Mechanistic insights into the regulation of neuronal SNAREs mediate membrane fusion: interplay between core fusion machinery and regulatory proteins

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Mechanistic insights into the regulation of neuronal SNAREs mediate membrane fusion --interplay between core fusion machinery and regulatory proteins

by

Xiaochu Lou

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

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2015

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Ca$^{2+}$ triggered exocytosis of synaptic vesicle releases neurotransmitter to synaptic cleft, which is critical for the ability of neurons to communicate with one another and essential for brain functions. The neuronal SNAREs syntaxin 1A, SNAP-25, and VAMP2 play a central role in fusion of synaptic vesicles with plasma membrane through assembly of SNARE complex in chasm of two membranes that bridges the synaptic vesicles and plasma membrane closely together even through fusion. However, to gain a precise temporal control of the release which is essential for healthy brain activities, other regulatory proteins such as synaptogamins, complexins, Munc18-1, and Munc13, are also required. Defining how each proteins interact and function together to orchestrate fast and synchronous synaptic vesicles fusion is essential for understanding the mechanism of neurotransmitter release and neuron communication. Though structural and functional studies have yielded extensive knowledge about physiological role and molecular mechanism of these proteins, a clear and comprehensive picture of the whole process from vesicles docking to Ca$^{2+}$ triggered fast fusion is still elusive.

In this work, we used recently developed total internal reflection (TIR) microscopy based single molecular assays to study the regulatory mechanism of synaptotagmin 1 (Syt1) and Munc18-1, which is significant and innovative that the new assays are capable of resolving SNARE assembly, vesicles docking, lipid mixing, hemifusion, and fusion pore opening steps, thereby facilitate to delineate the function of regulatory proteins onto individual fusion steps. Combining with site-directed spin labeling (SDSL) EPR and other biochemical and biophysical assays, our results showed that the linker region of Syt1 between its
transmembrane domain and cytoplasm C2 domains, which contains the basic amino acid-rich N-terminal region and the acidic amino acid-rich C-terminal region, is essential for its two signature functions: Ca\(^{2+}\)-independent vesicle docking and Ca\(^{2+}\)-dependent fusion pore opening. We also found that the polybasic region of Syt1, which is known important for SNARE-binding, may not be as essential for fusion pore opening as the Ca\(^{2+}\) binding and membrane penetration regions of Syt1. These results delineate multiple functions of Syt1 along the pathway of Ca\(^{2+}\)-triggered exocytosis in unprecedented detail. Moreover, we found that though Munc18-1 could promote SNARE assembly and lipid mixing in the absence of Syt1, the enhancement is abolished in the presence of Syt1 as well as that Munc18-1 didn’t affect fusion pore opening mediated by SNAREs and Syt1/Ca\(^{2+}\). This work shed some light on a puzzle for a long time that whether Munc18-1 is part of the central fusion machinery in neuronal system.
CHAPTER 1

GENERAL INTRODUCTION

1.1 Neurotransmitter Release

Neurotransmitter release is a critical step for normal brain function, which requires the Ca\(^{2+}\)-triggered membrane fusion between synaptic vesicle and pre-synaptic plasma membrane that releases neurotransmitters from pre-synaptic terminals. Neurotransmitter release requires three stages: 1, a dockings step that active synaptic vesicles filled with neurotransmitters are translocated to specialized area of plasma membrane called active zone; 2, a priming process involves all required reactions that allow the vesicles ready to release; 3, a fast fusion step that Ca\(^{2+}\) influx due to action potential arrival triggers fusion between vesicles and plasma membrane in highly cooperative manner (Figures 1) (Jahn and Fasshauer, 2012). In the past decades, intensive efforts have been carried out to delineate and understand machinery and regulatory proteins to execute fusion precisely and efficiently. This process is energy required and exactly drove by a serial of proteins such as SNAREs, synaptotagmin 1, Munc18-1, Munc13, complexin, and so on (Jahn and Scheller, 2006; Chapman, 2008; Sudhof and Rothman, 2009; Sudhof, 2013; Rizo and Xu, 2015). SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) is the central fusion machinery that pulling opposing membrane tightly together once assembled, which can be disassembled by ATPase NSF together with SNAP. Munc18-1 and Munc13 are important in preparing the SNARE for assembly. Complexin functions as a fusion clamp and may be displaced by Ca\(^{2+}\) sensor synaptotagmin 1 upon Ca\(^{2+}\) influx. However, a definitive mechanism of the synaptic vesicle fusion process is still elusive (Rizo and Xu, 2015).
1.2 SNAREs

The soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins mediate membrane fusion, which is essential for all exocytotic pathways (Rothman, 1994). SNAREs are a highly conserved set of proteins which contain 1 or 2 heptad-repeat sequences of 60~70 residues termed SNARE motif which is predicted to have a high probability to form coiled coil (Lupas, 1996; Jahn and Scheller, 2006).

The best characterized SNAREs are involved in neuro-exocytosis through synaptic vesicle fusion, which is governed by the synaptic vesicles (v-) SNARE VAMP2 (vesicle-associates membrane protein 2, also known as synaptobrevin 2) and plasma membrane (t-) SNAREs SNAP-25 (synaptosomal-associated protein of 25 kDa; no relation to SNAPs) and syntaxin 1A. These neuronal SNAREs were first individually identified from nervous system (Trimble et al., 1988; Baumert et al., 1989; Oyler et al., 1989; Südhof et al., 1989; Bennett et al., 1992), and then identified together as SNAP (soluble NSF-attachment protein) receptor (Söllner et al., 1993b). Neuronal SNAREs are critical component of the exocytotic machinery during synaptic vesicle fusion since they were identified as targets for botulinum and tetanus toxins, which specifically proteolyze SNAREs that block exocytosis (Jahn and Scheller, 2006). VAMP2 and syntaxin 1A each contains one SNARE motif connecting to a C-terminal transmembrane (TM) domain by a short linker-region and is targeted to the synaptic vesicle and the plasma membrane, respectively, by its C-terminus transmembrane domain. SNAP-25 contains two SNARE motifs and is attached to plasma membrane by palmitoylation-modified cysteine residues (Hess et al., 1992; Söllner et al., 1993a) (Figures 2). The three proteins are initially unstructured and assemble spontaneously into a stable and SDS-resistant ternary
complex with a melting temperature \( (T_m) \) of 90 °C \((\text{Söllner et al., 1993a; Hayashi et al., 1994; Fasshauer et al., 1997a; Fasshauer et al., 1997b})\), which is predicted through forming a four-helix coiled coil with 1:1:1 stoichiometry \((\text{Fasshauer et al., 1997b; Hanson et al., 1997})\). The ternary SNARE complex can be disassembled by ATPase NSF (N-ethylmaleimide-sensitive fusion protein), together with SNAPs in the presence of ATP and Mg\(^{2+}\) to recycle the SNAREs for another round of fusion \((\text{Söllner et al., 1993a; Hayashi et al., 1995; Otto et al., 1997})\). However, the orientation of these helices was not known initially. Originally, it is proposed that SNAREs together with NSF and SNAP consist the essential fusion machinery based upon a discovery of a 20S complex \((\text{Söllner et al., 1993b})\), and NSF disassembles the pre-assembled SNARE complex drive the membrane fusion. It was soon established that SNAREs alone can form stable complex in the absence of NSF and SNAP \((\text{Söllner et al., 1993a})\), and NSF is not necessary for fusion in yeast \((\text{Mayer et al., 1996; Nichols et al., 1997})\).

An electron microscopy work and a fluorescence resonance energy transfer (FRET) work showed that syntaxin 1A and VAMP2 aligned parallel in the complex with their transmembrane regions located at the same end \((\text{Hanson et al., 1997; Lin and Scheller, 1997})\). Soon after that, two independent works by electron paramagnetic resonance spectroscopy (EPR) and X-ray crystallography determined the core SNARE structure as parallel four-helix bundle \((\text{Figures 3})\) \((\text{Poirier et al., 1998; Sutton et al., 1998})\). The parallel orientation of two SNAP-25 helices were suggested to be connected by a 54-residue loop between the two helices, which allow the lipid anchor of SNAP-25 to align with the transmembrane domain of both syntaxin 1A and VAMP2 \((\text{Poirier et al., 1998; Sutton et al., 1998})\). The individual residues in heptad-repeat pattern can be designated \(a-g\), with the \(a\) and \(d\) positions generating a
hydrophobic α-helical contact surface (Figures 4) (Poirier et al., 1998). This was confirmed by the structure of the core SNARE complex, which contains multiple layers of interacting hydrophobic side chains. Moreover, a central ionic layer which is formed by one arginine side-chain from VAMP2 and three Glutamine side-chains from syntaxin 1A and SNAP-25 was also found (Figures 4). Then these features are found conserved in all SNARE complexes. Accordingly, SNARE motifs are also classified into R, Qa, Qb, and Qc types, which provides a more precise structural classification for most of the fusion reactions than the original target-(t-) and vesicle-(v-) SNAREs classification (Fasshauer et al., 1998; Jahn and Scheller, 2006).

Thereafter, a zippering model that SNARE assembly other than disassembly might drive the membrane fusion had been proposed (Jahn and Scheller, 2006). A more recently X-ray structure of neuro-SNAREs complex that includes the transmembrane regions of both syntaxin 1A and VAMP2 showed that both syntaxin 1A and VAMP2 form continuous helical structures throughout their SNARE motifs, linker regions, and transmembrane regions (Figure 3) (Stein et al., 2009). This process, by forming cis-SNARE complex, is proposed to pull the opposing membranes together and mediate fusion.

The most direct evidence to show SNAREs can mediate membrane fusion was first reported by a proteoliposome fusion work (Weber et al., 1998). Recombinant v- and t-SNAREs were reconstituted into lipid bilayer vesicles and could functionally assemble SANRE complex between the vesicles thus lead to fusion of the membrane. However, this process is spontaneous and slow (about 10 min half time) (Weber et al., 1998). It was also
shown that even one to three SNAREs are sufficient to fuse two membranes (Mohrmann et al., 2010; van den Bogaart et al., 2010; Sinha et al., 2011; Shi et al., 2012; Rizo and Xu, 2015).

All these work suggested that the transition from largely unstructured monomers to a tightly packed, energetically favored ternary complex following an N- to C- terminus zipping fashion (Pobbati et al., 2006; Sorensen et al., 2006) and bringing vesicle and membrane closely together is a key step in overcoming energy barriers for membrane fusion (Fasshauer et al., 1997b). Plasma membrane SNAREs syntaxin 1A and SNAP-25 form an acceptor intermediate precede to the VAMP2 binding (Pobbati et al., 2006). However, it is unclear how the energy is transfer to the membrane that promotes the fusion.

It is believed that SNARE assembly takes place in multiple steps (Fiebig et al., 1999) that mediates membrane fusion intermediates (Hernandez et al., 2012). It is easy to propose that the initially loosely structured v- and t-SNARES assemble to post fusion cis-SNARE four-helix bundle through a pre-fusion trans-SNARE intermediates. However, the trans-SNARE structure is hard to obtain (Jahn and Scheller, 2006). The trans-SNARE pairs was first defined as functional transient state which leads to the further fusion process (Ungermann et al., 1998) and clearly confirmed by the reconstitution work (Weber et al., 1998). On contact, the SNARE proteins assemble in trans starting at the N-terminus of the SNARE motifs. Recently, single molecule tweezers techniques captured an intermediate structure in which the N-terminal helical structure is robust while the C-terminal halves are not assembled yet (Gao et al., 2012; Min et al., 2013b; Zorman et al., 2014). An EPR investigation revealed further
details of the intermediate structure and single molecule (sm)FRET was used to monitor SNARE zippering (Shin et al., 2014; Lou et al., 2015). More significantly, the pre-fusion trans-SNARE intermediate is believed to be the main point of regulation by other key components of fusion machinery during the neurotransmitter release. Although it is thought that the neuronal SNAREs do not have the required regulatory function that confers the temporal on/off switching capability, they do contain according sequences to interplay with the regulatory proteins, which will be discussed in more detail below.

1.3 Munc18-1

Munc18-1 is a soluble neuronal protein that belongs to the Sec1/Munc18 (SM) family (Figure 5) (Hata et al., 1993), which participates and is essential for every SNARE-dependent fusion reactions (Sudhof, 2013). The essential role of Munc18-1 during neurotransmitter release was shown that deletion of Munc18-1 protein leads to complete loss of neurotransmitter release from synaptic vesicles (Verhage et al., 2000) and pre-incubation of Munc18-1 can accelerate membrane fusion significantly in recombinant SNAREs reconstituted vesicle assay (Shen et al., 2007). Though Munc18-1 is thought to control the vesicle docking and initial SNARE complex assembly, the molecular mechanism that why Munc18-1 is essential for neurotransmitter release is still unclear. Moreover, there is still debate whether it directly play in fusion or merely has regulatory functions (Rizo and Xu, 2015).

It has been shown that SNARE motifs from neuron SNARE proteins could also associate into other kinds of helix bundles different from the core complex four-helix bundle (Jahn and Scheller, 2006). One of the most significant of such complexes is acceptor complex
composed by 2 SNARE motifs from syntaxin 1A and both the N- and C-terminus SNARE motifs from SNAP-25 (Margittai et al., 2001; Xiao et al., 2001; Zhang et al., 2002a), which prevents the further binding of V-SNARE VAMP2 thus may be “dead-end” and not able to proceed to drive membrane fusion (Fasshauer and Margittai, 2004). Though an activate acceptor complex can be stabilized by a C-terminal fragment of SNARE motif of VAMP2 in vitro, which results in significant acceleration of SNARE assembly (Pobbati et al., 2006), according regulatory mechanism is needed for correct SNARE zippering in vivo but unsolved yet. Munc18-1 was proposed as the primary candidate.

Different to VAMP2 and SNAP-25 that don’t have extra domain, syntaxin 1A has an N-terminal peptide preceding to an evolutionarilty conserved N-terminal domain folding in to up-and-down antiparallel three-helix bundle (Fernandez et al., 1998), termed Habc domain. The Habc domain is connected to the SNARE motif by a flexible linker region (Figure 2). Moreover, it was later shown that syntaxin 1A is presented in a “closed” form in which its Habc domain could fold against its SNARE motif with the assistant of Munc18-1 (Figure 5) (Dulubova et al., 1999; Misura et al., 2000). This “closed” conformation of syntaxin 1A prevents core complex assembly which requires an “opening” syntaxin 1A conformation (Dulubova et al., 1999), which provides a regulatory basis for SNARE complex assembly. It has been shown that the N-terminal peptide and the Habc domain of syntaxin 1A play essential functions in synaptic vesicle fusion (Zhou et al., 2013b), which may be derived from their interplay with Munc18-1. The “closed” syntaxin-Munc18 is shown be opened by Munc13 to proceed to SNARE complex assembly (Ma et al., 2011). Other binding modes of syntaxin-Munc18 were recognized also that Munc18-1 may bind to the N-terminal peptide of syntaxin
1A in an “opening” conformation (Burkhardt et al., 2008; Sudhof, 2013) and bind to assembling SNARE complex (Xu et al., 2010; Sudhof, 2013) (Figure 5). Recently, a working model of how Munc18-1 switches interaction with SNARE proteins and functions in different fusion steps is proposed (Figure 6) (Rizo and Sudhof, 2012), and partially proved by in vitro reconstitution work (Ma et al., 2013). However, this model still not able to confirm that if Munc18-1 really functions as core fusion machinery and participates in the final fusion step by proving free energy. Whereas, more recently independent single vesicle assays showed that Munc18-1 may not play role during the final fusion steps but synaptotagmin 1/Ca\(^{2+}\) may take control of the SNARE mediated fusion pore opening (Lou et al., 2015; Zhang et al., 2015), which suggest further investigation is required.

### 1.4 Synaptotagmin 1

One of the most important features of neurotransmitter release is that fast and synchronous synaptic vesicle fusion (µs-ms scale) is triggered by Ca\(^{2+}\) influx upon action potential arrival (Neher and Zucker, 1993; Schneggenburger and Neher, 2000). The neuronal synaptotagmin 1 (Syt1, or originally named p65) is known as a major Ca\(^{2+}\)-sensor of fast synchronous neurotransmitter release (Brose et al., 1992; Geppert et al., 1994; Fernandez-Chacon et al., 2001; Chapman, 2008), which was identified widely distributed in neuronal and neural secretory tissue and characterized even earlier than neuronal SNAREs (Matthew et al., 1981; Perin et al., 1990; Perin et al., 1991a). Syt1 is an abundant and conserved synaptic vesicle protein that contains an intravesicular glycosylated N-terminal domain, a single transmembrane helical region, a putatively unstructured linker region (~60 amino acids) preceding to two tandem cytoplasmic Ca\(^{2+}\) binding C2 domains (termed as C2A
and C2B, respectively or C2AB to represent the cytoplasmic region of Syt1) (Figure 7) (Perin et al., 1991a). Both the C2 domains are homologous to the regulatory C2-domain of protein kinase C (PKC) with slightly more homologous to each other than to PKC (Perin et al., 1991b). The cytoplasmic C2 domains was first shown to bind to acidic phospholipids similar with PKC (Perin et al., 1990) that suggests a role in mediating membrane interaction during synaptic vesicle exocytosis.

Syt1 was shown to specifically bind Ca\(^{2+}\) and phospholipids in mutually dependent manner through both of its C2 domains independently (Brose et al., 1992; Davletov and Sudhof, 1993; Chapman and Jahn, 1994; Desai et al., 2000). Atom structure works by X-ray and NMR showed that both C2A and C2B fold into eight-stranded β-barrels and with Ca\(^{2+}\)-binding pockets formed by two protruding loops, and three Ca\(^{2+}\) were incorporated in C2A and two Ca\(^{2+}\) were incorporated in C2B, respectively, through the interaction with a set of acidic amino acids in the Ca\(^{2+}\) binding loops (Figure 7) (Sutton et al., 1995; Ubach et al., 1998; Fernandez et al., 2001; Fuson et al., 2007). The Ca\(^{2+}\) binding may induce a conformational change to Syt1 as indicated from comparative structures of Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free Syt3 C2AB fragments (Sutton et al., 1999; Vrljic et al., 2010). Further studies showed that Syt1 could bind membrane in both Ca\(^{2+}\) dependent (Chapman and Jahn, 1994) and independent (Brose et al., 1992) manners although Syt1 selectively interacts with negatively charged lipid in both manners.

The functional importance of Syt1-Ca\(^{2+}\) interaction for synaptic vesicle fusion is supported by genetic and biochemical studies using various Ca\(^{2+}\) binding mutants, in which
the Ca\(^{2+}\) binding sites were disrupted by point mutations that neutralize the acidic Ca\(^{2+}\) binding ligands in each C2 domain, as well as the mutants alter Ca\(^{2+}\) sensing ability of Syt1.

However, C2A and C2B domain may not be just functional duplication, but they work cooperatively. Syt1 has both trans- and cis- membrane binding properties which was proposed important for its function through synaptic vesicles docking until fusion pore opening (Lee et al., 2010; Vennekate et al., 2012). Syt1 was also implicated to participate in synaptic vesicle docking as it was shown to bind the plasma membrane protein syntaxin and syntaxin/SNAP-25 complex (binary SNARE complex) in Ca\(^{2+}\) independent manner (Bennett et al., 1992; de Wit et al., 2009; Kim et al., 2012). Moreover, Syt1 was shown to bind to ternary SANRE complex in both Ca\(^{2+}\) independent and dependent manner (Sutton et al., 1999).

Very early works had proposed that Syt1 serves as a fusion clamp for SNARE-mediated fusion until Ca\(^{2+}\) influx triggering the synchronous fusion (Söllner et al., 1993a), which is supported by the genetic studies that spontaneous synaptic vesicle fusion was increased by Syt1 null strains in fly (DiAntonio and Schwarz, 1994; Littleton et al., 1994; Chapman, 2008) and recently work in vitro (Chicka et al., 2008).

How Syt1/Ca\(^{2+}\) triggers fast vesicle fusion, there are several models: 1, Syt1 could promote SNARE-mediated fusion by lowering the activation energy of bilayer-bilayer fusion through inducing high positive curvature in target membrane upon Ca\(^{2+}\)-dependent C2 domains membrane insertion (Martens et al., 2007). 2, Syt1 function as a clamp before Ca\(^{2+}\) influx. 3,
Syt1 displaced complexin from a metastable SNARE complex upon Ca\(^{2+}\) influx (Tang et al., 2006). However, a definitive description of the mechanism is still elusive.

1.5 Complexin

Complexin is a family of small soluble protein enriched in neurons, which was discovered by virtue of its tight binding to SNARE complexes (McMahon et al., 1995), and suggested as an essential regulator for normal transmitter release that the fast synchronous exocytosis is suppressed and spontaneous exocytosis is increased in complexin-deficient neurons (Reim et al., 2001). A seminal X-ray work shows that Cpx binds to the SNARE core, potentially stabilizing the four-helix bundle (Chen et al., 2002). However, the structure is not able to explain complexin’s function simply, and several alternative models have been proposed but all of them were either not verified experimentally or deemed unsatisfactory (Figure 8) (Giraudo et al., 2009; Maximov et al., 2009; Lu et al., 2010; Kimmel et al., 2011; Cho et al., 2014; Trimbuch et al., 2014; Krishnakumar et al., 2015), which suggests further study is needed.

1.6 Methodology

In the work of this thesis, we used recently developed total internal reflection (TIR) microscopy (Roy et al., 2008) based single molecular assays (Figure 9) (Kyoung et al., 2011; Diao et al., 2012b; Shin et al., 2014) to study the regulatory mechanism of Syt1 and Munc18-1, which is significant and innovative that the new assays are capable of resolving SNARE assembly (Bae et al., 2013), vesicles docking (Lai et al., 2015; Lou et al., 2015), lipid mixing (Yoon et al., 2006; Lou et al., 2015), and fusion pore opening and expansion steps (Lai et al.,
2013a), thereby facilitate to delineate the function of regulatory proteins onto individual fusion steps (Brunger et al., 2015). More innovatively, we developed a nanodisc sandwich that harbors a single SNAREpin (Figure 10) (Shin et al., 2014), which we believe best represents the pre-fusion SNAREpin that give us ability to research the structure and regulation of SNARE assembly in trans. We also used a well-established site-directed spin labeling (SDSL) EPR to study the local flexibility and/or conformational changes. Combining with other well applied biochemical and biophysical assays, we are able to understand the SNARE-mediated membrane fusion and its regulation in molecular level and step by step.

1.7 Thesis Organization

This thesis is organized into five chapters: a general introduction chapter (Chapter 1), three chapters describing the scientific results (Chapter 2-4), and a general summary chapter (Chapter 5). In chapter 2, we reported that the linker region of Syt1 between its transmembrane domain and cytoplasm C2 domains, containing the basic amino acid-rich N-terminal region and the acidic amino acid-rich C-terminal region, is essential for its two signature functions: Ca\(^{2+}\)-independent vesicle docking and Ca\(^{2+}\)-dependent fusion pore opening. In chapter 3, we found that the polybasic region of Syt1, which is known important for SNARE-binding, may not be as essential for fusion pore opening as the Ca\(^{2+}\) binding and membrane penetration regions of Syt1. These results delineate multiple functions of Syt1 along the pathway of Ca\(^{2+}\)-triggered exocytosis in unprecedented detail. Moreover, in chapter 4, we found that though Munc18-1 could promote SNARE assembly and lipid mixing in the absence of Syt1, the enhancement is abolished in the presence of Syt1 as well as that Munc18-1 didn’t affect fusion pore opening mediated by SNAREs and Syt1/Ca\(^{2+}\). This work shed some light on a puzzle for
a long time that whether Munc18-1 is part of the central fusion machinery in neuronal system.

The final chapter simply summarized the work we have accomplished and discussed some ongoing idea and future work.
1.8 Reference


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1.9 Figures and Legends

Figures 1. A simplified mechanistic model for Ca$^{2+}$-triggered synaptic vesicle fusion. (A) Synaptotagmin 1 (Syt1, purple) binds to t-SNARE, facilitating vesicle docking. (B) v-SNARE VAMP2 (blue) and t-SNARE zipper to form the pre-fusion SNAREpin, but the complex is clamped by complexin (Cpx, yellow). (C) Ca$^{2+}$ influx activates Syt1, which in turn, unclamps Cpx and turns on the fusion machinery to drive fusion pore opening. See text and references for more details.
Figures 2. Domain diagrams of the neuronal SNAREs syntaxin 1A, SNAP-25, and VAMP2. SNARE motifs are colored in red (syntaxin 1A), green (SNAP-25), and blue (VAMP2). In syntaxin 1A and VAMP2, the transmembrane regions are labeled TM and colored in yellow and the linker regions are labeled L and colored in grey. The Habc domain (white) of syntaxin 1A is depicted broken to indicate the longer length than the SNARE motif (red). The syntaxin 1A N-terminal peptide is labeled N (grey). In SNAP-25, the palmitoylated cysteines are denoted as lines. Numbers on the right above the diagrams indicate the length of the proteins.
Figures 3. Overall structures of SNARE complex. (A and B) Backbone ribbon drawing of the core SNARE complex: blue, VAMP2; red, syntaxin 1A; green SNAP-25. The SNARE motifs are aligned in parallel, and the N and C in round brackets indicate the N- and C-terminus of the protein respectively. (C) Model of cis-SNARE complex inserted in a POPC membrane. The figures are re-prepared with PyMOL using the PDB files 1SFC (A and B) and 3HD7 (C). See text and references for more details (Sutton et al., 1998; Stein et al., 2009).
Figures 4. Layer organization of the SNARE core complex. Each layer is generated by black line-connected Cα of according layer residues (bold in sequences listed below). The Cα ribbons are color as the same as Figure 2 and 3. The 0 or ionic layer residues are highlighted in bolded red characters. The figures are re-prepared with PyMOL using the PDB files 1SFC. See the text and reference for more detail (Sutton et al., 1998).
Figure 5. (A) Domain diagrams of Munc18-1. (B and C) Diagrams of Munc18-1, Habc domain of syntaxin 1A, SNARE core complex, and Munc18-1/syntaxin 1A complex. The figures are reproduced from Figure 2 in reference (Rizo and Sudhof, 2012), see the reference for detail.
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Figure 7. Domain and diagram of synaptotagmin 1. The figures are reproduced from Figure 3 in reference (Chapman, 2008), see the reference for detail.
Figure 8. Three structural models for the interaction between Cpx and the SNARE complex. (A) Cpx binds to the groove in the SNARE four-helix bundle. (B) The trans-insertion model. Cpx displaces the SNARE motif of v-SNARE locally. (C) The zig-zag model. Cpx cross-links two neighboring SNARE complexes.
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CHAPTER 2

THE SYNAPTOTAGMIN 1 LINKER MAY FUNCTION AS AN ELECTROSTATIC ZIPPER THAT OPENS FOR DOCKING BUT CLOSES FOR FUSION PORE OPENING


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2.1 Abstract

Synaptotagmin 1 (Syt1), a major Ca\(^{2+}\) sensor for fast neurotransmitter release, contains tandem Ca\(^{2+}\)-binding C2 domains (C2AB), a single transmembrane α-helix, and a highly charged 60-residue-long linker in between. Using the single vesicle docking and content mixing assay we found that the linker region of Syt1 is essential for its two signature functions: Ca\(^{2+}\)-independent vesicle docking and Ca\(^{2+}\)-dependent fusion pore opening. The linker contains the basic amino acid-rich N-terminal region and the acidic amino acid-rich C-terminal region. When the charge segregation was disrupted, fusion pore opening was slowed while docking was unchanged. Intramolecular disulfide cross-linking between N- and C-terminal regions of the linker or deletion of 40 residues from the linker reduced docking while enhancing pore opening, although the changes were subtle. The EPR analysis showed Ca\(^{2+}\)-induced line broadening reflecting a conformational change in the linker region. Thus, the results suggest that the electrostatically bipartite linker region may extend for docking and fold to facilitate pore opening.
2.2 Introduction

Neurotransmitter release at the synapse constitutes the fundamental basis for major brain functions including cognition, memory, and motor control. The precise temporal control of the release is essential for healthy brain activities. SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) are known to be the core fusion machinery in neuro-exocytotic pathways (Weber et al., 1998; Brunger, 2005; Jahn and Scheller, 2006). SNARE complex formation between the synaptic vesicle and the plasma membrane is mediated by the cognate coiled coil motifs on vesicle (v-) and target plasma membrane (t-) SNAREs: one such motif from t-SNARE syntaxin 1A, two from t-SNARE SNAP-25, and another from v-SNARE VAMP2 form a parallel four-stranded coiled coil, which brings about the apposition of two membranes (Poirier et al., 1998; Sutton et al., 1998; Stein et al., 2009). SNARE complex formation might proceed in sequential steps. Two or three distinct stages have been observed with optical or magnetic tweezers set-ups (Gao et al., 2012; Min et al., 2013a). It is however thought that SNARE proteins themselves do not have the required regulatory function that controls the timing of SNARE complex formation (Rizo and Rosenmund, 2008; Wickner and Schekman, 2008), which confers the temporal on/off switching capability for vesicle fusion. A vesicular protein synaptotagmin 1 (Syt1) is instead believed to be the key regulator, which senses the spike of the Ca\(^{2+}\)-level in response to the action potential and helps trigger fast vesicle fusion (Chapman, 2002; Sudhof, 2004; Rizo and Rosenmund, 2008).

At the molecular level, Syt1 contains a transmembrane domain, a putatively unstructured linker region (~60 amino acids) and two cytoplasm tandem C2 domains (C2AB)
that bind Ca$^{2+}$ (Perin et al., 1991a). Syt1 is thought to be involved both in docking and fusion pore opening (de Wit et al., 2009; Lee et al., 2010; Lai and Shin, 2012). In early steps, Syt1 binding to the binary t-SNARE (syntaxin 1A/SNAP-25) on the plasma membrane may mediate vesicle docking (de Wit et al., 2009; Kim et al., 2012; Lai and Shin, 2012; Vennekate et al., 2012). Recent *in vitro* experiments indicate that the negatively charged lipid PIP$_2$ plays a role in docking via the t-SNARE-Syt1 interaction (Kim et al., 2012). In response to the Ca$^{2+}$ influx, Syt1 inserts itself into the target plasma membrane (Arac et al., 2006; Hui et al., 2009), which triggers membrane fusion (Martens et al., 2007; Hui et al., 2009).

Mechanistically, C2AB is considered as the functional domain and has been widely used as a soluble substitute for Syt1 (Tucker et al., 2004; Schaub et al., 2006; Xue et al., 2008; Hui et al., 2011). However, recent studies have indicated that C2AB is not an adequate model to recapitulate important Syt1 functions (Stein et al., 2007; Wang et al., 2011). C2AB promotes fusion by aggregating vesicles in response to Ca$^{2+}$ and thereby enhancing v- and t-SNARE pairing (Xue et al., 2008; Diao et al., 2009a; Hui et al., 2011; Wang et al., 2011), while Syt1 is supposed to stimulate membrane fusion by the *trans*-interaction with the plasma membrane (Stein et al., 2007; Lee et al., 2010; Kyoung et al., 2011; Lai and Shin, 2012). For the *trans*-interaction the 60 residue-long linker region appears to be essential as an *in vitro* study suggests that Syt1 is a distance regulator reaching out to the plasma membrane using this long linker (van den Bogaart et al., 2011).

Here using the single vesicle docking and content mixing assay, which has the capacity to detect the docking and the fusion pore opening steps separately (Diao et al., 2012a; Kyoung
et al., 2013; Lai et al., 2013b), we found that the Syt1’s linker region is essential for both docking and fusion pore opening. The linker region is featured with the basic residue-rich N-terminal half and the acidic residue-rich C-terminal region. Interestingly, this feature is well conserved in all species from C. elegans to human. When the asymmetric charge distribution was disrupted by double or triple mutations, it hardly affected the docking step, but impaired fusion pore opening. However, disulfide cross-linking between the basic and the acidic regions reduced docking while it enhanced Ca\textsuperscript{2+} triggered fusion pore opening. Our site-directed spin labeling (SDSL) and EPR analysis of the linker showed that upon Syt1 binding to t-vesicles in the presence of Ca\textsuperscript{2+} it became motionally restricted. However, this conformational change was not seen when the charge segregation in the linker was disrupted. Thus, our results suggest that the flexible linker region of Syt1 undergoes conformational changes during vesicle fusion: it stretches out to mediate vesicle docking but folds to assist C2AB for fusion pore opening.

2.3 Materials and methods

2.3.1 Plasmid Constructs and Site-Directed Mutagenesis

DNA sequences encoding rat syntaxin 1A (amino acids 1-288 with three native cysteines C145, C271, and C272 replaced by alanines), rat VAMP2 (amino acids 1-116 with C103 replaced by alanine), rat SNAP-25 (amino acids 1-206 with four native cysteines C85, C88, C90, and C92 replaced by alanines), C2AB (amino acids 140-421), soluble syntaxin 1A (amino acid 191-266), and soluble VAMP2 (amino acids 1-96) were inserted into the pGEX-KG vector as N-terminal glutathione S-transferase (GST) fusion proteins. SNAP-25 was also inserted into pET-28b vector as N-terminal 6xHis tag fusion protein. Full-length 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 a C-terminal His-tagged protein. We used the Quick Change site-directed mutagenesis kit (Stratagene) to generate all Syt1 mutants including Syt1 K86E, K86E/K90E, K86E/K90E/K95E, E131K, E131K/E135K, E131K/E135K/E139K, C277A/G92C/G130C and Syt1 linker deletion mutant (Δ(99-140)aa). DNA sequences were confirmed by the Iowa State University DNA Sequencing Facility.

2.3.2 Protein expression and purification

The GST-tagged proteins were expressed in *E. coli* Rosetta (DE3) pLysS (Novagen). Details can be found in our previous work (Lai and Shin, 2012). The His-tagged proteins were expressed in an *E. coli* BL21 (DE3) (Novagen) and purified with the same protocol as previously described (Lai and Shin, 2012).

2.3.3 Membrane reconstitution

The lipid molecules used in this study are 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), cholesterol, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamin-N-(biotinyl) (biotin-DPPE). All lipids were obtained from Avanti Polar Lipids. 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine Perchlorate (DiI), 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindodicarbocyanine Perchlorate (DiD), and sulforhodamine B were obtained from Invitrogen.
For the bulk lipid mixing assay, the molar ratios of lipids were 15:61:20:2:2 (DOPS:POPC:cholesterol:PIP$_2$:DiI) for the t-SNARE-reconstituted (t-)vesicles, and 5:73:20:2 (DOPS:POPC:cholesterol:DiD) for the v-SNARE-reconstituted (v-)vesicles, respectively. For the single vesicle docking assays, 0.1% Biotin-DPPE was added in v-vesicles. The lipid mixture was first completely dried and then hydrated by dialysis buffer (25 mM HEPES, pH 7.4, 100 mM KCl). After five freeze–thaw cycles, protein-free large unilamellar vesicles (~100 nm in diameter) were prepared by extrusion through a 100 nm polycarbonate filter (Whatman). For membrane reconstitution, SNARE proteins and Syt1 were mixed with protein free vesicles at the protein to lipid molar ratio of 1:200 for each protein component (this ratio was kept for all experiments including the single vesicle content mixing assay) with ~0.8% OG in the dialysis buffer at 4°C for 15 min. The mixture was diluted two times with dialysis buffer and this diluted mixture was then dialyzed in 2 L dialysis buffer at 4°C for overnight. Details for reconstitution was discussed in our previous work (Lai and Shin, 2012).

For the single vesicle content mixing assay with the small sulforhodamine B content indicator, the lipid compositions were the same as those used in the single vesicle docking assay except that the fluorescent lipid dyes (DiI and DiD) were replaced by the equal amount of POPC. The lipid mixture was first completely dried and then hydrated by dialysis buffer, but a population of vesicles to make v-vesicles was hydrated in the presence of 50 mM sulforhodamine B. The overall vesicle preparation and protein reconstitution process was the same as above except that v-vesicles were always kept in the 50 mM sulforhodamine B prior to dialysis overnight. Free sulforhodamine B was removed using the PD-10 desalting column (GE healthcare) after dialysis.
2.3.4 Bulk lipid mixing assay

Reconstituted t- and v-vesicles were mixed at a ratio of 1:1. The final lipid concentration was 0.1 mM. The fluorescence intensity was monitored in two channels with the excitation wavelength of 530 nm and emission wavelengths of 570 and 670 nm for donor DiI and acceptor DiD, respectively. Fluorescence changes were recorded with the Varian Cary Eclipse model fluorescence spectrophotometer using a quartz cell of 100 μL with a 2 mm path length. All measurements were performed at 35 °C.

2.3.5 Single vesicle docking and content mixing assays

After coating the quartz surface with a solution of methoxy-polyethylene glycol (mPEG) and biotin-PEG molecules to eliminate non-specific binding of vesicles, the quartz slide was assembled into a flow chamber and coated with neutravidin (0.2 mg ml⁻¹). Following 30 minutes incubation at room temperature (~25 °C), the v-vesicles were immobilized on the PEG-coated surface. After two rounds of 200 μl buffer washing, the t-vesicles (100 ~ 200 nM) were injected into the flow chamber for 30 minutes pre-docking at room temperature. After washing out free t-vesicles, the docking probability was calculated by the ratio of docked t-vesicles and total anchored v-vesicles in the imaging area (45×90 μm²). The detail of single vesicle docking assay was reported in our previous work (Lai and Shin, 2012).

For real-time imaging of the small sulforhodamine B content release, the sulforhodamine B containing v-vesicles were immobilized on the PEG-coated surface. After two rounds of washing with 1 ml dialysis buffer, empty t-vesicles were injected into the
channel to make them bind to v-vesicles. After 30 minutes incubation at room temperature (~25 °C), the dialysis buffer with or without 500 µM Ca^{2+} was injected into the flow chamber at a speed of 33 µl sec^{-1} by a motorized syringe pump. The detail of the single vesicle content mixing assay was reported in previous work (Kyoung et al., 2011; Diao et al., 2012a; Lai et al., 2013b).

2.3.6 GST Pull-down assay

To form the binary complex, His-tagged SNAP-25 and GST-tagged soluble syntaxin 1A (191-266) cell lysates were mixed and loaded to Ni-NTA beads. The binary complex was purified following the same procedure described for His-tagged SNAP-25. Then the purified binary complex was loaded to Glutathione-agarose beads. After washing thoroughly with cleavage buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.8% OG) to remove unbound His-tagged SNAP-25, the beads immobilized with the binary complex were separated equally into 1.7 ml Eppendorf tubes. And then about equal amount of wild-type Syt1 or its mutants and equal volume of cleavage buffer were added to the immobilized binary complex and the mixture was incubated at 4 degree for 1h. The beads were then washed thoroughly with the cleavage buffer. 5xSDS-loading buffer (0.313 M Tris-HCl, pH 6.8, 10% SDS, 0.05% bromophenol blue, 50% glycerol, 0.5 M DTT ) was added to the samples and boiled for 10 min. Proteins were resolved on precast 12% SDS-PAGE and visualized by Coomassie blue staining. To test binding of wild-type Syt1 and its mutants to the ternary SNARE complex, purified soluble syntaxin 1A (191-266) was mixed with His-tagged SNAP-25 and GST-tagged soluble VAMP2 (1-96) cell lysates before loading to Ni-NTA beads. The ternary complex was purified following the same procedure described for His-tagged SNAP-25. Then the purified
ternary complex was loaded to Glutathione-agarose beads. After washed thoroughly with cleavage buffer to remove unbound His-tagged SNAP-25 and the binary complex, binding of wild-type Syt1 and its mutants was carried out following the same procedure as the immobilized binary complex, except that 500 μM Ca$^{2+}$ was added to the cleavage buffer all the time.

2.3.7 Cross-linking and gel filtration assays

Native cysteine at position 277 in wild-type Syt1 was mutated to Ala, and then Gly 92 and Gly 130 were mutated to Cys. 1 mM H$_2$O$_2$ was added to the cell lysate before loading to Ni-NTA beads, and then the protein was purified following the same procedure described for wild-type Syt1. To separate the intermolecular disulfide bonded oligomers from intramolecular disulfide-bond monomer the protein was concentrated and loaded to 10/300 GL Superdex 200 column (GE healthcare) on the Bio-rad biologic Duoflow system. Multi-peak Gaussian curves were fitted to the elution profile obtained from the gel filtration experiment using Origin 8.0 (Figure 4A). The sample was collected with the elution volume from 15 to 20 ml as monomer. The samples were analyzed on precast 12% SDS-PAGE and visualized by Coomassie blue staining (Figure 4A, inset).

2.3.8 Site-directed spin labeling and CW-EPR data collection

Single cysteine mutant G130C of wild-type Syt1 and its triple K-to-E and E-to-K mutants were labeled by (1-Oxyl-2,2,5,5-tetramethylpyrroline-3-methyl) methanethiosulfonate (MTSSL), and reconstituted on to v-vesicles with VAMP2. CW-EPR spectra were collected by using the Bruker ESP 300 spectrometer with a loop-gap resonator
(Medical Advances) and a low-noise microwave amplifier (Miteq) at room temperature. To examine the conformational changes derived from t-vesicles binding and Ca\(^{2+}\) binding, three times t-vesicles were added to v-vesicles with or without 1 mM Ca\(^{2+}\).

2.4 Results

2.4.1 Membrane-anchored Syt1 is essential for fusion pore opening

C2AB is often used as an alternative model for Syt1 in a variety of \textit{in vitro} studies (Tucker \textit{et al.}, 2004; Schaub \textit{et al.}, 2006; Xue \textit{et al.}, 2008; Hui \textit{et al.}, 2011). C2AB is shown to recapitulate some features of Syt1 functions such as Ca\(^{2+}\)-triggered enhancement of SNARE-driven lipid mixing, which has been later found to be via aggregating t- and v-vesicles in the presence of Ca\(^{2+}\) (Xue \textit{et al.}, 2008; Diao \textit{et al.}, 2009a; Hui \textit{et al.}, 2011; Wang \textit{et al.}, 2011). Here, we revisited the Ca\(^{2+}\) control of SNARE-dependent lipid mixing by C2AB and that by membrane-anchored Syt1. As demonstrated previously (Xue \textit{et al.}, 2008; Lee \textit{et al.}, 2010; Hui \textit{et al.}, 2011; Wang \textit{et al.}, 2011; Lai and Shin, 2012), both C2AB and Syt1 show strong Ca\(^{2+}\)-dependent stimulation of lipid mixing (Figure 1A and Supplementary Figure S1). Without SNAP-25 no lipid mixing was observed (Figure 1A), indicating that both lipid mixing cases are SNARE-dependent. In the absence of Ca\(^{2+}\), however, C2AB has no effect on lipid mixing while Syt1 has yet substantial stimulatory effect (Figure 1A). This Ca\(^{2+}\)-independent stimulation of lipid mixing by Syt1 may be mainly derived from the enhancement of vesicle docking (Supplementary Figure S3A), similar to the previously published work (Stein \textit{et al.}, 2007; Wang \textit{et al.}, 2011; Kim \textit{et al.}, 2012; Lai and Shin, 2012), due to the t-SNARE-Syt1-lipid interaction, which is absent for recombinant soluble C2AB.
Next, we compared the capacity of soluble C2AB to drive fusion pore opening with that of membrane-anchored Syt1. As shown previously (Lai et al., 2013b), SNAREs alone without Syt1 cannot efficiently open the fusion pore even in the presence of 1 mM Ca\(^{2+}\) (red bars in Figure 1B). Pore opening happens in less than 2% of docked vesicles. With 500 μM Ca\(^{2+}\) Syt1 stimulates fusion pore opening significantly, leading to pore opening for 15% of docked vesicles (black in Figure 1B). Surprisingly, however, when 2 μM C2AB was used instead of membrane-anchored Syt1, we did not observe efficient content mixing in the Ca\(^{2+}\) range up to 2 mM (grey in Figure 1B). When we reconstituted Syt1 into vesicles in the absence of VAMP2, little content mixing was observed in the presence of 1 mM Ca\(^{2+}\) (blue bar in Figure 1B), indicating that the stimulation of fusion pore opening by Syt1 and Ca\(^{2+}\) is strictly SNARE-dependent. On the other hand, when we reconstituted Syt1 into t-vesicles, we observed only very mild enhancement of content mixing (Supplementary Figure S3B). Thus, our results show that anchoring of Syt1 to the vesicular membrane is essential for efficient fusion pore opening.

2.4.2 The linker region of Syt1 is highly conserved but does not contribute to the binding to SNARE complexes.

The fact that C2AB does not reproduce two important functions of Syt1, Ca\(^{2+}\)-independent enhancement of docking and Ca\(^{2+}\)-dependent stimulation of fusion pore opening, shows that the linker region is indispensable for Ca\(^{2+}\)-triggered vesicle fusion. We now take a close look at its amino acid sequence. Strikingly, within the linker region the N-terminal half is enriched with positively charged amino acids while the C-terminal half is abundant in negatively charged amino acids (Figure 2A). Moreover, this feature of the bipolar charge
distribution is highly conserved from C. elegans to human and all of them have almost equal net charge although the amino acids in linker regions are not as highly conserved as those in the C2 domains in different species.

To test if this bipolar charge distribution in the linker region is important for the Syt1’s function, we made several mutants on the linker region to disrupt this asymmetric charge distribution by changing lysine to glutamic acid (K-to-E) or glutamic acid to lysine (E-to-K). We made single, double, and triple K-to-E mutants (K86E, K86E/K90E, and K86E/K90E/K95E mutants, respectively, Figure 2B). We also made single, double, and triple E-to-K mutants (E131K, E131K/E135K, and E131K/E135K/E139K mutants, respectively, Figure 2B). We tested if the bipolar charge distribution is important for the SNARE binding affinity of Syt1. As expected, in our pull-down assay all mutants showed similar binding abilities to t-SNAREs without Ca\(^{2+}\) and to the ternary SNARE complex in the presence of Ca\(^{2+}\) (Figure 2C), indicating that the linker region of Syt1 does not directly contribute to the interaction with SNARE complexes.

2.4.3 The bipolar charge distribution in the Syt1 linker plays a role in fusion pore opening.

To test if the asymmetric charge distribution in Syt1 linker region influences vesicle fusion, we investigated the effect of those Syt1 mutants we made (K86E, K86E/K90E, and K86E/K90E/K95E; E131K, E131K/E135K, and E131K/E135K/E139K) on vesicle docking and content mixing. The single vesicle pairing analysis shows that all mutations have negligible effect on vesicle docking (Figure 3A). In the content mixing assay with 500 μM
Ca\(^{2+}\), we did not observe any change in content mixing for single E-to-K or K-to-E mutants (Figure 3B, left).

However, for double or triple mutants we clearly observed the impairment of fusion pore opening (Figure 3B, middle and right) in the presence of 500 μM Ca\(^{2+}\). The quantitative analysis revealed that after 1 minute, content mixing for double mutants was reduced by 20~30% when compared with that of wild-type Syt1 while triple mutants had as much as 50% reduction (Figure 3C). Thus, our results suggest that the bipolar charge distribution contributes to fusion pore opening in a positive way.

2.4.4 Disulfide cross-linking in the linker reduces docking while enhancing fusion pore opening

By what mechanism does the bipartite linker region contribute to fusion pore opening? One can envision that electrostatic interaction between the basic N-terminal region and the acidic C-terminal region drives some folding of the linker region, which would effectively shorten the tether between C2AB and the transmembrane domain.

To further verify the hypothesis that folding and unfolding state of linker region of Syt1 regulate synaptic vesicle fusion, we designed a double-cysteine mutant of Syt1 G92C/G130C, which can lock the folded conformation by cross-linking the N-terminal to C-terminal regions by the intramolecular disulfide bond. The double-cysteine mutant was cross-linked with 1 mM H\(_2\)O\(_2\). Both intermolecular and intramolecular disulfide bonds formed in this process, in which intermolecular dimers or higher oligomers had larger molecular weights than the monomer with
the intramolecular disulfide bond. Thus, we were able to further purify the monomer component via gel filtration using a 10/300 GL Superdex 200 column (Figure 4A), which was confirmed by SDS-PAGE (Figure 4A, inset). The Syt1 mutant with the disulfide bond in its linker region ran slightly faster than the wide-type monomer, making it easy to confirm the intramolecular disulfide bond formation. Then, we reconstituted the purified intramolecular cross-linked Syt1 into v-vesicles.

We found that the cross-linked Syt1 mutants could reduce vesicle docking by about 40%. Although the changes appear to be statistically insignificant, the inhibition of vesicle docking could be recovered when the sample was treated with DTT within experimental errors (Figure 4B). Thus, the results suggest that the flexibility of the linker region is favorable for Syt1 in assisting vesicle docking.

In contrast, in the content mixing assay, cross-linked Syt1 enhanced fusion pore opening compared to wild-type Syt1 by about 50% (Figure 4C and 4D), which also could be negated by DTT. Thus, the results show that the folded linker region is preferable for Syt1/Ca\(^{2+}\) to drive opening of the fusion pore for content mixing.

To further substantiate this observation, we made the linker of Syt1 shorter by deleting 40 amino acids (Δ(99-140)aa). As expected, the truncated mutant of Syt1 reduced the vesicle docking by ~50%, but enhanced fusion pore opening by more than 40% (Figure 4E). Thus our results support the notion that an extended and flexible linker region is preferred for Syt1 to
induce vesicle docking to the plasma membrane while a shorter linker region is favored for Syt1 to drive fusion pore opening.

2.4.5 Syt1’s binding to t-vesicles in the presence of Ca\(^{2+}\) induces conformation change in the linker

To examine whether t-vesicles binding in the absence or presence of Ca\(^{2+}\) could induce conformation changes in the linker of Syt1, we used SDSL and the EPR analysis of spin-labeled Syt1 G130C mutant. We found that the linker was in flexible conformation when reconstituted on v-vesicles (black line in Figure 5A). Upon binding to t-vesicles in the absence of Ca\(^{2+}\), the linker region became slightly more flexible as the EPR lines got somewhat narrower (red line in Figure 5A). However, an opposite conformation change happened when Syt1 bound to t-vesicles with Ca\(^{2+}\) (blue line in Figure 5A), which indicated a conformation change from flexible to restricted. Interestingly, this conformational change was lost when the bipolar charge distribution of linker was disrupted by triple K-to-E or E-to-K mutants (Figure 5B and 5C), consistent with our result from the fusion assay.

2.5 Discussion

In this work our single vesicle docking and content mixing assays helped reconstitute two signature Syt1 functions in synaptic vesicle fusion successfully: Ca\(^{2+}\)-independent docking and Ca\(^{2+}\)-dependent content mixing (Lai and Shin, 2012; Lai et al., 2013b). When recombinant soluble C2AB, for which the linker and the transmembrane regions were deleted, was used instead, these two important Syt1 functions were both lost, consistent with the prediction by Chapman and coworkers (Wang et al., 2011).
The linker region is featured with the distinct charge segregation: positively charged amino acids are enriched in the N-terminal region while the negatively charged amino acids are clustered in the C-terminal region. Therefore, one might wonder if the linker region is capable of folding as an electrostatic zipper, which could regulate the distance between functional C2AB and the membrane anchor. Indeed, the molecular dynamics simulation for wild-type sequence reveals that the linker region can collapse due to electrostatic interactions (Supplementary Figure S5). When the charge segregation was disrupted by triple E-to-K or K-to-E mutations in the simulation, the linker region instead prefers to be in an extended conformation (Supplementary Figure S5).

Our single vesicle experiments showed that the double and triple E-to-K and K-to-E mutations impair content mixing to some extent. Thus, the results suggest that the folded conformation is favorable for content mixing. For double and triple mutants, the electrostatic interactions are weakened and the tendency for the linker to fold is reduced and consequently, content mixing is reduced. In contrast, the disulfide cross-linked mutant and the mutant with shortened linker both showed enhanced content mixing, which corroborated the argument.

For both disulfide cross-linked mutant and the mutant with the shortened linker, vesicle docking was reduced, although the changes were subtle. Thus, the data suggest that the extended linker region might be favored for docking. It is however intriguing that for the E-to-K or K-to-E mutants, docking was unchanged when compared to the wild-type Syt1. The results might suggest that the linker region of the wild-type Syt1 stays in an extended
conformation most of the time, despite it spends a small portion of time in the folded conformation.

Our EPR experiments provided some structural basis for the proposed mechanistic model for the linker region. The results showed that the Syt1 linker region prefers a flexible conformation during docking but a folded conformation during content mixing. However, for the triple K-to-E and E-to-K mutants, the ability to flex was lost.

For Syt1-mediated docking the longer linker would be much more effective. The 60 residue long linker can theoretically extend as much as 30 nm, which will allow C2AB to reach out to bind t-SNARE on the target membrane (de Wit et al., 2009; Kim et al., 2012). In contrast, for Ca\(^{2+}\)-triggered vesicle fusion the short linker would be the better because it would allow more efficient SNARE complex formation in a confined environment. Thus, to make the linker be effective for both docking and fusion it has perhaps evolved to become an electrostatic zipper that can extend to facilitate docking but fold to help SNARE assembly.

The conformation of the linker region may be in equilibrium between extended and folded conformation. Hypothetically, it populates extended conformation in the absence of Ca\(^{2+}\), thereby assisting Syt1-mediated docking (Figure 6A) (Diao et al., 2012a). But the equilibrium shifts to the folded conformation in the presence of Ca\(^{2+}\) to reduce the gap between two membranes (Figure 6B and 6C). Conceptually, the hypothetical model presented here is similar to the distance regulation model proposed by Jahn and Coworkers for Ca\(^{2+}\)-triggered stimulation of SNARE-dependent membrane fusion by Syt1 (van den Bogaart et al., 2011;
Park et al., 2012). In our work we propose that the linker region play a role in regulating the distance between two opposing membranes.

In summary, we show using single vesicle fusion assay that the linker region of Syt1 plays a subtle role in Ca$^{2+}$-independent docking as well as in Ca$^{2+}$-dependent content mixing. Such dual function might exist due to the capacity of the electrostatically bipartite linker region to extend and fold to some extent in response to Ca$^{2+}$ signal.
2.6 References


Kyoung M, Srivastava A, Zhang Y, Diao J, Vrljic M, Grob P, Nogales E, Chu S, and Brunger AT. In vitro system capable of differentiating fast Ca2+-triggered content mixing from


Sudhof TC. The synaptic vesicle cycle. *Annu Rev Neurosci*, 2004, 27, 509-547. PMID: 15217342


2.7 Figures and legends

Figure 1 Syt1 is a superior fusion pore opener to C2AB. (A) Bulk lipid mixing assays with C2AB (2 μM) or with reconstituted Syt1 (~0.5 μM). Initial rate of changes of the FRET efficiency (Supplementary Figure S1) was calculated (ΔE sec⁻¹). The black bar is the control with SNAREs only without Syt1. The blue bars represent lipid mixing in the presence of 2 μM C2AB with 100 μM Ca²⁺ (solid blue bar), or without Ca²⁺ (open blue bar), while the red bars represent the reaction in the presence of Syt1 with 100 μM Ca²⁺ (solid red bar), or without Ca²⁺ (open red bar). The grey bar is the negative control without SNAP-25. Results shown are mean ± S.D. from three independent experiments (*P<0.05). (B) The single vesicle content mixing assay with 2 μM C2AB at different Ca²⁺ concentrations (grey bars), or reconstituted Syt1
(black bar). Red bars represent SNAREs only with or without Ca\textsuperscript{2+}. Blue bar represents reconstituted Syt1 without VAMP2 in the presence of 1 mM Ca\textsuperscript{2+}. Results shown are mean ± S.D. from three independent experiments (**P<0.01). An exemplary image of acceptor and donor channels for content mixing and a typical time trace of dequenching of small sulforhodamine B are shown in Supplementary Figure S2. Summary of fusion events among docked vesicles are shown in Table S1.
Figure 2 Asymmetric distribution of charged amino acids on the linker region does not contribute to SNARE binding. (A) Multi-alignment of the Syt1 linker amino acid sequences from C. elegans to human shows that the Syt1 linker is highly conserved. Residues numbers of the rat Syt1 sequence are labeled. The positive charged residues Lys and Arg are shown in blue, and the negative charged residues Asp and Glu are shown in red. Net charges are shown on the right. The highly asymmetric distribution of charged residues is simply emphasized in
the upper bar. All amino acid sequences are obtained from the UniProtKB database with entry of Syt1_RAT (P21707), Syt1_HUMAN (P21579), Syt1_CHICK (P47179), Syt1_APLCA (P41823), Syt1_DROME (P21521), and Syt1_CAEEL (P34693). The muti-alignment is carried out by MutAlien. (B) The single, double, and triple K-to-E (K86E, K86E/K90E, and K86E/K90E/K95E) mutants and those of E-to-K (E131K, E131K/E135K, and E131K/E135K/E139K) are made to disrupt the asymmetric distribution of charged residues. (C) The charge-disrupting mutations do not alter Syt1’s binding abilities to SNARE complexes as shown by the GST pull-down assay for the binary complex and the ternary complex. Binary complex is formed between GST-tagged soluble Syntaxin 1A (191-266) and His-tagged SNAP-25. Ternary complex is formed among GST-tagged soluble VAMP2 (1-96), Syntaxin 1A (191-266), and His-tagged SNAP-25.
Figure 3 Asymmetric distribution of charged amino acids in the Syt1 linker region is important for fusion pore opening. (A) Normalized single vesicle docking probabilities for wild-type Syt1 and its mutants. Results shown are mean ± S.D. from three independent experiments. Summary of immobilized v-vesicles and docked t-vesicles are shown in Table S2. (B) Real time single vesicle content mixing with Syt1 single (left), double (middle) and triple (right) E-to-K or K-to-E mutants. The solid and dotted black lines represents wild-type Syt1 with Ca$^{2+}$ and without Ca$^{2+}$, respectively; the solid and dotted red lines represent Syt1 K-to-E mutants and the solid and dotted blue lines represent Syt1 E-to-K mutants. (C) Single vesicle content mixing events within 1 min. Black bars are without Ca$^{2+}$ while grey bars are in presence of 500 μM Ca$^{2+}$. Results shown are mean ± S.D. from three independent experiments (*P<0.05). Summary of fusion events among docked vesicles are shown in Table S1.
Figure 4 Disulfide cross-linking or deletion in the Syt1 linker region reduces docking but enhances pore opening. (A) FPLC profile shows the separation of the Syt1 monomer. (Inset) SDS-PAGE gels show purification and separation of Syt1 cross-linked mutants with or without DTT. Lane 1 for protein markers, lane 2 for wild-type Syt1, lane 3 for Syt1 cross-linked mutants eluted from Ni-NTA beads, and lane 4 for Syt1 cross-linked monomer. (B) Normalized docking probability for Syt1 cross-linked monomer mutant with or without DTT. Results shown are mean ± S.D. from three independent experiments. Summary of immobilized v-vesicles and docked t-vesicles are shown in Table S2. (C) Real-time content mixing with the cross-linked monomer mutant. The black lines represent wild-type Syt1 in the presence of 500 μM Ca$^{2+}$ without (solid), or with (dotted) 1 mM DTT. The red lines represent Syt1 cross-
linked monomer mutant in the present of 500 μM Ca\textsuperscript{2+} without (solid), or with (dotted) 1 mM DTT. (D) Bar graphs represent content mixing events within 1 min. Black and grey bars represent content mixing in the presence of 500 μM Ca\textsuperscript{2+} without and with 1 mM DTT, respectively. Results shown are mean ± S.D. from three independent experiments (*P<0.05). Bar graphs representing content mixing events in the absence of Ca\textsuperscript{2+} within 1 min were separately shown in Supplementary Figure S4. Summary of fusion events among docked vesicles are shown in Table S1. (E) Bar graphs represent the normalized docking probability (left) and content mixing events within 1 min (right) with the Syt1 linker deletion mutant. The black bars represent wild-type Syt1, and red bars represent Syt1 linker deletion mutant. Results shown are mean ± S.D. from three independent experiments (*P<0.05). Summary of fusion events among docked vesicles are shown in Table S1, and summary of immobilized v-vesicles and docked t-vesicles are shown in Table S2.
Figure 5 Asymmetric distribution of charged amino acids in the Syt1 linker region is important for the Ca$^{2+}$-dependent conformational change. First-derivative EPR spectra of spin-labeled (A) wild-type Syt1 and (B, C) its triple K-to-E and E-to-K mutants. The black lines show the EPR spectra of v-vesicles reconstituted with wild-type Syt1 or its triple K-to-E and E-to-K mutants, the red lines show the EPR spectra of v-vesicles reconstituted wild-type Syt1 or its triple K-to-E and E-to-K mutants bound to t-vesicles, and the blue lines show the EPR spectra of v-vesicles reconstituted wild-type Syt1 or its triple K-to-E and E-to-K mutants bound to t-vesicles in the presence of 1 mM Ca$^{2+}$. 
Figure 6 A hypothetical model summarizing the mechanism by which the Syt1 linker assists docking and fusion. For docking, the Syt1 linker region extends for C2AB to reach out t-SNARE residing on the plasma membrane surface (A). Upon Ca\(^{2+}\) arrival the linker region folds, reducing the gap between the vesicle membrane and the plasma membrane, which may facilitate SNARE complex formation (B). The SNARE complex and Syt1/Ca\(^{2+}\) drive fusion pore opening (C).
2.8 Supplementary information

Figure S1 Bulk lipid mixing assays with C2AB and reconstituted synaptotagmin 1. The change of FRET efficiency (E), which was calculated by the formula: $E = \frac{I_{DiD}}{I_{DiD} + I_{DI}}$, represents the lipid mixing. The black trace represents the reaction with SNAREs only, the with (solid blue) or without (dotted blue) Ca$^{2+}$ traces represent lipid mixing in the presence of C2AB, the (solid red) or without (dotted red) Ca$^{2+}$ traces show lipid mixing in the presence of Syt1, the dotted grey line is the control without SNAP-25.
Figure S2 The single vesicle content mixing assay. (A) Imaging area (45×90 μm²) before and after Ca²⁺ injection. Each spot represents a single vesicle containing 50 mM sulforhodamine B. (B) Real-time content mixing detection. The change of the fluorescence intensity of the sulforhodamine B containing vesicle was shown as a black trace. A sudden increase (red arrow) of the sulforhodamine B fluorescence signal due to dilution-induced fluorescence dequenching was observed when content mixing happened.
Figure S3 Syt1 needs to be on the v-vesicle side to be effective for vesicle docking and content mixing. (A) Single vesicle docking when Syt1 is on the v-vesicle side or on the t-vesicle side. Results shown are mean ± S.D. from three independent experiments (*P<0.05). Summary of immobilized v-vesicles and docked t-vesicles are shown in Table S2. (B) Single vesicle content mixing of sulforhodamine B when Syt1 is on the v-vesicle side or on the t-vesicle side. Results shown are mean ± S.D. from three independent experiments (*P<0.05). Summary of fusion events among docked vesicles are shown in Table S1.
Figure S4 Bar graph of the single vesicle content mixing with Syt1 linker mutants. Wild-type Syt1 and its mutants are not capable of triggering content mixing effectively in the absence of Ca\(^{2+}\). Results shown are mean ± S.D. from three independent experiments. Summary of fusion events among docked vesicles are shown in Table S1.
Figure S5 Representative conformations of the Syt1 linker. (A) wild-type. (B) K86E/K90E/K95E mutant. (C) E131K/E135K/E139K mutant. Color coding has no particular meaning. From the simulation we estimates the end-to-end distance for the linker $R_e \approx 1.6 \pm 0.3$ nm for wild-type Syt1, $4.4 \pm 1.6$ nm for the Syt1 K86E/K90E/K95E linker, and $5.5 \pm 1.0$ nm for E131K/E135K/E139K. $R_e$ of wild-type Syt1 is much shorter than the prediction of the freely jointed chain model wherein $R_e \approx 4$ nm (distance between amino acids is assumed as 0.5 nm). This discrepancy is attributed to the strong electrostatic attraction between charged amino acids and the stiffness of them. To help understand, we divide the linker region into three sub-parts, and measure $R_e$s of them; (1) positive rich part which is composed of first 19 amino acids (amino acids 80-98) at the linker region whose total charge is $+10$ e (part 1), (2) almost neutral middle part (net $+1$ e) which is composed of next 22 amino acids (amino acids 99-120) (part 2), and (3) negative rich (net $-8$ e) part containing last 21 amino acids (amino acids 121-141) (part 3).

Interestingly, the $R_e$ of wild-type Syt1 is even shorter than $R_e$ of part 2, which is about 1.9 nm (2.0 nm, 2.1 nm for part 1, and part 3). This indicates that part 1 and part 3 are folded by the charge interaction, like an electrostatic zipper (A). Noticeable conformational changes are found under even small modification in Syt1 amino acids sequence. Syt1 K86E/K90E/K95E
shows extended the end-to-end distance with respect to WT. Part 1 is shrunk to 1.3 nm, and the end-to-end distances of part 2, part 3 are 1.8 nm, 2.7 nm. The alternating sequence of positive and negative charges may provoke collapse of part 1 and part 2 into a globule while part 3 is still stretched as shown in (B). Syt1 E131K/E135K/E139K, whose net charge is +9 e, exhibits stretched conformations. $R_c$ is not much different from the summation of the end-to-end distance of sub-parts. The end-to-end distances of three parts are about 2.0 nm, 2.3 nm, 2.2 nm for part 1, part 2, and part 3. Thus, they may be approximated as a linear connection of these three parts like charged cylinder (C).
Methods

**Molecular dynamics simulation.** Atomic level molecular dynamics was performed within NVT ensemble using AMBER. For simplicity, only the linker regions of the wild-type Syt1 and two mutations (K86E/K90E/K95E, E131K/E135K/E139K) are considered. Salts and water are implicitly treated. Three independent 20 ns length molecular dynamics simulations at 2fs time steps are taken averaged for the data production. The end-to-end distance ($R_e$) is measured in order to characterize the linker conformation.
Table S1 Numbers of fusion events and total docked vesicles for single content mixing using the small content indicator, sulforhodamine B

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Table S2 Single vesicle docking with SNAREs, SNAREs/Syt1, and its mutants

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CHAPTER 3

MOLECULAR ORIGINS OF SYNAPTOTAGMIN 1 ACTIVITIES ON VESICLE DOCKING AND FUSION PORE OPENING


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3.1 Abstract

Synaptotagmin 1 (Syt1), a major Ca\textsuperscript{2+} sensor in neuroexocytosis, utilizes SNARE- and membrane-binding to regulate vesicle fusion, a required process for neurotransmitter release at the synapse. However, the mechanism by which Syt1 orchestrates SNARE- and membrane-binding to control individual vesicle fusion steps is still unclear. In this study, we used a number of single vesicle assays that can differentiate intermediates of neuroexocytosis, to focus on Syt1 mutants that might impair Syt1-SNARE/PIP\textsubscript{2} interaction, Ca\textsuperscript{2+}-binding, or membrane penetration. Our results show that, although putative Syt1-SNARE/PIP\textsubscript{2} coupling through the polybasic region of the C2B domain is critical for vesicle docking, its disruption does not affect content release. In contrast, Ca\textsuperscript{2+}-binding and membrane-penetration mutants significantly reduce content release. Our results thus delineate multiple functions of Syt1 along the pathway of Ca\textsuperscript{2+}-triggered exocytosis in unprecedented detail.
3.2 Introduction

In the presynapse, Ca\(^{2+}\)-triggered neurotransmitter release from synaptic vesicles is the key process for maintaining signal transduction in the neuronal system (Südhof, 2004). SNARE proteins, comprised of presynaptic t-SNAREs syntaxin 1A and SNAP-25 and vesicle v-SNARE VAMP2 (vesicle-associated membrane protein 2), function as the core fusion machinery in vesicle exocytosis (Poirier et al., 1998; Sutton et al., 1998; Weber et al., 1998; Stein et al., 2009). For tight control of neurotransmitter release by Ca\(^{2+}\), another vesicle protein, synaptotagmin 1 (Syt1), has been identified as a major Ca\(^{2+}\) sensor (Fernandez-Chacon et al., 2001; Chapman, 2002). It is believed that Syt1 plays a key role in synchronizing neurotransmitter release by sensing Ca\(^{2+}\) influx and subsequently triggering rapid fusion of the vesicle with the plasma membrane (Chapman, 2002; Südhof, 2004; Rizo and Rosenmund, 2008; Jahn and Fasshauer, 2012; Rizo and Sudhof, 2012).

At the molecular level, Syt1 contains an N-terminal transmembrane domain, a flexible linker region, and tandem Ca\(^{2+}\)-binding C2 (C2A and C2B) domains (Perin et al., 1991a). Cytoplasmic C2A and C2B domains share a β-sandwich structure containing a bottom loop region that can bind two and three Ca\(^{2+}\) ions, respectively (Sutton et al., 1995; Shao et al., 1996; Ubach et al., 1998). Syt1 can interact with t-SNARE and the ternary SNARE complex via the polybasic region on the C2B domain in both Ca\(^{2+}\)-independent and -dependent manners (Gerona et al., 2000; Earles et al., 2001; Zhang et al., 2002b; de Wit et al., 2009; Vrljic et al., 2010). Also, Syt1 could bind to the negatively charged lipid PIP\(_2\) (Phosphatidylinositol 4,5-bisphosphate) on the plasma membrane probably via the same polybasic region on the C2B domain in the absence of Ca\(^{2+}\), while the loop regions could penetrate into the acidic membrane
upon Ca\textsuperscript{2+} binding (Bai et al., 2004; Li et al., 2006; Kuo et al., 2009; Radhakrishnan et al., 2009; van den Bogaart et al., 2012).

In functional studies, both SNARE and membrane binding by the C2 domains have proven essential in achieving vesicle fusion (Rhee et al., 2005; Chapman, 2008; Lynch et al., 2008; de Wit et al., 2009; Lai et al., 2011; Kim et al., 2012; Vennekate et al., 2012). Mutations in the polybasic region resulted in the impairment of SNARE-mediated lipid mixing \textit{in vitro} as well as Ca\textsuperscript{2+}-triggered neurotransmitter release \textit{in vivo} (Li et al., 2006; Loewen et al., 2006; Gaffaney et al., 2008). Mutations in the Ca\textsuperscript{2+} binding sites revealed that Syt1 is the Ca\textsuperscript{2+} sensor and that Ca\textsuperscript{2+} binding to the loop region is required for vesicle exocytosis (Fernandez-Chacon et al., 2001; Hui et al., 2009; Hui et al., 2011; van den Bogaart et al., 2011; Vennekate et al., 2012). Finally, the loss and the gain of function mutations on the loop region showed that its membrane penetration is a critical step for Ca\textsuperscript{2+}-triggered membrane fusion (Shin et al., 2002; Rhee et al., 2005; Lynch et al., 2008; Hui et al., 2009; Yao et al., 2012). It has recently been shown that membrane attachment through its transmembrane domain is important for Syt1 to function properly as a Ca\textsuperscript{2+}-sensor (Wang et al., 2011; Lai and Shin, 2012; Vennekate et al., 2012; Lai et al., 2013c).

It is thought that vesicle fusion proceeds in at least three sequential steps: vesicle docking, hemifusion (or lipid mixing), and fusion-pore opening (or content release) (Xu et al., 2005; Jahn and Fasshauer, 2012; Diao et al., 2013b), and studies have shown that Syt1 might be involved in all three steps. Apparently, Syt1’s interactions with the SNARE complex and with the membrane are responsible for these regulations. For example, the putative Syt1-t-
SNARE/PIP_2 interaction assists docking of vesicles to the plasma membrane (de Wit et al., 2009; Kim et al., 2012; Honigmann et al., 2013). Syt1’s penetration into the membrane is likely to play roles in both lipid mixing and fusion-pore opening (Fernandez-Chacon et al., 2001; Shin et al., 2002; Rhee et al., 2005; Lynch et al., 2008; Hui et al., 2009; Hui et al., 2011; van den Bogaart et al., 2011; Vennekate et al., 2012; Yao et al., 2012). The Syt1-ternary SNARE interaction might regulate those later steps as well in response to the Ca^{2+} signal, although this has not been demonstrated experimentally. Because of experimental difficulties for previous ensemble fusion assays in resolving individual steps along the fusion pathway, many ambiguities still remain with respect to understanding the molecular origins of the Syt1’s involvement in individual steps of Ca^{2+}-triggered neuroexocytosis.

To address these issues, we generated polybasic region mutants, which are likely to impair the Syt1-SNARE/PIP_2 interaction, Ca^{2+} binding site mutants to hamper its Ca^{2+} affinity, and loop region mutants to alter its membrane penetration capacity. With well-established single vesicle assays (Yoon et al., 2008a; Diao et al., 2012a; Lai et al., 2013b; Lai et al., 2013c) that, can resolve the vesicle fusion process into docking and content release reflecting fusion pore opening, we dissected the effect of these mutations on individual fusion steps. The results show that Ca^{2+}-independent t-SNARE and PIP_2 binding, mediated by the polybasic region of Syt1, is essential for vesicle docking. To our surprise, however, content release is regulated only by the Ca^{2+}-dependent insertion of the loop region and Ca^{2+} binding sites of Syt1 into the membrane, and not at all by its SNARE/PIP_2 interaction.
3.3 Materials and Methods

3.3.1 Plasmid constructs and site-directed mutagenesis

DNA sequences encoding rat syntaxin 1A (amino acids 1-288 with three native cysteines C145, C271, and C272 replaced by alanines), rat VAMP2 (amino acids 1-116 with C103 replaced by alanine), rat SNAP-25 (amino acids 1-206 with four native cysteines C85, C88, C90, and C92 replaced by alanines), soluble syntaxin 1A (amino acid 191-266), and soluble VAMP2 (amino acids 1-96) were inserted into the pGEX-KG vector as N-terminal glutathione S-transferase (GST) fusion proteins. SNAP-25 was inserted into a pET-28b vector also as an N-terminal 6xHistidine (His)-tag fusion protein. Recombinant 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, (we denoted this recombinant form as wild-type Syt1 in this work and used it as the template for generating the point mutants) was inserted into a pET-28b vector as a C-terminal His-tagged protein. It has been previously shown that the deleted first 49 N-terminal residues does not affect the Syt1 function in exocytosis (Hui et al., 2009; Wang et al., 2011; Yao et al., 2012). The Quick Change site-directed mutagenesis kit (Stratagene) was used to generate all Syt1 mutants, including Syt1 EEE (L326/327/331E), Syt1 QQQ (L326/327/331Q), Syt1 Y311N, Syt1 2A(A) (M173A/F234A), Syt1 2A(B) (V304A/I367A), Syt1 4A (M173A/F234A/ V304A/I367A), Syt1 2W(A) (M173W/F234W), Syt1 2W(B) (V304W/I367W), Syt1 4W (M173W/F234W/V304W/I367W), Syt1-C2A* (D230A/D232A), Syt1-C2B* (D363A/D365A), and Syt1-C2A*B* (D230A/D232A/D363A/D365A). DNA sequences were confirmed by the Iowa State University DNA Sequencing Facility.
3.3.2 Protein expression and purification

The GST-tagged proteins were expressed in *E. coli* Rosetta (DE3) pLysS (Novagene). Details can be found in our previous work ([Lai and Shin, 2012; Lai et al., 2014b](#)). The His-tagged proteins were expressed in *E. coli* BL21 (DE3) (Novagen) and purified using previously-described protocol ([Lai and Shin, 2012; Lai et al., 2014a](#)).

3.3.3 Membrane reconstitution

The lipid molecules used in this study were 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), phosphatidylinositol-4,5-bisphosphate (PIP$_2$, from porcine brain), cholesterol, and 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamin-N-(biotinyl) (biotin-DPPE). All lipids were obtained from Avanti Polar Lipids. 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine Perchlorate (DiI), 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindodicarbocyanine Perchlorate (DiD), and sulforhodamine B were obtained from Invitrogen.

For the single vesicle docking assay, the molar ratios of lipids were 15:61:20:2:2 (DOPS:POPC:cholesterol:PIP$_2$:DiD) for the t-SNARE-reconstituted (t-)vesicles, and 5:73:20:2 (DOPS:POPC:cholesterol:DiI) for the v-SNARE-reconstituted (v-)vesicles, respectively. To fix v-vesicles on the TIR imaging surface coated with neutravidin, 0.1 mol% biotin-DPPE was added to the v-vesicle lipid mixture prior to the rehydration-freeze thaw-extrusion steps (see below). The lipid mixture was first completely dried and then rehydrated with dialysis buffer (25 mM HEPES, pH 7.4, 100 mM KCl). After five freeze–thaw cycles, protein-free large unilamellar vesicles (~100 nm in diameter) were obtained by extrusion.
through a 100 nm polycarbonate filter (Whatman). For membrane reconstitution, SNARE proteins and Syt1 were mixed with protein-free vesicles at a protein-to-lipid molar ratio of 1:200 for each protein component (this ratio was kept the same for all experiments, including the single vesicle content mixing assay) with ~0.8 % OG in the dialysis buffer at 4 °C for 15 min. The mixture was diluted by a factor of two with the dialysis buffer and this diluted mixture was then dialyzed overnight in 2 L dialysis buffer at 4 °C. Details for the reconstitution process were discussed in our previous work (Diao et al., 2012b; Lai and Shin, 2012).

For the single vesicle content mixing assay using a small sulforhodamine B content indicator, lipid mixtures were prepared as described for the single vesicle docking assay except that the fluorescent lipid dyes (DiI and DiD) were replaced by equal amounts of POPC. The t-vesicles were prepared as described earlier, except that 50 mM sulforhodamine B in the dialysis buffer was kept throughout all the sample preparation steps for the v-vesicles. Free sulforhodamine B was removed through dialysis and a further PD-10 column desalting step (GE healthcare).

Membrane reconstitution efficiency was confirmed using liposome co-sedimentation assay followed by a SDS-page analysis. Briefly, the aggregated protein was first removed after dialysis by centrifugation at 10,000 g for 10 min at 4 °C. Then the membrane-bound protein was pelleted through high-speed liposome sedimentation using an Airfuge Air-Driven Ultracentrifuge (Beckman) at 150,000 g for 30 min at 4 °C. Pelleted vesicles were re-suspended in the dialysis buffer and re-subjected to the centrifuge for twice more. Finally, the
pellets were re-suspended in 60 μl (about 1/3 of the initial volume) of dialysis buffer and analyzed by SDS-page.

### 3.3.4 Single vesicle binding assay

Slide preparation was the same as that in the vesicle docking assay. SNARE-free vesicles (with or without 2 mol% PIP₂) were immobilized on the PEG-coated surface. After two rounds of 200 μl dialysis buffer washing, Syt1 or its mutants reconstituted vesicles (Syt1-vesicles, DiD-labeled without PIP2, 100~200 nM) were injected into the flow chamber for 30 min docking at room temperature. After washing out free Syt1-vesicles, the docked Syt1-vesicles number in the imaging area (45 × 90 μm²) was counted (Supplementary Figs. S10A-C)

### 3.3.5 Single vesicle docking and content mixing assays

After coating the quartz surface with a solution of methoxy-polyethylene glycol (mPEG) and biotin-PEG molecules to eliminate non-specific binding of vesicles, the quartz slide was assembled into a flow chamber and coated with neutravidin (0.2 mg ml⁻¹). Following 30 minutes of incubation at room temperature (~25 °C), the v-vesicles were immobilized on the PEG-coated surface. After two rounds of 200 μl dialysis buffer washing, t-vesicles (100 ~ 200 nM in lipid concentrations) were injected at room temperature into the flow chamber for 30 min of docking. After washing out free t-vesicles, we acquired images (45 × 90 μm²) from 5 ~ 40 random locations within the flow chamber using 635 nm laser excitation for docked t-vesicles and 532 nm laser excitation for immobilized v-vesicles, respectively. All spots appeared in the green channel were considered as docked t-vesicles. The spots in the image
were identified by the smCamera program (kindly provided by Dr. Taekjip Ha’s group) based on the criteria: peak radius, 3 pixel; peak threshold/ data scaler, 1%. The docking probability was calculated as the ratio of average docked t-vesicles to average anchored v-vesicles (Supplementary Figs. S10D-F). Details of the single vesicle docking assay were reported in our previous work (Yoon et al., 2008b; Diao et al., 2009b).

For real-time imaging of small sulforhodamine B content release, sulforhodamine B containing v-vesicles was immobilized on the PEG-coated surface. After two rounds of washing using 1 ml dialysis buffer, t-vesicles were injected into the channel to make them bind to v-vesicles. After 30 min of incubation at room temperature (~25 °C), dialysis buffer, with or without 500 μM Ca$^{2+}$, was injected into the flow chamber by a motorized syringe pump at a speed of 33 μl sec$^{-1}$. The detail of the single vesicle content mixing assay was reported in previous work (Kyoung et al., 2011; Diao et al., 2012a; Lai et al., 2013b).

3.3.6 GST pull-down assay

To form a binary complex, His-tagged SNAP-25 and GST-tagged soluble Syntaxin 1A (191-266) cell lysates were mixed and loaded onto Ni-NTA beads. The binary complex was purified following the procedure described for His-tagged SNAP-25, and the purified binary complex was then loaded onto Glutathione-agarose beads. After washing thoroughly with a cleavage buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.8 % OG) to remove unbound His-tagged SNAP-25, the binary complex immobilized beads were separated into many equal parts in 1.7 ml Eppendorf tubes. Equal amounts of wild-type Syt1 or its mutants and cleavage buffer were added to the immobilized binary complex and the mixture was incubated at 4 °C for 1 h.
The beads were then thoroughly washed with the cleavage buffer. 5xSDS-loading buffer (313 mM Tris-HCl, pH 6.8, 10 % SDS, 0.05 % bromophenol blue, 50 % glycerol, 0.5 M DTT ) was added to the samples and the mixture was boiled for 10 min. Proteins were resolved on precast 12 % SDS-page and visualized using Coomassie blue staining. To test the binding of wild-type Syt1 and its mutants to the ternary SNARE complex, purified soluble Syntaxin 1A (191-266) was mixed with His-tagged SNAP-25 and GST-tagged soluble VAMP2 (1-96) cell lysates before loading onto Ni-NTA beads. The ternary complex was purified following the same procedure described for His-tagged SNAP-25. The purified ternary complex was then loaded onto Glutathione-agarose beads. After washing thoroughly with cleavage buffer to remove unbound His-tagged SNAP-25 and the binary complex, binding of wild-type Syt1 or its mutants was performed following the procedure described above for the binary complex, except that 500 μM Ca^{2+} was added to the cleavage buffer.

3.3.7 CD spectroscopy

The CD spectra were measured with an AVIV stop-flow Circular Dichroism spectropolarimeter at 190 to 260 nm using a cell with the 1 mm path-length. The sample containing 10 μM of either wild-type Syt1 or the Y311N mutant was measured at 25 °C. For the correction of the baseline error, the signal from a blank run with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na_{2}HPO_{4}, and 2 mM KH_{2}PO_{4}) containing 0.8% OG was subtracted from all the experimental spectra.

3.4 Results

3.4.1 Syt1 mutants and their SNARE interactions
Syt1 mutants were designed on the basis of three reported functions of Syt1 in synaptic vesicle exocytosis: its interaction with SNARE and/or PIP₂, Ca²⁺ binding, and membrane penetration. To alter these features of Syt1, we generated three groups of previously well-characterized Syt1 mutants. First, we intended to disrupt the SNARE/PIP₂-binding polybasic region of the C2B domain by changing three basic amino acids Lys326, Lys327, and Lys331 to either acidic amino acid Glu (EEE), or neutral amino acid Gln (QQQ) (Li et al., 2006; Loewen et al., 2006; Gaffaney et al., 2008). We note however that there might be other binding modes between SNAREs and Syt1 (Choi et al., 2010; Vrljic et al., 2010). Another mutant, Y311N, was generated, because, although Y311 is a residue buried inside the polybasic region, this mutation has been shown to affect Syt1’s binding to binary t-SNARE (Rickman et al., 2006; de Wit et al., 2009), thereby hampering vesicle docking (blue in Fig. 1A). We also note though that these mutations might disrupt the Syt1 structure, thereby impacting functions. Such disruption is especially concerning for partially buried position 311 (Rickman et al., 2006). The circular dichroism (CD) spectra however show that the overall folding was not affected significantly by the Tyr to Asn mutation at this position (Supplementary Fig. S1). Second, to block the Ca²⁺ binding capability, we mutated the amino acids Asp230 and Asp232 in the C2A domain to Ala (C2A*), the amino acids Asp363 and Asp365 in the C2B domain to Ala (C2B*), or generated all four mutations in the C2A and C2B domains (C2A*B*) (Ubach et al., 1998; Fernandez-Chacon et al., 2001; Fernandez et al., 2001; Hui et al., 2009; Hui et al., 2011; van den Bogaart et al., 2011; Vennekate et al., 2012) (red in Fig. 1A). Third, we generated loop region mutants to reduce the membrane penetration ability of Syt1. We mutated amino acids Met173 and Phe234 in C2A to Ala (2A(A)), amino acids Val304, Ile367 in C2B to Ala (2A(B)), or generated all four mutations in C2AB (4A) (Martens...
Conversely, to enhance membrane penetration ability, we changed these amino acids to Trp instead of Ala (2W(A), 2W(B), 4W) (Shin et al., 2002; Rhee et al., 2005; Lynch et al., 2008; Hui et al., 2009; Yao et al., 2012) (magenta in Fig. 1A).

Because it has been shown that Syt1 binding to the binary complex of t-SNAREs syntaxin 1A and SNAP-25 plays a role in vesicle docking prior to the rise of the Ca^{2+} level (de Wit et al., 2009), we first examined the Syt1 binding to the t-SNARE complex. The polybasic region mutants EEE, QQQ, and Y311N showed significant reduction of binding to the binary t-SNARE complex in the absence of Ca^{2+} (Fig. 1B). To further understand molecular mechanism of the t-SNARE-Syt1 interaction, we performed the GST pull-down assay in the present of 200 μM inositol 1,4,5-trisphosphate (IP_3) to mimic PIP_2 in solution. Our results showed that IP_3 reduces the interaction between wild-type Syt1 and the binary t-SNARE complex somewhat (Supplementary Figs. S2B and S2C), indicating that Syt1 binding to PIP_2 might compete with t-SNARE binding somewhat. Controls BSA and Ca^{2+} did not affect the interaction between Syt1 and the binary t-SNARE complex (Supplementary Figs. S2B and S2C), indicating that Syt1-binary t-SNARE interaction is specific and Ca^{2+}-independent.

On the other hand, it has been postulated that Syt1 binding to the ternary SNARE complex regulates Ca^{2+}-triggered opening of fusion-pores (Chapman, 2002). The polybasic region mutations reduced Syt1 binding to the ternary SNARE complex in the presence of Ca^{2+} (Fig. 1C). As controls, adding BSA or removing Ca^{2+} did not affect the interaction between Syt1 and the ternary SNARE complex (Supplementary Figs. S2D and S2E). IP_3 (200 μM)
did not alter the interaction between wild-type Syt1 and the ternary SNARE complex in the presence of 500 μM Ca\(^{2+}\) (Supplementary Figs. S2D and S2E). With these mutants we are now ready to test, with the single vesicle fusion assay whether or not there is direct correlation between Syt1-t-SNARE coupling and vesicle docking and, more importantly, between Syt1-ternary SNARE complex coupling and content release.

In contrast to these polybasic region mutations, neither the mutations in the Ca\(^{2+}\)-binding sites nor those in the membrane-penetrating loops affected the Syt1’s ability to bind the binary t-SNARE complex in the absence of Ca\(^{2+}\) or the ternary SNARE complex in the presence of Ca\(^{2+}\) (Figs. 1B and 1C).

### 3.4.2 Polybasic region mutations in Syt1 reduce PIP\(_2\)-binding and vesicle docking

To study the effect of the Syt1 mutants on individual fusion steps, we reconstituted wild-type Syt1 or its mutants and VAMP2 (molar ratio of 1:1) onto a population of vesicles (v-vesicles) (Supplementary Fig. S3). Premixed t-SNAREs (syntaxin 1A: SNAP-25 = 1:1.5) were reconstituted into another population of vesicles (t-vesicles) (Supplementary Fig. S3A). The wild-type Syt1 and its mutants had all similar reconstitution efficiencies when reconstituted together with VAMP2 to v-vesicles (Supplementary Fig. S3B).

Previously, we had shown that both binary t-SNARE and PIP\(_2\) play roles in Syt1-mediated vesicle docking (Kim et al., 2012). Therefore, we performed the single vesicle membrane-binding assay to examine Syt1’s binding to SNARE free vesicles (Supplementary Fig. S4A). In this assay, SNARE-free vesicles with 2 mol% PIP\(_2\) were immobilized (or
tethered) on the surface by the avidin-biotin conjugation (see Materials and Methods section), Syt1- or its mutants-reconstituted vesicles doped with 2 mol% fluorescence acceptor lipid DiD (Syt1-vesicles) were loaded, and the number of Syt1-vesicles docked (or bound) to the SNARE-free vesicles in the imaging area (45 × 90 μm²) were counted. We found that only the polybasic region mutants showed impaired binding to the PIP2-containing vesicles by as much as 60% in the absence of Ca²⁺ (Fig. 2A and Supplementary Fig. S5), showing that Syt1 binding to the negatively charged PIP₂ might contribute to vesicle docking, confirming our previous results (Kim et al., 2012).

Then, we tested the effect of these mutations on vesicle docking by applying the single vesicle docking assay with t-vesicles containing DiD and v-vesicles containing fluorescence donor lipid DiI (2 mol% each) (Supplementary Figs. S4B and S4C) (Lee et al., 2010; Diao et al., 2013a; Lai et al., 2013c). Our results showed that polybasic region-disrupted mutants Syt1 EEE and Y311N exhibited reduced vesicle docking by as much as ~70% when compared with wild-type Syt1. The QQQ mutant, however, had a mild inhibition by ~30% (Fig. 2D). Unlike the polybasic region mutants, the Ca²⁺ binding site and loop region mutants had negligible effects on vesicle docking (Figs. 2E and F). Similar to our previous work using solution single-vesicle assay, when PIP₂ was removed from t-vesicles, vesicle docking is reduced significantly (Supplementary Fig. S6)(Kim et al., 2012), suggesting that the Syt1-PIP₂ interaction may well be the dominant force for vesicle docking. When the control IP₃ or BSA was injected into the chamber together with t-vesicles, vesicle docking was not affected (Supplementary Fig. S6). Although our results may not be sufficient to pinpoint the detailed
binding mechanism, they establish a direct correlation between Syt1-SNARE/PIP2 coupling and vesicle docking, and show that the polybasic region of Syt1 plays a role in vesicle docking.

3.4.3 Ca^{2+} binding and the penetration of the loop region of Syt1 into the membrane are essential for content release

Ca^{2+} triggered neurotransmitter release at the synapse requires a fusion-pore encompassing the vesicle membrane and the plasma membrane. To study the effect of various Syt1 mutations on the fusion-pore opening step, we first examined the Ca^{2+} triggered insertion of Syt1 into the membrane. This time PIP2 was removed from SNARE-free vesicles and Syt1-vesicles were injected into the chamber in the presence of 500 μM Ca^{2+}. We found that both Ca^{2+} binding site mutants and alanine mutants on the loop region showed impaired vesicle docking (Supplementary Fig. S7), consistent with the previous report that Ca^{2+} bridges Syt1 and the membrane and both C2A and C2B domains of Syt1 bind to the membrane in the presence of Ca^{2+} (Honigmann et al., 2013).

Then we applied the single vesicle content mixing assay and monitored the diffusion of sulforhodamine B from a v-vesicle to an empty t-vesicle, that results in a sudden increase of the fluorescence signal due to dilution-induced fluorescence dequenching (Lai et al., 2013b; Lai et al., 2013c) (Supplementary Fig. S8A). We used v-vesicles reconstituted with Syt1 and VAMP2 in a 1:1 molar ratio for wild-type Syt1 and its mutants, although the vesicles containing Syt1 and VAMP2 at the 1:4 molar ratio also exhibited robust content mixing (Supplementary Fig. S9). The polybasic region mutants EEE, QQQ, and Y311N showed no difference in term of the fusion percentage among docked vesicles from the wild-type Syt1 in
Ca$^{2+}$-triggered content release (Fig. 3A and Supplementary Fig. S8B). We note that the disruption of polybasic region by these mutations could still affect neurotransmitter release in fast (submillisecond) time scale after the Ca$^{2+}$ influx in vivo. However, the Ca$^{2+}$ binding sites mutants Syt1-C2A*, Syt1-C2B*, and Syt1-C2A*B* exhibited a decrease in Ca$^{2+}$-triggered content release by 30, 60, and 70 %, respectively (Fig. 3B and Supplementary Fig. S8C). The results indicate that Ca$^{2+}$ binding to the C2 domain is important for Ca$^{2+}$-triggered content release. We notice that the mutation in C2B is more severely disruptive than those in C2A, consistent with previous findings (Chapman, 2008).

Next, we tested loop region mutants that either reduce or enhance the membrane penetration ability of the loops, depending on the side-chain size of the corresponding amino acids. We observed an anticipated inhibition of content release for alanine mutants (2A(A), 2A(B), 4A) by 40%, 20%, 70% (Fig. 3C and Supplementary Fig. S8D), respectively. The results thus indicate that membrane penetration of Syt1 is important for opening fusion pores. In contrast, we observed no enhancement for two tryptophan mutants (2W(A), 2W(B)), although the 4W mutant was able to increase content mixing by 20% (Fig. 3C and Supplementary Fig. S8D). As controls, in the absence of Ca$^{2+}$ none of these mutants supported content mixing effectively (pink bars in Fig. 3 and dotted lines in Supplementary Fig. S8).

Our results thus show that the polybasic region that mediates Syt1-SNARE/PIP$_2$ coupling plays a role in vesicle docking but not in the final fusion step. It appears, however,
that Ca\textsuperscript{2+}-binding and the loop penetration into the membrane are important elements in content release although they have minimal effect on vesicle docking.

3.5 Discussion

In this work, our single vesicle fusion assay revealed that mutations in the polybasic region in Syt1 cause reduced SNARE and PIP\textsubscript{2} binding and result in an apparent decrease in vesicle docking, but produce little change in the content release. It has been long speculated that Syt1-SNARE interaction plays a critical role in Ca\textsuperscript{2+}-triggered exocytosis (Chapman, 2008). Our results show that the coupling between the polybasic region of Syt1 and SNARE/PIP\textsubscript{2} is limited to vesicle docking (Fig. 4) and does not extend its influence on to the final content release step.

However, the Syt1-ternary SNARE complex coupling might still play an important role in regulating Ca\textsuperscript{2+}-triggered exocytosis. A current mechanistic model predicts that complexins, a family of small presynaptic proteins, bind to the SNARE complex and inhibit full zippering, thereby clamping membrane fusion (Schaub et al., 2006; Tang et al., 2006; Giraud et al., 2009; Krishnakumar et al., 2011; Krishnakumar et al., 2013; Yang et al., 2013). It has been thought that Syt1 would unclamp the complexin clamp in the presence of Ca\textsuperscript{2+}, requiring Syt1’s binding to the SNARE complex (Schaub et al., 2006; Tang et al., 2006). It thus appears that Syt1-ternary SNARE coupling is required for replacing the complexin clamp. In a separate note, since polybasic region mutants cannot completely block the Syt1/ternary SNARE interaction (Fig. 1 and Supplementary Fig. S2) we cannot rule out the possibility that there
might be another unidentified region mediating the Syt1/ternary SNARE interaction besides the polybasic region (Choi et al., 2010; Vrljic et al., 2010).

Although the Syt1-SNARE/PIP$_2$ interaction has little influence on fusion pores, our results show that Syt1’s membrane insertion is indeed critical for Ca$^{2+}$-triggered opening of the fusion (Fig. 4), consistent with the conclusion of many previous in vivo and in vitro studies (Chapman, 2002, 2008). For Syt1 it is unclear if SNARE- and membrane-binding is sequential. Our previous work raised the possibility that Syt1 simultaneously binds the SNARE complex and the PIP$_2$-containing membrane (Kim et al., 2012; Lai and Shin, 2012). Here, based on experiments involving Syt1 mutants, we show this concurrent SNARE/PIP$_2$ interaction through the polybasic region on Syt1 in the absence of Ca$^{2+}$, might be essential for synaptic vesicle docking.

How might the Syt1’s insertion into the membrane promote the opening of fusion-pores for content release? McMahon and Chapman have independently shown that Syt1 has the capacity to induce a positive curvature of the bilayer (Martens et al., 2007; Hui et al., 2009). They demonstrated that this newly discovered function is well correlated with the ability to stimulate lipid mixing (or hemifusion) with Ca$^{2+}$. It is also possible that this curvature-inducing ability of Syt1 might provide some thrust to overcome the energy barrier for pore opening. Alternatively, it has long been thought that transmembrane domains of fusion proteins, including those in viral membrane fusion as well as in intracellular membrane fusion, play a critical role in driving the fusion-pore (Tong et al., 2009; Hernandez et al., 2012). It has been shown that Syt1 binds the basic membrane proximal linker region of SNAREs where PIP$_2$
molecules can cluster (Honigmann et al., 2013). We speculate that this interaction might activate transmembrane domains to drive fusion pore opening, although more work is needed to support this idea. Very recently, Südhof and coworkers have disputed the critical involvement of SNARE transmembrane domains for Ca\(^{2+}\)-triggered exocytosis, warranting further work in this area (Zhou et al., 2013a).

In summary, using a series of single vesicle assays (Kyoung et al., 2011; Diao et al., 2012b; Kyoung et al., 2013; Lai et al., 2013b; Lai et al., 2013c) resolving the individual fusion steps in the single vesicle fusion event, we have shown that Syt1-SNARE/PIP\(_2\) coupling through the polybasic region has little to do with content release although it plays a significant role in vesicle docking. On the other hand, our results demonstrate that Ca\(^{2+}\)-induced insertion of Syt1 into the membrane is essential for content release. Our results thus delineate multiple functions of Syt1 along the pathway of Ca\(^{2+}\)-triggered exocytosis.
3.6 References


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3.7 Figures and Legends

**Figure 1. SNARE binding of Syt1 mutants.** (A) Ribbon diagram of the cytoplasmic domain of Syt1 (C2AB). The model is derived from the crystal structural of the cytoplasmic domain of Syt1 (C2AB, PDB id: 2R83) (Fuson et al., 2007). The C2B domain was rotated about 180° in the horizontal plane relative to C2A and the linker between C2A and C2B to show the Ca²⁺ binding loops of both domains in the same plane. Two important Ca²⁺ binding residues (D230/D232) on the C2A domain and the other two Ca²⁺ binding residues (D363/D365) on the C2B domain are shown as red sticks. Four residues proposed to be important for membrane insertion (M173/ F234 on C2A, V304/ I367 on C2B) are shown in magenta. Four residues on the C2B domain (K326/K327/K331/Y311) that are proposed to bind the SNARE complex, are shown in blue. (B and C) GST pull-down assays of SNARE-Syt1 binding. Mutations in the
polybasic region of the C2B domain (EEE, QQQ, and Y311N) hamper SNARE binding while the Ca$^{2+}$ binding (C2A*, C2B*, and C2A*B*) and loop region mutations (2A(A), 2A(B), 4A, 2W(A), 2W(B), and 4W) mutations do not change the SNAREs binding capability of Syt1. The binary complex is formed between GST-tagged soluble syntaxin 1A (191-266) and His-tagged SNAP-25 and the ternary complex is formed among GST-tagged soluble VAMP2 (1-96), syntaxin 1A (191-266), and His-tagged SNAP-25.
Figure 2. Interaction between the polybasic region of the C2B domain and PIP$_2$ is important for vesicle docking. (A-C) PIP$_2$ binding abilities of wild-type Syt1 and its mutants with alterations at the polybasic region, Ca$^{2+}$ binding sites, and the loop region. (D-F) Single vesicle docking probabilities of wild-type Syt1 and its mutants with alterations at the polybasic region, Ca$^{2+}$ binding sites, and the loop region. The fraction is defined as the number of docked t-vesicles divided by the number of immobilized v-vesicles. Results are shown as the mean ± S.D. (*$P < 0.05$, n=3, and n.s. means ‘not significant’).
Figure 3. Content mixing of wild-type Syt1 and its mutants. The bar graphs represent single vesicle content mixing events within 1 min. of the fusion reaction. Pink bars represent the cases without Ca$^{2+}$ while red bars represent the cases with 500 μM Ca$^{2+}$. Content mixing probabilities are for wild-type Syt1 and its mutants in the polybasic region (A), for the Ca$^{2+}$ binding sites (B), and for the loop region (C). The fraction is defined as the number of content mixing events divided by the number of docked vesicles. Results shown represent the mean ± S.D. (*$P < 0.05$, n=3 and n.s. means ‘not significant’).
Figure 4. A mechanistic model for multiple synaptotagmin 1 functions along the vesicle fusion pathway. Before Ca$^{2+}$, the polybasic region of the C2B domain interacts with t-SNAREs and PIP$_2$ molecules to promote docking of synaptic vesicles. Upon calcium arrival, the membrane association and insertion of Ca$^{2+}$-bound C2A and C2B domains become critical for content release.
3.8 Supplementary Information

**Supplementary Figure S1.** The CD spectra for wild-type Syt1 (red) and Syt1 Y311N (blue) show no major changes of the secondary structure by the Tyr to Asn mutation at position 311.
Supplementary Figure S2. GST pull-down assay in the present of IP3 and BSA. (A) Analysis of input wild-type Syt1 and its polybasic region mutants (EEE and QQQ) on SDS-page. (B) SDS-page analysis of the GST pull-down assay between the binary t-SNARE complex and Syt1. (C) Normalized binding abilities of Syt1 and its mutants to the binary t-SNARE complex. (D) SDS-page analysis of the GST pull-down assay between the ternary SNARE complex and Syt1. (E) Normalized binding abilities of Syt1 and its mutants to the ternary t-SNARE complex. The experiments were repeated two times independently and samples from each trial are analyzed twice by SDS-page. Image acquisition and band analysis
were performed using the ChemiDOC system (Bio-Rad). Results shown represent the mean ± S.D. We note that error bars for EEE and QQQ in the presence of BSA is large, which may be due to the smearing of BSA bands.
Supplementary Figure S3. Membrane reconstitution. (A) Analysis of reconstituted t-vesicles and v-vesicles on SDS-page. After reconstitution, the t-vesicles contain t-SNAREs (Syntaxin 1A: SNAP-25) at nearly 1:1 molar ratio (lane 1), and the v-vesicles contain Syt1 and VAMP2 at 1:4 molar ratio (lane 2) or 1:1 molar ratio (lane 3). SNARE proteins were kept at the protein-to-lipid ratio of 1:200 unless otherwise noted. (B) Membrane reconstitution efficiencies of wild-type Syt1 and its mutants together with VAMP2 at 1:1 molar ratio.
Supplementary Figure S4. Single-vesicle membrane binding and docking assays. (A) Schematic diagrams of the membrane binding (A) and single-vesicle docking (B) assays. (C) Imaging area (45×90 µm²) after washing out free DiD-labeled vesicles. Each spot in the left channel represents a single DiI-labeled vesicle excited by green laser (532 nm), and each spot in the right channel represents a single DiD-labeled vesicle excited by red laser (635 nm).
Supplementary Figure S5. Syt1 binding to the membrane through the polybasic region in the absence of Ca^{2+}. (A) The representative images of docked DiD-labeled vesicles reconstituted with Syt1 or its mutants to the PIP_2 containing SNARE-free vesicles in the absence of Ca^{2+} (red channel, Fig. S2C). (B) Bar graph of membrane binding abilities of DiD-labeled vesicles with or without wild-type Syt1. Membrane binding ability is defined as normalized count number of membrane-bound vesicles. Results shown represent the mean ± S.D. (n=3).
Supplementary Figure S6. Single-vesicle docking in the present of IP$_3$ and BSA. Single-vesicle docking probabilities of immobilized v-vesicles containing VAMP2 and wild-type Syt1 to t-vesicles containing 2% PIP$_2$ (black bar, as the same lipid composition as Figure 2A), t-vesicles without PIP$_2$ (red bar, replacing PIP$_2$ with the equal amount of POPC), t-vesicles containing 2% PIP$_2$ in the presence of 200 μM IP$_3$ (blue bar), and t-vesicles containing 2% PIP$_2$ in the presence of 2 μM BSA (dark cyan bar). Results shown represent the means ± S.D. from at least 10 screens in three independent measurements.
Supplementary Figure S7. Syt1 binding to the membrane through Ca\textsuperscript{2+} binding sites and the loop region in the presence of Ca\textsuperscript{2+}. (A) The representative images of docked DiD-labeled vesicles reconstituted with wild-type Syt1 or its mutants to SNARE-free vesicles (no PIP\textsubscript{2}) in the presence of 500 μM Ca\textsuperscript{2+} (red channel, Fig. S2C). (B) Bar graphs of membrane binding abilities in the presence of 500 μM Ca\textsuperscript{2+} for wild-type Syt1 and its mutants altered at the polybasic region, Ca\textsuperscript{2+} binding sites, and the loop region, and a bar graph of membrane binding
abilities of DiD-label vesicles with or without wild-type Syt1 (bottom right). Membrane binding ability is defined as normalized count number of membrane-bound vesicles. Results shown represent the mean ± S.D. (n=3).
Supplementary Figure S8. Single-vesicle content-mixing experiments. (A) Schematic illustration of the single-vesicle content-mixing assay. Cumulative counts of vesicle fusion events in real time that show content mixing for wild-type Syt1 (black lines) and its polybasic region mutants (red lines for QQQ, cyan lines for EEE, and blue lines for Y311N) (B), its Ca$^{2+}$ binding site mutants (red lines for C2A*B*, cyan lines for C2B*, and blue lines for C2A*) (C), and its loop region mutants (red lines for 4W and cyan lines for 4A) (D). The solid and dotted lines represent experiments with and without 500 μM Ca$^{2+}$, respectively. The cumulative time plots for wild-type Syt1 in (B)-(D) are slightly different from one another, indicating the variation among different batches of experiments.
Supplementary Figure S9. Single-vesicle content-mixing activities at different Syt1 concentrations. (A) Cumulative counts of vesicle fusion events in real time that show content mixing between t- and v-vesicles incorporated with wild-type Syt1 and VAMP2 at 1:1 (solid red line) or 1:4 molar ratios (solid black line) in the presence of 500 μM Ca^{2+}. The dashed lines represent experiments in the absence of Ca^{2+}. (B) The bar graph represents fusion pore opening probabilities in first 60 seconds of the fusion reaction. Black bars are without Ca^{2+} while red bars are with 500 μM Ca^{2+}. Results shown represent the mean ± S.D. (n=3).
Supplementary Figure S10. Data analysis strategy in single-vesicle membrane binding and docking assays. (A-C) Non-specific and specific binding of DiD-Syt1 vesicle to PEG
surface. (A) Non-specific binding of DiD-Syt1 vesicle to neutravidin surface is not significant. (B) Specific binding of DiD-Syt1 vesicles to surface-immobilized SNARE-free unlabeled vesicles. (C) Bar graph of non-specific and specific binding. Results shown represent the mean ± S.D. (n=5). (D-F) No significant difference of docking probabilities between independent counting and co-localization counting. (D) Independent counting of immobilized DiI-v-vesicles by green laser excitation and that of docked DiD-t-vesicles by red laser excitation. (E) Co-localization counting. The green and red channels were overlapped through the smCamera program-generated map files. The white circles indicate co-localized vesicles. (F) Docking fraction obtained from independent and co-localization counting methods. Results shown represent the means ± S.D. (n=10).
Supplementary Table S1. Single vesicle-vesicle docking assay with t-SNARE-vesicles and v-SNARE/Syt1-vesicles. ( # of screens means the number of images taken for analysis.)

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Supplementary Table S2. Numbers of content mixing events using the small content indicator, sulforhodamine B in the absence and presence of Ca\textsuperscript{2+}.

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CHAPTER 4
SYNAPTOTAGMIN 1 IS AN ANTAGONIST FOR MUNC18-1 IN SNARE-ZIPPERING


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4.1 Abstract

In neuroexocytosis, SNAREs and Munc18-1 may consist of the minimal membrane fusion machinery. Consistent with this notion, we observed, using single molecule fluorescence assays, that Munc18-1 stimulates SNARE zippering and SNARE-dependent lipid mixing in the absence of a major Ca\(^{2+}\)-sensor synaptotagmin 1 (syt1), providing the structural basis for the conserved function of SM (Sec1/Munc18) proteins in exocytosis. However, when full-length syt1 is present no enhancement of SNARE zippering and no acceleration of Ca\(^{2+}\)-triggered content mixing by Munc18-1 are observed. Thus, our results show that syt1 acts as an antagonist for Munc18-1 in SNAREs zippering and fusion pore opening. Although the SM family may serve as part of the fusion machinery in other exocytotic pathways, Munc18-1 may have evolved to play a different role such as regulating syntaxin 1a in neuroexocytosis.
4.2 Introduction

Essential to the functional connectivity in the central nerve system is neurotransmitter release at synapses which requires fusion of vesicles to the presynaptic plasma membrane. Vesicle fusion is an energetically costly process because it needs to overcome the energy barrier for merging two stable membranes to a single bilayer (Chernomordik and Kozlov, 2003; Martens and McMahon, 2008).

The required fusion energy would have to be provided by the protein machinery. It is thought that SNAREs and Munc18-1 constitute the minimal fusion machinery (Maximov et al., 2009; Rizo and Sudhof, 2012). The SNARE complex formed between vesicle (v-) SNARE and target plasma membrane (t-) SNARE is considered the core of the fusion machine (Söllner et al., 1993b; Poirier et al., 1998; Sutton et al., 1998; Weber et al., 1998; Brunger, 2005; Jahn and Scheller, 2006; Maximov et al., 2009; Stein et al., 2009; Rizo and Sudhof, 2012). Further, the binding of Munc18-1 to the SNARE complex would provide an additional energetic boost in driving vesicle fusion (Shen et al., 2007; Deak et al., 2009; Diao et al., 2010).

There is ample evidence that SNAREs belong to the minimal fusion machinery. Treatment of the presynapse with the clostridial toxins, which specifically cleave SNARE proteins, abolishes neurotransmitter release completely (Schiavo et al., 1992; Blasi et al., 1993; Keller and Neale, 2001; Breidenbach and Brunger, 2005). Furthermore, proteoliposomes reconstituted with SNAREs only support lipid mixing, demonstrating that SNAREs alone can drive membrane fusion (Weber et al., 1998; Bhalla et al., 2006; Pobbati...
et al., 2006; Stein et al., 2007; Yoon et al., 2008a; Lee et al., 2010; Shi et al., 2012). Similarly, it was shown that Munc18-1 accelerates SNARE-mediated proteoliposome fusion, supporting the notion that Munc18-1 is part of the minimal fusion machine (Shen et al., 2007; Yu et al., 2013).

Meanwhile, Munc18-1 appears to have another important function to regulate the SNARE assembly. Munc18-1 binds to the Habc domain of t-SNARE syntaxin 1a and prevents syntaxin 1a from the premature binding to another t-SNARE SNAP-25 that might lead to a nonproductive t-SNARE complex (Fernandez et al., 1998; Dulubova et al., 1999; Misura et al., 2000; Ma et al., 2011; Ma et al., 2013).

Although the Munc18-1 function to protect syntaxin 1a is supported by a variety of evidence its role as part of the fusion machine is debatable. The proteoliposome fusion assay (Shen et al., 2007; Yu et al., 2013), on which this proposition relies heavily, did not include synaptotagmin 1 (syt1), a major Ca$^{2+}$-sensor for synaptic vesicle fusion (Fernandez-Chacon et al., 2001; Chapman, 2002; Rizo and Rosenmund, 2008). Syt1 is a vesicular protein, consisting of tandem Ca$^{2+}$-binding C2 domains and a transmembrane helix (Perin et al., 1991a). Syt1 interacts with the core SNARE complex as well as phospholipids (de Wit et al., 2009; Hui et al., 2009; Kim et al., 2012; Lai and Shin, 2012; Lai et al., 2013c). Provided that syt1 cooperates with the SNARE complex intimately during the moment of fusion it is difficult to envision how Munc18-1 might gain access to the core complex simultaneously (Shin, 2013).
In this work, we investigated, using single molecule (sm) FRET, the conformational changes of a trans-SNARE complex (or SNAREpin) assembled between two nanodisc membranes (Shin et al., 2014) induced by Munc18-1. We also studied the effect of Munc18-1 on SNARE-dependent proteoliposome lipid mixing and on the Ca\textsuperscript{2+}-triggered fusion pore opening in well-defined in vitro settings (Kyoung et al., 2013; Lai et al., 2013c) to dissect the Munc18-1 function in the presence of syt1. Our results show that although Munc18-1 has the capacity to stimulate SNARE complex formation and SNARE-dependent lipid mixing, syt1 largely negates such positive effects on membrane fusion, suggesting that syt1 acts as an antagonist for Munc18-1.

4.3 Materials and Methods

4.3.1 Plasmid constructs and site-directed mutagenesis

DNA sequences encoding rat syntaxin 1a (amino acids 2-288 with three native cysteines C145, C271, and C272 replaced by alanines), rat VAMP2 (amino acids 1-116 with C103 replaced by alanine), soluble rat VAMP2 (Vps, amino acids 1-94), and rat SNAP-25 (amino acids 1-206 with four native cysteines C85, C88, C90, and C92 replaced by alanines) were inserted into the pGEX-KG vector as N-terminal glutathione S-transferase (GST) fusion proteins. Full-length 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) and full-length rat Munc18-1 were inserted into pET-28b vector as C-terminal His-tagged proteins. We used the Quick Change site-directed mutagenesis kit (Stratagene) to generate all mutants including syntaxin 1a I203C and V241C and VAMP2 Q33C and A72C. DNA sequences were confirmed by the Iowa State University DNA Sequencing Facility.
4.3.2 Protein expression and purification

Protein expression and purification were described previously (Lai et al., 2013a; Shin et al., 2014). Briefly, all recombinant proteins were expressed in E. coli BL21 (DE3). GST-tagged proteins, syntaxin 1a, SNAP-25, VAMP2, and Vps were purified by affinity chromatography using Glutathione-Agarose beads (Sigma-Aldrich) and were cleaved from beads with thrombin (0.02 unit μl⁻¹, Sigma-Aldrich) in PBS or PBS with 0.8% (w/v) octyl β-D-glucopyranoside (PBS-OG) for membrane proteins. His-tagged proteins, apoA1, syt1, and Munc18-1 were purified by Ni-NTA agarose beads (Qiagen). His-tagged apoA1 and Munc18-1 were eluted with 200 mM imidazole (Sigma-Aldrich) in PBS. Munc18-1 was further dialyzed in 2 L PBS buffer at 4 °C overnight after elution. Syt1 was eluted with 25 mM HEPES pH 7.4 with buffer containing 400 mM KCl, 250 mM imidazole, 0.8% OG, and 1 mM EDTA.

4.3.3 Lipid mixture preparation

The lipid molecules used in this study are 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), cholesterol, 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamin-N-(biotinyl) (biotin-DPPE), and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (biotin-PEG-DSPE). All lipids were obtained from Avanti Polar Lipids. 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindocarbocyanine Perchlorate (DiI), 1,1′-Dioctadecyl-3,3,3′,3′-Tetramethylindodicarbocyanine Perchlorate (DiD), and sulforhodamine B were obtained from
Invitrogen. The desired amounts of lipids were first mixed in a glass tube and the mixture was then completely dried under vacuum.

**4.3.4 Fluorophore-labelling of the single cysteine mutants**

The fluorophore-labelling of the single cysteine mutants of syntaxin 1a and VAMP2 was described previously (Lai et al., 2014b; Shin et al., 2014). Briefly, the mutants were purified as described above for syntaxin 1a and VAMP2, except that the cleavage buffer contained 2.5 mM DTT. For smFRET of the trans-SNAREpin, cysteine mutants of syntaxin 1a (I203C or V241C) and VAMP-2 (Q33C or A72C) were desalted with the PD-10 column (GE healthcare) to eliminate free DTT and then incubated with 10x molar excess of maleimide-derivative fluorophores Cy5 and Cy3 (GE healthcare), respectively, at 4 °C overnight. Unreacted free fluorescence labels were removed by the PD-10 column. The labelling efficiency of each mutant was measured with spectrophotometry (Beckman). Extinction coefficients of Cy5 (250,000 M⁻¹ cm⁻¹ at 650 nm) and Cy3 (150,000 M⁻¹ cm⁻¹ at 552 nm) were used to calculate concentrations of fluorophores. The detergent compatible Lowry assay (DC assay, Bio-Rad) was used to determine the protein concentration. The labelling efficiencies were 46% and 40% for syntaxin 1a I203C and V241C while they were 55% and 62% for VAMP2 Q33C and A72C, respectively.

**4.3.5 Reconstitution and purification of t- and v-discs**

The mixture of POPC, DOPS, cholesterol, PIP₂, and biotin-PEG-DSPPE with the molar ratio of 62.9:15:20:2:0.1 for t-discs and the mixture of POPC, DOPS, and cholesterol with the molar ratio of 75:5:20 for v-discs were dried and resuspended in Tris150-EDTA buffer (10
mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4). The lipid compositions were chosen to emulate those of the presynaptic and the vesicle membranes at least for major lipid components. 3 μl of each of the 50 mM lipid mixture was dissolved in sodium cholate (the final concentration of 50 mM). Then, t- (the binary complex of syntaxin 1a and SNAP-25) or v-SNARE (VAMP-2 with or without syt1 with the molar ratio of 1:1) and apoA1 protein were added to the detergent-solubilized lipid mixture. The molar ratio of lipid, SNARE(s), and apoA1 was 160:0.5:2. The self-assembly of SNARE-incorporated nanodiscs was initiated by the rapid removal of sodium cholate by treating the sample with 50% (w/v) SM-2 Bio-Beads (Bio-rad). The t- or v-discs were then purified through gel-filtration using Superdex™ 200 GL 10/30 column (Amersham Biosciences).

4.3.6 FRET measurements of trans-SNAREpins

After coating the quartz surface with a solution of methoxy-polyethylene glycol (mPEG) and biotin-PEG molecules (100:1), the quartz slide was assembled into a flow chamber and coated with streptavidin (0.2 mg ml⁻¹). The t-discs containing Cy5-labeled sytnaxin 1a) were immobilized on the surface by flowing in 100-200 nM nanodisc solution. Then 1 μM Munc18-1 or equal volume buffer was flown in and the sample was incubated for 10 min at room temperature (~25 °C). Five times molar excess of v-discs containing Cy3-labeled VAMP2 without or with syt1 with 1 μM Munc18-1 or equal volume buffer were then added to the flow chamber and the sample was incubated for 25 min at 37 °C to allow trans-SNAREpin formation. All TIR experiments were performed at room temperature in the presence of the oxygen scavenger system (0.4 % (w/v) glucose (Sigma), 4 mM Trolox (Calbiochem), 1 mg/ml glucose oxidase (Sigma), 0.04 mg/ml catalase (Calbiochem)) in
Tris150-EDTA buffer. The smFRET measurements were carried out on a prism-type TIRF setup, which is based on the inverted microscope (IX71, Olympus) with the laser exposure time of 200 ms. A solid-state laser at 532 nm was used to excite the Cy3-labeled v-discs and measure FRET, and a Helium-Neon (HeNe) laser at 635 nm was used to check the presence of the Cy5-labeled t-discs. The Cy3 and Cy5 fluorescence emissions were collected by a water immersion lens (UPlanSApo 60x/1.20w, Olympus) and split by a dichroic mirror (T660lpxr, chroma), which has a threshold at 635 nm wavelength. Both Cy3 and Cy5 emissions were then imaged with the imaging area of 45×90 μm² side by side on an electron multiplying charged-coupled device camera (iXon DU897E, Andor Technology) which has high quantum efficiency in the 450-700 nm range. The collected images were analyzed by smCamera (kindly provided by Dr. Taekjip Ha’s group). FRET efficiencies \( E \) were obtained by

\[
E = \frac{I_A}{I_A + I_D}
\]

where \( I_A \) and \( I_D \) represent the acceptor and donor fluorescence intensities. More details about the experimental setup can be found in a recent review paper \( \text{(Roy et al., 2008)} \), and representative traces can be found in our previous work \( \text{[Figure 4 in reference (Shin et al., 2014)}] \).

### 4.3.7 Proteoliposome reconstitution

For the bulk and single lipid mixing assays, the molar ratios of lipids were 15:62:20:2:1:0.1 (DOPS:POPC:cholesterol:PIP\(_2\):DiI:Biotin-DPPE) for the t-SNARE-reconstituted (t-) vesicles, and 5:74:20:1 (DOPS:POPC:cholesterol:DiD) for the v-SNARE-reconstituted (v-) vesicles, respectively. For the t-vesicles without PIP\(_2\), equal molar amount of POPC was used instead. The lipid mixture was first completely dried under vacuum and
then hydrated by dialysis buffer (25 mM HEPES, pH 7.4, 100 mM KCl). After five freeze–thaw cycles, protein-free large unilamellar vesicles (~100 nm in diameter) were prepared by extrusion through a 100 nm polycarbonate filter (Whatman). For membrane reconstitution, SNARE proteins and syt1 were mixed with protein free vesicles at the protein to lipid molar ratio of 1:200 for each protein component (this ratio was kept for all experiments including the single vesicle content mixing assay) with 0.8% OG in the dialysis buffer at 4°C for 15 min. The liposome/protein mixture was diluted 2 times with dialysis buffer for t-vesicles, and then the diluted t-vesicles were dialyzed in 2 L of dialysis buffer at 4 °C overnight. For v-vesicles, the mixture was diluted twice with dialysis buffer containing 1 mM EDTA and dialyzed in 2 L of dialysis buffer with EDTA at 4 °C overnight. Details for reconstitution were described in our previous work (Lai et al., 2013a; Lai et al., 2013c).

For the single-vesicle content-mixing assay with the small sulforhodamine B content indicator, the lipid compositions were as the same as those used in the single vesicle docking assay except that the fluorescent lipid dyes (DiI and DiD) were replaced by the equal amount of POPC and 2% PIP2 was incorporated into the t-vesicles. The lipid mixture was first completely dried and then hydrated with dialysis buffer. A population of vesicles intended for v-vesicles was hydrated in the presence of 20 mM sulforhodamine B (SRB). The overall vesicle preparation and protein reconstitution process was the same as above except that v-vesicles were always kept throughout in the 20 mM SRB prior to dialysis overnight. Remaining free SRB was removed using the PD-10 desalting column (GE healthcare) after dialysis. Details for reconstitution were described in our previous work (Lai et al., 2013a; Lai et al., 2013c).
4.3.8 Single vesicle docking and lipid-mixing assays

The t-vesicles with the final lipid concentration of 1 μM were flown into the chamber and immobilized on the PEG-coated surface through the streptavidin-to-biotin lipid conjugation with 30-minute incubation at room temperature (~25 °C). After two rounds of washing with 200 μl dialysis buffer, 1 μM Munc18-1 or equal volume of dialysis buffer was flown into the chamber and the sample was incubated for another 30 minutes at room temperature. Then, the v-vesicles (3 μM) with or without 1 μM Munc18-1 were injected into the flow chamber and the sample was again incubated for 30 minutes at 37 °C. After washing off free v-vesicles using dialysis buffer containing 1 μM Munc18-1 or equal volume of dialysis buffer, movies were acquired by taking 100 consecutive frames with the 100 ms exposure time from five randomly chosen imaging areas using the same TIRF setup described above. The first 60 frames were taken by using 532 nm laser excitation for DiI-labeled t-vesicles and these data were used to calculate the FRET efficiency and the following 40 frames were taken by using 635 nm laser excitation to verify the presence of DiD-labeled v-vesicles. The non-specifically bound v-vesicles were excluded from the analysis.

4.3.9 Single-vesicle content-mixing assay

The t-SNARE vesicles (125 μM) were immobilized on the PEG-coated surface through the streptavidin-to-biotin lipid conjugation for 20 min. Following several rounds of washing with 200 μL of dialysis buffer, the v-vesicles containing 20 mM SRB (10 μM) were injected into the flow chamber and the sample were incubated at room temperature for 10 min for docking. After washing out the unbound v-vesicles with dialysis buffer containing 1mM EDTA,
additional 20 min incubation was followed by the injection of 500 μM Ca\(^{2+}\) using the motorized syringe pump. Movies are acquired using the same TIRF setup as described above with 532 nm excitation for SRB, and the stepwise jump in the fluorescence emission intensity due to fluorescence dequenching of SRB was recorded as the signal for content mixing, the representative images and traces can be found in our previous work [Figure S2 in reference (Lai et al., 2013c) and Figures 1B and S6B in reference (Lai et al., 2013a)]. The details of TIR fluorescence microscope imaging and the data analysis of the single-vesicle content mixing assay were described in detail elsewhere (Diao et al., 2012b).

4.4 Results

4.4.1 Munc18-1 promotes SNARE zippering without synt1, but not in the presence of synt1

It has been previously shown that Munc18-1 can bind to the SNARE four helix bundle but it has not yet been demonstrated that such binding actually stimulates SNARE zippering (Dulubova et al., 2007). To investigate the effects of Munc18-1 on the conformation of the trans-SNAREpin with smFRET we prepared the N-terminal FRET pair VAMP2 Q33C and syntaxin 1a I203C labeled with the donor dye Cy3 and the acceptor dye Cy5 (NN), respectively. We also prepared a C-terminal FRET pair, Cy3-labeled VAMP2 A72C and Cy5-labeled syntaxin 1a V241C (CC) (Fig. 1A). For the detection of FRET with total internal reflection fluorescence (TIRF) microscopy the t-SNARE reconstituted nanodiscs (t-discs) were tethered on the imaging surface using the streptavidin-to-biotin lipid conjugation. The v-SNARE reconstituted nanodiscs (v-discs) were then flown into the flow cell to allow docking and trans-SNAREpin formation (Fig. 1B) (Shin et al., 2014).
For the SNAREpin with the NN FRET pair we observed a dominant FRET distribution peaked at the FRET efficiency $E \sim 0.9$ (Fig. 1C, left), indicating that the helical structure is robust at the N-terminal region. We observed a small low FRET population (~8%) that might reflect non-specific binding of nanodiscs on the surface (Fig. 1C, left). The possibility of antiparallel SNARE assembly (Weninger et al., 2003) could be ruled out because out-of-register combinations (NC and CN) populated mid-FRET dominantly ($E \sim 0.4$) [See Figure 2C in reference (Shin et al., 2014)] but we did not observe an appreciable mid FRET population for the NN pair. In contrast, the SNAREpin with the CC FRET pair gave two major low and high FRET distributions with some population at mid-FRET [Fig. 1C, upper-right, see the reference (Shin et al., 2014) for the discussion of the mid-FRET population]. The low FRET peak might reflect a half-zipped SNAREpin, while the high and mid FRET peaks represent a fully-zipped SNAREpin (Shin et al., 2014). When Munc18-1 (1 µM) was added, for the CC FRET pair we observed the shift of the FRET distribution from low to high, reflecting that Munc18-1 promoted formation of the fully-zipped SNAREpin (Fig. 1C, lower-right, Fig. 1E), which was Munc18-1 dependent instead of non-specific crowing effect (Fig. 1F). The association between a t-disc and a v-disc was SNARE-dependent as evidenced by the significant reduction of the association in the presence of soluble VAMP2 (Fig. 1G).

Now, to find out if such promotion of SNARE zippering by Munc18-1 still happens in the presence of syt1 we incorporated syt1 to the v-disc in the molar ratio of 1:1 to VAMP2. With syt1 we still observed a dominant high FRET distribution for the NN FRET pair (Fig. 1D, upper-left), identical to that observed in the absence of syt1. Interestingly, however, we
did not observe any change in the distribution of the CC FRET population at all (Fig. 1D right, and Fig. 1H). Thus, the results suggest that syt1 works as an antagonist to Munc18-1 to interact with SNAREpin, which abrogates the enhancement of SNARE zippering by Munc18-1.

4.4.2 Syt1 and Munc18-1 are mutually antagonistic in SNARE-dependent lipid mixing

Since Munc18-1 stimulated SNARE zippering in the absence of syt1, we tested if Munc18-1 could stimulate lipid mixing as it was previously observed by Rothman and coworkers (Shen et al., 2007). To measure lipid mixing with the single vesicle assay we immobilized vesicles carrying t-SNARE on the imaging surface (t-vesicles) and vesicles carrying v-SNARE (v-vesicles) was added to the flow cell to allow vesicle docking (Fig. 2A). The lipid dyes DiI and DiD were separately incorporated into t- and v-vesicles, respectively to detect FRET due to lipid mixing (Fig. 2B-E). Without Munc18-1 lipid mixing was slow: only around 30% of docked vesicles showed lipid mixing after 30 min incubation (gray bars in Fig. 3A and Fig. 3C). However, when Munc18-1 was present lipid mixing was accelerated significantly: 65% of docked vesicles were lipid-mixed (blue bars in Fig. 3A and Fig. 3C), consistent with previously reported results (Diao et al., 2010). Moreover, we observed that Munc18-1 could also stimulate the docking between v-vesicles and t-vesicles, which is SNARE-dependent (Fig. 3D).

On the other hand, when syt1 was incorporated into the v-vesicles in the molar ratio of 1:1 to VAMP2 we observed great enhancement of lipid mixing. After 30 min incubation nearly all docked vesicles were lipid-mixed (gray bars in Fig. 3B and Fig. 3C). The stimulation of SNARE-dependent lipid mixing and vesicle docking by syt1 (Fig. 3D) was previously reported
and not surprising (Lai et al., 2013c). What is surprising is however, when we added Munc18-1 the stimulation of SNARE-dependent vesicle docking and lipid mixing by syt1 is reduced. We observed about 80% of docked vesicles to have lipid mixing (blue bars in Fig. 3B and Fig. 3C), and the docking was reduced by as much as 50% (Fig. 3D). As both SNARE and PIP2 were known to be important for the Ca2+-independent vesicle docking and lipid mixing, we removed PIP2 from t-vesicles. Without PIP2 Munc18-1 behaved the same as it did when PIP2 was present (Fig. 3, E and F), and the docking was reduced even further by as much as 70% (Fig. 3F) in the presence of syt1. Therefore, our results suggest that Munc18-1 is again antagonistic to syt1 in SNARE-dependent vesicle docking and lipid mixing.

4.4.3 Munc18-1 has little effect on Ca2+-triggered content mixing

Although SNAREs alone as well as SNAREs together with syt1 can support lipid mixing, they are not effective in driving content mixing or fusion pore formation unless Ca2+ is present (Lai et al., 2013a; Lai et al., 2013c). As such, in vitro Ca2+-triggered content mixing assay offers a more stringent test for the competition between Munc18-1 and syt1 to gain access to the SNAREpin.

To measure content mixing we incorporated sulforhodamine B into the v-vesicles in an in vitro setup depicted in Fig. 2A except for lipid dyes (Fig. 4A). After vesicle docking Ca2+ (500 µM) was flown into the flow cell and the intensity jump of the fluorescence signal due to fluorescence dequenching was detected as a signal for content mixing (Lai et al., 2013a). The cumulative time plot shows that approximately 13.5% of docked vesicle experience content mixing with a half time of ~30 sec (Fig. 4B), which is both syt1 and SNARE dependent.
Interestingly though, addition of Munc18-1 showed little change in the cumulative time plot in the range of 100-1000 nM, although there was some reduction of content mixing at 1 µM of Munc18-1 (Fig. 4B), which is dependent on the Ca\(^{2+}\) (Fig. 4C). Thus, our result demonstrates that Munc18-1 does not stimulate Ca\(^{2+}\)-triggered fusion pore opening, suggesting that the SNAREpin is not accessible to Munc18-1 in the presence of syt1.

Similarly, we also note that in this assay the vesicle docking became significantly reduced as the Munc18-1 concentration was increased (Fig. 4D and Table 1). We interpret the reducing of vesicle docking as the consequence of dissociation of heterodimeric t-SNARE into the individual components syntaxin 1a and SNAP-25 (Ma et al., 2013; Zhang et al., 2015), which would reduce vesicle docking mediated by the interaction between syt1 and t-SNARE (de Wit et al., 2009; Kim et al., 2012). Therefore, it appears that Munc18-1 has the ability to both improve and prevent SNARE complex formation in a concentration dependent manner.

4.5 Discussion

In this work, we observed, using smFRET and single vesicle lipid mixing, that Munc18-1’s positive influence on SNARE zippering and lipid mixing is robust in the absence of syt1. Our results are consistent with those of Rothman and coworkers (Shen et al., 2007) and show that Munc18-1 indeed has the capacity to drive the completion of SNARE zippering by acting on the partially-zipped (N-terminally-assembled but C-terminally-frayed) SNARE complex. Rothman and coworkers (Shen et al., 2007) also showed that the incubation at low temperature, which presumably promotes N-terminal zippering of SNAREs, is required for the
enhancement of SNARE-dependent lipid mixing by Munc18-1, again consistent with our results from smFRET on the trans-SNAREpin between two apposed nanodiscs.

Surprisingly, however, when syt1 is present the stimulation of SNARE zippering by Munc18-1 disappears completely while its effect on lipid mixing is reduced significantly. Most importantly, we did not observe any acceleration of content mixing (or fusion pore opening) by Munc18-1. These findings suggest that syt1 competes with Munc18-1 for the access to the SNAREpin and acts as an antagonist for Munc18-1 in SNAREpin binding. It is not clear if syt1 binds the SNARE complex in replacement of Munc18-1. We cannot rule out the possibility that syt1 binds to lipids (most likely PIP2) in the close vicinity of SNARE complex, which in turn expel Munc18-1 from the SNARE complex. However, it has been shown that Munc18-1 could stimulate lipid mixing in the presence of syt1 in the recent work employing SUV/GUV (Parisotto et al., 2012; Parisotto et al., 2014), even without pre-incubation on ice. These results appear to be at odds with our data, warranting further investigation.

Neurotransmitter release is tightly regulated by Ca\textsuperscript{2+} and happens in less than 1 msec upon the Ca\textsuperscript{2+} influx (Borst and Sakmann, 1996; Jahn and Fasshauer, 2012). Thus, it appears to be necessary for Ca\textsuperscript{2+}-sensor syt1 to gain intimate access to the SNAREpin (Chapman, 2002). In fact, it is shown that syt1 has the capacity to bind the SNARE core and negatively charged lipids clustered at the immediate vicinity of the SNARE complex (Kim et al., 2012; Honigmann et al., 2013).
With the necessity of the synt1’s presence at the nearest neighbor of the SNAREpin it is hard to envision how Munc18-1 gains full access to the SNAREpin simultaneously. Meanwhile, Munc18-1 binding to the SNARE core is relatively weak with the binding constant of \( \sim 1 \mu M \) (Xu et al., 2010) which may not be sufficiently strong to hold onto the SNARE core in competition with synt1.

Alternatively, Munc18-1’s binding to the Habc domain of syntaxin 1a is much stronger with the binding constant of \( \sim 10 \) nM (Misura et al., 2000; Burkhardt et al., 2008). This binding stabilizes the ‘closed’ form of syntaxin 1a, which does not allow the premature binding of syntaxin 1a to SNAP-25 (Ma et al., 2013). A controlled binding between syntaxin 1a and SNAP-25 is necessary because their free-binding likely leads to the formation of the 2:1 complex which is known to be the non-productive dead-end product (Fasshauer et al., 1997b; Xiao et al., 2001). Recently, it was shown that Munc13 plays a role in relieving syntaxin 1a from the inhibitory Munc18-1 capping (Ma et al., 2011; Ma et al., 2013).

The notion that Munc18-1 is part of the minimal fusion machinery is largely based on two experimental observations. Firstly, the knockout of Munc18-1 abolishes neurotransmitter release completely (Verhage et al., 2000; Gerber et al., 2008; de Wit et al., 2009). The severity of the knockout phenotype could be explained equally well by the losing of inhibitory capping on syntaxin 1a. Secondly, Munc18-1 has the capacity of accelerating SNARE-dependent proteoliposome fusion significantly (Shen et al., 2007; Yu et al., 2013). The caveat here is however that synt1 is not included in those experiments. Our results argue that Munc18-
1 may not be part of the minimal fusion machinery and it rather plays an important regulatory role in controlling the syntaxin 1a binding to SNAP-25, at least in neuro-exocytosis.

There are however many exocytotic systems where vesicle fusion is not regulated by Ca$^{2+}$ and syt1-like molecules (Schekman, 1992; Bonifacino and Glick, 2004). Also, there are systems where the Habc-like domain is not present in syntaxin 1a-analogs (Dulubova et al., 2001; Dulubova et al., 2002). In such cases, the Sec1/Munc18’s main function may be to stimulate vesicle fusion via its binding to the SNAREpin. The analysis by Shen et al. suggests that the SNAREpin binding may be the evolutionarily conserved function for the Sec1/Munc18 family (Yu et al., 2013). However, at the top of the evolution may be neuroexocytosis. Thus, we speculate that the evolutionary pressure to implement the tight Ca$^{2+}$ control of vesicle fusion might have diverted the Munc18-1’s role elsewhere.
4.6 References


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Figure 1 Effects of Munc18-1 on the conformation of the trans-SNAREpin detected by smFRET. (A) Fluorescent dye-labeled positions on syntaxin 1a and VAMP2. I203C or V241C of syntaxin 1a was labeled with Cy5 (light blue saw-tooth circle) and Q33C or A72C of VAMP2 was labeled with Cy3 (red saw-tooth circle). The Habc domain (white) of syntaxin 1a is depicted broken to indicate the longer length than the SNARE motif (red). (B) The TIRF
microscope setup for the smFRET detection of the trans-SNAREpin conformation. The t-discs carrying Cy5-labeled syntaxin 1a (red and white) and SNAP-25 (green) was immobilized on the quartz surface through the biotin (yellow square) and streptavidin (purple, cross-shaped) conjugation and then allowed to interact and form the trans-SNAREpin with the v-discs carrying Cy3-labeled VAMP2 (blue) with or without synaptotagmin 1 (syt1) (not shown here). Distributions of the FRET efficiency for the NN and CC FRET pairs in the absence (upper panel) or presence (lower panel) of Munc18-1 without syt1 (C) and with syt1 (D), respectively. (E) Plot of the normalized FRET populations in the absence of syt1 using the cutoff FRET efficiencies \(E\) of \(E \leq 0.4\) for low FRET, \(0.4 < E < 0.72\) for medium FRET, and \(E \geq 0.72\) for high FRET. (F) The relative changes in the ratio of high-to-low FRET populations for the CC pair in the presence of increasing amount of Munc18-1 or 2 μM BSA without syt1. (G) Docking ability of the CC pair in the absence and presence of 10 μM soluble VAMP2 (Vps, amino acids 1-94). (H) Plot of the normalized FRET populations in the presence of syt1 using the cutoff FRET efficiencies \(E\) of \(E \leq 0.4\) for low FRET, \(0.4 < E < 0.72\) for medium FRET, and \(E \geq 0.72\) for high FRET. Error bars denote the S.D. of three independent experiments, and movies were recorded for the analysis from more than 5 randomly selected screens in each experiment. * \(P < 0.05\), assessed using the two-sample \(t\)-test, unless otherwise specified.
Figure 2 Schematic diagram and representative traces of the single-vesicle docking and lipid mixing assay. (A) Schematic diagram of the single-vesicle docking and lipid mixing assay. T-vesicles reconstituted with SNAP-25/syntaxin 1a were immobilized on the surface of the flow cell. V-vesicles reconstituted with VAMP2 or VAMP2 together with syt1 (VAMP2:syt1=1:1) were flown into the flow cell with or without 1 μM Munc18-1. (B) Representative imaging areas of DiI (green framed, left) and DiD (red framed, right) emission under 532 nm excitation for DiI-labeled t-vesicles. Each green circle in the left represents emission from an immobilized t-vesicle and the fluorescence emission was analyzed as shown on the right. Three representative spots were chosen as shown in yellow circles and labeled with a, b, and c. (C) The trace of spot (a) represents immobilized t-vesicle only. (D) The trace
of spot (b) represents docking of v-vesicle onto the immobilized t-vesicle without significant lipid mixing. (D) The trace of spot (c) represents docking of the v-vesicle onto the immobilized t-vesicle with significant lipid mixing. The green bar represents a 532 nm excitation for FRET and red bar represents a 635 nm excitation for checking the presence of docked vesicles.
Figure 3 Munc18-1 and syt1 are mutually antagonistic in SNARE-dependent lipid mixing. (A) Normalized distributions of FRET efficiencies for immobilized t-vesicles and docked VAMP2-vesicles (grey), VAMP2-vesicles in the presence of 10 μM Vps (red), VAMP2-vesicles in the presence of 1 μM Munc18-1 (blue), and VAMP2-vesicles in the presence of 10 μM Vps and 1 μM Munc18-1 (dark cyan). (B) Normalized distributions of FRET efficiencies for immobilized t-vesicles and docked VAMP2/syt1-vesicles (grey), VAMP2/syt1-vesicles in the presence of 10 μM Vps (red), VAMP2/syt1-vesicles in the presence of 1 μM Munc18-1 (blue), and VAMP2/syt1-vesicles in the presence of 10 μM Vps and 1 μM Munc18-1 (dark cyan). (C) Lipid-mixing efficiencies quantified based on the percentages of normalized high-FRET population (E~0.5-1) (Yoon et al., 2006). Bars are normalized with respect to the number of the docked vesicles to take into account the
differences in docking probabilities (D). (E) Lipid-mixing efficiencies quantified based on the percentages of the normalized high-FRET population (E~0.5-1) (Yoon et al., 2006) for the t-vesicles without PIP2. Bars are normalized with respect to the number of the docked vesicles (F). Error bars denote the S.D. of three independent experiments, and movies were recorded for analysis from more than 5 randomly selected screens in each experiment. * P<0.05, assessed using the two-sample t-test, unless otherwise specified.
Figure 4 Effects of Munc18-1 on Ca^{2+}-triggered fusion pore opening in *in vitro* content mixing assay. (A) Schematics of the *in vitro* content mixing assay. (B) Quantitative comparison of the cumulative time content mixing percentage of the total docked population at various concentrations of Munc18-1 (Ca^{2+} injection at 10 s). The control, without Munc18-1, is depicted with red circles. (C) Plot of the content mixing percentage at 120s in the absence of Ca^{2+}. (D) *In vitro* single vesicle docking. Individual v-vesicles that tethered onto the t-vesicles on the imaging area were counted. The experiments were performed by incubating the samples in the presence of the specified Munc18-1 concentrations. The data were normalized against the control which was obtained in the absence of Munc18-1. Error bars in (B-D) denote
the S.D. of three independent experiments, and movie was recorded for analysis from one randomly selected screen in each experiment.
4.8 Supplementary Information

Table 1. Numbers of docked vesicles for content mixing using Sulforhodamine B

<table>
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<tr>
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CHAPTER 5
GENERAL SUMMARY

5.1 General Conclusion

Structure conserved SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins mediate membrane fusion, which is essential for all exocytosis pathways (Rothman, 1994). However, the remarkable character of the synaptic vesicle exocytosis process in neuron is that precise temporal control of the release is essential for healthy brain activities. Sec1/Munc18 (SM) proteins, similar as the SNAREs, are critical for most types of intercellular membrane traffic (Sudhof and Rothman, 2009; Rizo and Xu, 2015), Munc18-1, the member of SM protein family in neuron, is proposed to be required for every step of membrane fusion (Shen et al., 2007; Rizo and Sudhof, 2012), and provide the neuronal SNAREs mediated synaptic vesicle fusion with ability of both specificity and fast rate (Shen et al., 2007; Ma et al., 2013). However, in these work models, the other protein, synaptotagmin 1 (Syt1) is missed. Syt1, which has two tandem Ca$^{2+}$-binding C2 domains, therefore is identified as a major Ca$^{2+}$ sensor for synaptic vesicle fusion (Perin et al., 1991; Fernandez-Chacon et al., 2001; Chapman, 2008). The arrival of action potential at the presynaptic terminal causes the Ca$^{2+}$ influx, which binds to Syt1 and together trigger vesicle fusion. However, the mechanism of this is still elusive. Intensive studies have been carried out in the past decades. It is proposed that Ca$^{2+}$ binding to Syt1 would (1) relieve Syt1’s fusion clamp effect (Chicka et al., 2008), (2) displace inhibitory Cpx from the SNARE core (Tang et al., 2006), (3) insert into the membrane and destabilize or curvature the bilayer at the fusion site (McMahon et al., 1995; Hui et al., 2009), (4) cross-link the opposing membranes and
short the distance that accelerates the fusion rate (Arac et al., 2006; Lee et al., 2010). However, most of the previous work is relied on the ensemble vesicle fusion assays, which is not possible to dissect fusion steps in detail. Moreover, the working model of Munc18-1 functioning as core fusion machinery has not been characterized yet. Therefore, it is necessary to study whether Munc18-1 really provides energy directly at the final fusion pore opening stage, and how the proteins like SNAREs, Munc18-1, Syt1, and Complexin interact together.

In our work, we first focused on the linker region of Syt1 between its transmembrane region and cytoplasm C2 domain (Lai et al., 2013). This linker region is highly unstructured, but has a basic residue-rich N-terminal half and an acidic residue-rich C-terminal region. Interestingly, this feature is well conserved in all species from C. elegans to human. Using site-mutagenesis and cross-linking combining with the smFRET, our results suggested that the flexible linker region of Syt1 may function as a distance regulator which undergoes conformational changes during vesicle fusion: it stretches out to mediate vesicle docking but folds to assist C2AB for fusion pore opening. A very recently work using a fluorescence-lifetime based inter-membrane distance ruler suggested a similar mechanism as ours (Lin et al., 2014).

Next, we re-examined a serial of characterized mutants of Syt1, which might impair Syt1-SNARE/PIP2 interaction, Ca$^{2+}$-binding, or membrane penetration (Lai et al., 2015). The results show that Ca$^{2+}$-independent t-SNARE and PIP2 binding, mediated by the polybasic region of Syt1, is essential for vesicle docking. To our surprise, however, content release is regulated only by the Ca$^{2+}$-dependent insertion of the loop region and Ca$^{2+}$ binding sites of
Syt1 into the membrane, and not at all by its SNARE/PIP2 interaction. This work shed some light on the mechanism of how Syt1/Ca2+ coupling triggers fusion pore opening, although more work is still necessary.

Although there is a proposed working model of Munc18-1’s role in all steps of fusion, but it has not been well characterized yet. One of the reason is because of the shortage of the ensemble bulk fusion assays used and missing of the full-length Syt1 in the previous work. Using our well developed *in vitro* single vesicle fusion assay, we are able to revisit Munc18-1’s function. Our results show that although Munc18-1 has the capacity to stimulate SNARE complex formation and SNARE-dependent lipid mixing, syt1 largely negates such positive effects on membrane fusion, suggesting that syt1 acts as an antagonist for Munc18-1 (Lou *et al.*, 2015). Similar result is shown by the other work independently (Zhang *et al.*, 2015), which suggested that Munc18-1 may not alter the fusion rate mediated by neuronal SNAREs, Syt1, and complexin. At together, these works suggest that although the SM family may serve as part of the fusion machinery in other exocytotic pathways, Munc18-1 may have evolved to play a different role such as regulating syntaxin 1A in neuroexocytosis.

### 5.2 Future Direction

The regulatory proteins are important for the synaptic vesicle fusion for the ability of precise temporal control of the fusion pore opening. However, the neuronal SNAREs are the core fusion machinery and may provide the free energy source. The C terminus of SNAP-25 is well known to be the target of botulinum neurotoxins (BoNT/A and BoNT/E) that block neurotransmitter release *in vivo* (Blasi *et al.*, 1993; Binz *et al.*, 1994). We propose that the
truncated SNAP-25 mutants will disrupt the assembly of SNARE core complex and then inhibit the initial fusion pore opening. Therefore, by aiding of these mutants, one of our ongoing work is using our recently developed smFRET based single nanodisc sandwich to examine the SNARE complex assembly and using the single vesicle fusion assays to delineate the effect of SNARE complex assembly on each step of fusion. In the other hand, the assembly of SNARE core complex provides the energy source to bridge the two membranes, which is also thought to zipper all the way through the transmembrane regions to drive the membrane fusion. However, the function of the juxtamembrane regions is still unclear. Therefore, we are also interest to decipher the function of the linker region.

Secondary, Zinc, which the secondary abundant transition metal in the body after iron is essential for normal cellular function, was also thought as a neuro-toxin. Previous work had suggested that Zn$^{2+}$ can be released from nerve terminals after transient global ischemia, sustained seizures, and head trauma, which would result in elevations of Zn$^{2+}$ in the extracellular space in CNS and become neurotoxic. A key step of Zn$^{2+}$ induced neuronal death appears to be excessive influx across the plasma membrane largely through Voltage-gated Ca$^{2+}$ channels. However, the detail mechanism of Zn$^{2+}$'s neuro-toxicity is still unknown. We propose that that Zn$^{2+}$ toxicity may derive from the Zn$^{2+}$ effect on the activity zone during high level influx, and our preliminary data showed that Zn$^{2+}$ may affect the promote SNAREs and Syt1 docked vesicles at both lipid mixing and content mixing levels. Moreover, the stimulatory effect of Zn$^{2+}$ is even stronger than Ca$^{2+}$, and also different to Ca$^{2+}$. We are going to clarify the mechanism that might be very new and exciting.
Moreover, because release underlies cognition and behavior, toxic agents that undermine the release of neurotransmitter might lead to the symptoms of neurodegenerative diseases such as Parkinson’s and Alzheimer’s. Comprehending the mechanism of how neurotoxin affects neurotransmitter release might benefit for developing new therapeutic strategy. Though the α-synuclein monomer functions as a chaperone for promoting SNARE complex formation (Burré et al., 2010) it becomes a toxic agent when misfolded into oligomers (Conway et al., 2000), causing Parkinson’s disease and dementia with Lewy body. Very recently, our group discovered that the pathophysiological origin of the toxicity of α-synuclein oligomers might be its intervention of the SNARE fusion machinery (Choi et al., 2013). we also found that α-synuclein monomers can inhibit vesicle fusion at high concentrations (Lai et al., 2014). However, the chaperone effect of α-synuclein has not been understood well in vitro yet. Recently, using our developed single-vesicles docking assays, we found the recombinant α-synuclein has a bitonic dose-response function for SNARE-dependent vesicle docking. Our results showed that α-synuclein could enhance docking at lower concentration through a trans-interaction between negatively charged lipid and VAMP2, whereas it might inhibit docking due to its effect on vesicle clustering.

5.3 Reference


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This thesis is dedicated to the memory of my beloved father, Hezhen Lou, I miss him every day!