Investigation of SNARE mediated membrane fusion and its regulation by optimized single molecule method

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Investigation of SNARE mediated membrane fusion and its regulation by optimized single molecule method

by

Yicheng Zhu

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Biochemistry

Program of Study Committee:
Yeon-Kyun Shin, Major Professor
Amy Andreotti
Sanjeevi Sivasankar

Iowa State University
Ames, Iowa
2016

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DEDICATION

To my mom (Yi, Yuanxiu) and dad (Zhu, Deyuan) who are always being supportive.
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Firstly, I would like to thank my major professor, Dr. Yeon-Kyun Shin to teach me to be independent, proud of what I did and always be aggressive. Those are important lessons I learned in my life. Thanks for all my committee members, and professor who attend my final oral defense to give me the chance to share my progress on my science journey.

I want to thank to my lab mate for sharing the ideas and conducting wonderful discussion. Also, I would like to thank to my friends, your inspiration, your support and your being around is my irreplaceable power to go though my graduate study.

Lastly I want to thanks myself and all the graduate students who is conducting independent research, graduate college is tough, and thanks all of you to unlock the wonderful door of acknowledge by your brave heart.
ABSTRACT

Neurotransmitter release going through synaptic vesicle cycle is one key step how signal is transported in our brains. The mechanism on molecular level has been under development and debated for decades. Many milestones have been made including, the identification of SNARE as core assembly machinery, the clarification of synaptotagmin as calcium sensor, the recognition of NSF and SNAP as disassembly apparatus, the determination of complexin and SM protein as regulatory protein. However, the sequence of their involvement in synaptic vesicle cycle, the relationship between the structure and psychological function, microscale fusion mechanism and are under further investigation. This puzzle is completing with effort from international groups and our group. Complexin as one regulatory protein, has been found owning both inhibitory and facilitatory function. This dual function adds more complication to identify the role of complexin in membrane fusion. Research groups get either inhibitory or facilitatory function based on the experimental condition, which is contradictory. Also, single molecule FRET mixing assay has been adopted widely as one method to isolate membrane fusion system in vitro to give more detailed information on step-by-step mechanism. One major method in single molecule FRET, content mixing, faces obstacles by slow time scale and low fusion percentage. By looking deeper into complexin function, we optimize content mixing and for the first time we observed complexin showing both inhibitory and facilitatory role in a concentration dependent manner.
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CHAPTER 1
GENERAL INTRODUCTION

1.1 Exocytosis and neurotransmitter release

The release of neurotransmitter from synapse is one major step in how signal is transported from one neuron to the other neuron. This process is highly regulated, Calcium dependent and extremely fast that fusion could occur less than 1ms. (Betz et al., 1992; Ryan et al., 1993; Brose, 2008) Neurotransmitter, after synthesis, will be incorporated into synaptic vesicle (SV). One complete SV cycle, from neurotransmitter uptake to budding, will go through seven intermediate steps: translocation, docking, priming, fusion/exocytosis, endocytosis, fission, endosome fusion, budding. (Figure 1) (Südhof, 1995)

The first three steps, starting from SV uptaking neurotransmitter, ending with neurotransmitter release, needs the SV membrane to fuse with presynaptic plasma membrane. (Jahn and Scheller, 2006) During docking, SV is docked to the active site close to calcium channel, but it is not competent enough to finish fusion. The priming phase includes SV becomes more competent. During fusion/exocytosis, an action potential (AP) triggers this step, causing neurotransmitter releasing in a short (ms) time scale. (Rastaad et al., 1992; Hessler et al., 1993; Rosenmund et al., 1993)

1.2 SNARE in neurotransmitter release

Back to 1988-1992, N-ethylmaleimide-sensitive factor(NSF) and soluble NSF attachment protein(SNAP) have been identified as soluble constituent that are required for mammalian SV transport. (Wilson et al., 1989; Clary et al., 1990) Meanwhile, VAMP and
syntaxin (SYX) have been proposed to be receptors for NSF and SNAP. (Trimble et al., 1988; Baumert et al., 1989; Bennett et al., 1992) Later on, VAMP, located at SV, SN25 and SYX, located at presynaptic plasma membrane, were shown to be target of the botulinum and tetanus neurotoxin. (Schiavo et al., 1992; Blasi et al., 1993; Blasi et al., 1993) Since botulinum and tetanus neurotoxin are well-known to inhibit the communication between neurons, its target, VAMP, SN25 and SYX are thought to be major player in neurotransmitter release. The soluble property, combined with the ATP-driven disassembly nature of NSF and SNAP, leaves the role of membrane anchored VAMP, SN25 and SYX (together called: soluble N-ethylmaleimide-sensitive factor attachment protein receptor or SNARE complex) to be assembly to drive the membrane fusion. (Hanson et al., 1997; Lin et al., 1997) (Figure 2.a) Then, the crystal structure of SNARE complex showed highly twisted and parallel four helical bundle, (Figure 2.b) further confirmed the “zippering” model of SNARE function. (Sutton et al., 1998; Fasshauer et al., 1998)

SNARE complex contain three proteins: two at the plasma membrane side named SN25 and SYX, one at the SV side named VAMP. The conserved part among three proteins calls SNARE motif, which is 60-70 conserved amino acids length helical arranged in heptad repeats (assigned as a-g). (Poirier et al., 1998) For each individual helical, a and d residues point towards each other, forming a hydrophobic contacting surface with 16 layers in total (layer -7 to layer +8). Residues at layer 0 are conserved among specifies by assigning one arginine (R) residue to VAMP and three glutamine (Q) to SYX and SN25. (Figure 2.c) Thus, v – SNARE could be called R SNARE while t – SNARE could be called Qa, Qb, Qc SNARE. SYX and VAMP has a transmembrane (TM) domain link to the C-terminal of SNARE motif by a short linkage of ~ 10 amino acids lengths. SN25 has two SNARE motifs
connected by a 54-residue loop, anchored to membrane by palmitoylation-modified cysteine residues. (Sutton et al., 1998) SNARE motif zipper start from N to C terminal. (Pobbati et al., 2006)

Once zipper, SNARE proteins form SDS-resistant bundle with 1:1:1 stoichiometry, overcoming the energy barrier from two membranes electrostatic repulsion and bring two membranes closer. (Jahn et al., 2006) SNARE under zipper process with two TM domain being contributed by two membranes called trans-SNARE, while fully zippered SNARE with all TM domain in one membrane, called cis-SNARE. (Sørensen, 2004) The zipper model is further confirmed by the X-ray structure of SN25, SYX 1a (with TM domain) and VAMP2 (with TM domain) formed continuous helical bundle (cis – SNARE) throughout SNARE motif, linker region and transmembrane domain. (Stein et al., 2009) (Figure 3)

The zipper of SNARE motif is not one step procedure. Initially, plasma membrane protein SYX and SN25 forms a 1:1 helical bundle in which SN25 contribute two SNARE motifs, possibly holding by a “place-holder” like SM protein to avoid formation of “dead-end”, waiting the unstructured SV protein VAMP to bind. (Pobbati et al., 2006) Before the pore open, membranes will go through a hemifusion metastable state with the exchange of out leaflet lipid but without the aqueous connection. (Xu et al., 2005; Reese et al., 2005; Giraudo et al., 2005) At this stage, trans-SNARE is subject to regulation of the regulatory proteins like Synaptotagmin (SYT) and Complexin (CPX). (Südhof, 2002; Reim et al., 2001) (Figure 4) This is consistent with the idea all the vesicles synchronized in the beginning, generating a ready release pool (RRP), then release most of the content at short time scale when AP arrives. However, this intermediate is not directly proved by
experimental observation. Later on, a few in vitro experiment provide direct or indirect
evidence for this hemifusion state by using optical tweezers, EPR and cell fusion assay.\textbf{(Gao et al., 2012; Min et al., 2013; Zorman et al., 2014; Shin et al., 2014)} In early EPR
experiment, half TM domain of v-SNARE motif has been truncated to achieve the mimic
hemifusion state. Later, two specific of SNARE has been attached to two nanodiscs and
detected under EPR, giving a considerable (~15\%) population of hemifusion. For optical
tweezer, a delicately controlled force has been applied to tear apart C-terminal of SNARE
bundle to generate the intermediate state. After Ca\(^{2+}\) influx, the trans SNARE will finish
zippering and form a cis SNARE.

\subsection*{1.3 Complexin}

After identifying SNARE complex as the core machinery, in in vitro experiment,
membrane could fuse but with a half-time longer than 10 min with SNARE protein only.\textbf{(Weber et al., 1998)} This fact showed that, by conducting \textit{Ca}^{2+} dependent and fast
exocytosis, other regulatory protein is required in addition to SNARE complex.
Crystallization results from co-crystallization complexin (Cpx) with SNARE complex
showed the central alpha helix of Cpx directly binds to the groove between VAMP 2 and
SYX 1a.\textbf{(Chen et al., 2002)} Also Cpx can bind to the SYX/SN25 bundle.\textbf{(Maximov et al.,
2009; Weninger et al.,2008)} These results provide insight on that Cpx may participate in
two stages of fusion: 1. Act as a clamp to arrest SNARE complex to prevent fusion which is
later activated by SYT at prefusion state\textbf{(Huntwork et al., 2007; Giraudo et al., 2006;
Tang et al., 2006)} 2. to stabilize SNARE complex in high fusogenic state at post-priming
step\textbf{(Reim et al., 2001; Xue et al., 2007; Schaub et al., 2006).}
Numerous efforts have been put on understanding the role of complexin in vesicle fusion including the usage of liposomes, cells with flipped SNAREs, PC12 cells, different kinds neurons, chromaffin cells and *etc.* as artificial or real fusion machinery. However, a controversy conclusion is achieved on the role of complexin to be both inhibitory and facilitatory. Cpx exerts different role depending on the method used, the concentration of protein, cell type and the fragment of protein (Ono et al., 1998; Tokumaru et al., 2001; Roggero et al., 2007; Itakura et al., 1999; Liu et al., 2007).

While Cpx seems to conduct a complicated role in neurotransmitter release, it has a relatively simple four-domains structure, including an N-terminal domain (NTD), an accessory helix domain, a central helical domain and a C-terminal domain (CTD). (Figure 5)

Multiple studies showed individual domain of Cpx could display either inhibitory or facilitatory function. (Trimbuch and Rosenmund, 2016)

Crystal structure revealed that central helical domain has a high affinity, binding to the groove between SYX and VAMP interface in an antiparallel way. (Chen et al., 2002) (Figure 5) This binding is essential for Cpx function since the deletion of central helical domain or point mutants on central helical domain that impede binding will completely stop spontaneous and evoked fusion.

Based on the crystal structure, the accessory helical domain “hangs” above the VAMP C–terminal domain and exhibits an inhibitory function based on the structure-function analysis. (Martin et al., 2011; Xue et al., 2007; Xue et al., 2009) A few mechanisms have been provoked about this inhibitory function. (Figure 6) The “superclamp” model has been proposed by substituting charged residues on accessory helical with hydrophobic residues, which increase hydrophobic interaction. Both *in vitro* cell-cell fusion
assay and cross-species rescue experiment showed the hydrophobic “superclamp” mutants have more clamping ability, seemingly drawing a positive correlation between hydrophobicity and inhibitory effect. (Giraudo et al., 2009; Cho et al., 2014) Secondly, electrostatic model states the repulsive forces between equally charged membranes (SV membrane and presynaptic plasma membrane) will be tuned by accessory helical charge. This state is supported by the introduction of negatively or positively charged residue which induced strong reduction or enhancement in spontaneous release. (Trimbuch et al., 2014) In the third model, the helical structure of accessory helical domain itself provides stability to the central helical domain by using C. elegans. However, the link between stabilizing function and inhibitory role of accessory helical is missing here. (Radoff et al., 2014) In summary, though three different models have been proposed for explanation of inhibitory role of accessory domain, neither of them could be accepted. Except Cpx, the other regulatory proteins like Munc 13 and Munc 18 have been studied too.

1.4 Synaptotagmin

In total, 14 kinds of Synaptotagmin (SYT) have been identified in mammals, while SYT 1 is exclusively expressed on SV. (Südhof, 2002) SYT has a glycosylated N-terminal domain, one single helical TM domain, followed by a putatively unstructured ~60 amino acids linker region, then two tandem cytoplasmic Ca\(^{2+}\) binding domain named C2A and C2B (C2AB used to represent the cytoplasmic region and always served as a binding competitor with SYT). (Perin et al., 1991) (Figure 7)
During exocytosis, AP will trigger a high local concentration of Ca\(^{2+}\) at active site, implying the involvement of Ca\(^{2+}\) sensor during this step. The sensor should have high affinity for Ca\(^{2+}\), and also undergo a conformational change after binding to Ca\(^{2+}\). Combined with the facts that (1) SYT has C2A and C2B Ca\(^{2+}\) binding domain and undergoes a Ca\(^{2+}\) binding conformational change (Brose et al., 1992; Davletov et al., 1993; Davletov et al., 1994), (2) SYT knockout mice showed severely impaired Ca\(^{2+}\) evoked neurotransmitter release (Geppert et al., 1994), and (3) In SYT knockout experiment, Ca\(^{2+}\) independent release could still be triggered (Geppert et al., 1994); SYT has been selected as potential Ca\(^{2+}\) sensor candidate to trigger fast, synchronized, Ca\(^{2+}\) evoked neurotransmitter release.

However, not only just binding to Ca\(^{2+}\), SYT has multiple binding sites in Ca\(^{2+}\) independent or dependent manner, suggesting potential roles of SYT other than involvement in fusion. SYT was identified to bind to t-SNARE protein SYX and SYX/SNAP-25 complex in Ca\(^{2+}\) independent manner, which suggests SYT might have a role during docking. (Shao et al., 1997) Also, SYT could bind to SNARE complex in a Ca\(^{2+}\) dependent and independent way. (Bowen et al., 2005; Bai et al., 2004; Chapman et al., 1995) Additionally, SYT could bind to negatively charged lipid (e.g. PIP2) on plasma membrane in Ca\(^{2+}\) dependent way by incorporation of two C2 domains. (Chapman and John, 1994; Heidelberger et al., 1994)

The other remaining controversy which is inconclusive is the effort to try to mimic the machinery in vitro can not achieve the physiological concentration level of Cpx, level of Ca\(^{2+}\) sensitivity (uM) and level of synchronization(ms). Those add uncertainty to correctly identify the relationship between the results above and the real role of regulatory protein.
1.5 Single molecule mixing assay

Single molecule based techniques make it possible to identify the properties by directly “looking at the thing” (Feynman, 1992), bringing the investigation of biological system to a whole new level. They are widely applied to the research of motor protein, DNA replication, recombination, protein folding and so on. (Ha, 1996) Single molecule fluorescence resonance energy transfer (sm-FRET) is one popular single molecule based technique. The resonance energy transfer efficiency between donor and acceptor (called FRET pair) can be represented as \( E = \frac{1}{1+(R/R_0)^6} \), while R is the internal distance between donor and acceptor and \( R_0 \) is Förster radius when \( E \) equals to 0.5. (Figure 8.a) The six power of R gives FRET a high resolution as function of distance. FRET signal is commonly recorded by total internal reflection (TIR) spectroscopy. Because the evanescent light can only stimulate the imaging area as thick as 100 -200 nm, only the molecule attached to the surface could be detected, thus background is highly reduced. However, time scale is one restriction since detective CCD can only take single image as fast as 100ms. (Figure 8.b)

Single molecule method gives ability to track individual behaviors instead of the average of bulk numbers of molecules (on the order of Avogadro’s number), thus gives more information for system with variability. That SV fused with plasma membrane goes through three stages- docking, hemifusion and fusion makes sm – FRET a perfect method to clarify its mechanism. For performing the sm -FRET experiment, a pair of fluorophores (FRET pair) is needed, in which energy transfer from donor to acceptor. FRET pairs can be applied to two SNARE proteins or to SNARE complex-regulatory protein to detect the protein-protein interaction. (Weninger et al., 2008; Li et al., 2007) This gives dynamic structural
information, however, the choose of labeling site, the size of label dye should be cautious to shield the result highly from facts.

Comparing with labeling protein, labeling membrane has advantages at the feasibility of labeling membrane and the fast time scale of membrane two-dimensional diffusion. (Schlessinger et al., 1977) Firstly, lipid mixing assay has been introduced. Two species of vesicles, namely t-vesicle and v-vesicle, harboring two dyes, once fused, the FRET signal between two dyes increases as dye distance decreases, served as indicator of membrane fusion. (Weber et al., 1998; Liu et al., 2005; Liu et al., 2008) (Figure 9.a) However, later lipid mixing assay has been found to give more than 90% lipid mixing even with less than 2% content mixing. (Chan et al., 2009) In this case, whether a pole formed or not can’t be ensured by lipid mixing FRET as indicator, thus put lipid mixing method under suspicious.

In content mixing assay, a high concentration soluble dye or a Cy3/Cy5 dual labelled DNA will be introduced inside the content of one vesicle. Because of high concentration or close distance of Cy3/Cy5, vesicle showed low signal because of dye self-quenching, which could be recovered by the dilution or introduction of target DNA, which achieved by pore open and by vesicle aqueous content diffusion from one to the other. (Diao et al., 2012) This method conquers the disadvantage that lipid-mixing can’t distinguish pore openness (Figure 9.b; Figure 9.c) Right now, both lipid mixing and content mixing are utilized to clarify the mechanism of membrane fusion under different condition and emphasis.

Though efforts have been made to mimic natural membrane fusion as much as possible. However, the results are dimmed including the mixing percentage and time-scale. In this report, those disadvantage are highly improved by introducing nM level of CPX to the system.
1.6 Figures

**Figure 1 Nine steps of membrane fusion.** Start from SV uptake neurotransmitter, typical SV will go through translocation, docking, priming, fusion, endocytosis, fission, endosome fusion, budding. Image modified from (Jahn et al., 2012)
Figure 2 Structure of SNARE  
a) Domain structure of SNARE. All Q and R SNARE will share the highly conserved SNARE motif. For Qa-SNARE (SYX), showed Habc domain at N terminal as the first three cylinder.  
b) Crystal structure of SNARE four helical bundle  
c) The 16 hydrophobic layer of SNARE complex, also show the side chain at layer -2, -1, 0 and +1. Image modified from (Jahn and Scheller, 2006)
Figure 3 Model of cis-SNARE insert into POPC bilayer. Helical bundle formed continuously along the SNARE motif, linker region and TM domain. Image modified from (Stein et al., 2009)

Figure 4 Hemifusion state as intermediate state during membrane fusion. Notice under hemifusion state, which is after docking and before pore open, lipid from inner membrane leaflet contact and interchange. Image modified from (Jahn et al., 2006)
**Figure 5 Structure of CPX.** Upper figure show domain structure of CPX. From N terminal to C terminal, it has NTD, accessory helix (AH) domain, center helix domain (CH) and CTD. Lower image show CPX center helical domain binds to the groove of VAMP and SYX, while the CPX accessory helical domain “hang” above VMAP C terminal SNARE motif. Image modified from *(Trimbuch et al., 2016)*
Figure 6 Models of inhibitory role of CPX accessory helical domain. Left, accessory helical binds to C-terminal of SYX/SN25 bundle by hydrophobic strength thus competitively retard VAMP zippering. Middle, accessory helical repulse two membranes away from each other by its relatively positive charge. Right, stabilization effect to center helical bundle. Image modified from (Trimbuch et al., 2016)

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1.7 List of reference


NEUROTRANSMITTER RELEASE BY PROTEOLYTIC CLEAVAGE OF SYNAPTOBREVIN. *Nature*, 359(6398), 832-835. doi:10.1038/359832a0


CHAPTER 2

PREINCUBATION OF T-SNARES WITH COMPLEXIN I INCREASE CONTENT-MIXING EFFICIENCY


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Author Contributions

##J.K. and Y.Z. contributed equally to this work.

2.1 Introduction

Complexins (Cpx) are a family of small proteins that are specifically localized at the presynapse to regulate neurotransmitter release.1-2 Cpx is thought to modulate both spontaneous and evoked release in the neuron. There is strong evidence that the deletion of Cpx reduces evoked exocytosis significantly,3-8 although controversy surrounds the proposition that Cpx suppresses spontaneous release.4, 6, 7, 9-12

The effect of Cpx on neuroexocytosis is of great interest because changes in Cpx could elicit the disruption of the exocytosis patterns, which could affect behavioral and
cognitive activities. While the causal role is yet to be elucidated, indeed apparent changes in Cpx levels have been observed in schizophrenia as well as in neurodegenerative diseases such as Parkinson’s and Alzheimer’s. 13

It is generally believed that synaptic vesicle fusion, required for the neurotransmitter release, is mediated by soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs). 14 Additionally, a major Ca$^{2+}$ sensor synaptotagmin 1 (Syt1) 15, 16 and the SNARE binding Cpx are considered to be two principal regulatory components that orchestrate fast Ca$^{2+}$-triggered vesicle fusion. 17-19

Toward our understanding of the roles that Cpx plays in neuroexocytosis, in vitro membrane fusion assays have made contributions. 19-20 In these assays, SNARE proteins and Syt1 are appropriately reconstituted into the two populations of liposomes and fusion between two respective proteoliposomes is monitored spectroscopically. Of particular interest is the single-vesicle content-mixing assay which is capable of dissecting docking, lipid mixing, and content-mixing steps along the fusion pathway. 21-22 The assay revealed that Cpx stimulates and synchronizes Ca$^{2+}$-triggered vesicle fusion while inhibiting spontaneous fusion. Moreover, it was shown that Cpx accelerates the rate of vesicle docking. 25 Although the results from single vesicle fusion assays have revealed important features of the Cpx function in exocytosis there are a few shortcomings. The assay requires high Ca$^{2+}$ to produce appreciable yields of vesicle fusion, 21 which is still not comparable to the highly efficient evoked vesicle fusion in the neuron. 26, 27 Moreover, it was shown that overexpression of Cpx reduces Ca$^{2+}$-triggered exocytosis in cells, 28, 29 which is not explainable with the qualitative data accumulated so far. This raises some concerns whether the single-vesicle
content-mixing assay, in its current form, is sufficiently robust to faithfully recapitulate the essential features of Cpx function in the neuroexocytosis.

In this work, we find that the pretreatment of t-SNARE with Cpx improves the efficiency of vesicle fusion dramatically and recovers the natural high \( \text{Ca}^{2+} \)-sensitivity. Moreover, with this improved method, we discover that Cpx stimulates \( \text{Ca}^{2+} \)-triggered exocytosis in a concentration-dependent manner below 100 nM. But the trend reverses its course above 200 nM and shows the dose-dependent decrease in the higher concentration range, resulting in a bell-shaped response curve. Thus, our results describe how the change in the Cpx level might affect the neurotransmitter release quantitatively.

### 2.2 Material and method

#### 2.2.1 Plasmid construct and site-directed mutagenesis.

DNA sequences encoding rat Syntaxin 1A (amino acids 1–288 with three native cysteines replaced by alanines), VAMP2 (amino acids 1–116 with C103 replaced by alanines), soluble VAMP2 (VpS, amino acids 1–94), SNAP-25 (amino acids 1–206 with four native cysteines replaced by alanines), rat complexin I (Cpx, amino acids 1–134), truncation mutant Cpx 27 (amino acids 27–134), and double mutant Cpx M5E/K6E were inserted into the pGEX-KG vector as N-terminal GST fusion proteins. Rat synaptotagmin 1 (Syt1, amino acids 50–421 with four native cysteines C74, C75, C77 and C79 replaced by alanines and another C82 replaced by serine) was inserted into pET-28b vector as C-terminal His-tagged proteins. DNA sequences were confirmed by the Iowa State University DNA Sequencing Facility.
2.2.2 Protein expression and purification.

VAMP2, SNAP-25, syntaxin 1a, VpS, Cpx, and Cpx mutants were expressed as GST fusion proteins. Escherichia coli BL21 Rosetta (DE3) pLysS (Novagen) was used to express the recombinant GST fusion proteins. The cells were grown in LB medium at 37 °C with ampicillin (100 μg/mL) until the ~0.6–0.8 absorbance at 600 nm. Isopropyl β-D-1-thiogalactopyranoside (0.3 mM final concentration) was then added to induce protein expression. The cells were grown for another 12 h at 16 °C. The cell pellets were then harvested via centrifugation at 6000g for 10 min. The pellets were resuspended in PBS at pH 7.4 containing 2 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 2 mM EDTA, and 2 mM dithiothreitol. Transmembrane proteins required 0.5% Triton X-100, 0.05% Tween 20, and 10% N-lauroylsarcosine additionally in the buffer. Cells were broken up via sonication immersed in an ice bath. The supernatant was collected by centrifugation at 15000g for 20 min. The glutathione-agarose beads in buffer were added and nutated at 4 °C for 2 h. The unbound proteins were then washed out, and the GST fusion proteins were cleaved off from the beads by thrombin (Sigma-Aldrich) at room temperature for 2 h. Thrombin cleavage buffer for membrane proteins contained 50 mM Tris-HCl, 150 mM NaCl, and, pH 8.0 and 1% n-octyl glucoside.

Syt1 (amino acids 51–421) was expressed with the C-terminal 6-histidine-tag in E. coli BL21 Rosetta (DE3) pLysS and purified with the aforementioned protocol except for using Ni-NTA beads (Qiagen). Elution was carried out with buffer of 25 mM HEPES, 400 mM KCl, 500 mM imidazole, and 0.8% OG. Purified proteins were examined with 15% SDS-PAGE, and the purity was at least 90% for all of the proteins.
2.2.3 Proteoliposome Reconstitution.

We used the following lipid molecules to make proteoliposomes: 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), phosphatidylinositol-4,5-bisphosphate (PIP2, from porcine brain), and cholesterol. All lipids were obtained from Avanti Polar Lipids. In reconstituting the SNAREs into liposomes, molar ratios of lipids were 15:63:20:2:0.1 (DOPS: POPC: cholesterol: PIP2: biotin-DPPE) for the t-vesicles, and 5:75:20 (DOPS: POPC: cholesterol) for the v-vesicles, respectively. The lipids were mixed in the chloroform soluble state and dried in a glass tube with nitrogen gas and stored overnight in a desiccator under house vacuum. The t-vesicle lipid film was resuspended with HEPES buffer (25 mM HEPES, 100 mM KCl, pH 7.4), whereas v-vesicle lipid film was resuspended with HEPES buffer containing 20 mM SRB (Invitrogen). After 10 freeze-thaw cycles between hot water and liquid nitrogen, large unilamellar vesicles (~100 nm in diameter) were prepared by extrusion through the polycarbonate filter (Avanti Polar lipids). The t-SNAREs were mixed with liposomes (10 mM in total lipid concentration), while VAMP2 and Syt1 were reconstituted with SRB (20 mM)-containing liposomes for ~15 min. We used a 200:1 lipid/protein molar ratio for all reconstitution. The liposome/protein mixture was diluted 2 times with the HEPES buffer and then dialyzed in 2 L dialysis buffer at 4 °C overnight. For the v-vesicles, free SRB was removed using the PD-10 desalting column (GE healthcare) after dialysis.

2.2.4 Single vesicle content-mixing assay and docking assay.

The imaging quartz surface (25 × 75 × 1.0 mm) was PEGylated with the PEG and PEG-biotin mixture with a 40:1 molar ratio (Laysan Bio). The imaging surface was divided
to form 10 independent flow chambers. The flow chambers were incubated with streptavidin (0.2 mg/mL, Sigma-Aldrich) for 10 min followed by thorough washing. A mixture containing 125 μM t-vesicles with 100 nM Cpx in HEPES buffer was introduced into the flow chamber, and the t-vesicles were allowed to be immobilized on the PEG-coated surface while maintaining 100 nM Cpx concentration. After 15 min incubation, unbound t-vesicles were washed using HEPES buffer containing 100 μM Cpx. A mixture of 100 nM Cpx and v-vesicles (20 mM SRB) in HEPES buffer was injected, and the sample was incubated in the flow chamber for 10 min to allow vesicle–vesicle docking. The unbound v-vesicles were washed out using HEPES buffer containing 100 nM Cpx. The channels were then imaged and Ca\(^{2+}\) was injected (1.2 mL/1 min) into the flow chamber while recording via TIR microscope. A stepwise jump in the fluorescence intensity was detected as an indication of content-mixing, which was the result of the SRB dequenching. The details of TIR microscope imaging and single molecule data analysis have been reported in our previous work.\(^{24}\) The time when the stepwise increase was observed was recorded manually and plotted onto a histogram with the bin size of 1 s. The histogram was then fitted with a single exponential decay in order to obtain the first-order time constant.

The single vesicle docking experiment was performed identical to the content-mixing assay prior to Ca\(^{2+}\) injection. Once the v-vesicles, with VAMP2 and Syt1, and t-vesicles were incubated and washed with buffer containing the appropriate Cpx concentration, we took multiple images and the immobilized spots were counted and plotted on a histogram. To ensure quality control, the full range of Cpx concentrations was performed on a single PEG slides, with multiple replicates.
2.3 Results

2.3.1 Single-vesicle content-mixing assay.

Previously, the effect of Cpx on Ca\(^{2+}\)-triggered exocytosis has been studied with the single-vesicle content-mixing assay, initially in our group \(^{24}\) and later, extensively in Brunger’s group. \(^{24}\) Although the experiments have recapitulated some essential features of the Cpx function, there have been two apparent shortcomings. First, the assay requires unusually high Ca\(^{2+}\); several hundreds of μM instead of biologically relevant tens of μM. Second, the outcomes do not explain why the release decreases when Cpx is overexpressed in cells. \(^{28,29}\)

In the single-vesicle content-mixing assay based on total internal reflection (TIR) microscopy, the t-SNARE-carrying liposomes (t-vesicles) were tethered to the imaging surface followed by the docking of v-SNARE plus Syt1-carrying liposomes (v-vesicles) onto the t-vesicles (Figure 1a). In all previous experiments, Cpx was premixed with v-vesicles, and the mixture was flown into the flow cell while t-vesicles were not pretreated with Cpx. This time, however, realizing that Cpx might interact with t-SNAREs, \(^{30}\) we pretreated t-vesicles with Cpx before injecting v-vesicles which were also premixed with Cpx. All subsequent washing of untethered excess vesicles was conducted in the presence of Cpx such that the Cpx concentration would remain constant throughout the experiment. The v-vesicles were encapsulated with ∼20 mM sulforhodamine B (SRB) for the fluorescence detection of content-mixing. \(^{22}\) Subsequent Ca\(^{2+}\) injection into the flow chamber promotes vesicle-vesicle fusion that results in content-mixing. Content-mixing induces a step-like sudden rise of the fluorescence intensity (Figure 1b) as a result of dequenching of the SRB fluorescence due to the fusion-induced dilution.
2.3.2 Bell-shaped response of Ca\textsuperscript{2+}-triggered vesicle fusion to Cpx.

In the absence of Cpx, a mere \(~12\%\) of the docked vesicles exhibited content-mixing when triggered by 500 \(\mu\text{M} \text{Ca}^{2+}\), which has been seen consistently in previous studies.\textsuperscript{23,24} This is apparently short of reproducing in vivo synaptic vesicle fusion in which almost all vesicles in the readily releasable pool fuses with the plasma membrane when stimulated with only \(~10 \mu\text{M} \text{Ca}^{2+}\).\textsuperscript{26,27} However, when we introduced 100 nM Cpx into our system, we observed a significant increase in content-mixing population. Over two-thirds of the docked vesicle pairs exhibited content-mixing at 500 \(\mu\text{M} \text{Ca}^{2+}\) (Figure 2a). In a control experiment, Mg\textsuperscript{2+} was not capable of promoting content mixing at all, indicating that Ca\textsuperscript{2+} and the Ca\textsuperscript{2+}-sensor Syt1 played roles in triggering the membrane fusion reaction. On the other hand, while we observed a slight decrease at 4 \(\mu\text{M} \text{Cpx}\), there was no obvious change in vesicle docking probability 0–800 nM Cpx, indicating that Cpx may be specifically involved in the fusion pore opening step (Figure S1).

Having such high content-mixing percentage in the presence of Cpx, we ask if physiologically relevant Ca\textsuperscript{2+} conditions (10 \(\mu\text{M}\)) could trigger appreciable content-mixing which had not been previously achieved.\textsuperscript{23,24} As expected from previously reported results, content-mixing was hardly observable in the absence of Cpx at 10 \(\mu\text{M} \text{Ca}^{2+}\). However, in the presence of 100 nM Cpx, we observe \(~45\%\) content-mixing among docked vesicles (Figure 2b). Our results show that not only does Cpx significantly increase the probability of vesicle fusion but also dramatically improves the Ca\textsuperscript{2+} sensitivity in our in vitro assay. Such an improvement was observed only when t-vesicles were pretreated with Cpx.
Previously, it was shown that Cpx alone, even in the absence of a major \( \text{Ca}^{2+} \)-sensor Syt1, could trigger SNARE-mediated lipid mixing in response to \( \text{Ca}^{2+} \). Similarly, we found that Cpx alone was able to trigger content mixing with \( \text{Ca}^{2+} \) (Figure 1a,b). However, membrane fusion with Cpx alone was less efficient and slower than it was when both Cpx and Syt1 were present. Particularly, under physiological 10 \( \mu \text{M} \) \( \text{Ca}^{2+} \), the fusion efficiency and time scale was approximately 3 times less and 6 times slower, respectively.

As we increase the Cpx concentration from 0 up to 100 nM, we are able to observe a steep enhancement of \( \text{Ca}^{2+} \)-triggered content-mixing. Specifically, with 10 \( \mu \text{M} \) \( \text{Ca}^{2+} \), the yield of content-mixing increased from \(~1\%\) to \(~45\%\) as we increased the Cpx concentration from 0 to 100 nM.

Surprisingly, however, as we further increase the Cpx concentration above 200 nM the stimulating effect gradually diminishes in a concentration dependent manner. At 200, 400, 800, and 4000 nM Cpx, we observed approximately 44\%, 20\%, 8\%, and 1\% content-mixing, respectively. Thus, our results demonstrate that Cpx elicits a bell-shaped response on \( \text{Ca}^{2+} \)-triggered vesicle fusion, an ascending trend under low concentrations (below 100 nM) but descending trend under high concentrations (above 200 nM). We also observed a similar bell-shaped curve for 500 \( \mu \text{M} \) \( \text{Ca}^{2+} \) with slight increase in yields over the entire Cpx concentrations. The overall slight lift of the response curve for 500 \( \mu \text{M} \) \( \text{Ca}^{2+} \) compared to 10 \( \mu \text{M} \) \( \text{Ca}^{2+} \) was sort of expected and in fact is quite consistent with the in vivo observation that the fusion efficiency is effectively saturated with only a small increase above 10 \( \mu \text{M} \) \( \text{Ca}^{2+} \).
2.3.3 Cpx contributes little to the synchronization of Ca$^{2+}$-triggered vesicle fusion.

In neurons, when Cpx was deleted by knockout the amplitude of excitatory postsynaptic potential (EPSC) decreases significantly, but the time scale of EPSC changes little in cultured mammalian neurons. In the previous experiments by Brunger and co-workers, approximately a factor of 2−4 enhancement in the time scale of synchronization was observed for Cpx. This appears to be inconsistent with the observations in mammalian neurons.

Here, we revisit the time scale changes with Cpx using the newly improved assay (Figure 3a). When the time scales of synchronization are examined as a function of Cpx we observed little variation over the entire range of Cpx concentrations studied (Figure 3b, c). The results suggest that Cpx is not involved in the synchronization of Ca-triggered vesicle fusion. While there is still controversy, our results are more in favor of the proposition that Cpx may not be involved in clamping and synchronization of vesicle fusion in mammalian neurons. However, we do note that an approximately 2 fold increase in synchronization was observed with 500 µM when compared to 10 µM Ca$^{2+}$.

2.3.4 The N-terminal of Cpx is essential for the enhancement of the fusion probability.

It was previously reported that the first 26 residues of Cpx facilitate the enhancement of fusion probability in mouse neurons. Moreover, the efficacy of Cpx was completely lost when Met5 and Lys6, which are considered to contribute in forming the N-terminal α helix, was mutated to glutamate.

We prepared two Cpx mutants, Cpx 27 in which N-terminal 26 amino acids were deleted and a double point mutant Cpx M5E/K6E, in order to verify the role of the N-
terminal region with our newly improved single vesicle fusion assay (Figure 4). The content-mixing assay was performed identically except for using the mutants instead of the wild-type at each step. In coherence with the in vivo results, Cpx 27 and Cpx M5E/K6E failed to promote vesicle fusion recapitulating the critical role of the N-terminal region of Cpx in stimulating Ca\(^{2+}\)-triggered exocytosis.

As a control, we tested if membrane fusion was SNARE-dependent using soluble VAMP2 lacking the transmembrane domain (VpS, amino acids 1–94). VpS has been frequently used to verify the SNARE-dependency of the fusion reaction. We incubated VpS (20 µM) along with the v-vesicles and Cpx wild-type (Figure 4). In the presence of VpS, content-mixing was hardly observed, confirming that the fusion reaction was indeed SNARE-dependent.

### 2.4 Discussion

Although the role of Cpx in synaptic membrane fusion has been highly controversial, it is generally agreed that Cpx stimulates evoked exocytosis. The results from our improved single-vesicle content-mixing assay are fully consistent with this notion.

What is new and interesting though is that the stimulatory effect of Cpx reverses its course after cresting at \(\sim\)150 nM, thus showing a bell-shaped dose response curve. Previously, it has been somewhat mysterious why overexpression of Cpx in cells results in reduced evoked exocytosis despite its established positive role. Our results demonstrate, in a well-defined environment, that there is indeed a dose-dependent decrease of Ca-triggered vesicle fusion at high concentrations above 200 nM. Thus, if overexpression changed the
Cpx level in the regime of 200 nM, a few µM one would observe the reduction of evoked exocytosis. Thus, the bell-shaped dose response for Cpx reconciles seemingly paradoxical results that both knockout and overexpression studies show the reduction of evoked exocytosis.

Our improved single-vesicle content-mixing assay made it possible to obtain the dose response curve for Cpx in Ca- triggered vesicle fusion. The dramatic improvement of the fusion efficiency over the previous work is apparent in our results. The Ca-sensitivity was increased to the natural level, and thus, the assay can now operate at physiological-relevant 10 µM Ca. We point out that the only tweak, compared to the previous studies, was the pretreatment of t-vesicles with Cpx prior to vesicle docking. Why would the pretreatment of t-SNARE with Cpx affect so much the fusion outcomes? There might be two possible scenarios. Scenario one is that when Cpx is delivered during or after docking the SNARE complex is not freely accessible by Cpx any more due to the steric crowding at the fusion site. This would in turn reduce the effectiveness of Cpx in regulating the SNARE function. Scenario two is that Cpx may have the capacity to prime t-SNAREs by a yet unknown mechanism. For instance, it is possible that Cpx might play a role in converting the inactive 2:1 complex to the active 1:1 complex. However, these postulations are purely speculative, warranting further experiments.

Intriguingly, we observe little change in the synchronization kinetics of vesicle fusion over the entire Cpx concentration rage of 0–4 µM. Our results are quite consistent with those from the whole cell patch clamp conducted with cultured mammalian neurons. The results suggest that Cpx may not be involved in the clamping and synchronization of exocytosis. However, the caveat of our experiments is that the time scale is still 3 orders of magnitude
slower than what is normally observed in vivo. Thus, it is possible that our assay does not faithfully reproduce the synchronization kinetics of vesicle fusion. We note that some slower kinetics was observed in the absence of Cpx in Drosophila suggesting the variation of the Cpx function among different organisms.

Our results show that Cpx alone, in the absence of Ca-sensor Syt1, can trigger SNARE-mediated content mixing in response to $\text{Ca}^{2+}$. Intriguingly, Cpx does not have an apparent Ca-sensing module or domain. However, it was previously shown that Cpx binds the membrane in the presence of $\text{Ca}^{2+}$. We wonder if this Ca-mediated Cpx binding to the membrane is relevant to the Ca-sensing capacity in our in vitro membrane fusion assay. We note however that the biological relevance of the $\text{Ca}^{2+}$-sensing activity of Cpx has not been established, warranting further investigation.

In conclusion, we have vastly improved the single-vesicle fusion assay and show that Cpx modulates evoked exocytosis with an unusual bell-shaped response curve. This quantitative description, which is not easily obtainable with knockout or overexpression studies in cellular environments, not only helps to understand the Cpx function in neuroexocytosis but also to understand the relationship between changes in Cpx and mental diseases associated with aberrant neurotransmitter release.
2.5 Figures

Figure 1. Figure caption. *In vitro* single-vesicle content-mixing assay with Cpx. (a) Schematics of the *In vitro* single-vesicle content-mixing assay with Cpx. The flow chamber maintains constant Cpx concentration throughout the experiment by pre-treating t-vesicles with Cpx prior to immobilization to the imaging surface. After the t-vesicles are immobilized on the imaging surface, unbound t-vesicles are washed out with buffer containing the designated Cpx concentration. Subsequent docking and washing of unbound v-vesicles are also performed in the presence of Cpx. Once the v-vesicles and t-vesicles are docked in the presence of Cpx, we inject Ca\(^{2+}\) into the flow chamber to evoke content-mixing which is detected by a sudden step-wise increase of fluorescent intensity. (b) A representative fluorescent intensity time trace is shown. The blue arrow indicates the time of Ca\(^{2+}\) injection and the red arrow depicts content-mixing.
Figure 2. Figure caption. Bell-shaped response of Ca$^{2+}$-triggered SNARE-dependent vesicle fusion in vitro to Cpx. (a) Cumulative fusion-probability with 500 µM Ca$^{2+}$ in the presence (black line) and absence (red line) of 100 nM Cpx. The blue line is with 100 nM Cpx only, without Syt1. Controls using 500 µM Mg$^{2+}$ in the presence of Cpx with and without Syt1 are shown in green and magenta (overlapped with green), respectively. (b) Cumulative fusion-probability triggered with either 10 µM Ca$^{2+}$ or 10 µM Mg$^{2+}$. (c) Total content-mixing percentage among docked vesicle pairs over 60 s period in the presence of 0, 50, 100, 200, 400, 800 and 4000 nM Cpx triggered by 500 (black line) and 10 µM Ca$^{2+}$ (red line) respectively. Error bars are standard deviations (S.D.) obtained from five independent data acquisitions with independently prepared samples.
Figure 3. Figure caption. First order time-constant for content-mixing exhibits little change with various Cpx concentrations. (a) Representative plot of content-mixing events triggered by 500 µM Ca²⁺ versus time in the presence of 100 nM Cpx (blue bars). The data was fitted by the first order kinetics with the time constant of ~2.6 s (red line). (b) Histogram of the first order time constant for content-mixing with 0, 50, 200, 400, 800 and 4000 nM Cpx triggered by 10 µM Ca²⁺. The time constants for 0 and 4000 nM Cpx are not determined due to insufficient fusion events. (c) Histogram of the first order time constant for content-mixing with 0, 50, 200, 400, 800 and 4000 nM Cpx triggered by 500 µM Ca²⁺. Error bars and standard deviations (S.D.) are obtained from three independent data acquisitions with independently prepared samples.
Figure 4. Figure caption. The N-terminal region of Cpx is necessary for the enhancement of content-mixing. Histogram of the content-mixing percentage among docked vesicle pairs triggered by 10 µM Ca^{2+} in the presence of 100 nM Cpx wild-type, Cpx 27 and Cpx M5E/K6E. Content-mixing with 100 nM Cpx and 20 µM VpS are also shown as a control. Error bars are standard deviations (S.D.) obtained from five independent data acquisitions with independently prepared samples.
2.6 Reference


CHAPTER 3

SUMMARY AND FUTURE DIRECTION

3.1 Summary

It’s widely accepted SNARE as the core machinery for membrane fusion. SNARE only mediated fusion is slow and not synchronized, prompting the role of regulatory protein. By using overexpression, knock down or knock out method could gain limit information, it’s ideal to separate the membrane fusion system in vitro to gain the step-by-step mechanism. Mixing assay, developed as one in vitro fusion system, has suffered defect by its low fusion percentage and slow time scale. By looking at the content mixing data, its low fusion percentage add bias to interpret the data. Also, Complexin, with four domain structure, shows up both inhibitory and facilitatory role. In this study, by incorporating nM level of complexin in the system, the content mixing percentage increase extremely from less than 10% to around 70% percent at 500Um Ca2+ concentration. Also, by using the optimized system, fusion percentage showed an interesting concentration dependent trend about CPX. The fusion event peaks at between 100-200 nM CPX. Above 200 nM CPX, the fusion percentage drops quickly till 4uM CPX. This gives possible explanation about why CPX inhibits fusion when it’s overexpressed but still work as promoter from the knockout experiment.

3.2 Future direction

Though the fusion percentage has been highly improved, the time scale stays around 5s at 500uM Ca^{2+}, far away from ms scale. One hypothesis for the slow time scale is under content mixing assay, the fusion happens between sphere to sphere. However, in the psychological condition, fusion happens between sphere and planar membrane. To test this
hypothesis out, different membrane could be utilized like supported bilayer. Also, based on the concentration dependent response of CPX role to complexin concentration, it’s possible to revisit the old content mixing data about CPX to further clarify function of CPX in membrane fusion.