STRUCTURAL STUDIES OF COMPLEXIN-SNARE INTERACTIONS

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DEDICATION

I would like to thank my family and friends who supported me

STRUCTURAL STUDIES OF COMPLEXIN-SNARE INTERACTIONS

by

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STRUCTURAL STUDIES OF COMPLEXIN-SNARE INTERACTIONS

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Vesicular neurotransmitter release is mediated by exocytosis of synaptic vesicles at the presynaptic active zone of nerve terminals. The Ca^{2+}-triggered release process is extremely fast, lasts less than half a millisecond, and is tightly regulated by Ca^{2+}. Action potentials cause Ca^{2+} influx through voltage-gated Ca^{2+} channels, which in turn triggers synaptic vesicle fusion. The typical sub-millisecond latency between an action potential and neurotransmitter release and the precise timing of the release process are crucial for information processing in the nervous system. To achieve this exquisite regulation, many proteins are involved.

The goal of our investigations was to delineate interactions between complexin and SNARE components that lead to the formation of a primed minimal fusion machinery. We have generated and used new constructs of short forms of synaptobrevin and complexin, as well as constructs of SNAP-25 and syntaxin that have previously been shown to be part of the minimal fusion machinery.

With NMR spectroscopy, the use of the short synaptobrevin constructs has led to experimental results suggesting at least two key intermediates during the docking/priming process that are independent of complexin. We found evidence for a modular assembly of the full SNARE complex. In the absence of Syb2-CT, the N-terminal half of SNARE complex forms a four-helix bundle, while the C-terminal half, starting just after the polar layer, is disordered. In the presence of the Syb2-CT, however, both halves of the SNARE complex form a four-helix bundle. It is interesting to note that Syb2 residues 29-84 are sufficient for formation of the fully assembled SNARE complex. This evidence strongly suggests the existence of at least two intermediates during the docking priming reaction.

Furthermore, with NMR spectroscopy, we have found new evidence that complexin can bind to the t-SNARE complex, in contrast to earlier evidence suggesting that complexin regulates the fully assembled SNARE complex. We demonstrated that Cpx-FL binds the t-SNARE complex SN1/SN3/Syx1a(188-259) in solution, as was suggested for membrane-bound t-SNAREs. Note, however, that the t-SNARE complex does not contain the large complexin-binding interface provided by Syb2. Furthermore, we found that Cpx-FL also binds t-SNARE sub-complexes formed by SN1/SN3, and

SN1/Syx1a(188-259), while very little binding was observed between Cpx-FL and Syx1a(188-259) alone. This finding is particularly interesting, because the crystal structure of the fully assembled SNARE complex does not suggest any binding between Cpx26-83 and either SN1 or SN3, whereas the only common component in all of the above experiments was SN1 domain.

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LIST OF DEFINITIONS

1D	One – dimensional
2D	Two – dimensional
3D	Three - dimensional
C.elegans	*Caenorhabditis elegans*
CSA	Chemical shift anisotropy
DAG	Diacylglycerol
DD	Dipole-dipole interaction
DMSO	dimethylsulfoxide
DTT	Dithiothreitol
E.coli	*Eschericheria coli*
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-tetraacetic acid
EM	Electron microscopy
EPR	Electron paramagnetic resonance
FPLC	Fast performance liquid chromatography
FRET	Fluorescence resonance energy transfer
GAP	GTPase activating factor
GST	Glutathione-S-transferase
HSQC	Heteronuclear single quantum correlation
IPTG	Isopropyl β-D-thiogalactopyranoside

ITC	Isothermal titration calorimetry
Ka	Association constant
Kd	Dissociation constant
LB	Luria broth
NMR	Nuclear magnetic resonance
NSF	N-ethylmaleimide-sensitive factor
OD	Optical density
PAGE	Polyacrylamide gel elecgtrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PMSF	Phenylmethylsulphonyl fluoride
ppm	Parts per million
RIM	Rab3-interacting molecule
RIM-BP	RIM-binding protein
rms	Root mean square
rpm	Revolution per minute
SDS	Sodium dodecylsulfate
SM	Sec1/Munc18
SNAP	Soluble NSF attachment protein
SNAP-25	Synaptosome-associated protein of 25 kDa
SNARE	SNAP receptor

TCA	Trichloroacetic acid
TCEP	Tris(2-carboxyethyl) phosphoine
TeNT	Tetanus toxin
TEV	Tobacco etch virus
Tris	Tris (hydroxymethyl) amonomethane
t-SNARE	Target membrane SNARE
UV	ultraviolet
VAMP	Vesicle-associated membrane protein
v-SNARE	Vesicle SNARE

CHAPTER 1

GENERAL INTRODUCTION

1.1 Signal Transduction in Neurons

The nervous system is a highly integrated and coordinated system which is specialized in monitoring and responding to a great variety of stimuli and regulating all aspects of bodily activities using electrochemical signals. The Nervous systems are found in many multicellular animals but differ greatly in complexity between species. Main components of the nervous system are composed of nerves called neurons.

1.1.1 The Nervous System and the Neuron

The nervous system is a complex, highly organized network of billions of neurons and even more neuroglia. The structures that make up the nervous system include the brain, spinal cord, nerves, ganglia, enteric plexuses, and sensory receptors. The neurons are the functional unit of the nervous system and can be divided into hundreds or even thousands of subgroups depending on their size, location, and shape. Most neurons have three different parts: (1) a star-like cell body or soma, (2) broad dendrites emerging from one pole, and (3) a fine axon emerging from the other pole (Figure 1.1). The cell body contains a nucleus surrounded by cytoplasm; within the cytoplasm, typical organelles such as lysosomes, mitochondria, and the Golgi complex can be found. Two kinds of processes or extensions emerge from the cell body of a neuron: multiple dendrites and a single axon. Dendrites (=little trees) are the receiving or input portions of a neuron. They usually are short, tapered, and highly branched. The

single axon of a neuron propagates nerve impulses toward another neuron, a muscle fiber, or a gland cell. This asymmetric shape gives the neuronal network a direction. The cell body receives input signals from dendrites and passes them to the following neuron by an action potential. Neurons form specialized structures called synapses to pass the signal to the following neuron (Figure 1.2). Chemical synapses allow the neurons of the central nervous system to form interconnected neural circuits. They are important for the biological computations that underlie perception and thought. The distinctive feature of these synapses is that the action potentials arriving at the presynaptic terminal lead to the release of a chemical messenger. The presynaptic terminals are distinct, specialized structures located at the terminal branches of the axon. They are characterized by an active zone, a region where the presynaptic plasma membrane comes in close contact with the postsynaptic plasma membrane, and an associated cluster of vesicles (De Camilli P, 2001). One neuron transmits signals to the dendrites of other neurons through its terminals. This transmission is accomplished by releasing the neurotransmitter.

1.1.2 Action Potential and Synaptic Transmission

An action potential or nerve impulse is a sequence of rapidly occurring electrical events that take place in two phases. During the initial, depolarizing phase, the negative membrane potential decreases toward zero and eventually becomes positive. Then, the repolarizing phase restores the membrane potential to the resting state of -70 mV (Jessell and Kandel, 1993). If the membrane potential is reduced by a threshold of 15 mV to -55 mV, an action potential is generated. Different neurons may have different thresholds for generation of an action potential, but the threshold in any one neuron usually is constant.

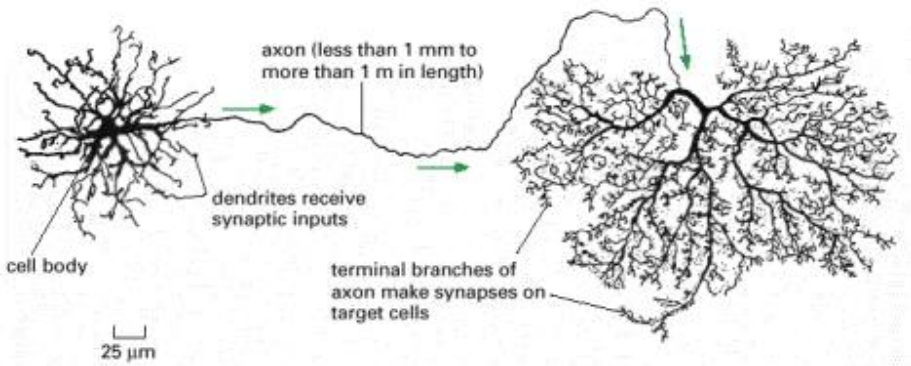


Figure1.1 Structure of a typical neuron. The neuron shown is a typical neuron of a vertebrate such as the retina of a monkey. The *arrows* indicate the direction in which signals are transmitted. The size of neurons are various, and longest and largest neurons in a human extend for about 1 m and have an axon diameter of 15 µm (Drawing of neuron from B.B. Boycott, in Essays on the Nervous System) (Colonnier, 1974).

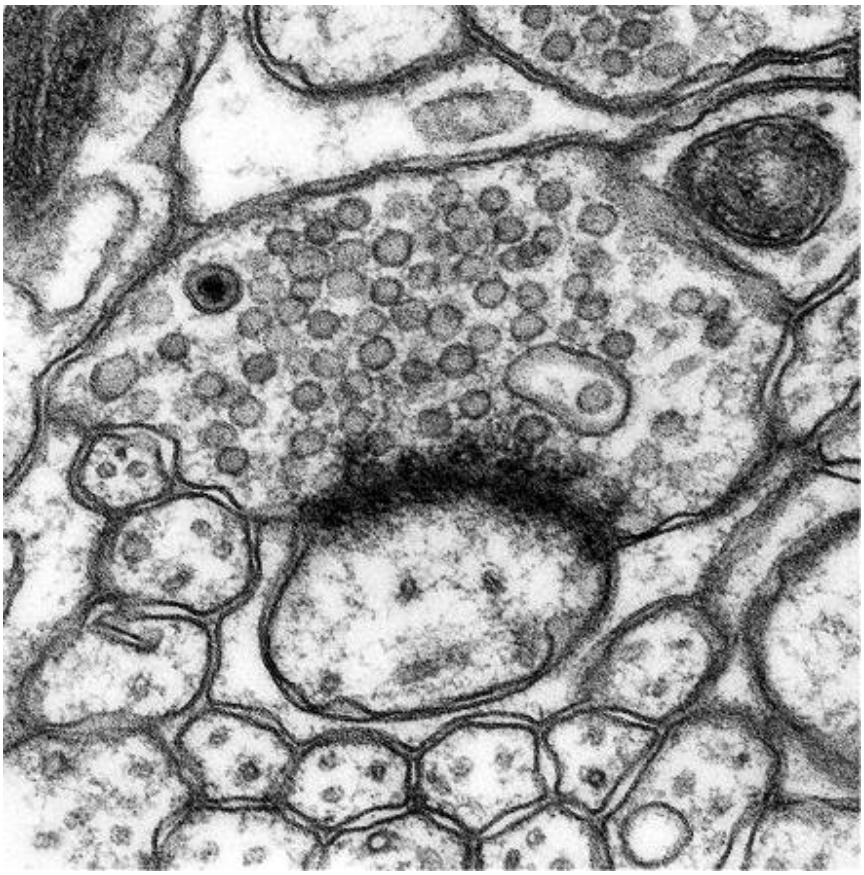


Figure 1.2 Ultrastructure of the synapses. A electron micrograph a synapse showing the pre- and postsynaptic elements with synaptic specializations. The actual points of synaptic contact are indicated by the arrows. The presynaptic compartment is densely populated by small, synaptic vesicles that store and secrete neurotransmitters (Liza, 2004).

There are no big or small action potentials in one nerve cell. Therefore, the neuron either does not reach the threshold or a full action potential is fired. During an action potential, two types of voltage-gated channels open and then close. These channels are present mainly in the plasma membrane of the axon and axon terminal. The Na^+ channels are the first channels that open; they allow Na^+ to rush into the cell, resulting in the depolarizing phase. Then K^+ channels open, allowing K^+ to flow out, producing the repolarizing phase. When the membrane potential reaches to 40 mV during the action potential, this depolarization of the membrane is followed by a rapid repolarization (Hodgkin, 1964; Huxley, 1964; Katz and Miledi, 1968). Together, the depolarization and repolarization phases last about 1 millisecond in a typical neuron.

1.1.3 Calcium Dependence of Neurotransmitter Release

To communicate information from one part of the body to another, an action potential must travel from where it arises at a trigger zone to the axon terminals. The arrival of the action potential at the presynaptic terminal opens voltage-gated Ca^{2+} channels at the synapse and causes a sudden increase of intracellular Ca^{2+} concentration. This acute Ca^{2+} influx triggers the actual release of neurotransmitters with an exquisite temporal specificity through the process of membrane fusion.

A central question is to understand the tight coupling between the increase in intracellular Ca^{2+}, and the activation of the exocytotic synaptic vesicle fusion machinery. The probability of vesicle fusion increases drastically in the 200 microseconds following Ca^{2+} influx and returns to much lower levels within 1 millisecond. In order to ensure the temporal fidelity of synaptic transmission, Ca^{2+} regulated neurotransmitter release is

uniquely rapid and precise, a fact that distinguishes it from other membrane trafficking processes. Intensive studies of synaptic proteins by multiple approaches have yielded insights into the molecular basis of the various steps that lead to neurotransmitter release.

Action potential-evoked vesicle release has at least two components, a fast synchronous component that dominates at low-frequency stimulation, and a slower asynchronous component that dominates at high-frequency stimulation. Compared to other Ca^{2+}-regulated biological processes, both components of release are rapid, because Ca^{2+} triggers synchronous release in as little as 100 microseconds, and asynchronous release in 10-50 milliseconds. Both release components are Ca^{2+}-dependent, with similar apparent Ca^{2+} cooperativities but different apparent Ca^{2+}-affinities, suggesting that multiple Ca^{2+}-sensors control release.

A number of key proteins involved in neurotransmitter release have been discovered, and extensive studies have yielded crucial insights into the molecular basis for the different steps that lead to release. But many aspects of the mechanism of exocytosis and its regulation by Ca^{2+} remain obscure. Soluble NSF attachment receptor proteins (SNAREs) are composed of the vesicle protein synaptobrevin and the presynaptic plasma membrane proteins syntaxin and SNAP-25. These presynaptic SNAREs are essential for synaptic transmission. They form a four-helix core complex during exocytotic processes. Ca^{2+}-sensors interact with SNAREs to promote synaptic exocytosis. Thus, the identification of Ca^{2+}-sensors is crucial for unveiling the mechanisms governing synaptic transmission. Synaptotagmin-1 is currently one of the best characterized proteins shown to act as a major Ca^{2+}-sensor that triggers exocytosis in

the forebrain. Recent studies showed that complexin are also involved in the Ca^{2+}-triggered step of neurotransmitter release.

1.1.4 Synaptic Vesicle Cycle

Synaptic vesicles are independent functional organelles that are made of a lipid bilayer in which transport proteins specific to each type of neurotransmitter are inserted. Fusion of the synaptic vesicle membrane with the plasma membrane to release its contents into the synaptic cleft is an essential step for signal transmission. In order to achieve highly precise spatial and temporal regulation, a complex hierarchy of protein machinery is involved. Synaptic vesicles filled with neurotransmitters dock at specialized areas of the presynaptic plasma membrane known as the active zone. Docking is a recognition event that determines the fusion specificity of a particular vesicle and is followed by a priming reaction that makes the vesicles ready to fuse. In this priming step, the protein of synaptic vesicle and the plasma membrane probably form a complex to prepare for fusion. The resulting primed state may already involve partial membrane fusion (hemifusion); however the actual neurotransmitter release requires complete membrane fusion and opening of the fusion pore. Complete membrane fusion is triggered only when an arriving action potential causes an increase in the intracellular Ca^{2+} concentration. The released synaptic vesicles are then retrieved from the plasma membrane by endocytosis, refilled with fresh neurotransmitter, and trafficked back to rejoin the existing pool of 200-500 vesicles to wait for the next cycle (Südhof, 1995, 2004) (Figure 1.3).

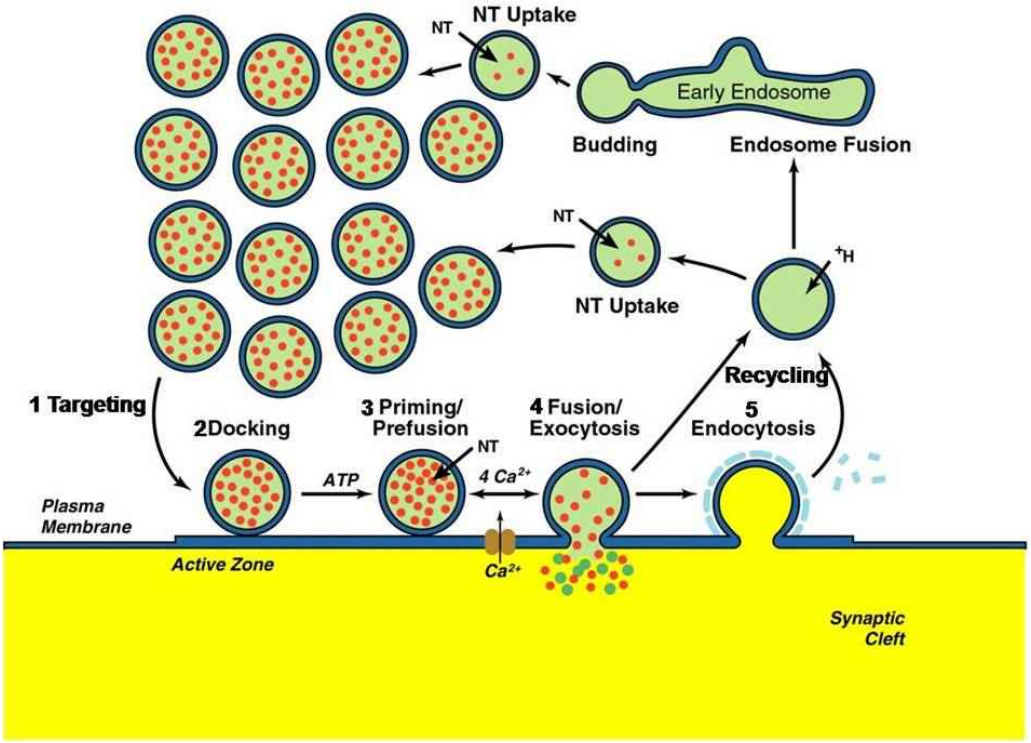


Figure 1.3 Synaptic vesicle cycle. The synaptic vesicle cycle is tightly regulated by a cascade of protein-protein interactions. Synaptic vesicles are filled with neurotransmitters and form the reserve pool of the vesicle cluster. Filled vesicles dock at the active zone. Then, the docked vesicles undergo a priming step, which makes the vesicles ready for Ca^{2+} triggering. After fusion-pore opening, the fused synaptic vesicles are retrieved back for recycling. (Südhof, 2004).

1.1.5 Membrane Trafficking and Membrane Fusion

Membrane trafficking within eukaryotic cells is a highly regulated process, which is vital for the maintenance of distinct subcellular compartments, and a variety of secretion processes (Rothman, 1994; Schekman, 1996). Membrane trafficking along the exocytic and the endocytic pathways of eukaryotic cells is mediated by a series of vesicular intermediates. The fact that vesicular transport is vectorial suggests that each step is mediated by specific targeting, docking, and fusion events. The molecular machinery for this process is conserved from yeast to neurons (Bennett and Scheller, 1993; Clary et al., 1990). Synaptic vesicle exocytosis is basically a membrane fusion process. Because of the repulsive force from negatively charged heads of phospholipids on the outer surface of the two opposing membranes, membrane fusion does not occur spontaneously. Forces such as electrostatic repulsion and repulsive hydration force push the membranes away from each other. The two opposing lipid bilayers need to approach within 3 nm for the membrane merger to occur (Helm, 1993). When two membranes are placed in close proximity, which is necessary for fusion, the energy barrier is very high.

The membrane interior can be exposed more when there are imperfections in the membranes. Increased hydrophobic interactions in the carbon chains of the membrane lipids cause disorder in membrane packing such as the extreme membrane curvature, local changes in membrane lipid composition and insertion/de-insertion of hydrophobic peptides into the membranes, which helps to overcome the energy barrier for fusion. To overcome this energy barrier with an exquisite temporal specificity through the process of membrane fusion, there might be some proteins to catalyze a fusion reaction. These proteins mediate the initial recognition of the membranes that are destined for fusion, pull

the membranes close together to destabilize the lipid/water interface and to initiate mixing of the lipids and more importantly guarantee rigorous regulation in space and time.

1.2 Proteins that are Essential for Synaptic Vesicle Exocytosis

Vesicular neurotransmitter release is mediated by exocytosis of synaptic vesicles at the presynaptic active zone of nerve terminals. The Ca^{2+}-triggered release process is extremely fast, lasts less than half a millisecond, and is tightly regulated by Ca^{2+}. Action potentials cause Ca^{2+} influx through voltage-gated Ca^{2+} channels, which in turn triggers synaptic vesicle fusion. The typical sub-millisecond latency between an action potential and neurotransmitter release and the precise timing of the release process are crucial for information processing in the nervous system. Entensive studies have led to the identification of numerous proteins involved in neurotransmitter release (Augustine et al., 1996; Jahn and Südhof, 1999; Lin and Scheller, 2000; Südhof, 1995).

1.2.1 SNARE-mediated Membrane Fusion

The SNARE proteins are important components of the evolutionarily conserved membrane fusion machinery in all steps of the secretory and endocytic pathways. SNAREs were first identified as vital to neurotransmission as they are the specific targets for proteolysis by clostridial neurotoxin (Blasi et al., 1993; Blasi, 1993; Link et al., 1992; Schiavo et al., 1993). Clostridial neurotoxins are produced by the pathogenic bacterium *Clostridium botulinum* and cause paralysis by hydrolyzing peptide bonds in SNARE proteins. Since their discovery, the SNAREs have been subjects of intense biochemical

and structural study. Key to understanding the function of SNAREs in membrane fusion was the discovery that different sets of SNAREs, that are present in two opposing membranes, associate into complexes that are subsequently disassembled upon binding to SNAPs and NSF through the ATPase activity of NSF to recycle the SNAREs.

Extensive biochemical and structural studies revealed many important characteristics of the SNARE proteins. All SNARE proteins share a common heptad repeat of about 60-70 peptides called the SNARE motif which tends to form a coiled coil structure (Bock et al., 2001; Hayashi et al., 1994; Jahn and Südhof, 1999) (Figure 1.4). Complex formation is mediated by the SNARE motifs, and is associated with conformational and free-energy changes. They are mostly unstructured as monomers (Dulubova, 1999; Fasshauer et al., 1997; Hazzard et al., 1999). However, when appropriate sets of SNAREs are combined, the SNARE motifs spontaneously associate to form helical core complexes. Preceeding synaptic vesicle fusion, the synaptic vesicle protein synaptobrevin 2 and the two target plasma membrane proteins syntaxin 1a and SNAP-25 form the SNARE complex, a bundle of four α-helices, each of which is contributed by a single SNARE motif of the SNARE proteins. These partially-assembled membrane-bridging complexes are called trans-SNAREs. This four helix bundle is extremely stable and is also sodium dodecyl sulfate (SDS) resistant up to 90°C (Fasshauer et al., 1998; Hayashi et al., 1994). The SNARE core complex is also resistant to proteolysis by botulinum and tetanus neurotoxins. The extraordinary stability of the complexes helps to overcome the repulsive force between the two membranes and bring them close in space.

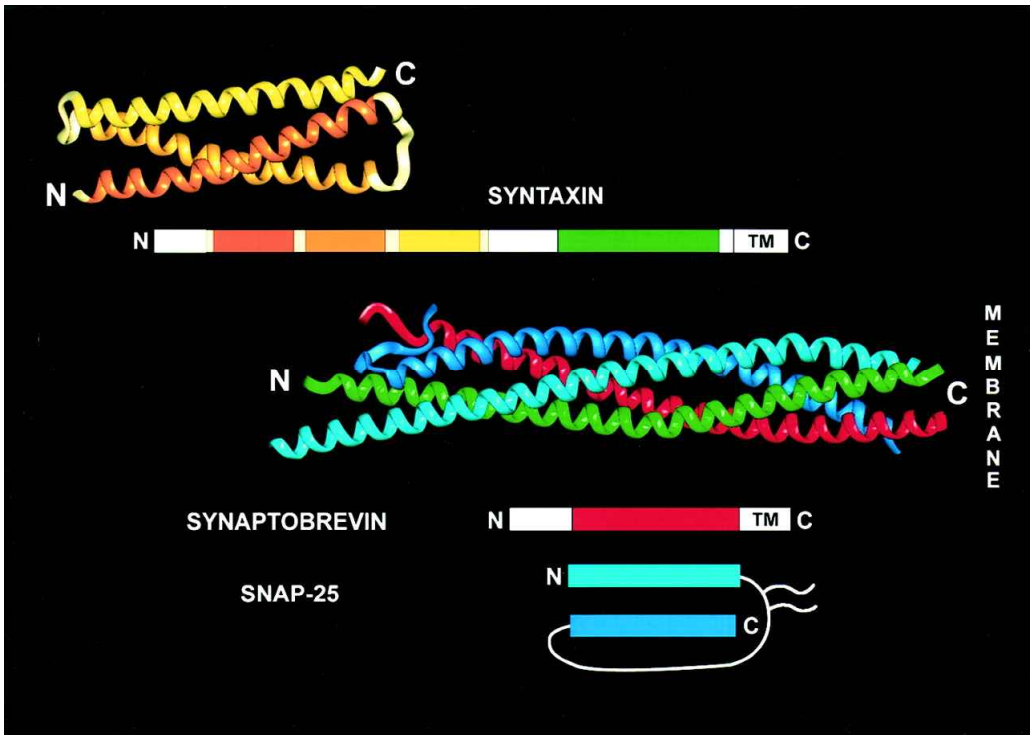


Figure 1.4 Schematic diagrams of the linear arrangement of the domains and structures of the neuronal SNARE proteins (modified from (Fernandez et al., 1998; Sutton et al., 1998)).

Syntaxin 1a and synaptobrevin contain one SNARE motif each that precedes a C-terminal transmembrane (TM) region, while SNAP-25 contains two SNARE motifs (Fernandez et al., 1998). In addition, syntaxin 1a contains a three-helix bundle called the H_{abc} domain that is preceded by a short N-terminal sequence. In isolated syntaxin 1a, the H_{abc} domain folds onto the SNARE motif, forming a 'closed conformation' that hinders SNARE complex formation and also can form a tight complex with neuronal SM protein Munc18-1 (Dulubova, 1999). With Munc18 bound, syntaxin is locked in the closed conformation and cannot bind the other two SNAREs to form a complex. Thus, syntaxin 1 must undertake a conformational change to switch between its complex with Munc18-1 and the core complex. Munc13 is one candidate that could help syntaxin to fulfill this goal.

SNAP-25 contains two SNARE motifs (the N-terminal and the C-terminal SNARE motifs designated as SN1 and SN3, respectively) with a very long joint loop between them to ensure the parallel alignment of the two SNARE motifs within the same molecule when forming the SNARE complex. Although SNAP-25 has no transmembrane region, SNAP-25 still can attach to the membrane by four palmitoylated cysteine residues in the joint loop (Hess et al., 1992). Recent studies shows that a flexible linker with these cysteine residues between two SNARE motifs (SN1 and SN3) may play a role in fast exocytosis (Nagy et al., 2008).

Structural studies reveal that the core complex is highly twisted, with salt bridges on the surfaces and conserved leucine-zipper-like layers at the center. The overall length of the core complex is about 120 Å (Sutton et al., 1998). The interactions in the core of the bundle are mostly hydrophobic except for an ionic layer formed in the middle of the

four-helical bundle, known as the zero-layer or polar layer. This layer contains one arginine residue from synaptobrevin and three glutamine residues from each of the other SNARE motifs and is suggested to help the core complex to zip correctly during assembly. The interior residues of the SNAREs are more conserved than the surface residues and the zero-layer is conserved all over the SNARE family. The strict conservation of the charged residues at the zero-layer, also called polar layer, led to another classification of SNAREs as the Q-SNAREs (containing glutamine in the zero-layer position) and the R-SNAREs (containing arginine in the zero-layer position). In spite of low sequence homology, a mammalian endosomal SNARE complex remarkably shows very similar properties to the neuronal SNARE complex, (Antonin et al., 2002). The interior residues of the neuronal core complex are mostly hydrophobic; however, the surface residues are highly charged. Syntaxin 1a and both SNARE motifs of SNAP-25 are negatively charged whereas synaptobrevin 2 is positively charged.

SNARE complex assembly from the membrane distal N-terminus to the membrane proximal C-terminus was proposed to provide the driving force to overcome the energy barrier for membrane fusion (Figure 1.5). Evidence for this 'zippering mechanism' has been obtained, and reconstitution studies led to the proposal that the SNAREs constitute a minimal membrane fusion machinery (Weber et al., 1998). This model has been accepted by many researchers and reinforced by the observation that 'flipped SNAREs' at the cell surface can induce intercellular fusion (Hu et al., 2003). However, the structural and energetic basis for this minimal model is unclear and other studies found that the neuronal SNAREs are not able to induce membrane fusion using an improved reconstitution most researchers agree that SNARE complex assembly plays a

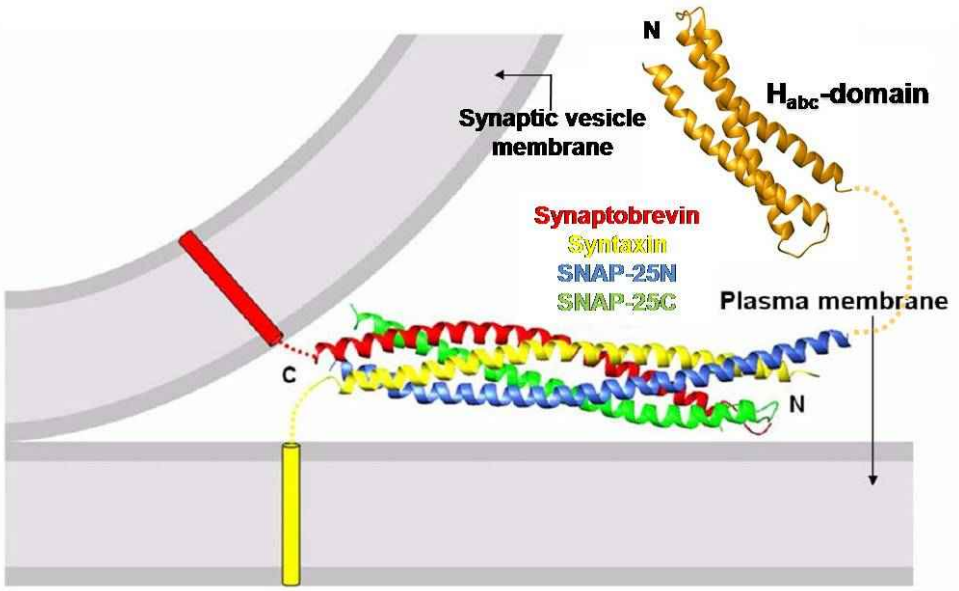


Figure 1.5 SNARE complexes assemble progressively from an N- to C-terminal. SNARE complex assembly from the membrane distal N-terminus to the membrane proximal C-terminus was proposed to provide the driving force for membrane fusion (modified from (Rizo and Südhof, 2002; Sutton et al., 1998)).

central role in membrane fusion (Chen et al., 2004; Hu et al., 2002; Kweon et al., 2003; Rizo, 2003; Rizo and Südhof, 2002; Weber et al., 1998). It is certainly clear that this mechanism alone does not suffice for the demands imposed by fast Ca^{2+}-triggered synaptic vesicle fusion and the involvement of additional factors is likely required for accomplishing the extreme speed and exquisite Ca^{2+}-sensitivity of vesicle fusion at the synapse (Rosenmund et al., 2003). In the search for these factors, numerous studies employing biochemical methods and yeast-two-hybrid technology discovered a large number of SNARE-interacting proteins (Jahn and Scheller, 2006). In most cases, the function of the respective SNARE-interacting proteins is unknown, and even in the cases where a function has been assigned to a putative SNARE binding protein, the functional significance of its interaction with SNARE proteins is often unclear. Thus, detailed structural information and rigorous assessment of the relevance of these SNAREs interactions are required.

1.2.2 Munc18-1

Sec1/munc18 (SM) proteins are 600-700 residue cytosolic proteins with homology throughout their entire sequence (Gallwitz and Jahn, 2003; Rizo and Südhof, 2002; Toonen and Verhage, 2003). Like SNAREs, SM proteins are indispensable for all membrane trafficking events in all species, from yeast to mammals, which suggests that they constitute an evolutionary conserved, central aspect of the fusion machinery (Hanson et al., 1997; Sudhof, 2004; Verhage and Toonen, 2007). SM proteins are essential for regulated exocytosis, as null mutations in SM genes in a wide variety of species result in a loss of vesicle fusion reactions (Jahn, 2000). The first SM protein, named unc-18, was identified in *C.elegans* (Brenner, 1974). In particular, the functional importance of the neuronal SM protein involved in neurotransmitter release, munc18-1 (also referred to as n-Sec1 or reb-Sec1), has been shown by the total abrogation of spontaneous, sucrose-induced and Ca^{2+}-triggered release observed in munc18-1 knockout mice, as monitored with electrophysiological measurements (Verhage et al., 2000). Munc18-1, the mammalian homologue of UNC-18, was identified through its tight interaction with syntaxin 1 (Garcia et al., 1994; Hata et al., 1993; Pevsner et al., 1994). This finding suggested that Munc18-1 is functionally coupled to the SNAREs and forms part of the fusion machinery (Hata et al., 1993), but this interaction was found to require the closed conformation of syntaxin-1 (Dulubova, 1999) and prevents SNARE complex formation (Yang et al., 2000). Moreover, X-ray crystallography data from the munc18-1-syntaxin 1 dimer reveals a horseshoe shaped molecule, which forms a central cavity of 15 Å, surrounding syntaxin 1 in a ‘closed’ (SNARE complex incompatible) conformation (Misura et al., 2000). In addition, NMR studies indicate that the N-terminal domain is an

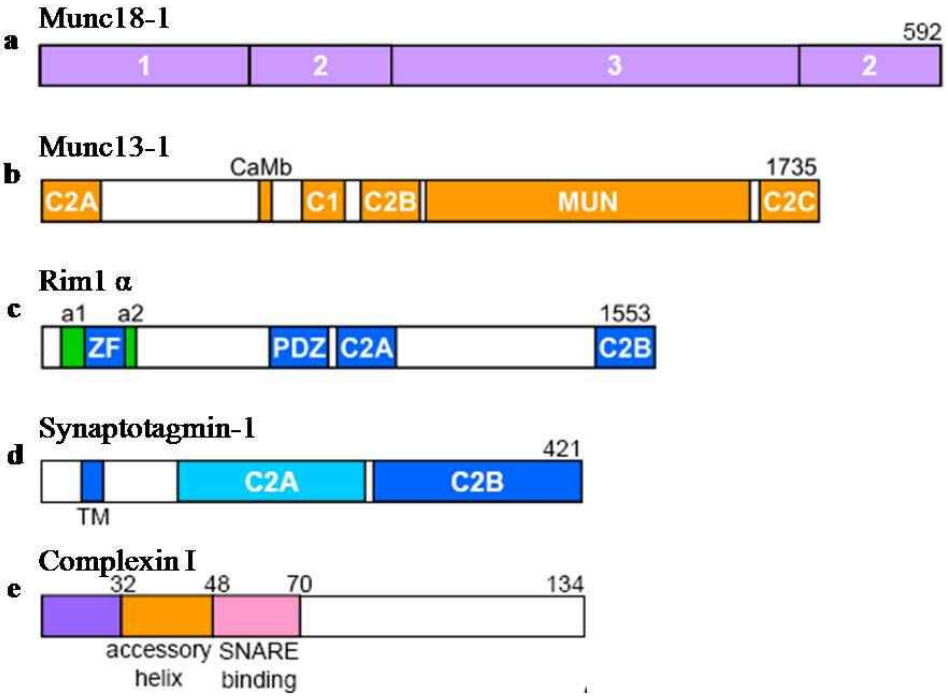


Figure 1.6 Schematic diagrams of synaptic proteins involved in synaptic vesicle exocytosis. (a) Munc18-1. (b) Munc13-1. (c) RIM1α. (d) Synaptotagmin-1. (e) Complexin I. The number of residues of each protein is indicated above each diagram on the right.

autonomously folded domain (Dulubova et al., 2003). Although it was proposed that munc18-1 may somehow assist in SNARE complex assembly to explain its critical function in release, our mechanistic understanding of the function of munc18-1 has been complicated by the discrepancy between structural and biochemical data. Overexpression and microinjection experiments (Dresbach et al., 1998) (Wu, 1998), suggest an inhibitory role for munc18-1, but such role contrasts with the munc18-1 knockout mice phenotype.

Recent studies both *in vitro* and with brain lysates provided compelling evidence that munc18-1, like most other SM proteins, also binds assembled SANRE complexes (Connell E, 2007; Dulubova et al., 2007; Latham et al., 2007; Rickman et al., 2007; Shen et al., 2007). In addition to its interaction with the coiled-coil domain of syntaxin 1, munc18-1 interacts with the syntaxin 1 N-terminus, which was not resolved in the crystal structure. This interaction is compatible with SNARE complex formation and even essential because either deletion of the first 6-24 amino acids or the L8A mutation in syntaxin 1 prevents the interaction between munc18-1 and the SNARE complex (Connell E, 2007; Latham et al., 2007; Rickman et al., 2007; Shen et al., 2007). Thus, munc18-1 is becoming an established component of the SNARE complex, and SNARE complex binding is now a general feature of SM proteins. In all cases, syntaxin 1a N-terminus is required for this interaction but the exact sequence of events is not known. It could be that the N-terminal binding precedes the SNARE binding (Hu et al., 2007) or that it even promotes SNARE complex formation (Shen et al., 2007). Binding to assembled SNARE complexes was first reported for the yeast ortholog (Sec1p), which is currently the only SM protein that does not bind to monomeric syntaxin (Sso1p). The Sec1p-SNARE interaction does not require the N-terminal peptide, which argues that Sec1p uses a

distinct SNARE binding mode and suggests that, at least in yeast, SM proteins can promote fusion without N-terminal interactions.

1.2.3 Munc13s and RIMs

Two large proteins at the active zone of neurons, unc13/munc13 and RIM (Rab3-interacting protein), are suggested to mediate the disassembly of syntaxin/munc18 complex to enable the switch of syntaxin from closed to open conformation preparing it for core complex formation (Betz et al., 1997). Invertebrate unc13 (Maruyama and Brenner, 1991) and mammalian munc13s (Brose et al., 1995) constitute a family of large (ca. 200 kDa), phorbol ester dependent proteins that act as targets of the DAG second messenger signaling pathway (Betz et al., 1997; Brose et al., 1995; Lackner et al., 1999; Maruyama and Brenner, 1991). The major mammalian isoform, munc13-1, contains one DAG/phorbol ester binding C_1 domain, three C_2-domains (called C_2A-C_2C domains) and a MUN domain (Basu et al., 2005; Brose et al., 1995). This architecture is largely conserved in the unc13/munc13 family, but the N-terminal region is variable. RIM1α, originally identified as a Rab3 effector, has multiple functions beyond a Rab effector role with functional domains such as a ZF domain, a PDZ domain, and C_2 domains (called C_2A, C_2B). These protein domains adopt characteristic β-sandwich structures and commonly function as Ca^{2+} binding modules(Rizo and Sudhof, 1998), but the RIM and munc13 C_2 domains are Ca^{2+}-independent (Dai et al., 2005; Guan et al., 2007; Lu et al., 2006) except the Munc13 C_2B domain.

Double knockout of munc13-1 and the closely related munc13-2 leads to total abrogation of spontaneous, sucrose-induced and Ca^{2+}-triggered release in the forebrain of

mice. The critical role of unc13/munc13s in release is played by its MUN domain, which is sufficient to rescue release in Munc13-1/2 double knockout mice (Basu et al., 2005), and likely arises from defects in vesicle priming. In addition to this fundamental function, munc13-1 mediates different forms of presynaptic plasticity. Thus, munc13-1 is responsible for DAG/phorbol ester-dependent augmentation of release (Betz et al., 1998; Rhee et al., 2002), and some forms of Ca^{2+}-dependent short-term plasticity depend on a calmodulin-binding sequence and the C_2B-domain of munc13-1 (Junge et al., 2004; Rosenmund et al., 2002). Moreover, the C_2A-domain of munc13-1 binds to α-RIMs (Betz et al., 2001), which are large Rab3 effectors of the active zone that are also involved in synaptic vesicle priming and in different forms of short- and long-term presynaptic plasticity.

The above observations suggest that regulation of the efficiency of release by different agents such as Ca^{2+}, DAG and Rab3/GTP during diverse presynaptic plasticity processes may converge on the activity of the MUN domain in synaptic vesicle priming, but the mechanism of action of the MUN domain is still unclear. The abrogation of release observed in unc13 nulls in *C.elegans* can be partially rescued by overexpression of a syntaxin 1 mutant that is constitutively open. This finding led to a widespread model whereby munc13-1 helps to open syntaxin 1, allowing SNARE complex formation. Biochemical proof of this model was never provided, and the Munc13-1 MUN domain does not bind to isolated syntaxin 1(Basu et al., 2005; Guan et al., 2008), but the MUN domain does bind to membrane anchored SNARE complexes and syntaxin 1/SNAP-25 hetorodimers (Guan et al., 2008; Weninger et al., 2008). These results support the idea that Munc13s function in opening syntaxin 1 but perhaps by a mechanism different to

that originally envisioned, acting in concert with Munc18-1 to form the syntaxin 1/SNAP-25 heterodimer and promote vesicle priming. It is also worth noting that the protein CAPS, which also contains a MUN domain (Basu et al., 2005), has also been implicated in vesicle priming, suggesting that priming may depend generally on MUN domains. An interaction of the N-terminal region of unc13 with unc18 was also proposed to medicate the release of unc18 from syntaxin 1, but release can be rescued in munc13-1/2 double knockout mice without this N-terminal region. These bulky domains could play a direct role in fusion through their interaction with the SNARE complex, but this notion has not been tested. The restricted localization of Munc13 and RIM to active zone ensures that neurotransmitter release occurs only at the right place.

1.2.4 Synaptotagmin as a Calcium Sensor

Among many potential calcium-binding proteins localized to synaptic active zones, synaptotagmin-1 is now well-described as the primary calcium sensor for SNARE-dependent fusion (reviewd in (Koh and Bellen, 2003)). Synaptotagmin-1, a brain-specific vesicular transmembrane protein, is the Ca^{2+} sensor in neurons, as it has been observed that it is selectively required for fast Ca^{2+} -dependent neurotransmitter release (Geppert et al., 1994; Perin et al., 1990). Synaptotagmin-1 is an intrinsic membrane protein and has a conserved structure within vertebrates and invertebrates.

The observation that the synchronous, fast component of Ca^{2+}- dependent neurotransmitter release is severely decreased in the synaptotagmin knock-out mouse, whereas asynchronous, slow release is unaffected, demonstrated that synaptotagmin-1 function is required for Ca^{2+} triggering of neurotransmitter release (Geppert et al., 1994).

In Ca^{2+}-dependent interactions between synaptotagmin-1 and SNARE complexes, synaptotagmin-1 acts at the Ca^{2+} triggering step of release and SNARE complexes are believed to contribute to membrane fusion during this last step. A recent report revealed that synaptotagmin-1 can displace complexins from SNARE complexes in a Ca^{2+}-dependent manner (Tang et al., 2006) which suggests that such displacement works as control switches for fast synaptic vesicle exocytosis. However, it has not been investigated in the reconstituted proteoliposomes system, which is more physiologically relevant.

1.2.5 Complexins Regulate Ca^{2+}-Evoked Release

Particularly interesting among the SNARE-interacting proteins are complexins, which are also known as synaphins and constitute a family of small (15-20 kDa) proteins of about 130-160 amino acids. In addition to synaptotagmin, complexins are also implicated in Ca^{2+}-regulated neurotransmitter release (McMahon et al., 1995). The mammalian complexin family contains four closely related isoforms, complexin I, complexin II, complexin III and complexin IV (abbreviated as complexin I-IV), with distinct but overlapping cellular expression patterns. The two major isoforms, complexin I and II, are soluble proteins, whereas complexin III and IV are C-terminally farnesylated on a CAAX-box motif, which regulates their synaptic targeting (Reim et al., 2005).

The first two complexins (complexin I and complexin II) were identified and cloned by virtue of their tight interaction with the core complex (Ishizuka et al., 1995; McMahon et al., 1995; Takahashi et al., 1995). These two isoforms are highly homologous and share 86% of amino acid sequences. Complexin I is specifically expressed in the central

nervous system while complexin II is also present in non-neuronal tissues. Both complexin I and II are enriched in neurons and mRNA expression patterns are very similar in many different neuron types (McMahon et al., 1995). Recently complexin III and IV were found in mammals and share 58% identity with each other but share only limited homology (24-28% identity) with complexin I and II (Reim et al., 2005).

It has been shown that complexins compete with α-SNAP for binding to the SNARE complex, but the physiological relevance of this competition is not well understood (McMahon et al., 1995). Biochemical studies revealed that complexin I and II interact exclusively via a central α-helical domain with assembled SNARE complexes, but that they do not bind individual SNARE components. However, a single molecule spectroscopy study recently showed that complexin I has an ability to bind to and stabilize binary complexes of syntaxin and SNAP-25 (t-SNARE) (Weninger et al., 2008).

In solution, complexins are largely unstructured, but contain an α-helical region in the middle of the sequence that is responsible for their specific binding to the SNARE complex (Pabst et al., 2000). Previous nuclear magnetic resonance (NMR) spectroscopy and X-ray crystallography studies from our laboratory validated many of the earlier biochemical data. In brief, these studies show that residues 29-86 contain helical structure, that the 48-70 helix binds to a groove along the preformed SNARE complex, and that the N- and C-terminal ends of complexin appear to be mostly unstructured (Bracher et al., 2002; Chen et al., 2002; Pabst et al., 2000) (Figure 1.6).

The crystal structure of complexin and SNARE complexes suggested a mechanism that complexins may facilitate the transformation of a loosely assembled SNARE complex to a complete tight four helix bundle. By binding and stabilizing the SNARE

complex, complexins may induce a strain on the participating membranes in which the respective SNARE proteins reside (synaptobrevin on synaptic vesicles, syntaxin and SNAP-25 on plasma membrane), and activate vesicles into a state ready for fusion. Since the currently available structural data were obtained with the core SNARE complex and a fragment of complexin containing only the accessory helix domain and SNARE binding domain, the complexin-SNARE complex does not provide information on the N-terminal and C-terminal complexin sequences nor the protein domains flanking the core SNARE complex. Recent studies of truncated complexin show that SNARE complex binding of complexin I via its central α-helix is necessary, but, unexpectedly, not sufficient for its key function in promoting neurotransmitter release (Xue et al., 2007). Through the interplay between distinct functional domains, complexin I carries out a crucial role in fine-tuning Ca^{2+}-triggered fast neurotransmitter release.

The functional importance of complexins has been demonstrated by the lethal phenotype observed in the complexin I/II double knockout mice. Complexins are necessary for a positive role in synaptic vesicle fusion, as neurons lacking both complexin I and II show a selective impairment in the Ca^{2+}-triggered fast, synchronous neurotransmitter release, but not the hypertonic sucrose-triggered release (Reim et al., 2001). Conversely, complexin also seems to have a negative role in fusion because the introduction of excess amount of recombinant complexin or their fragments into presynaptic neurons of *Aplysia* buccal ganglia, either by transfection or microinjection, led to the inhibition of evoked neurotransmitter release (Ono et al., 1998). In addition, when complexin peptides were microinjected into the presynaptic terminals of giant squid axons, the peptides caused a potent inhibition of evoked transmitter release without

affecting presynaptic resting or action potentials (Tokumaru et al., 2001). Ironically, the dialetic functions of complexin may provide the complexities to regulate the synaptic exocytosis more tightly and precisely.

In addition, complexins have been found to be involved in several neurological disorders, such as schizophrenia, bipolar disorder and Huntington's disease (Eastwood, 2000; Edwardson et al., 2003; Morton and Edwardson, 2001). Thus, understanding the function of complexins will not only help to elucidate the regulatory mechanisms of neurotransmitter release, but also to potentially provide clues on therapeutic strategies and reagents for these human neurological diseases.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials and Methods

2.1.1 Expression and Purification of Recombinant Protein

DNA constructs encoding GST fusion proteins of the complexin 1 fragment (residues 26-83) and the SNARE motifs of rat synaptobrevin 2 (29-93, hereafter abbreviated Syb2(29-93)), rat syntaxin 1a (188-259), and human SNAP-25 (residues 11-82 and 141-203, both containing an additional Trp residue at the C terminus to facilitate detection, abbreviated as SN1 and SN3, respectively) were prepared using standard PCR cloning techniques, and subcloned into the pGEX-KT expression vector (Hakes and Dixon, 1992) were prepared in our laboratory (Chen et al., 2002). In addition, based on crystal structure (Chen et al., 2002), truncated synaptobrevin constructs (Syb2(29-76), Syb2(29-68), and Syb2(29-62)) in different length were designed and generated by the standard PCR cloning techniques. An additional Trp residue was introduced to each construct at the N terminus to facilitate the protein quantification. All plasmids were transformed into Escherichia coli BL21 (DE3) cells for protein expression. Every construct was maintained as a lab glycerol stock (40% glycerol) at -80°C. For a typical one liter bacteria culture, a single colony of the clone was picked off from an LB agar/ampicillin plate and grown overnight in the appropriate selectable liquid medium (e.g., LB amp) in a shaker at 250rpm at 37°C. Proper amount of this seed culture, usually 10-20ml, was transferred to 1 liter LB media containing 100μg/ml ampicillin. The flask

was incubated at 37°C in the shaking incubator at the speed of 250rpm utill OD_{600} reached 0.6-0.8. And then the temperature was lowered to 22-25°C and 0.4 mM IPTG (isopropyl β-D-thiogalactopyranoside, from Sigma) was added to induce protein expression. After at least 16 hours of induction, cells were harvested by centrifugation at 4500 rpm for 45min in swing buckets with a rotor JS 4.2 (Model J6-MI centrifuge, Beckman Instuments) and resuspended in phosphate buffer saline (PBS) solution containing 2mM EDTA, 5mM EGTA, 0.5mM AEBSF and 10ul/ml sigma protease inhibitor cocktail (Sigma). The cell suspension was frozen with liquid nitrogen and stored in -80°C.

For protein purification, the frozen cells were thawed and passed through an EmulsiFlex-C5 cell disruptor (Avensin) at 10000 -14000 psi for 3 to 4 times and spun at 19000 rpm for 30 min in a JA-20 rotor with Beckman centrifuge (model J2-21). Supernatants were mixed with glutathione Sepharose 4B (Amersham Pharmacia Biotech.) and incubated for 2 hours at the room temperature or overnight at 4°C in the coldroom. GST fusion proteins were bound tightly to the beads, whereas unbound proteins and non-specifically bound proteins were removed by extensive washing sequentially with 25ml PBS, 50ml PBS (1% triton X-100), 50ml PBS (1M NaCl) and 25ml PBS for several times. For Syb2s and SN1, a benzonase (0.25 units/ul) treatment was used to remove all forms of DNA and RNA. The resin was then extensively washed with PBS buffer and then washed again with 3 ml thrombin cleavage buffer (50mM Tris, pH8.0, 200 mM NaCl, 2.5 mM $CaCl_2$ and 1 mM DTT) for three times. A treatment with 5-7 units/ml thrombin at room temperature was followed to remove the GST moiety . Proteins were eluted with corresponding elution buffers and further purified by gel

filtration (Superdex-75 Hiload 16/60 column, Amersham Pharmacia Biotech.). The purity of the preparation was assessed by SDS-PAGE and Coomassie blue staining.

Rat complexin I (26-83) (abbreviated as Cpx26-83) was subcloned into the expression vector pGEX-KT for expression as a GST fusion protein by Kovrigin E., a former postdoctoral fellow in our laboratory (Chen et al., 2002). The plasmid was transformed into *Escherichia coli* BL21 (DE3) cells for protein expression. Glycerol stock (40% glycerol) of the transformed cells was kept in -80°C. The expression and purification procedures for Cpx26-83 were similar to those for the SNARE motifs as described above, except that Cpx26-83 after the cleavage of GST was eluted with 20 mM sodium acetate buffer (pH4.5), and the eluted Cpx26-83 was further purified by ion exchange chromatography. Briefly, a linear gradient of salt (0 to 500 mM NaCl) was used to elute Cpx26-83 from the Source S column (Amersham Pharmacia Biotech.). Cpx26-83 eluted at 300mM salt and purify of the preparation was assessed by SDS-PAGE and Coomassie blue staining.

2.1.2 NMR Sample Preparation

NMR signals of large molecules with molecular masses greater than 20 kDa are highly overlapped. Taking advantage that the core complex (32 kDa) was assembled with four individually expressed and purified SNARE fragments, I prepared four NMR samples with only one SNARE motif ^{2}H or/and ^{15}N labeled in each sample. Thus, I was able to observe one SNARE motif at one time in NMR experiments, which decreased the number of the observable resonance fourfold and dramatically relieved the signal-overlap problem. Some samples were deuterium labeled to reduce the dipolar relaxation and

improve the spectral sensitivity. Uniform ^{15}N- labeling was achieved by growing the bacteria in $^{15}NH_4Cl$ as the sole nitrogen source. Perdeuteration was achieved by growing bacteria using D_2O as the solvent.

The high stability of the core complex enabled facile preparation by simply mixing the purified isotope labeled SNARE motif with 1.2 equivalents of each of the other three purified unlabeled SNARE motifs, followed by an overnight incubation at room temperature. The small amount of unassembled fragments was removed by extensive concentration/dilution with a Millipore concentrator (10 kDa cutoff). This procedure produced pure SNARE complex as judged by SDS-PAGE. Note that the complex is SDS resistant, and the absence of isolated SNARE bands in the gel provide a reliable method to assess the purity of the complex. The final concentration of the core complex was about 200 uM according to UV_{280}, and the buffer condition for NMR experiments was 20 mM Tris (pH 7.4) and 150 mM NaCl.

2.1.3 Complexin/ SNARE Preparation

Assembly of the SNARE core complex followed the same procedure as described above. The complexin/SNARE complex was prepared by addition of 1.2 equivalents of Cpx26-83 to preassembled SNARE complex. An extensive concentration/dilution step with Millipore concentrator (10 kDa cutoff) was used to eliminate the small amount of unassembled fragments and at the same time change the sample buffer to final conditions. The observation of only one set of cross-peaks in the ^{1}H-^{15}N TROSY-HSQC spectra of the different samples prepared provided further evidence of the purity of both complexes.

2.1.4 NMR Spectroscopy

All NMR experiments were performed at 25.6 °C on a Varion INOVA600 spectrometers with samples dissolved in 20 mM Tris (pH 7.4), 150 mM NaCl, and 0.1 mM TCEP, using H_2O/D_2O 95:5 (v/v) as the solvent. The 5% (v/v) D_2O was included to provide the lock signal for the control of the long term stability of the magnetic field. For each sample, I acquired and analyzed one-dimensional (1D) spectra and TROSY-based-HSQC spectra with acquisition times between 1 and 12 hours. All NMR data were processed with the program NMRPipe (Delaglio, 1995) and analyzed with the program NMRView (Johnson, 1994).

CHAPTER 3

STRUCTURAL STUDIES OF THE INTERACTION BETWEEN COMPLEXIN I AND THE SNARE COMPLEX

3.1 INTRODUCTION

Assembly of the SNARE complex happens in multiple steps, so understanding how this process is regulated is a central question in synaptic vesicle fusion. To gain further insights, additional proteins that interact with the SNARE complex need to be studied. Complexin binds the SNARE complex via a ~40 amino acid central helical domain which binds a groove between syntaxin and synaptobrevin 2 (Bracher et al., 2002; Chen et al., 2002). Deuterium exchange experiments showed that complexin especially stabilized the C-terminal of the synaptobrevin 2 SNARE motif, consistent with a role in stabilizing a particular assembly conformation of the SNARE complex (Chen et al., 2002). Interestingly, the membrane proximal region of complexin forms a second helix, also called accessory helix, which bends away from the SNARE complex (Chen et al., 2002). Previous studies in vivo and in vitro allowed us to reach the conclusion that complexin may function as a clamp in both facilitatory and inhibitory way and could directly accelerate an otherwise slow step in SNARE complex assembly (Giraudo et al., 2006; Reim et al., 2001; Reim et al., 2005; Schaub et al., 2006; Tang et al., 2006; Xue et al., 2007). However, the exact mechanism of the clamp function is still under investigation. Therefore, my research project was focused on the structural and functional studies of the interaction between the central peptide (Cpx26-83) and the SNARE

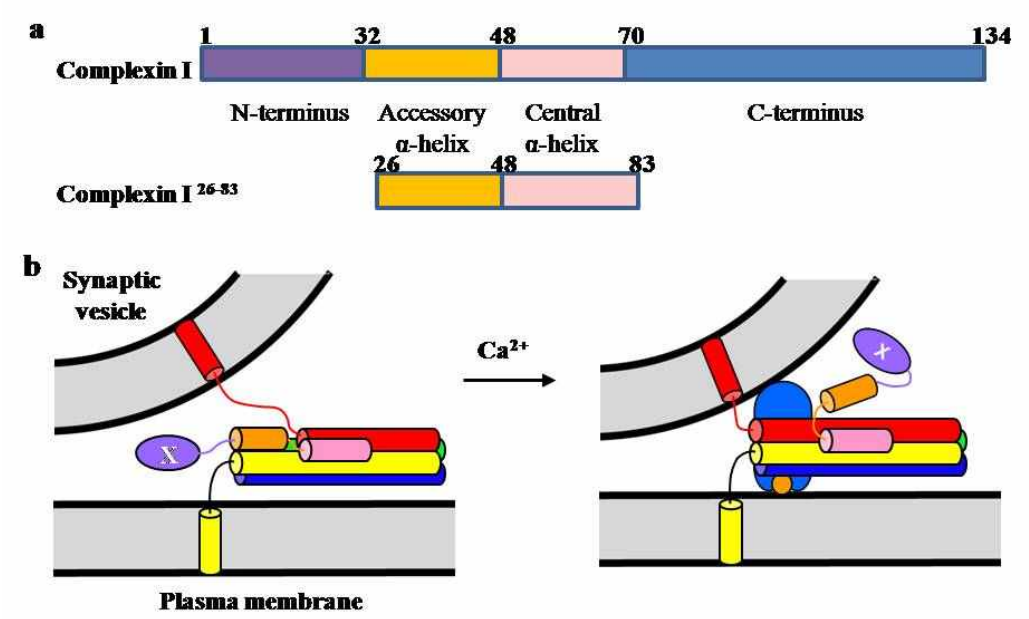


Figure 3.1 Complexins and their coupling to SNAREs. (a) Schematic domain diagram of complexin-I. Shorter construct of complexin, Cpx26-83, shown in this figure is used for crystallography with core complex (Chen et al., 2002). (b) Models of how complexin functions as a clamp by replacing the binding region of synaptobrevin at the membrane proximal end of the four helix bundle with the accessory α-helix of complexin on the C-terminal side of the SNARE complex-binding region. An accessory α-helix on the C-terminal side of the SNARE complex–binding region indicated in orange has an inhibitory effect on fast synaptic exocytosis through binding to the C-terminus of the SNARE complex. Unidentified interaction of N-terminus of complexin, indicated as an oval in purple with an X, is critical for a facililatory role of complexin (Xue et al., 2007).

complex. The specific hypothesis of my thesis project is that complexin functions as a clamp by replacing the binding region of synaptobrevin at the membrane proximal end of the four helix bundle with the accessory α-helix of complexin on the C-terminal side of the SNARE complex-binding region (Figure 3.1).

3.2 RESULTS

3.2.1 Purification of the SNARE Motifs and Assembly of the Core Complex

Previous studies from our lab have shown that complexin binds tightly to the SNARE core complex containing the SNARE motifs only in an antiparallel, α-helical conformation to the groove between the synaptobrevin and syntaxin SNARE motifs of the core complex. The binding does not cause any overall conformational change in the core complex except some slight side chain re-orientations. The core complex used in the previous binding studies was composed of syntaxin 1a (191-253), synaptobrevin 2 (29-93), SN1, and SN3, which correspond to the minimal sequences involved in the SNARE complex assembly (Sutton et al., 1998). Although the deletion of six residues at the C-terminus of syntaxin SNARE motif facilitated the NMR study of the core complex by reducing the aggregation of the assembled SNARE complex (Margittai et al., 2001), in this study, syntaxin 1a (188-259) (Sutton et al., 1998) was used since the C-terminus of syntaxin SNARE motif may play a important role in binding to the complexin accessory helix. Stoichoiometric amounts of the SNARE motifs assembled almost quantitatively into a SDS-resistant SNARE complex.

Therefore, we started to characterize the interaction between complexin I (26-83) and the truncated core complex (TCC) containing the truncated synaptobrevin ((Syb2(29-76), Syb2(29-68), and Syb2(29-62)) in solution using biochemical and biophysical methods (Figure 3.2).

In order to assemble the SNARE complex, the SNARE protein/motifs were expressed and purified separately. The gel filtration profiles of the SNARE proteins through the Superdex 75 Hiload 16/60 column (Amersham Pharmacia Biotech.) are shown in Figure 3.3. The SNARE motifs of SNAP-25 (SN1 and SN3) and synaptobrevin 2 also eluted earlier than globular proteins with similar molecular weight (Figure 3.3a, 3.3b, and 3.3d), because they do not have tertiary structures (Fasshauer et al., 1997). Syx1a(188-259) eluted with an apparent molecular weight of 36 kDa (Figure 3.3c), which is consistent with the observation that the SNARE motif of syntaxin forms homotetramers in solution (Misura et al., 2001).

A typical SNARE complex assembly reaction was carried out by mixing 0.8:1:1:1 stoichiometry of syx1a(188-259), SN1, SN3, and synaptobrevin 2, and incubating overnight at room temperature. Samples were taken at different time points to check the assembly level of the ternary SNARE complex. Since the ternary SNARE complex is SDS-resistant and only denatures above 90 °C, the assembly of the ternary SNARE complex can be easily evaluated by comparing the sample with or without boiling and observing the existence of SDS-resistant SNARE complex in the non-boiled sample. The assembly efficiency for the TCC type of SNARE complex is >80%. The TCC with Syb2(29-62) does associate as well, but the resulting complex is not SDS-resistant, and we therefore cannot accurately determine the assembly efficiency by SDS

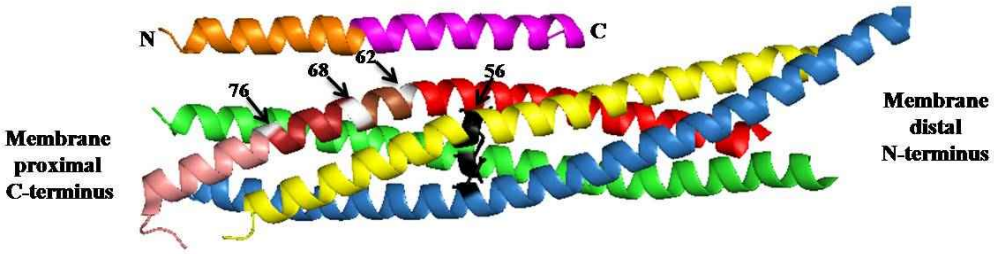


Figure 3.2 Structure of the complexin/SNARE complex. (a) Ribbon diagram of complexin/SNARE complex. The color coding is as follows. Syx1a(191-253), yellow; Syb2, red; SN1, blue; SN3, green; Cpx26-83, orange and pink (Chen et al., 2002). Using structural information, we generate various mutant constructs of synaptobrevin such as Syb2(29-76), Syb2(29-68), and Syb2(29-62). Ionic '0' layer of the synaptic fusion complex is labeled in black and side chains involved in the layer are shown as sticks.

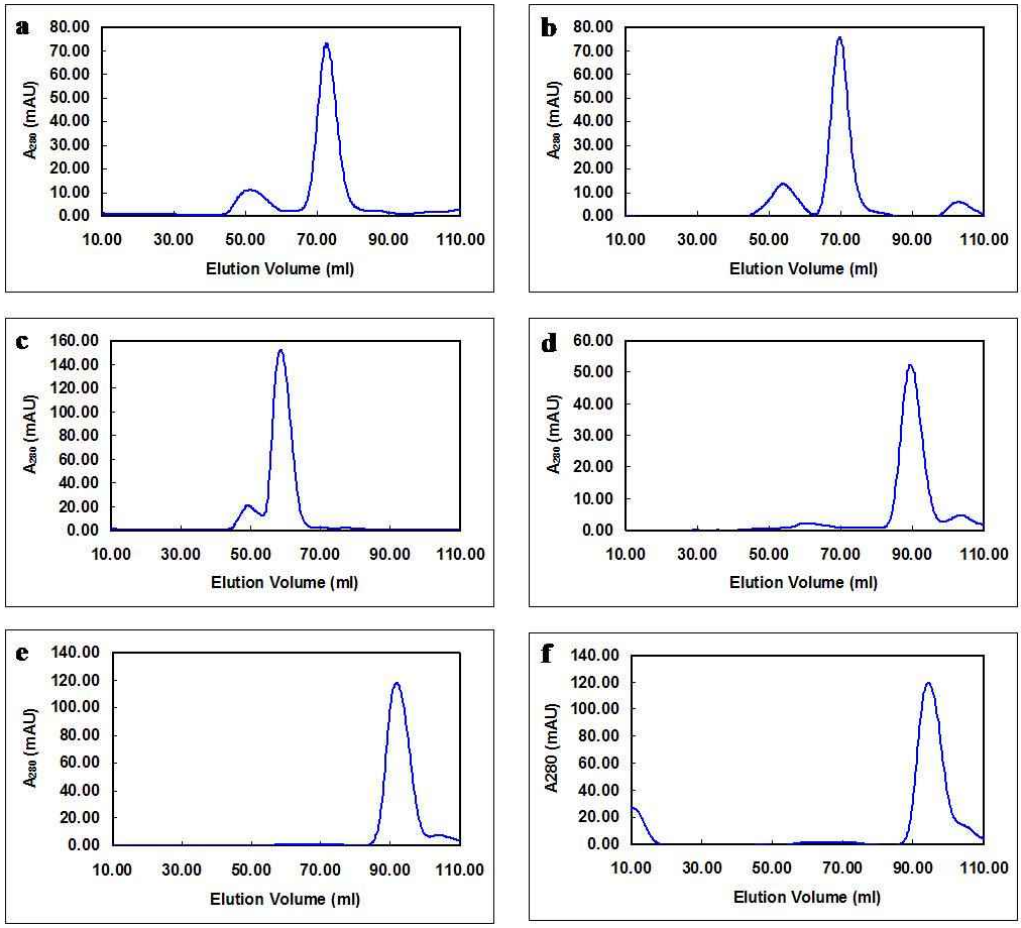


Figure 3.3 Purification of the SNARE proteins. Gel filtration profiles of (a) SN1, (b) SN3, (c) Syx1a(188-259), (d) Syb2(29-76), (e) Syb2(29-68), (f) Syb2(29-62) through the Superdex 75 Hiload 16/60 column (Amersham Pharmacia Biotech.).

PAGE. However, the gel filtration data suggests that assembly efficiency is approximately 50%.

The assembled SNARE complex was pooled and subjected to further purification by gel filtration chromatography with a Superdex 200 5/150 GL (Amersham Pharmacia Biotech.). The gel filtration chromatogram is shown in Figure 3.4.

It has been shown that the syntaxin SNARE motif and SN1 can also form a very tight 2:2 parallel four-helix bundle. This type of complex is extremely stable once formed and it can be a kinetic trap for the assembly of the ternary SNARE complex. Therefore, the mixing order of SNARE proteins for the assembly reaction is crucial to assure the proper SNARE complex formation. A typical order of the mixing in an assembly reaction was first, SN3, then, the syntaxin SNARE motif, followed by the synaptobrevin SNARE motif, and finally, SN1.

3.2.2 One Dimensional NMR for Evaluation of the Quality of the Core Complex Assembly

A typical 1D ^{1}H NMR spectrum of the SNARE complex is shown in Figure 3.5. The intensity and line-shape of the methyl group in 1D spectrum is very informative. Therefore, protein-protein interactions can be detected by measuring the intensity changes of the strongest methyl resonance (SMR) of proteins. Most proteins of SMR are observed at 0.8-0.9 ppm, and its intensity usually increases linearly with the concentration of the sample (Araç et al., 2003). The severe resonance overlap in the region facilitates detection of the SMR at low micromolar and even sub-micromolar protein concentrations. However, if sample aggregates, the line width of the methyl

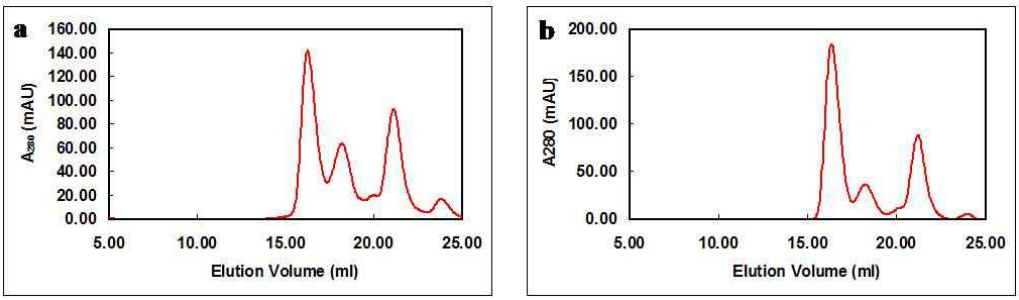


Figure 3.4 Purification of the SNARE complex. Gel filtration profiles of the assembled SNARE complex with (a) ^{15}N-labeled SN3 and (b) ^{15}N-labeled Syx1a(188-259) thruogh gel filtration chromatography with a Superdex 200 5/150 GL (Amersham Pharmacia Biotech.).

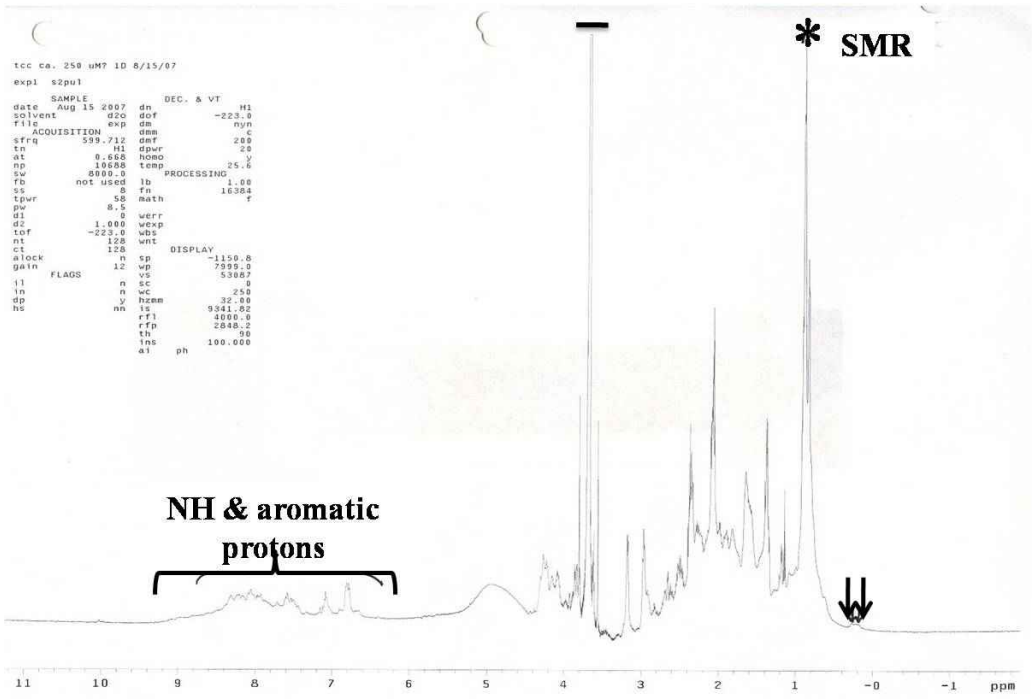


Figure 3.5 1D NMR spectrum of the SNARE complex. The signals under bracket are mainly from NH and aromatic protons. The peaks indicated by an asterisk is the strongest methyl resonance (SMR) signal. The intensity of the SMR was used to estimate the protein concentration. The arrows indicate two well-isolated methyl resonances, whose line widths indicate the aggregation state of the core complex sample.

group will increase and the intensity of the SMR will decrease with molecular mass. Generally, the quality of the 1D spectrum can be used as an indicator for the formation of the core complex. Therefore, 1D NMR spectroscopy was performed to verify the quality of the core complex and to measure the approximate concentration of proteins. Information from 1D NMR is then used as reference for TROSY-HSQC experiments to verify the quality of the core complex containing different amounts of proteins.

3.2.3 Binding Studies of the Complexin/SNARE complex by TROSY based NMR Experiments

Nuclear magnetic resonance (NMR) is used as a spectroscopic technique. NMR techniques monitor the absorption of energy associated with transitions of nuclei between adjacent nuclear magnetic energy levels (Wüthrich, 1986). Basically, it measures the signals of radio-frequencies that have been emitted by the nuclei. However, the signal decays exponentially with a characteristic time constant, the transverse relaxation time, T_2, that is inversely proportional to the line width of the resonances in the spectrum, and that depends on not only molecular size but also molecular motion.

The molecular weight of complexin/truncated core SNARE complex will be about 37 kDa which is already close to the upper limit of conventional NMR methods (Clore and Gronenborn, 1994). Together with the fact that core complex shows an elongated cylindrical structure and has even slower tumbling rate and shorter relaxation time than its molecular mass, regular ^{1}H-^{15}N heteronuclear single quantum coherence (HSQC) experiments could not yield good quality spectra due to the broadened linewidths, and consequently low intensity and poor sensitivity. Therefore, we took

advantage of the recent development of transverse-relaxation optimized spectroscopy (TROSY) technique, which constructively utilizes the interference between dipole-dipole (DD) coupling and chemical shift anisotropy (CSA) to suppress transverse nuclear spin relaxation (Fernádez and Wider, 2003; Pervushin et al., 1997). Briefly, there are two major sources of interfering relaxation mechanisms for diamagnetic macromolecules: one depends on the dipole-dipole interaction (DD) between the protons and the other depends on the chemical shift anisotropy (CSA) of the protons. The DD relaxation is independent of the static magnetic field; however, the CSA increases with larger magnetic fields. DD and CSA interfere with each other, and under certain circumstances, they can almost completely cancel each other and consequently eliminate the transverse relaxation effects originated from them. The ^{1}H nuclei of amide groups have a scalar coupling with the corresponding ^{15}N nucleus instead of the natural isotope ^{14}N, and the NMR signals split into two components. Each component has different line widths, which directly demonstrates the relaxation interference (Figure 3.6b). In conventional NMR experiments , the two lines are collapsed into one line by a technique called ‘decoupling’, at the cost of averaging the relaxation rates (Figure 3.6a). For small molecules, this is not a problem, but for large molecules the signal may be attenuated due to the contribution of the more rapidly relaxing resonance line. However, the TROSY–based NMR technique exclusively selects the component with the slowest relaxation rate and narrowest linewidths, and eliminates the fast relaxing component (Figure 3.6c). Theoretically, the complete cancellation of DD and CSA for this component occurs at ^{1}H frequencies near 1 GHz due to the fact that the DD interaction is independent of the magnetic field whereas the CSA increases with

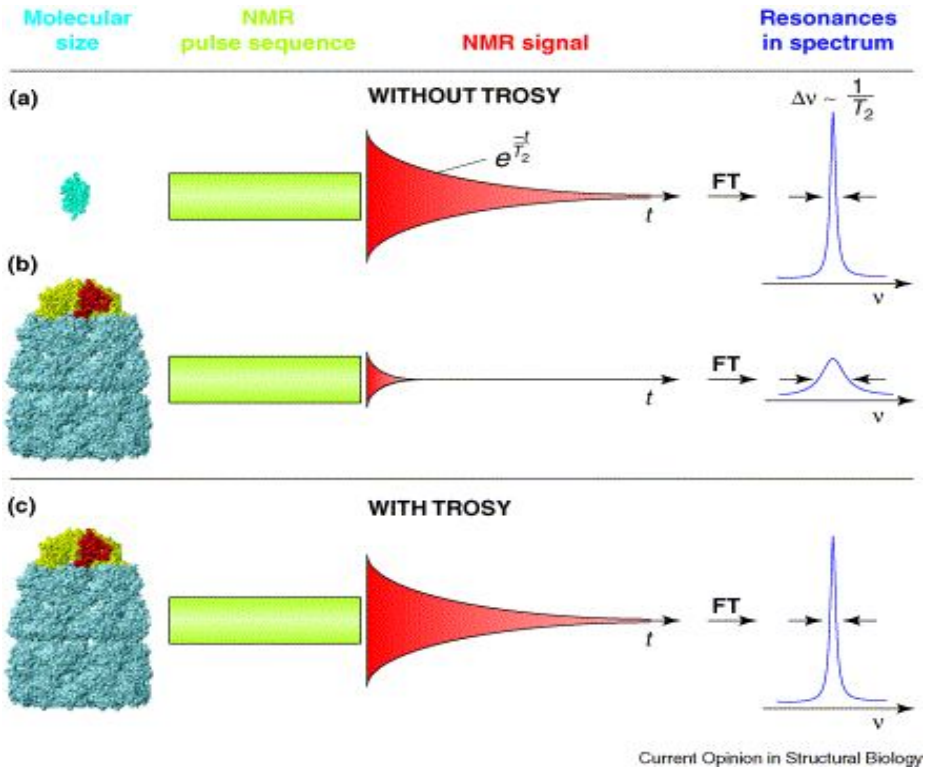


Figure 3.6 NMR spectroscopy with small and large molecules in solution. (a) Small molecules in solution relaxes slowly, resulting in long transverse relaxation time (T_2). A large T2 value corresponds to narrow line width ($\Delta\nu$) after Fourier transformation (FT) of the NMR signal. (b) Larger molecules have a smaller T_2, so the decay of the NMR signal is faster. This results both in weaker signals and in broad lines in the spectra. (c) TROSY-based NMR substantially can reduce the transverse relaxation, resulting in improved spectral resolution and sensitivity for large molecules (Fernádez and Wider, 2003).

higher magnetic fields. Therefore, a higher magnetic field will reinforce the TROSY effect. At first glance, the TROSY technique disregards 3/4 of the potential signal, but in the case of large molecules, slower relaxation readily compensates for the loss of signal and improves the spectral sensitivity substantially during the pulse sequence and the data acquisition, enabling the measurement of high-quality spectra for these systems (Pervushin et al., 1997) (compare Figure 3.6b and 3.6c). Moreover, due to the low gyromagnetic ratio of the deuteron ($\gamma D/\gamma H = 0.15$), replacement of the protons with deuterons will significantly decrease amide proton transverse relaxation rate and attenuate the DD interaction, gaining the increased sensitivity and improving the resolution of the spectra. However, perdeuteration method still has the size limit of 50 kDa (Reviewed in (Gardner and Kay, 1998)).

Larger molecules and macromolecular complexes have another complication of NMR spectroscopy due to the large number of resonances, resulting in more crowded spectra and lower resolution. For the truncated core complex (TCC) in the presence or absence of Cpx26-83, selective isotope labeling can be easily carried to alleviate this problem. The proper choice of isotope-labeling will significantly reduce the number of resonances in spectrum, thus simplifying the data acquisition and processing. For mapping the binding region in the TCC, for instance, each SNARE motif can be individually labeled and assembled with non-labeled corresponding fragments, which will reduce the significant number of crosspeaks in NMR spectra.

In previous NMR studies in our laboratory, the full-length complexin I was shown to be largely unfolded, except for a helical region in the middle of its sequence , providing a binding region for the SNARE complex (Pabst et al., 2000). This helical

region (residue 29-86) can be divided into two regions: a stable α-helix (residues 29-64) and another helix (65-86) with a lower tendency. Consistently, deletion mutagenesis also showed that complexin binds to the core complex through its central region (Pabst et al., 2000).

Based on the above results, to gain insight into the region(s) of the central peptide (Cpx26-83) involved in binding to the SNARE complex, we first acquired TROSY-enhanced ^{1}H-^{15}N HSQC spectra (Pervushin et al., 1997) of ^{2}H-^{15}N Cpx26-83 fragment in the presence or absence of unlabeled short SNARE complex. These experiments were done by a former graduate student Xiaocheng Chen in our laboratory and she was able to acquire high quality NMR spectra of the core complex at 600 MHz. Similar to the ^{1}H-^{15}N HSQC experiment, ^{1}H-^{15}N TROSY-HSQC spectra can be considered like protein fingerprints. In addition, the cross-peak dispersion observed in ^{1}H-^{15}N TROSY-HSQC spectra is indicative of the presence or absence of a well-defined tertiary or quaternary structure in the protein.

Briefly, the ^{1}H-^{15}N HSQC spectrum of ^{2}H-^{15}N labeled Cpx26-83 showed poor chemical shift dispersion like full length complexin, suggesting the absence of tertiary structure. Upon addition of the unlabeled core complex to the ^{2}H-^{15}N labeled Cpx26-83, a striking dispersion of a subset of the cross-peaks was observed and the dispersed cross-peaks exhibited generally more severe broadening, which is indicative of the formation of quaternary contacts (Chen et al., 2002). The region directly involved in binding to the SNARE complex is residues 50-70 of complexin. The widespread chemical shift changes induced by the Cpx26-83/SNARE complexin interaction can be explained by the helix stabilization in the binding region of the Cpx26-83 and the subsequent propagation of the

helical conformation beyond the binding region. The chemical shifts of the N-termianl 16 residues of Cpx26-83 bound to the SNARE complex are very similar to those observed in free complexin. Consistently, crystal structure show that the N-terminal of Cpx26-83 bends away from the core complex suggesting that there is no direct interaction (Chen et al., 2002).

To gain insight into the region(s) of the SNARE complex involved in binding to Cpx26-83, ^{1}H-^{15}N TROSY-HSQC spectra of the core complex containing only one of the four SNARE motifs labeled with ^{2}H-^{15}N were acquired. Many cross-peaks are moved in the spectra of Syx191-253 and Syb2(29-93) in the core complex, whereas only small number of crosspeaks are moved for the SNAP-25 SNARE motifs. Thus, the NMR studies suggested that complexin binds to the interface formed by syntaxin and synaptobrevin in the core SNARE complex (Chen et al., 2002; Pabst et al., 2000).

3.2.4 Binding Studies of Complexin/SNARE Complex Containing a Shorter Synaptobrevin.

Complexin binds the SNARE complex via a ~40 amino acid central helical domain which binds a groove between syntaxin and synaptobrevin 2 (Bracher et al., 2002; Chen et al., 2002). Deuterium exchange experiment showed that complexin especially stabilized the C-terminal third of the synaptobrevin 2 SNARE motif, consistent with a role in stabilizing a particular assembly conformation of the SNARE complex (Chen et al., 2002). Interestingly, the membrane proximal region of complexin forms a second helix which bends away from the SNARE complex (Pabst et al., 2000). Previous studies in vivo and in vitro allowed us to reach the conclusion that complexin may function as a

fusion clamp in both facilitatory and inhibitory way and could directly accelerate an otherwise slow step in SNARE complex assembly as well. However, the exact mechanism of the clamp function is still under investigation. Therefore, my research project was focused on the structural and functional studies of the interaction between the central peptide (Cpx26-83) and the SNARE complex. The specific hypothesis of my thesis project is that complexin functions as a clamp by replacing the binding region of synaptobrevin at the membrane proximal, C-terminal end of the four-helix bundle with an accessory α-helix of complexin. In order to investigate this hypothesis, we performed the following experiments (Figure 3.1).

Based on the previous results, we initiated a more detailed characterization of the Cpx26-83/SNARE complex interaction using different combinations of isotopically labeled and unlabeled samples of the minimal SNARE fragments and Cpx26-83. In addition, we used truncated synaptobrevin 2 fragments, (Syb2(29-76), Syb2(29-68), and Syb2(29-62) to investigate the exact interaction between the Cpx26-83 fragment and each type of truncated core SNARE complex (TCC) by ^{1}H-^{15}N TROSY-based HSQC experiment. The ^{1}H-^{15}N TROSY-HSQC spectrum of isolated Cpx26-83 shows poor chemical shift dispersion due to the absence of tertiary structure (Figure 3.7a, black contours and (Chen et al., 2002)); however, significant dispersion of a subset of the complexin cross-peaks was observed upon addition of the unlabeled TCC containing Syb2(29-76) (TCC76) (Figure 3.7b, red contours) This dispersion and broadening effect arises from the formation of quaternary contacts between Cpx26-83 and TCC. A practically superimposable spectrum was acquired when ^{2}H-^{15}N-labeled Cpx26-83 was bound to the core SNARE complex (with full-length synaptobrevin). These results led us

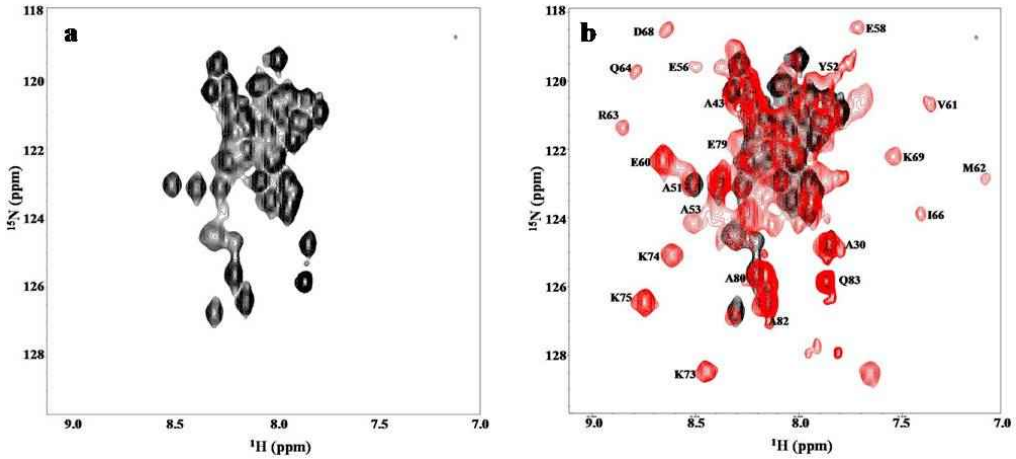


Figure 3.7 ^{1}H-^{15}N TROSY-HSQC spectra of ^{15}N-labeled Cpx26-83 in the absence and presence of TCC76. (a) ^{1}H-^{15}N TROSY-HSQC spectra of the isolated ^{15}N-labeled Cpx26-83 (black contours). (b) ^{1}H-^{15}N TROSY-HSQC spectra of the ^{15}N-labeled Cpx26-83 bound to the unlabeled TCC76 (red contours). The HSQC spectrum of ^{15}N-labeled Cpx26-83 bound to the unlabeled TCC76 is practically superimposable with the spectrum of Cpx26-83 bound to the minimal core complex (Chen et al., 2002).

to conclude that the C-terminal truncation of the synaptobrevin SNARE motif does not affect the binding to Cpx26-83.

To gain insight into the region(s) of the SNARE complex involved in binding to Cpx26-83, we acquired ^{1}H-^{15}N TROSY-HSQC spectra of samples of TCC containing fragments Syb2(29-76), Syb2(29-68), and Syb2(29-62), respectively, assembled with the ^{1}H-^{15}N-labeled C-terminal SNARE motif of SNAP-25 (SN3). Based on our hypothesis, we expected to see peak-shifts in SN3 after addition of complexin, indicative of a binding event between complexin and the SNARE complex, where binding of the complexin accessory helix replaces synaptobrevin 2. In detail, we expected to see that progressive truncation of the Syb-CT allows for increasingly more binding between the complexin accessory helix and the SNARE complex, particularly in regions of the SNARE complex that were previously occupied by Syb2-CT. However, we did not see any significant peak shifts upon complexin addition (Figure 3.7c), suggesting that the complexin accessory helix does not substitute for Syb2-CT to any detectable degree.

3.2.5 Mapping of Complexin binding site on SNARE Complex Containing a Shorter Synaptobrevin.

While we could not confirm that the complexin accessory helix can substitute for Syb2-CT, another surprising and unexpected result arose from this experiment. The ^{1}H-^{15}N TROSY-HSQC spectra of ^{15}N-labeled SN3 SNARE complex in the presence or absence of Cpx26-83 showed that almost half of SN3 is unstructured, if any of 3 truncated synaptobrevin fragments, Syb2(29-76), Syb2(29-68), and Syb2(29-62), respectively, is used to replace Syb2(29-93) in the core complex (Figure 3.8). The data of

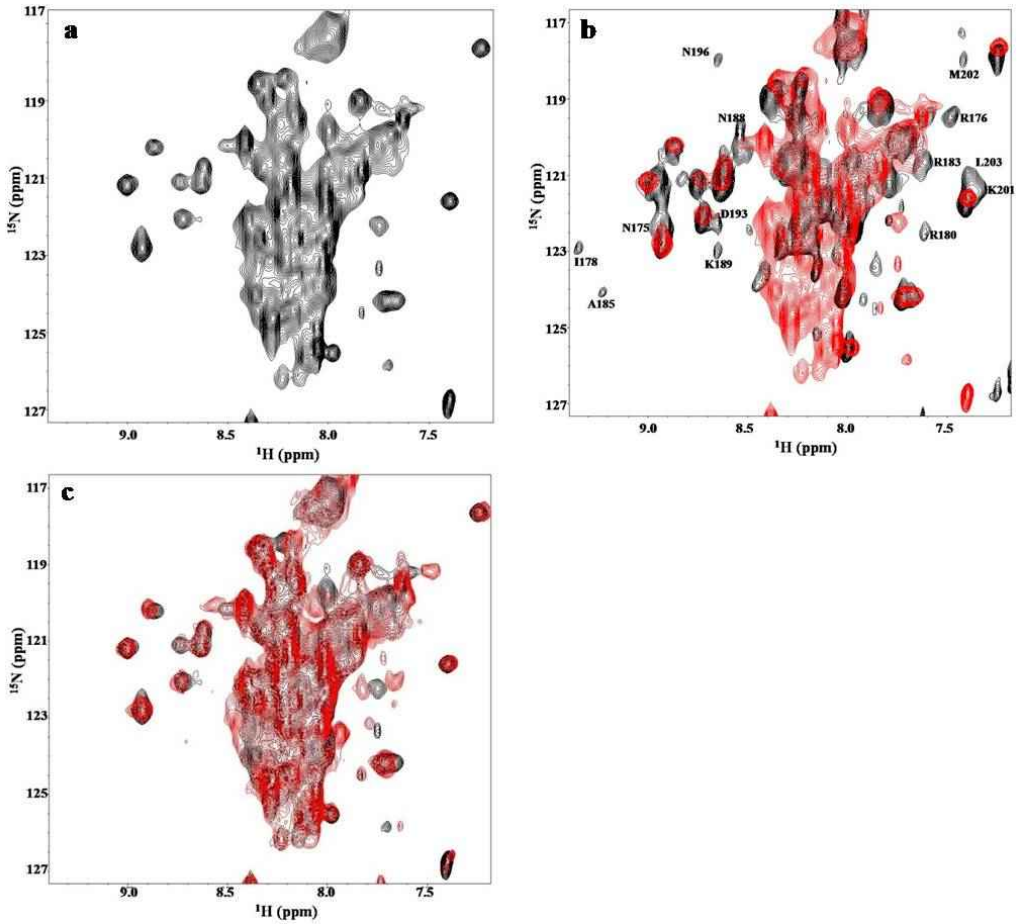


Figure 3.8 ^{1}H-^{15}N TROSY-HSQC spectra of TCC complex with ^{15}N-labeled SN3. (a) ^{1}H-^{15}N TROSY-HSQC spectra of the isolated ^{15}N-labeled SN3 (black contours). (b) Compared to TROSY-HSQC spectra of ^{2}H-^{15}N-SN3 core complex, most cross-peaks from residues of the C-terminal part of ^{15}N-labeled SN3 in TCC76 (red contours) are disappeared. (c) TROSY-HSQC spectra of ^{15}N-labeled SN3 in the absence (black contours) and presence of Cpx26-83 (red contours).

TCC68 and TCC62 are not shown. These results led us to conclude that the core complex may have two cooperative folding units, with the membrane-distal N-terminal unit of the four-helix bundle that can be formed in the absence of both complexin and the Syb2 C-terminus (residues 76-93; Syb2-CT).

To confirm that the C-terminal part of the entire four-helix bundle is disordered in the absence of Syb2-CT, we labeled Syx1a(188-259) with ^{15}N and performed the ^{1}H-^{15}N TROSY-HSQC experiment under the same conditions. As expected, we found that most cross-peaks from residues of the C-terminal part of the syntaxin SNARE-motif disappeared, suggesting that this region is disordered as well (Figure 3.9). Taken together, these findings suggest that the membrane-proximal part of the entire four-helix bundle is disordered in the absence of Syb2-CT.

It is further interesting to note that the region of disorder starts at the respective polar layers of both SN3 and syntaxin 1a, residue Gln 174 and residue Gln 226, respectively. As the polar layers of all the components of the four-helix bundle align closely in three-dimensional space (Sutton et al., 1998) (Figure 3.2), this finding strongly suggests that the polar layers of the components form an interface between the two folding cassettes of the core SNARE complex. Moreover, the truncation of only 20 residues at the Syb2-CT is enough to produce disorder of the second module in entire SNARE complex. This may suggest that the interactions within the second module are intrinsically weaker, and could therefore be more tightly regulated. In conjunction with other studies (Chen et al., 2002) the existence of such an interface may imply the existence of at least two intermediates during the formation of the core SNARE complex. The first step might be the formation of a stable four-helix bundle N-terminal to the polar

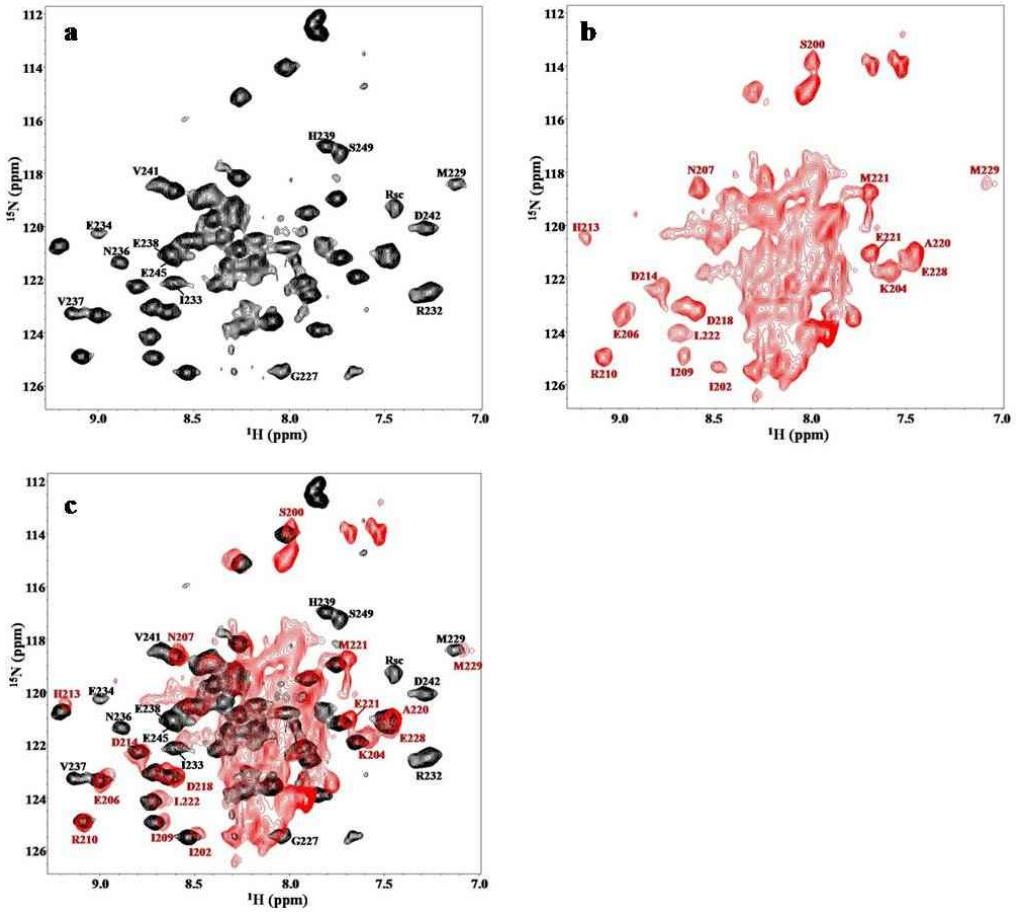


Figure 3.9 ^{1}H-^{15}N TROSY-HSQC spectra of TCC complex with ^{15}N-labeled Syx1a fragment. (a) ^{1}H-^{15}N TROSY-HSQC spectra of ^{15}N-labeled Syx1a(191-253) fragment in minimal core complex containing fragments Syb2(29-93) as a reference. These experiments were done by a previous graduate student Xiaocheng Chen from our laboratory. (b) ^{1}H-^{15}N TROSY-HSQC spectra of ^{15}N-labeled Syx1a(188-259) fragment in TCC76. (c) Superposition of ^{1}H-^{15}N TROSY-HSQC spectra of ^{15}N-labeled Syx1a(188-259) fragment in TCC (red contours) with the spectrum of ^{1}H-^{15}N TROSY-HSQC spectra of ^{15}N-labeled Syx1a(191-253) fragment in core complex (black contours). Most cross-peaks from residues of the C-terminal part of the Syntaxin SNARE-motif disappeared, suggesting that this region is disordered as well.

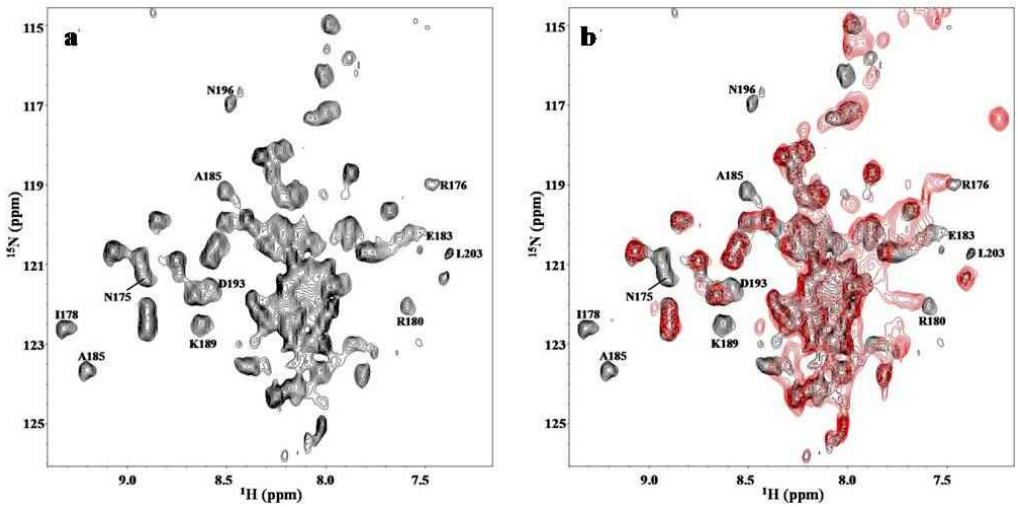


Figure 3.10 ^{1}H-^{15}N TROSY-HSQC spectra of TCC84 complex with ^{2}H-^{15}N -labeled SN3 fragment. (a) ^{1}H-^{15}N TROSY-HSQC spectra of ^{2}H $^{-15}$N-labeled SN3 fragment in TCC84. (b) Superposition of ^{1}H-^{15}N TROSY-HSQC spectra of ^{2}H-^{15}N-labeled SN3 in TCC84 (black contours) with the spectrum of ^{1}H-^{15}N TROSY-HSQC spectra of ^{2}H $^{-15}$N-labeled SN3 in TCC76 (red contours). Almost all the ^{1}H $^{-15}$N TROSY-HSQC cross-peaks from TCC84 reappeared in the spectra, suggesting that Syb2(29-84) was long enough to form the full four-helix bundle.

layers, while the second step could be the "zippering up" toward the C-terminus of the entire core SNARE complex. In such a two-state priming model, the first intermediate can be considered as an energetically inexpensive and stable platform for a second, high-energy priming step. Formation of the second, fusion-competent intermediate can be reversible and tightly regulated, and brings vesicles to closely similar energy levels. From this second intermediate, fusion can occur in a Ca^{2+}-dependent manner, and the narrow energy spread of the second intermediate allows for precise timing of the fusion reaction of an ensemble of vesicles.

To further define the minimal sequences of synaptobrevin to form the full four-helix bundle, we generated another deletion mutant of synaptobrevin, Syb2(29-84), by the standard PCR cloning techniques. We recorded ^{1}H-^{15}N TROSY-HSQC spectra of ^{15}N-labeled SN3 under the same conditions as before. In comparison to the same experiment with Syb2(29-76), almost all the ^{1}H-^{15}N TROSY-HSQC cross-peaks from SN3 reappeared in the spectra, suggesting that Syb2(29-84) was long enough to form the full four-helix bundle. Furthermore, the same set of the SN3 crosspeaks reappeared as with Syb2(29-93), suggesting that the Syb2(29-84) construct is sufficient to form the fully assembled core complex intermediate (Figure 3.10).

3.2.6 Binding Studies of Complexin/t-SNARE Complex by HSQC NMR Method

In a further set of experiments, we decided to investigate whether our failure to provide support to our initial hypothesis could be attributed to the lack of the N-terminal 25 residues of complexin. The first step for such an investigation is to verify the binding of complexin to its known target, SNARE complex. However, due to a recent series of

studies collectively showed binding of complexin with the membrane-anchored t-SNARE complex (composed of Syntaxin/SNAP-25 complex) (Guan et al., 2008; Weninger et al., 2008), rather than only with the fully assembled SNARE complex (Syntaxin/SNAP-25, Synaptobrevin). Therefore, we decided to investigate the association of full-length complexin (Cpx-FL) with the t-SNARE complex and its components in solution.

For this purpose, we produced ^{15}N-labeled full-length complexin and monitored the change in the crosspeaks by ^{1}H-^{15}N TROSY-HSQC upon addition of various t-SNARE components. The t-SNARE complexes we used for these experiments are SN1/SN3 complex, SN1/ Syx1a(188-259) complex, Syx1a(188-259) alone, and the full t-SNARE complex of SN1/SN3/ Syx1a(188-259). As expected from previous experiments with Cpx26-83, the ^{1}H-^{15}N TROSY-HSQC spectrum of isolated full-length complexin shows poor chemical shift dispersion due to the absence of tertiary structure. Upon addition of pre-assembled, full t-SNARE complex, we found that many cross-peaks had disappeared, suggesting that complexin can indeed bind to the full t-SNARE complex. The ^{1}H-^{15}N HSQC spectra of complexin in the absence (black contours) and presence (red) of the unlabeled t-SNARE complex are shown in Figure 3.11. Very similar results were obtained by addition of SN1/SN3 complex, as well as SN1/Syx1a(188-259) complex. By contrast, little change was observed upon addition of Syx1a(188-259) alone. Taken together, these results suggest, that complexin can bind to the t-SNARE complex, and that this association is mediated mainly by SNAP-25 SN1 domain, but not by syntaxin. Although we cannot formally conclude that SN1 is sufficient for complexin binding to the t-SNARE complex, the SN1 domain is the only common component found in all the t-SNARE complexes that we have found to bind complexin. It is also important

to note that the observed interactions are likely to be formed by the N-terminal of complexin, as the previously suggested crystal structure of the complexin/SNARE complex does not suggest any direct interactions between Cpx26-83 and either SN1 or SN3. Such a model, in which the N- and C-termini of complexin can bind, perhaps independently, to different SNARE complex components, could also accommodate cooperativity between both modules of complexin, allowing for more specific and tight regulation than would be possible with independent protein. This idea may be supported by other findings that suggest a facilitatory role for the N-terminus of complexin, but an inhibitory role for the complexin accessory helix (Xue et al., 2007). However, further experiments are necessary to conclusively identify the binding sites of t-SNARE complex to complexin and to obtain support for this model.

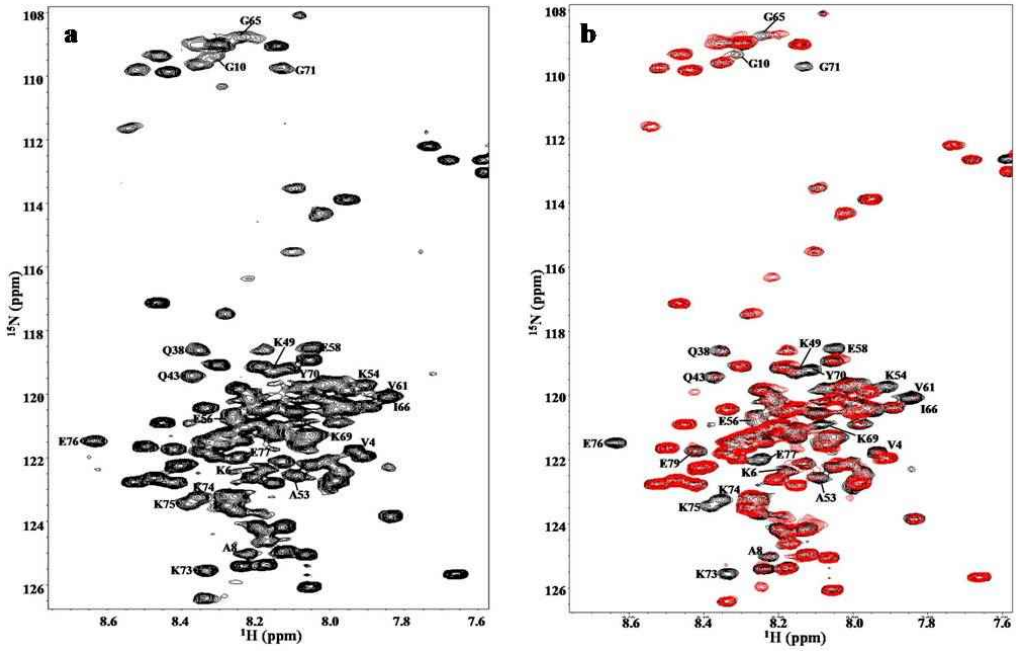
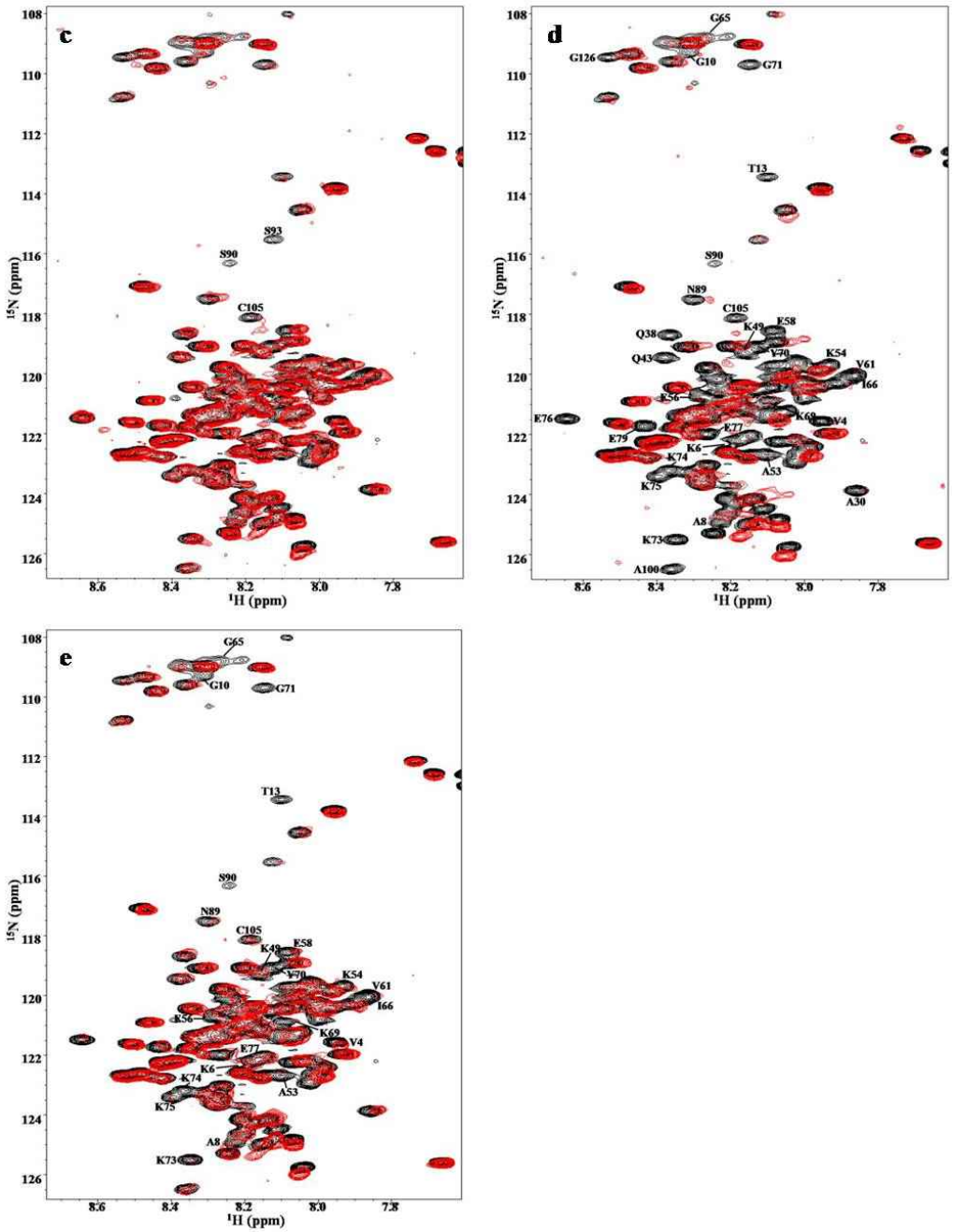


Figure 3.11 ^{1}H-^{15}N TROSY-HSQC spectra of full length complexin in the absence and presence of various t-SNARE components. (a) ^{1}H-^{15}N HSQC spectra of the isolated ^{15}N-labeled Cpx-FL(black contours). (b) ^{1}H-^{15}N HSQC spectra of the ^{15}N-labeled Cpx26-83 bound to the unlabeled SN1/SN3 (red contours). (c-e) are shown in following page. (c) ^{1}H-^{15}N HSQC spectra of the ^{15}N-labeled Cpx26-83 bound to the unlabeled Syx1a(188-259) alone (red contours). (d) ^{1}H-^{15}N HSQC spectra of the ^{15}N-labeled Cpx26-83 bound to the unlabeled Syx1a(188-259)/SN1(red contours). (e) ^{1}H-^{15}N TROSY-HSQC spectra of the ^{15}N-labeled Cpx26-83 bound to t-SNARE complex containing the unlabeled Syx1a(188-259)/SN1/SN3 (red contours).


c
d
e
^{15}N (ppm)
^{1}H (ppm)
S93
S90
C105
G65
G126
G10
G71
T13
N89
Q38
Q43
K49
E58
V70
K54
V61
I66
E56
K69
V4
E76
E77
E75
K6
K74
A53
K75
A30
A8
K73
A100

CHAPTER 4

DISCUSSION AND SUMMARY

4.1 Discussion and Summary

The goal of our investigations was to delineate interactions between complexin and SNARE components that lead to the formation of a primed minimal fusion machinery. We have generated and used new constructs of short forms of synaptobrevin and complexin, as well as constructs of SNAP-25 and syntaxin that have previously been shown to be part of a minimal fusion machinery. The use of the short synaptobrevin constructs has led to experimental results suggesting at least two key intermediates during the docking/priming process that are independent of complexin. Furthermore, we have found new evidence that complexin can bind to the t-SNARE complex, in contrast to earlier evidence suggesting that complexin regulates the fully assembled SNARE complex (Pabst et al., 2002). Together, these data suggest new key interactions between components of the SNARE complex, as well as a model for the docking/priming step preceding vesicle fusion.

4.1.1 v-SNARE / t-SNARE interactions

Our initial goal was to find evidence supporting a model in which complexin regulates the docking/priming reaction by binding to partly assembled SNARE complexes. For this purpose, we generated short fragments of the v-SNARE synaptobrevin 2 and of complexin. Our initial hypothesis was that such regulation occurs through the displacement of the Syb2-CT by the complexin accessory helix, but we found

no evidence that complexin binding is affected by the presence or absence of the Syb2-CT module.

More importantly, however, we found evidence for a modular assembly of the full SNARE complex. In the absence of Syb2-CT, the N-terminal half of SNARE complex forms a four-helix bundle, while the C-terminal half, starting just after the polar layer, is disordered. In the presence of the Syb2-CT, however, both halves of the SNARE complex form a four-helix bundle. It is interesting to note that Syb2 residues 29-84 are sufficient for formation of the fully assembled SNARE complex. This evidence strongly suggests the existence of at least two intermediates during the docking priming reaction. The first intermediate is formed by the N-terminal module of the SNARE complex, up to and including the polar layer. The second intermediate consists of both N- and C-terminal modules of the SNARE complex folded to a four-helix bundle. Some evidence for such modules has been provided by deuterium exchange studies (Chen et al., 2002), that found that complexin binds more tightly to the N-terminal SNARE module than to the C-terminal module. Other studies suggest a "progressive zippering model" for the assembly of the C-terminal end of the full SNARE complex (Siddiqui et al., 2007). In the context of these studies, our results suggest that the N-terminal, membrane distal module of the SNARE complex is formed early during the docking reaction, while formation of the C-terminal, membrane-proximal module could be through "zippering up" from the polar layer towards the membrane, ultimately leading to membrane fusion.

4.1.2 Complexin / t-SNARE interactions

Previous studies have suggested that complexin binds to the fully assembled SNARE complex (Pabst et al., 2000). The crystal structure of the Cpx26-83/SNARE complex suggests that the binding surface for complexin is formed mainly by Syb2(29-96), and to a lesser extent by Syx1a(188-259), supporting the notion that the fully assembled SNARE complex is required for complexin binding. However, several recent studies suggested that complexin can bind the t-SNAREs, without the requirement for the v-SNARE component (Guan et al., 2008; Weninger et al., 2008). In light of this new data, we decided to investigate, using full-length complexin, rather than Cpx26-83 as before, the interactions between full-length complexin (Cpx-FL) and the t-SNARE complex components SN1, SN3 and Syx1a(188-259). We were able to confirm by NMR that Cpx-FL binds the t-SNARE complex SN1/SN3/Syx1a(188-259) in solution, as was suggested for membrane-bound t-SNAREs (Guan et al., 2008; Weninger et al., 2008). Note, however, that the t-SNARE complex does not contain the large complexin-binding interface provided by Syb2. Furthermore, we found that Cpx-FL also binds t-SNARE sub-complexes formed by SN1/SN3, and SN1/Syx1a(188-259), while very little binding was observed between Cpx-FL and Syx1a(188-259) alone. This finding is particularly interesting, because the crystal structure of the fully assembled SNARE complex does not suggest any binding between Cpx26-83 and either SN1 or SN3, whereas the only common component in all of the above experiments was SN1 domain. One possibility to explain these results is that SNARE complex assembly is highly dynamic, with components able to bind in different relative orientations (Weninger et al., 2008), and that complexin can bind to and distinguish different sets of SNAREs (Pabst et al., 2000). However, it is also tempting to speculate that the N-terminal portion of complexin forms

another SNARE-binding domain that can loop around to bind to t-SNARE components on the same complex, or extend to bind t-SNARE components on other, adjacent SNARE complexes. One implication of such a model with a bivalent complexin is that this interaction could cluster several closely associated SNARE complexes in a spatially defined manner. Even more interestingly, complexins ability to bind different sub-components of the same SNARE complex may enable it to distinguish between different aggregation states of the SNARE complex. In this case, complexin could play the role of a highly specific regulator for assembly and priming of a fusion-competent SNARE complex. Although this model is very attractive to explain complexins complexities, it will be necessary to conduct further experiments to validate these ideas.

BIBLIOGRAPHY

Antonin, W., Fasshauer, D., Becker, S., Jahn, R., and Schneider, T.R. (2002). Crystal structure of the endosomal SNARE complex reveals common structural principles of all SNAREs. Nat Struct Mol Biol *9*, 107-111.

Araç, D., Murphy, T., and Rizo, J. (2003). Facile Detection of Protein-Protein Interactions by One-Dimensional NMR Spectroscopy. Biochemistry *42*, 2774-2780.

Augustine, G.J., Burns, M.E., DeBello, W.M., Pettit, D.L., and Schweizer, F.E. (1996). Exocytosis: Proteins and Perturbations. Annual Review of Pharmacology and Toxicology *36*, 659-701.

Basu, J., Shen, N., Dulubova, I., Lu, J., Guan, R., Guryev, O., Grishin, N.V., Rosenmund, C., and Rizo, J. (2005). A minimal domain responsible for Munc13 activity. Nat Struct Mol Biol *12*, 1017-1018.

Bennett, M.K., and Scheller, R.H. (1993). The Molecular Machinery for Secretion is Conserved from Yeast to Neurons. Proceedings of the National Academy of Sciences *90*, 2559-2563.

Betz, A., Ashery, U., Rickmann, M., Augustin, I., Neher, E., Südhof, T.C., Rettig, J., and Brose, N. (1998). Munc13-1 is a presynaptic phorbol ester receptor that enhances neurotransmitter release. Neuron *21*, 123-136.

Betz, A., Okamoto, M., Benseler, F., and Brose, N. (1997). Direct Interaction of the Rat unc-13 Homologue Munc13-1 with the N Terminus of Syntaxin. J Biol Chem *272*, 2520-2526.

Betz, A., Thakur, P., Junge, H.J., Ashery, U., Rhee, J.-S., Scheuss, V., Rosenmund, C., Rettig, J., and Brose, N. (2001). Functional Interaction of the Active Zone Proteins Munc13-1 and RIM1 in Synaptic Vesicle Priming. Neuron *30*, 183-196.

Blasi, J., Chapman, E.R., Link, E., Binz, T., Yamasaki, S., Camilli, P.D., Südhof, T.C., Niemann, H., and Jahn, R. (1993). Botulinum neurotoxin A selectively cleaves the synaptic protein SNAP-25. Nature *365*, 160-163.

Blasi, J., Chapman, ER., Yamasaki, S., Binz, T., Niemann, H., Jahn, R. (1993). Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. EMBO J *12*, 4821-4828.

Bock, J.B., Matern, H.T., Peden, A.A., and Scheller, R.H. (2001). A genomic perspective on membrane compartment organization. Nature *409*, 839-841.

Bracher, A., Kadlec, J., Betz, H., and Weissenhorn, W. (2002). X-ray structure of a neuronal complexin-SNARE complex from squid. The Journal of biological chemistry *277*, 26517-26523.

Brenner, S. (1974). THE GENETICS OF CAENORHABDITIS ELEGANS. Genetics *77*, 71-94.

Brose, N., Hofmann, K., Hata, Y., and S?hof, T.C. (1995). Mammalian Homologues of Caenorhabditis elegans unc-13 Gene Define Novel Family of C(2)-domain Proteins. J Biol Chem *270*, 25273-25280.

Chen, X., Tomchick, D.R., Kovrigin, E., Araç, D., Machius, M., Südhof, T.C., and Rizo, J. (2002). Three-Dimensional Structure of the Complexin/SNARE Complex. Neuron *33*, 397-409.

Chen, Y., Xu, Y., Zhang, F., and Shin, Y.-K. (2004). Constitutive versus regulated SNARE assembly: a structural basis. The EMBO journal *23*, 681-689.

Clary, D.O., Griff, I.C., and Rothman, J.E. (1990). SNAPs, a family of NSF attachment proteins involved in intracellular membrane fusion in animals and yeast. Cell *61*, 709-721.

Clore, G.M., and Gronenborn, A.M. (1994). Structures of larger proteins, protein-ligand and protein-DNA complexes by multidimensional heteronuclear NMR. Protein Sci *3*, 372-390.

Colonnier, M. (1974). Spatial interrelationshps as physiological mechanisms in the central nervous system, (Oxford, UK, Clarendon Press).

Connell E, D.F., Broersen K, Gatsby N, Peak-Chew SY, Rickman C, Davletov B. (2007). Mechanism of arachidonic acid action on syntaxin-Munc18. EMBO Rep *8*, 411-419.

Dai, H., Tomchick, D.R., Garcia, J., Sudhof, T.C., Machius, M., and Rizo, J. (2005). Crystal Structure of the RIM2 C2A-Domain at 1.4 Å Resolution. Biochemistry *44*, 13533-13542.

De Camilli P, H.V., Takei K, Mugnaini E., ed. (2001). The structure of synapses. (Baltimore, MD., Johns Hopkins Univ. Press).

Delaglio, F., Grzesiek, S., Vuister, G.W., Zhu, G., Pfeifer, J., Bax, A. (1995). NMRPipe: a multidimensional spectral processing system based on UNIX pipes. J Biomol NMR *6*, 277-293.

Dresbach, T., Burns, M.E., O'Connor, V., DeBello, W.M., Betz, H., and Augustine, G.J. (1998). A Neuronal Sec1 Homolog Regulates Neurotransmitter Release at the Squid Giant Synapse. J Neurosci *18*, 2923-2932.

Dulubova, I., Khvotchev, M., Liu, S., Huryeva, I., S?dhof, T., and Rizo, J. (2007). Munc18-1 binds directly to the neuronal SNARE complex. Proc Natl Acad Sci U S A.

Dulubova, I., Sugita, S., Hill, S., Hosaka, M., Fernandez, I., Südhof, T.C., and Rizo, J. (1999). A conformational switch in syntaxin during exocytosis: role of munc18. The EMBO journal *18*.

Dulubova, I., Yamaguchi, T., Araç, D., Li, H., Huryeva, I., Min, S.-W., Rizo, J., and Südhof, T.C. (2003). Convergence and divergence in the mechanism of SNARE binding by Sec1/Munc18-like proteins. Proceedings of the National Academy of Sciences *100*, 32-37.

Eastwood, S., Harrison, PJ.. (2000). Hippocampal synaptic pathology in schizophrenia, bipolar disorder and major depression: a study of complexin mRNAs. Molecular psychiatry *5*, 425-432.

Edwardson, J.M., Wang, C.-T., Gong, B., Wyttenbach, A., Bai, J., Jackson, M.B., Chapman, E.R., and Morton, A.J. (2003). Expression of Mutant Huntingtin Blocks Exocytosis in PC12 Cells by Depletion of Complexin II. J Biol Chem *278*, 30849-30853.

Fasshauer, D., Eliason, W.K., Brunger, A.T., and Jahn, R. (1998). Identification of a minimal core of the synaptic SNARE complex sufficient for reversible assembly and disassembly. Biochemistry *37*, 10354-10362.

Fasshauer, D., Otto, H., Eliason, W., Jahn, R., and Brunger, A. (1997). Structural Changes Are Associated with Soluble N-Ethylmaleimide-sensitive Fusion Protein Attachment Protein Receptor Complex Formation. Journal of Biological Chemistry *272*, 28036-28041.

Fernádez, C.a., and Wider, G. (2003). TROSY in NMR studies of the structure and function of large biological macromolecules. Current Opinion in Structural Biology *13*, 570-580.

Fernandez, I., Ubach, J., Dulubova, I., Zhang, X., Südhof, T.C., and Rizo, J. (1998). Three-Dimensional Structure of an Evolutionarily Conserved N-Terminal Domain of Syntaxin 1A. Cell *94*, 841-849.

Gallwitz, D., and Jahn, R. (2003). The riddle of the Sec1/Munc-18 proteins - new twists added to their interactions with SNAREs. Trends in Biochemical Sciences *28*, 113-116.

Garcia, E.P., Gatti, E., Butler, M., Burton, J., and Camilli, P.D. (1994). A Rat Brain Sec1 Homologue Related to Rop and UNC18 Interacts with Syntaxin. Proceedings of the National Academy of Sciences *91*, 2003-2007.

Gardner, K.H., and Kay, L.E. (1998). THE USE OF 2H, 13C, 15N MULTIDIMENSIONAL NMR GTO STUDY THE STRUCTURE AND DYNAMICS OF PROTEINS. Annual Review of Biophysics and Biomolecular Structure *27*, 357-406.

Geppert, M., Goda, Y., Hammer, R.E., Li, C., Rosahl, T.W., Stevens, C.F., and S?dhof, T.C. (1994). Synaptotagmin I: a major Ca2+ sensor for transmitter release at a central synapse. Cell *79*, 717-727.

Giraudo, C.G., Eng, W.S., Melia, T.J., and Rothman, J.E. (2006). A Clamping Mechanism Involved in SNARE-Dependent Exocytosis. Science *313*, 676-680.

Guan, R., Dai, H., and Rizo, J. (2008). Binding of the Munc13-1 MUN Domain to Membrane-Anchored SNARE Complexes. Biochemistry *47*, 1474-1481.

Guan, R., Dai, H., Tomchick, D.R., Dulubova, I., Machius, M., Sudhof, T.C., and Rizo, J. (2007). Crystal Structure of the RIM1α C2B Domain at 1.7 Å Resolution. Biochemistry *46*, 8988-8998.

Hakes, D.J., and Dixon, J.E. (1992). New vectors for high level expression of recombinant proteins in bacteria. Analytical Biochemistry *202*, 293-298.

Hanson, P.I., Roth, R., Morisaki, H., Jahn, R., and Heuser, J.E. (1997). Structure and Conformational Changes in NSF and Its Membrane Receptor Complexes Visualized by Quick-Freeze/Deep-Etch Electron Microscopy. Cell *90*, 523-535.

Hata, Y., Slaughter, C.A., and Sudhof, T.C. (1993). Synaptic vesicle fusion complex contains unc-18 homologue bound to syntaxin. Nature *366*, 347-351.

Hayashi, T., McMahon, H., Yamasaki, S., Binz, T., Hata, Y., S?dhof, T.C., and Niemann, H. (1994). Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. The EMBO journal *13*, 5051-5061.

Hazzard, J., S?dhof, T.C., and Rizo, J. (1999). NMR analysis of the structure of synaptobrevin and of its interaction with syntaxin. Journal of biomolecular NMR *14*, 203-207.

Helm, C., Israelachvili, JN. (1993). Forces between phospholipd bilayers and relationship to membrane fusion. Methods Enzymol *220*.

Hess, D.T., Slater, T.M., Wilson, M.C., and Skene, J.H. (1992). The 25 kDa synaptosomal-associated protein SNAP-25 is the major methionine-rich polypeptide in rapid axonal transport and a major substrate for palmitoylation in adult CNS. J Neurosci *12*, 4634-4641.

Hodgkin, A. (1964). The ionic basis of nervous conduction. Science *145*, 1148-1154.

Hu, C., Ahmed, M., Melia, T.J., Sollner, T.H., Mayer, T., and Rothman, J.E. (2003). Fusion of Cells by Flipped SNAREs. Science *300*, 1745-1749.

Hu, K., Carroll, J., Fedorovich, S., Rickman, C., Sukhodub, A., and Davletov, B. (2002). Vesicular restriction of synaptobrevin suggests a role for calcium in membrane fusion. Nature *415*, 646-650.

Hu, S.-H., Latham, C.F., Gee, C.L., James, D.E., and Martin, J.L. (2007). Structure of the Munc18c/Syntaxin4 N-peptide complex defines universal features of the N-peptide binding mode of Sec1/Munc18 proteins. Proceedings of the National Academy of Sciences *104*, 8773-8778.

Huxley, A. (1964). Excitation and conduction in nerve: quantitative analysis. Science *145*, 1154-1159.

Ishizuka, T., Saisu, H., Odani, S., and Abe, T. (1995). Synaphin: A Protein Associated with the Docking/Fusion Complex in Presynaptic Terminals. Biochemical and Biophysical Research Communications *213*, 1107-1114.

Jahn, R. (2000). Sec1/Munc18 Proteins: Mediators of Membrane Fusion Moving to Center Stage. Neuron *27*, 201-204.

Jahn, R., and Scheller, R. (2006). SNAREs--engines for membrane fusion. Nature reviews Molecular cell biology *7*, 631-643.

Jahn, R., and Südhof, T.C. (1999). MEMBRANE FUSION AND EXOCYTOSIS. Annual Review of Biochemistry *68*, 863-911.

Jessell, T.M., and Kandel, E.R. (1993). Synaptic transmission: A bidirectional and self-modifiable form of cell-cell communication. Cell *72*, 1-30.

Johnson, B.A.a.B., R. A. (1994). NMRView: a computer program for the visualization and analysis of NMR data. J Biomol NMR *4*, 603-614.

Junge, H.J., Rhee, J.-S., Jahn, O., Varoqueaux, F., Spiess, J., Waxham, M.N., Rosenmund, C., and Brose, N. (2004). Calmodulin and Munc13 Form a Ca2+ Sensor/Effector Complex that Controls Short-Term Synaptic Plasticity. Cell *118*, 389-401.

Katz, B., and Miledi, R. (1968). The role of calcium in neuromuscular facilitation. J Physiol *195*, 481-492.

Koh, T.-W., and Bellen, H.J. (2003). Synaptotagmin I, a Ca2+ sensor for neurotransmitter release. Trends in Neurosciences *26*, 413-422.

Kweon, D.-H., Kim, C.S., and Shin, Y.-K. (2003). Regulation of neuronal SNARE assembly by the membrane. Nat Struct Mol Biol *10*, 440-447.

Lackner, M.R., Nurrish, S.J., and Kaplan, J.M. (1999). Facilitation of Synaptic Transmission by EGL-30 Gq[alpha] and EGL-8 PLC[beta]: DAG Binding to UNC-13 Is Required to Stimulate Acetylcholine Release. Neuron *24*, 335-346.

Latham, C.F., Osborne, S.L., Cryle, M.J., and Meunier, F.A. (2007). Arachidonic acid potentiates exocytosis and allows neuronal SNARE complex to interact with Munc18a. Journal of neurochemistry *100*, 1543-1554.

Lin, R.C., and Scheller, R.H. (2000). MECHANISMS OF SYNAPTIC VESICLE EXOCYTOSIS. Annual Review of Cell and Developmental Biology *16*, 19-49.

Link, E., Edelmann, L., Chou, J.H., Binz, T., Yamasaki, S., Eisel, U., Baumert, M., S?hof, T.C., Niemann, H., and Jahn, R. (1992). Tetanus toxin action: Inhibition of neurotransmitter release linked to synaptobrevin proteolysis. Biochemical and Biophysical Research Communications *189*, 1017-1023.

Liza, B. (2004). Biography of Pietro De Camilli. Proceedings of the National Academy of Sciences of the United States of America *101*, 8259-8261.

Lu, J., Machius, M., Dulubova, I., Dai, H., dhof, T.C., Tomchick, D.R., and Rizo, J. (2006). Structural Basis for a Munc13–1 Homodimer to Munc13–1/RIM Heterodimer Switch. PLoS Biology *4*, e192.

Margittai, M., Fasshauer, D., Pabst, S., Jahn, R., and Langen, R. (2001). Homo- and Heterooligomeric SNARE Complexes Studied by Site-directed Spin Labeling. J Biol Chem *276*, 13169-13177.

Maruyama, I.N., and Brenner, S. (1991). A Phorbol Ester/Diacylglycerol-Binding Protein Encoded by the Unc-13 Gene of Caenorhabditis Elegans. Proceedings of the National Academy of Sciences *88*, 5729-5733.

McMahon, H.T., Missler, M., Li, C., and Südhof, T.C. (1995). Complexins: Cytosolic proteins that regulate SNAP receptor function. Cell *83*, 111-119.

Misura, K.M.S., Scheller, R.H., and Weis, W.I. (2000). Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. Nature *404*, 355-362.

Misura, K.M.S., Scheller, R.H., and Weis, W.I. (2001). Self-association of the H3 Region of Syntaxin 1A. IMPLICATIONS FOR INTERMEDIATES IN SNARE COMPLEX ASSEMBLY. J Biol Chem *276*, 13273-13282.

Morton, A.J., and Edwardson, J.M. (2001). Progressive depletion of complexin II in a transgenic mouse model of Huntington's disease. Journal of neurochemistry *76*, 166-172.

Nagy, G., Milosevic, I., Mohrman, R., Wiederhold, K., Walter, A.M., and Sorensen, J.B. (2008). The SNAP-25 Linker as an Adaptation Towards Fast Exocytosis. Mol Biol Cell, E07-12-1218.

Ono, S., Baux, G., Sekiguchi, M., Fossier, P., Morel, N.F., Nihonmatsu, I., Hirata, K., Awaji, T., Takahashi, S., and Takahashi, M. (1998). Regulatory roles of complexins in neurotransmitter release from mature presynaptic nerve terminals. European Journal of Neuroscience *10*, 2143-2152.

Pabst, S., Hazzard, J.W., Antonin, W., Südhof, T.C., Jahn, R., Rizo, J., and Fasshauer, D. (2000). Selective Interaction of Complexin with the Neuronal SNARE Complex. DETERMINATION OF THE BINDING REGIONS. J Biol Chem *275*, 19808-19818.

Pabst, S., Margittai, M., Vainius, D., Langen, R., Jahn, R., and Fasshauer, D. (2002). Rapid and Selective Binding to the Synaptic SNARE Complex Suggests a Modulatory Role of Complexins in Neuroexocytosis. J Biol Chem *277*, 7838-7848.

Perin, M.S., Fried, V.A., Mignery, G.A., Jahn, R., and Südhof, T.C. (1990). Phospholipid binding by a synaptic vesicle protein homologous to the regulatory region of protein kinase C. Nature *345*, 260-263.

Pervushin, K., Riek, R., Wider, G., and Wuthrich, K. (1997). Attenuated T2 relaxation by mutual cancellation of dipole-dipole coupling and chemical shift anisotropy indicates an avenue to NMR structures of very large biological macromolecules in solution. Proceedings of the National Academy of Sciences *94*, 12366-12371.

Pevsner, J., Hsu, S., and Scheller, R.H. (1994). n-Sec1: A Neural-Specific Syntaxin-Binding Protein. Proceedings of the National Academy of Sciences *91*, 1445-1449.

Reim, K., Mansour, M., Varoqueaux, F., McMahon, H.T., Südhof, T.C., Brose, N., and Rosenmund, C. (2001). Complexins regulate a late step in Ca2+-dependent neurotransmitter release. Cell *104*, 71-81.

Reim, K., Wegmeyer, H., Brandstatter, J., Xue, M., Rosenmund, C., Dresbach, T., Hofmann, K., and Brose, N. (2005). Structurally and functionally unique complexins at retinal ribbon synapses. The Journal of Cell Biology *169*, 669-680.

Rhee, J.S., Betz, A., Pyott, S., Reim, K., Varoqueaux, F., Augustin, I., Hesse, D., Su?dhof, T.C., Takahashi, M., Rosenmund, C., *et al.* (2002). **β** phorbol ester- and diacylglycerol-induced augmentation of transmitter release is mediated by Munc13s and not by PKCs. Cell *108*, 121-133.

Rickman, C., Medine, C.N., Bergmann, A., and Duncan, R.R. (2007). Functionally and Spatially Distinct Modes of munc18-Syntaxin 1 Interaction. J Biol Chem *282*, 12097-12103.

Rizo, J. (2003). SNARE function revisited. Nat Struct Mol Biol *10*, 417-419.

Rizo, J., and Südhof, T. (2002). Snares and Munc18 in synaptic vesicle fusion. Nature reviews Neuroscience *3*, 641-653.

Rizo, J., and Sudhof, T.C. (1998). C2-domains, Structure and Function of a Universal Ca2+-binding Domain. J Biol Chem *273*, 15879-15882.

Rosenmund, C., Rettig, J., and Brose, N. (2003). Molecular mechanisms of active zone function. Current opinion in neurobiology *13*, 509-519.

Rosenmund, C., Sigler, A., Augustin, I., Reim, K., Brose, N., and Rhee, J.-S. (2002). Differential Control of Vesicle Priming and Short-Term Plasticity by Munc13 Isoforms. Neuron *33*, 411-424.

Rothman, J.E. (1994). Mechanisms of intracellular protein transport. Nature *372*, 55-63.

Schaub, J., Lu, X., Doneske, B., Shin, Y.-K., and McNew, J. (2006). Hemifusion arrest by complexin is relieved by Ca2+-synaptotagmin I. e structural & molecular biology *13*, 748-750.

Schekman, R., Orci, L.. (1996). Coat proteins and vesicle budding. Science *271*.

Schiavo, G., Santucci, A., Dasgupta, B.R., Mehta, P.P., Jontes, J., Benfenati, F., Wilson, M.C., and Montecucco, C. (1993). Botulinum neurotoxins serotypes A and E cleave SNAP-25 at distinct COOH-terminal peptide bonds. FEBS Letters *335*, 99-103.

Shen, J., Tareste, D.C., Paumet, F., Rothman, J.E., and Melia, T.J. (2007). Selective Activation of Cognate SNAREpins by Sec1/Munc18 Proteins. Cell *128*, 183-195.

Siddiqui, T.J., Vites, O., Stein, A., Heintzmann, R., Jahn, R., and Fasshauer, D. (2007). Determinants of Synaptobrevin Regulation in Membranes. Mol Biol Cell *18*, 2037-2046.

Sudhof, T.C. (2004). THE SYNAPTIC VESICLE CYCLE. Annual Review of Neuroscience *27*, 509-547.

Südhof, T.C. (1995). The synaptic vesicle cycle: a cascade of protein-protein interactions. Nature *375*, 645-653.

Südhof, T.C. (2004). THE SYNAPTIC VESICLE CYCLE. Annual Review of Neuroscience *27*, 509-547.

Sutton, R.B., Fasshauer, D., Jahn, R., and Brunger, A.T. (1998). Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4[thinsp]A resolution. Nature *395*, 347-353.

Takahashi, S., Yamamoto, H., Matsuda, Z., Ogawa, M., Yagyu, K., Taniguchi, T., Miyata, T., Kaba, H., Higuchi, T., Okutani, F.*, et al.* (1995). Identification of two highly homologous presynaptic proteins distinctly localized at the dendritic and somatic synapses. FEBS Letters *368*, 455-460.

Tang, J., Maximov, A., Shin, O.-H., Dai, H., Rizo, J., and S?dhof, T. (2006). A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. Cell *126*, 1175-1187.

Tokumaru, H., Umayahara, K., Pellegrini, L.L., Ishizuka, T., Saisu, H., Betz, H., Augustine, G.J., and Abe, T. (2001). SNARE complex oligomerization by synaphin/complexin is essential for synaptic vesicle exocytosis. Cell *104*, 421-432.

Toonen, R.F.G., and Verhage, M. (2003). Vesicle trafficking: pleasure and pain from SM genes. Trends in cell biology *13*, 177-186.

Verhage, M., Maia, A.S., Plomp, J.J., Brussaard, A.B., Heeroma, J.H., Vermeer, H., Toonen, R.F., Hammer, R.E., van den Berg, T.K., Missler, M.*, et al.* (2000). Synaptic Assembly of the Brain in the Absence of Neurotransmitter Secretion. Science *287*, 864-869.

Verhage, M., and Toonen, R.F.G. (2007). Regulated exocytosis: merging ideas on fusing membranes. Current Opinion in Cell Biology *19*, 402-408.

Weber, T., Zemelman, B.V., McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Söllner, T.H., and Rothman, J.E. (1998). SNAREpins: minimal machinery for membrane fusion. Cell *92*, 759-772.

Weninger, K., Bowen, M.E., Choi, U.B., Chu, S., and Brunger, A.T. (2008). Accessory Proteins Stabilize the Acceptor Complex for Synaptobrevin, the 1:1 Syntaxin/SNAP-25 Complex. Structure *16*, 308-320.

Wu, M.N., Littleton, J.T., Bhat, M.A., Prokop, A., Bellen, H.J. (1998). ROP, the Drosophila Sec1 homolog, interacts with syntaxin and regulates neurotransmitter release in a dosage-dependent manner. The EMBO journal *17*, 127-139.

Wüthrich, K. (1986). NMR of proteins and nucleic acids (New York, Wiley).

Xue, M., Reim, K., Chen, X., Chao, H.-T., Deng, H., Rizo, J., Brose, N., and Rosenmund, C. (2007). Distinct domains of complexin I differentially regulate neurotransmitter release. Nat Struct Mol Biol *14*, 949-958.

Yang, B., Steegmaier, M., Gonzalez, L.C., Jr., and Scheller, R.H. (2000). nSec1 Binds a Closed Conformation of Syntaxin1A. J Cell Biol *148*, 247-252.