Supplementary Information for

Complexin-1 regulated assembly of single neuronal SNARE complex

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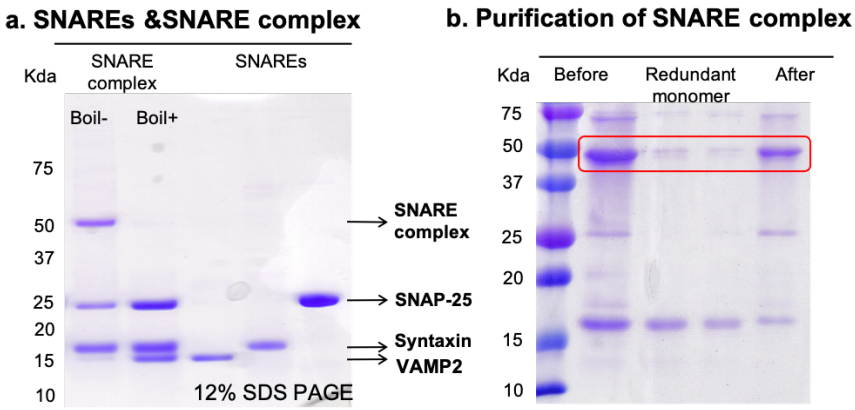
1. Purification and pre-assemble of proteins

The synaptic SNARE complex consists of VAMP2 (1-92, C2A, Q36C), syntaxin 1 (172-265, C173A, L209C), and SNAP25 (1-206). The proteins were then expressed in E. coli BL21(DE3) cells and purified as described in the manual of ChampionTM pET SUMO Expression System (Invitrogen).

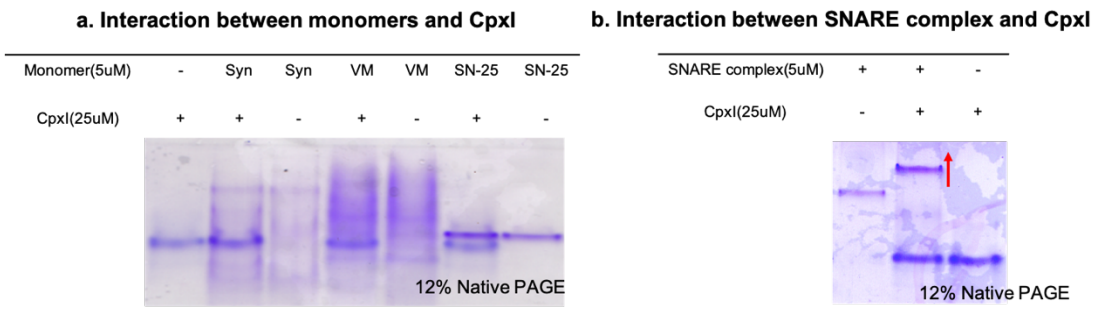
Ternary SNARE complexes were formed by mixing syntaxin, SNAP25 and VAMP2 proteins with 3:4:5 molar ratios in 25 mM HEPES, 150 mM NaCl, 2mM TCEP, pH 7.7 and the mixture was incubated at 4 °C for 30mins. Formation of the ternary complex was confirmed by SDS polyacrylamide gel electrophoresis. Excessive SNARE monomers or binary complexes were removed from the ternary complex by further purification through Ni-NTA resin using the His-Tag on the SNAP25 molecule.



Supplementary Figure 1. Purification and pre-assemble of proteins. **a** Purification and pre-assemble of SNARE Complex, formation of the ternary complex was confirmed by SDS polyacrylamide gel electrophoresis. **b** Purification of SNARE complex by Ni-NTA, SNARE complex was marked by red box.

2. Interaction test between SNAREs and CpxI

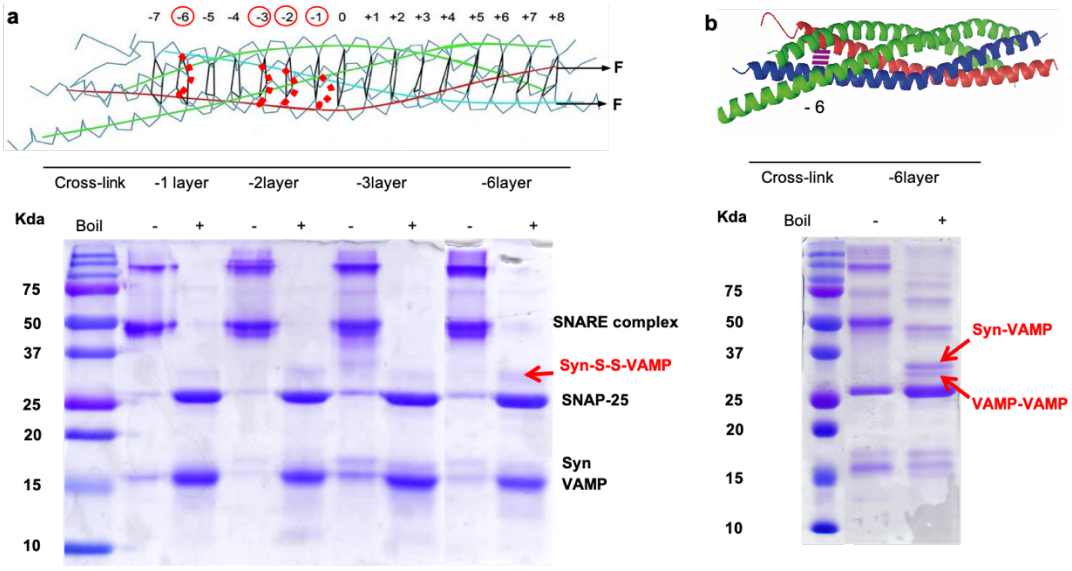
The Binding assay between SNARE monomers and complexin is completed [FigS2a]. For we did not see significant difference on the gel, complexin may do not binding to any SNARE monomers. The binding assay about complexin and SNARE complex on native-page is completed [FigS2b]. The mole ratio of complexin: SNARE is 5:1. We can see that the complex-SNARE complex shows a new band on the native page. CpxI could bind with SNARE complex.



Supplementary Figure 2. Interaction test between SNAREs and CpxI. **a** Binding interaction between SNARE monomers and complexin-1. **b** Binding interaction between SNARE and CpxI on native page. CpxI could bind with SNARE complex.

3. Middle cross-linked constructs of SNARE complex

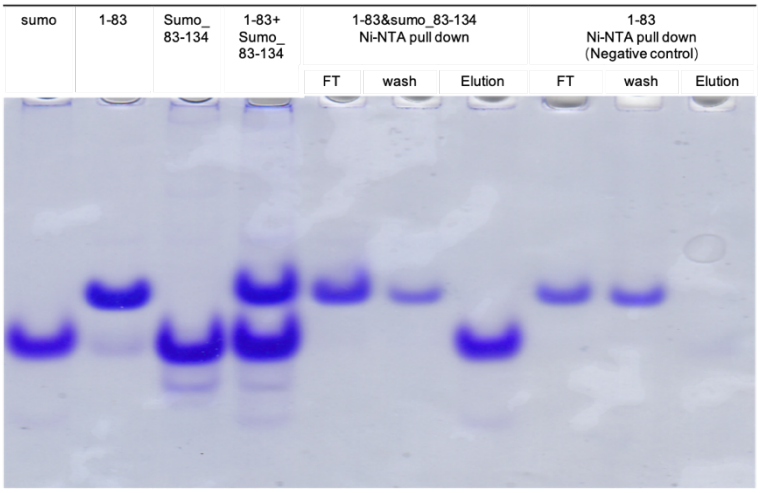
The half-zipped state of the N-terminal cross-linked SNARE complex is too short, so we need built a new SNARE pulling system with sable and long half-zipper state. To get four different middle-cross-linked SNARE complex, we design and purify four different mutants of syntaxin, and four different mutants of VAMP. The point mutations of syntaxin and vamp were chosen by pre-design on the base of X-ray structure of SNARE complex. The cysteine of wild-type syntaxin and vamp is mutant into alanine, and new cysteine is mutant at new sites. And we incubate the SNARE complex on 34 ℃, over 8 hours with shaking of 300rpm to test the formation of intramolecular disulfide bond between syntaxin and VAMP. The red arrow at 30 kda show the supported band, which confirm the existence of the middle-cross-link we need. Then we optimized the salt ion concentration, PH, temperature, oscillation speed and duration for the formation of disulphide bond, and finally selected the buffer of 150mM NaCl, PH8.5, and incubated at 300rpm at 34℃ for more than 8 hours. The connection efficiency as shown in Figure 3b is obtained (since the molecular weight of the complex is twice that of the syntaxin-VAMP, the connection efficiency of the -6layer cross-linked SNARE complex is about 50%).



Supplementary Figure 3. Boil assay of intramolecular disulfide bond on SDS page. **a** -1, -2, -3, -6 on the top symbolizes corresponding layer of SNARE complexes. Red dashed lines repensent the disulfide bond between cysteines. **b** The efficiency of disulfide bond generation between Syntaxin and VAMP of -6layer SNARE complex was optimized.

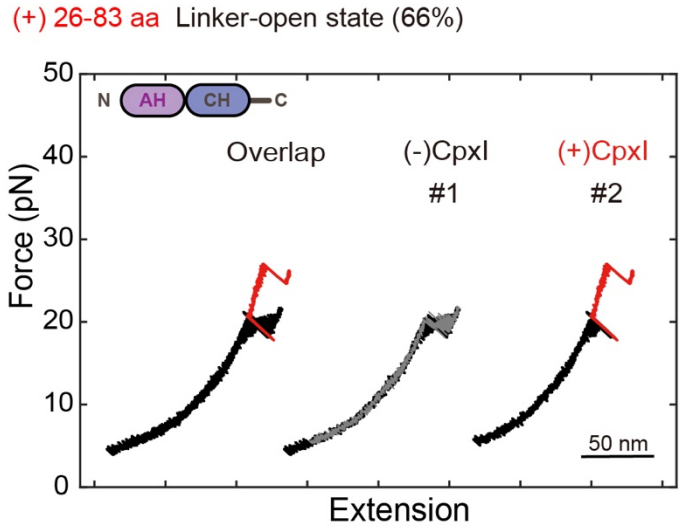
4. No interaction between 1-83 aa and 83-134 aa

In order to test whether mixed CpxI 1-83 aa and 83-134 aa would interact in advance, I conducted Ni-NTA pull-down experiment. The 1-83 aa without his Tag was mixed with sumo_83-134 aa with 6* His Tag for half an hour, and then incubated with Ni-NTA for 1 hour. Collect the post-cleaning solution of the flow through fluid and the low imidazole buffer, and finally elute with the high imidazole buffer. Obviously, almost all 1-83 aa flowed out before elution (Supplementary Figure 4, lane 5 and 6, FT: Flow Through), while there was no 1-83 in the hyperimidazole eluent, only SUMO_83-134 aa (Supplementary Figure 4, lane 7), proving that there was no interaction between 1-83 aa and 83-134 aa.



Supplementary Figure 4. Ni-NTA pull-down assay of interaction between 1-83 aa and 83-134 aa. Mixture of sumo_83-134 aa (with 6*his-tag) and 1-83 aa was incubated with Ni-NTA for 1 hour. Then we collected the flow-through and wash solution， finally eluted the beads by buffer with 250 mM imidazole. Obviously, almost all 1-83 aa flowed out before elution, indicated that there was no interaction between 1-83 aa and 83-134 aa.

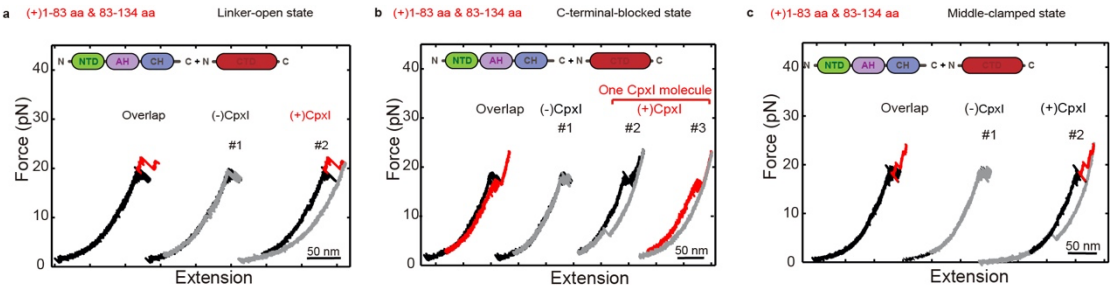
5. The stabilization function of CpxI NTD



Supplementary Figure 5. The stabilize function of CpxI NTD. In the presence of 8μM 26-83aa, 57.5% of 66 transition-state molecule changed their state after the addition of Cpx. FECs of single SNARE complexes under constant forces showing that 66% of

them was stabilized to linker-open state after (red) the addition of 8 μM 26-83aa in real time.

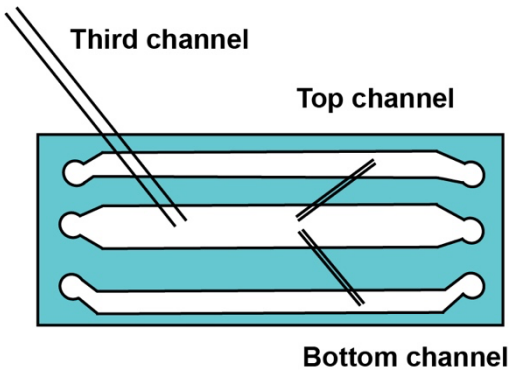
6. CpxI CTD can inhibit the assemble of C-terminal of SNARE complex and stabilize the N-terminal of SNARE complex.



Supplementary Figure 6. CpxI CTD can inhibit the assemble of C-terminal of SNARE complex and stabilize the N-terminal of SNARE complex. **a-c** FECs of single SNARE complexes under constant forces showing SNARE unfolding kinetics before (black) and after (red) the addition of 8 μM 1-83aa and 8/16/24 μM 83-134aa in real time.

7. Chamber fabrication

Standard coverslides were cleaned with dishwashing liquid, then washed with clean water, drained, and ultrasonic cleaned in deionized water for 5 minutes. After rinsing in deionized water, the slides were ultrasonic cleaned in anhydrous ethanol for 5 minutes, and then dried. The glass slide was cut with 6 inlets/outlets using the Laser engraving machine. Meanwhile, the parafilm was cut into three channels as described in Supplementary figure 7.



Supplementary Figure 7. Sketch map of chamber cell. It is constructed by sandwiching one parafilm between two slides，with parafilm thickness as the sample storage space.

The custom chamber was made using the following steps：

(1) Place one parafilm on the slide without laser engraving, then mount the glass pipettes to form the connection channels between the top/bottom channels with the main channel. Put another pipette to serve as the protein channel if necessary. Then put on top of them another parafilm and coverslide, and gently press on the assembly. Such that the parafilms and the capillary are sandwiched in between.

(2) The sample pool is turned over and placed on the metal hot plate to melt the parafilm, and the appropriate pressure is applied to make the slide and the melted parafilm tightly bonded for about 20 minutes, and then it can be cooled after taking out.

(3) Assemble the chamber cell according to the diagram and install it on the optical tweezers instrument. Fill the chamber cell with 0.2% NaN_3 to prevent bacterial growth when finish the experiment.

When assembling the chamber, be careful to test the leakage using clean water, and make sure there will be no air bubbles inside the channels. The tiny air bubbles can be removed by gently tapping on the plastic tubing to generate gentle vibration.

8. Mutated protein sequence

8. 1 VAMP2 (1-92, Q36C)

SAGGMSATAATVPPAAPAGEGGPPAPPPNLTSNRRLQQTCAQVDEVVDI MRVNVDKVLERDQKLSELDDRADALQAGASQFETSAAKLKRKYWWKN GGSGNGSGGLCTPSRGGDYKDDDDK

8. 2 Syntaxin (172-265, L205C)

SAGGNPAIFASGIIMDSSISKQALSEIETRHSEIIKCENSIRELHDMFMDMA MLVESQGEMIDRIEYNVEHAVDYVERAVSDTKKAVKYQSKARRKKGGS GNGGSGSGLNDIFEAQKIEWHEDYKDDDDK

8. 3 SNAP25 (1-206, C85S/C88S/C80S/C92S)

MAEDADMRNELEEMQRRADQLADESLESTRRMLQLVEESKDAGIRTLV MLDEQGEQLERIEEGMDQINKDMKEAEKNLTDLGKFSGLSVSPSNKLKS SDAYKKAWGNNQDGVVASQPARVVDEREQMAISGGFIRRVTNDARENE MDENLEQVSGIIGNLRHMALDMGNEIDTQNRQIDRIMEKADSNKTRIDE ANQRATKMLGSG

The sequences with underline were the native sequences, red marked amino acids were mutated amino acids, the FLAG tag (DYKDDDDK) at the C-terminal was used for protein purification, but was not used in this assay. SAGG at N-terminal and GGSGNGSGG at C-terminal were protein linkers, which were designed to increase the flexibility of protein.

9. Primers for the preparation of DNA handles

Primer sequences for the biotin- and digoxigenin-modified 2260-bp handles were:

SHP2 Primer: /5ThioMC6-D/ AAA TCG ACG CTC AAG TCA GAG GTG

Dual-DIG Primer: /5'Doubler Amino + DIG/ ATC ATC CAA GGC TGA GCC TGC AGG

10. Oxygen scavenging system

Name	Stock concentration	Stock buffer	Temperature(°C)	Working concentration
Glucose	400 mg/ml	Water	-20	250 mg/ml
Glucose Oxidase	50 mg/ml	50 mM NaAc (pH 5.1)	-20	37 mg/ml

Catalase	20 mg/ml	50 mM KPB (pH 7.0)	4	1.7 mg/ml

Supplementary Table 1. Experiments were carried out at room temperature (22 °C) in the HEPES buffer (25 mM of HEPES, 150 mM NaCl, 0.02% CA630, pH 7.4), supplemented with 2% oxygen scavenging system (for reduce light damage to hold for longer time).