COMPETITION BETWEEN SYNAPTOTAGMIN 1 AND COMPLEXIN FOR SNARE COMPLEX BINDING, CONTROLS FAST SYNAPTIC VESICLE EXOCYTOSIS

APPROVED BY SUPERVISORY COMMITTEE

Thomas C. Südhof, M.D., Advisor

Jane Johnson, Ph.D., Committee Chair

Jose Rizo-Rey, Ph.D.

Leon Avery, Ph.D.

Gang Yu, Ph.D.

DEDICATION

I would like to thank the members of my Graduate Committee and my parents.

COMPETITION BETWEEN SYNAPTOTAGMIN 1 AND COMPLEXIN FOR SNARE COMPLEX BINDING, CONTROLS FAST SYNAPTIC VESICLE EXOCYTOSIS

by

JIONG TANG

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May, 2007

ACKNOWLEDGEMENTS

First, I would like to thank my mentor, Dr. Thomas C. Südhof, who gave me enormous instructions and support during the past five years. What impressed me most is his passion and enthusiasm for science that have always encouraged me to pursue my goals. From him, I learned not only how to design and perform experiments, but also to address science in a systematic way and in different angles. It would have been impossible to finish my training and research work without him.

I would like to give my thanks to all the people in Tom's lab. They have always been friendly and generous to help me. Dr. Ok-Ho Shin, a bench mate for 5 years, gave me a lot of advice on details of experiments. Drs. Weiping Han and Zhiping Pang raised many good suggestions through a lot of discussions. My project is incorporation with Drs. Ok-Ho Shin, Anton Maximov in our lab and Han Dai from Dr. Rizo's lab. I would really like to thank them for their help and excellent work.

I would also like to thank my committee members, Drs. Jane Johnson, Jose Rizo-Rey, Leon Avery and Gang Yu for their insightful criticism and important suggestions. In addition, I want to thank Drs. Ege Kavalali and Kim Huber, who gave me tremendous help in my qualification exam in which I have learned a great deal during this process.

I would like to thank my parents and brother for their love and encouragement.

COMPETITION BETWEEN SYNAPTOTAGMIN 1 AND COMPLEXIN FOR SNARE COMPLEX BINDING, CONTROLS FAST SYNAPTIC VESICLE EXOCYTOSIS

Publication No. _____

Jiong Tang, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2007

Supervising Professor: Thomas C. Südhof, M.D.

Calcium binding to synaptotagmin 1 triggers fast exocytosis of synaptic vesicles that were primed for release by SNARE complex assembly. Besides synaptotagmin 1, fast Ca^{2+}-triggered exocytosis requires complexins. Synaptotagmin 1 and complexins both bind to assembled SNARE complexes, but it is unclear how their functions are coupled.

To clarify previous debates on calcium dependent and independent binding between synaptotagmin 1 and SNARE proteins, I systematically examined the interactions between

synaptotagmin 1 and purified SNARE monomer, heterodimer and core complex separately. This would avoid the problem of doing binding assays in an undefined protein mixture. We found the calcium dependency of synaptotagmin 1 and SNARE interactions relied on the accurate binding conditions that include protein concentration and ionic strength. In addition, at physiological conditions, calcium dependent binding is favored.

Based on this system, I discovered the competition between complexin and synaptotagmin 1 for SNARE complex binding. Although in hydrophilic environment, complexin shows much higher affinity for SNARE complex than synaptotagmin 1, synaptotagmin 1 can more efficiently replace complexin from membrane embedded SNARE complex in a strictly calcium dependent manner.

Expression of synaptic vesicle targeted complexin (by fusion to synaptobrevin 2) in cultured cortical neurons severely blocks fast synchronous release, but not asynchronous release, which is very similar to that of synaptotagmin 1 knockout mice. Based on electrophysiological data and biochemical confirmation of competition, we suggest that the phenotype could result from the replacement of synaptotagmin 1 from SNARE complex by local high concentration of fused complexin.

We propose our model as: complexin binding promotes the assembly of SNARE complex and further stabilizes it. As a result, vesicles are activated into a “superprimed” metastable state, and are clamped at the same time waiting for triggering signals.

Synaptotagmin 1 replaces complexin and releases this clamp through SNARE complex binding upon calcium entry. The simultaneous binding of synaptotagmin 1 with SNARE complex and phospholipids finally triggers membrane fusion and vesicle release.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Tang, J., Maximov, A., Shin, OH., Dai, H., Rizo, J., Südhof, TC. (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. **Cell.** 126, 1175-87.

Deak, F., Shin, OH, Tang, J., Hanson. P., Ubach, J., Jahn, R., Rizo, J., Kavalali, ET., Südhof, TC. (2006) Rabphilin regulates SNARE-dependent re-priming of synaptic vesicles for fusion. **EMBO J.** 25, 2856-66

Chen, X., Tang, J., Südhof, TC., Rizo, J. (2005) Are neuronal SNARE proteins Ca2+ sensors? **J Mol Biol.** *347*, 145-58.

Shin, OH., Rhee, JS., Tang, J., Sugita, S., Rosenmund, C., Südhof, TC. (2003) Sr2+ binding to the Ca2+ binding site of the synaptotagmin 1 C2B domain triggers fast exocytosis without stimulating SNARE interactions. **Neuron.** *37*, 99-108.

Jia, J., Amanai, K., Wang, G., Tang, J., Wang, B., Jiang, J. (2002) Shaggy/GSK3 antagonizes Hedgehog signalling by regulating Cubitus interruptus. **Nature.** *416*, 548-52.

Sugita, S., Saito, F., Tang, J., Satz, J., Campbell, K., Südhof, TC. (2001) A stoichiometric complex of neurexins and dystroglycan in brain. **J Cell Biol.** *154*, 435-45.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

BoNT	botulinum neurotoxin
BSA	bovine serum albumin
Co-IP	co-immunoprecipitation
CpxI	complexin I
CpxII	complexin II
DMEM	Dulbecco's Modified Eagle Medium
MEM	minimal essential medium
FPLC	fast performance liquid chromatography
GST	glutathione-S-transferase
Kd	dissociation constant
NSF	N-ethylmaleimide-sensitive factor
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
SNAP	soluble NSF attachment protein
SNAP-25	synaptosome-associated protein of 25 kDa
SNARE	soluble NSF attachment receptor element
SNARE core complex	SNARE complex containing only SNARE motifs
SN1	the N-terminal SNARE motif of SNAP-25, 11-82
SN3	the C-terminal SNARE motif of SNAP-25, 141-203
Syb 2	rat synaptobrevin 2
Syt 1	rat synaptotagmin 1

Synt	rat syntaxin 1A
TeNT	tetanus neurotoxin

Chapter I: Introductions

1.1 Overview of nervous system

General structure of the brain and nerve cells

The human brain contains more than 10^{11} cells and is composed of 2 large groups of cells: neurons and glia. Although glial cells far outnumber neurons, they have less diversity and consist of 4 classes of cells: astrocytes, oligodendrocytes (schwann cells) and microglia. Glial cells predominate in the vertebrate nervous system and function as support, isolation and maintenance of a stable environment. Neurons, with at least a thousand different types, forming thousands of connections with other neurons, are the major functional components of the brain.

A typical neuron has 3 morphologically defined regions: the cell body (also called soma); dendrites and axon. Fitting with its information processing function, the soma receives input signals from dendrites (usually more than one) and passes them along to the next neuron by an action potential. Neurons form specialized structures called synapses, at sites of contact for communication. Synapses are composed of presynaptic terminals, postsynaptic spines protruding from the shaft of dendrites and synaptic cleft between them.

Synaptic transmission

When an action potential invades a presynaptic terminal, it opens voltage-dependent calcium channels. Calcium entry evokes synaptic vesicle fusion and neurotransmitter release within milliseconds. Binding of neurotransmitter with postsynaptic receptors induces conformational change and opening of ligand-gated channels, followed by the generation of postsynaptic currents. After exocytosis, vesicles undergo endocytosis and recycling. Neurotransmitter will be transported into vesicles for the next round of release after acidification of vesicles. In this way, information is relayed from one neuron to the next.

In most synapses, Ca^{2+} influx is mediated by P/Q- or N-type Ca^{2+} channels. Calcium triggered two kinetically different forms of release: fast synchronous release which arises as short as 50 μs after calcium transient develops; and slow asynchronous release with over 1s duration (Fig. 1.1). Both forms of release are strictly calcium dependent, but may have different forms of mechanism. Not all action potentials will induce synaptic vesicle release. In most cases, only 10~20% can succeed. In adaptation to their functions, some neurons, such as the Calyx of Held, can form a huge synaptic terminal with ~500-600 independent synaptic contacts to guarantee the reliability and accuracy of synaptic transmission. The relationship between action potential and synaptic transmission is also a subject to change due to intrinsic messengers and extracellular signals.

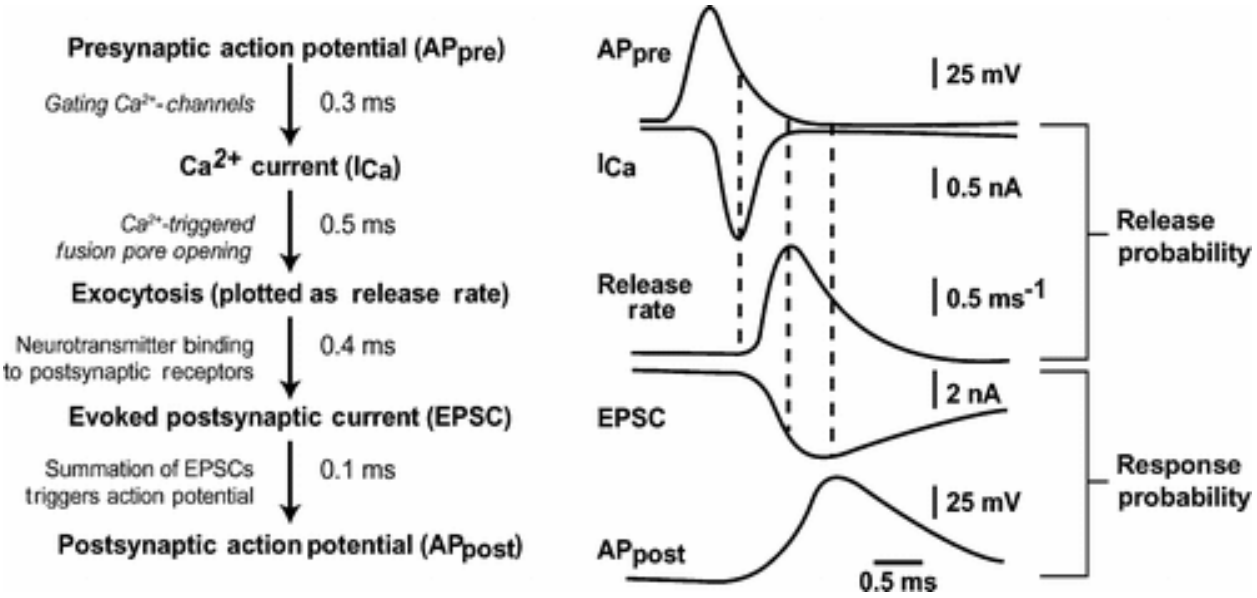


Figure 1.1 Reaction sequence and timing of synaptic transmission. The principal reactions with the associated time constants are shown on the left, and traces from the corresponding reactions in the calyx of Held synapses are illustrated on the right. The time calibration bar at the bottom applies to all traces. (Südhof, 2004)

Synaptic vesicle recycle

To fulfill the purpose of fast and repetitive release, synaptic vesicles undergo a trafficking cycle in the presynaptic terminal (Figure 1.2) which can be divided into sequential steps. First, neurotransmitters are filled into synaptic vesicles by specific transporters (step 1); then synaptic vesicles are relocated towards the active zone along actin filaments (step 2); after which they are docked at the active zone (step 3), and undergo an ATP-required process called priming (step 4) to be converted into a competent status for Ca^{2+}-triggered release (step 5).

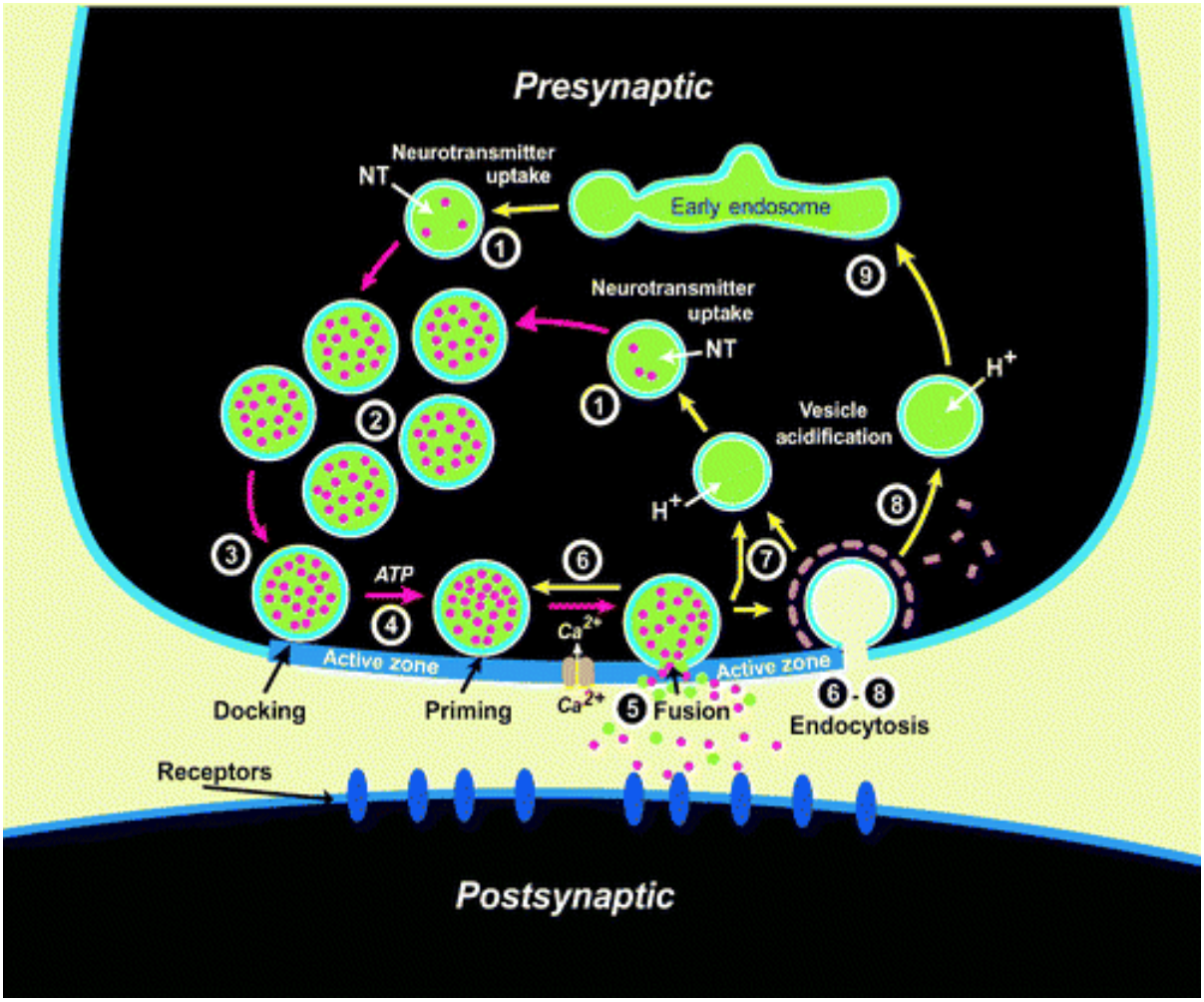


Figure 1.2 The synaptic vesicle cycle. Synaptic vesicles are filled with neurotransmitters by active transport (step 1) and form vesicle clusters that may represent the reserve pool (step 2). Filled vesicles dock at the active zone (step 3), where they undergo a priming reaction (step 4) that makes them competent for Ca^{2+} triggered release (step 5). After fusion-pore opening, synaptic vesicles undergo endocytosis and recycle *via* several routes: local reuse (step 6), fast recycling without an endosomal intermediate (step 7), or clathrin-mediated endocytosis (step 8) with recycling *via* endosomes (step 9). Steps in exocytosis are indicated by red arrows and steps in endocytosis and recycling by yellow arrows. (Südhof, 2004)

After fusion-pore opening and neurotransmitter release, synaptic vesicles endocytose and recycle probably by three different pathways: (*a*) “kiss-and-stay”, vesicles are reacidified and refilled with neurotransmitters at the same fusion site without undocking, thus remaining in the readily releasable pool and maintaining their competence status (step 6); (*b*) "kiss-and-run", vesicles endocytose and recycle locally for regeneration (step 7); or (*c*) vesicles undergo clathrin-mediated endocytosis (step 8) and reacidify to refill with neurotransmitters either directly or after passing through an endosomal intermediate (step 9).

Membrane fusion

The ultimate purpose of synaptic transmission is the fusion of synaptic vesicles with the plasma membrane to release neurotransmitters. Membrane fusion can not happen by itself, due to the repulsive force from negatively charged heads of phospholipids on the outer surface of the two opposing membranes. The energy barrier is very strong especially when two membranes get close to the distance necessary for fusion. Once the force is overcome, a nonbilayer metastable transition state is reached which will then lead to the formation of an aqueous fusion pore. Then the fusion pore expands followed by the completion of the fusion reaction.

There are two proposals for the formation of the fusion pore (Almers and Tse, 1990; Lindau and Almers, 1995). First, proteins form an oligomeric ring structure along the fusion pore between the two opposing membranes for subsequent phospholipids invasion to cover

the newly exposed amphiphilic surfaces; and second, the fusion pore is primarily lined by phospholipids with the help of proteins to reduce activation energy and spatially organize the fusion.

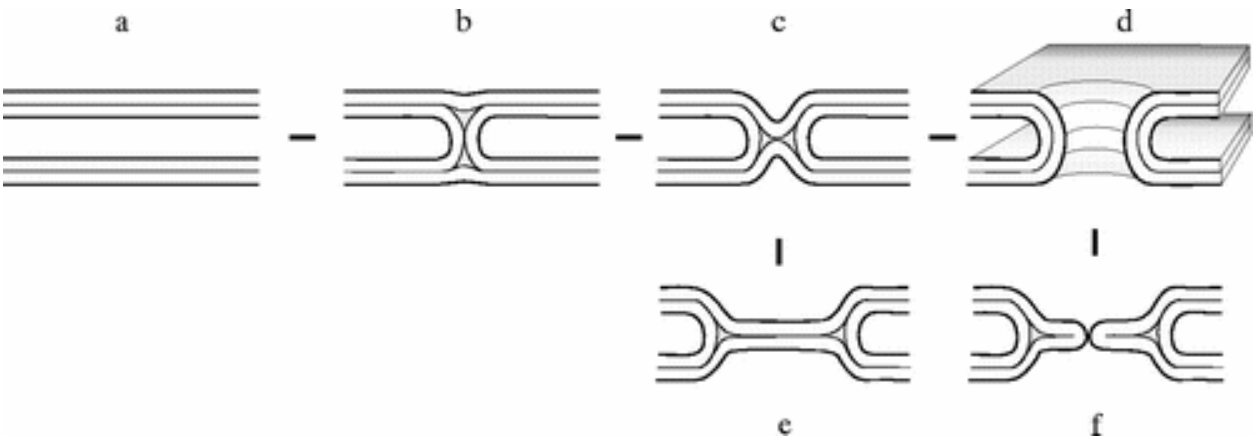


Figure 1.3 Intermediates in membrane fusion: (*a*) membranes separated; (*b*) fusion stalk; (*c*) transbilayer contact; (*d*) opening of the fusion pore; (*e*) hemifusion diaphragm; (*f*) pore formation in the diaphragm. (Jahn & Südhof, 1999)

The current model for fusion begins with a stalk intermediate in which the proximal monolayers are connected by a highly bent stalk, and the distal monolayers are pulled toward each other that is often referred as hemifusion states. The uncertainty of the model is whether the contact of the two distal monolayers will result in the expansion and formation of a disk-like single bilayer which is energetically unfavorable without stabilizing factors or direct break of the connection to form a fusion pore (Fig 1.3). No matter which are correct, the central step is the open of the fusion pore.

1.2 Essential Proteins for exocytosis

There are more than 1000 proteins in presynaptic terminals, among them hundreds are thought to be involved in synaptic transmission. Several groups of proteins have been demonstrated to play key roles in vesicles docking, priming and fusion. The following part will mainly discuss SNAREs, synaptotagmin 1 and complexin.

SNAREs

General introductions

SNAREs form a large family of proteins sharing a common ~60 residue sequence known as SNARE motif which has a high tendency to form coiled coil domain. SNAREs are the most basic components for all membrane fusion events studied in yeast and higher eukaryotes, suggesting a highly essential and conserved function (Jahn et al., 1999; Rizo et al., 2002). Based on their localization, SNAREs were originally divided into 2 groups: t-SNAREs (on target membrane) and v-SNAREs (on trafficking vesicle membrane).

The major forms of SNAREs in the central nervous system include: synaptic vesicle protein synaptobrevin/VAMP (vesicle associated membrane protein); plasma membrane protein syntaxin and membrane associated protein SNAP-25 (synaptosomal-associated protein of 25kD). As a common feature for SNAREs, synaptobrevin, syntaxin and SNAP-25 can form a very tight SNARE core complex mediated by the 4 SNARE motifs from these 3 proteins (Fig 1.4).

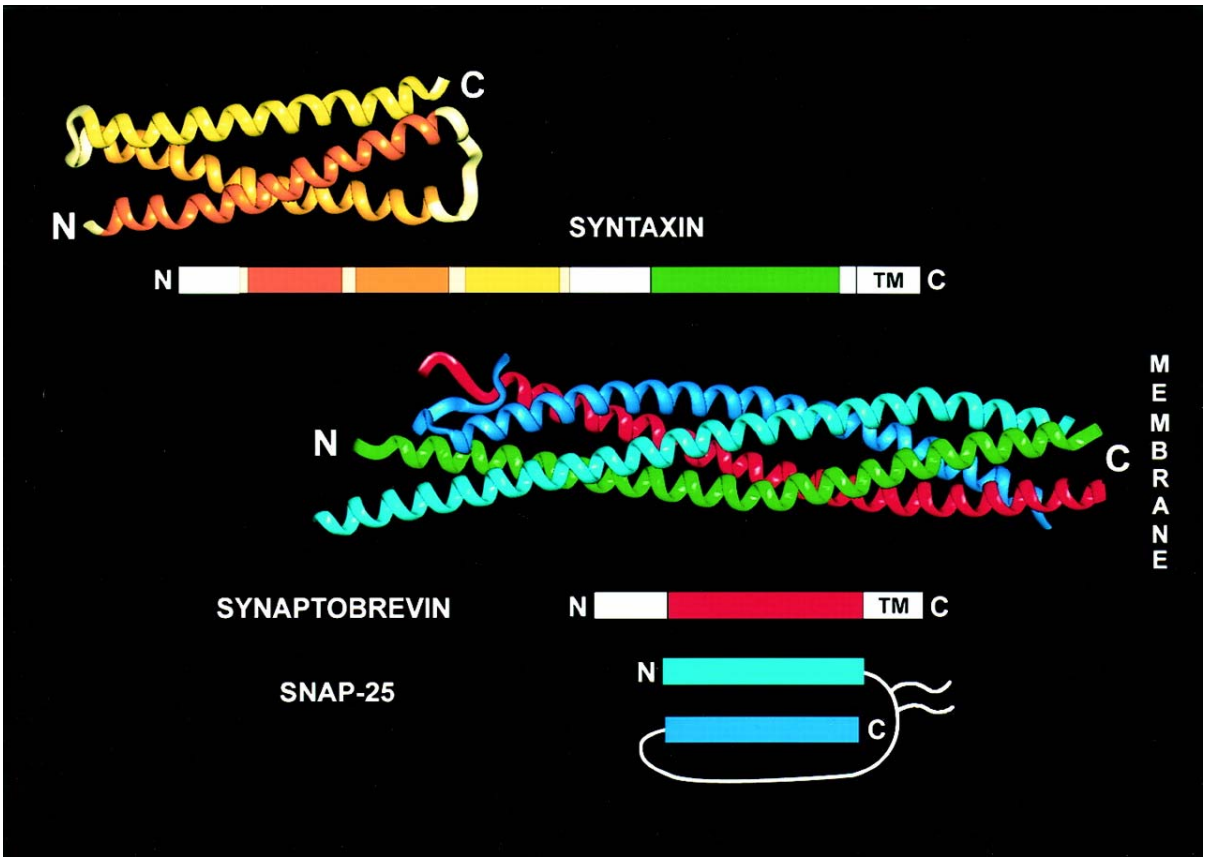


Figure 1.4 Structures of the neuronal SNARE proteins. Syntaxin has automatically folded N-terminal Habc domain and C terminal SNARE motif (green). In the middle is ribbon diagram of core complex. Bottom shows the relative position of SNARE motif within synaptobrevin (red) and SNAP-25 (dark and light blue). For comparison, the linear arrangement of the domains is shown for each protein in like colors. (Jahn & Südhof, 1999)

Synaptobrevin and syntaxin only possess one SNARE motif each. In synaptobrevin, the SNARE motif and transmembrane region are flanked by short N terminal proline-rich

sequences that do not form independent domains. Besides syntaxin and SNAP-25, synaptobrevin also binds synaptophysin, another abundant synaptic vesicle protein. This interaction prevents synaptobrevin from forming SNARE complex with syntaxin and SNAP-25 (Calakos et al., 1994; Edelmann et al., 1995), and is considered as a mechanism to regulate vesicle release.

Different from synaptobrevin, syntaxin owns a very large and conserved N terminal which can form a tight complex with neuronal SM protein Munc18-1. The N terminal contains an antiparallel three-helix bundle (Fernandez et al., 1998, Fig 1.4), known as Habc domain. In isolated syntaxin 1, the Habc domain folds back onto the SNARE motif, forming a "closed conformation" that is required for Munc18-1 binding, but differs from the "open conformation" of syntaxin 1 in the core complex. With Munc18 bound, syntaxin is locked in closed conformation and can't 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 (Betz et al., 1997).

SNAP-25 contains two SNARE motifs (the N-terminal and the C-terminal SNARE motifs designated as SNN and SNC, respectively) with a very long joint loop between them to ensure the parallel alignment of 2 SNARE motifs within same molecule when forming SNARE complex. Although without transmembrane region, SNAP-25 still can attach to the membrane by four palmitoylated cysteine residues in the joint loop.

The SNARE core complex

The SNARE core complex is formed by the 4 SNARE motifs from synaptobrevin, syntaxin and SNAP-25. Mostly unstructured in isolation, they can form a very tight parallel 4 helix bundle on assembly. SNARE core complex is unusually stable. It can not be denatured by SDS and is resistant to proteolysis by botulinum and tetanus neurotoxins (Hayashi et al., 1994). The crystal structure reveals the bundle is highly twisted and contains salt bridges on the surface and conserved leucine-zipper-like layers at the center. The interior residues are more conserved than the surface residues since the surface could provide additional scaffold for binding of regulatory proteins localized at different organelles. Both the interior residues and the whole helix structure of the bundle are highly conserved throughout the SNARE family (Rizo et al., 2002). Embedded within these leucine-zipper layers is an ionic layer (called zero layer) consisting of one arginine and three glutamine residues that contributes from each of the four alpha-helices (R from synaptobrevin and Q from syntaxin and SNAP-25). This led to the definition of Q- and R- SNAREs (Sutton et al., 1998; Fasshauer et al., 1998) and the proposal that all core complexes consist of four-helix bundles formed by three Q-SNAREs and one R-SNARE. This proposal is supported by reconstitution studies (McNew et al., 2000; Parlati et al., 2000) and especially the crystal structure of endosomal SNARE complex which is remarkably similar to that of the neuronal complex (Antonin et al., 2002).

Assembly of SNARE Core Complexes

SNARE motifs readily assemble into SNARE complex in hydrophilic solutions. However, with membrane restriction, the endogenous SNAREs may only form partial complex. *In vitro*, other combinations of SNARE motifs can also generate 4 helix bundle, such as: syntaxin SNARE motif itself (Misura et al., 2001); syntaxin and SNAP-25 binary complex with 2:1 ratio (Xiao et al., 2001); and syntaxin SNARE motif and SNAP-25 N terminal SNARE motif with 1:1 ratio (Misura et al., 2001). Thus, the assembly of SNARE motifs *in vitro* doesn't always follow the rule of 1 R-SNARE with 3-Q SNAREs. Endogenous vesicles contain different SNAREs and are targeted to different membrane structures. The accurate transport of various vesicles suggests that SNARE motifs themselves are not sufficient to guarantee the specificity for SNAREs recognition. Other factors, such as flanking region of SNARE motifs, transmembrane domain of SNAREs, or other associated proteins could be involved in this process.

Disassemble of SNARE complex

The extreme stability of the SNARE complex to overcome the energy barrier and pull the two membrane structures close also needs special chaperone proteins to dissemble the complex by ATP hydrolysis. After fusion, cis-SNARE complex need to be separated to its original tran-SNAREs for the next round of fusion. α-SNAP (soluble NSF attachment protein) is an adaptor protein and can selectively bind to SNARE core complex followed by NSF (*N*-ethyl maleimide sensitive factor) binding and dissociation of the complex. Both NSF and α-SNAP are structurally and functionally conserved proteins and are essential SNARE complex chaperone in almost all intracellular transport steps. NSF is a hexamer, belonging to

the AAA protein superfamily. Each subunit is composed of three distinct domains: an N-terminal domain (N-domain) responsible for substrate binding and two nucleotide binding domains, D1 for ATP hydrolysis and D2 for holding subunit together.

Function and mechanism

The importance of SNAREs in synaptic transmission was discovered by the findings that SNAREs are the target proteins of clostridial neurotoxins which can inhibit synaptic transmission by cleaving SNARE proteins at specific sites. The abolishment of evoked release, but with spontaneous miniature release still remaining in genetic deletion of SNAREs in mice, *Drosophila* and *C. elegans* indicate SNARE proteins are essential for the major form of vesicle release. The fact that these SNARE proteins are localized on opposing membranes and can form a big complex with NSF and SNAPs complex suggests that SNAREs may mediate vesicle docking and targeting (the SNARE hypothesis). Although experiments have proved that SNAREs are not required for vesicle docking (Südhof et al., 1993; Broadie et al., 1995), the crystal structure of the SNARE complex promotes the generation of another attractive model: the formation of the stable SNARE complex can overcome the energy barrier of the two membrane structures and directly cause vesicle fusion (Fig 1.5). Reconstitution experiments with SNAREs incorporated into separate liposomes showed SNARE complex itself can drive vesicles to fuse (Weber et al., 1998). However, the low efficiency and long time scale indicate that this minimal fusion machinery itself is not sufficient to perform all the physiological functions. Inactivation of NSF can increase SNARE complex and neurotransmitter release on stimulation, suggesting that the SNARE

complex formation and vesicle fusion can be separated into two steps. And core complex formation precedes vesicles exocytosis. In summary, the model can be modified as: SNARE complex formation pull vesicles close to membrane and make it ready for triggering. Other factors control the last step of membrane fusion.

Of course this is not the only model. There is evidence that SNARE complex assembly coincides with fusion, although the fusion rate is too slow in this *in vitro* liposome fusion assay (Hu et al., 2002). Other data have also indicated that the core complex assembly does not coincide with fusion-pore opening by showing that trans-complex could be disrupted without inhibiting the subsequent mixing of vacuolar contents (Ungermann et al., 1998).

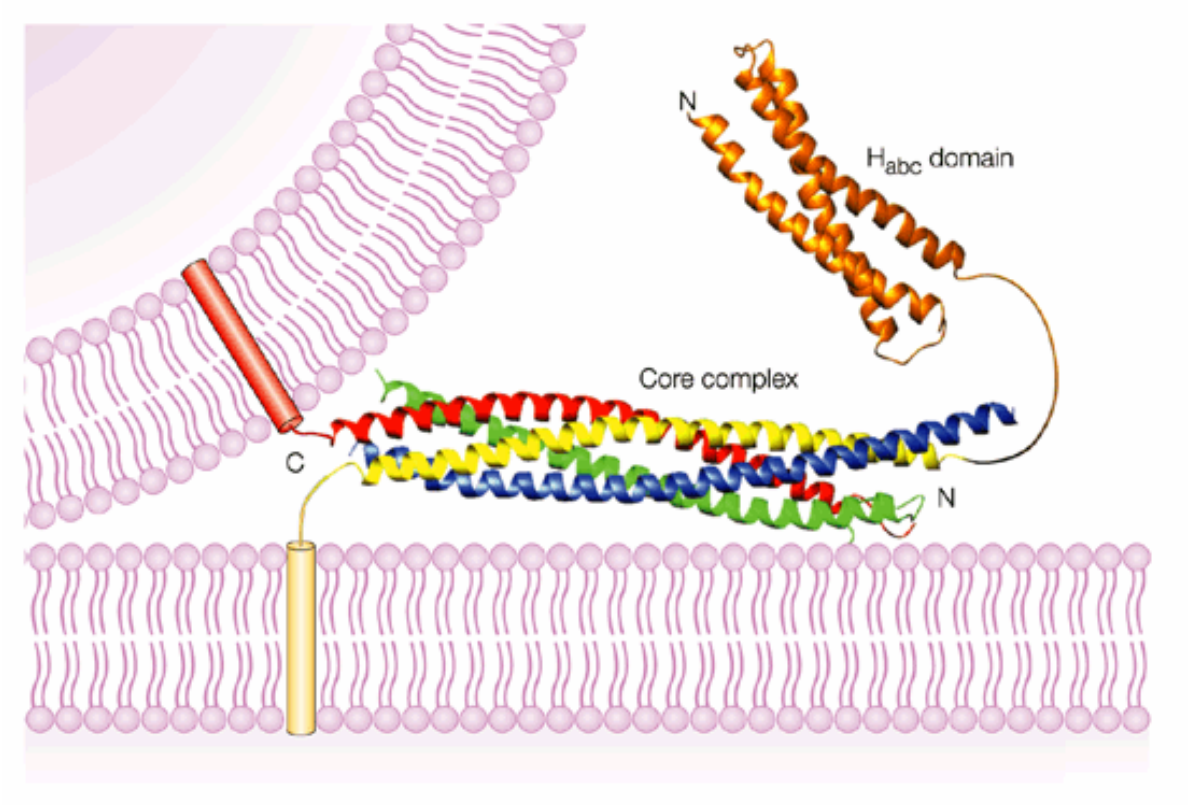


Figure 1.5 Model of the neuronal SNAREs assembled into the core complex. The ribbon diagrams represent the crystal structure of the core complex and the NMR structure of the amino-terminal Habc domain of syntaxin 1. The Habc domain is colored in orange and the SNARE motifs are color coded as follows: synaptobrevin, red; syntaxin 1, yellow; SNAP-25 amino terminus, blue; SNAP-25 carboxyl terminus, green. The cylinders represent the transmembrane regions of synaptobrevin and syntaxin 1, which are inserted into the synaptic vesicle and plasma membranes, respectively. (Rizo & Südhof, 2002)

Synaptotagmin 1

General structure of Synaptotagmin 1:

Synaptotagmin 1 was discovered by two monoclonal antibodies raised against rat synaptic membrane proteins. It is an abundant vesicle protein and originally named p65 from its apparent molecule weight on SDS-PAGE gel (Matthew et al., 1981). As a type I membrane protein, synaptotagmin1 has a short glycosylated intravesicular N-terminal followed by transmembrane sequence, linker region and two C_2 domains (C_2A and C_2B), which are homologous to the second domain of protein kinase C (Perin et al., 1991). Synaptotagmin 1 is a highly conserved protein with 97% identity between human and rat. Among different species, synaptotagmin 1 exhibits a selective conservation of the two C_2 domains. These C_2 domains are slightly more homologous to each other than to protein kinase C, and the difference between the repeats are conserved in evolution, suggesting that they might be functionally different.

The structures of C_2A and C_2B are very similar. Both are composed of a β-sandwich containing eight β-strands with loops emerging from two ends. Calcium selectively binds to the top loops (1 and 3). As shown is Fig. 1.6, C_2A domain binds 3 Ca^{2+} ions *via* 5 aspartates and 1 serine which are widely separated in the primary sequence (Ubach et al., 1998). Without serine on loop3, C_2B domain only binds two Ca^{2+} ions (Fernandez et al., 2001). Besides that, C_2B domain has two additional α-helices, one between the 7th and 8th β-strand, and the other at the very end (Fernandez et al., 2001, Fig 1.7).

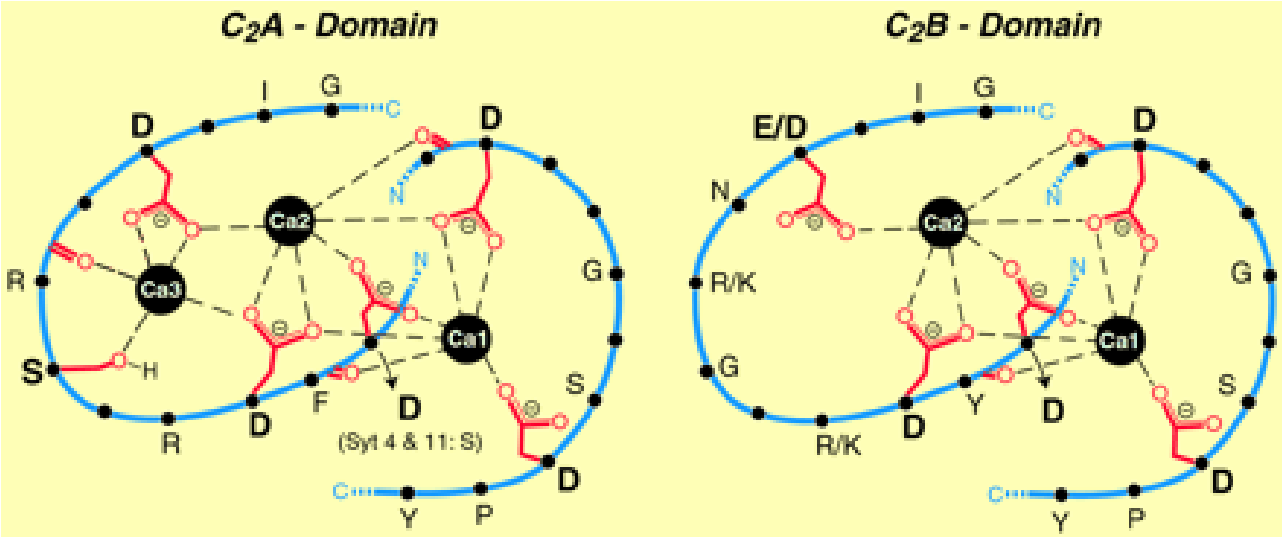


Figure 1.6 Structure of the Ca^{2+}-binding sites of the C_2A- and C_2B-domains of synaptotagmins. The diagram shows a generic model for synaptotagmin Ca^{2+}-binding sites that are based on the structure of the C_2A- and C_2B-domains of synaptotagmin 1. The Ca^{2+}-binding sites at the top of the synaptotagmin C_2-domains are formed by loops 1 (right blue line) and 3 (left blue line). The C_2A-domain ligates three Ca^{2+} ions *via* five

aspartates and one serine residue, whereas the C_2B-domain lacks the binding site for Ca3 and ligates only two Ca^{2+} ions. (Südhof, 2002)

Calcium and Phospholipids binding

Although the C_2 domains of synaptotagmin 1 can bind calcium, the intrinsic calcium affinity is very low (K_d >1 mM for C_2A and >0.3 mM for C_2B-domain), and can be tremendously enhanced (~10,000 folds) in the presence of negatively charged phospholipids which probably provide additional coordination sites for incomplete calcium binding sphere (Fernandez et al., 2001). Ca^{2+}-dependent phospholipid binding to the synaptotagmin-1 C_2A domain involves a combination of electrostatic interactions between basic residues and the phospholipid head groups, coordination of the Ca^{2+} ions by these head groups and insertion of hydrophobic residues into the lipid bilayer (Zhang et al., 1998; Bai et al., 2002; Gerber et al., 2002). All these residues are located at the top loops of the sandwich (loops 1–3). As expected from its homology, the top loops of the C_2B domain share similar structural features with those of the C_2A domain. Calcium binding to C_2 domains doesn't cause significant conformational change, suggesting C_2 domains function as electrostatic switches (Ubach et al., 1998; Fernandez et al., 2001). Calcium binding triggers the simultaneous penetration of the C_2A and C_2B domain (Hui et al., 2006), and both C_2A and C_2B have similar orientations with deeper penetration into the bilayer interior compared to that of single C_2 domain (Frazier et al., 2003; Rufener et al., 2005; Herrick et al., 2006). However, the composition and metal ion can also affect the phospholipid binding of synaptotagmin 1. PIP and PIP_2 can

increase both calcium dependent and independent phospholipid binding, and the latter is mediated by the polybasic region of C_2B domain (Bai et al., 2004; Li et al., 2006).

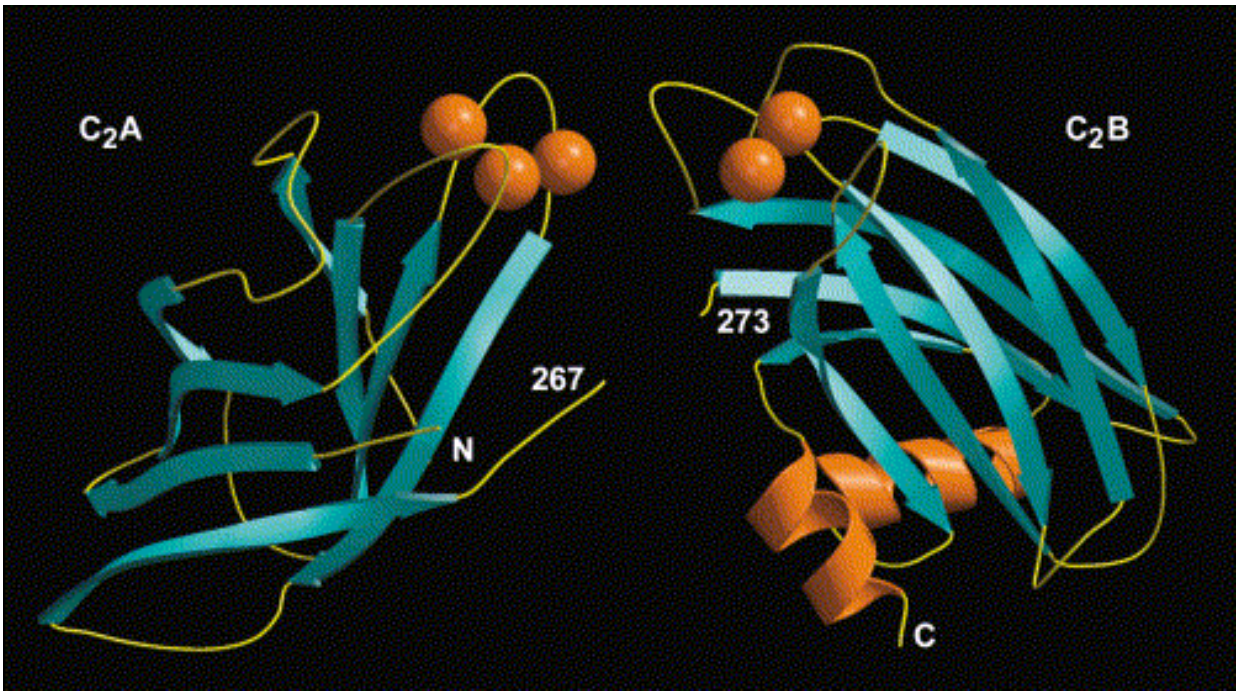


Figure 1.7 Ribbon diagrams of the C_2A- and C_2B-domains of synaptotagmin 1. C_2A and C_2B domain are oriented with their Ca^{2+} binding sites in close proximity. The proximity of the C terminus of the C_2A-domain (267) to the N terminus of the C_2B-domain (273) shows that this orientation can be easily reached. (Fernandez et al., 2001)

Interactions with SNAREs

In addition to forming Ca^{2+}-dependent complex with phospholipid, synaptotagmin 1 also bind to SNARE proteins. For SNARE monomers, synaptotagmin 1 has no interaction with synaptobrevin (Schiavo et al., 1997; Tang et al., 2006). The binding between syntaxin

and synaptotagmin 1 is clearly calcium dependent (Li et al., 1995; Chapman et al., 1995; Shin et al., 2003). Syntaxin is composed of N terminal Habc domain and C terminal SNARE motif. Both of them have been reported to have calcium dependent interactions with synaptotagmin 1 separately in different assay, but can never be detected together (Chapman et al., 1995; Kee et al., 1996; Shao et al., 1997). In addition to SNAP-25, SNARE heterodimer and core complex have also been shown to have both calcium dependent and independent interactions with synaptotagmin 1 (Schivao et al., 1997; Zhang et al., 2002; Rickman et al., 2003; Ernst et al., 2003; Bai et al., 2004). The corresponding binding region on SNAREs is still not completely clear. C_2A domain of synaptotagmin 1 is involved in syntaxin and SNAP-25 binding, but no direct interaction with synaptobrevin 2 (Shin et al., 2003). Although some studies described that the polybasic region of C_2B domain is responsible for the calcium independent binding (Rickman et al., 2004), the possibility that this could be mediated by the contaminated proteins sticking to the polybasic region raised questions (Ubach et al., 2001). C_2AB domain functions in a cooperative manner, and its SNARE binding pattern is different from that of individual C_2 domain. Therefore, it is critical to resolve the structure of whole C_2AB binding to SNAREs. FRET assay has revealed that the C_2B domain and the linker region are important for calcium dependent C_2AB binding with SNARE complex (Bowen et al., 2005). Further details will need to be clarified with the help of solving the structure of the whole C_2AB domain (Montes et al., 2006).

Calcium dependent self-association

Recombinant C_2B domain, but not C_2A domain can pull down synaptotagmin 1 and 2 from brain lysate in a calcium dependent manner which may have essential role for synaptotagmin 1 to mediate vesicle fusion (Sugita et al., 1996; Chapman et al., 1996). Unlike the calcium independent dimerization by transmembrane region, this interaction is mediated by C_2B domain. However, individual C_2B domain alone lost this ability to form complex while it still can bind calcium, which make this interaction unclear (Ubach et al., 2001).

Other binding partners

Synaptotagmin 1 can interact with AP-2 (adaptor for clathrin coating) with high affinity. Acute inactivation of synaptotagmin 1 by CALI (chromophore-assisted laser inactivation) in live neurons suggests it is important for vesicle endocytosis (Poskanzer et al., 2003). Because inositol phosphate can bind many proteins, and its binding to synaptotagmin 1 is mediated by the polybasic region on C_2B, whether the inhibitory effect on vesicle release is specific for synaptotagmin 1 is unknown, although there is evidence that its binding can block t-SNAREs interactions with synaptotagmin 1 (Rickman et al., 2004).

Mechanism of synaptotagmin 1 functions

All the biochemical studies of synaptotagmin 1 indicate it as the best candidate for calcium sensor of vesicle release. Consistent with this hypothesis, neurons from synaptotagmin 1 knockout mice showed severe abolishment of fast synchronous release, but no change for asynchronous release (Geppert et al., 1994). Moreover, a point mutation (R233Q) in synaptotagmin 1 C_2A domain that decreases the overall apparent calcium affinity

to half also induce the same shift in the calcium affinity for exocytosis (Fernandez-Chacon et al., 2001). Different from the phenotype of the SNARE (synaptobrevin 2) knockout mice which non-specifically abolish almost all fusion events (Schoch et al., 2001; Deak et al., 2004), the selectivity of synaptotagmin 1 function further confirms that SNARE proteins broadly mediate most of the vesicle fusion, and synaptotagmin 1 is the calcium sensor only for fast synchronous release.

A crude model for vesicle fusion can be raised as: synaptotagmin 1 can be involved in the cycle shortly after vesicle docking. During the process of SNARE zippering, synaptotagmin 1 may facilitate this process. Whether by calcium independent binding to SNAP-25 and syntaxin heterodimer or to loosely assembled SNARE complex is unknown. Synaptotagmin 1 could have certain extent of calcium independent interactions with SNARE complex or phospholipids. The approximate of synaptotagmin 1 to membrane and SNARE complex can fasten its reaction upon calcium binding to trigger vesicle fusion. There are several focused points that still need to be elucidated. Firstly, both calcium dependent phospholipids binding and SNARE complex binding can be the driving force for vesicle fusion. Although some results favor that phospholipids binding of synaptotagmin 1 is essential (Shin et al., 2002; Shin et al., 2003), the importance of SNARE complex binding is not neglected (Bai et al., 2004). A key problem is the difficulty to find a mutant of synaptotagmin 1 which selectively affect only one of the interactions. Moreover, probably synaptotagmin 1 could bind simultaneously to SNARE complex and membrane together to trigger release, although the opposite result also existed (Davis et al., 1999; Arac et al.,

2003). Secondly, the importance of the cooperativity of C_2A and C_2B domains has been underestimated for a long time. Mutations in single C_2A domain showed great decrease in phospholipid binding and exhibited almost no change in C_2AB context (Fernandez-Chacon et al., 2002). This also affects the conclusions based on the biochemical results tested only from mutations on single C_2A or C_2B, but not whole C_2AB domain (Mackler et al., 2002; Robinson et al., 2002). With these concerns, those conclusions need to be reevaluated with more experiments. Third, the conciliation of calcium dependent and independent binding of SNAREs and phospholipids binding is not completed. Calcium independent binding site on synaptotagmin 1 was within the polybasic region, close to the bottom and far from the top calcium binding loop. Synaptotagmin 1 doesn't bind to phospholipids by these two sites at the same time unless the membrane is bended (Herrick et al., 2006). If synaptotagmin 1 binds to phospholipids before calcium entry with both ends immobilized (N terminal on vesicle and C_2B on plasma membrane), whether synaptotagmin 1 by itself still can change its orientation to make the top loop insert into membrane is questionable. This issue is also applied to SNARE binding.

Other isoforms of synaptotagmin

The synaptotagmin family of proteins contains more than a dozen isoforms, most of them with unknown functions. According to sequence similarity and properties, they can be classified into several groups. Here, I only discussed a few of them. Synaptotagmin1 and 2- the most closely related isoforms, are localized on synaptic vesicles and secretory granules, and have similar functions as calcium sensors for fast release (Geppert et al., 1994; Pang et

al., 2006). In the brain, they have complementary distribution. Synaptotagmin 7 is alternatively spliced and is mainly expressed on plasma membrane in brain. Although synaptotagmin 7 C_2 domains have 10-20 folds higher calcium affinity than synaptotagmin 1, its function in synaptic transmission is still unclear. Synaptotagmin 4 has a conserved substitution of an aspartate for a serine in C_2A domain, which abolishes calcium binding for rat synaptotagmin 4. But *Drosophila* synaptotagmin 4 still binds calcium and can functionally replace synaptotagmin 1 in *Drosophila* (Robinson et al., 2002).

Most synaptotagmins can bind calcium with different affinity. Combined with their distribution on different organelles in neurons and neuroendocrine cells, they could function as sensor to regulate different processes (Südhof, 2002).

Complexins

General introductions

Complexins are small soluble presynaptic proteins, first purified as two isoforms (I and II) by co-immunoprecipitation with SNARE proteins. Their name comes from the feature that they preferentially bind to SNARE complex, but not SNARE monomer or heterodimer (McMahon et al., 1995).

Composed of 134 amino acids, complexins are highly charged proteins. They are highly conserved, with 100% identity among mouse, rat and human complexin II. Recently,

complexin III and IV were discovered by the human genome search. They have higher molecular weight (158 and 160 amino acids, respectively) and less homology compared to complexin I and II (Reim et al., 2005).

Complexin I and II are highly enriched in the brain. In addition, low levels of complexin II was found ubiquitously expressed in many tissues, such as kidney, muscle and lung. Their expression covers most regions of the brain with some overlaps, but in different patterns (McMahon et al., 1995). In cultured neurons, within the first 1~2 weeks, complexin immunoreactivity was observed mainly in the cell soma, most correlated with syntaxin and SNAP-25 staining, unlike the puncta distribution of vesicle proteins like synaptotagmin 1 and synaptobrevin 2. After 4 weeks of maturation, complexin I was primarily localized in synapses surrounding the cell soma and thick neurites, while complexin II mainly concentrated in synapses of thin neurites (McMahon et al., 1995; Takahashi et al., 1995; Ono et al., 1998). These developmental changes in complexin agree with the observations *in vivo* by immunohistochemistry (Ono et al., 1998). Unlike complexin IV, which is exclusively expressed in retina, complexin III has certain level of expression in cerebral cortex and hippocampus (Reim et al., 2005).

Structure and biochemical features

Isolated complexins have no tertiary structure except a remarkable stable N terminal α helix structure (Pabst et al., 2000; Chen et al., 2002). Although this N terminal α helix structure doesn't bind to SNARE complex, it serves to nucleate the helix formation of

complexin upon interacting with SNARE complex, resulting an anti-parallel binding of complexin to the groove between syntaxin and synaptobrevin (Pabst et al., 2002; Chen et al., 2002; Bracher et al., 2002). Complexin binding is mediated by the central region from residue 48~70, and the binding helps to stabilize the SNARE complex (Chen et al., 2002).

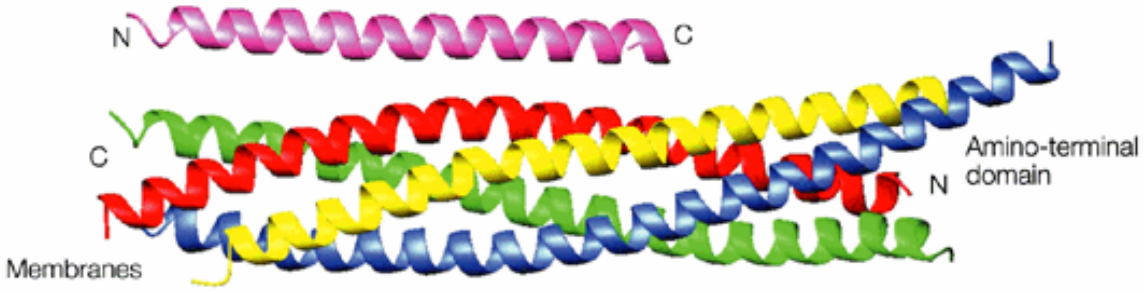


Figure 1.8 Three-dimensional structure of the complexin-SNARE complex. Complexin is colored in pink and the SNARE motifs are colored as in Fig 1.4. The approximate locations of the membranes and the N terminal domain of syntaxin are indicated. (Rizo & Südhof, 2002)

Biochemically, till now, complexins only bind to SNARE core complex. Studies have shown that it competes with α-SNAP, but not synaptotagmin 1 for SNARE complex binding (McMahon et al., 1995). The affinity between complexin and SNARE complex also varies according to the environment. With SNARE complex embedded in membrane, the binding affinity for complexin drops dramatically compared to that in solution (Pabst et al, 2002; Bowen et al., 2005). The stabilizing effect of complexin on SNARE complex doesn't block

vesicle recycling, since NSF still can efficiently disassemble SNARE complex in the presence of complexin (Pabst et al., 2002) with the help of α-SNAP. Presumably α-SNAP has a higher affinity to SNARE complex than complexin.

Mechanism of complexin function

Up-regulation of complexin by over-expression in PC12 and chromaffin cells (Itakura et al., 1999) or direct protein injection into neurons (Ono et al., 1998) suppresses vesicle release, suggesting an inhibitory effect in synaptic transmission. However, electrophysiological results from complexin(I/II) double knock out mice showed a selective defect in synchronous release with asynchronous release untouched, an phenotype very similar to synaptotagmin 1 knockout mice (Geppert et al., 1994), indicating an activating function of complexin (Reim et al., 2001). Miniature amplitude and frequency, sucrose induced readily releasable pool size has no significant change in these double knockout mice, except that the calcium affinity for exocytosis get right shifted, suggesting decreased release probability. This contradictory phenotype can not be simply explained by the difference between neuronal and non-neuronal system, because knock down of complexin in mast cells also impairs vesicle exocytosis (Tadokoro et al., 2005).

Promotion of SNARE complex full assembly and further stabilization can't explain why complexin is involved in the last step for calcium dependent vesicle fusion, since complexin doesn't need calcium for SNARE binding. The phenotypic similarity between synaptotagmin 1 and complexin knockout mice neurons shed light on the direction to

investigate how complexin regulate synaptic transmission. Two groups have established an *in vitro* fusion assay system to unravel the mechanism of complexin function by either reconstituting all SNARE proteins into liposome or expressing flipped SNARE proteins on cell surface. In both systems, complexin inhibited cell or liposome fusion, which can be rescued by the addition of synaptotagmin 1 and calcium (Giraudo et al., 2006; Schaub et al., 2006). However, at the molecular level, they did not give convincing evidence to elucidate the mechanism.

Chapter II: Interactions between synaptotagmin 1 C_2AB domains and SNARE proteins

2.1 Introductions

As discussed in previous chapter, to relay calcium signal to membrane fusion, synaptotagmin 1 need to interact with other fusion components to trigger vesicle release. Besides calcium dependent phospholipid binding, the interaction between synaptotagmin 1 and SNARE proteins also play a key role in driving vesicle fusion.

The interaction between synaptotagmin 1 and SNARE proteins have been extensively studied by many groups using different assays. A few commonly recognized conclusions have been reached that includes the following: (1), synaptotagmin 1 has no binding to synaptobrevin 2 (Schiavo et al., 1997; Tang et al., 2006); and (2), syntaxin can bind to synaptotagmin 1 in a calcium dependent manner (Chapman et al., 1995; Li et al., 1995; Shin et al., 2003; Bai et al., 2004; Tang et al., 2006). However, controversies still remain unresolved, mainly for the observation of both calcium dependent and independent interactions of synaptotagmin 1 with SNAP-25 and SNARE complex (Schiavo et al., 1997; Zhang et al., 2002; Shin et al., 2003; Rickman et al., 2003; Ernst et al., 2003; Bai et al., 2004; Tang et al., 2006).

There are two major caveats for most of the experiments. First, considering the fact that SNARE proteins can form different complexes, it is difficult to tell which form of

SNARE protein bind to synaptotagmin 1 when using mixed protein resources such as brain lysate or transfected cell lysate. Second, immunoblot was widely used for protein detection as evidence for binding. This sensitive and non-linear technique makes it difficult to judge the efficiency and reliability of the binding.

To partially overcome these problems, we adopt the recombinant protein system and GST affinity chromatography to investigate the relationship between synaptotagmin 1 and SNARE proteins. The key point is to make all SNARE proteins from monomer, dimer to complex, and purify them individually. With uniform and defined components, we can get an accurate conclusion and binding parameters such as stoichiometry and binding affinity values.

2.2 Material and Methods

2.2.1 Recombinant proteins purification

All SNARE motifs and synaptotagmin 1 C_2AB domains were cloned by PCR and put into pGex-KT vector to make GST fusion proteins including rat syntaxin (180~264, 1~264, 1~180), bovine synaptobrevin 2 (1~96), rat SNAP-25A (11~82 and 141~203, abbreviated as SN1 and SN3, respectively), rat synaptotagmin 1 C_2AB (140~421). Synaptotagmin 1 construct was made by Dr. Ok-Ho Shin. SN1 and SN3 constructs were from Dr. Rizo-Rey's lab and syntaxin and synaptobrevin 2 constructs were from Tom's lab. Plasmids were transformed into *Escherichia coli* BL21 cells for protein expression.

Transformed bacterial was inoculated into 25ml of LB medium for overnight incubation. About 10ml of culture was transferred to 1L of LB medium for amplification until OD_{600} reaches between 0.6~0.8. IPTG (0.1~0.5mM) was added to induce GST fusion protein expression after the culture was cooled down to room temperature. Bacterial was collected after induction at room temperature for 5~6 hours. Cells were then directly broken for protein purification or frozen at -80°C for future use. All LB medium contains 100μg/ml of ampicillin.

Bacterial were broken by passing through emulsiflex French pump after being resuspended in lysis buffer (PBS with 2mM EDTA, 2mM EGTA, 1mM PMSF, 5mM DTT, 1μg/ml pepstatin, 1μg/ml leupeptin and 2μg/ml aprotinin). Cell lysates were separated from insoluble debris by spinning at 18,000 rpm for half an hour in JA-20 rotor. Supernatant was mixed with 600μl of 50% slurry of glutathione sepharose 4B beads (Amersham, equilibrated in PBS, with 0.05% NaN_3) and incubated overnight at 4°C for GST fusion protein binding. Bound GST proteins were washed once with buffer A (PBS plus 1M NaCl), followed by PBS washes before subject to Benzonase treatment (1000U/L culture, 4°C for 2 hrs) to remove tightly bound DNA and RNA. After beads were further washed alternatively by buffer A and PBS for 3 times, GST fusion proteins were eluted with 10mM glutathione (reduced form, in 50mM Tris-Cl, pH 7.9) or cleaved by 0.5U/ml thrombin (Roche, in 50mM Tris-Cl, pH 7.9, 150mM NaCl and 2.5mM $CaCl_2$).

For all chromatography purification, low salt buffer contains 50mM Hepes, 100mM NaCl, 4mM EGTA and high salt buffer contains the same composition but with 1M NaCl. All columns are equilibrated with low salt buffer. All the pre-loaded column, empty column and beads are from Amersham Pharmacia Biotech.

GST-synaptotagmin 1 C_2AB fusion protein was eluted from beads and further purified by passing through Mono S column (by Acta FPLC system) to separate contaminants. Fractions with pure GST-synaptotagmin 1 were combined and concentrated by filter (Pierce). After dialysis in standard buffer (50mM Hepes, 100mM NaCl, 4mM EGTA, pH 7.2), protein concentration was determined by BCA kit (Pierce) and frozen at -80°C in aliquots.

All SNARE motifs were cleaved from GST-beads. Proteins were incubated with glutathione sepharose 4B beads to remove GST and other non-specific bound proteins. After spinning down all insoluble materials, proteins were passed through Mono S or Mono Q column for further purification. SN1 and SN3 were applied to Mono Q column. They do not bind to the column and will appear in flow-through fraction. Synaptobrevin 2 (1~96) was first pass though Mono Q with part of the contaminants bound to beads and then to Mono S to separate from other proteins. Syntaxin was directly applied to Mono Q and eluted by high salt buffer. Fractions with target proteins were concentrated and dialyzed in the same standard buffer as above. Aliquot proteins were frozen at -80°C.

2.2.2 Mini SNARE complex formation and purification

The four SNARE motifs were mixed at the same molar ratio to form the complex. Since syntaxin SNARE motif and SN1 can form a stable complex, and the SNARE complex need to overcome energy barrier to break it, SNARE motifs were mixed in the following order as syntaxin, SN3, synaptobrevin 2 and SN1. After incubation at 4°C for 24 hrs, samples were passed through gel filtration column Superdex 75 (Amersham Pharmacia Biotech). Taking advantage that the mini SNARE complex is SDS resistant, the purity of the complex can be easily monitored by running SDS-PAGE gel without boiling the samples. SNARE complex fractions were collected and further applied to Mono S column. All fractions were combined with pure mini SNARE complex after SDS-PAGE gel confirmation. Protein concentration was measured after dialysis in standard buffer.

2.2.3 Affinity chromatography assay

All recombinant proteins for binding assay were thawed on ice. They were centrifuged at 14,000 rpm for 10 minutes to remove denatured insoluble proteins. About 20μl of 50% slurry of glutathione sepharose 4B beads were washed once with 1ml binding buffer of 50mM Hepes, 100mM NaCl, 4mM EGTA, 0.1% Triton X-100, pH 7.2. For standard experiments, ~ 30μg GST-synaptotagmin 1 was attached to beads by rocking at 4°C for 2.5 hrs with total volume of 200μl. Beads with proteins were washed two times with 1ml binding buffer (standard binding buffer, with or without additional 1mM free $CaCl_2$), and mini SNARE complex was incubated with synaptotagmin 1 for 2 hrs at 4°C. This was followed by washing 3 times in corresponding binding buffer. Bound proteins were eluted with 1x SDS

loading buffer and visualized by Coomassie Blue staining or immunoblotting after separation on SDS-PAGE gels.

2.2.4 Measure of binding affinity

To measure binding affinity between synaptotagmin 1 and mini SNARE complex, binding assays were performed with fixed amount of GST-synaptotagmin 1 and increasing concentration of SNARE complex. Bound SNARE complex was separated on SDS-PAGE gel and stained with Coomassie blue. Gels are dried, scanned and analyzed using ImageQuant 5.2 on a Storm instrument (Amersham). Free mini SNARE complex concentration was calculated by subtracting bound SNARE complex from total amount added to reactions. Data were analyzed by Prism software to get binding curve and Kd value. All experiments were repeated 3 times for statistical analysis.

2.2.5 Formation of mini SNARE complex on GST-synaptobrevin 2

We immobilized ~20μg of GST-synaptobrevin 2 on 10μl of glutathione beads and reconstituted the SNARE complex on the beads *in situ* by incubation with a two-fold excess of SNAP-25 and syntaxin SNARE motifs overnight at 4°C.

2.2.6 Generation of SNARE polyclonal antibodies

Syntaxin 1~264 was purified as mentioned above. 150μg of protein was injected into a rabbit every week for 4 times. Rabbit blood was collected every two weeks, and the first bleed was taken 1 week after the last injection.

2.2.7 Immunoprecipitation.

One unstripped rat brain (~1.5 g/brain; Pel-Freez Biologicals) was homogenized with a tissue homogenizer (Thomas Scientific) in 30ml of buffer containing 50mM Hepes-NaOH pH 6.8, 100mM NaCl, 4mM EGTA, protease inhibitor cocktail (Roche), 1mM PMSF, and 1mM DTT. 1% Triton X-100 was added, proteins were extracted for 1 hr at 4°C with rocking, insoluble proteins were removed by centrifugation (150,000xg for 1 hr), and the supernatant was used for experiments. Immunoprecipitation reactions were performed with polyclonal syntaxin 1 antibodies either U6250 or U6251 (15µl serum), polyclonal synaptobrevin 2 antibody P939 (15µl serum), attached to protein A-Sepharose beads (10µl; Pharmacia) in a 1ml volume of binding buffer (50mM Hepes-NaOH pH 6.8, 100mM NaCl, 4mM EGTA, 2mM $MgCl_2$, 0.5% Triton X-100 in the presence or absence of 1mM free Ca^{2+}) containing 0.4mg protein of 1% Triton X-100 soluble brain lysates. Binding reactions were incubated at 4°C for 2 hrs with rocking, beads were washed six times with 1ml of binding buffer, and bound proteins were eluted with SDS-PAGE sample buffer. After separation on SDS-PAGE, immunoprecipitated proteins were analyzed by immunoblotting using ^{125}I-labeled secondary antibody to quantify protein amounts.

2.2.8 Protein quantification

Protein bands were transferred to nitrocellulose membrane after separation on SDS-PAGE gel. Membrane was incubated with 10% milk in TBST buffer for 1 hr to block non-specific binding. Primary antibodies was diluted 5,000~10,000 times in 5% milk in TBST

buffer to blot the membrane. After washing 3 times by TBST, ^{125}I-labelled secondary antibodies were incubated with membrane for overnight (with 0.05% NaN_3). Before drying the membrane for exposure, membrane was washed 5~6 times to remove unbound secondary antibody. Screen was scanned by Storm and protein was quantified by Imagequant 5.2 software.

2.3 Results

2.3.1 Purification of GST-synaptotagmin 1, SNARE motifs and mini SNARE complex.

SNARE motifs are highly purified after chromatography purification, as shown in Fig 2.1A. Ion exchange chromatography can remove the major contaminant proteins (Data not shown). The efficiency of mini SNARE complex formation doesn't reach 100%, even after over 24 hr incubation. Unassembled SNARE monomer can be removed by gel filtration and ionic exchange chromatography, and the purity of mini SNARE complex is over 90% (Fig 2.1B). The dissociation of mini SNARE complex into 4 components after boiling also confirmed the identity of this complex (Fig 2.1C).

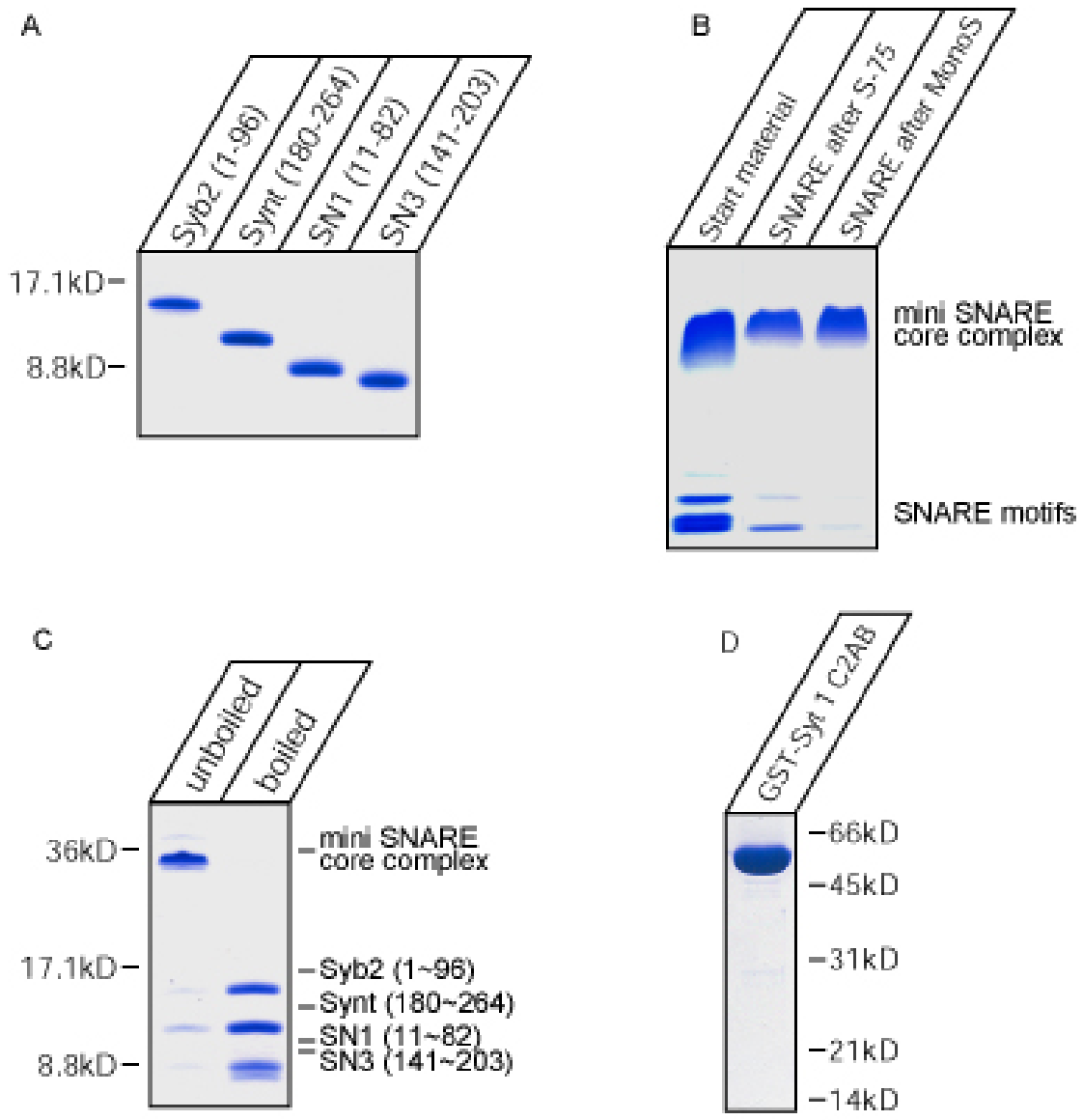


Figure 2.1 Purification of SNARE motifs, mini SNARE complex and synaptotagmin 1 C_2AB domain. A, purified SNARE motifs separated on Tricine gel; B, mini SNARE complex (SDS resistant) get further purified after gel filtration (Superdex-75) and ionic exchange chromatography (Mono S); C, mini SNARE complex can be disassembled to original 4 SNARE motifs after boiling; D, purified GST-synaptotagmin 1 C_2AB.

2.3.2 Titration of the binding between synaptotagmin 1 and mini SNARE complex

Both calcium dependent and independent interactions between synaptotagmin 1 and SNARE complex have been reported (Ernst et al., 2003; Bai et al., 2004). In our binding conditions, with 50mM Hepes, 100mM NaCl, 4mM EGTA, 0.1% TritonX-100, pH 7.2, synaptotagmin 1 C_2AB domain can bind avidly to mini SNARE complex (Fig 2.2A) and calcium has very little effect on the binding (Fig 2.2B), which suggests the possibility of synaptotagmin 1 binding to SNARE complex before the entry of calcium. As a positive control, we measured binding of SNARE complexes to immobilized GST-complexin 1 fusion protein because the interaction of complexin with SNARE complexes is well characterized (McMahon et al. 1995; Pabst et al., 2002). The binding result here also confirmed the strong interaction between complexin and SNARE complex (Fig 2.2C).

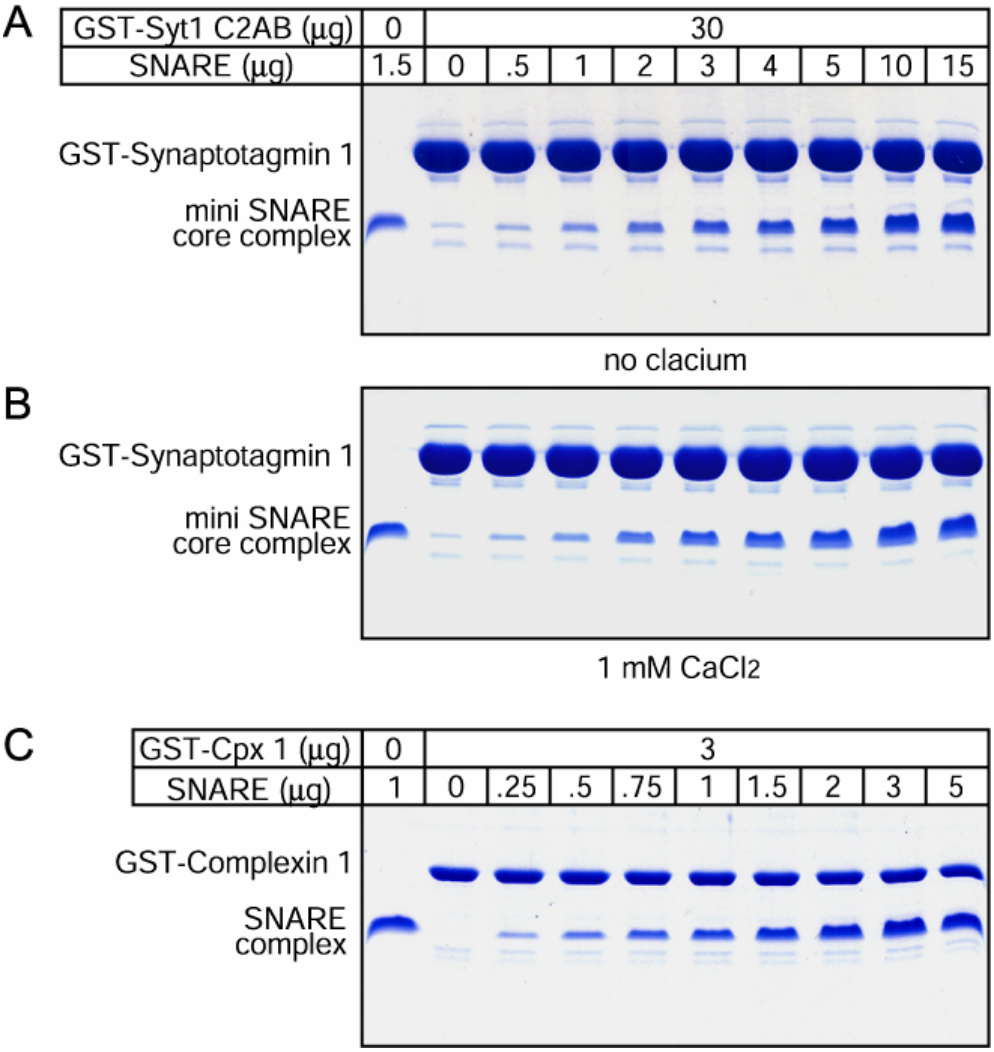


Figure 2.2 Binding of mini SNARE complex to immobilized synaptotagmin 1 and complexin. A-B. Increasing concentration of mini SNARE complex was incubated with 30 µg of immobilized synaptotagmin 1 C_2AB with (B) and without (A) 1mM of free calcium; C, Binding between GST-complexin 1 and increased amount of mini SNARE complex. All samples are not boiled to maintain mini SNARE complex.

2.3.3 Binding specificity of interactions between synaptotagmin 1 and mini SNARE complex

The specificity of binding was examined by performing the same pull down assay with all the combinations of SNARE motifs without forming and purifying any complex beforehand. As shown in Fig 2.3, interactions can only be detected between synaptotagmin 1 and those combinations that can form t-SNARE heterodimer or mini SNARE complex. By Coomassie staining, syntaxin N terminal (1~180) can not bind to synaptotagmin 1 even in the presence of mini SNARE complex consistent with previous results that N terminal is essential for Munc18 binding and syntaxin adopt the open conformation to form SNARE complex. For monomers of SNARE motifs, no binding was detected at all by Coomassie Blue staining (Fig 2.3 top panel).

Previous results showed calcium dependent synaptotagmin binding between either N terminal Habc domain or C terminal SNARE motif separately. Our binding results displayed calcium dependent synaptotagmin 1 binding to both N terminal (1~180) and C terminal SNARE motif (180~264) of syntaxin by immunobloting detection (Fig 2.3 bottom panel, left part). For the first time, we reconciled previous data by showing both binding, although very weak. Unable to be detected by western blot indicated neither SNARE motifs nor synaptotagmin C_2AB are sticky, thus the non-specific binding can be maximally avoided (Fig 2.3 bottom panel, right part).

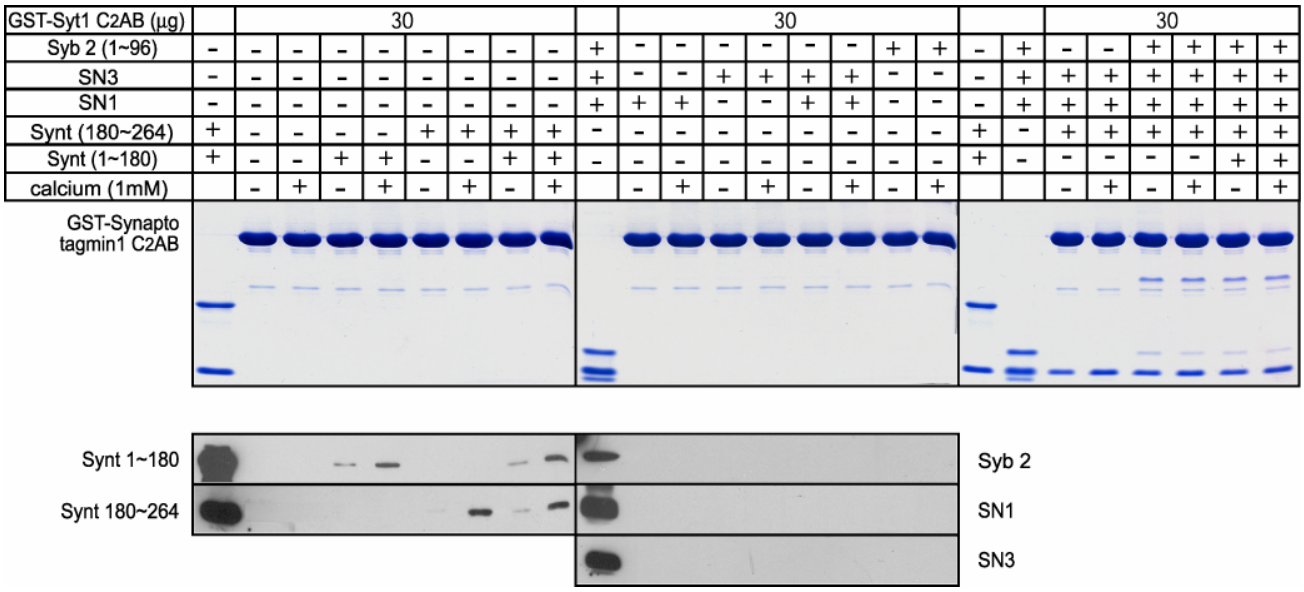


Figure 2.3 Binding of synaptotagmin 1 to different combinations of SNARE motifs. Combinations of SNARE motifs and syntaxin N terminal are incubated with synaptotagmin 1 as indicated. SNARE proteins are mixed together without pre-incubation. Experiments were performed with and without 1mM free calcium. Panels show representative Coomassie Blue stained gels (on the top), and western blot results (on the bottom).

2.3.4 Stoichiometry and Kd value of synaptotagmin 1 and SNARE core complex binding

To measure the binding affinity and stoichiometry between synaptotagmin 1 and mini SNARE complex, 30μg of GST-synaptotagmin 1 C_2AB was immobilized on 10μl of glutathione beads and incubated with increasing concentration of mini SNARE complex with or without 1mM of free calcium (Fig. 2.4 A-B). Synaptotagmin 1 binds to SNARE complex with a 2:1 molar ratio which is consistent with the concept that synaptotagmin 1 can form dimer *in vivo* by its trans-membrane region (Perin et al., 1991). Kd value indicates that the binding between synaptotagmin 1 and SNARE complex is strong, and at least in this binding

condition, is mainly calcium independent (Fig 2.4, table 1.1). Complexin binding to SNARE complexes was Ca^{2+}-independent (data not shown), and exhibited an affinity and stoichiometry similar to those previously described using solution methods (Pabst et al., 2002, table 1.1). Thus, in spite of the fact that our measurements were performed with a solid-phase assay, the estimated parameters approximate those observed by other approaches.

	no calcium		1mM calcium	
n=3	Kd value (nM)	Stoichiometry (GST protein/SNARE)	Kd value (nM)	Stoichiometry (GST protein/SNARE)
GST-Synaptotagmin 1 /mini SNARE complex	256 ± 18	2.3 ± 0.2	224 ± 7	2.0 ± 0.1
GST-Complexin 1 /mini SNARE complex	36 ± 6	1.0 ± 0.0	N/A	N/A

Table 1.1 Quantification of binding parameters. Experiments were repeated for 3 times independently, and data were shown as mean ± SEM. The stoichiometry represented the molar ratio of GST-fusion proteins to mini SNARE complex.

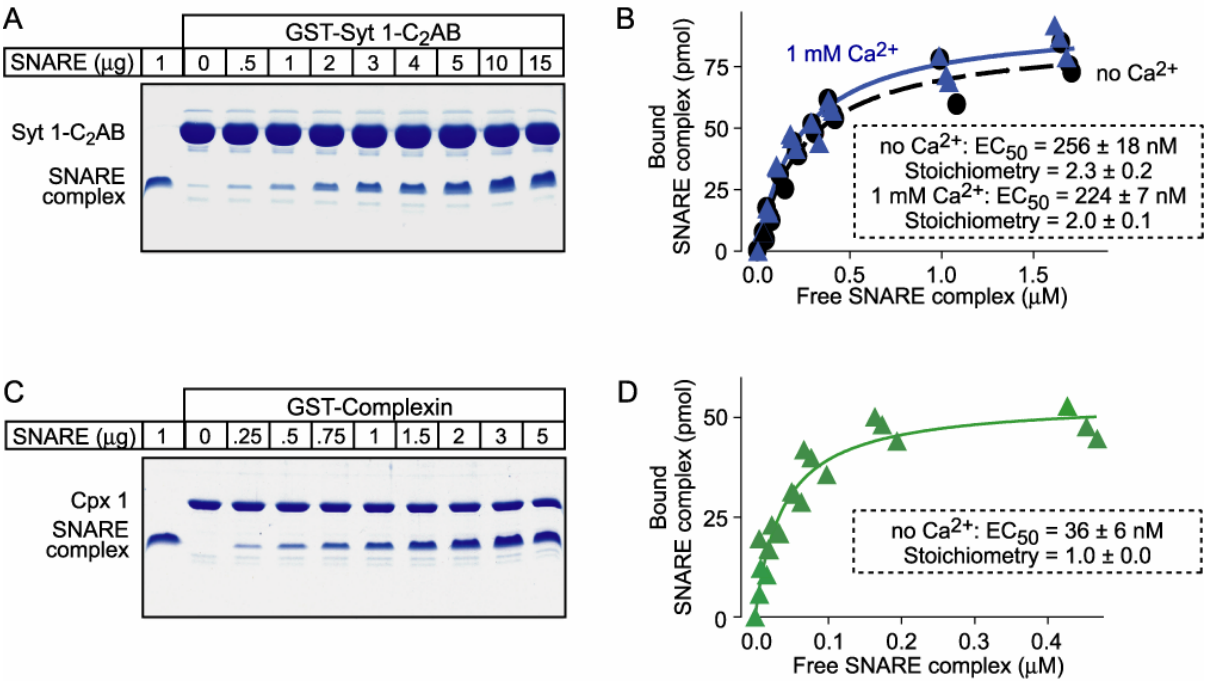


Figure 2.4 Titration of SNARE complex binding to immobilized synaptotagmin 1 C_2-domains and to immobilized complexin. A-B. Binding of increasing concentrations of purified assembled 'mini' SNARE complexes to 30µg immobilized synaptotagmin 1 C_2-domains without (dark circle) and with 1mM (blue triangle) of calcium. C-D. Binding of mini-SNARE complexes to immobilized GST-complexin. Panels on the left show representative Coomassie-stained gels, and on the right summary graphs of quantitations (means ± SEMs; n=3). Average stoichiometries and affinities calculated are shown in the boxes.

2.3.5 Comparison of separated SNARE motifs and full length SNARE proteins (or whole cytoplasmic region) for synaptotagmin 1 binding.

As already shown in Fig 2.4, neither N terminal Habc region nor SNARE motif of syntaxin has strong binding to synaptotagmin 1 by themselves. However, whole cytoplasmic region (1~264) binds avidly to synaptotagmin 1 in a calcium dependent manner (Fig 2.5A). Small truncation at C terminal (1~253) and LE mutant have no significant effects on the interaction. Full length SNAP-25 mainly displayed a high calcium independent affinity to synaptotagmin 1, which is similar to previous published reports (Fig 2.5B, Schiavo et al., 1997).

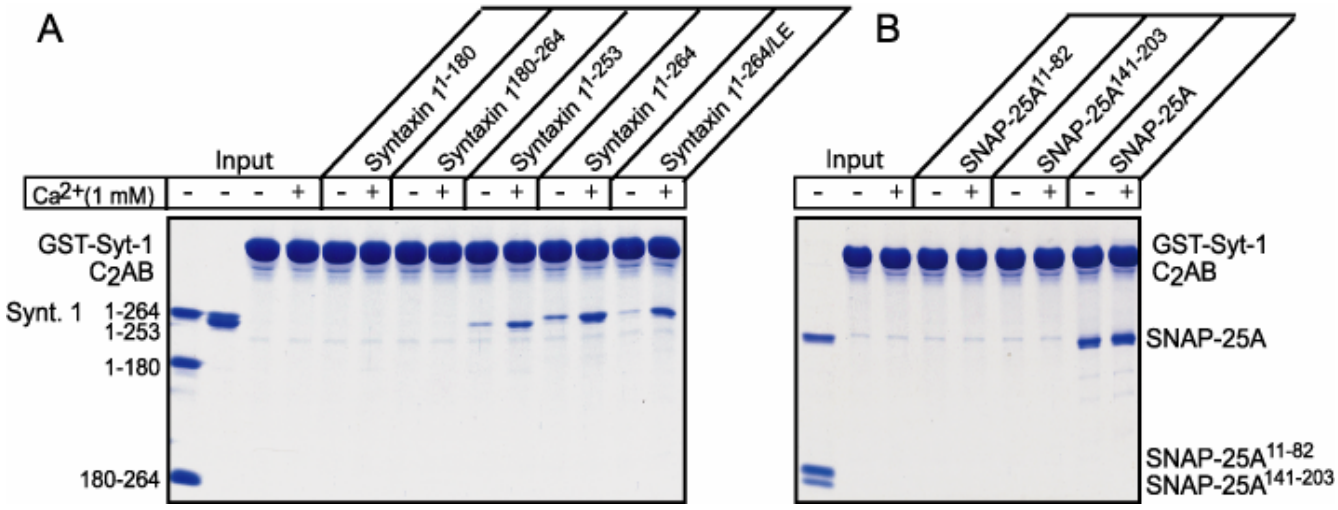


Figure 2.5 Comparison of binding of SNARE motifs and full length SNAREs to synaptotagmin 1. A, Fragments and mutant of syntaxin: 1-180, 180-264, 1-253, 1-264, 1-264 (LE) were mixed with synaptotagmin C_2AB; B, binding between different fragments of SNAP-25 (separate N and C terminal SNARE motifs and full length SNAP-25) and synaptotagmin 1. Calcium dependency was also examined here.

The binding between synaptotagmin 1 and full length SNAREs were further investigated by measuring the stoichiometry and binding affinity (Kd value). Synaptotagmin 1 at 30µg was incubated with increasing concentration of syntaxin and SNAP-25 until saturation. Experiments were carried out in the presence of 1mM of calcium since the interaction between syntaxin and synaptotagmin 1 is calcium dependent. Binding assay and subsequent analysis was the same as before. With no surprise, both syntaxin and SNAP-25 showed 1:1 binding ratio to synaptotagmin 1, with SNAP-25 exhibiting higher affinity (Fig 2.6, Table 1.2).

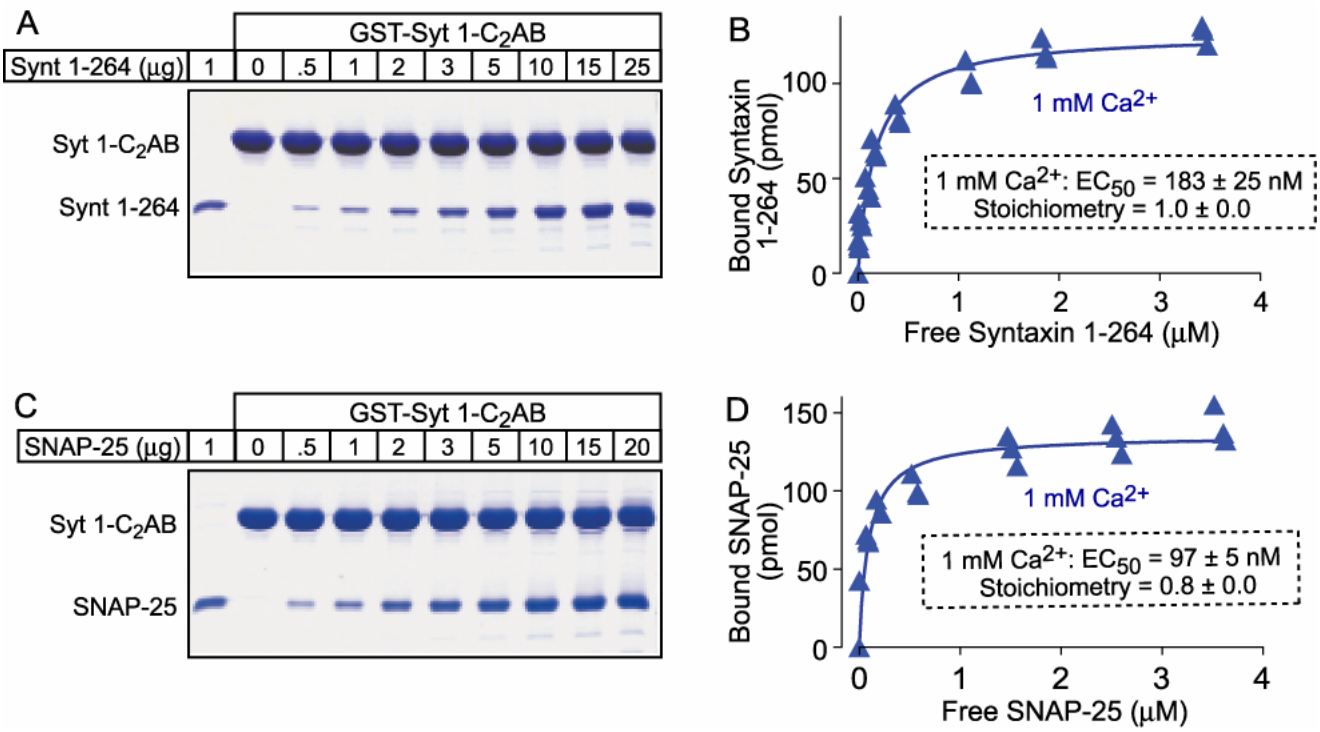


Figure 2.6 Titration of syntaxin 1-264 and SNAP-25 binding to immobilized synaptotagmin 1 C_2AB in the presence of 1mM of calcium. A-B, Binding of increasing concentrations of syntaxin 1-264 to immobilized synaptotagmin 1 C_2AB. C-D, Binding of SNAP-25 to

immobilized synaptotagmin 1. Panels on the left show representative Coomassie Blue-stained gels, and on the right summary graphs of quantitations (means ± SEMs; n=3). Average stoichiometries and affinities calculated are shown in the boxes.

	1mM calcium	
n=3	Kd value (nM)	Stoichiometry (GST protein/SNARE)
GST-Synaptotagmin 1 /Syntaxin 1-264	183 ± 25	1.0 ± 0.0
GST-Complexin 1 /SNAP-25	97 ± 5	0.8 ± 0.0

Table 1.2 Binding affinity (Kd) and stoichiometry for t-SNAREs. Experiments were repeated for 3 times independently, and data were shown as mean ± SEM. The stoichiometry represented the molar ratio of GST-fusion proteins to syntaxin 1-264 or SNAP-25 in the presence of 1mM of free calcium.

2.3.6 Interactions between synaptotagmin 1 and full length (or whole cytoplasmic region) SNARE proteins.

Results in Figure 2.5 clearly demonstrated that the full length (or whole cytoplasmic region) SNAREs behave quite differently from the individual SNARE motif. To clarify the interactions between synaptotagmin 1 and SNAREs more closely to its native status, we performed a binding assay with combinations of full length SNAREs. 30 and 7.5 μg of GST-synaptotagmin 1 C_2AB was immobilized for the assay.

The interactions between SNARE monomers and synaptotagmin 1 have already been discussed in previous experiments (Fig 2.3 and 2.5). Forming heterodimer has no significant effect on SNAP-25 binding, but greatly increased the calcium independent binding of syntaxin. Even syntaxin and SNAP-25 can form helix bundle, it is not as stable as SNARE complex and not SDS resistant. Synaptobrevin still has no binding when combined with either syntaxin or SNAP-25, indicating synaptobrevin does not have interactions with single t-SNAREs unless all of them are present to form SNARE complex. With all the SNARE proteins together, they can form large SDS resistant SNARE complex (the bands on top of synaptotagmin 1 in both panels) and bind to synaptotagmin 1. Calcium has little effect similar to that of mini SNARE complex. Since all the samples were not boiled before separation on SDS-PAGE gel, the existence of synaptobrevin bands indicated the binding between synaptotagmin 1 and loosely assembled SNARE complex which is not SDS resistant.

Another interesting finding is that with less synaptotagmin 1, SNAP-25 has more calcium dependence for the interaction. This effect can also be expanded to all reactions with SNAP-25 involved in (compare Fig 2.7A and B).

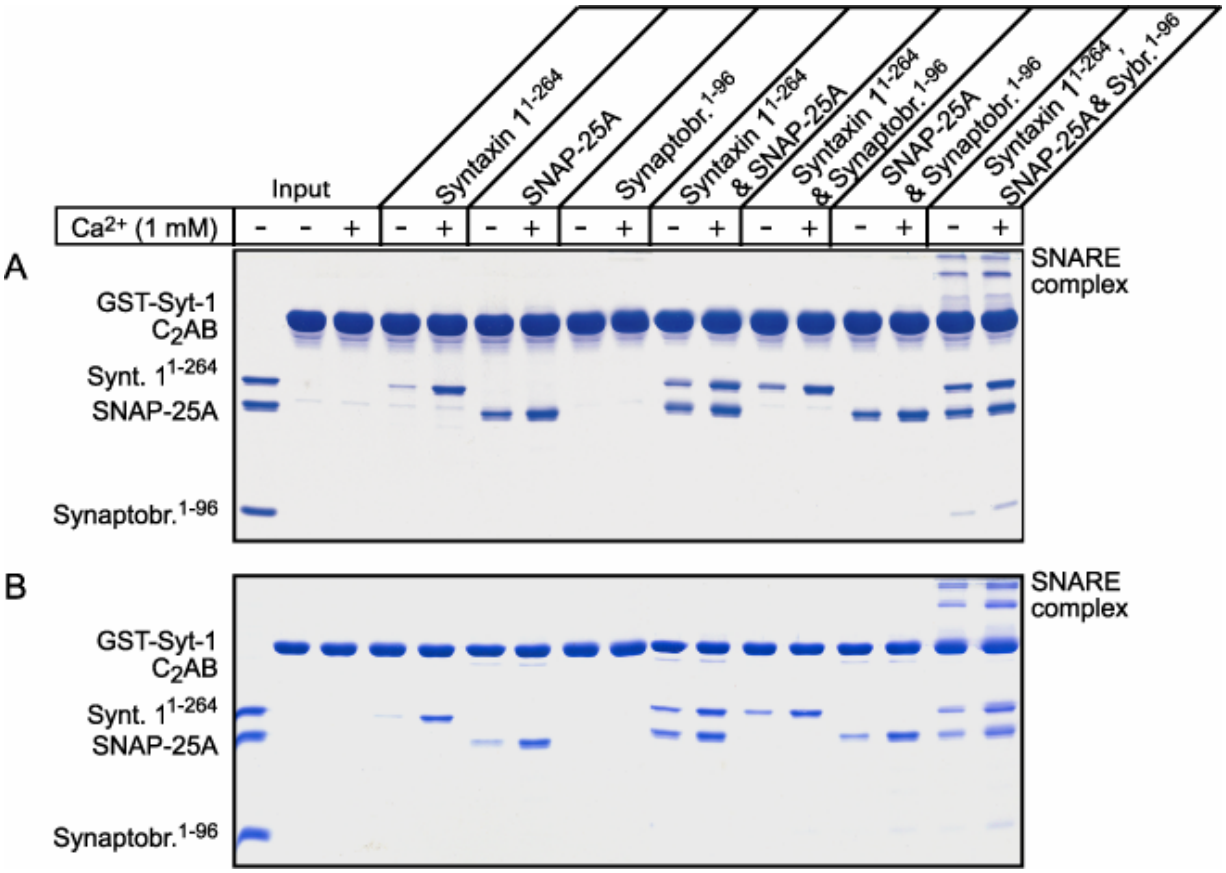


Figure 2.7 Interactions between synaptotagmin 1 C_2AB domain and combinations of full length SNARE proteins. SNAREs are incubated with different amount of GST-synaptotagmin 1: (A), 30μg and (B), 7.5μg with the indicated combinations. Note SNARE complex itself can oligomerize, revealed by the higher molecular weight bands on top of synaptotagmin 1 C_2AB.

2.3.7 Effect of synaptotagmin 1 density on interactions with SNAREs

Almost all results we displayed indicated that the interaction between synaptotagmin 1 and SNARE complex are mainly calcium independent. However, calcium dependent

binding was clearly described by many groups. These results in Fig 2.7 revealed a new point to address.

Very little has been described as to how many GST-synaptotagmin 1 should be used for pull down assay. When immobilized on beads, the density of proteins and charge distribution are unknown. It is possible that GST-synaptotagmin 1 could form oligomeric complex due to high local concentration on beads which can increase the calcium independent binding by forming larger contact surface and charge density. To test this hypothesis, I titrated the amount of GST-synaptotagmin 1 C_2AB by attaching 7.5, 15, 22.5, 30, 45μg of synaptotagmin 1 to 10μl of beads, and mixed them with mini SNARE complex, syntaxin 1-264 and SNAP-25 at saturating concentrations with and without calcium.

Syntaxin displayed a consistent calcium dependent binding (Fig 2.8.B) in spite of the increase of synaptotagmin 1 density. With less protein on beads, SNAP-25 and mini SNARE complex had an obvious calcium dependent binding pattern. With higher synaptotagmin 1 density, they showed more calcium independent binding. The strong calcium independent binding here is more than the corresponding increase of signals due to more synaptotagmin 1 on beads. Although this is not a convincing demonstration that local concentration of synaptotagmin 1 can affect its binding affinity to SNAREs, this experiment suggested another possibility to explain the discrepancy for the calcium dependency arguments.

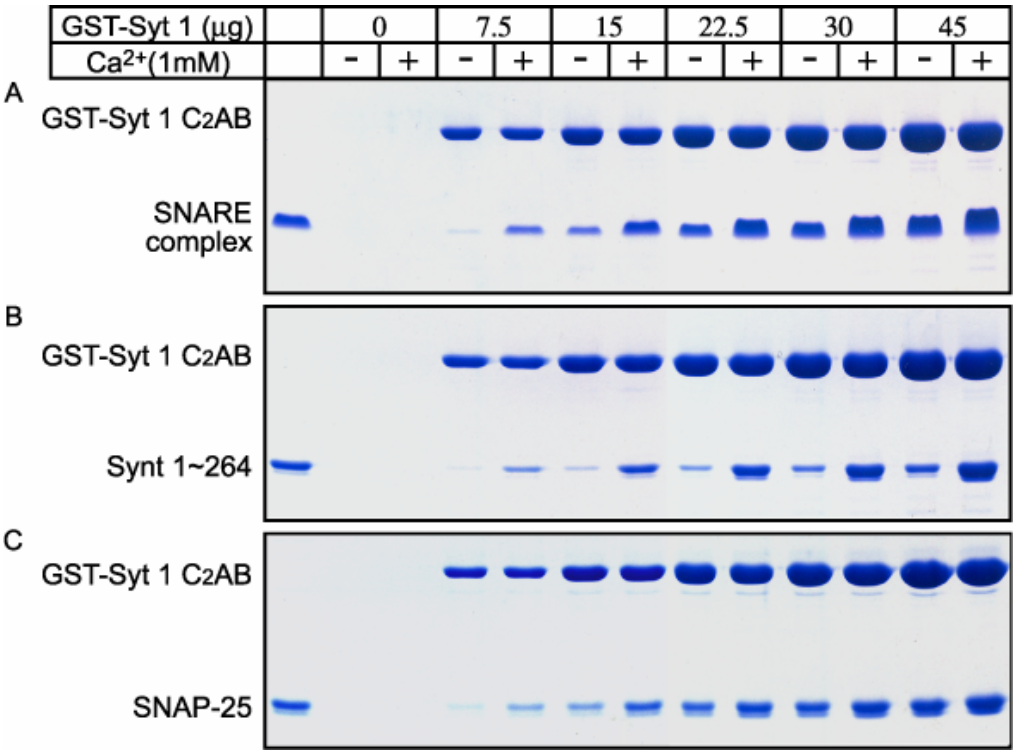


Figure 2.8 Effect of synaptotagmin 1 density on interactions with SNAREs. Different amount of synaptotagmin 1 C_2AB (7.5, 15, 22.5, 30, 45μg) was immobilized on 10μl of beads, and then incubated with saturating concentration of SNARE proteins for binding: (A), mini SNARE complex; (B), Syntaxin 1-264 and (C), SNAP-25. All experiments were performed with and without 1mM of free calcium.

2.3.8 Binding of purified synaptotagmin 1 to SNARE complexes at different salt concentration.

Up to now, we have examined the effects of several parameters of proteins on binding. Besides proteins themselves, binding conditions could greatly affect the final results. Among all the variables, we focused on how ionic strength could change the calcium dependency of the interactions. To achieve this goal, we tested our hypothesis with both

recombinant and native proteins. With recombinant proteins, we immobilized both synaptotagmin 1 and SNARE complex on beads to pull down corresponding partners. For native proteins, we used antibodies for synaptobrevin 2 and syntaxin. All these actions were aimed to reduce bias.

To examine Ca^{2+}-dependent and independent binding of synaptotagmin 1 to SNARE complexes, we immobilized GST-synaptobrevin 2 on glutathione beads, reconstituted the SNARE complex on beads *in situ* with recombinant SNARE motifs from SNAP-25 and syntaxin 1, and measured the Ca^{2+}-dependent binding of recombinant C_2AB domain from synaptotagmin 1 (residues 140-421) in buffers containing 50-200mM NaCl (Fig 2.9A-B). As a negative control, we used beads containing only GST-synaptobrevin 2 alone and detected no binding of synaptotagmin 1 (data not shown). We found that synaptotagmin 1 binding to SNARE complexes was activated at low, and was abolished at high ionic strengths. Binding was strictly Ca^{2+}-dependent at intermediate physiological ionic strengths, but became at least partially Ca^{2+}-independent at low ionic strengths (Fig 2.9A-B).

The same experiment performed in the reverse orientation with immobilized GST-synaptotagmin 1 yielded similar results, except that more Ca^{2+}-independent binding was observed (Fig 2.9C-D). Similar to what have been revealed in Fig 2.8, decrease in protein concentration of both synaptotagmin 1 and SNARE complex shifted the binding curve to the left. Now, more calcium dependent binding was observed at intermediate ionic strength.

Viewed together, these data showed that synaptotagmin 1 binds to assembled SNARE complexes in a precarious balance dictated by the ionic strength and Ca^{2+} concentration, and under physiological conditions, Ca^{2+}-dependent binding is favored.

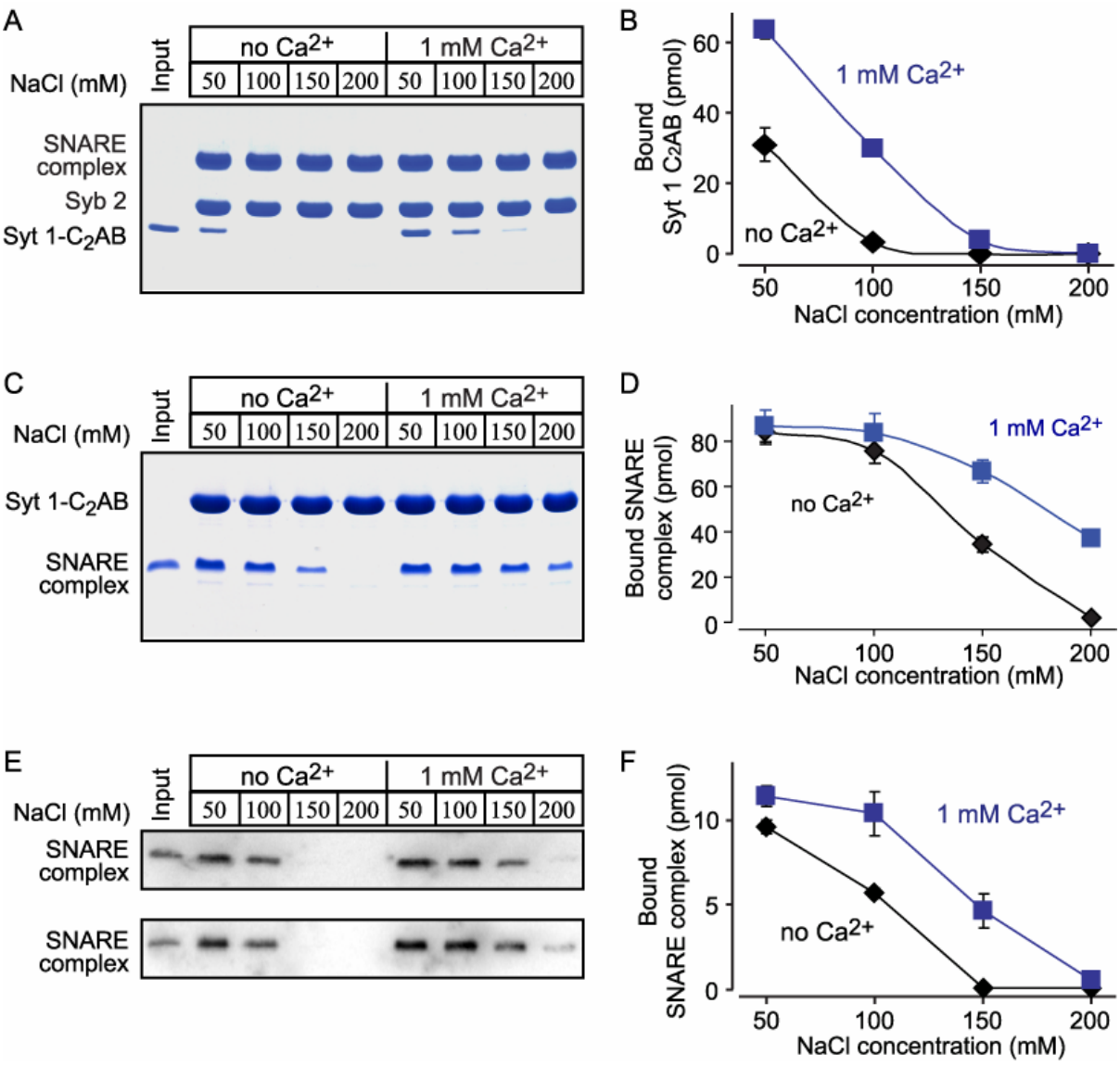


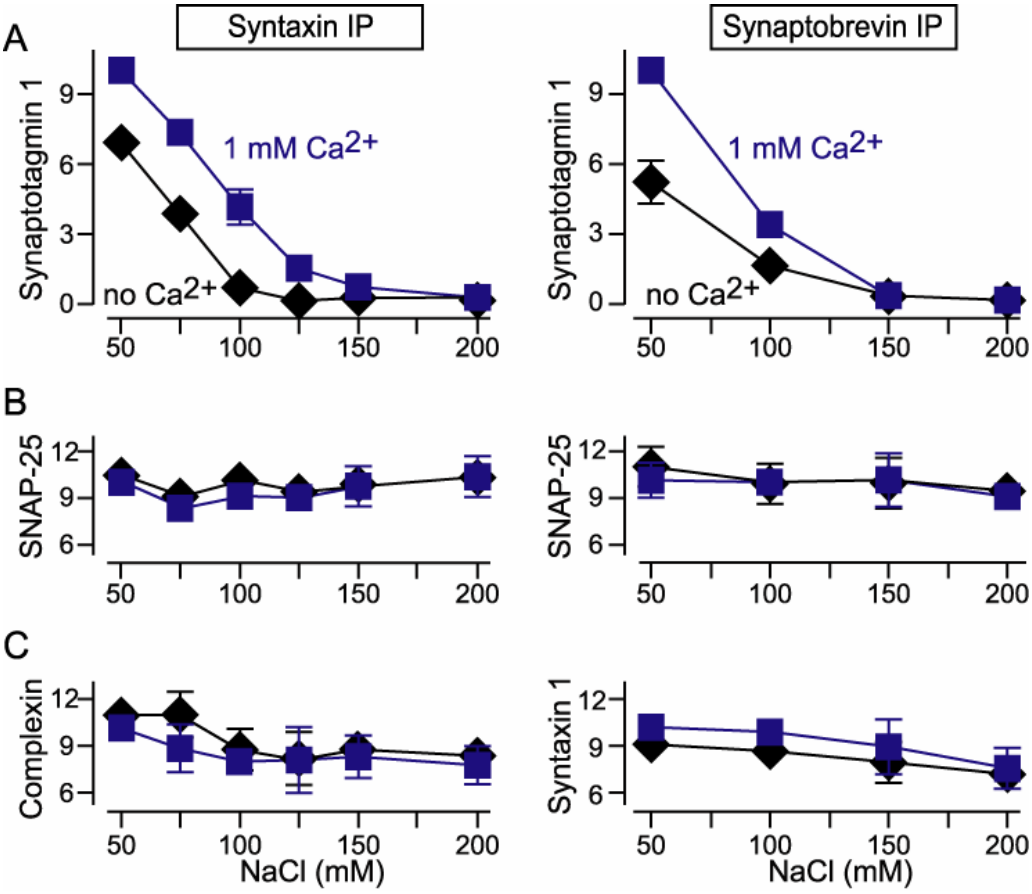
Figure 2.9 Binding of recombinant synaptotagmin 1 to mini SNARE complexes at different salt concentration. A-B. Effect of ionic strength on the binding of the double C_2-domain

fragment from synaptotagmin 1 to immobilized SNARE complexes by excess GST-synaptobrevin 2. Binding was carried out with and without 1 mM free Ca^{2+}. C-D. Effect of ionic strength on the binding of 690nM mini SNARE complex to synaptotagmin 1 C_2-domains (30μg) immobilized as a GST-fusion protein. Bound proteins were measured by scanning of Commassie-stained SDS-gels. E-F. Same as in C-D, except that 7.5μg of GST-synaptotagmin 1 was attached to beads and incubated with 69nM of SNARE complex. Bound proteins were quantified by immunoblotting using ^{125}I-labeled secondary antibodies. Data shown are means ± SEMs; n=3.

2.3.9 Characterization of the Ca^{2+}-dependent binding of native synaptotagmin 1 to brain SNARE complexes.

To study synaptotagmin 1 binding to SNARE complexes with native proteins, we immunoprecipitated brain proteins that were solubilized in Triton X-100 (Fig 2.9). We immunoprecipitated SNARE complexes with syntaxin 1 or synaptobrevin 2 antibodies, measured the amounts of co-immunoprecipitated proteins by quantitative immunoblotting with ^{125}I-labeled secondary antibodies at different ionic strengths in the presence and absence of Ca^{2+}, and normalized the protein amounts for the immunoprecipitated protein to correct for differences in yield. We found that similar to the binding of recombinant proteins, binding of native synaptotagmin 1 to SNARE complexes was largely independent of Ca^{2+} at low ionic strength, predominantly Ca^{2+}-dependent at intermediate physiological ionic strength, and inhibited at high ionic strength (Fig 2.10A).

Control experiments showed there was no significant change in SNARE proteins amount in the immunoprecipitation complexes performed using either U6251 (syntaxin) or P939 (synaptobrevin 2) antibodies (Fig 2.10B). Quantitations revealed that syntaxin 1 and synaptobrevin antibodies immunoprecipitated equivalent amounts of the total syntaxin 1, SNAP-25, synaptobrevin 2, and complexins from brain homogenates.



by Ok-Ho Shin

Figure 2.10 Immunoprecipitation analysis of effect of ionic strength and Ca^{2+} on synaptotagmin 1 binding to native brain SNARE complexes. SNARE complexes were immunoprecipitated from rat brain lysate at the indicated NaCl concentrations by antibodies

to syntaxin 1 or synaptobrevin 2 with and without 1 mM free Ca^{2+}. A. synaptotagmin 1 bound to SNAREs was quantified by ^{125}I-labeled secondary antibody. B-C. SNAP-25 and complexin were measured as internal control.

2.4 Discussions

In summary, the interactions between synaptotagmin 1 and SNAREs depend on several parameters, including binding regions within proteins, local concentration of proteins, buffer ionic strength, metal ion (like Ca^{2+} or Mg^{2+}) and other undiscovered factors. Based on all the results we have, several conclusions can be drawn:

First, even under the most favorable binding condition (high density of GST-synaptotagmin 1 on beads with relative low salt concentration and the presence of calcium), there was no strong interaction between individual SNARE motifs and synaptotagmin 1 C_2AB domains. Either with the flanking region to form a complete SNARE protein (full length syntaxin and SNAP-25) or with other SNARE motifs (syntaxin and SNAP-25 SNARE motifs together), can have stoichiometry binding with synaptotagmin 1. This conclusion suggests that synaptotagmin 1 can have direct contact with syntaxin, SNNAP-25 and syntaxin-SNAP-25 complex on plasma membrane in the presence of calcium, which could be one pathway to position vesicles to the active zone.

Second, Both syntaxin N terminal Habc domain and C terminal SNARE motif exhibited weak calcium dependent binding with synaptotagmin 1, which reconciles previous

conflicting data and suggests that the co-binding of N and C terminal of syntaxin is critical for a strong interaction with synaptotagmin 1.

Third, We showed that synaptotagmin 1 binding to SNARE complexes is controlled by a precarious balance of protein concentration, ionic strength and Ca^{2+}, providing an explanation for contradictions between previous studies about Ca^{2+}-dependence of synaptotagmin 1 binding to SNARE complexes. High local concentration of synaptotagmin 1 on synaptic vesicles could render at least some Ca^{2+}-independent binding (Fig 2.2, 2.4), especially at low ionic strength. At physiological ionic strength, synaptotagmin 1 binds to SNARE complexes in a Ca^{2+}-regulated manner (Fig 2.9-2.10). At high ionic strength, no synaptotagmin 1 binding to SNARE complexes is observed under any condition (Figs. 2.9-2.10). The strong Ca^{2+}-dependence of synaptotagmin 1 binding to SNARE complexes implies that there is little binding between them before the entry of calcium.

However, synaptotagmin 1 and SNARE proteins are membrane or membrane-associated proteins. They could behave differently in a membrane-embedded status. In addition, the restriction on membrane by the transmembrane region could limit the accessibility of the region close to membrane. In solution, protein or protein complex are in free orientation, and they have the chance to contact with each other from any angles which could led to the observation of some interactions that can never happen *in vivo*.

Chapter III: Competition between complexin and synaptotagmin 1 for SNARE complex binding

3.1 Introductions

Both positive and negative effects of complexin on vesicle release have been described, leaving the function and mechanism of complexin still elusive. Complexin binds tightly with SNARE complex at 1:1 ratio. It competed with α-SNAP for SNARE complex, but not with synaptotagmin 1. The electrophysiological results of complexin double knock out mice exhibits a selective decrease of calcium dependent fast synchronous release. However, the asynchronous release and readily releasable pool size are not affected at all, a phenotype very similar to that of synaptotagmin 1 knockout mice, which suggests these two proteins may function in a same pathway. Since binding of complexin to SNARE complex is completely calcium independent, the impairment of calcium dependent release of vesicles led us to link the function of complexin to some calcium dependent effect.

With these two concerns in mind, we want to reinvestigate the relationship between complexin and synaptotagmin 1. Previous results have already showed that complexin and synaptotagmin 1 can be co-immunoprecipitated with each other (McMahon et al., 1995). However, the previous co-immunoprecipitations were analyzed by sensitive but non-quantitative immunoblotting methods which would make trace amounts of bound protein (which could, for example, be present because of SNARE-complex oligomerization) appear to represent significant binding.

The availability of purified mini SNARE complex and its strong binding with GST-synaptotagmin 1 makes it easy to test the relationship. If complexin competes with synaptotagmin 1 for SNARE complex binding, it will form complexin-SNARE complex and block further binding to synaptotagmin 1. We can predict the appearance of additional complexin band on gel in case of co-binding, with SNARE complex still binding to synaptotagmin1.

By adding exogenous complexin to co-immunoprecipitation assay with SNARE antibody from brain lysate, we could examine the competition with native synaptotagmin 1 and SNARE complex. In collaboration with Dr. Rizo's lab, we were able to test the competition in more physiological conditions with SNARE complex embedded in membrane.

3.2 Method and Materials

3.2.1 Making constructs and protein purification

Rat complexin 2~134 was inserted into pGex-KG vector at XbaI/Hind III sites by previous postdoctoral fellow in our lab. I did mutagenesis on this construct and made several mutants: R48A, R59A, R48/R59A, R48/R59/K69/Y70A. GST-complexin truncations 41~134 was also prepared on pGex-KT vector at BamH I site.

All forms of GST-complexins were expressed and purified by the same protocol described in 2.2.1. After eluted from beads, fusion proteins were passed through gel filtration column (Superdex-75) for further purification. For those need to be cleaved, they were passed through Mono Q column after pre-clear of remaining GST. Pure complexins was left in flow through fractions with contaminants bound to column. All proteins were concentrated and dialyzed in standard buffer after examination of purity. Concentration was determined by BCA kit before aliquot and subject to -80°C for storage.

3.2.2 Competition between recombinant synaptotagmin 1 and complexin for mini SNARE core complex binding.

All the procedures are same as in 2.2.3 except that complexins were added to the binding system in addition to mini SNARE complex at the same time with indicated concentration. Binding assay was performed with 50mM Hepes, 100mM NaCl, 4mM EGTA, 0.1% Triton X-100, pH 7.2 with total volume of 200µl. After quantification, SNARE complex signal was normalized to that of control (without addition of complexin) for plot.

3.2.3 Co-Immunoprecipitation competition.

Generally, the immunoprecipitation reactions were performed the same as in 2.2.7. Here, in addition to polyclonal syntaxin-1 antibodies, either U6250 or U6251 (15µl serum) and polyclonal synaptobrevin 2 antibody P939 (15µl serum), and monoclonal synaptobrevin 2 antibody Cl69.1 (10µl ascite) was attached to protein A- or G-Sepharose beads (10µl; Pharmacia). Binding reactions were incubated at 4°C for 2 hr with rocking with the addition

of various recombinant complexin proteins. Beads were then washed six times with 1ml of binding buffer, and bound proteins were eluted with SDS-PAGE sample buffer. After separation on SDS-PAGE, immunoprecipitated proteins were analyzed by immunoblotting using ^{125}I-labeled secondary antibody to quantify protein amounts. The changes of complexins, α-SNAP, and synaptotagmin 1 in the immunoprecipitates were normalized to that of control (100% = amount pulled down without exogenous recombinant complexin 1).

3.2.4 Preparation of planar supported proteolipid bilayer and microfluidic channels.

Purified full length SNAP-25, a fragment of syntaxin 1A containing its SNARE motif and transmembrane region (residues 183-288), and the synaptobrevin cytoplasmic region (residues 1-96) were prepared as previously described (Chen et al., 2005; Chen et al., 2006). These proteins were used to assemble SNARE complexes in the presence of 1% β-octylglucoside. Liposomes containing a lipid composition that resembles that of synaptic vesicles (41% POPC, 32% DPPE, 12% DOPS, 5% PI and 10% Cholesterol, by weight) were prepared by extrusion, and SNARE complexes were reconstituted into these preformed liposomes as described previously (Chen et al., 2006) with a 1:1000 protein:lipid ratio. Microfluidic channels of 200μm width, 200μm height and 2.5cm length were formed using standard soft lithography techniques (Xia and Whitesides, 1998). Briefly, PDMS stamps were formed by curing Sylgard 184 at 65 °C for 5 hrs on silicon masters with the desired pattern. Cured PDMS stamps were removed from the masters, and inlets/outlets were punched with an 18 gauge blunt needle. Coverslips were plasma-oxidized with a glow discharge unit for 3 min under vacuum. PDMS stamps were then firmly pressed down

against the coverslips to form a reversible, leak-tight seal. Lanes of supported bilayers within the microfluidic channels were formed using the vesicle fusion method (Brian and McConnell, 1984).

3.2.5 Analysis of complexin binding by confocal fluorescence microscopy.

A complexin I fragment (residues 26-83) containing a V61C substitution was labeled with BODIPY-FL. BODIPY-FL fluorophores were imaged on a Leica (Wetzlar, Germany) confocal fluorescence microscope (TCS SP2) using a HC PL APO CS 10x, 0.4 numerical aperture confocal scanning objective, and a 488 nm argon laser excitation with emission light collected between 500 and 560 nm. In a typical experiment, 50nM BODIPY-FL labeled complexin was incubated with a deposited supported bilayer for 15 min, and unbound complexin was washed out with buffer (25mM HEPES, 100mM KCl, 0.1mM EGTA, 0.3mM TCEP). An unlabeled synaptotagmin 1 fragment containing its two C_2-domains (residues 140-421) was then added in the presence of either 1 mM EDTA or 1mM Ca^{2+} and incubated with the bilayer for 10 min, followed by a wash with buffer containing either 1mM EDTA or 1mM Ca^{2+}, respectively. Control experiments where complexin was added to supported bilayers lacking reconstituted SNAREs were used to measure background fluorescent that might result from non-specific binding of complexins to the bilayers. The amount of complexin bound to SNARE complexes on the bilayer was quantified with Image J (NIH, MD). Briefly, fluorescence intensity in separate squares of 100 μm x 100 μm within the same image was integrated and the resulting values were averaged. The competition titration data were analyzed with Origin 6.0 and fitted to a Dose-response model.

3.3 Results

3.3.1 Recombinant synaptotagmin 1 and complexin compete for SNARE complex binding.

Since synaptotagmin 1 and complexin both bind to SNAREs, we asked whether they compete with each other for binding. We tested full length wild type complexin 1, which can tightly bind to SNARE complexes (Fig 2.2C).

Titrations revealed that SNARE complexes were quantitatively displaced from synaptotagmin 1 by full-length complexin at submicromolar concentrations. The addition of more complexin (until several folds at molar ratio) to the binding system led to the formation of more complexin-SNARE complex and less free mini SNARE complex, which resulted in the decreased pull down of SNARE complex by synaptotagmin 1 (Fig 3.1A). Excess amount of complexin didn't completely block the binding of synaptotagmin 1, probably due to an equilibrium reached between synaptotagmin 1 and complexin. The formation of SNARE complex oligomer can also account for the co-existence of complexin and synaptotagmin 1 by binding to different SNARE complex subunit within the same oligomer.

Complexin selectively binds to SNARE complex with very little interactions with syntaxin. As expected, it didn't interfere with the bindings between synaptotagmin 1 and t-SNAREs, either monomers or heterodimer (Fig 3.1B). The competition (Fig 3.1A) was observed independent of whether Ca^{2+} was present or absent, presumably because we carried

out these experiments under saturating conditions that render SNARE binding to synaptotagmin 1 Ca^{2+}-independent.

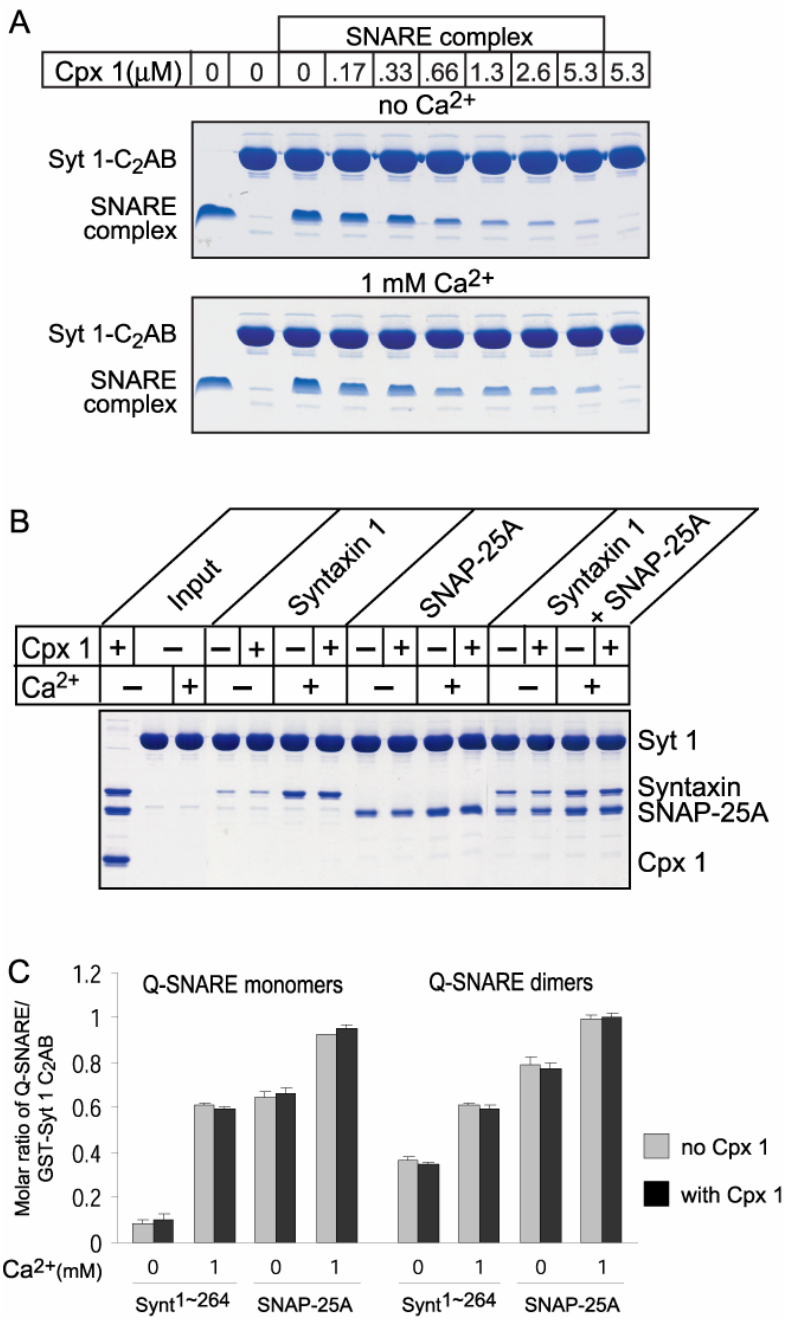


Figure 3.1 Complexin inhibit synaptotagmin 1 for SNARE complex binding, but not for SNARE monomer and heterodimer. A. 5μg (0.7μM) of mini SNARE complex, as well as

increased concentration of complexin was mixed with immobilized synaptotagmin 1 C_2AB for binding in the presence of 0 or 1mM of free calcium. B. Single t-SNAREs and t-SNARE heterodimer binding to synaptotagmin 1 with excess amount of complexin. C. Quantification of the competition results of B. Grey bar is without complexin and black bar means with additional complexin.

3.3.2 Mutant complexin without SNARE complex binding ability is unable to inhibit synaptotagmin 1 for SNARE complex binding.

To confirm the specificity of the competition, we made a series of mutations focused on the central region of complexin, which mediated the binding to SNARE complex. The N terminal part of complexin in not involved in SNARE complexin binding. As expected, the deletion of the first 40 residues doesn't change the 1:1 ratio binding (Fig 3.2A). R48, R59 residues directly form salt bridge with synaptobrevin. Single mutation of either one doesn't have visible effect on SNARE complex binding. However, double mutant (R48/59A) greatly decreases the SNARE complex binding affinity of complexin (Fig 3.2A). Since complexin binds to the groove of SNARE complex between synaptobrevin and syntaxin, mutation of residues on complexin for syntaxin binding could further decrease the binding. This hypothesis was supported by the addition of mutations K69/Y70A (residues responsible for syntaxin contact and are conserved among different species) to the original double mutant. The final Cpx^{4M} (R48/R59/K69/Y70A) has no interaction with SNARE complex at all by Coomassie staining (Fig 3.2A).

The following competition experiments with all these recombinant mutant complexins confirmed that the inhibitory effect of complexin on synaptotagmin 1 for SNARE complex binding completely depends on its binding to SNARE complex. The decreased affinity of complexin with SNARE complex correspondingly resulted in lower inhibitory effect on synaptotagmin 1 binding (Fig 3.2B). Interestingly, the exception is that N-terminally truncated complexin displaced only 50% of the SNARE complexes with unknown reason (Fig 3.2B-C).

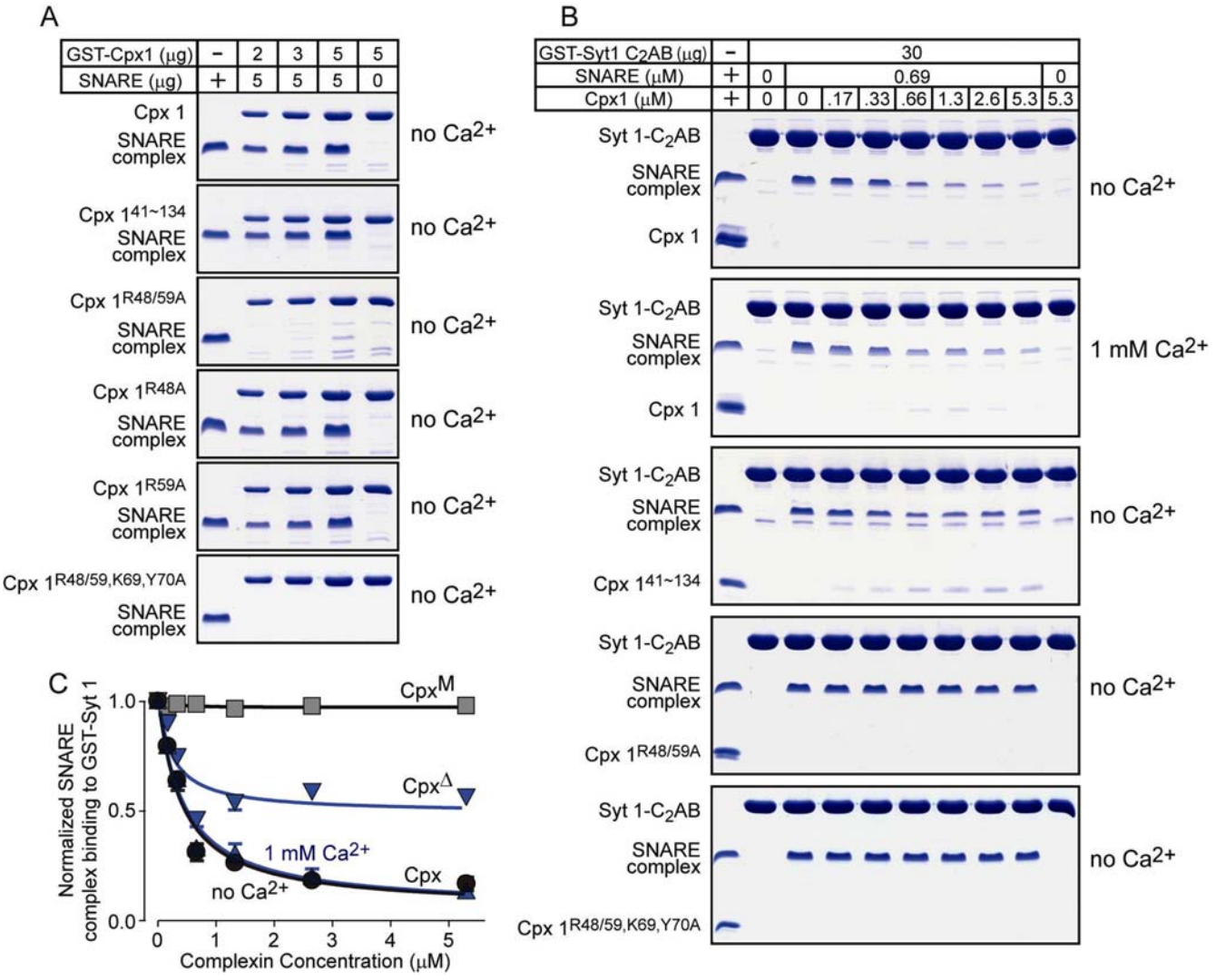


Figure 3.2 Mutations in the SNARE-binding sequence of complexin 1 abolish its binding to SNARE complexes and its ability to compete with synaptotagmin 1 for SNARE complex binding. A. Comparison of the binding of assembled mini SNARE complex to full length wildtype complexin 1 (Cpx 1), N-terminally truncated complexin 1 (Cpx $1^{41\text{-}134}$), and various complexin point mutants (Cpx $1^{R48A/59A}$, Cpx 1^{R48A}, Cpx 1^{R59A}, and Cpx $1^{R48/R59/K69/Y70A}$). B. Effect of increasing concentrations of purified wildtype or mutant complexins (same as used in panel A, but without the GST moiety) on the binding of SNARE complexes to immobilized synaptotagmin 1 C_2-domains. C. Quantification results and inhibition curve for full length (dark circle for no calcium, blue triangle for 1mM calcium), truncated (inverted blue triangle) and mutant (grey square) competition. Cpx^{Δ}=(Cpx $1^{41\text{-}134}$), Cpx^{M}=(Cpx $1^{R48/R59/K69/Y70A}$). Bound mini SNARE complex was normalized to that of control (without exogenous recombinant complexin).

3.3.3 Complexin displaces synaptotagmin 1 from native brain SNARE complexes.

To determine whether complexin and synaptotagmin 1 also compete with each other for binding to native SNARE complexes in solution, we performed the SNARE immunoprecipitations in the presence of increasing concentrations of recombinant complexins (Fig 3.3A). Without additions, the immunoprecipitates contained endogenous complexins. After addition of excess exogenous complexins, only exogenous complexins were present in the immunoprecipitated SNARE complex (note that the N-terminally truncated complexin 1 still binds effectively to SNARE complexes, indicated by the asterisk). Rab3 and synaptophysin, analyzed as control proteins, were not co-immunoprecipitated.

We quantitated the displacement of synaptotagmin 1 from native SNARE complexes by complexin, and also examined α-SNAP binding because α-SNAP is known to compete with complexin for binding to SNAREs (McMahon et al., 1995). Complexin binding was determined as a control to ensure that the exogenous complexin is incorporated into the precipitated complex. Nanomolar complexin concentrations displaced >80% of synaptotagmin 1 from the immunoprecipitated SNARE complexes. In contrast, complexin was less effective in displacing α-SNAP from SNARE complexes (Fig 3.3B), consistent with the idea that α-SNAP has a higher affinity for SNARE complex than complexin and synaptotagmin 1. In these experiments, the complexin concentrations required for displacement of synaptotagmin 1 from SNARE complexes were lower than in the recombinant protein experiments (Fig 3.1A), possibly because the synaptotagmin 1 concentration is lower in brain homogenates, or because complexin binding to SNARE complexes is facilitated when SNARE proteins contain transmembrane regions (Hu et al., 2002; however, see Bowen et al., 2005).

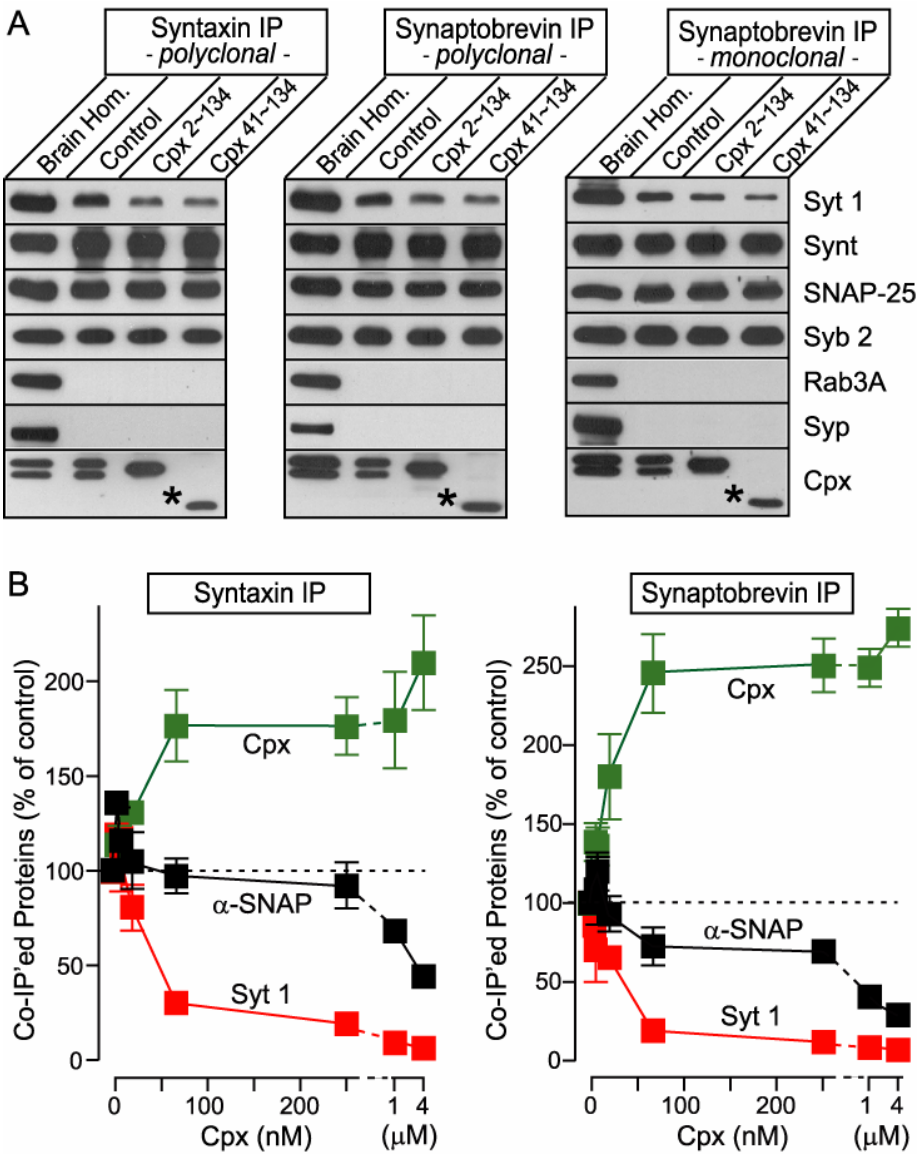


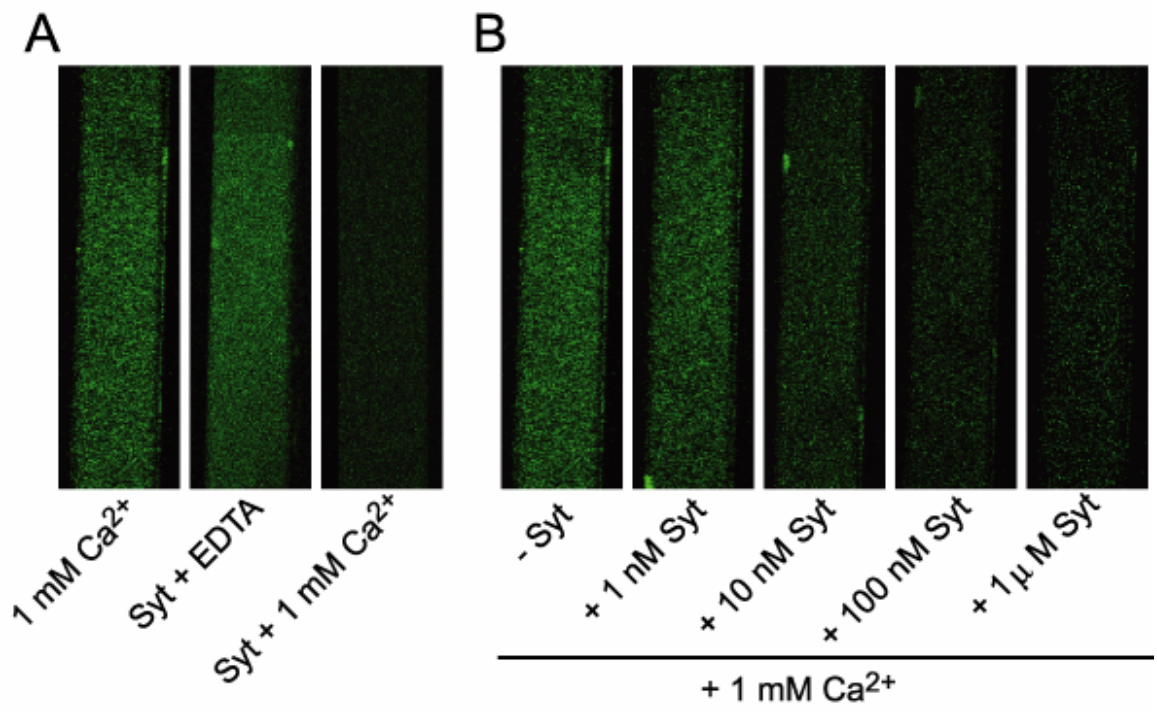
Fig 3.3B by Ok-Ho Shin

Figure 3.3 Recombinant complexin inhibit synaptotagmin 1 for native SNARE complex binding. A. Representative immunoprecipitations from brain homogenates with monoclonal and polyclonal synaptobrevin 2 antibodies (right and central panel) and polyclonal syntaxin 1 antibodies (left panel) either without additions, or after addition of full length (Cpx^{2-134}) or N-terminally truncated complexin 1 (Cpx^{41-134}; both 13.2 μM). Immunoprecipitates were

immunoblotted with antibodies as indicated on the right (Cpx; asterisk = position of the truncated complexin that is co-immunoprecipitated). B. Amounts of complexins, α-SNAP, and synaptotagmin 1 co-immunoprecipitated with SNARE complexes in 1 mM Ca^{2+} in the presence of increasing concentrations of exogenous complexin 1. The relative amounts of complexins, α-SNAP, and synaptotagmin 1 in the immunoprecipitates were measured by quantitative immunoblotting (means ± SD; n=4 independent experiments).

3.3.4 Ca^{2+}-dependent displacement of complexin by synaptotagmin 1 in a membrane attached environment.

SNARE proteins and synaptotagmin 1 are normally embedded in membranes, whereas all experiments up to now were performed in a non-membranous environment. To examine the role of Ca^{2+} in the binding of synaptotagmin 1 to SNARE complexes in a physiological membranous environment, and to test whether synaptotagmin 1 can displace complexin from SNARE complexes (and not only complexin displace synaptotagmin 1), we reconstituted membrane-bound SNARE complexes into a supported bilayer deposited within a microfluidic channel. We then bound fluorescently labeled complexin to the complexes, and measured the ability of recombinant synaptotagmin 1 to displace complexin from the SNARE complexes as a function of Ca^{2+} (Fig 3.4).

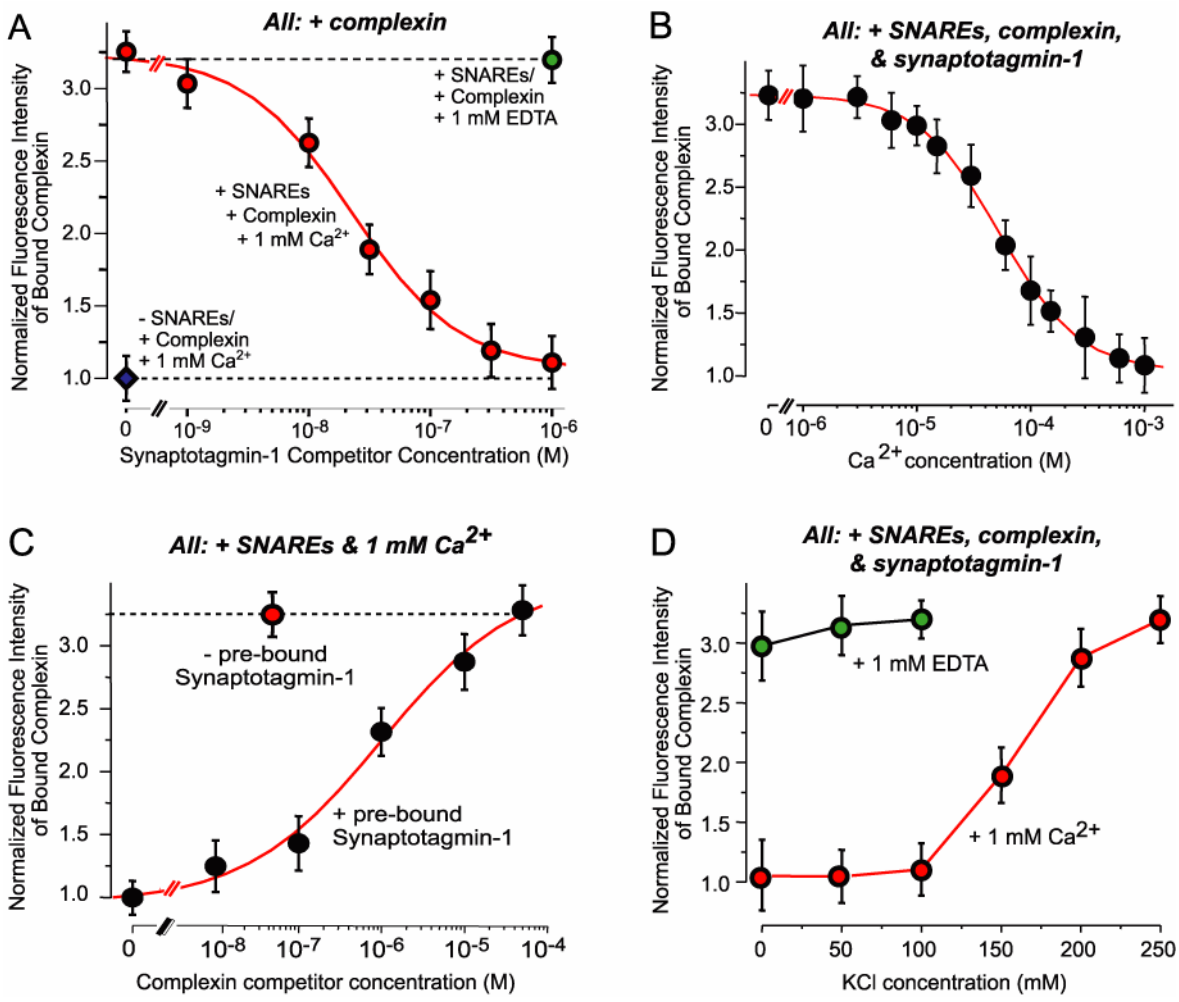


by Han Dai

Figure 3.4 Replacement of labeled complexin from membrane embedded SNARE complex by synaptotagmin 1 C_2AB. A. Confocal micrographs of supported bilayers containing reconstituted SNARE complexes loaded with 50nM fluorescent complexin (residues 26-83). Bilayers were deposited in microfluidic channels, and washed with buffer containing indicated additions (Syt = 1 μM synaptotagmin 1 cytoplasmic region). B. An analogous experiment in which SNARE complexes loaded with fluorescent complexin were washed with Ca^{2+}-containing buffer and increasing concentrations of synaptotagmin 1 as indicated.

Synaptotagmin 1 quantitatively displaced complexin from SNARE complexes in the presence, but not the absence of Ca^{2+}. In displacing complexin, synaptotagmin 1 exhibited an EC_{50} of 23 ± 1nM (Fig 3.5A) and a micromolar apparent Ca^{2+}-affinity (EC_{50} = 53 ± 14μM;

Fig 3.5B). All of these experiments were performed in a nearly physiological salt solution (100mM KCl and 25mM HEPES-NaOH), suggesting that in a membranous environment, synaptotagmin 1 powerfully dislodges complexin from SNARE complexes. To demonstrate the system worked in both ways (complexin in a membranous environment can in turn displace synaptotagmin 1), we measured the binding of fluorescently labeled complexin to membrane-attached SNARE complexes containing pre-bound synaptotagmin 1 (Fig 3.5C). Without pre-bound synaptotagmin 1, 50nM complexin quantitatively bound to the SNARE complexes. With pre-bound synaptotagmin 1, by contrast, micromolar complexin concentrations were required for binding, with an apparent affinity that is ~40-fold lower than that of the displacement of complexin by synaptotagmin 1 (EC_{50} = 1.0 ± 0.2μM). Similar to the GST-pulldowns and immunoprecipitations experiments, high ionic strength inhibited the displacement of complexin from SNARE complexes by 1μM synaptotagmin 1 (Fig 3.5D). However, low ionic strength (0 and 50mM KCl) did not enable synaptotagmin 1 to displace complexin from SNARE complexes in a Ca^{2+}-independent manner. This suggests that in a membranous environment, lowering the ionic strength does not activate Ca^{2+}-independent binding of synaptotagmin 1 to SNARE complexes. Overall, these data show that under close to physiological conditions in a membranous environment, synaptotagmin 1 binds to SNARE complexes in a strictly Ca^{2+}-dependent manner and effectively displaces complexin from SNARE complexes.



by Han Dai

Figure 3.5 Synaptotagmin 1 displaces complexin from membrane-attached SNARE complexes in a Ca^{2+}-dependent manner. A. Titration of synaptotagmin 1 for displacement of pre-bound fluorescent complexin from SNARE complexes. Average fluorescence intensities measured under each condition were normalized to the control in which fluorescently labeled complexin was added to a supported bilayer lacking SNARE complexes (blue diamond). Data were fitted to a dose-response curve (EC_{50} = 23 ± 5nM synaptotagmin 1; Hill coefficient = -0.96 ± 0.02 [n=3 independent experiments]). B. Ca^{2+} titration of the

displacement of fluorescent complexin from SNARE complexes by 1μM synaptotagmin 1 (EC_{50} = 53 ± 14μM Ca^{2+}; Hill coefficient: -1.24 ± 0.37). C. Displacement of pre-bound synaptotagmin 1 from SNARE complexes by fluorescent complexin. The displacement of synaptotagmin 1 bound to the reconstituted SNARE complexes was measured in 1mM Ca^{2+} as the binding of increasing concentrations of fluorescent complexin (EC_{50}= 1.00 ± 0.65μM; Hill coefficient = -0.53 ± 0.18). As a control, the red circle indicates the amount of 50nM fluorescent complexin bound in the absence of pre-bound synaptotagmin 1. D. Effect of ionic strength on the displacement of bound complexin from SNARE complexes by synaptotagmin 1. Experiments were performed in the presence of 1mM Ca^{2+} (red circles) or 1 mM EDTA (green circles).

3.4 Discussions

The present studies showed there is competition between synaptotagmin 1 and complexin for SNARE complex binding. The competition is dependent on the relative binding affinity between them, which is also greatly affected by the binding conditions and environment.

As indicated by the data above, in hydrophilic solution, cis mini SNARE complex has a much higher affinity for complexin than for synaptotagmin 1 in both recombinant and native protein experiments (Fig 3.1 and 3.3). However, when SNARE complex was embedded in the membrane similar to their native status, the affinity for synaptotagmin 1 binding was greatly enhanced by calcium, probably to a certain extent by the presence of

negatively charged phospholipids. In contrast, complexin binding is much reduced (Bowen et al., 2005), resulting in the suggestion that synaptotagmin 1 can more potently replace complex in a calcium dependent manner (~40-fold) (Fig 3.4 and 3.5).

In the last chapter, we showed that at low ionic strength, synaptotagmin 1 binding to SNARE complexes is largely independent of Ca^{2+} in solution (Fig 2.2, 2.9, 2.10). However, there was no corresponding tight calcium independent binding between synaptotagmin 1 and membrane attached SNARE complex, revealed by its inability to replace complexin at low ionic strength (Fig 3.5D). Although the absence of a high local concentration could partially explain this, it is possible that synaptotagmin 1 and SNARE complex interaction remains Ca^{2+}-dependent in a membranous environment.

With a native membraneous environment and physiological ionic strength, displacement of complexin from SNARE complexes by synaptotagmin 1 is absolutely dependent on Ca^{2+} (Fig 3.4A), and the apparent Ca^{2+}-affinity for this displacement is in the physiological range of neurotransmitter release (~50μM; Fig 3.5B).

The combination of all these results together suggested in a native environment, there is strict Ca^{2+}-dependent interactions between synaptotagmin 1 and SNARE complexes and the binding is strong enough for the subsequent displacement of complexin. This conclusion implies that complexin binding to SNARE complexes dominates prior to the action potential-driven local increase in Ca^{2+} at the terminal that leads to release.

Of course, this explanation is still far from being completely proved. Many problems still remain unsolved in this system. Synaptotagmin 1 was never tested as a membrane protein in all these assays, and we don't know whether and how this could affect the binding. SNARE complex was never examined as a 'trans' complex formed between two membrane structures, and the effect on complexin and synaptotagmin 1 binding is also unknown.

The future direction should be focused on the interactions with all proteins in their native status, although the exact local conditions are not clearly defined. Experiments have been carried out with membrane embedded SNARE proteins to reconstitute the fusion events *in vitro*. Progress has been achieved to improve the slow rate of liposome fusion, and the calcium dependence introduced by synaptotagmin 1 was also described (Tucker et al., 2004). But more work still needs to be done to completely figure out the mechanism.

Chapter IV: Physiological functions of the competition between complexin and synaptotagmin 1

4.1 Introductions

The functional analysis of complexin showed contradictory results, from which it is difficult to make a conclusion. To directly compare the results from secretory cells and neurons is also not reasonable. Since complexin is mainly expressed in central nervous system, we think the results from experiments performed in its native environment might be closer to its real function in synaptic transmission.

Our strategy is to fuse complexin N terminal to synaptobrevin 2 to make a fusion protein. By expressing this fusion protein in cultured cortical neurons, we can perform a series of electrophysiological experiments to examine the effect of complexin on synaptic transmission. The advantages of this idea are: (1), complexin is soluble synaptic protein. In cultured neuronal culture, it takes 3~4 weeks to get enriched into synapse, which increases the time and difficulty of the experiment. However, synaptobrevin 2, as vesicle protein, can very easily be targeted to synaptic vesicle within 2 weeks *in vitro* culture. The fusion of complexin to synaptobrevin 2 can concentrate complexin more efficiently and early to synapse. (2), Complexin bind to SNARE complex in an anti-parallel manner. Inserting a flexible linker region between complexin and synaptobrevin 2 could allow complexin to fold back and bind to SNARE complex. Since complexin doesn't bind to synaptobrevin 2 itself, the intracellular interactions guarantee the local concentration of complexin is high enough to

replace endogenous synaptotagmin 1 from SNARE complex, but not interfere with other interactions.

To test the practicability of this strategy, I made two sets of fusion constructs: (1) Lenti-virus infection, complexin was put before full length of synaptobrevin 2 with linker region between them. Venus was added at the C terminus of synaptobrevin 2 as a marker. (2), Complexin was fused to cytoplasmic region of synaptobrevin 2 (1-96) with the identical linker region, but without venus at the C terminus. By making SNARE complex with complexin fused to synaptobrevin 2, we can examine whether the incorporated complexin can bind intracellularlly to SNARE complex and inhibit synaptotagmin 1 binding.

4.2 Methods and Materials

4.2.1 Making complexin-synaptobrevin (Cpx-Syb) constructs

I took advantage of the linker region between GST and target protein on pGexKG vector by putting complexin to BamH I site and synaptobrevin 2 into EcoR I site. Considering this linker region may not be long enough, I repeatedly insert synthesized oligo sequences into Xma I site just after BamH I. All the constructs for final use contained 3 copies of the oligos and the original linker region with the total length at 147 bp (including enzyme cleavage site).

4.2.2 HEK cell transfection to make lentivirus containing Cpx-Syb fusion constructs.

HEK 293T cells were maintained in T-75 flasks using complete DMEM medium containing L-Glutamine, 10% FBS and P/S plated. Cells were split into T-25 flasks 1 day before transfection and density was adjusted to get 70-90% confluence the next day. Prepare the DNA mix as: in tube 1, add 167μl of MEM (minimal essential medium) plus 1.7μg of each plasmid (Shuttle vector with target gene on it + VSVg + CMVΔ8.9); in tube 2, mix 20μl FUGENE (Roche) with 334μl of MEM, and incubate 5 min at room temperature. Mix content of tube 1 and 2 and incubated for 15 min at room temperature. The mixture was added to HEK cells after changing its medium to neuronal culture medium without Ara-C. Allow 48-60 hrs for virus amplification and package, supernatant containing viruses were passed through 0.44μm filter and applied to neurons at 250μl each well.

4.2.3 Neuronal cultures preparation and lentivirus infection.

Primary cortical neurons were isolated from E18 or P1 pups of wildtype rats or mice or synaptotagmin 1 or synaptobrevin 2 deficient mice, and dissociated by trypsin digestion. Neurons were plated on Matrigel-coated circular glass coverslips and cultured in MEM (Gibco) supplemented with B27 (Gibco), glucose, transferrin, fetal bovine serum, and Ara-C (Sigma). Neurons were infected with lentiviruses encoding full length synaptobrevin 2, soluble complexin (Cpx) or complexin-synaptobrevin-venus fusion proteins (Cpx-Syb) at 5 days *in vitro* and analyzed at 14-16 days *in vitro*. For all experiments, expression of fusion proteins in neurons was confirmed by immunoblotting and Venus fluorescence.

4.2.4 Immunocytochemistry.

Cortical neurons infected with lentiviruses containing complexin-synaptobrevin-venus were performed immunocytochemistry at 14-16 days *in vitro*. All steps are in room temperature except pointed out. Cells were rinsed in PBS, followed by fixation with cold methanol in -20°C for 10 min. After washing with PBS for 5 min, neurons were blocked with 3% milk and 0.1% saponin/PBS (PBSS) for 15 min. Primary antibody was incubated with cells for 1 hr, followed by 3 times washing with PBSS for 5 min each before subject to secondary antibody for 45 min. Coverslips with neurons were mounted after another 3 times of washing by PBSS. Digital images were collected with LSM510 software (Carl Zeiss Microimaging Inc.) and processed with Adobe Photoshop software.

4.2.5 Electrophysiology.

Inhibitory postsynaptic currents (IPSCs) were evoked by current injections (900μA for 1ms) *via* a local extracellular electrode (FHC, Inc.), and recorded in a whole-cell mode using Multiclamp 700A amplifier (Axon Instruments, Inc.). All experiments were performed at room temperature. The frequency, duration, and magnitude of the extracellular stimulus were controlled with a Model 2100 Isolated Pulse Stimulator (A-M Systems, Inc.). The whole-cell pipette solution contained 135mM CsCl, 10mM HEPES, 1mM EGTA, 1mM Na-GTP, 4mM Mg-ATP and 1mM QX-314 pH 7.4. The bath solution contained 140mM NaCl, 5mM KCl, 2mM $CaCl_2$, 0.8mM $MgCl_2$, 10mM HEPES-NaOH pH 7.4, and 10mM glucose. Excitatory AMPA and NMDA-receptor dependent currents were suppressed by addition of 50μM AP-5 and 20μM CNQX to the bath solution. For recording of spontaneous miniature IPSCs (mIPSCs) 0.001mM TTX was added to the bath solution to suppress spontaneous

firing. In all experiments, the holding potential was –70mV. IPSCs were sampled at 10 kHz and analyzed off-line using pClamp9 (Axon Instruments, Inc.) and Origin7 (Mocrocal Inc.) software. All statistical analyses were performed with the Students' t-test (* corresponds to $p<0.001$).

4.3 Results

4.3.1 Construction and expression of complexin-synaptobrevin fusion protein in cortical culture.

A series of Cpx-Syb constructs were prepared as indicated in Fig 4.1A with different complexins: full length, wild type Cpx, truncated wild type Cpx^{Δ} (without first 40 residues), and truncated mutant $Cpx^{\Delta M}$ (lacking first 40 residues and with mutations R48/R59/K69/Y70A). Using truncated complexin is to avoid interference and also could inhibit synaptotagmin 1 more efficiently, as suggested from the immunoprecipitation experiments with truncated complexin, which is contradictory to the result of recombinant protein competition experiment (Fig 3.2 and 3.3). Single soluble complexin was also constructed as a control, with venus attached to C terminus as a tag.

We infected the dissociated cortical neurons at day 5 *in vitro* with lentivirus containing Cpx-Syb. After two weeks, proteins were collected by adding 60µl of 1x SDS gel loading buffer to each well after PBS washing. Cell lysates were boiled to disassemble SNARE complex before subject to separation on SDS-PAGE gel. We compared the

expression level of several key proteins by western blot (Fig 4.1B). The results indicated there is no significant change for the expression of all the endogenous proteins tested. The exogenous Cpx-Syb protein was expressed at similar level compared to endogenous synaptobrevin (Fig 4.1B), but was over 5 folds higher than endogenous complexin (data not shown).

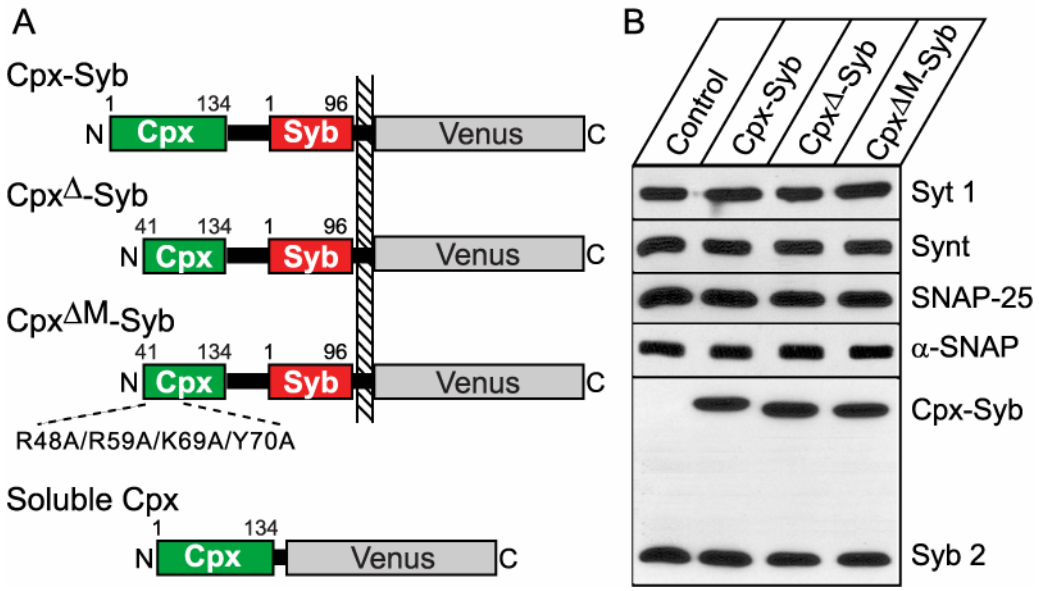


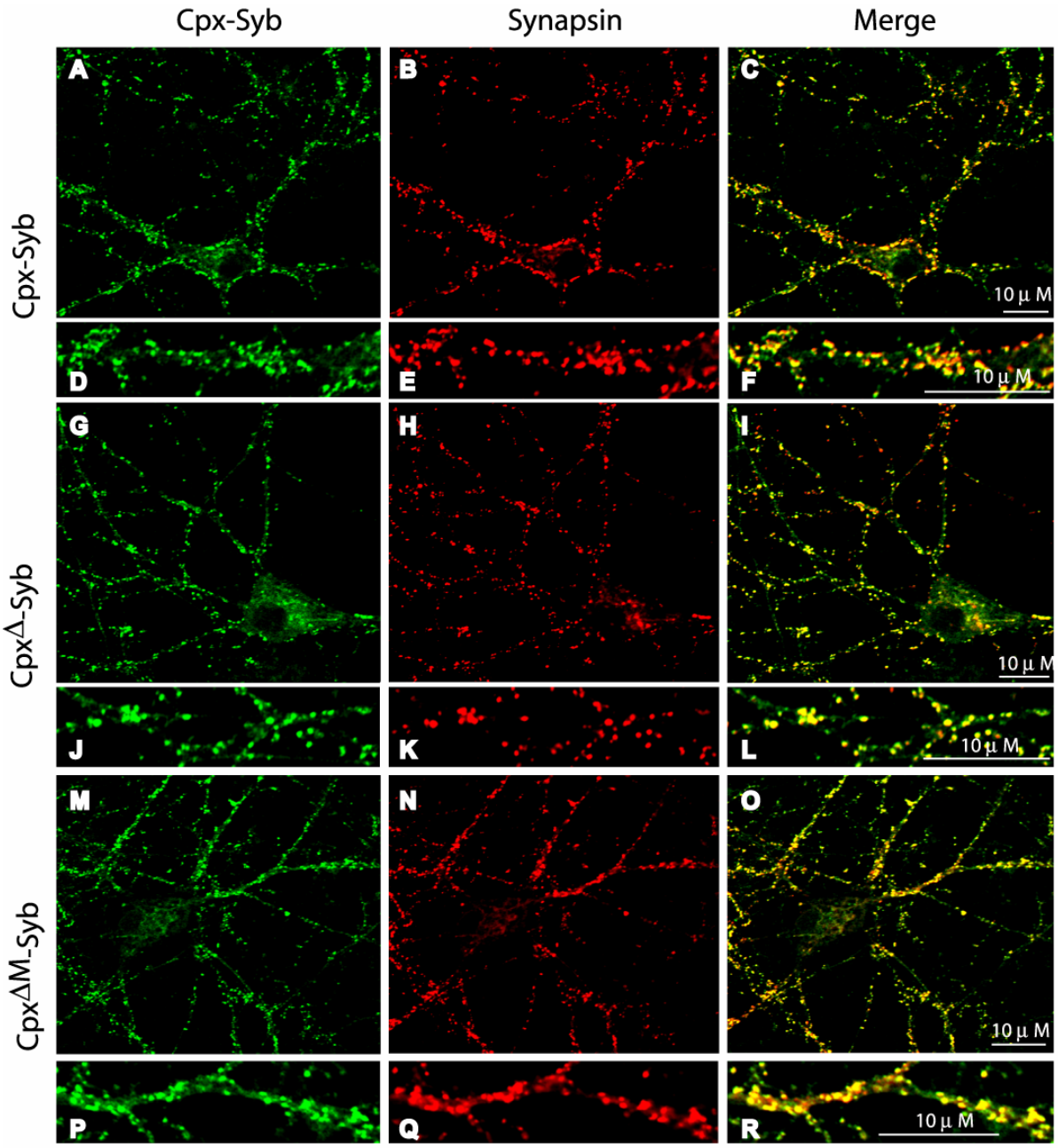
Figure 4.1 Construction and expression of complexin-synaptobrevin fusion protein in cultured cortical neurons: A. Schematic diagram of the structure of complexin (Cpx) and complexin-synaptobrevin fusion proteins. Expressed proteins contain full length complexin 1 (Cpx and Cpx-Syb), N-terminally truncated wild type complexin 1 (Cpx^{Δ}-Syb; residues 41-134), and N-terminally truncated mutant complexin 1 that lacks SNARE binding ($Cpx^{\Delta M}$-Syb with the R48A/R59A/K69A/Y70A mutation). *B.* Immunoblot analysis of various synaptic

proteins (as indicated on the right) and complexin-synaptobrevin fusion proteins in cortical neurons infected with Cpx-Syb lentiviruses.

4.3.2 Localization of complexin-synaptobrevin fusion protein.

To investigate whether Cpx-Syb fusion protein is targeted to synapse as expected, we performed immunocytochemistry to examine the localization of the fusion protein. The self-containing tag venus was compared with synaptic marker synapsin to check the extent of overlapping.

As shown in Fig 4.2, there is more than 95% overlap of the green (venus) and red (synapsin) signals. Almost all the red puncta was accompanied by green fluorescence. In addition to synapses, fusion protein also scattered with low level in cell soma. This suggested the fusion proteins mainly followed the expression pattern and time scale of synaptobrevin, but not complexin, as discussed in previous introduction.



by Anton Maximov

Figure 4.2 Synaptic localization of complexin-synaptobrevin fusion protein in neurons. Neurons were infected with lentivirus expressing (A-F) complexin-synaptobrevin fusion proteins with a full length wild type complexin sequence (Cpx-Syb), (G-L) a truncated wild

type complexin sequence (Cpx^{Δ}-Syb), or (M-R) a truncated mutant complexin sequence ($Cpx^{\Delta M}$-Syb). Synapses were visualized by antibody to synapsin as a synaptic marker (red), and the localization of the complexin-synaptobrevin fusion protein was measured by the endogenous fluorescence (green) from the venus moiety that was fused to all proteins.

4.3.3 Complexin-synaptobrevin fusion protein inhibits synaptotagmin 1 binding to the mini SNARE complex which it is incorporated into.

As mentioned in discussion, we first need to confirm that the fused complexin was able to fold back to bind the SNARE complex and then block synaptotagmin 1 binding. The SNARE complex was reconstituted by mixing SNARE motifs from syntaxin and SNAP-25 with the complexin-synaptobrevin fusion protein (Fig 4.3A) containing a wild-type complexin sequence (Cpx-Syb SNARE), or with point mutations (R48/R59/K69/Y70A) that abolish SNARE complex binding (Cpx^{M}-Syb SNARE). The binding of purified SNARE complexes containing the wild type or mutant complexin-synaptobrevin fusion proteins to immobilized synaptotagmin 1, α-SNAP, and complexin was assayed as a function of Ca^{2+}. Note that the SNARE complexes containing wildtype and mutant complexin-synaptobrevin fusion proteins exhibit distinctly different apparent sizes by SDS-PAGE.

The first step is to examine whether fused complexin can fold back and bind the mini SNARE complex which it is fused to. Wild type complexin fused SNARE complex has very little binding to immobilized complexin compared to mutant complexin fused complex,

indicating the intracellular complexin binds tightly to SNARE complex and prevent exogenous complexin binding (Fig 4.3B, bottom panel). As expected, only SNARE complex with wild type complexin can block synaptotagmin1 binding, but not the mutant one, indicating the specificity of this inhibitory effect (Fig 4.3B, top panel). Finally, this new complex, no matter with wild type or mutant complexin, has no effect on α-SNAP binding (Fig 4.3B, middle panel), which is the only known factor binding to SNARE complex besides complexin and synaptotagmin 1.

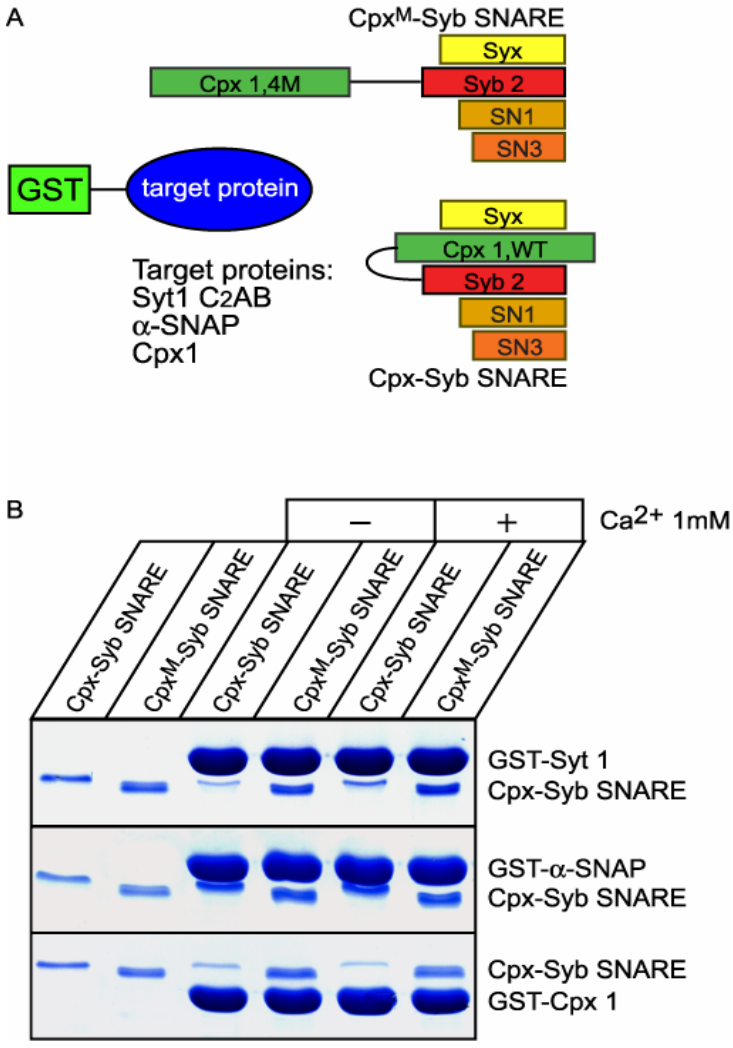
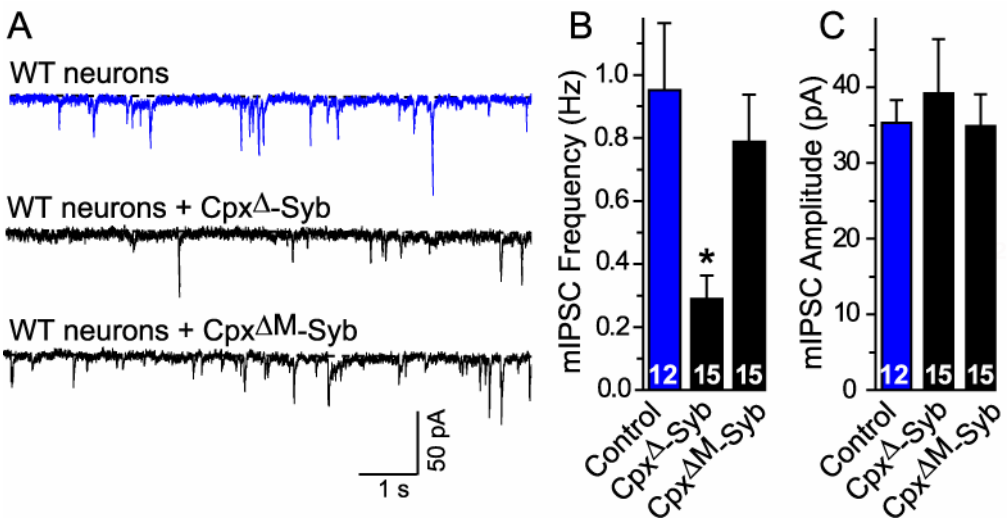


Figure 4.3 Fusion of complexin to synaptobrevin inhibits binding of mini SNARE complexes to immobilized synaptotagmin 1. A. Diagram of the structure of the minimal SNARE complex containing the complexin-synaptobrevin fusion protein (Syx = syntaxin SNARE motif; Syb = synaptobrevin SNARE motif; SN1 and SN3, first and second SNARE motifs of SNAP-25). B. Binding of the minimal SNARE complex containing the wild type (Cpx-Syb SNARE) or mutant complexin-synaptobrevin fusion protein (Cpx-Syb^{M} SNARE) to immobilized GST-fusion proteins containing the synaptotagmin 1 C_2-domains (GST-Syt 1), full length α-SNAP (GST-α-SNAP), or full length complexin (GST-Cpx) as a function of calcium.

4.3.4 Miniatures were reduced by the expression of complexin-synaptobrevin fusion protein in cortical culture.

We analyzed the effect of the complexin-synaptobrevin fusion protein on synaptic transmission using whole-cell voltage-clamp recordings on day 14-16 in culture when neurons display robust postsynaptic responses to extracellular stimulation (Maximov and Südhof, 2005). Recordings of spontaneous release events revealed that the wild-type but not the mutant fusion protein reduced the rate of spontaneous mini events ~3-fold (Fig 4.4A-B), but had no effect on the size of spontaneous mini events (Fig 4.4C), consistent with a presynaptic role in release.



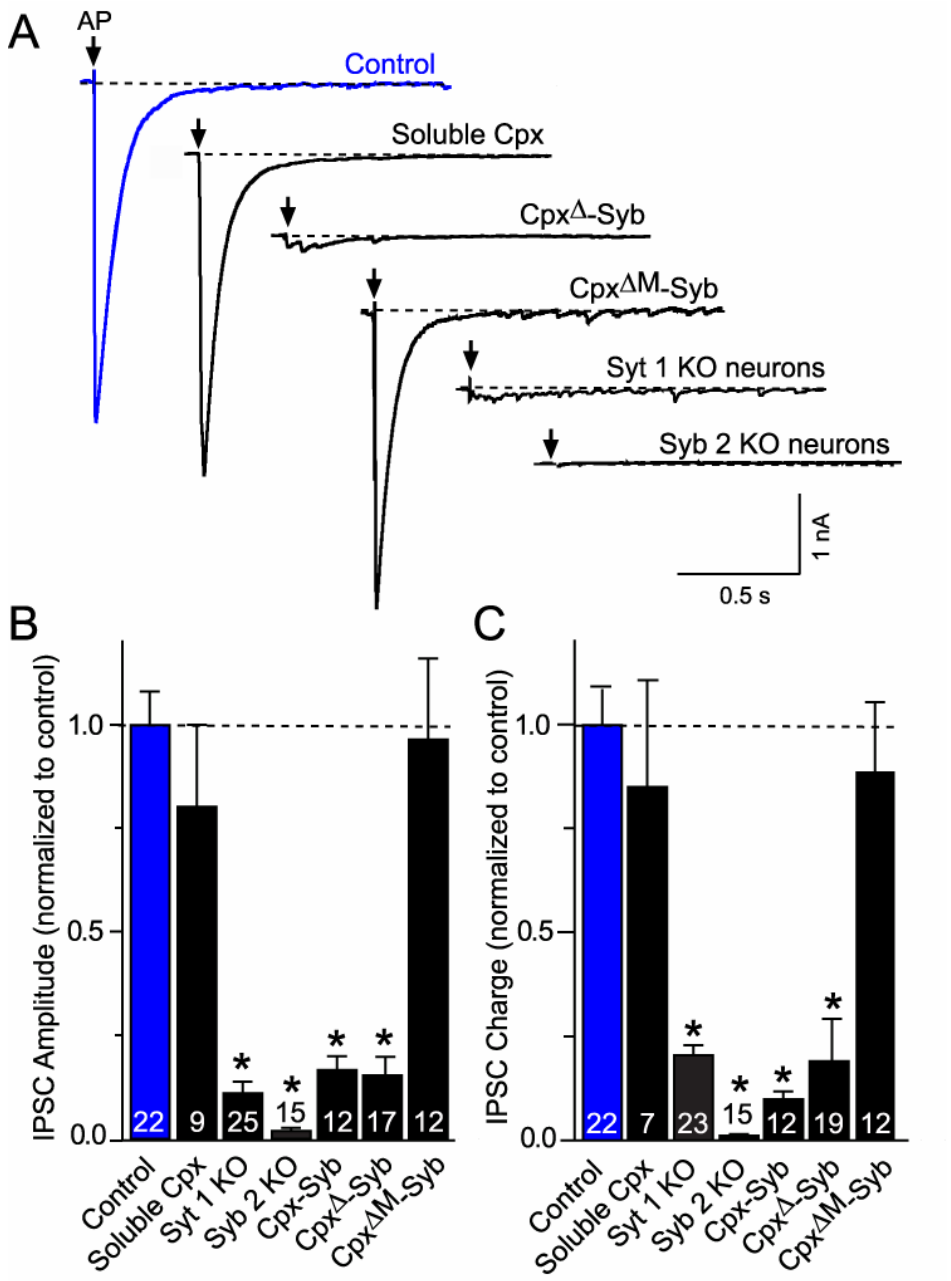
by Anton Maximov

Figure 4.4 Effect on miniatures by the expression of complexin-synaptobrevin fusion protein. A. Representative traces of miniature inhibitory postsynaptic currents (mIPSCs) monitored from non-infected wild type neurons or wild type neurons expressing Cpx^{Δ}-Syb or $Cpx^{\Delta M}$-Syb fusion proteins. Scale bars apply to all traces. B- Average frequency and C-amplitude of mIPSCs monitored in non-infected neurons or neurons expressing Cpx^{Δ}-Syb or $Cpx^{\Delta M}$-Syb (means ± SEMs; n = number of neurons analyzed in three independent cultures are indicated in the bars).

4.3.5 The complexin-synaptobrevin fusion protein inhibits fast Ca^{2+}-triggered neurotransmitter release.

To determine which modes of release are perturbed by the local increase in the concentration of complexin in synapses, we measured inhibitory postsynaptic currents (IPSCs), and compared IPSCs obtained in non-infected wild type neurons, in neurons from synaptotagmin 1 and synaptobrevin 2 KO mice, and in wild type neurons expressing Cpx-

Syb proteins. Initially, we analyzed responses to single action potentials (Fig 4.5A). Expression of wild type full length or N-terminally truncated complexin-synaptobrevin fusion proteins led to an 80% depression of the amplitude and the charge transfer of the IPSCs (Fig 4.5B-C). The control complexin-synaptobrevin fusion protein with a mutation that inactivates SNARE complex binding by complexin had no significant effect on the size of the IPSCs, demonstrating that the SNARE-binding activity of complexin is responsible for the inhibitory effect. The inhibitory effect of the wild type complexin-synaptobrevin protein was indistinguishable from that of the synaptotagmin 1 deletion, but less than that of the synaptobrevin 2 deletion, presumably because the asynchronous release remains in the synaptotagmin 1 KO, but is depressed together with the synchronous release in synaptobrevin 2 KO mice (Geppert et al., 1994; Deak et al., 2004).



by Anton Maximov

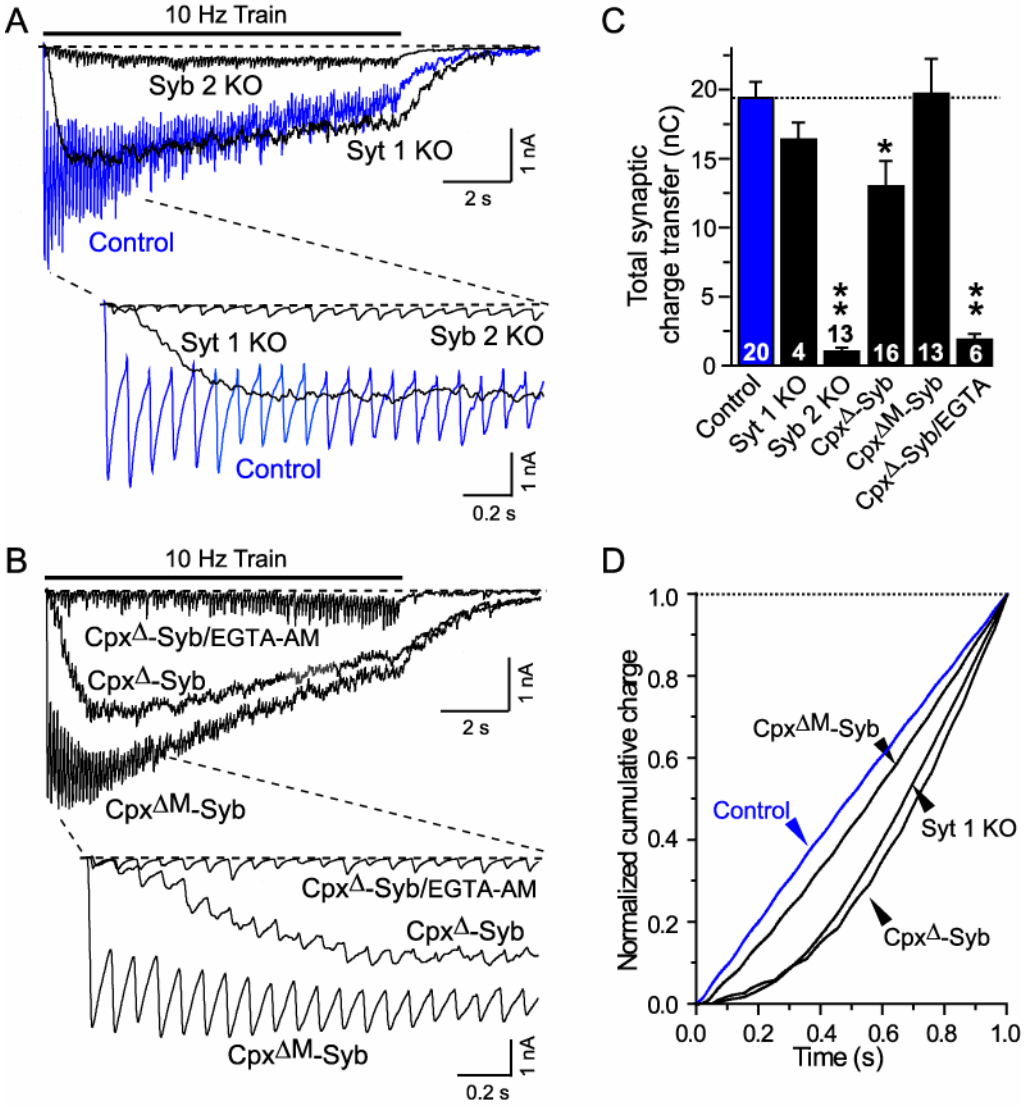
Figure 4.5 Expression of a complexin-synaptobrevin fusion protein in wild type neurons impairs synaptic responses triggered by single action potentials. A. Representative IPSCs recorded from non-infected wild type neurons, synaptotagmin 1 and synaptobrevin 2

deficient neurons, and wild type neurons infected with lentiviruses encoding soluble complexin, Cpx^{Δ}-Syb, or $Cpx^{\Delta M}$-Syb. IPSCs were triggered by isolated AP's at 0.1Hz. Scale bars apply to all traces. B-C. Average amplitudes (B) and total charge transfers integrated over 1.5s (C) of IPSCs recorded from wild type neurons, synaptotagmin 1 and synaptobrevin 2 deficient neurons, and wild type neurons expressing the indicated complexin-synaptobrevin fusion proteins (numbers of neurons analyzed in each group are indicated in the bars; data are from at least two independent cultures; values are normalized for the control analyzed in the same experiment).

4.3.6 Constitutive complexin binding to SNARE complexes does not block asynchronous release.

As we all know, calcium dependent synaptic transmission can be divided into 2 components: fast synchronous release and slow asynchronous release. It is important to know whether the expression of the complexin-synaptobrevin fusion protein fully impairs exocytosis similar to the deletion of synaptobrevin 2, or only specifically blocks fast synchronous exocytosis triggered by Ca^{2+}-binding to synaptotagmin 1. To distinguish between these two possibilities, we examined the effects of the complexin-synaptobrevin fusion proteins on release triggered by trains of action potentials (100APs at 10Hz). In synaptobrevin 2 KO neurons, synaptic responses remain suppressed during high-frequency stimulus trains (Deak et al., 2004), with a total amount of release that is <5% of the release of wild type controls over the entire stimulus train (Fig 4.6A). In synaptotagmin 1 deficient neurons, in contrast, synaptic responses are only initially

abnormal during a high-frequency stimulus train, but quickly reach wild type levels, and the total amount of release over the entire train is not significantly different from wild type neurons (Maximov and Südhof, 2005).



by Anton Maximov

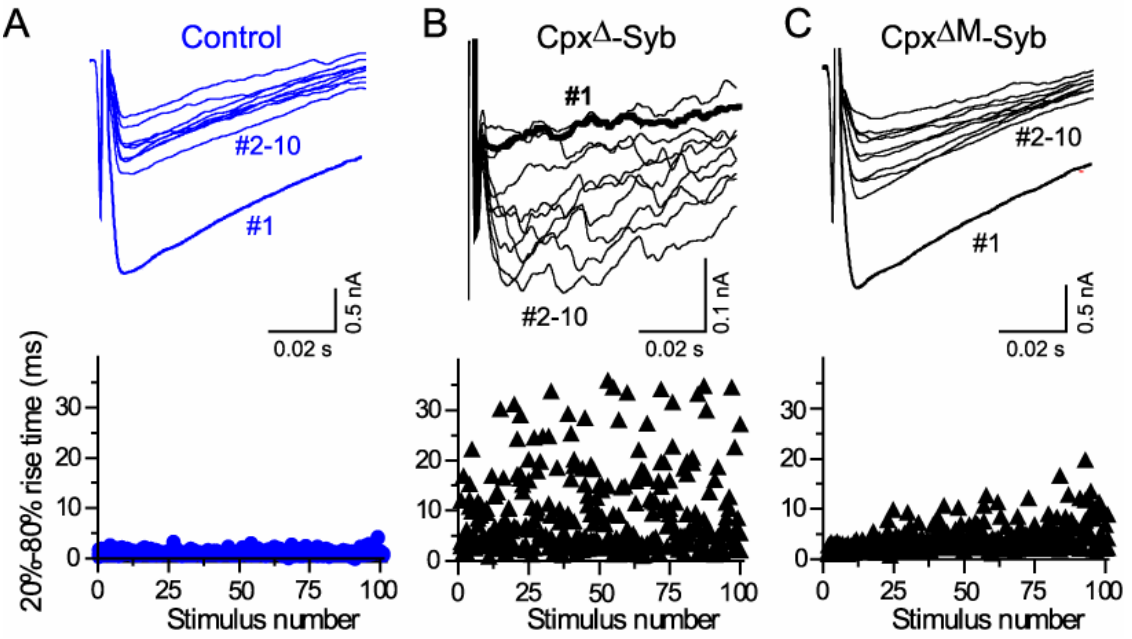
Figure 4.6 Expression of a complexin-synaptobrevin fusion protein causes loss of fast Ca^{2+}-triggered release but not of asynchronous Ca^{2+}-triggered release. A. Representative IPSCs during a 10Hz stimulus train for 10s from wild type neurons (WT), synaptotagmin

1 deficient neurons (Syt 1 KO) or synaptobrevin 2 deficient neurons (Syb 2 KO). B. IPSCs monitored in the same conditions from non-treated wild type neurons expressing Cpx^{Δ}-Syb and $Cpx^{\Delta M}$-Syb, or neurons expressing Cpx^{Δ}-Syb and treated for 5 min with 0.1mM EGTA-AM. In panels A and B, expanded lower traces illustrate the initial onsets of high frequency responses (filtered at 50Hz to remove the stimulus artifacts). Scale bars apply to all traces. C. Total synaptic charge transfer during a 10Hz stimulus train for 10s monitored in non-infected wild type, synaptotagmin 1 KO, and synaptobrevin 2 KO neurons or neurons infected with Cpx-Syb lentiviruses (analyzed neuron numbers are indicated in the bars; *$p<0.01$; **$p<0.001$). D. Average plots of the normalized cumulative charge transferred during the first 1 sec of 10Hz stimulation shown on panels A and B.

Analysis of the release produced during high-frequency trains in neurons that express complexin-synaptobrevin proteins revealed a pattern indistinguishable from that observed in synaptotagmin 1 KO neurons: the initial fast synchronous responses were absent, but subsequent asynchronous release that becomes dominant after 3-5 action potentials was normal (Fig 4.6B). Quantitation of the total synaptic charge transfer during the stimulus train confirmed that in contrast to the synaptobrevin 2 KO neurons, neurons lacking synaptotagmin 1 or expressing the complexin-synaptobrevin fusion protein exhibited nearly normal asynchronous release (Fig 4.6C). This release is indeed asynchronous because it is blocked by EGTA-AM (Fig 4.6B) as previously demonstrated for asynchronous release in wild-type neurons (Lu and Trussell, 2000) and synaptotagmin

1 KO neurons (Maximov and Südhof, 2005). Moreover, plots of the time course of cumulative charge transfer during the stimulus train demonstrate that the initial 4-5 action potentials induce little synaptic charge transfer in either synaptotagmin 1 KO neurons or wild type neurons expressing the wild type complexin-synaptobrevin protein. Later action potentials, however, induce normal synaptic charge transfers in these neurons (Fig 4.6D).

In synaptotagmin 1 KO neurons the initial responses were dramatically desynchronized, a reasonable result from the abolishment of fast synchronous release. Since the neurons expressing Cpx-Syb fusion proteins displayed a phenotype so similar to that of synaptotagmin 1 KO neurons, we also analyzed the responses from train stimulation in these neurons. The synchronization can be reflected by the uniform of rising time of the IPSCs. Only synaptotagmin 1 KO neurons and wild type neurons expressing the wild type complexin-synaptobrevin protein but not the mutant complexin-synaptobrevin protein exhibit desynchronization (Fig 4.7A-C). This is evident from the fact that the first 10 responses during the stimulus train were highly synchronized in wild type control neurons and neurons expressing mutant complexin-synaptobrevin fusion protein, as evidenced by the uniformly short rise times (although the mutant complexin-synaptobrevin fusion protein did have a moderate effect on rise times, Fig 4.7C). In contrast, neurons expressing wild type complexin-synaptobrevin fusion protein exhibited massive desynchronization as reflected in a scattering of the rise times (Fig 4.7B).



by Anton Maximov

Figure 4.7 Desynchronization of responses from train stimulations. A-C. Top: aligned segments of the initial 10 IPSCs during a 10Hz stimulus train to illustrate that the synchronous responses become irregular in neurons expressing Cpx^{Δ}-Syb, but not in $Cpx^{\Delta M}$-Syb. Bottom: plots of the 20-80% rise times of individual IPSCs triggered during a 10 Hz stimulus train in control wild-type neurons and in neurons expressing Cpx^{Δ}-Syb or $Cpx^{\Delta M}$-Syb. For each group, data are from three different neurons.

4.4 Discussions

The expression of complexin-synaptobrevin 2 in wild type neurons caused selective inhibition of fast synchronous release, but with asynchronous release largely unchanged. Fusion protein didn't block the binding of α-SNAP to SNARE complex (Fig 4.3), therefore it would have little effect on SNARE complex disassembly and vesicle

regeneration. As a result, a very mild effect (Fig 4.6C) on total charge transfer by train stimulation indicated the release pool size, as well as the docking and priming process is not affected by the exogenous fusion protein. Thus, the reduction of fast synchronous release implied the decrease of release probability, which was consistent with the observation of facilitation for the second and third IPSCs during train stimulations (data not shown). Based on our biochemical data (Fig 4.3), the introduction of complexin-synaptobrevin fusion protein led to a specific replacement of synaptotagmin 1 from SNARE complex, which can be considered as an alternative way to functionally "knockout" synaptotagmin 1 by preventing it from contacting with SNARE proteins. This was a reasonable explanation for why the phenotype is so similar to that of synaptotagmin 1 KO mice.

At first glance, the reduction of miniatures and IPSCs, as well as the biochemical data can be used as evidence for complexins serving as fusion clamps. A similar hypothesis was advanced in experiments in which transfected cells that display surface-exposed SNARE proteins or liposomes that contain reconstituted SNARE proteins are fused *in vitro*; in both cases, addition of excess complexin inhibited fusion, consistent with a 'clamp' function for complexin (Giraudo et al., 2006; Schaub et al., 2006). However, the clamp hypothesis is contradictory to the complexin KO phenotype which demonstrates that complexins are activators, not inhibitors of fusion (Reim et al., 2001). It is possible that the KO analyses are misleading because of compensatory effects, or the

in vitro fusion experiments don't represent a normal synaptic membrane fusion because of the use of very artificial system.

The crystal structure of complexin and SNARE complexes (Chen et al., 2002) suggests 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 SNARE complex, complexins may induce a strain on the participating membranes in which the respective SNARE proteins reside (synaptobrevin on synaptic vesicles while syntaxin and SNAP-25 on plasma membrane), and activate vesicles into a state ready for fusion triggering.

We suggest an alternative interpretation for complexin function and also a new model for calcium dependent synaptic transmission (Fig 4.8). According to this hypothesis, complexins, by binding to SNARE complexes that were partially assembled during priming, force completion of SNARE-complex assembly and thereby transform vesicles into a 'superprimed' metastable state. Ca^{2+}-binding to synaptotagmin 1 triggers vesicle fusion by inducing the simultaneous binding of synaptotagmin 1 to SNARE complexes and to phospholipids, thereby displacing complexins and pulling the fusion pore open (see model in Fig 4.8).

This hypothesis suggests that primed vesicles containing partly assembled SNARE complexes are either substrates for asynchronous Ca^{2+}-triggered release, or are

activated by complexin binding to SNARE complexes which transforms them into a substrate for Ca^{2+}-triggered synchronous release. This hypothesis still includes a 'clamp' function for complexins that, however, is secondary to their role in activating SNARE complexes and does not involve clamping of SNARE complexes as such, but only clamping of activated SNARE complexes produced by complexin binding in the first place. In this model, complexin and synaptotagmin 1 function in same pathway for synchronous release, but not involved in the other asynchronous process, which easily explain the similarity of phenotype between KO mice (step 4-6 in Fig 4.8).

The interplay between SNARE complexes, complexins and synaptotagmin 1 is probably an evolutionarily old universal mechanism as all organisms with a nervous system appear to express both synaptotagmin 1 and complexin (see for example Dykes et al., 2004). Moreover, this mechanism may be modulated by changing the affinity of either complexin or synaptotagmin 1 for SNARE complexes as suggested by their phosphorylation (Hill et al., 2006), a modulation that could cause dramatic changes in the efficacy of synaptic transmission and remains to be evaluated.

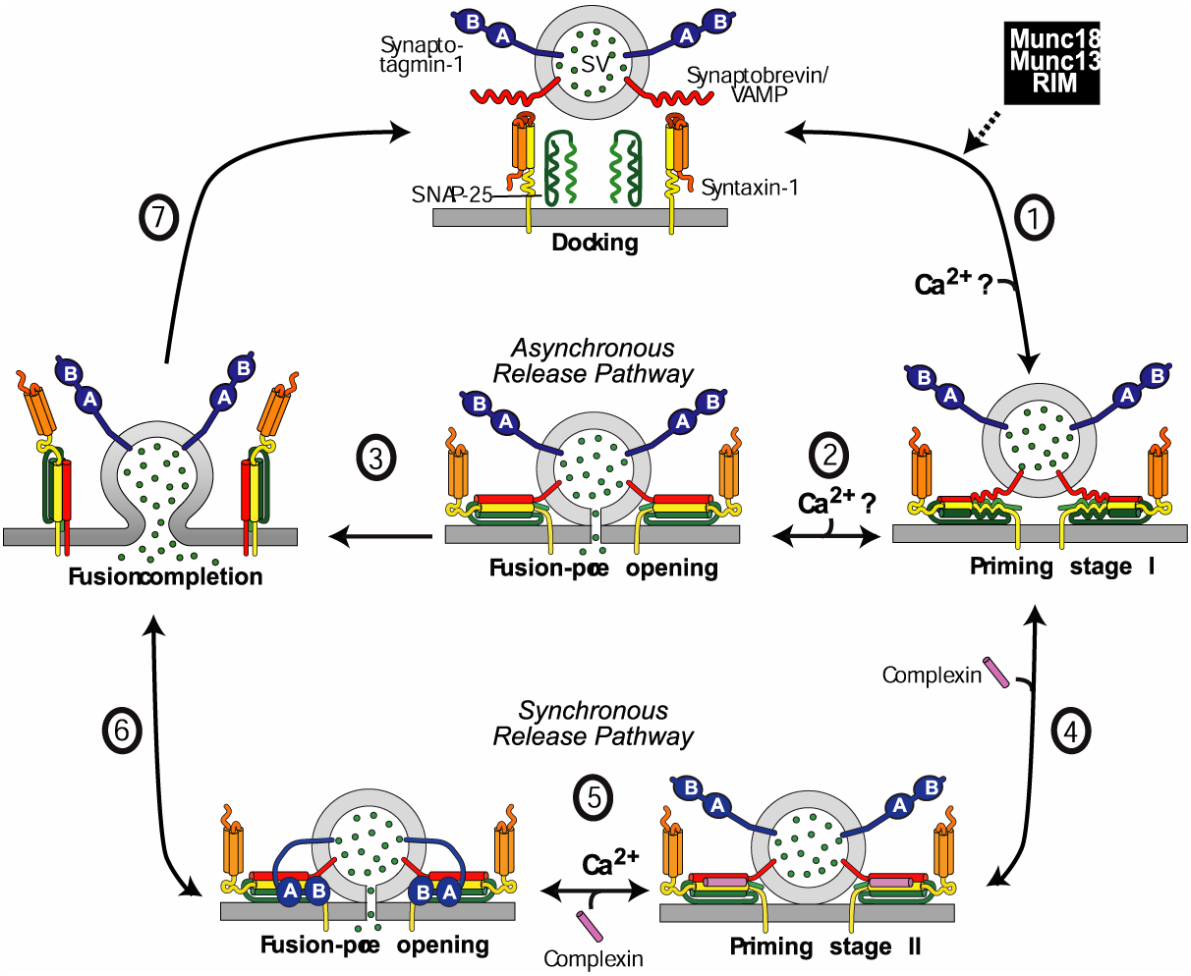


Figure 4.8 Model for complexin and synaptotagmin 1 function in Ca^{2+}-triggered release. Docked vesicles containing unassembled SNARE complexes (top) are primed for release by partial SNARE complex assembly catalyzed by Munc18, Munc13, and RIM (step 1). The resulting primed vesicles form the substrate for two release pathways: asynchronous release in which full assembly of the SNARE complexes leads to fusion-pore opening followed by complete fusion (steps 2 and 3), or synchronous release in which 'superpriming' by binding of complexins to assembled SNARE complexes (step 4) activates and freezes SNARE complexes in a metastable state (referred to as priming

stage 2). This stage is then substrate for fast Ca^{2+}-triggering of release when Ca^{2+}-binding to synaptotagmin 1 induces its binding to phospholipids and to SNARE complexes, with the latter reaction displacing complexin and resulting in fusion pore opening (step 5). Again, opened fusion pores can then dilate to complete fusion (step 6), although both steps 2 and 5 are potentially reversible, i.e. lack of dilation of the fusion pore could lead to 'kiss-and-run' exocytosis in these pathways. Note that steps 1 and 4 are also probably reversible, with a much faster forward than backward speed. It is likely that step 1 is Ca^{2+}-dependent, but it is unclear whether or not step 2 is Ca^{2+}-dependent since it is possible that asynchronous release is Ca^{2+}-dependent solely because Ca^{2+} accelerates step 1, and step 2 has a finite probability. Thus the nature of Ca^{2+}-triggering of asynchronous release could operate either at the priming or at the actual fusion step.

BIBLIOGRAPHY

Arac, D., Murphy, T., Rizo, J. (2003) Facile detection of protein-protein interactions by one-dimensional NMR spectroscopy. Biochemistry. 42, 2774-80.

Archer, D.A., Graham, M.E., and Burgoyne, R.D. (2002) Complexin regulates the closure of the fusion pore during regulated vesicle exocytosis. J Biol Chem. *277*, 18249-18252.

Antonin, W., Dulubova, I., Arac, D., Pabst, S., Plitzner, J., Rizo, J., Jahn, R. (2002) The N-terminal domains of syntaxin 7 and vti1b form three-helix bundles that differ in their ability to regulate SNARE complex assembly. J Biol Chem. *277*, 36449-56.

Bai, J., Wang, P., Chapman, E.R. (2002) C2A activates a cryptic Ca(2+)-triggered membrane penetration activity within the C2B domain of synaptotagmin I. Proc Natl Acad Sci. *99*, 1665-70.

Bai, J., Wang, C.T., Richards, D.A., Jackson, M.B., Chapman, E.R. (2004) Fusion pore dynamics are regulated by synaptotagmin*t-SNARE interactions. Neuron. *41*, 929-42.

Banerjee, A., Kowalchyk, J.A., DasGupta, B.R., and Martin, T.F. (1996) SNAP-25 is required for a late postdocking step in Ca^{2+}-dependent exocytosis. J. Biol. Chem. *271*, 20227-20230.

Bennett, M.K., Calakos, N., and Scheller, R.H. (1992) Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. Science *257***,** 255-259.

Betz, A., Okamoto, M., Benseler, F., Brose, N. (1997) Direct interaction of the rat unc-13 homologue Munc13-1 with the N terminus of syntaxin. J Biol Chem. *272*, 2520-6.

Bowen, M.E., Weninger, K., Ernst, J., Chu, S., and Brunger, A.T. (2005) Single-molecule studies of synaptotagmin-1 and complexin binding to the SNARE complex. Biophys. J. *89*, 690-702.

Bracher, A., Kadlec, J., Betz, H., and Weissenhorn, W. (2002) X-ray structure of a neuronal complexin-SNARE complex from squid. J. Biol. Chem. *277*, 26517-23.

Brian, A. A., and McConnell, H. M. (1984) Allogeneic stimulation of cytotoxic T cells by supported planar membranes. *Proc. Natl. Acad. Sci. U.S.A. 81*, 6159-63.

Broadie, K., Prokop, A., Bellen, H.J., O'Kane, C.J., Schulze, K.L., Sweeney, S.T. (1995) Syntaxin and synaptobrevin function downstream of vesicle docking in *Drosophila.* Neuron. *15*, 663-673

Brose, N., Petrenko, A.G., Südhof, T.C., Jahn, R. (1992) Synaptotagmin: a calcium sensor on the synaptic vesicle surface. Science. *256*, 1021-5.

Brunger, A.T. (2005) Structure and function of SNARE and SNARE-interacting proteins. Q Rev Biophys. *38*, 1-47.

Calakos, N., Scheller, R.H. (1994) Vesicle-associated membrane protein and synaptophysin are associated on the synaptic vesicle. J Biol Chem. *269*, 24534-7.

Chapman, E.R., and Jahn, R. (1994) Calcium-dependent interaction of the cytoplasmic region of synaptotagmin-1 with membranes. Autonomous function of a single C2-homologous domain. J Biol Chem. *269*, 5735-41.

Chapman, E.R., An, S., Edwardson, J.M., Jahn, R. (1996) A novel function for the second C2 domain of synaptotagmin. Ca2+-triggered dimerization. J Biol Chem. *271*, 5844-9.

Chen, X., Tomchick, D.R., Kovrigin, E., Arac, D., Machius, M., Südhof, T.C., and Rizo, J. (2002) Three-dimensional structure of the complexin/SNARE complex. Neuron *33*, 397-409.

Chen, X., Tang, J., Südhof, T.C., Rizo, J. (2005) Are neuronal SNARE proteins Ca2+ sensors? J Mol Biol. *347*, 145-58.

Chen, X., Arac, D., Wang, T.M., Gilpin, C.J., Zimmerberg, J., and Rizo, J. (2006) SNARE-Mediated Lipid Mixing Depends on the Physical State of the Vesicles. Biophys. J. *90*, 2062-2074.

Dai, H., Shin, O.H., Machius, M., Tomchick, D.R., Südhof, T.C., Rizo, J. (2004) Structural basis for the evolutionary inactivation of Ca2+ binding to synaptotagmin 4. Nat Struct Mol Biol. *11*, 844-9.

Davis, A.F., Bai, J., Fasshauer, D., Wolowick, M.J., Lewis, .JL., Chapman, E.R. (1999) Kinetics of synaptotagmin responses to Ca2+ and assembly with the core SNARE complex onto membranes. Neuron. *24*, 363-76.

Davletov, B.A., and Südhof, T.C. (1993) A single C2 domain from synaptotagmin I is sufficient for high affinity Ca2+/phospholipid binding. J Biol Chem. *268*, 26386-90.

Deak, F., Schoch, S., Liu, X., Südhof, T.C., and Kavalali, E.T. (2004) Synaptobrevin is essential for fast synaptic-vesicle endocytosis. Nat. Cell Biol. *6*, 1102-8.

Dulubova, I., Sugita, S., Hill, S., Hosaka, M., Fernandez, I., Sudhof, T.C., Rizo, J. (1999) A conformational switch in syntaxin during exocytosis: role of munc18. EMBO J. *18*, 4372-82.

Dykes, I.M., and Davies, J.A. (2004) Cloning and expression of a leech complexin. Gene Expr. Patterns *4*, 93-97.

Edelmann, L., Hanson, P.I., Chapman, E.R., Jahn, R. (1995) Synaptobrevin binding to synaptophysin: a potential mechanism for controlling the exocytotic fusion machine. EMBO J. 14, 224-31.

Ernst, J.A., Brunger, A.T. (2003) High resolution structure, stability, and synaptotagmin binding of a truncated neuronal SNARE complex. J Biol Chem. *278*, 8630-6.

Fasshauer, D., Eliason, W.K., Brunger, A.T., Jahn, R. (1998) Identification of a minimal core of the synaptic SNARE complex sufficient for reversible assembly and disassembly. Biochemistry. *37*, 10354-62.

Fasshauer, D., Sutton, R.B., Brunger, A.T., Jahn, R. (1998) Conserved structural features of the synaptic fusion complex: SNARE proteins reclassified as Q- and R-SNAREs. Proc Natl Acad Sci U S A. *95*, 15781-6.

Fernandez, I., Ubach, J., Dulubova, I., Zhang, X., Südhof, T.C., Rizo, J. (1998) Three-dimensional structure of an evolutionarily conserved N-terminal domain of syntaxin 1A. Cell. *94*, 841-9.

Fernandez, I., Arac, D., Ubach, J., Gerber, S.H., Shin, O., Gao, Y., Anderson, R.G.W., Südhof, T.C.,and Rizo, J. (2001) Three-dimensional structure of the synaptotagmin 1 C_2B-domain: Synaptotagmin 1 as a phospholipid binding machine. Neuron *32*, 1057-1069.

Fernandez-Chacon, R., Konigstorfer, A., Gerber, S.H., Garcia, J., Matos, M.F., Stevens, C.F., Brose, N., Rizo, J, Rosenmund, C., and Südhof T.C. (2001) Synaptotagmin I functions as a calcium regulator of release probability. Nature *410*, 41-49.

Fernandez-Chacon, R., Shin, O.H., Konigstorfer, A., Matos, M.F., Meyer, A.C., Garcia, J., Gerber, S.H., Rizo, J., Südhof, T.C., Rosenmund, C. (2002) Structure/function analysis of Ca2+ binding to the C2A domain of synaptotagmin 1. J Neurosci. *22*, 8438-46.

Frazier, A.A., Roller, C.R., Havelka, J.J., Hinderliter, A., Cafiso, D.S. (2003) Membrane-bound orientation and position of the synaptotagmin I C2A domain by site-directed spin labeling. Biochemistry. *42*, 96-105.

Geppert, M., Goda, Y., Hammer, R.E., Li, C., Rosahl, T.W., Stevens, C.F., and Südhof, T.C. (1994) Synaptotagmin I: A major Ca^{2+} sensor for transmitter release at a central synapse. Cell *79*, 717-727.

Gerber, S.H., Rizo, J., Südhof, T.C. (2002) Role of electrostatic and hydrophobic interactions in ca(2+)-dependent phospholipid binding by the c(2)a-domain from synaptotagmin I. Diabetes *51* Suppl 1, S12–18.

Giraudo, C.G., Eng, W.S., Melia, T.J., and Rothman, J.E. (2006) A Clamping Mechanism Involved in SNARE-Dependent Exocytosis. Science. *313*, 676-80.

Goda, Y., and Stevens, C.F. (1994) Two components of transmitter release at a central synapse. Proc. Natl. Acad. Sci. U. S. A. *91*, 12942-12946.

Hayashi, T., McMahon, H., Yamasaki, S., Binz, T., Hata, Y., Südhof, T.C., Niemann, H. (1994) Synaptic vesicle membrane fusion complex: action of clostridial neurotoxins on assembly. EMBO J. *13*, 5051-61.

Herrick, D.Z., Sterbling, S., Rasch, K.A., Hinderliter, A., Cafiso, D.S. (2006) Position of synaptotagmin I at the membrane interface: cooperative interactions of tandem C2 domains. Biochemistry. *45*, 9668-74.

Hill, J.J., Callaghan, D.A., Ding, W., Kelly, J.F., and Chakravarthy, B.R. (2006) Identification of okadaic acid-induced phosphorylation events by a mass spectrometry approach. Biochem. Biophys. Res. Commun. *342*, 791-799.

Hu, C., Ahmed, M., Melia, T.J., Sollner, T.H., Mayer, T., Rothman, J.E. (2003) Fusion of cells by flipped SNAREs. Science. *300*, 1745-9.

Hu, K., Carroll, J., Fedorovich, S., Rickman, C., Sukhodub, A., Davletov, B. (2002) Vesicular restriction of synaptobrevin suggests a role for calcium in membrane fusion. Nature. *415*, 646-50.

Hu, K., Carroll, J., Rickman, C., and Davletov, B. (2002) Action of complexin on SNARE complex. J. Biol. Chem. *277*, 41652-41656.

Hui, E., Bai, J., Chapman, E.R. (2006) Ca2+-triggered simultaneous membrane penetration of the tandem C2-domains of synaptotagmin I. Biophys J. *91*, 1767-77.

Ishizuka, T., Saisu, H., Odani, S., and Abe, T. (1995) Synaphin: a protein associated with the docking/fusion complex in presynaptic terminals. Biochem. Biophys. Res. Commun. *213*, 1107-1114.

Itakura, M., Misawa, H., Sekiguchi, M., Takahashi, S., and Takahashi, M. (1999) Transfection analysis of functional roles of complexin I and II in the exocytosis of two different types of secretory vesicles. Biochem Biophys Res Commun. *265*, 691-696.

Jahn, R., Südhof, T.C. (1999) Membrane fusion and exocytosis. Annu Rev Biochem. *68*, 863-911.

Jahn, R., Lang, T., Südhof, T.C. (2003) Membrane fusion. Cell. 112, 519-33.

Katz, B. (1969) The release of neural transmitter substances. *Liverpool: Liverpool University Press*

Kee, Y., Lin, R.C., Hsu, S.C., Scheller, R.H. (1995) Distinct domains of syntaxin are required for synaptic vesicle fusion complex formation and dissociation. Neuron. *14*, 991-8.

Kee Y, Scheller RH. (1996) Localization of synaptotagmin-binding domains on syntaxin. J Neurosci. *16*, 1975-81.

Li, C., Davletov, B.A., Südhof, T.C. (1995) Distinct Ca2+ and Sr2+ binding properties of synaptotagmins. Definition of candidate Ca2+ sensors for the fast and slow components of neurotransmitter release. J Biol Chem. *270*, 24898-902.

Li, C., Ullrich, B., Zhang, Z.Z., Anderson, R.G.W., Brose, N., and Südhof, T.C. (1995) Ca^{2+}-dependent and Ca^{2+}-independent activities of neural and nonneural synaptotagmins. Nature *375*, 594-599.

Littleton, J.T., Bai, J., Vyas, B., Desai, R., Baltus, A.E., Garment, M.B., Carlson, S.D., Ganetzky, B., Chapman, E.R. (2001) synaptotagmin mutants reveal essential functions for the C2B domain in Ca2+-triggered fusion and recycling of synaptic vesicles in vivo. J Neurosci. *21*, 1421-33.

Lu, T., and Trussell, L.O. (2000) Inhibitory transmission mediated by asynchronous transmitter release. Neuron *26*, 683-694.

Mackler, J.M., Drummond, J.A., Loewen, C.A., Robinson, I.M., Reist, N.E. (2002) The C(2)B Ca(2+)-binding motif of synaptotagmin is required for synaptic transmission in vivo. Nature. *418*, 340-4.

Matthew, W. D., Tsavaler, L., and Reichardt, L. F. (1981) Identification of a synaptic vesicle-specific membrane protein with a wide distribution in neuronal and neurosecretory tissue. J. Cell Biol. *91*, 257-269

Maximov, A., and Südhof, T.C. (2005) Autonomous function of synaptotagmin-1 1 in triggering asynchronous release independent of asynchronous release. Neuron *48*, 547-554.

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

McNew, J.A., Parlati, F., Fukuda, R., Johnston, R.J., Paz, K., Paumet, F., Sollner, T.H., Rothman, J.E. (2000) Compartmental specificity of cellular membrane fusion encoded in SNARE proteins. Nature. *407*, 153-9.

Misura, K.M., Scheller, R.H., Weis, W.I. (2000) Three-dimensional structure of the neuronal-Sec1-syntaxin 1a complex. Nature. *404*, 355-62.

Misura, K.M., Scheller, R.H., Weis, W.I. (2001) Self-association of the H3 region of syntaxin 1A. Implications for intermediates in SNARE complex assembly. J Biol Chem. *276*, 13273-82

Misura, K.M., Gonzalez, LC Jr, May, A.P., Scheller, R.H., Weis, W.I. (2001) Crystal structure and biophysical properties of a complex between the N-terminal SNARE region of SNAP25 and syntaxin 1a. J Biol Chem. *276*, 41301-9.

Montes, M., Fuson, K.L., Sutton, R.B., Robert, J.J. (2006) Purification, crystallization and X-ray diffraction analysis of human synaptotagmin 1 C2A-C2B. Acta Crystallograph Sect F Struct Biol Cryst Commun. *62*, 926-9.

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. Eur. J. Neurosci. *10*, 2143-2152.

Pabst, S., Hazzard, J.W., Antonin, W., Südhof, T.C., Jahn, R., Rizo, J., Fasshauer, D. (2000) Selective interaction of complexin with the neuronal SNARE complex. Determination of the binding regions. J Biol Chem. *275*, 19808-18.

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*, 7838-48.

Pang, Z.P., Sun, J., Rizo, J., Maximov, A., Südhof, T.C. (2006) Genetic analysis of synaptotagmin 2 in spontaneous and Ca2+-triggered neurotransmitter release. EMBO J. *25*, 2039-50.

Parlati, F., McNew, J.A., Fukuda, R., Miller, R., Sollner, T.H., Rothman, J.E. (2000) Topological restriction of SNARE-dependent membrane fusion. Nature. 407, 194-8.

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.

Perin, M.S., Brose, N., Jahn, R., Südhof, T.C. (1991) Domain structure of synaptotagmin (p65) J Biol Chem. *266*, 623-9.

Poirier, M.A., Xiao, W., Macosko, J.C., Chan, C., Shin, Y.K., Bennett, M.K. (1998) The synaptic SNARE complex is a parallel four-stranded helical bundle. Nat Struct Biol. *5*, 765-9.

Poskanzer, K.E., Marek, K.W., Sweeney, S.T., Davis, G.W. (2003) Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis in vivo. Nature. *426*, 559-63.

Reim, K., Mansour, M., Varoqueaux, F., McMahon, H.T., Südhof, T.C., Brose, N., and Rosenmund, C. (2001) Complexins regulate a late step in Ca^{2+}-dependent neurotransmitter release. Cell *104*, 71-81.

Reim, K., Wegmeyer, H., Brandstatter, J.H., Xue, M., Rosenmund, C., Dresbach, T., Hofmann, K., Brose, N. (2005) Structurally and functionally unique complexins at retinal ribbon synapses. J Cell Biol. *169*, 669-80.

Rhee, J.S., Li, L.Y., Shin, O.H., Rah, J.C., Rizo, J., Südhof, T.C., and Rosenmund, C. (2005) Augmenting neurotransmitter release by enhancing the apparent Ca^{2+} affinity of synaptotagmin-1 1. Proc Natl Acad Sci U S A. *102*, 18664-9.

Rickman, C., Davletov, B. (2003) Mechanism of calcium-independent synaptotagmin binding to target SNAREs. J Biol Chem. *278*, 5501-4.

Rickman, C., Archer, D.A., Meunier, F.A., Craxton, M., Fukuda, M., Burgoyne, R.D., and Davletov, B. (2004) Synaptotagmin-1 interaction with the syntaxin/SNAP-25 dimer is mediated by an evolutionarily conserved motif and is sensitive to inositol hexakisphosphate. J. Biol. Chem. *279*, 12574-12579.

Rizo, J., Südhof, T.C. (2002) Snares and Munc18 in synaptic vesicle fusion. Nat Rev Neurosci. *3*, 641-53.

Robinson, I.M., Ranjan, R., Schwarz, T.L. (2002) Synaptotagmins I and IV promote transmitter release independently of Ca(2+) binding in the C(2)A domain. Nature. *418*, 336-40.

Rufener, E., Frazier, A.A., Wieser, C.M., Hinderliter, A., Cafiso, D.S. (2005) Membrane-bound orientation and position of the synaptotagmin C2B domain determined by site-directed spin labeling. Biochemistry. *44*, 18-28.

Schaub, J.R., Lu, X., Doneske, B., Shin, Y.K., and McNew, J.A. (2006) Hemifusion arrest by complexin is relieved by Ca^{2+}-synaptotagmin I. Nat. Struct. Mol. Biol., *13*, 748-750

Schiavo, G., Stenbeck, G., Rothman, J.E., and Söllner, T.H. (1997) Binding of the synaptic vesicle v-SNARE, synaptotagmin, to the plasma membrane t-SNARE, SNAP-25, can explain docked vesicles at neurotoxin-treated synapses. Proc. Natl. Acad. Sci. U.S.A. *94*, 997-1001.

Schlüter, O., Schmitz, F., Jahn, R., Rosenmund, C., and Südhof, T.C. (2004) A complete genetic analysis of neuronal Rab3 function. J. Neurosci. *24*, 6629-6637.

Schoch, S., Deak, F., Konigstorfer, A., Mozhayeva, M., Sara, Y., Südhof, T.C., Kavalali, E.T. (2001) SNARE function analyzed in synaptobrevin/VAMP knockout mice. Science. *294*, 1117-22.

Shao, X., Davletov, B.A., Sutton, R.B., Südhof, T.C., Rizo, J. (1996) Bipartite Ca2+-binding motif in C2 domains of synaptotagmin and protein kinase C. Science. *273*, 248-51.

Shao, X., Li, C., Fernandez, I., Zhang, X., Südhof, T.C., Rizo J. (1997) Synaptotagmin-syntaxin interaction: the C2 domain as a Ca2+-dependent electrostatic switch. Neuron. *18*, 133-42.

Shin, O.H., Rizo, J., Südhof, T.C. (2002) Synaptotagmin function in dense core vesicle exocytosis studied in cracked PC12 cells. Nat Neurosci. *5*, 649-56.

Shin, O.H., Rhee, J.S., Tang, J., Sugita, S., Rosenmund, C., Südhof, T.C. (2003) Sr2+ binding to the Ca2+ binding site of the synaptotagmin 1 C2B domain triggers fast exocytosis without stimulating SNARE interactions. Neuron. *37*, 99-108.

Söllner, T., Bennett, M.K., Whiteheart, S.W., Scheller, R.H., and Rothman, J.E. (1993) A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion. Cell *75*, 409-418.

Sorensen, J.B. (2005) SNARE complexes prepare for membrane fusion. Trends Neurosci. *28*, 453-455.

Südhof, T.C., De Camilli, P., Niemann, H., Jahn, R. (1993) Membrane fusion machinery: insights from synaptic proteins. Cell. *75*, 1-4.

Südhof, T.C. (2004) The synaptic vesicle cycle. Annu. Rev. Neurosci. *27*, 509-547.

Sugita, S., Hata, Y., Südhof, T.C. (1996) Distinct Ca(2+)-dependent properties of the first and second C2-domains of synaptotagmin I. J Biol Chem. *271*, 1262-5.

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 A resolution. Nature. *395*, 347-353.

Tadokoro, S., Nakanishi, M., Hirashima, N. (2005) Complexin II facilitates exocytotic release in mast cells by enhancing Ca2+ sensitivity of the fusion process. J Cell Sci. *118*, 2239-46.

Takahashi, S., Yamamoto, H., Matsuda Z., Ogawa, M., Yagyu, K., Taniguchi, T., Miyata, T., Kaba, H., Higuchi, T., Okutani, F. and Fujimoto, S. (1995) Identification of two highly homologous presynaptic proteins distinctly localized at the dendritic and somatic synapses. FEBS Lett. 368, 455-60.

Tang, J., Maximov, A., Shin, O.H., Dai, H., Rizo, J., Südhof, T.C. (2006) A complexin/synaptotagmin 1 switch controls fast synaptic vesicle exocytosis. Cell. *126*, 1175-87.

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.

Trommershauser, J., Schneggenburger, R., Zippelius, A., Neher, E. (2003) Heterogeneous presynaptic release probabilities: functional relevance for short-term plasticity. Biophys J. *84*, 1563-79.

Tucker, W.C., Weber, T., Chapman, E.R. (2004) Reconstitution of Ca2+-regulated membrane fusion by synaptotagmin and SNAREs. Science. 304, 435-8.

Ubach, J., Zhang, X., Shao, X., Südhof, T.C., Rizo, J. (1998) Ca2+ binding to synaptotagmin: how many Ca2+ ions bind to the tip of a C2-domain? EMBO J. 17, 3921-30.

Ubach, J., Lao, Y., Fernandez, I., Arac, D., Südhof, T.C., Rizo, J. (2001) The C2B domain of synaptotagmin I is a Ca2+-binding module. Biochemistry. *40*, 5854-60.

Ungermann, C., Sato, K., Wickner, W. (1998) Defining the functions of trans-SNARE pairs. Nature. *396*, 543-8.

Weber, T., Zemelman, B.V., McNew, J.A., Westermann, B., Gmachl, M., Parlati, F., Sollner, T.H., Rothman, J.E. (1998) SNAREpins: minimal machinery for membrane fusion. Cell. 92, 759-72.

Xia, Y. and Whitesides, G. (1998) Soft lithography Annu. Rev. Mater. Sci. *28*, 153-184.

Xiao, W., Poirier, M.A., Bennett, M.K., Shin, Y.K. (2001) The neuronal t-SNARE complex is a parallel four-helix bundle. Nat Struct Biol. *8*, 308-11.

Xu, T., Rammner, B., Margittai, M., Artalejo, A.R., Neher, E., Jahn R. (1999) Inhibition of SNARE complex assembly differentially affects kinetic components of exocytosis. Cell. *99*, 713-22.

Yamada, M., Saisu, H., Ishizuka, T., Takahashi, H., Abe, T. (1999) Immunohistochemical distribution of the two isoforms of synaphin/complexin involved in neurotransmitter release: localization at the distinct central nervous system regions and synaptic types. Neuroscience. *93*, 7-18.

Yang, B., Steegmaier, M., Gonzalez, L.C. Jr, Scheller, R.H. (2000) nSec1 binds a closed conformation of syntaxin1A. J Cell Biol. *148*, 247-52.

Yoshida, A., Oho, C., Omori, A., Kuwahara, R., Ito T., and Takahashi, M. (1992) HPC-1 is associated with synaptotagmin-1 and omega-conotoxin receptor. J. Biol. Chem. *267*, 24925-24928

Zhang, X., Kim-Miller, M.J., Fukuda, M., Kowalchyk, J.A., Martin, T.F. (2002) Ca2+-dependent synaptotagmin binding to SNAP-25 is essential for Ca2+-triggered exocytosis. Neuron. *34*, 599-611.

VITAE

Jiong Tang was born in KunShan, JiangSu, China on April 14, 1976, the second son of Haifeng Tang and Yuefang Zhang. After completing his work in KunShan High School, JiangSu in 1993, he entered Fudan University at ShangHai. He received the degree of Bachelor of Science with a major in Biochemistry in June, 1997. During the following three years he was a graduate student in the Institute of Genetics in Fudan Universtiy and was awarded the degree of Master of Science in June, 2000. Since August 2000, he is a graduate student for Ph.D. degree in UT Southwestern Medical Center at Dallas.

Permanent Address: Rm 502. No. 26 South Renmin Rd.
KunShan/JiangSu 215300
People's Republic of China