldrich), and purified proteins underwent buffer exchange (150 mM NaCl, 50 mM Tris-HCl, 1 mM CaCl, 1mM DTT) using centrifugal filtration (Amicon).

SNARE pull-downs

Drosophila head lysates were prepared from whole adult heads in TS lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton, with Complete mini protease inhibitors used as directed (Sigma)). Adult heads were collected from ~6 ml of Canton S adult flies. Heads were homogenized and then underwent 3 rounds of 10x sonication. After centrifugation

at 8000 rpm for 15 minutes, supernatant was collected. Purified recombinant protein on glutathione sepharose beads (see above) were washed with TS buffer. After "preclearing" the head lysates with GST-only beads, head lysates were added to purified protein beads and incubated for 1 hour at 4^{o} with continuous rotation. After incubation, beads were washed 5x with TS buffer. GST and pull-down proteins were eluted using 100 μL of protein loading buffer. Half of the eluted proteins were subject to heat shock (2 min at 100^{o}C) in order to dissociate SDS-resistant SNARE complexes that may have been pulled-down by recombinant proteins. Heat shocked and unshocked samples were analyzed by Western blot. Pull-down of SNARE complexes was detected by anti-syntaxin antibody (8C3, DHSB, Iowa) and anti-GST 800 (600-132-200, Rockland).

Liposomal Flotation Assay

Liposomal flotation assays were preformed as previously described (Wragg et al., 2013). Briefly, lipids mixtures composed of 70% 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC) and 30% 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS) (Avanti Polar Lipids) were dried under a stream of N_2 gas with subsequent removal of residual solvent under a vacuum for 1 hour. Lipid was rehydrated in buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM $CaCl_2$, 1 mM DTT) to a final lipid concentration of 4 mM. Liposomes were generated by treating with 10 cycles of freeze thawing. ~100 nm liposomes were generated by extruding lipid mixture ~20 times through 400 nm then 200 nm pore-sized polycarbonate films.

Purified protein and liposomes were incubated at room temperature for 1 hr. Approximately 1 μg of each protein sample was used and incubated with liposomes at a protein:liposome molar ratio of approximately 4:1. A sucrose gradient (10 mM HEPES, 150 mM NaCl, 1mM $CaCl_2$) containing 500 μL of sample mixed with 500 μL of 60% sucrose was overlaid sequentially with 1 ml 25% sucrose, 1 ml 20% sucrose, and then 500 μL of buffer. Samples were subject to ultracentrifugation in an MLS-50 rotor (Beckman) at 35, 000 rpm. Fractions at the interface between the top layer and 20% sucrose layer were run on 12% SDS-PAGE gel followed by Western blot analysis. Proteins were detected using anti-GST antibody (600-132-200, Rockland).

Protein constructs, expression and purification

SNARE motif of Drosophila Syntaxin and SNAP25. The SNARE domains of *Drosophila* (*Dm*) Syntaxin and SNAP25 were cloned into a pCDFDuet-1 vector, respectively. The resulting plasmids were GST-PreScission-dmSyntaxin (containing Syntaxin residues 194 to 265), GST-PreScission-SNAP25N (containing SNAP25 residues 18 to 89), and GST-PreScission-*Dm*SNAP2n5C (containing *Dm* SNAP25 residues 149 to 211). The details of these plasmids, and protein expression and purification were similar to that was previously described (Cho et al., 2014). 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 were collected and washed. The GST tag was cleaved by incubating the protein (attached to glutathione beads) with PreScission 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.

Cytosolic domain of Dm neuronal VAMP. Cytosolic domain (residues 1 to 115) of *Dm* neuronal VAMP (n-syb) was cloned in to a pET28a vector that contains N-terminal His_6-SUMO tag. The details of the plasmid, and protein expression and purification were similar to that was previously described (Li et al., 2014). The plasmid was expressed in Rosetta2 (DE3) *Escherichia 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 His_6-SUMO tag was cleaved by incubating the protein (attached to Nickel-NTA beads) with SUMO protease. The 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.

Cpx variants. Full length wildtype Cpx and its mutant Cpx^{S126D} were cloned into a pGEX expression vector containing an N-terminal GST tag and a Thrombin cleavable site. The details of these plasmids, and protein expression and purification were similar to that was described previously(Cho et al., 2014; Li et al., 2014). The N-terminal GST tag was cleaved by incubating the protein (attached to glutathione beads) with Thrombin (from bovine plasma, Sigma-Aldrich).

Isothermal Titration Calorimetry (ITC) measurements

To study the interactions between postfusion, full-zippered *Dm*SNARE complex and *Dm*Cpx variants, DmSyntaxin (residues 194 to 265), *Dm*SNAP25N (residues 18 to 89), DmSNAP25C (residues 149 to 211) and the complete cytosolic domain from *Drosophila* VAMP (residues 1 to 115) were mixed together at a 1:1.2:1.2:1.2 molar ratio and incubated at 4^{o}C overnight to form the full-zippered *Dm*SNARE complex. Before ITC experiments, full *Dm*SNARE complex and Cpx variants (WT Cpx and Cpx^{S126D}) were purified by gel filtration using a regular or HiLoad Superdex 75 column (GE Healthcare Life Sciences) and PBS (phosphate buffered saline, pH 7.4: 137 mM NaCl, 3 mM KCl, 10 mM sodium phosphate dibasic, 2 mM potassium phosphate monobasic) with 0.25 mM TCEP as the running buffer, respectively. Peak fractions were pooled and concentrated. Cpx variants and full *Dm*SNARE complex were then dialyzed in the same flask against 4 liters of PBS buffer with 0.25 mM TCEP for 4 hours at 4^{o}C and then dialyzed against another 4 liters of fresh PBS buffer with 0.25 mM TCEP overnight at 4^{o}C. The concentrations of dialyzed proteins were determined by using the Thermo Scientific Pierce Bicinchoninic Acid (BCA) protein assay kit with BSA as the standard.

ITC experiments were performed with a Microcal ITC200 instrument similarly to that was described before (Li et al., 2014). Typically, about 200 μL of Cpx solution was loaded into the sample cell and about 60 μL of SNARE solution was loaded into the syringe. 180-second 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 SNARE in the injection. All ITC experiments were carried out at 23°C to match

the temperature (T) at which all 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 (ΔH), 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 (ΔG), and the binding entropy (ΔS) were calculated using the thermodynamic equations:

$$K_D = {}^{1}/_{K_a}$$

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

Supplemental Figure Legends

Figure S1 (refers to Discussion): WT and $Cpx^{S126A/D}$ transgene rescues localize normally at *Drosophila* NMJs. UAS-Cpx transgenes were expressed using the pan-neuronal driver *elav*-C155 in the *cpx* null background. (A) Cpx transgenes are localized and enriched at NMJ boutons similarly to endogenous Cpx (control Cpx^{PE}). Cpx protein was detected using anti-Cpx antibody (green) and costained with anti-HRP (magenta) to mark motor neurons at muscle 6/7 NMJs. Scale bar = 20 μm (B) Transgene expressed $Cpx^{S126A/D}$ mutant proteins localize similarly to transgene expressed WT Cpx and endogenous Cpx proteins at muscle 4 NMJs. Cpx was detected using anti-Cpx (green) and costained using anti-BRP (magenta), a marker for presynaptic active zones. Scale bars = 10 μm in the main panels and 2 μm in the inset. (C) Cpx rescues express at similar levels to endogenous Cpx. Western blots were performed using transgenic and control (Cpx^{PE}) adult fly head extracts. Cpx protein was detected using anti-Cpx (top panel), and anti-tubulin (bottom panel) was used as a loading control.

Figure S2 (Refers to Discussion): Phosphomimetic Cpx^{S126D} does not alter its association with SNARE complexes compared to WT Cpx. Recombinant GST-tagged WT Cpx, $Cpx^{K74A,\ Y75A}$, and Cpx^{s126D} were purified and incubated with adult fly brain extracts. Pulldown of SNARE complexes was assayed using anti-syntaxin antibody with and without boiling to distinguish the SDS-resistant SNARE complexes and syntaxin monomers. The central helix SNARE-binding mutant $Cpx^{K74A,\ Y75A}$ that abolishes SNARE association was used as a control. GST alone was also included as an additional control. Secondary antibody also detected GST tagged proteins, demonstrating similar GST protein loading across all pull down conditions.

Figure S3 (Refers to Discussion): WT Cpx and phosphomimetic Cpx^{S126D} exhibit similar binding properties with SNARE complexes. (A) ~91 μM *Drosophila (Dm)* SNARE complex was titrated into ~5.6 μM WT Cpx. (B) ~75 μM *Dm* SNARE complex was titrated into ~5.4 μM Cpx^{S126D}. (*Upper panels*) Raw data in power vs. time during the injection is presented. (*Lower panels*) Integrated heat of each injection normalized by the moles of injectant vs. the molar ratio between SNARE and WT Cpx or Cpx^{S126D}, as indicated, 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. Thermodynamic parameters for each binding reaction are listed in Table S3.

Figure S4 (Refers to Discussion): WT Cpx and phosphomimetic Cpx^{S126D} recombinant proteins similarly associate with phospholipids. Samples were analyzed by Western blot using anti-GST antibody to detect recombinant proteins. (*left blot*) Input samples are shown demonstrating equal loading for GST alone, WT Cpx, Cpx^{S126D}, and CTD Cpx. (*right blot)* Liposome flotation assay was performed by sucrose gradient (30, 25, 20, 0%) using GST alone, WT Cpx, Cpx^{S126D}, and C-terminal deletion (CTD) Cpx consisting of amino acids 1-87 lacking the proposed C-terminal amphipathic region. Top three fractions between the top buffer 0 % buffer and the 20% sucrose fraction were collected and analyzed by Western blot. Differences in WT Cpx and Cpx^{S126D} association with liposomes were not detected. CTD Cpx associated with liposomes, but slightly to a lower degree, suggesting additional domains in *Drosophila* Cpx that mediate protein/lipid interactions.

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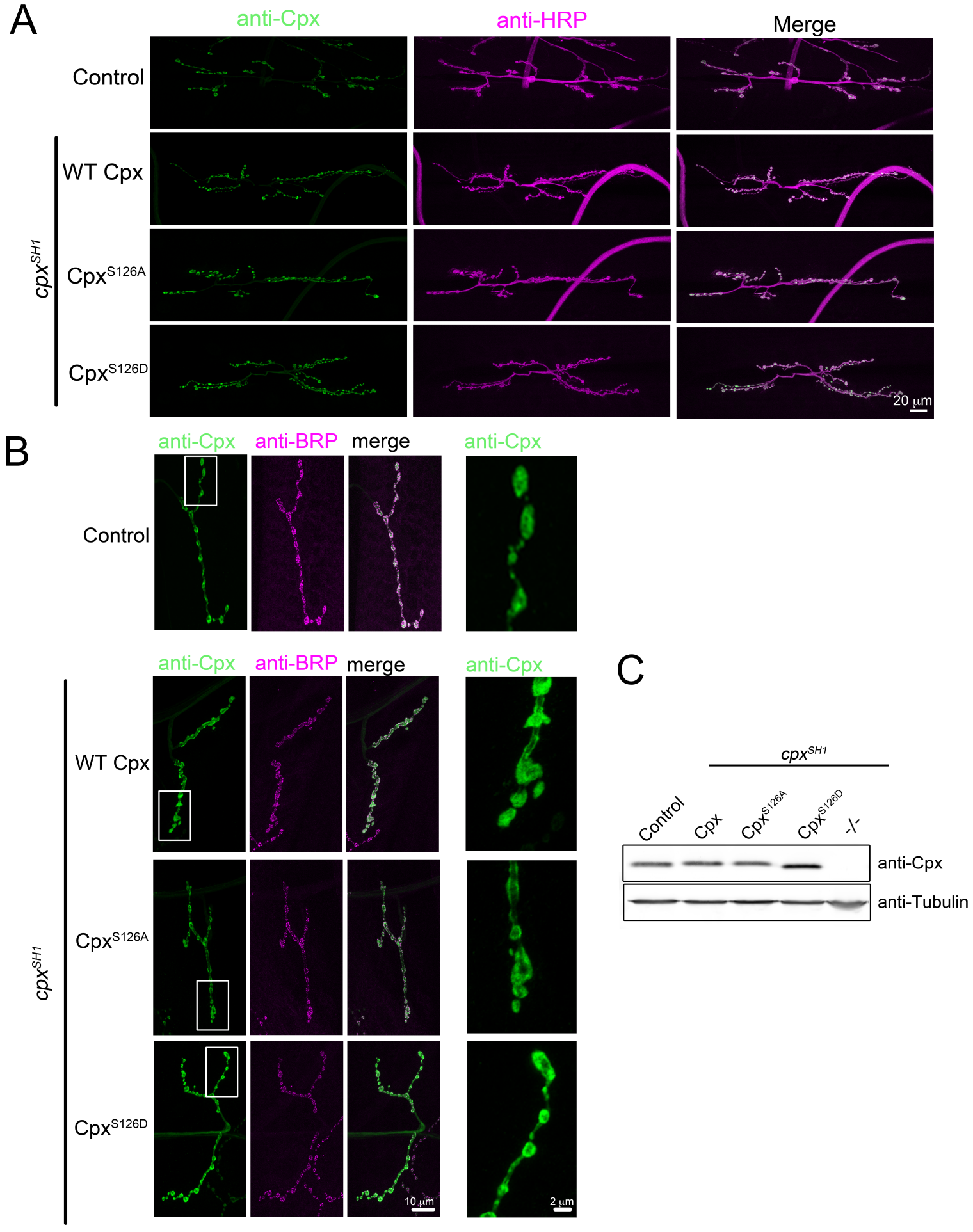
Figure S1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merge
anti-Cpx
Control
WT Cpx
CpxS126A
CpxS126D
cpxSH1
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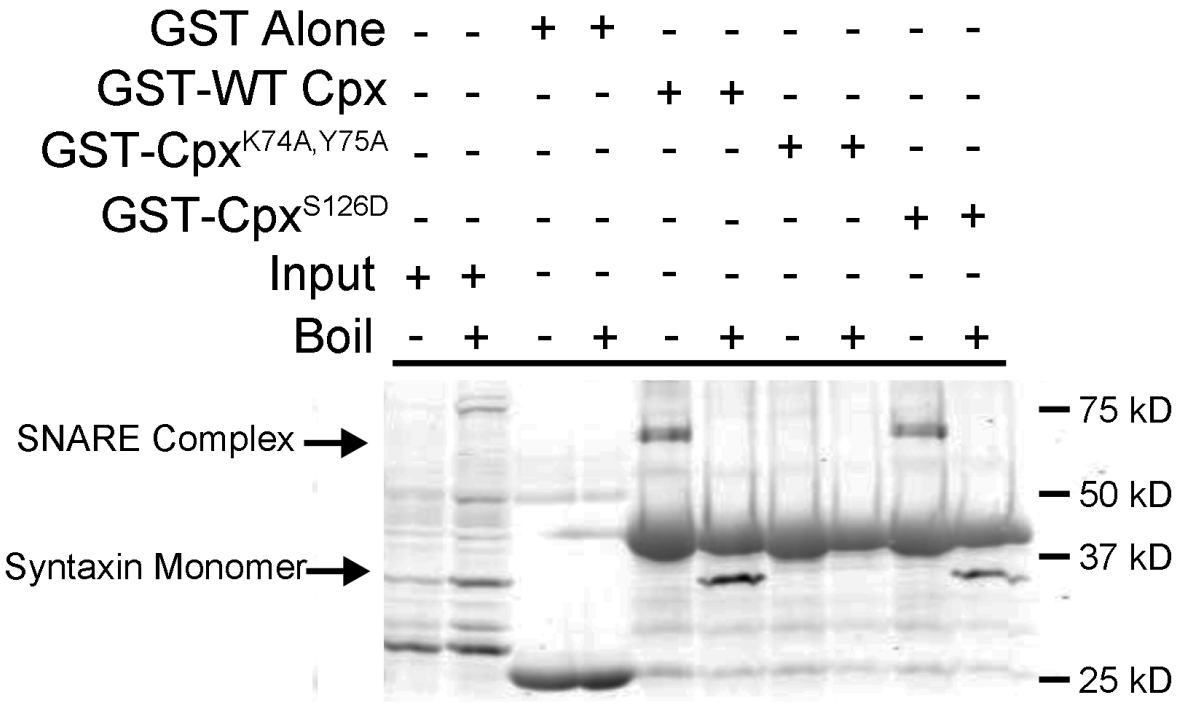
-/-
anti-Cpx
anti-Tubulin

Figure S2


GST Alone - - + + - - - - - -
GST-WT Cpx - - - - + + - - - -
GST-Cpx^{K74A,Y75A} - - - - - - + + - -
GST-Cpx^{S126D} - - - - - - - - + +
Input + + - - - - - - - -
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SNARE Complex
Syntaxin Monomer
75 kD
50 kD
37 kD
25 kD

Figures S3

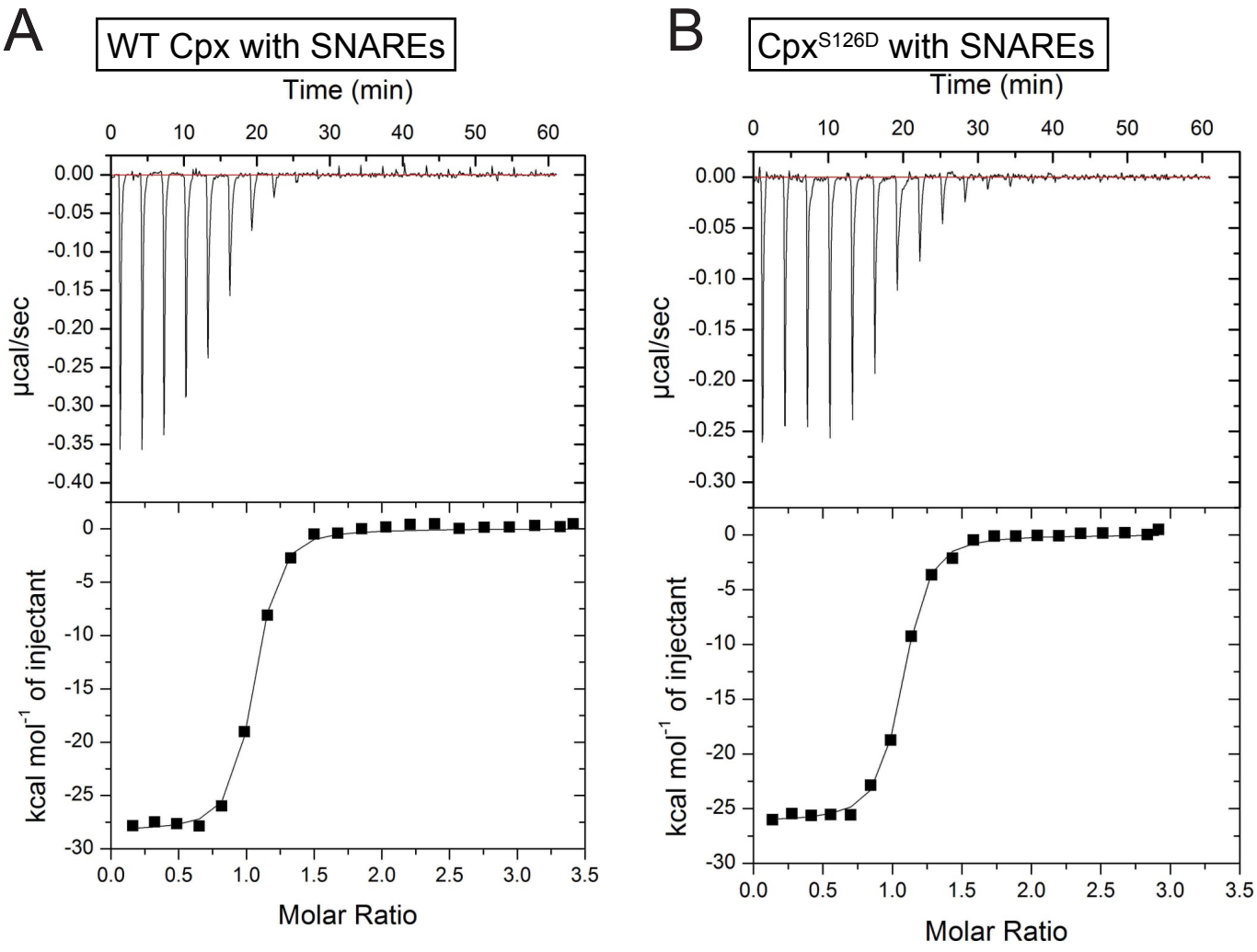
A
WT Cpx with SNAREs
Time (min)
µcal/sec
kcal mol-1 of injectant
Molar Ratio
B
CpxS126D with SNAREs
Time (min)
µcal/sec
kcal mol-1 of injectant
Molar Ratio

Figure S4

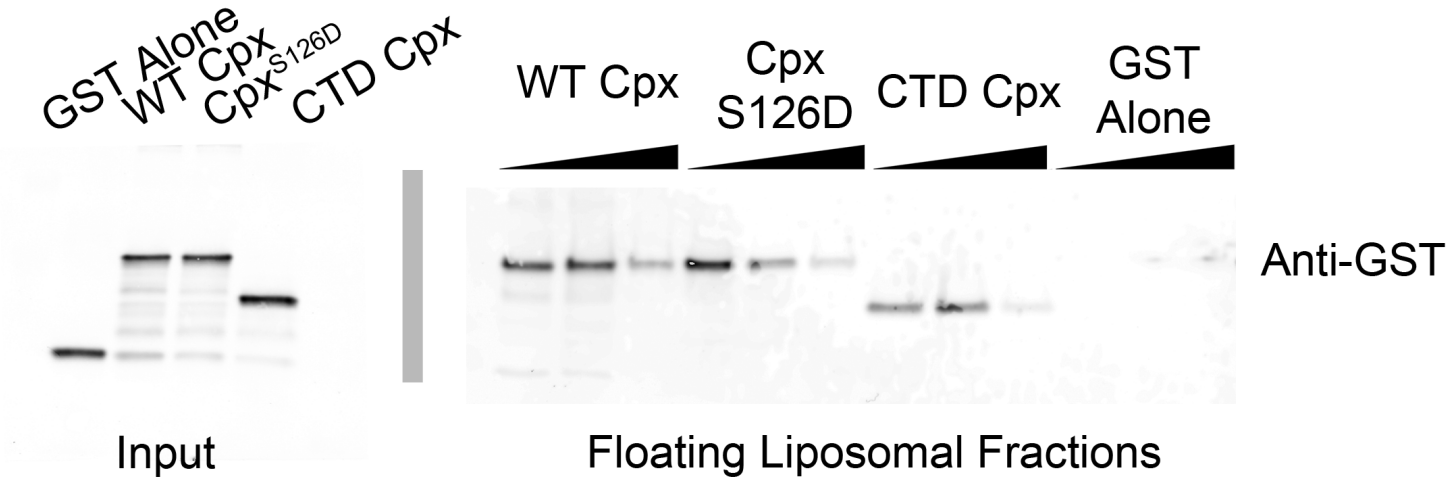
GST Alone
WT Cpx
Cpx S126D
CTD Cpx
WT Cpx
Cpx S126D
CTD Cpx
GST Alone
Anti-GST
Input
Floating Liposomal Fractions

Table S1: Summary of Synaptic Growth Data at *Drosophila* Muscle 6/7 NMJs (A) Refers to Figures 1D and 1E, (B) Refers to Figures 2B and 2C, (C) Refers to Figures 2D and 2E (D) Refers to Figures 2F and 2G (E) Refers to 5F and 5G, (F) Refers to 7A and 7B (G) Refers to 7C and 7D.

Table S1 (A)

Genotype	Temp (C)	Bouton Number	n
$w^{1118}; \frac{\text{c164-GAL4}}{+}$	25°	89.2 ± 3.8	18
$w^{1118}; \frac{\text{c164-GAL4}}{+}; \frac{\text{Cpx RNAi}}{+}$	25°	120.8 ± 5.5	13

Table S1 (B)

Genotype	Temp (C)	Bouton Number	n
w^{1118}	25°	87.8 ± 3.9	16
$w^{1118};; syx^{3\text{-}69}$	25°	124.8 ± 4.4	10
$w^{1118};; syt\ 4^{BA1}$	25°	83.3 ± 4.1	7
$w^{1118};; syx^{3\text{-}69}, syt\ 4^{BA1}$	25°	80.2 ± 2.3	6

Table S1 (C)

Genotype	Temp (C)	Bouton Number	n
$w^{1118};; Cpx^{PE}$	25°	77.8 ± 4.7	9
$w^{1118};; cpx^{SH1}$	25°	106.3 ± 4.2	16
$w^{1118};; Syt\ 4^{PE}$	25°	83.6 ± 3.2	10
$w^{1118};; syt\ 4^{BA1}$	25°	77.5 ± 4.0	13
$w^{1118};; syt\ 4^{BA1}, cpx^{SH1}$	25°	92.6 ± 2.6	19

Table S1 (D)

Genotype	Temp (C)	Bouton Number	n
$w^{1118};; Cpx^{PE}$	25°	71.1 ± 2.8	26
$w^{1118};; cpx^{SH1}$	25°	119.2 ± 5.0	18
$w^{1118};; \frac{wit^{A12}}{wit^{B11}}$	25°	45.1 ± 2.2	15
$w^{1118};; \frac{cpx^{SH1},\ wit^{A12}}{cpx^{SH1},\ wit^{B11}}$	25°	42.4 ± 2.2	12
$w^{1118};; \frac{cpx^{SH1},\ wit^{A12}}{cpx^{SH1}}$	25°	79.4 ± 3.1	18

Table S1 (E)

Genotype	Temp (C)	Bouton Number	Muscle Area (μm^2)	Normalized Bouton Number (x $1x10^5$)	n
w^{1118};; Cpx^{PE}	25^o	78.8 ± 2.1	121261 ± 3159	65.6 ± 1.8	28
C155;; WT Cpx, cpx^{SH1}	25^o	88.8 ± 3.3	100498 ± 2287	88.7 ± 3.0	24
C155;; Cpx^{S126A}, cpx^{SH1}	25^o	87.4 ± 2.5	106219 ± 1762	83.1 ± 2.9	27
C155;; Cpx^{S126D}, cpx^{SH1}	25^o	122.3 ± 4.4	115661 ± 2467	105.8 ± 3.2	25
w^{1118};; cpx^{SH1}	25^o	118.2 ± 3.0	99957 ± 4918	115.8 ± 4.3	8

Table S1 (F)

Genotype	Temp (C)	Bouton Number	Muscle Area (μm^2)	Normalized Bouton Number (x $1x10^5$)	n
C155	25^o	73.1 ± 1.8	114449 ± 1913	64.3 ± 1.8	30
C155; $\frac{\text{CA-PKA}}{+}$	25^o	105.2 ± 3.9	94512 ± 2171	111.6 ± 3.6	33
C155; $\frac{\text{CA-PKA}}{+}$; $\frac{\text{WT Cpx, } cpx^{SH1}}{cpx^{SH1}}$	25^o	102.6 ± 4.5	91252 ± 2585	115.6 ± 5.8	19
C155; $\frac{\text{CA-PKA}}{+}$; $\frac{Cpx^{S126A}, cpx^{SH1}}{cpx^{SH1}}$	25^o	85.3 ± 2.9	100892 ± 2964	85.4 ± 3.1	24
C155; $\frac{\text{CA-PKA}}{+}$; $\frac{Cpx^{S126D}, cpx^{SH1}}{cpx^{SH1}}$	25^o	126.5 ± 4.2	118417 ± 2604	106.3 ± 3.5	18
C155; $\frac{\text{CA-PKA}}{+}$; cpx^{SH1}	25^o	130.8 ± 5.2	106711 ± 1696	122.8 ± 5.0	12

Table S1 (G)

Genotype	Temp (C)	Bouton Number	Muscle Area (μm^2)	Normalized Bouton Number (x $1x10^5$)	n
w^{1118};; Cpx^{PE}	25^o	81.4 ± 1.9	101814 ± 4527	83.5 ± 3.8	32
w^{1118};; Cpx^{PE}	29^o	111.2 ± 4.1	109014	106.3 ± 5.2	36

			5156		
C155;; WT Cpx, *cpx*^{SH1}	25^{o}	86.4 ± 2.6	88726 ± 2594	99.7 ± 3.8	36
C155;; WT Cpx, *cpx*^{SH1}	29^{o}	111.8 ± 3.1	91882 ± 3090	128.8 ± 5.1	28
C155;; Cpx^{S126A}, *cpx*^{SH1}	25^{o}	85.3 ± 2.4	92680 ± 3016	93.8 ± 2.8	35
C155;; Cpx^{S126A}, *cpx*^{SH1}	29^{o}	80.8 ± 3.0	94201 ± 3500	88.4 ± ± 3.7	36
C155;; Cpx^{S126D}, *cpx*^{SH1}	25^{o}	124.6 ± 5.4	110589 ± 1648	112.6 ± 4.4	20
C155;; Cpx^{S126D}, *cpx*^{SH1}	29^{o}	126.4 ± 5.1	111902 ± 2484	113.6 ± 5.4	17

Table S2: Summary of Mini Frequencies Recorded from Indicated Genotypes at Muscle 6 (Refers to Figures 2A-G)

Genotype (n)	Mini Frequency (Hz)
w^{1118};; Cpx^{PE} (6)	2.6 ± 0.7
w^{1118};; $Syt\ 4^{PE}$ (8)	2.7 ± 0.4
w^{1118};; $syx^{3\text{-}69}$ (8)	14.2 ± 1.3
w^{1118};; $syt\ 4^{BA1}$ (9)	3.4 ± 0.4
w^{1118};; $syx^{3\text{-}69}$, $syt\ 4^{BA1}$ (7)	14.9 ± 1.1
w^{1118};; $syt\ 4^{BA1}$, cpx^{SH1} (4)	76.9 ± 3.0
w^{1118};; $\frac{cpx^{SH1},\ wit^{A12}}{cpx^{SH1},\ wit^{B11}}$ (5)	71.2 ± 2.2
w^{1118};; $\frac{cpx^{SH1},\ wit^{A12}}{cpx^{SH1}}$ (3)	69.7 ± 2.3

Table S3: Thermodynamic Properties of *Drosophila (dm)* SNARE Complex Binding with dm WT Cpx and Phosphomimetic Cpx^{S126D} (Refers to Discussion and Figure S3)

Interaction	Stoichiometric coefficient, N	K_D (nM)	ΔH (kcal·mol^{-1})	ΔS (cal·mol$^{-1}\cdot{}^{o}C^{-1}$)	ΔG (kcal·mol^{-1})	ΔG (k_BT)
WT dmCpx / postfusion dmSNARE	0.99 ± 0.01	39 ± 5	-28.3 ± 0.2	-61.6 ± 1.1	-10.0 ± 0.1	17.1 ± 0.1
dmCpx S126D/ postfusion dmSNARE	1.01 ± 0.01	41 ± 5	-26.2 ± 0.2	-54.7 ± 0.9	-10.0 ± 0.1	17.0 ± 0.1