CHARACTERIZATION OF SYNAPTOTAGMIN 1 FUNCTION IN CALCIUM DEPENDENT NEURONAL EXOCYTOSIS

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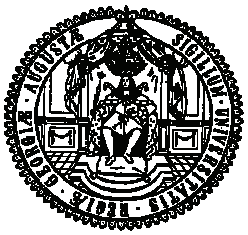

Göttingen, der 20. März 2007

Referent: Prof. Reinhard Jahn

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Eidesstattliche Erklärung:

Hiermit erkläre ich an Eides Statt, dass ich die vorliegende Arbeit: “Characterization of Synaptotagmin 1Function in Neuronal Exocytosis“ selbständig verfasst ohne unerlaubte Hilfe angefertigt habe.

Göttingen, 20. März 2007

Anand Radhakrishnan

For my parents…

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The work presented here was only possible due to contributions from many people both in the lab as well as outside.

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Table of Content

List of Figures & Tables

Introduction

Methods

Results

Discussion

Appendix 1

Appendix 2

Abbreviations

aa.	amino acid
BoNT	Botulinum Neurotoxin
CFP	Cyan Fluorescent Protein
DPTA	Diethylenetriaminepentaacetic Acid
DsRed	Discosoma Red (Fluorescence dye)
DTT	Dithiothreitol
EC_{50}	Median Effective Concentration (required to induce a 50% effect)
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
EPR	Electro-paramagnetic Resonance
FPLC	Fast Protein Liquid Chromatography
FRET	Fluoroscence Resonance Energy Transfer
GST	Glutathione-S-Transferase
His6	Hexa-histidine tag
HSQC	Heteronuclear Single Quantum Correlation
IAANS	2-(4'-(iodoacetamido)anilino)naphthalene-6-sulfonic acid
IAEDANS	5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid
ITC	Isothermal Titration Claorimetry
kDa	kilodalton
kT/e	Electrostatic Potential Unit
NMR	Nuclear Magnetic Resonance
O.D.	Optical Density
PAGE	Polyacrylamide Gel Electrophoresis
PC	Phosphatidylcholine
PC12	Phaeochromocytoma 12 cells
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PE	Phosphatidylethanolamine
pI	Isoelectric point
PIP_2	Phosphatidylinositol-(4,5)bis-phosphate

PKC	Protein Kinase C
PMSF	Phenylmethanesulfonyl Fluoride
PS	Phosphatidylserine
rpm	rotations per minute
SDS	Sodium Dodecylsulphate
SNAP 25	Synaptosomal Associated Protein, 25kDa
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptor
SV	Synaptic vesicle
TCEP	Tris[2-carboxyethyl] phosphine
TMD	Transmembrane Domain
TRPE	Texas red Phosphatidylethanolamine
YFP	Yellow Fluorescence Protein

Abstract

Synaptotagmin 1 is a synaptic vesicle protein believed to be the calcium sensor at the neuronal synapse. The protein consists of two calcium dependent membrane binding domains known as the C2 domains. Previous studies have shown that deletion of Synaptotagmin 1 results in a loss of calcium dependent secretion at the synaptic terminal. More recently, much work has focused on unravelling the molecular mechanism of Synaptotagmin 1 function. However, due to its highly dynamic and fast action, the biochemical assays required to elucidate its precise function have remained somewhat limited. Conventional methods, such as GST pulldowns and immunoprecipitation lack sufficient resolution to capture the protein "in action". Currently, it is hypothesised that upon calcium influx into the presynaptic terminal, Synaptotagmin rapidly binds to lipid membrane resulting in the fusion of the synaptic vesicle with the plasma membrane. Recent evidence has also suggested that Synaptotagmin binds to the SNARE complex. The SNARE proteins are a superfamily of proteins that drive membrane fusion. There are two classes of SNARE proteins, the Q-SNARE and the R-SNARE, which forms a four helical bundle, required for the merger of opposing membranes. However, the role and mechanism of the SNARE complex interaction with Synaptotagmin remains unclear. Another SNARE-interacting protein that has been implicated in calcium dependent exocytosis is Complexin. The exact interplay between Complexin and Synaptotagmin is largely unknown although Complexin has been suggested to regulate the interaction of Synaptotagmin with the SNARE complex. Given as such, the major aim of this project was to develop biochemical tools to enable a more precise characterisation of the molecular mechanism of Synaptotagmin action. Due to the inherent functional complexity of the protein, assays that could better define the distinct interactions of Synaptotagmin with its binding partners were developed. The first section of this dissertation focuses on the calcium binding to the C2 domains. This aspect was explored using a method known as isothermal titration calorimetry (ITC). ITC allows the direct determination of parameters such as affinity (K), enthalpy (ΔH), entropy (ΔS) and Gibbs free energy (ΔG) using a single titration experiment. By employing this method, calcium titration into Synaptotagmin revealed that the two C2 domains of Synaptotagmin (namely C2A and C2B) binds calcium with divergent mechanisms and are independent of each other. Furthermore, no additional weak calcium binding sites were evident when calcium was

titrated to the C2 domains rendered inactive by site-directed mutagenesis. The second section of this thesis details the use a fluorescence resonance energy transfer (FRET)-based method used to study the binding of Synaptotagmin to liposome membranes. This approach was employed to examine the potential effects of lipid composition and phosphatidylserine (PS) density on Synaptotagmin binding. Interestingly, increasing the amount of PS in the liposome membrane appeared to correspond with an increase the affinity of Synaptotagmin to PS hinting that microdomains might play a role in Synaptotagmin function. The final section of the thesis attempts to further clarify the interaction of Synaptotagmin with the membrane fusion machinery, i.e. SNARE complex. This was achieved using complementary gel-based and fluorescence-based assays to probe the function of the Synaptotagmin – SNARE interaction. Based on the results presented herein, the binding of Synaptotagmin to SNAREs was exclusively observed for both the binary and ternary SNARE complex. In contrast to previous reports, binding of Synaptotagmin to individual Q-SNARE proteins was not detected under the assay conditions outlined in this study. It has also been suggested that Synaptotagmin might play a role in assisting SNARE complex assembly. However based on the findings presented in this study it appears highly unlikely given that the addition of Synaptotagmin both in the absence and presence of calcium does not significantly affect the kinetics of SNARE complex assembly. Finally, the potential role of Complexin in the interplay between Synaptotagmin and the SNARE complex was studied using a FRET-based assay. Synaptotagmin was able to bind to the SNARE complex in the presence of Complexin, but only in the presence of calcium. In the absence of calcium, the interaction between Synaptotagmin and the SNARE complex was abolished. Collectively, the findings of this thesis provide important new insights into the function of Synaptotagmin. However, future work remains to elucidate the exact molecular details of Synaptotagmin action in calcium-dependent exocytosis.

Kurzfassung

Es wird vermutet, dass das Protein Synaptotagmin 1, das in der Membrane des synaptischen Vesikels sitzt, der Kalziumsensor in der neuronalen Synapse ist. Synaptotagmin 1 hat zwei sogenannte C2 Domänen, die kalziumabhängig Membranen binden. Es ist gezeigt worden, dass die Deletion von Synaptotagmin zum Verlust der kalziumabhängigen Sekretion an der Synapse führt. In letzter Zeit haben sich Arbeiten darauf konzentriert den molekularen Wirkungsmechanismus von Synaptotagmin aufzuklären. Die hochdynamische und schnelle Wirkweise dieses Proteins stellt jedoch hohe Anforderungen an biochemische Methoden zu seiner Untersuchung, die von konventionelle Methoden ohne zeitliche Auflösung wie GST-Bindungsstudien und Immunuopräzipitation nicht erfüllt werden. Zur Zeit wird eine Hypothese favorisiert, nach der der Einstrom von Kalzium in die präsynaptische Zelle die schnelle Bindung von Synaptotagmin an die Lipidmembran bewirkt. Dies wiederum führt zur Fusion des synaptischen Vesikels mit der Plasmamembran. Daneben wurde auch gezeigt, dass Synaptotagmin an den neuronalen SNARE-Komplex bindet. SNARE Proteine bilden eine Proteinsuperfamile, die an der Membranfusion beteiligt ist. Sie wird in zwei Klassen unterteilt, die Q-SNAREs und die R-SNAREs, die das für das Verschmelzen zweier Membranen benötigte Vier-Helix-Bündel bilden. Die Funktion der Interaktion Synaptotagmins mit dem SNARE-Komplex ist jedoch weitgehend ungeklärt. Complexine bilden eine weitere Familie von Proteinen, die mit dem neuronalen SNARE-Komplex interagieren and an der Regulation der kalziumabhängigen Exozytose beteiligt sind. Das Zusammenspiel von Complexin und Synaptotagmin ist weitgehend unverstanden, auch wenn vorgeschlagen wurde, dass Complexin die Interaktion von Synaptotagmin mit dem SNARE-Komplex reguliert. Der Hauptzweck dieser Arbeit ist daher die Entwicklung biochemischer Methoden zur genaueren Charakterisierung des molekularen Wirkungsmechanismus von Synaptotagmin. Wegen der funktionellen Komplexität Synaptotagmins wurden Methoden entwickelt, die es erlauben, Interaktionen mit verschiedenen Bindungspartnern getrennt voneinander zu betrachten. Im ersten Abschnitt dieser Dissertation beschäftige ich mich mit der Kalziumbindung durch die C2 Domänen. Dieser Aspekt wurde durch isothermale Titrationskalorimetrie (ITC) untersucht. ITC erlaubt die direkte Bestimmung thermodynamischer Parameter einer Interaktion wie der Affinität, der Enthalpy, der Entropie und der Freien Enthalpy aus einem einzigen

Titrationsexperiment. Durch Anwendung dieser Methode wurde gezeigt, dass die beiden C2 Domänen Synaptotagmins (genannt C2A und C2B) Kalzium über verschiedene Mechanismen binden und voneinander unabhängig agieren. In Experimenten mit Mutanten von Synaptotagmin, in denen die bekannten Kalziumbindungsstellen mutiert waren, wurde außerdem gezeigt, dass Synaptotagmin keine weiteren Kalziumbindungsstellen geringerer Affinität aufweist. Der zweite Abschnit behandelt Untersuchungen zur Bindung von Synaptotagmin an Liposomenmembranen. Dazu wurden Methoden verwendet, die auf dem Phänomen des *fluorescence resonance energy transfer* (FRET) beruhen. Es wurde der Einfluß der Lipidkomposition und der Dichte von Phosphatidylserin (PS) auf die Bindung von Synaptotagmin untersucht. Dabei wurde festgestellt, dass eine Erhöhung der PS-Menge in der Liposomenmebran zu einer erhöhten Affinität für die Bindung von Synaptotagmin führt. Dies ist ein Hinweis dafür, dass die Bildung von Mikrodomänen eine Rolle bei der Interaktion von Synaptotagmin mit der Membran spielen könnte. Im letzten Abschnitt wurde versucht, das Zusammenspiel von Synaptotagmin mit der Fusionmaschinerie, den SNARE Proteinen, näher zu beleuchten. Um diese Interaktion zu untersuchen, wurden gelelektrophoretische und fluoreszenzspektroskopische Methoden verwendet. Dabei wurde gezeigt, dass Synaptotagmin ausschließlich mit binären und ternären SNARE Komplexen interagiert. Frühere Untersuchungen konnten nicht bestätigt werden, in denen eine Bindung von Synaptotagmin an einzelne Q-SNAREs gezeigt worden war. Auch die Behauptung, Synaptotagmin würde bei der Assemblierung der SNARE Proteine assistieren, konnte nicht bestätigt werden: Synaptotagmin beeinflusste die Kinetik der Assemblierung weder in der Gegenwart noch in Abwesenheit von Kalzium. Zuletzt wurde der Einfluß von Complexin auf die Synaptotagmin-SNARE Interaktion durch FRET-basierte Methoden untersucht. Es wurde gezeigt, dass Synaptotagmin den SNARE Komplex in der Gegenwart von Complexin bindet, jedoch nur bei Anwesenheit von Kalzium. In Abwesenheit von Kalzium interagierte Synaptotagmin hingegen nicht mit dem SNARE Komlex, wenn Complexin zugegen war . Diese Arbeit präsentiert wichtige neue Einblicke in die Funktionsweise von Synaptotagmin. Zukünftige Arbeiten müssen sich nun mit der Aufklärung des exakten molekularen Wirkungsmechanismus von Synaptotagmin bei der kalziumabhägigen Exozytose beschäftigen.

1. Introduction

1.1 Calcium-dependent exocytosis and the hunt for a neuronal calcium sensor

Electrical signals conducted through the axon of a neuron allow the fast transmission of information through the neural network. However, the gap between two neurons inhibits this electrical signal propagation. This gap, also known as the synapse, uses chemical-based signal transducers, known as neurotransmitters to transfer information in discreet quanta from one neuron to the other (9).

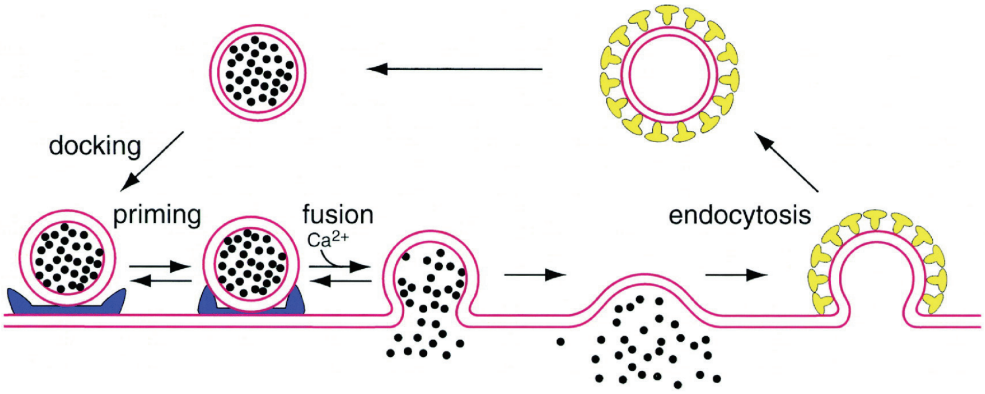


Figure I1: **The Synaptic Vesicle (SV) Cycle.** Adapted from (1).

Neurotransmitters are sequestered into synaptic vesicles (SV), which are 30-50nm in size and dock in a specialized region called the active zone at the pre-synaptic plasma membrane (10). At the active zone, the SVs undergo a series of maturation steps to make them ready for fusion. These steps are collectively known as priming (11). The fusion of the vesicle is triggered by the influx of Ca^{2+} ions controlled by voltage-activated-calcium channels. Since calcium plays an important role in the final exocytotic step, calcium sensing is an important regulatory factor in the SV membrane fusion event (9). The putative protein regulator for this event and the founding member of the Synaptotagmin family of proteins is a synaptic vesicle protein called Synaptotagmin 1. Its importance in calcium dependent neuronal exocytosis became evident when genetic-based tools were used to delete the gene encoding this protein.

1.2 Genetic-based analysis of Synaptotagmin 1

Synaptotagmin 1 was first discovered in 1981 when a monoclonal antibody raised against the synaptic junctional complex detected a 65 kDa antigen, which was localized to the synaptic vesicle (12) and later, to the large dense-core vesicles in PC12 cells (13). However, the identity of this antigen remained obscure until it was sequenced and named Synaptotagmin 1 (14).

In *Drosophila*, a mutation in the gene that causes the disruption of calcium sensing (*AD3* mutant; Y334N), showed a significantly inhibited calcium induced secretion at the synapse (15). *C. elegans* Synaptotagmin 1 deletion mutants also showed a similar phenotype as its *Drosophila* counterpart. Exocytosis in this *C. elegans* mutant was inhibited, causing an accumulation of acetylcholine. The mutants also showed severe behavioural abnormalities in its locomotion, feeding and defecation, indicative of a disturbed synaptic function. However, the mutants were still capable of coordinated motor movements showing that neurotransmitter release was not completely abolished (16).

Exocytosis in the mouse knockout was studied using electrophysiology and amperometry. The former measures the increase in the membrane surface area and the latter measures the amount of the neurotransmitter released and the kinetics of the release. Using these methods, the calcium dependent release was observed to consist of an initial exocytotic burst (synchronous) and more sustained phase (asynchronous). When the Synaptotagmin gene was deleted, the synchronous release was completely abolished but the sustained phase remained. The total number of vesicles and also the total amount of neurotransmitter released remained similar to the wild- type, confirming that the phenotype observed was only due to a defect in the release and not in the pre-exocytotic (i.e. docking and priming) stages of exocytosis (17).

1.3 Domain Structure of Synaptotagmin 1

The Synaptotagmin family of proteins are defined as proteins that contain an N-terminal transmembrane domain (TMD), a variable linker and two C-terminal C2 domains (18) (19). Currently there are, based on database searches, 16 isoforms of this protein identified in humans and mouse (19). Here, we focus our attention on the first

isoform of this family of proteins, Synaptotagmin 1, hereby referred to as Synaptotagmin, since this isoform plays an important role in SV exocytosis.

1.3.1 C2 domains

The C2 domains of Synaptotagmin have been described as the functional end of the protein. C2 domains are common modules of 130-140 residues that were initially defined as the "second constant sequence" in protein kinase C isoforms (20). Atomic structures at 1.9 Å revealed that the C2 domains are composed of a single Greek-key motif organized as an eight-stranded anti-parallel β-sandwich with flexible loops on top and at the bottom (21). There are two topologies of C2 domains classified based on the connectivity of the β-strands. The Synaptotagmin C2 domains are classified as type 1 C2 domains (22). However, the different topologies of the C2 domains have yet to be shown to have any regulatory or functional importance (23).

C2 domains, in general, have been described to be able to bind negatively charged lipid membrane in a calcium-dependent manner (reviewed in (23)). The calcium ions bind in a cup-shaped depression formed by the N- and C-terminal loops of the C2 key motifs (24). This calcium binding sites are rather unusual because the residues that coordinate the binding are widely separated in the primary sequence and the coordination spheres for the ions are incomplete (25) (26). This incomplete coordination sphere can be occupied by anionic phospholipids via a calcium bridge (27) (28). Based on NMR data, calcium binding on the C2A domain of Synaptotagmin is coordinated by amino acid residues, D172, D178, D230, D232, S235 and D238, which bind three calcium ions (5). The C2B calcium binding is coordinated by amino acids D303, D309, D363, D365 and D371 and binds two calcium ions (6) (Figure I2, I3 & I4). By titrating calcium into the C2 domains and measuring the changes in the chemical shift of the calcium coordinating residues in a Heteronuclear Single Quantum Correlation (HSQC) (^{15}N-^{1}H correlation) experiment, the apparent affinity for calcium of these C2 domains were calculated. The C2A domain had an apparent affinity of ~60-75 μM, ~400-500 μM and more than 1 mM for the three calcium binding sites respectively (5). The two calcium binding sites of C2B had similar intrinsic calcium affinities, which are ~300-600 μM (6).

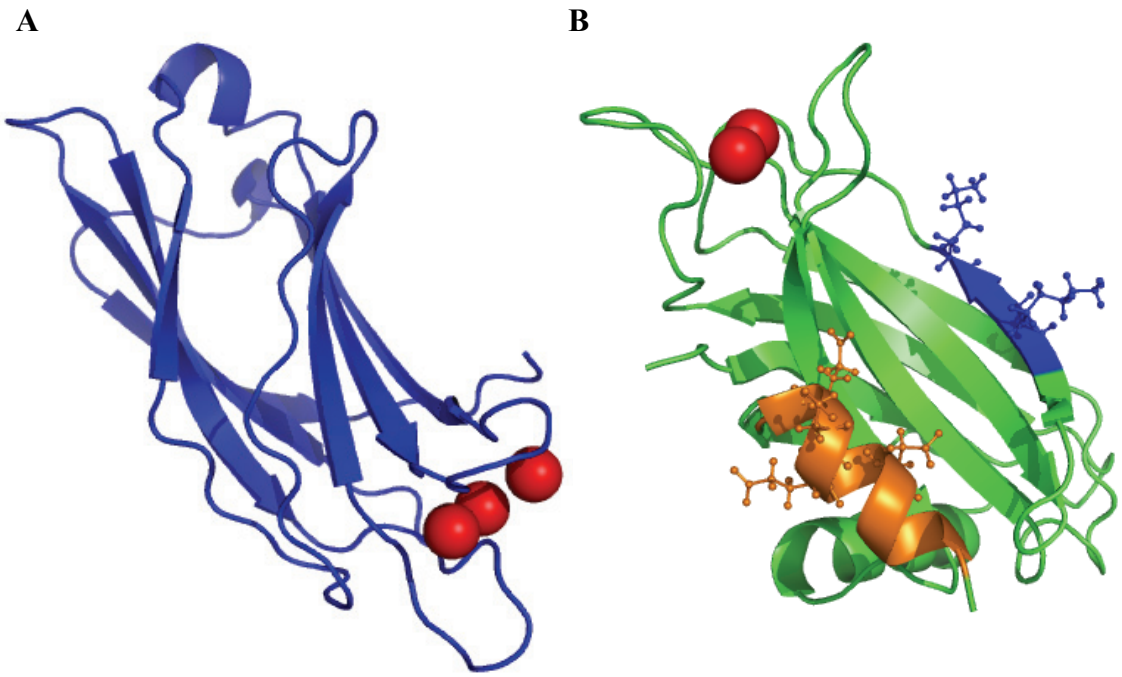


Figure I2: C2 domains of Synaptotagmin. The solution structure of the C2A domain (PDB ID: 1BYN) (**A**) and the crystal structure of the C2B domain (PDB ID: 1UOW) (**B**) in their calcium (red sphere) bound form. C2A domain binds two calcium ions and the C2B domain binds three. The Lysine-rich region of the C2B domain is highlighted in blue (**B**).

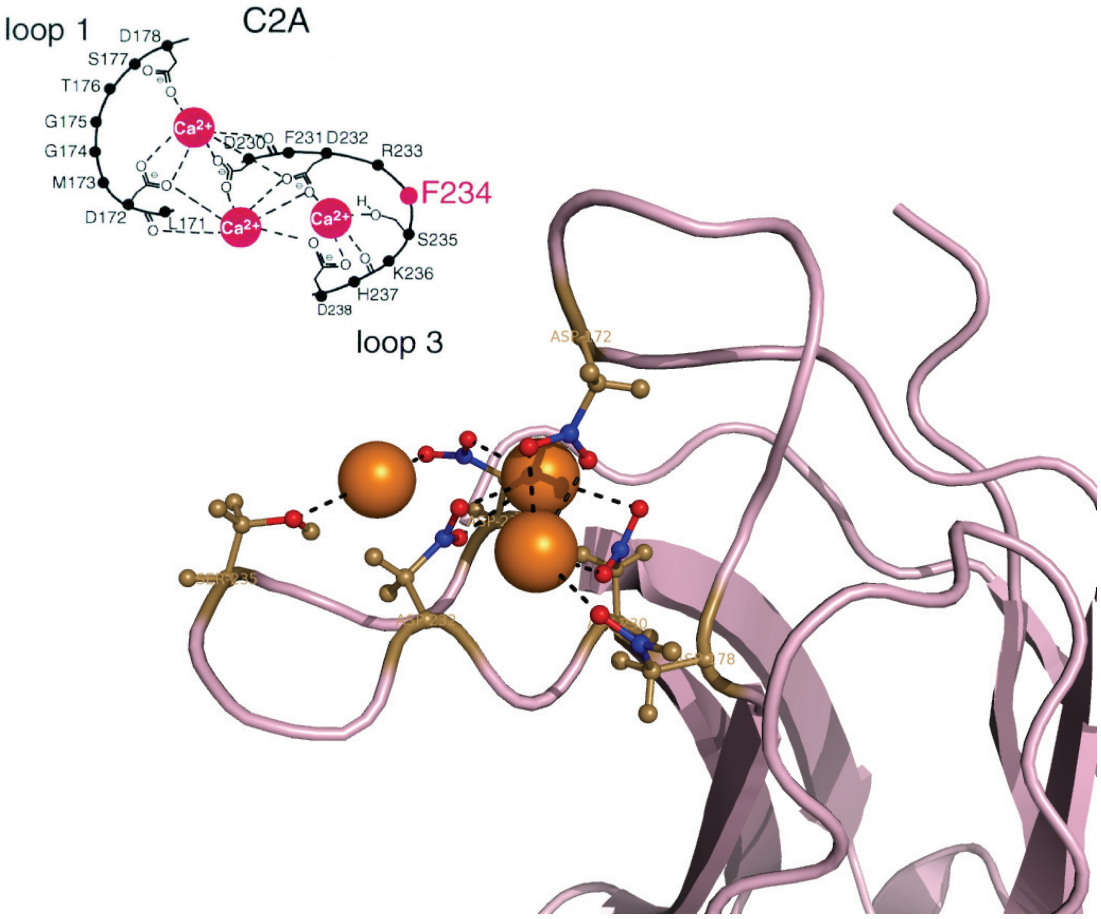


Figure I3: Calcium coordination of the C2A domain. Calcium ions (orange spheres) are coordinated by negatively charged amino acids present in the calcium-binding pocket. The insert shows the amino acids involved as a schematic (1). The blue atoms are the secondary carbons of the aspartate side chain and the red atoms are oxygen atoms that coordinate the calcium binding (5).

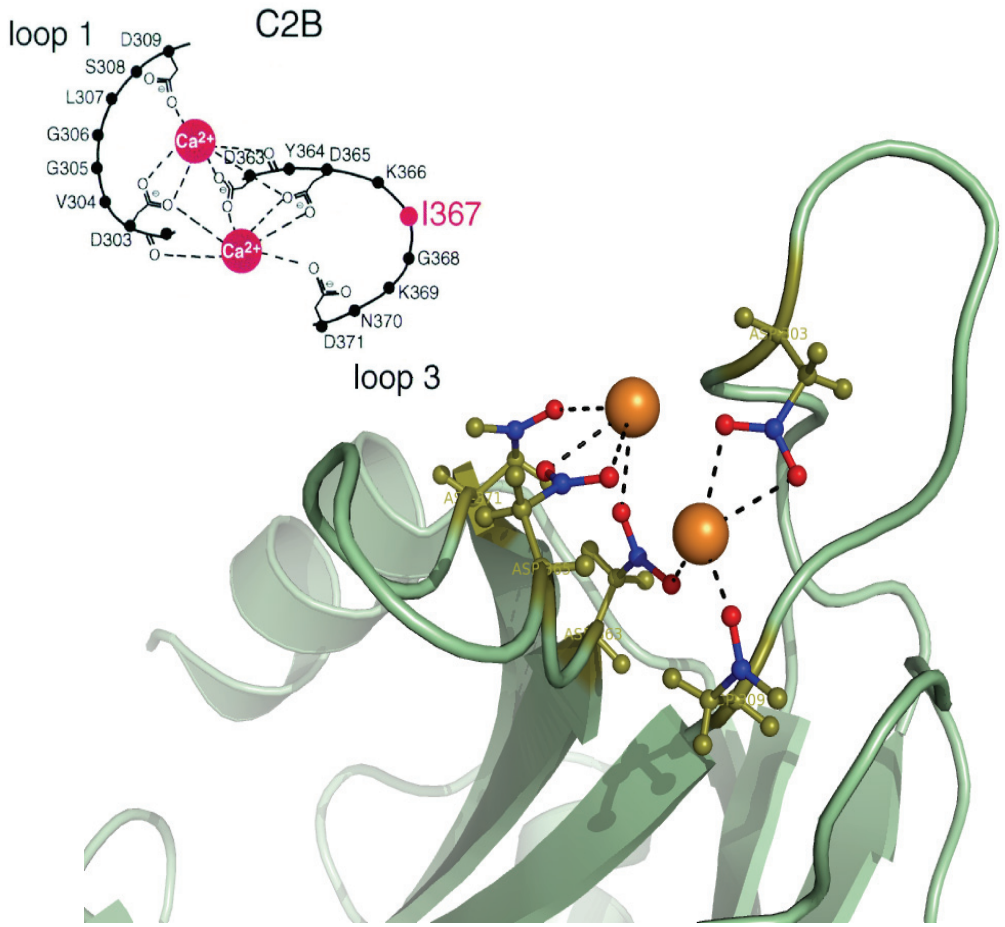


Figure I4: Calcium coordination of the C2B domain. Calcium ions (orange spheres) are coordinated by negatively charged amino acids present in the calcium-binding pocket. The insert shows the amino acids involved as a schematic (1). The blue atoms are the secondary carbons of the aspartate side chain and the red atoms are oxygen atoms that coordinate the calcium binding (6).

1.4 Calcium-dependent Phospholipid Binding

The binding of calcium to C2 domains induces, in turn, the binding of these domains to membranes with negatively charged phospholipids. In the case of Synaptotagmin, this was observed for the first time in a study using the lipid-blot assay (14). In this assay, Synaptotagmin expressed in bacteria was run on a SDS-PAGE gel. The gel was then blotted onto a nitrocellulose membrane. Liposomes, either with or without phosphatidylserine (PS), labeled with radioactive lipids were incubated with the nitrocellulose membrane. After washing the excess liposomes off, the membrane was exposed to film. Using this assay, Perin *et. al.* observed the binding of Synaptotagmin to membranes containing PS. The calcium dependence of this binding was proven in a separate study using the intrinsic tryptophan fluorescence of Synaptotagmin in a FRET assay with dansyl- labeled lipids (29). The addition of calcium induced changes in the FRET signal that could be fully reversed using EGTA to remove the calcium.

1.4.1 Membrane penetration and Cooperativity of Synaptotagmin C2 domains

Synaptotagmin was shown to penetrate the membrane bilayer upon calcium binding. This was shown using an assay that utilized tryptophan introduced by point mutations to different regions of the individual C2A domain of Synaptotagmin, and liposomes that were reconstituted using phosphatidylcholine (PC) labeled with a fluorescence quencher called doxyl at different positions on the fatty acid chain. The penetration of Synaptotagmin was studied based on the degree of tryptophan quenching observed. Using this assay, since the doxyl label was place on different carbons of the fatty acid chain, it was possible to estimate the depth of penetration, which was determined to be $1/6^{th}$ into the membrane bilayer (30).

The penetration of the C2 domains of Synaptotagmin was further confirmed using site-directed spin labelling (SDSL) and electro-paramagnetic resonance spectroscopy (EPR). Using this method, an arbitrary number called the depth parameter (Φ) was calculated. This parameter describes the distance of a specific nitroxide label from the lipid bilayer. Based on calibration curves and previous work (31), Φ values that are more negative than -2 are associated with sites that lie greater than 5Å from the lipid phosphates

(7). Loops 1 and 3 of both the isolated C2A and C2B domain of Synaptotagmin was found to have the most negative Φ values, indicating the deepest penetration (7) (8). Based on the depth parameter, the positions of the domains were modelled relative to the lipid bilayer (Figure I5). The penetration of the tandem C2AB domains of Synaptotagmin indicated a deeper penetration as compared to the single isolated domains. This was especially seen for the spin label positions on the C2B domain (I367 and K369), which hints that the C2B domain needs the C2A domain for more efficient lipid penetration (32).

In another study, this cooperativity between the domains in lipid binding was shown using Synaptotagmin labeled with the fluorescence dye IAEDANS. The intensity of this dye is dependent on its local environment, the more hydrophobic the environment, the higher the intensity observed. Using this assay, the isolated C2B domain was shown to be able to only weakly bind the membrane as opposed to the C2A domain that could function as an independent membrane-binding domain. However, when the C2B domain is attached to the C2A domain, even if the C2A domain has been mutated so that it is unable to bind calcium and subsequently the membrane, the C2B domain is able to bind the membrane with high affinity (33). Both these separate studies highlight the importance of using the tandem domains in trying to understand the function of Synaptotagmin. Although the cooperativity of the two C2 domains has been studied, it is still unknown what function this cooperativity serves in exocytosis. Additionally, since the cooperativity was observed in assays which measure calcium-dependent lipid binding, it is still largely unknown whether this cooperativity of the C2 domains is from either the calcium binding or lipid binding events in Synaptotagmin.

Synaptotagmin has been suggested to function as an electrostatic switch, whereby calcium binding induces the electrostatic potential of the C2 domain to become more positive, inducing the binding and subsequent penetration to negatively charged phospholipid membrane (34). This was supported further when molecular dynamic simulation for the C2A domain showed a large shift in the electrostatic potentials upon calcium binding (35). However, there exists some controversy to this mechanism of calcium dependent membrane binding of C2 domains. When the negatively charged calcium binding residues (aspartate) of protein kinase C β-II were mutated into residues with a positive charge (arginine), this protein was expected to be able to bind negatively charged membranes in the absence of calcium, i.e. a consitutively “on” state. However, this was not the case, suggesting that there might be some important local conformational

changes or structural stabilization, which remains undetected, occurring after calcium binding (36).

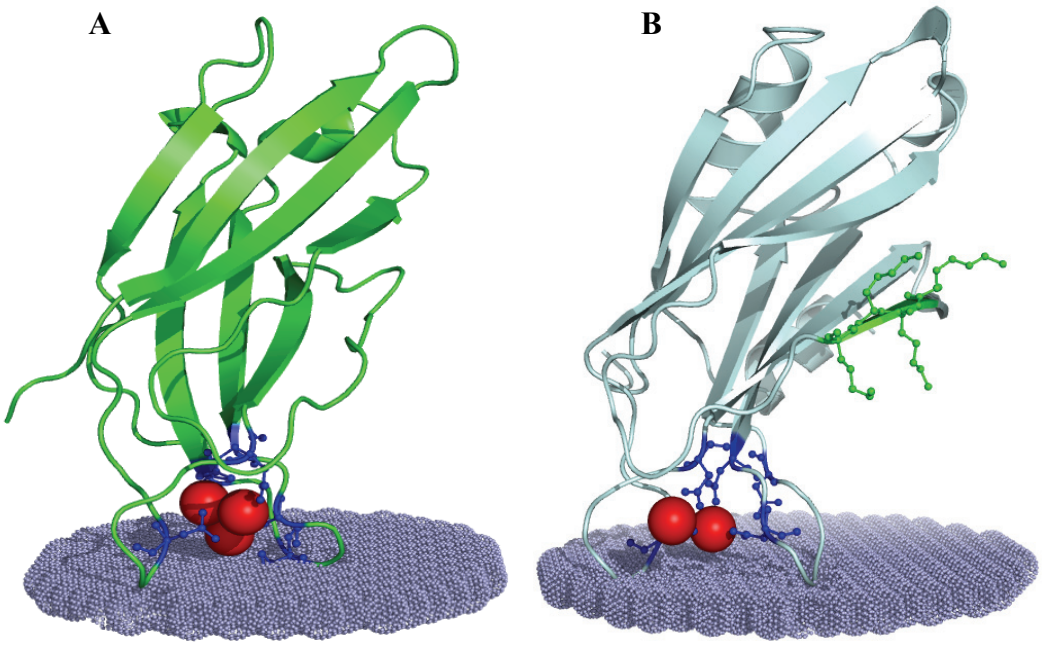


Figure I5: **Membrane penetration of C2A (A) and C2B (B) domain upon calcium binding as modelled by studies using Electro-paramagnetic resonance (EPR).** The Lysine-rich patch is highlighted green on the C2B molecule (2). Based on data from (7) and (8).

1.4.2 Effect of Phosphatidyl Inositol (4,5)bisphosphate (PIP_2) on Synaptotagmin

Phosphatidylinositol (4,5) bisphosphate (PIP_2) has been shown to be the most abundant form of phosphoinositides on the mammalian plasma membrane (typically 1% of total lipids) (37). The first indication of the importance of PIP_2 in calcium dependent exocytosis was highlighted in a study that used bacterially expressed phospholipase C to decrease phosphotidylinositols on the plasma membrane by cleaving PIP_2 to IP_3 and diacylglycerol, which resulted in an inhibition of calcium dependent secretion (38). Synaptotagmin was only much later implicated in this inhibition when GST labeled C2B domain of Synaptotagmin bound PIP_2 containing liposomes (39). The location of the binding site on the C2B domain has been mapped to the Lysine-rich region (K326 & K327) (40) when mutants containing alanine instead of lysine was not able to bind to PIP_2 anymore. Additionally, in the absence of PIP_2, the amount of calcium required for liposome binding of Synaptotagmin also increased. This was tested using a sedimentation assay where Synaptotagmin is incubated with the liposomes in the presence of different amounts of calcium. These liposomes are then sedimented using ultra-centrifugation and the amount of protein present in the pellet is determined using Coomassie-blue stained gels. It was postulated that PIP2 binding of Synaptotagmin is important in the modulation of the apparent calcium affinity during release (40).

In the C2 domain of Protein Kinase C-α (PKCα), binding of PIP_2 was localized to a poly-basic cluster, not unlike that found in Synaptotagmin C2B domain, K197 & K199 on the β3 strand and K209 & K211 on the β4 strand of this C2 domain. Using immuno-fluorescence, it was observed that when PIP_2 binding is abolished via mutation of this poly-basic cluster, PKC is unable to relocate to the membrane. Therefore, based on this result, PIP_2 is believed to be important for the localization of C2 domains to the plasma membrane (21). The proposed model for the role of PIP_2 in Synaptotagmin as well as C2 domains in general, is still heavily debated.

1.5 Interaction of Synaptotagmin with the SNARE complex

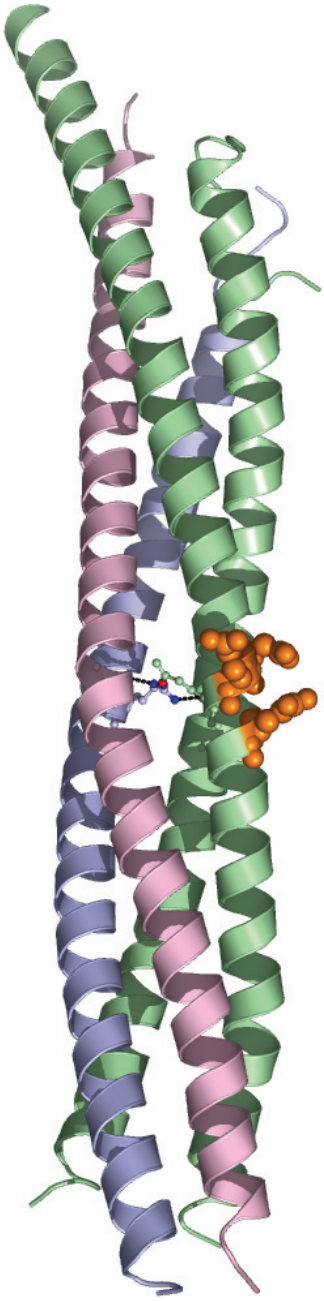
Soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) is a family of proteins that is believed to be responsible in membrane fusion. The SNARE proteins can be divided into two classes, the Q-SNARE and the R-SNAREs. This classification is based on the amino acids present at the 0-layer located in the centre of the SNARE four-helical bundle (Figure I6). In the case of neuronal SNAREs, which is the only class of SNAREs discussed here, Syntaxin 1A (Syntaxin) and SNAP 25 are the Q-SNAREs, located on the plasma membrane and VAMP-2 or Synaptobrevin-2 (Synaptobrevin) is the R-SNARE, located on the synaptic vesicle. In order to carry out its function, the SNARE complex is believed to form an acceptor complex known as the binary complex on the plasma membrane, which consists of Syntaxin and SNAP25. The formation of the SDS-resistant ternary complex occurs upon the arrival of Synaptobrevin in the vesicular membrane. The four helical SNARE bundle is formed by a zippering mechanism from the N- to the C-terminal of the SNARE complex (41). It is still unclear at which point of the SNARE pathway Synaptotagmin is functioning (reviewed in (42)).

The first description of the binding of Synaptotagmin to the SNAREs was done in an early study that also identified Syntaxin as an important member of the membrane fusion machinery (43). In this study, an antibody against Synaptotagmin was shown to co-immunoprecipitate Syntaxin. Although this early binding study showed that Synaptotagmin bound to the isolated Syntaxin molecule, other studies since then have also implicated the isolated SNAP 25 (44) (45), the binary (46) and ternary SNARE complexes (47) in Synaptotagmin binding.

The binding of Synaptotagmin to the SNARE complex and the importance of this interaction in calcium-dependent exocytosis *in vivo* was highlighted in a number of studies. The cleavage of SNAP 25 using BoNT A (aa. Q197) and E (aa. R180) were shown to inhibit calcium dependent exocytosis in PC 12 cells (48). However, the BoNT A-induced inhibition could be reversed by increasing the calcium concentration to induce exocytosis, hinting a connection between SNAP 25 and Synaptotagmin. Since both these toxins cleave at the C- terminal of SNAP 25, these results also prompted the notion that this region of SNAP 25 molecule might be important for the *in vivo* function of Synaptotagmin. When mutations were introduced at the C-terminus of SNAP 25, calcium-dependent exocytosis was inhibited (49). In this study, a BoNT-E resistant SNAP 25

mutant (D179K) was first generated. This allowed the introduction of the mutated protein in a background where all the wild-type SNAP 25 is eliminated by the co-expression of BoNT E. Two C-terminal mutations (D186K and/or D193K) were introduced to study the phenotype of the SNAP25 mutants in calcium dependent exocytosis by monitoring the secretion of human growth hormone (hGh). The rescue of secretion was severely suppressed when the C-terminal mutations were present (D186K and D193K). This failure to rescue the PC12 secretion was further mapped to a very late step in exocytosis indicating that the loss of SNAP 25 function is indeed due to a defective calcium dependent exocytosis step. The importance of the Synaptotagmin – SNARE interaction was also shown by the replacement of charged amino acids on the surface of SNAP 25 (D58A, E170A & Q177A) and over-expression of this construct in chromaffin cells, which caused the calcium triggered secretion to be significantly reduced despite the ability of this mutant to form SDS-resistant ternary complex (50). These *in vivo* experiments suggest that the SNAP 25 molecule is an important component in the interaction of Synaptotagmin with the SNARE complex. However, it is unclear if the interaction is with the isolated SNAP 25 or the SNAP 25 in the ternary complex.

A



B

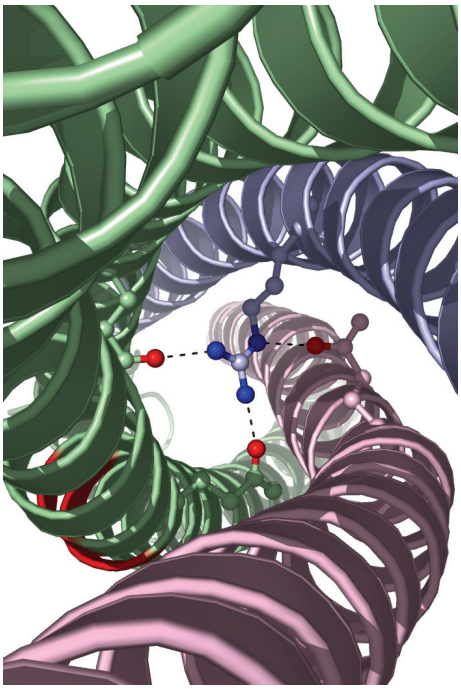


Figure I6: Crystal structure of the SNARE ternary complex. Syntaxin, SNAP25 and Synaptobrevin are colored light red, light green and light blue respectively. The orange balls represent the putative binding site as suggested by (3) on the SNAP25 molecule (D51, E52 & E55) (**A**). The 0-layer (Synaptobrevin R56, Syntaxin Q226, SNAP 25 Q53 and Q174) is shown (**B**) with the R-SNARE nitrogen labeled blue and the Q-SNARE oxygen labeled red.

1.5.1 Biochemical Characterization of Synaptotagmin - SNARE binding

The implication of the Synaptotagmin-SNARE complex binding is important since this interaction would be the crucial link between the membrane fusion machinery, the calcium sensor and calcium-dependent lipid mixing. Although a vast number of studies have been performed since the discovery of the Synaptotagmin – Syntaxin interaction (43) to understand its mechanism, the molecular details of this interaction is still unclear resulting in the existence of a large amount of contradicting data. Synaptotagmin has been shown before to be able to bind to the Q-SNAREs, Syntaxin and SNAP 25, in all stages of the SNARE assembly process (44) (48) (47) (51) (46). The binding of Synaptotagmin to the isolated Syntaxin was characterized using a biochemical pull-down assay with immobilized anti-Syntaxin antibodies. Rat brain detergent extract was incubated with immobilized antibodies on sepharose beads and after a number of washing steps, the bound proteins were visualized using western blotting (52). Synaptotagmin was present in stoichiometric amounts and the addition of calcium enhanced the binding. The presence of Synaptobrevin was also described but whether Synaptotagmin was bound to Syntaxin in the ternary complex or the isolated Syntaxin was not further addressed. In a separate study, the binding site for Synaptotagmin was localized to a C-terminal region of Syntaxin H3 domain (53). This was done by truncating the Syntaxin molecule and testing the binding of this truncated molecule to immobilized GST-Synaptotagmin.

Synaptotagmin was also shown to bind to SNAP 25 using a GST pull down assay (44) (45). Similar to the Syntaxin binding, the binding to SNAP 25 was also stoichiometric and enhanced in the presence of calcium. Conversely, a separate study using brain-purified Syntaxin and SNAP 25 showed that Synaptotagmin is only able to bind the binary complex and not the isolated Q-SNAREs (54). SNAP 25 was immobilized on an anti-SNAP 25 antibody column and either Syntaxin or Syntaxin and Synaptobrevin were added to this immobilized SNAP 25 to form the binary and ternary complex respectively. Brain-purified Synaptotagmin was then incubated with these columns and after washing, the bound protein was analyzed using SDS-PAGE and Coomassie-blue staining. Synaptotagmin was detected only on the binary and ternary complex beads but not to the isolated SNAP 25 beads indicating binding to the binary and ternary SNARE complex and not the isolated SNAP 25. The binding of Synaptotagmin to the SNARE ternary complex

was initially showed by the immunoprecipitation of Synaptotagmin and the SNARE complex with anti-Synaptobrevin antibody in both the presence and absence of calcium (47). Additionally, the binding sites for the lipid membrane and the SNARE complex were suggested to be different since simultaneous binding of SNARE ternary complex and liposomes were observed using a method which first immunoprecipitated Synaptotagmin with the SNARE complex and subsequently tested the binding of this quaternary complex with radioactively labelled liposomes. A recent study has mapped the binding site for Synaptotagmin to the SNARE complex to a relatively high resolution (3). In this study, Synaptotagmin was expressed as a GST fusion protein and immobilized on a GST sepharose column. The immobilized Synaptotagmin was then incubated with solutions containing, either the single isolated SNAREs or in combinations of the binary and ternary complex. After washing, the bound proteins were visualized using a Coomassie-blue-stained SDS PAGE gel. As in a previous study (54), this assay showed binding of Synaptotagmin only to the binary or ternary complex but not to the single isolated SNAREs. Additionally, the binding of Synaptotagmin also did not alter the stoichiometric SNARE complex formation indicating no influence on the SNARE assembly. Mutations introduced to an acidic patch on the SNAP 25 molecule, close to the 0-layer (D51K, E52K and E55K), completely abolished binding of Synaptotagmin to the ternary complex.

Similar to the mapping studies done on the SNARE proteins, the binding site determination on the Synaptotagmin molecule is equally confusing. Studies have suggested that both the isolated C2A as well as the C2B domain can bind the SNARE proteins. Binding studies with the C2A domain done using NMR (55) as well as GST pull down assays (56) showed weak binding to the SNARE complex. However, other studies have shown that the C2A domain is unable to bind SNARE proteins (57). The C2B domain, on the other hand, is currently believed to be important for SNARE binding. In vivo studies using mutated C2B domain showed a large inhibition of calcium dependent exocytosis in *Drosophila* (58) (51). Biochemical mapping studies have showed that the Lys-rich patch on this domain (Figure I2) is important and when mutated to alanines, is unable to bind to SNARE proteins (46). This is rather puzzling since the binding site for PIP_2 has also been localized to the same Lys-rich patch (40). Additional evidence for the importance of this site is that mutations introduced in this Lysine-rich patch (K326A & K327A) is unable to inhibit exocytosis in a cracked PC12 cell assay believed to be due to its failure to bind to the SNARE complex (45). Nevertheless, a FRET assay between

Synaptotagmin and the labeled SNAP 25 showed that mutating amino acids from this Lysine-rich patch (K326A & K327A) still allowed SNARE binding albeit with a reduced binding affinity, from 3μM to 10μM (59). Since the specific assembly stage at which Synaptotagmin binds to the SNARE complex is still unknown, this latter study causes confusion about the binding site of the SNARE proteins on the Synaptotagmin molecule. An interesting observation is that when both domains are present, the binding of Synaptotagmin to the SNARE complex seems to be stronger (EC_{50} = 3 μM vs. 10 μM for isolated C2 domains) (56).

The functional relevance of Synaptotagmin – SNARE complex interaction is also currently unresolved and largely confusing. A number of different hypothesis have been suggested for the function of Synaptotagmin. These include:

i. Synaptotagmin assists in either the binary or ternary complex assembly
ii. Synaptotagmin – SNARE complex, in its bound state controls the dynamics of the fusion pore
iii. SNARE proteins assist Synaptotagmin in membrane binding by functioning as a tether to bring the C2 domains closer to the membrane (reviewed in (1)).

The influence of Synaptotagmin in the fusion process was shown using the *in vitro* liposome fusion assay. The SNAREs, by themselves, have been shown to be able to fuse membranes in this assay (60). Liposomes containing different fluorescently labeled lipids that constitute a FRET pair, with a Syntaxin-SNAP25 complex inserted into its membrane, are mixed with another population of liposomes with unlabeled lipids that contain Synaptobrevin in its membrane. The dilution of the lipids, due to the fusion of one population of liposomes with the other, causes the FRET signal to be reduced. The rate of fusion is monitored by measuring the intensity of the donor signal, which increases due to the reduction in the FRET between the labeled lipids. The speed at which the fusion of the two liposome populations happens in this assay is far slower when compared to the *in vivo* neuronal exocytosis. However when Synaptotagmin is added to the liposome fusion assay, the reaction rate is accelerated, suggesting that the regulatory factors, such as Synaptotagmin, is necessary for the fast exocytosis observed in the synapse (61) (62). This acceleration has been suggested to be due to an increase in the speed of SNARE complex assembly. However, this interpretation of the result is still unresolved.

Recent studies have suggested that other proteins, such as Complexin might also be necessary to gain understanding of Synaptotagmin function (63) (64). Based on genetic analysis, deletion of the Complexin gene has been shown to reduce the calcium sensitivity of the synaptic exocytosis (65) indicating that Complexin might have a role in the Synaptotagmin induced calcium-dependent exocytosis.

1.5.2 Complexin

Complexin is a 15 kDa cytosolic protein that was identified and found to be enriched in the presynaptic terminal (66, 67) (68). Complexin exists as two highly homologous isoforms, Complexin 1 and 2. Deletion of either of these isoforms in mice exhibits only a mild phenotype but the deletion of both isoforms led to the mice dying at birth. Upon closer inspection of the exocytosis at the synapse using electrophysiology, the evoked release of neurotransmitter was reduced by approximately 60% in the double knock out mice, reminiscent of the Synaptotagmin deletion mutant. However, in the case of Complexin, the mutant phenotype could be rescued by increasing the amount of extracellular calcium used in the experiment. No morphological differences in the active zone were observed in these mice indicating that the reduction of the evoked response was due to the inhibition of calcium dependent-exocytosis (65).

Using *in-vitro* approaches such as GST binding assays and NMR, both of the Complexin isoforms were found to be able to bind only to the SNARE ternary complex and not the isolated SNARE components (69). The crystal structure of the SNARE – Complexin complex has shown that Complexin binds in an anti-parallel α-helical confirmation to the groove between Syntaxin and Synaptobrevin (4) (Figure I7). This α-helical portion of Complexin is a fragment between aa. 26-83 and the structure of the rest of the protein is still unknown. The binding kinetics between Complexin and the SNARE ternary complex was determined to be rapid (in the order of $\sim 10^7$ $M^{-1}s^{-1}$). The binding affinity is in the lower nanomolar range, as determined using Isothermal Titration Calorimetry (ITC). Based on these observations, Complexin was suggested to have a role in the post priming stage of exocytosis (70). The mechanism of Complexin's function is still largely unknown. The following models were suggested as being the putative functional roles of Complexin (65):

i. Complexin as the calcium sensor
ii. Complexin could increase the affinity of Synaptotagmin to calcium
iii. Complexin might increase the affinity of Synaptotagmin, in its calcium bound state, to the SNARE complex
iv. Complexin might have a role in increasing the kinetics of SNARE assembly or disassembly.

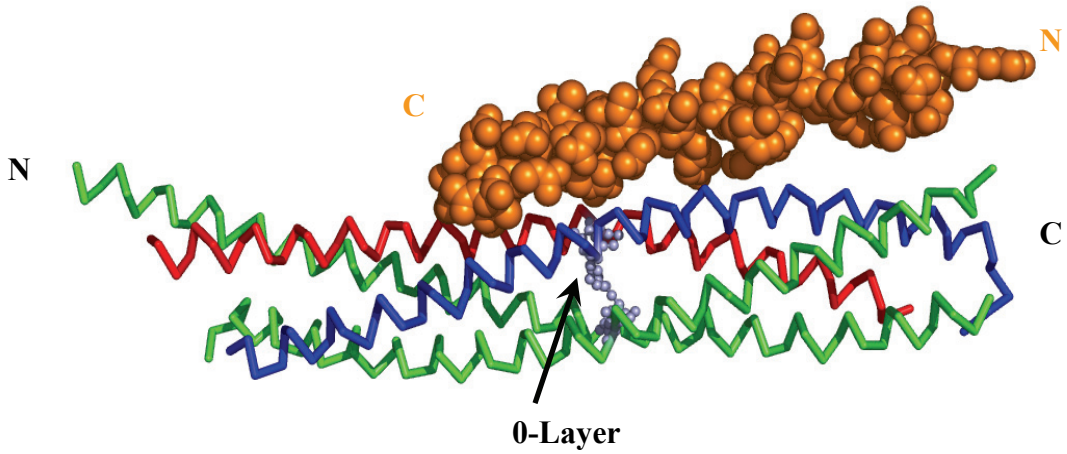


Figure I7: Crystal Structure of Complexin bound to the SNARE complex. Complexin binds the SNARE complex with a high affinity in α-helical confirmation to the groove between Syntaxin and Synaptobrevin Complexin is coloured orange and the 0-layer of the SNARE complex is coloured light blue (4).

1.6 Mechanism of calcium dependent exocytosis: Integrated *in vitro* approaches

The final step of calcium dependent exocytosis at the synaptic terminal is an extremely fast and highly dynamic reaction. This is due to the highly regulated nature of this membrane fusion event. Therefore, in order to gain a better understanding of the process, the different components (i.e. SNARE machinery, Synaptotagmin, Complexin and lipid membrane) need to be studied collectively. This interplay between Complexin, Synaptotagmin and the SNARE complex was shown in two separate studies that proposed Complexin acts as a clamp that is released by the calcium bound Synaptotagmin. In the first study (63), SNARE proteins were ectopically expressed on the HeLa cell surface. Two populations of cells were used, the first expressing the R-SNARE, Synaptobrevin on the surface and DsRed in the cytoplasm (v-cells) and the second population was expressing the Q-SNAREs, Syntaxin and SNAP25 on the surface and CFP in the nucleus of the cells (t-cells). Complexin, Synaptotagmin and nYFP (expressed only in the nucleus) were co-transfected in the v-cells. With the aid of confocal imaging, the cell-to-cell fusion was quantified by counting the number of cells that expressed both CFP and YFP in the nucleus and additionally contained DsRed in the cytoplasm, indicating fusion between the v-cells and the t-cells. These "fused cells" were then quantified and expressed as percentage to the total cells, indicating the percentage of fusion.

The percentage of fusion was markedly reduced in the presence of soluble Complexin (~50% reduction). This incomplete inhibition by Complexin was suggested to be due to low local concentrations of Complexin. This was overcome using Complexin attached to the cell surface via a glycerolphosphatidylinositol (GPI) anchor. In this case, the fusion was completely abolished. The addition of Synaptotagmin to the assay could not restore the cell-to-cell fusion blocked by Complexin. However, when phosphatidylinositol-specific phospholipase C (PI-PLC) was added to cleave the GPI-anchor and thus remove the Complexin bound to the surface of the cell, Synaptotagmin was able to restore the cell-to-cell fusion. This alleviation of the Complexin inhibition by Synaptotagmin was only observed in the presence of calcium (~200 μM) and only when Synaptotagmin was present in the v-cell membrane. Mutation of a calcium-binding residue on the C2A domain (D178N) reduced the calcium responsiveness and mutations in the calcium binding site of the C2B domain (D303N, D309N) completely abolished this rescue.

The second study to propose a similar mechanism was based on methods employing the use of a micro-channel assay and electrophysiology (64). The micro-channel assay was done using a microfluidic cell that consisted of a channel (0.2 mm width) containing supported bilayer with immobilized SNARE complex. The Complexin fragment (26-83) labelled with the fluorescent dye, BODIPY, was added to this immobilised SNARE complex. The channel was then washed with buffer containing 100 mM KCl and either 1 mM calcium, 1 μM Synaptotagmin and EDTA or 1 μM Synaptotagmin and 1 mM calcium, followed by a washing step. For every condition, the intensity of the fluorescence Complexin was measured using confocal imaging. The amount of fluorescent Complexin was significantly less when the channel was washed with Synaptotagmin in the presence of calcium, indicating that Complexin was displaced from the SNARE complex in this condition.

In order to understand at which release phase (i.e. synchronous or asynchronous) Synaptotagmin and Complexin were important, constructs where Synaptobrevin (residues 41-134) was fused to Complexin were made. This forced Complexin to bind only to the SNARE ternary complex. The constructs used in the study were, wild-type Complexin and a Complexin mutant (R48A, R59A, K69A, Y70A) that lacks SNARE binding capabilities. Both these Complexin constructs were fused to Synaptobrevin. The constructs were then, transfected into wild-type cultured cortical neurons using the lentivirus system. The wild-type Complexin that is fused to Synaptobrevin suppressed the synchronous component of the release. After three to five action potentials the asynchronous release, defined as the sustained release rate observed after the exocytotic burst caused by calcium (synchronous release), became dominant and was later determined to be similar to the wild type. Conversely, the mutant Complexin construct without SNARE complex binding ability, fused to Synaptobrevin, also showed a asynchronous release profile that was similar to the wild-type control. This suggested that Complexin function was not important in the asynchronous release stage.

While these studies managed to provide some interesting insights into the functional interplay between Synaptotagmin, Complexin and the SNARE complex, the biochemical details of this interaction is still largely confusing. Assays that can provide kinetic and affinity information on the binding between the different binding partners would be extremely useful in understanding the interplay between these three protein

components of calcium dependent exocytosis and additionally the protein-lipid interplay in this system.

1.7 Objective

Synaptotagmin 1 has been implicated as the calcium sensor for neuronal exocytosis in the synapse after the extensive characterization of the deletion mutants in mouse, worm and fly (17) (16) (15). However, despite many years of study, the molecular mechanism of the protein's function is still unknown. This is largely due to a massive lack in robust and versatile biochemical assays. The main aim of this study is to develop assays that could be used to provide some insights into the protein's mechanism of action. To do this, the final calcium-dependent neuronal exocytosis step has been deconstructed to three separate events, which are calcium binding, lipid binding and SNARE complex binding, which could occur sequentially or simultaneously.

Calcium binding in Synaptotagmin is mediated via its two C2 domains. C2 domains of Synaptotagmin have been studied before using NMR to a high structural resolution (5) (6). Based on these studies, the C2 domain has been shown to coordinate three calcium ions and the C2B, two calcium ions. However, it is still unknown whether the two domains cooperate in their calcium binding. The tandem C2 domain construct is difficult to study using NMR due to intrinsic limitations of the method with regards to the molecular weight of the proteins studied. The two C2 domains of Synaptotagmin have been shown to exhibit both structural and functional divergence (reviewed in (1)). Whether this divergence is translated into the intrinsic calcium binding mechanism of the two domains is yet to be shown. Finally, the existence of a weak calcium binding site other than the calcium binding "jaw" on the C2 domains is also another question that has not been answered satisfactorily. This study attempts to address these questions using Isothermal Titration Calorimetry (ITC), a method that allows the determination of binding isotherms in a chemical reaction (see Appendix 1 for details).

The lipid binding function of Synaptotagmin has been extensively studied in the past years (reviewed in (1)). Based on this review, studies have shown that Synaptotagmin binds to the negatively charged phospholipids (phosphatidylserine (PS) and phosphatidylinositol-(4,5)bisphosphate (PIP_2)) upon calcium binding. The binding mechanism is believed to be due a dramatic change in the electrostatic potential of the C2 domains (35) causing it to dip into the membrane (30). Additionally, the site of exocytosis has been known to be organized into discreet regions known as microdomains (71). The content in these microdomains and functional relevance are still largely unknown. This

study has attempted to tackle this question by studying the effect of PS density on Synaptotagmin function. Additionally, PIP_2 have been shown to be able to modulate the function of Synaptotagmin. The functional modulation is currently unclear but two diverse hypotheses have been proposed, which include; PIP_2 is responsible to modulate the calcium sensitivity of Synaptotagmin (40) or PIP_2 is responsible to localize Synaptotagmin to the plasma membrane (21). This study attempts to address the influence of PIP_2 on the binding affinity of Synaptotagmin to the membrane and how this effect of PIP_2 changes when the density of PS in the membrane is altered.

The final part of the study addresses the binding of Synaptotagmin to the membrane fusion machinery, the SNARE complex. In the recent years, mounting *in vivo* and *in vitro* studies have begun to show that the binding of Synaptotagmin to the SNARE complex is extremely important for the function of Synaptotagmin (reviewed in (1)). While some basic biochemical characterization has so far been undertaken, the detailed study of this protein-protein interaction has proven to be extremely difficult. Here, assays to functionally characterize the interaction between Synaptotagmin and the SNARE complex is developed and used to obtain a better understanding of the role of Synaptotagmin in calcium-dependent exocytosis.

2. Methods

2.1 Plasmid Construction

All constructs used here were generated using the Synaptotagmin 1 wild-type cytoplasmic domain (97-421) construct in the pET28a vector, already existing in the lab. The pET28a vector was chosen because the recombinant protein is expressed with a His_6-tag that is located at the N-terminus of the recombinant protein that would allow the relatively simple purification of the protein using agarose beads coated with nickel ions (Ni^{2+}-NTA), that bind to the His_6-tag. The isolated, single C2 domains C2A (97-273) and the C2B (262-421) domains were generated in a single step polymerase chain reaction (PCR) with *Pfu* polymerase (Promega) (Figure M1) (as described in (69)). The PCR products were then cloned into the expression vector, pET28a, via an *Nde1 / Xho1* cleavage site. The list of primers and the primer pairs used in the different PCR reactions are listed in Table M1 & M2.

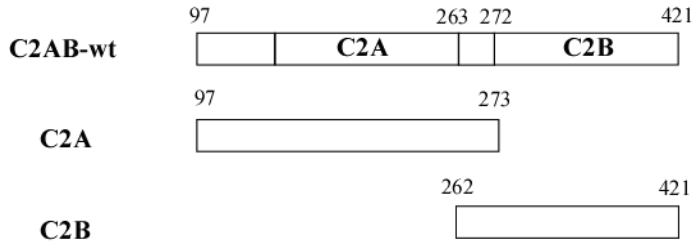


Figure M1: Single C2 domain constructs. The numbers denote the amino acid position relative to the full length (1-421, SWISS-PROT Accession No.: P21707) construct.

This single-step PCR protocol was also used to generate the tandem domain calcium mutants. Full-length Synaptotagmin (1-421) calcium mutants were obtained as a gift from Jacob Sorenson. These mutants were unable to bind calcium in the C2A (D178A, D230A, D232A; denoted as C2A*B), C2B (D309A, D363A, D365A; denoted as C2AB*) or both the C2 (D178A, D230A, D232A, D309A, D363A, D365A; denoted as C2A*B*) domains. These full-length constructs were used as a template to clone the soluble

Synaptotagmin tandem domain (97-421) constructs with the calcium mutations. This was done using the Start and Stop primers (Table M1) in a single PCR step, which were then cloned into the pET28a (*Nde 1 / Xho 1*) expression vector.

The generation of the single-cysteine mutants were done in two PCR steps (summarised in Figure M2). The first PCR step amplified two separate fragments of the construct (i.e. the 5' fragment and the 3' fragment). These fragments contained an overlapping region where the single point mutation would be found. In the second PCR step, the fragments are used as the template to amplify the entire construct (Figure M2). Synaptotagmin 1 (97-421) only contained one wild-type cysteine (C277) which was exchanged to a serine first before generating Synaptotagmin 1 (97-421) S217C and S342C single cysteine mutants.

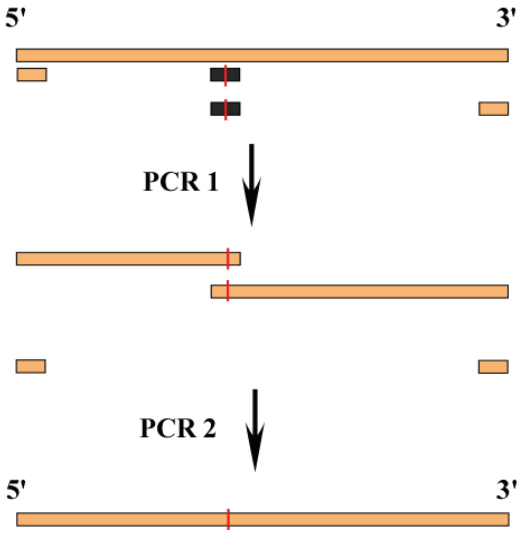


Figure M2: Two-step PCR strategy to introduce point mutation in the Synaptotagmin (97-421) construct. In the 1st PCR step, two fragments (5' and 3') are produced. These fragments are then used as a template to produce the entire (97-421) construct with the desired point mutation in the 2nd PCR. The point mutation is denoted as a red line.

Primer name	Primer sequence (5'-3')
Start	ATA TGC TCA TAT GGG AAA GAA GGC CAT TAA C
Stop	GGT CCT CGA GTT ACT TCT TGA CAG CCA G
C2A_R	GGT CCT CGA GTT ACA GTT TCT CTT GCT CTT CTT TCT CAG CGC T
C2B_F	ATA TGC TCA TAT GCA GAG CGC TGA GAA AGA AGA GCA AGA GAA A
277S_F	GAG AAA CTG GGT GAC ATC TCC TTC TCC CTC CGC TAC GTC
277S_R	GAC GTA GCG GAG GGA GAA GGA GAT GTC ACC CAG TTT CTC
217C_F	ACT TTC AAG GTA CCC TAC TGC GAA TTA GGT GGC AAA ACC C
217C_R	GGG TTT TGC CAC CTA ATT CGC AGT AGG GTA CCT TGA AAG T
342C_F	AAC CCC TAC TAC AAC GAG TGC TTC AGC TTT GAA GTT CGC T
342C_R	ACG GAA CTT CAA AGC TGA AGC ACT CGT TGT AGT AGG GGT T

Table M1: Primers used in the production of the mutants and fragments used in this study.

Construct	PCR step	Forward	Reverse
C2A	1	Start	C2A_R
C2B	1	C2B_F	Stop
Ca^{2+} binding mutants	1	Start	Stop
Syt C277S	1 2	Start 277_F	277_R Stop
Syt (C277S) S217C	1 2	Start 217_F	217_R Stop
Syt (C277S) S342C	1 2	Start 342_F	342_R Stop

Table M2: Primer pairs in the PCR reactions to produce the mutants and fragments used in this study.

2.2 Protein Expression and Purification

The expression of proteins used in this study was done using a standard protocol (72). Any deviation from this standard protocol is mentioned in the text. SDS PAGE was done as described in Schägger *et. al.* (73). All columns used in the purification were purchased from Amersham Pharmacia. Ni-NTA agarose was purchased from Qiagen.

The DNA construct was first transfected into *E. coli* BL21 (DE3) electro-competent cells (1.8 kV, 200 Ω and 25 μF). These cells are resuspended in 1 ml LB and incubated at 37 ºC on a tabletop thermal shaker for 1 hour. Then, the transfected bacteria were plated on a LB plate containing the antibiotic, kanamycin and incubated at 37 ºC overnight. A colony is picked from this plate and inoculated in LB containing kanamycin (30 μg/ml). This inoculated culture is incubated overnight in a shaking incubator at 37 ºC.

The expression of the protein is done in a 2 L Erlenmeyer flask containing 600ml of LB with kanamycin (30 μg/ml). Approximately 10ml of overnight culture is used to inoculate the expression culture. The expression culture is then incubated at 37 ºC until confluence ($O.D_{600}$ = 0.8 – 1), at which point, it is induced with 0.5 mM IPTG and left overnight at 25 ºC. The cells are harvested by centrifugation (4000xg) for approximately 15 minutes at 4 ºC. The medium is discarded and the bacterial pellet is resuspended in the Ni-wash buffer (20 mM Tris pH7.4, 500 mM NaCl, 8 mM Imidazole). Approximately 15 ml buffer is used for each flask. This resuspended pellet is stored in the -20 ºC freezer.

In order to purify the expressed protein, the frozen, resuspended pellet is first thawed before adding 1 mM PMSF, 1 mM magnesium chloride, DNase and lysozyme. DNase and lysozyme are initially dissolved in the Ni-wash buffer before adding it into the bacterial suspension to enhance the mixing of these compounds with the bacterial suspension. This mixture is incubated on ice for 10 minutes before adding 1% (v/v) Triton-X100 and incubating it for another 10 minutes. The suspension is then sonicated to break open the cells (3 times 40 seconds each). In the case of the SNARE proteins, after the sonication step, 6 M urea is added to the suspension. The suspension is sonicated another 3 times for 40 seconds each. This second sonication step is important since as the urea dissolves, it causes the solution to increase in viscosity.

This mix is then centrifuged at 45,000 rpm with the Ti45 rotor in the ultracentrifuge (Beckmann). The supernatant is incubated with Ni^{2+}-NTA agarose (Qiagen) (1.5 ml per expression flask) for approximately two hours at 4 ºC. The agarose and

supernatant suspension is then poured into a BioRad Econo-column (2.8 x 13 cm) (Bio-Rad). The flow-through is collected for the SDS-PAGE analysis. The agarose beads are washed using the Ni-wash buffer (approximately 20 times column volume (CV) of buffer) and eluted using 50 ml Ni-elution buffer (20 mM Tris pH7.4, 500 mM NaCl, 400 mM Imidazole). The eluate is then dialysed against 2 L of dialysis buffer (20 mM Tris pH7.4, ***x*** **mM** NaCl, 1 mM DTT). The amount of sodium chloride (NaCl) in the dialysis buffer is dependent on the protein. For the SNARE proteins, 50 mM NaCl is used for SNAP 25, 100 mM NaCl for Syntaxin and Synaptobrevin and for the Synaptotagmin dialysis, 150 mM NaCl is used. The dialysis is done overnight at 4 ºC with constant stirring. Thrombin (250 μg per 5 ml of eluate) is added to the protein solution in the dialysis bag to cleave the His_6-tag. The thrombin cleavage is confirmed using SDS-PAGE.

The eluate is then filtered through a 0.45 μm filter and loaded onto an ion exchange column using the FPLC system (AKTA; Amersham Pharmacia Biosci.). The choice of column depends on the isoelectric point of the protein. For the SNARE proteins, SNAP25 and Syntaxin were purified using the anion exchange column (Mono Q) and Synaptobrevin was purified using the cation exchange column (Mono S). All the Synaptotagmin constructs were purified over the Mono S column except for the isolated C2A domain, which was purified over the Mono Q. After loading, the proteins were eluted using a linear gradient of NaCl (buffer A: 20 mM Tris pH7.4, 1 mM DTT and buffer B: 20mM Tris pH 7.4, 1 M NaCl). During this gradient, 2 ml fractions were collected and analysed using SDS-PAGE. Peak fractions were pooled and the protein concentration was determined by either the Bradford assay (74) or absorption at 280 nm (Section 2.5) before snap-freezing and storage in the –80 °C freezer.

The same purification protocol was also used to purify the SNARE proteins with the trans-membrane domain. However, detergents were used in the buffers; 0.01 % *n*-Dodecyl-β-D-maltoside (DDM) was used for the Ni-wash, Ni-elution and dialysis buffers and 15 mM CHAPS was used in the ion exchange buffers. A typical purification experiment for Synaptotagmin C2AB (96-421) is shown in Appendix 2.

2.3 Protein labelling

The purified single cysteine proteins were dialysed twice, 1L each time, once for 2 hours and once overnight, in the labelling buffer (50 mM HEPES pH7.4, 500 mM NaCl, 100 μM TCEP). If membrane proteins are labelled, then 0.01 % DDM was added to the dialysis buffer. The dialysis buffer is degassed under vacuum and subsequently under a constant N_2 stream before dialysis. This is to avoid any soluble O_2 in the buffer that would oxidize the cysteine and thus, reduce the efficiency of the labelling. The dialysed protein solution was added to the lyophilized Alexa 488 (Invitrogen) dye since this fluorescence dye is water-soluble. As for the Texas Red (Invitrogen) and IAANS (Invitrogen) dye, it was first dissolved in an organic solvent (2,4-dimethyl formamide) and added to the protein solution. Conventionally 10-fold molar excess of dye to protein was used in the labelling reaction. The labelling reaction was left at room temperature for 2 hours and subsequently, the labelled protein was separated from the free dye over a Sephadex G50 desalting column. The SNARE proteins were labelled in the presence of ~6 M urea to avoid precipitation. The labelled proteins were determined to be free from unincorporated label by SDS-PAGE and stored in the -80 ºC freezer.

2.4 Lipid Mixes and Liposomes

All lipids were purchased from Avanti Polar Lipids except for the Texas Red labelled phosphatidylethanolamine (TRPE), which was purchased from Invitrogen. Lipid mixes were made by dissolving lyophilized lipids in Toulene:Ethanol (1:1). The pure lipids are mixed with either 0 or 30 mole% phosphotidylserine (PS) (Table M3). The total lipid concentration is 13.5 mM. For unlabeled lipid mixes, the TRPE is omitted and the PE amount is increased to 20%.

The lipids are then dried under vacuum or a constant nitrogen gas stream until the organic solvents have evaporated. This dried lipid film is resuspended in the lipid buffer (50 mM HEPES pH 7.4, 500 mM NaCl, 5% (w/v) Sodium Cholate). Liposomes containing Phosphatidylinositol (4,5)bisphosphte (PIP_2) is prepared the same way. 1 mole% of PIP_2 was added to the lipid mixes with the amount of PC adjusted to compensate this addition (Table M3A).

The 0 and 30% PS containing lipid mixes are then diluted in appropriate volumes to obtain the desired PS concentrations to a final volume of 60 µl (Table M3B). To make liposomes, the lipid mixes with the appropriate PS concentrations are mixed with an equal volume of either the lipid buffer, for liposomes without protein or a 50 µM of the appropriate protein solution for proteo-liposomes (Protein : Lipid = 1 : 270). Detergent removal to form liposomes from the lipid mixes was done using a gel filtration column (Fast Desalting PC 3.2 /10, Amersham Pharmacia Biosci.) on the SMART system (Amersham Pharmacia Biosci.) with 50 mM HEPES pH 7.4, 150 mM NaCl as the separation buffer.

A

	mole %	
	No PS	**30% PS**
Phosphatidylcholine (PC)	70	40
Phosphatidylethanolamine (PE)	17	17
Texas Red PE	3	3
Cholesterol	10	10
Phosphatidylserine (PS)	0	30

B

PS (mole %)	Dilution (Vol, µl) (in 60µl)	
	No PS	**30% PS**
0	60	0
5	50	10
10	40	20
15	30	30
20	20	40
25	10	50
30	0	60

Table M3: Lipid Mixes. Components of the lipid mixes are mixed in appropriate amounts (listed in mole %) (**A**). The lipid mixes are then diluted to a final volume of 60µl for the different PS concentrations (**B**).

2.5 Absorption Spectroscopy

The labelling efficiencies of the labelled proteins were determined using the Shimadzu (UV-2401 PC) spectrophotometer as outlined by the manufacturer. The extinction coefficient of the labels is as provided by the manufacturer ($\varepsilon(\text{Alexa 488})_{593nm}$ = 72,000 $M^{-1}cm^{-1}$, $\varepsilon(\text{Texas Red})_{582nm}$ = 112,000 $M^{-1}cm^{-1}$, $\varepsilon(\text{IAANS})_{326nm}$ = 27,000 $M^{-1}cm^{-1}$). The labelling efficiency was calculated as the ratio of the amount of dye to the amount of protein using the following equation:

$$\frac{A_X}{\varepsilon} \quad X \quad \frac{MW}{c\ (mg/ml)} = \frac{moles\ of\ dye}{moles\ of\ protein}$$

Where, A_x is the absorbance of the dye measured at a specific *x* nm wavelength, ε the molar extinction coefficient, MW the molecular mass of the protein and c the concentration of the protein in mg/ml.

For the labelled lipids, the phosphatidylserine (PS) concentration in the liposomes were estimated by first measuring the concentration of the TRPE in the liposome mix. This was done by diluting the TRPE liposomes in the lipid buffer (50 mM HEPES pH7.4, 500 mM NaCl, 5% (w/v) Sodium Cholate) and measuring the absorbance at 593 nm. The calculation of the TRPE liposomes is done using the Beer-Lambert law (A = εlc, where A is the absorbance value, ε is the extinction coefficient ($\varepsilon(TRPE)_{593nm}$ = 107,000 $M^{-1}cm^{-1}$), l the pathlength (cm) and c the concentration in M). The estimation of PS concentration is possible by an extrapolation from the TRPE concentration since the mole ratios of the lipids in the lipid mix are known. Protein concentration is determined in a similar way except the absorption at 280nm is measured in the presence of 6M guanidine hydrochloride (to completely denature the protein) and using the Beer-Lambert equation, the concentration was determined.

2.6 SNARE ternary complex formation

The unlabelled SNARE ternary complex was assembled from the individual SNAREs in the mole ratio of 1 : 2 : 2 (Syntaxin : SN25 : Synaptobrevin) overnight and separated from the SNARE protein not in the complex by ion exchange chromatography (Mono Q, Amersham Pharmacia Biosci.). For the labelled complexes, the single cysteine protein, either SNAP25 (1-206) 48C or Syntaxin (183-288) 225C, were first labelled with the appropriate fluorescence dye and incubated overnight on ice with the respective SNARE components in the same ratio as above. These complexes were not purified over the Mono Q column due to large amount of protein loss during separation. The complexes were visualized on a SDS-PAGE gel first, before further experiments were done.

2.7 Native PAGE

Native PAGE is used to separate proteins based on their intrinsic net charge (72). This net charge, when measured at pH7 is called the isoelectric point. The differential migration of a complex, as compared to the individual proteins, is detected in native PAGE since the complex has a different net charge as compared to the individual proteins. The proteins used in this experiment were incubated overnight in equal amounts (5 μg), with or without 5 mM calcium chloride. The native gels are made using the following recipe (for 2 gels):

8%	
Lower Gel Buffer (pH8.8)*	2.5ml
dH_2O	4.7ml
30% Polyacrylamide Solution	2.7ml
TEMED	5μl
10% APS	100μl

3.75%	
Upper Gel Buffer (pH6.8)**	1.25ml
dH_2O	3.1ml
30% Polyacrylamide Solution	0.625ml
TEMED	10μl
10% APS	100μl

*Lower gel buffer (1.5 M Tris-HCl pH8.8)

**Upper gel buffer (0.5 M Tris-HCl pH6.8)

The lower gel is poured first (~3.4ml) into a pre-assembled gel casting system (BioRad). It is extremely important to wash the gel casting trays as well as the tanks extensively to remove any residual SDS. Butanol-saturated-water is added to the surface of lower gel to ensure that the after polymerization, the lower gel has a flat surface. Once the lower gel has completely polymerized, the upper gel is poured and the comb to form the wells is inserted. The protein samples are mixed with equal volume of the native sample buffer (30% Glycerol (w/v), 0.05% Coomassie-blue blue G-250 (Serva), 150 mM Tris/HCl pH 7.0) and loaded into the wells. The gel is then ran at 120 V in running buffer (2.5 mM Tris, 19 mM Glycine), until the interface migrates out of the gel. The proteins are visualized using Coomassie-blue staining.

2.8 Isothermal Titration Calorimetry (ITC)

ITC was used to measure the intrinsic calcium binding properties of Synaptotagmin. This experiment was adapted from a study on the intrinsic calcium binding properties of C2 domains from protein kinase C (75). The buffer used in the experiment was 50 mM HEPES pH 7.4, 150 mM NaCl, 5 mM β-Mercaptoethanol. Before the experiment was done, the buffer was first pre-treated with a metal ion chelator to remove any residual calcium ions. This chelator, known as Chelex-100 (Bio-Rad) is a styrene divinylbenzene resin that is coated with iminodiacetate ions that act as the chelating group. The iminodiacetate group has a higher selectivity for divalent ions (Ca^{2+}, Ba^{2+} etc.) as compared to monovalent ions (Na^{+}, K^{+} etc.) (Approximately 5000 to 1).

The resin was first washed by rinsing it with 2 L of ddH_2O and 1 L of ITC buffer (pH 7.4). The washed resin is then added directly to the ITC buffer and left overnight at 4 ºC. Customarily, 120 g of resin is used for 2 L of buffer. The resin is removed from the buffer by filtering it under vacuum through a glass frit filter. After adjusting the pH of the buffer, the buffer is filtered through a 0.45 μm filter, under vacuum. The dialysis of the proteins used in the ITC experiment is done twice, each time in 1 L buffer, once overnight and once for 2 hours. The proteins' concentrations are determined by measuring the absorption at 280 nm. The ligand solution, which is 10mM calcium chloride, is made by diluting a 1 M stock solution with the ITC buffer from the second dialysis step. This buffer is also used for any further protein dilutions required in the experiment.

The ITC experiments were done on a MicroCal calorimeter. The calorimeter sample cell was filled with 50 μM or 25 μM of protein and 10 mM calcium chloride solution in the syringe. The titration was done by injecting 10 μl of the ligand solution 30 times into the macromolecule solution in the sample cell with a spacing of 250 s between the injections and the stirring speed of 300 rpm. For the purpose of analysis, the calcium chloride solution was defined as the ligand. The ITC raw data was then analysed using the Origin 7.0 software package (Originlabs) with a special add-in for the ITC data analysis (MicroCal Inc.).

2.9 Fluorescence Spectroscopy

All fluorescence measurements were done at 25ºC on the Fluorolog fluorometer (JobinYvon) using 3ml quartz cuvettes with 1cm pathlength (Hellma). The buffer used for all measurements, unless otherwise stated, was 50 mM HEPES pH 7.4, 150 mM NaCl. The analysis of the data obtained was done on both Microsoft Excel (Microsoft Corp.) and Origin 7.0 software package (Originlabs).

2.9.1 Interaction of Synaptotagmin with the lipid membrane

The interaction of Synaptotagmin with the lipid membrane was determined using a FRET assay between Synaptotagmin labelled at position 342 with Alexa 488 dye and liposomes containing Texas Red labelled phosphotidylethanolamine (TRPE). The Synaptotagmin labelled at position-342 with Alexa 488 (λ_{ex} = 488 nm, λ_{em} = 520 nm) was diluted in 40ml of buffer to a final concentration of 0.2 μM and pre-incubated in the 25 ºC water-bath for approximately 30 minutes to pre-equilibrate the protein and buffer solution to the experimental temperature. 2.5 ml of this diluted protein solution was then added to the cuvette. The slit-widths were adjusted to 2 nm and the integration time was 0.5 s. The intensity of the emission at 520 nm was recorded. The Texas Red-labelled liposomes were diluted 10-fold, in the same buffer, before its titration to the protein solution until saturation was reached. In the case when SNARE proteins were inserted into the liposome membrane, the proteins were first purified to form a ternary complex before the insertion using detergent removal by gel filtration at a protein to lipid ratio of 1 : 270. The data was analysed by averaging the intensity counts obtained for each titration point. These average intensity counts (F) are then normalised to a baseline value (F_0) obtained before the first injection (F_0/F). This allows plotting the data on the same axis for all conditions thereby enabling a direct comparison of the different conditions. The normalised intensity values are plotted against the PS concentration.

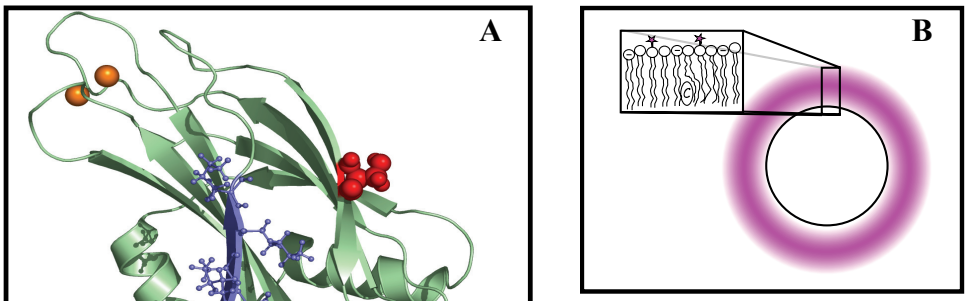


Figure M3: FRET assay between Synaptotagmin labelled at position 342 with Alexa- 488 (A; Red sphere) and liposomes with Texas Red-PE (B) was used to study lipid binding of Synaptotagmin.

2.9.2 Fluorescence measurements of SNARE complex labelled with SNAP25 48IAANS

The individual SNARE proteins were incubated at a ratio of 2 : 1 : 2 (Syntaxin H3 (183-262) : SNAP25 (1-206) (48IAANS) : Synaptobrevin (1-96)) with a final concentration of 0.8 μM of the labelled SNAP 25 (Figure M5). The mix was left overnight on ice to allow SNARE ternary complex formation. SDS-PAGE was used to confirm the presence of the ternary SNARE complex. The experiment was done by adding 25 μl of the mix into a cuvette filled with 2.5 ml of the fluorometer buffer. The slits were set to 2.5 nm for all the channels with an integration time of 0.5 s. The labelled protein was excited at 320 nm and a spectrum was recorded from 350 nm to 600 nm (maximum emission at 460 nm). Then, Synaptotagmin 1 (1 mM), calcium (0.5 mM) and EGTA were added sequentially in that order and each time a spectra was recorded. The effects of bivalent ions were tested by replacing calcium in the above scheme with either strontium or magnesium. The experiment was also done for different Syntaxin fragments. In this case, these fragments were incubated with the labelled SNAP 25 and Synaptobrevin and the experiment was carried out as described above.

The calcium titrations were done in a buffer with 50 mM HEPES pH 7.2, 150 mM NaCl containing 10 mM DPTA. The amount of total calcium needed to obtain a certain concentration of free calcium was calculated using the IGOR software (with the

assistance of Jacob Sorenson). Calcium is titrated into a cuvette containing 2.5 ml buffer, 25 µl labelled ternary complex mix and 1 µM Synaptotagmin until saturation. The emission intensity at 460 nm was measured after each addition of calcium and normalised to the baseline, which is the measurement recorded before the titration, to obtain a titration curve. The affinity of calcium required for the Synaptotagmin – SNARE protein interaction was determined by calculating the half maximal binding concentration (EC_{50}) from the titration curve.

The amount of Synaptotagmin needed for the observed signal change was determined by titrating Synaptotagmin into a cuvette containing 2.5 ml buffer, 25 µl labelled ternary complex mix and 0.5 mM calcium, until saturation. The emission intensity was measured at 460 nm and the affinity of the interaction was determined by determining the EC_{50} of the reaction.

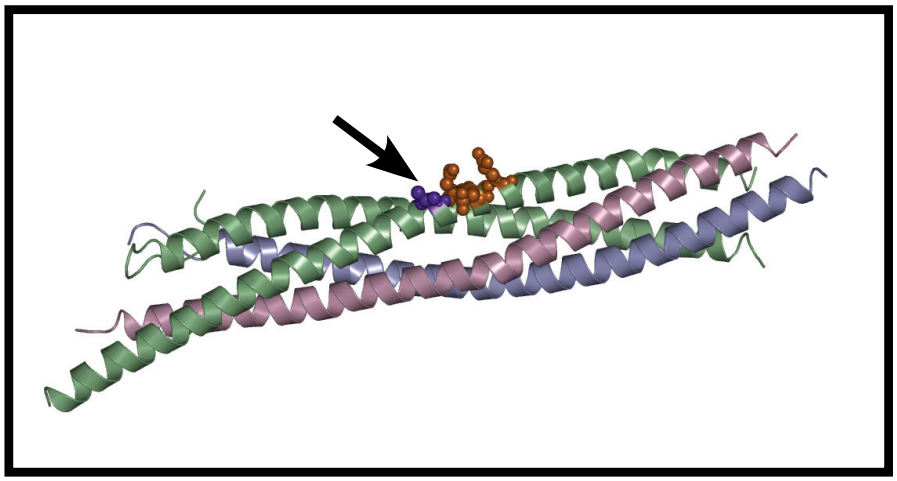


Figure M5: SNAP 25 position 48 used for the IAANS assay is indicated with an arrow. The putative binding site (3) D51, E52 and E55 on SNAP 25 is indicated by the orange spheres.

2.9.3 Fluorescence Anisotropy

2.9.3.1 SNARE assembly kinetics

The SNARE assembly kinetics was measured by monitoring the anisotropy of the Texas Red (λ_{ex} = 590 nm, λ_{em} = 610 nm) dye. The slits for all the following experiments were set to 5nm and the integration time was 1s. The binary complex assembly kinetics was monitored using Texas Red coupled to SNAP 25 at position-130. After running a baseline kinetic measurement for 10µl (~0.2 µM) of the labelled protein in a cuvette with 2.5ml buffer, 0.7 µM of unlabelled Syntaxin H3 (180-262) was added to the labelled SNAP25 and the kinetic measurement was started. To test whether Synaptotagmin or calcium exerted an influence on the assembly kinetics, either 1mM calcium, 1.5 µM Synaptotagmin C2AB or both were added to the labelled protein before baseline measurement and the assembly kinetics recorded upon Syntaxin H3 addition.

The ternary complex assembly kinetics was measured using two separate assays. The first was using the Synaptobrevin (1-96) labelled at position-28 with Texas Red and the second was Synaptobrevin (1-87), C-terminally labelled at position 88 with the same dye. Either 10µl or 5µl of the Synaptobrevin (1-96) 28TR or Synaptobrevin (1-87) 88TR respectively, was added to a cuvette filled with 2.5ml buffer. After an initial baseline measurement, 0.7 µM of unlabelled SNAP25 was added and another baseline measurement was taken. Then, 0.7 µM of Syntaxin H3 was added to the cuvette and the intensity change was recorded to measure the assembly kinetics of the ternary complex formation. The influence of Synaptotagmin and calcium was measured by adding either 1 mM calcium, 1.5 µM Synaptotagmin or both to the labelled Synaptobrevin and measuring the baseline and subsequently measuring the kinetics after addition of Syntaxin H3. The anisotropy values measured is then plotted against time.

2.9.3.2 Interaction of Complexin to the SNARE complex

The binding of unlabelled ternary complex to Complexin 2 labelled at position 39 with Oregon Green (λ_{ex} = 488 nm, λ_{em} = 520 nm) was previously reported in

another study (70). This experiment was done for both ternary complexes with and without the transmembrane domain. The buffer used in this experiment was 50 mM HEPES pH 7.4, 150 mM NaCl and 15 mM CHAPS. Labelled Complexin was added into a cuvette containing 2.5 ml buffer with 1 mM calcium chloride. After the baseline anisotropy was measured, 1 μM of the respective ternary complex and 1 μM of Synaptotagmin C2AB were added sequentially and for each condition, the anisotropy value was recorded. The analysis of the data was done by setting the baseline anisotropy to 100% and any further increase, normalised to this 100% value (Figure M4).

2.9.3.3 Binding of Synaptotagmin to the SNARE ternary complex (+TMD)

The anisotropy of the SNARE complex containing Alexa 488 labelled Syntaxin (aa. 225) was used to monitor the binding of Synaptotagmin to the SNARE complex with its transmembrane domains. 0.2 μM of this SNARE complex is added to 2.5 ml fluorometer buffer and the baseline anisotropy measured. After each sequential addition of Synaptotagmin (0.5 μM), calcium (1 mM) and EGTA (24 μl of 0.05 M stock), the anisotropy values were recorded. The experiment was done with the different isolated domains of Synaptotagmin as well as testing the effect of calcium and strontium. The data was analysed as shown in Figure M4.

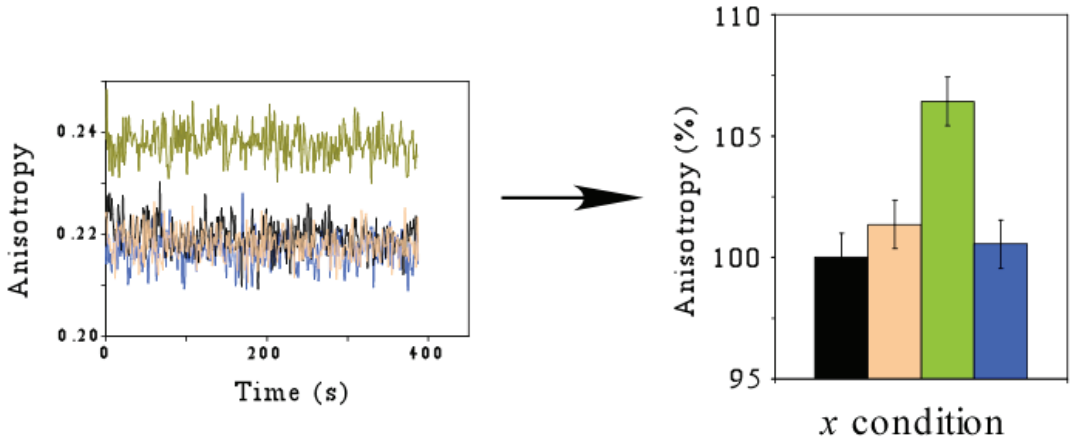


Figure M4: Analysis of Fluorescence anisotropy data. Fluorescence anisotropy was measured over time. This measurement was averaged to produce a single bar for each sequential addition of a particular component (e.g. Ca^{2+}, EGTA etc.). In order to compare the different conditions, the anisotropy baseline value was then set at 100% and all other values were normalised and expressed as the percentage to this baseline. The error bar shown is the standard error of mean (SEM) for each of the added component as a measure for the signal fluctuation.

2.9.4 Interaction of Synaptotagmin to the SNARE complex: A FRET-based approach

Fluorescence resonance energy transfer (FRET) measurements were done between Synaptotagmin and the SNARE complex with the transmembrane domain. The Synaptotagmin was labelled at position 217 with Texas Red and the SNARE complex labelled at position-225 of Syntaxin with Alexa 488, inserted into liposome membrane (Figure M6). In this case, the label on the SNARE complex is the FRET donor dye and the Texas Red on Synaptotagmin is the acceptor dye. The experiment was done in a cuvette containing 2.5 ml fluorometer buffer with 2.5 nm slit-widths for both the excitation and emission channels with an integration time of 0.2 s. The donor (Alexa 488) was excited at the wavelength of 488 nm and a spectrum was collected from 500 nm to 700 nm. The spectrum was collected only in the presence of 0.1 μM of the labelled ternary complex to determine the baseline of the donor. Subsequently, a spectrum is recorded after each sequential addition of 0.2 μM Synaptotagmin 217TR, 1 mM calcium, 60 μl 0.05 M EGTA and 0.1 μM Complexin. The analysis of the data was done by plotting the normalised (F_0/F) of the maximum donor emission (520 nm) as a bar diagram (Figure M7).

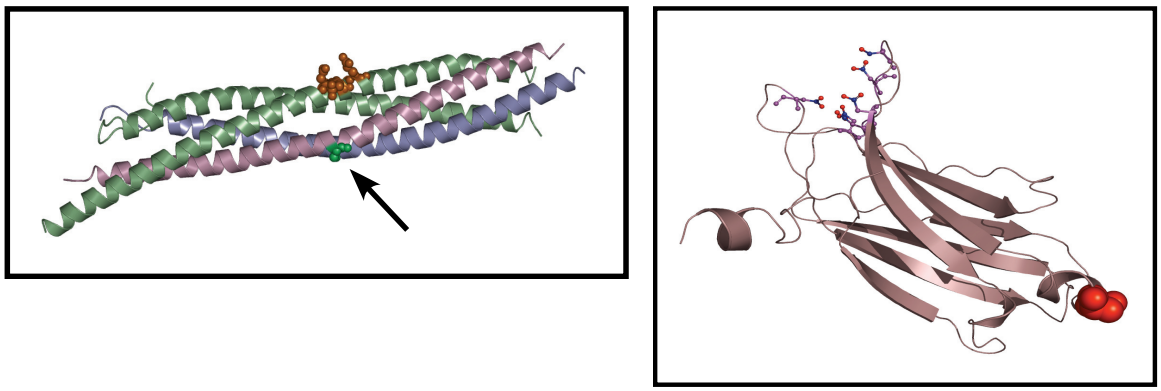


Figure M6: FRET pair between Synaptotagmin and the SNARE complex. The SNARE complex was labelled at position 225 on the Syntaxin molecule with the Alexa-488 (A; indicated with the arrow) as the donor and Synaptotagmin was labelled at position 217 with Texas Red (B; Red spheres). The putative binding site (3) D51, E52 and E55 on SNAP 25 is indicated by the red spheres.

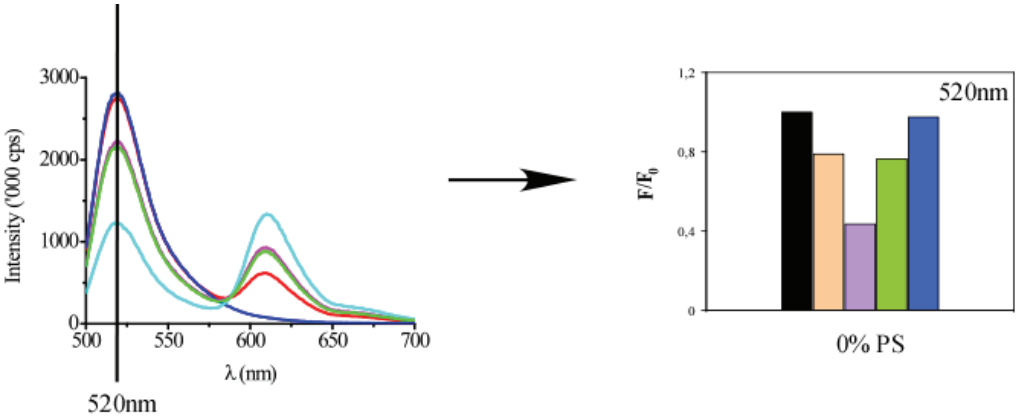


Figure M7: Analysis of the data obtained from the FRET assay between Synaptotagmin and the SNARE complex. The absolute intensity counts from the donor channel (λ = 520nm) was normalised to the baseline (when no acceptor was present) (F/F_0) and plotted as a bar diagram. Each bar represents the sequential addition of the components (e.g. Ca^{2+}, EGTA etc.) at a particular condition (e.g. 0% PS liposomes).

3. Results

3.1 Calcium binding of the Synaptotagmin C2 domains

Calcium binding of Synaptotagmin C2 domains has been studied before using NMR (26) (5) (6). The number of calcium ions as well as the coordinating residues for each of the Synaptotagmin C2 domain was outlined in these studies. However, NMR-based studies are limited by the size of the macromolecule used in the experiment. Therefore the intact protein cannot be studied using this method. The calcium affinity estimated from NMR experiments was done by measuring the changes in the chemical shifts upon calcium addition. This method of determining the affinity only provide a rough estimate and also binding studies using NMR is unable to provide other information such as enthalpy, entropy and free energy of binding which are important to elucidate the function of the protein.

Isothermal titration calorimetry (ITC) was used previously to investigate the calcium binding properties of the C2 domains from the classical protein kinase C (PKC) isoforms (α, β, γ) (75) and phospholipase D isoforms (76) (for details on the method, please refer to Appendix 1). These studies were the basis for the assay that was developed here. The PKC C2 domain study was carried out with single, isolated C2 domain (75). In order to reproduce the experiment as it was done in this study, the C2A (97-273) and C2B (262-421) domains of Synaptotagmin were cloned, expressed and purified separately. The purified proteins were then dialysed against the ITC buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 5 mM β-mercaptoethanol), which was pre-treated with Chelex-100 resin to remove any residual calcium in the buffer solution.

The ITC experiments were done at 25 ºC with the Synaptotagmin C2 domain in the sample cell and 10 mM calcium chloride solution in the syringe. The 10 mM calcium chloride ligand solution was prepared by diluting a 1 M calcium chloride standard stock solution with the second dialysis buffer. The calcium chloride solution was titrated in 10μl volumes 30 times with the spacing time of 250 s between each titration point. The titration curve observed in the experiment was confirmed to be due to the binding of calcium to Synaptotagmin and not the dilution of the calcium solution, by running a

control experiment where calcium was titrated into the sample cell containing buffer (data not shown).

3.1.1 Calcium binding of the isolated Synaptotagmin C2 domains

The ITC result of the titration of calcium to the single, isolated C2A domain (aa. 97-273) is shown in Figure R1A. The upper panel shows the electrical energy measurements, which is the raw data collected during the experiment. The lower panel shows the integrated peaks of this raw data, which is the heat evolved per titration step. The integrated data was fitted using a single site model. This model assumes that all the *n* number of ligands bind to the binding site with an equal affinity. Therefore, in the case of the C2A domain of Synaptotagmin, although it was reported that the three calcium ions bound with different affinities to the calcium-binding site (5), using the single-site model, only the overall affinity of calcium binding to Synaptotagmin C2A domain can be determined. In addition, the number of ligand bound to the macromolecule (n) was fixed to three for the C2A and two for the C2B domains (based on NMR studies) in the fitting of the integrated ITC data.

When calcium was titrated into the C2A domain, heat was absorbed and caused an increase in the electrical power flowing into the sample cell, as is evident from the positive raw data peaks. This absorption of energy is due to the endothermic chemical reaction of the calcium binding to the C2A domain. The enthalpy (ΔH) of the reaction is 2100 cal/mole with an apparent affinity (K_d) of 230 μM. The entropy (ΔS) of the system is approximately 24 cal and based on these values, the Gibbs free energy (ΔG) was calculated to be -5 kcal. Calcium titration into the C2B domain (aa. 262-421) (Figure R1B) showed a completely different behaviour as compared to the C2A domain. The binding of calcium to the C2B domain caused energy to be released, causing the amount of electrical energy flowing into the sample cell to be reduced to reach equilibrium with the reference cell. This behaviour causes the raw data peaks to have negative values and is characteristic of an exothermic chemical reaction. The apparent affinity (470 μM) is also significantly lower as compared to the isolated C2A domain. The amount of heat released (ΔH) is -2600 cal/mole. The entropy (ΔS) of the system is rather low at 6 cal and based on these the calculated free energy (ΔG) is approximately -5 kcal. These data indicate that the calcium

binding mechanisms to the two domains are divergent and seem to be independent. However, in order to confirm this independence, the calcium titration was repeated using the tandem Synaptotagmin C2 domain wild type construct and also the tandem construct with either one or both C2 domains rendered inactive through site-directed mutagenesis on the calcium binding site.

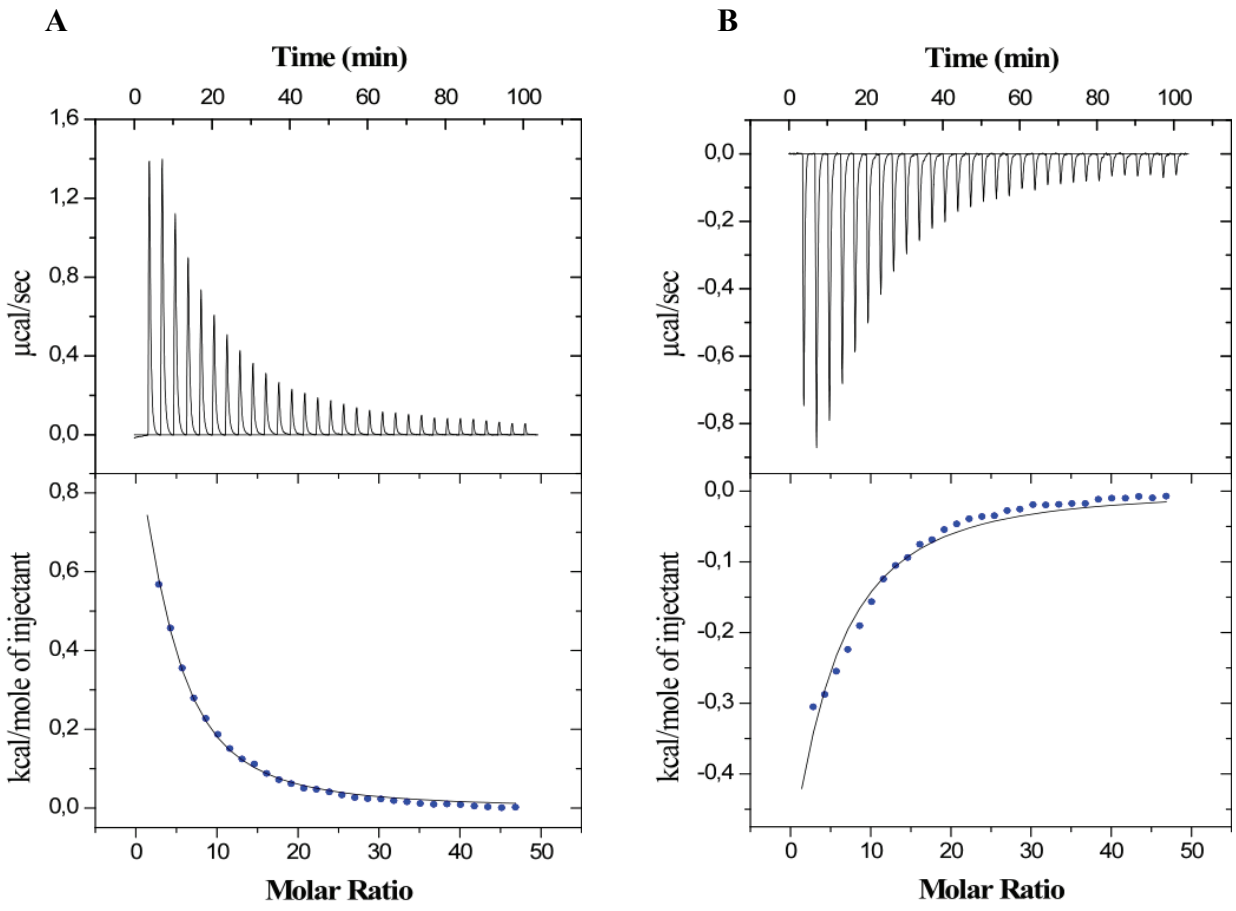


Figure R1: Isothermal Titration Calorimetry of isolated C2A (A) and C2B (B) domains. 10 mM calcium chloride solution was titrated into 50 µM protein in the sample cell to study the calcium binding behaviour of these domains. The upper panel shows the raw data and the lower panel shows the integrated raw data peaks (blue scatter diagram) and the appropriate fitting (solid line) using the single binding site model. The C2A domain exhibits an endothermic binding profile as opposed to the C2B, which exhibits an exothermic binding profile.

3.1.2 Calcium binding of Synaptotagmin with the tandem C2 domains

Titrating calcium into the Synaptotagmin wild type tandem C2AB (Figure R2A) protein showed an endothermic reaction (Figure R2). The apparent affinity of this reaction was determined to be 250 μM and 170 μM when 25 μM and 50 μM of protein were used in the cell. The enthalpy of this reaction is 700 cal/mole and 370 cal/mole for the 25 and 50 μM proteins respectively. The entropy for both reactions is 18 cal and the calculated free energy for both is -5 kcal. Although the affinity and enthalpy values vary, depending on the amount of protein used in the sample cell, the variation is rather small and could be caused by fitting problems due to the low signal strength of the calcium titration to this protein. Additionally, this variation could also be caused by incorrect protein determination. Remarkably, when the isolated C2A and C2B domain integrated curves are mathematically summed, the resulting curve overlaps well with the measured Synaptotagmin C2AB integrated curve indicating that the domains are acting independently in calcium binding (Figure R2B).

The experiments was next done with the Synaptotagmin C2A*B (D178A, D230A & D232A) and C2AB* (D309A, D363A & D365A) constructs, which contain mutations that render one of the domain inactive in the tandem domain construct, to confirm this independence of the two C2 domains. The exothermic calcium titration profile of the Synaptotagmin C2A*B (Figure R3A) construct was similar to the isolated C2B domain (Figure R1B). However, upon closer inspection, the apparent affinity was 270 μM, which is higher than the isolated C2B domain and the enthalpy was approximately -1600 cal/mole. The calculated entropy and free energy was 11 cal and -5 kcal respectively. Calcium titration to the C2AB* construct showed an endothermic profile similar to the isolated C2A domain. The apparent affinity was 260 μM when 25 μM of protein was used and 360 μM when 50 μM protein was used. The enthalpy was approximately 2000 cal/mole with a calculated entropy and free energy of 22 cal and -5 kcal respectively. The thermodynamic parameters obtained in the ITC experiments are summarized in Table R1 and R2.

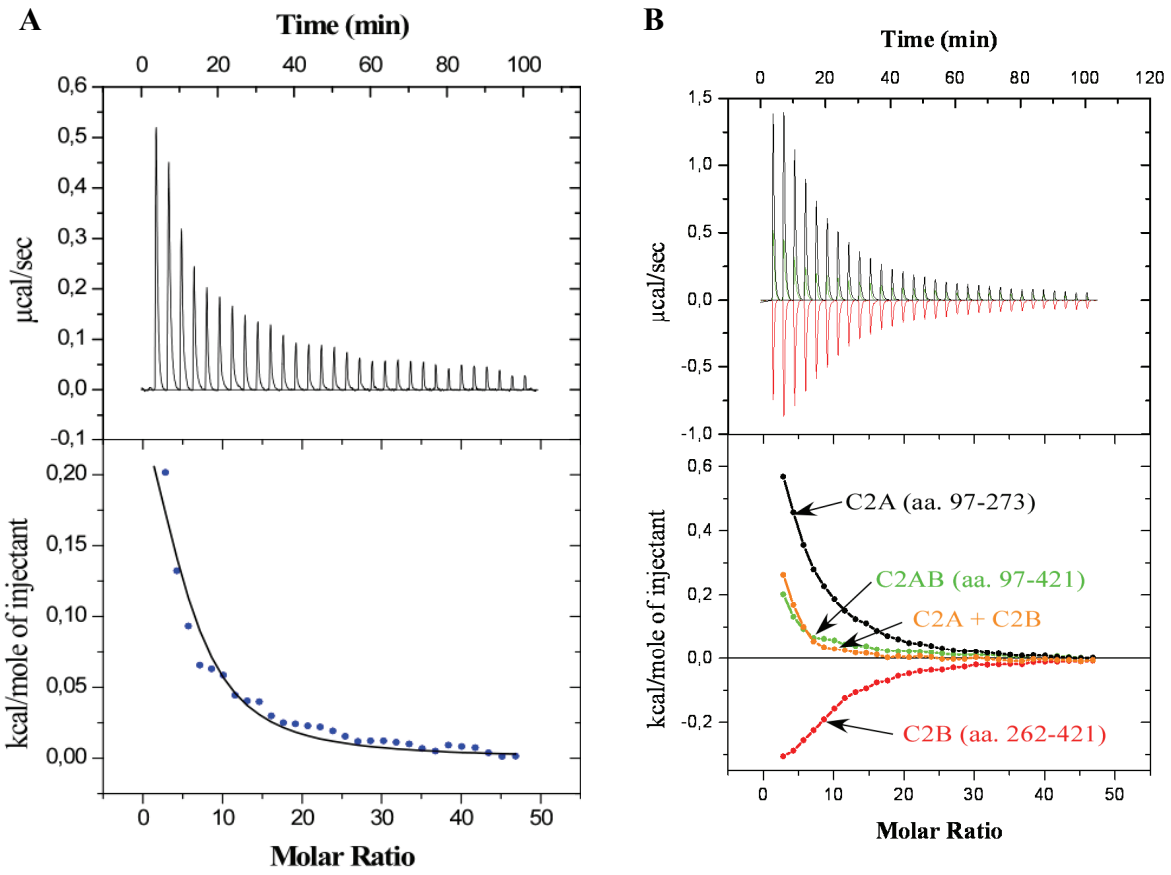


Figure R2: Isothermal Titration Calorimetry of wild-type tandem Synaptotagmin C2AB. 10 mM calcium chloride solution was titrated into 50 µM tandem wild-type Synaptotagmin C2AB in the sample cell **(A)**. The upper panel shows the raw data and the lower panel shows the integrated raw data peaks (blue scatter diagram) and the appropriate fitting (solid line) using the single binding site model. Remarkably, the heat evolved from calcium binding to the C2AB tandem domain overlays well with the curve representing the sum of the heat evolved by the C2A and C2B isolated domains **(B)**.

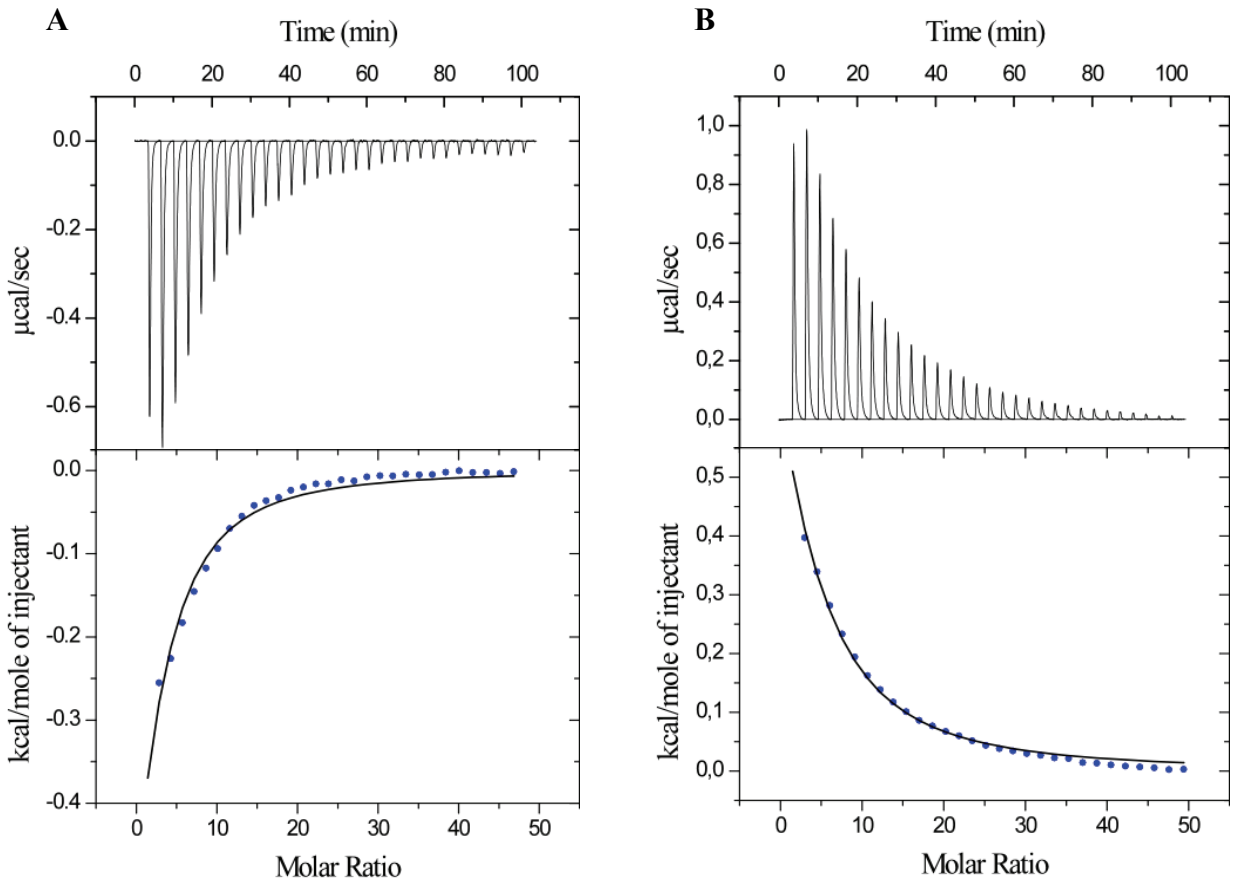


Figure R3: Isothermal Titration Calorimetry of the single C2 domain calcium-binding mutants. 10 mM calcium chloride solution was titrated into 50 µM protein in the sample cell to study the calcium binding behaviour of the tandem C2AB construct with the calcium coordinating aspartates in either the C2A (C2A*B) **(A)** or the C2B (C2AB*) **(B)** domains mutated to alanines to abolish calcium binding in the respective domains. The upper panel shows the raw data and the lower panel shows the integrated raw data peaks (blue scatter diagram) and the appropriate fitting (solid line) using the single binding site model. When the C2A domain is mutated, the protein behaves identical to the single C2B domain and vice versa. This supports further the idea that the two domains bind calcium independent of each other.

Do any other calcium binding sites other than the calcium binding jaw of the C2 domains exist? In order to answer this question, mutations to neutralize the key aspartate residues in both the C2 domain were done (D178A, D230A, D232A, D309A, D363A, and D365A). These mutations would cause both the calcium binding domains to lose their calcium binding abilities. Therefore any energy fluctuation observed during calcium titration would be due to calcium binding to sites other than the calcium binding jaw. However, when the experiment was done, no binding was detected (Figure R4) indicating that calcium binding only occurs at the calcium binding jaws of the Synaptotagmin C2 domains. The other possibility is that the protein, due to six point mutations could be unfolded. This however, is not the case because circular dichroism (CD) measurements, which is able to provide information on the secondary structure content of a protein, showed intact secondary structure elements similar to the wild type protein indicating a properly folded protein (data not shown).

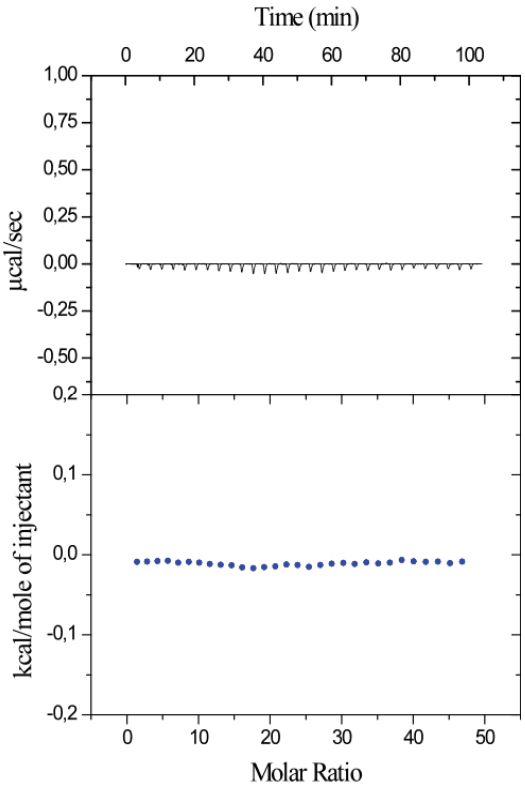


Figure R4: Isothermal Titration Calorimetry of the double C2 domain calcium-binding mutant. 10 mM calcium chloride solution was titrated into 50 µM protein in the cell to study the calcium binding behaviour of the tandem C2AB construct with calcium coordinating aspartates in both the C2A and the C2B (C2A*B*) domains mutated to alanines to abolish calcium binding. The upper panel shows the raw data and the lower panel shows the integrated raw data peaks (blue scatter diagram) and the appropriate fitting (solid line) using the single binding site model. No detectable binding could be observed suggesting that there are no other weak calcium binding sites apart from the calcium binding jaw of the C2 domains.

25μM protein in the cell	C2A	C2B	C2AB	C2AB*	C2A*B
N	3	2	5	3	2
K	4.17E3 ± 1.8E2	2.11E3 ± 2.0E2	3.91E3 ± 2.7E2	3.74E3 ± 1.9E2	3.67E3 ± 2.6E2
K_d (μM)	240 ± 11	474 ± 42	256 ± 17	267 ± 13	272 ± 18
ΔH (cal/mole)	2319 ± 35.2	-2567 ± 106	699.7 ± 22.9	1891 ± 34.9	-1734 ± 45.1
ΔS (cal)	24.3	6.61	18.8	22.7	10.5
ΔG (kcal)	-4.92	-4.54	-4.90	-4.87	-4.86

Table R1: Summary of thermodynamic parameters obtained from the calcium titration to 25 μM of the different Synaptotagmin constructs. All values were obtained after fitting the integrated scatter plots using the single binding site model.

50μM protein in the cell	C2A	C2B	C2AB	C2AB*	C2A*B
N	3	2	5	3	2
K	4.27E3 ± 1.5E2	2.13E3 ± 1.7E2	5.91E3 ± 9.1E2	2.79E3 ± 1.1E2	3.63E3 ± 3.0E2
K_d (μM)	234 ± 8	470 ± 36	169 ± 23	358 ± 14	275 ± 21
ΔH (cal/mole)	2142 ± 26.3	-2660 ± 93.4	372 ± 18.5	1991 ± 31.9	-1596 ± 49.2
ΔS (cal)	23.8	6.31	18.5	22.4	10.9
ΔG (kcal)	-4.95	-5.38	-5.14	-4.68	-4.84

Table R2: Summary of thermodynamic parameters obtained from the calcium titration to 50 μM of the different Synaptotagmin constructs. All values were obtained after fitting the integrated scatter plots using the single binding site model.

3.2 Lipid Binding

Calcium binding to the C2 domains induces a subsequent binding to lipid membranes (reviewed in (23)). In the case of Synaptotagmin, calcium-dependent lipid binding plays a central role in the final exocytotic step at the neuronal synapse. In the past number of years, many studies have focused on understanding the exact binding mechanism of Synaptotagmin to the membrane. These studies have shown that binding to the membrane is calcium dependent (29) and Synaptotagmin C2 domains are bound to negatively charged phospholipids, such as PS (14). Additionally, upon calcium binding, the C2 domains of Synaptotagmin have been shown to penetrate the bilayer (30) and this penetration is deeper when both domains are present (32). Studies from PKC have shown that PIP_2, which is enriched in the plasma membrane, is important for the localization of PKC to the plasma membrane (77).

Based on these studies, it seems that C2 domains, in general, are heavily influenced by the lipid composition of the target membrane. Therefore, the main aim of this section was to study the effect of lipid membrane composition on the interaction of Synaptotagmin to the membrane. Since it is difficult to modulate the lipid composition of membranes *in vivo*, an *in vitro* assay to study the effect of different lipid compositions in the binding of Synaptotagmin to membranes was developed. The *in vitro* assay was based on a previous FRET-based assay between the tryptophans residues of Synaptotagmin, as the donor fluorophore, and dansylated-PE, as the acceptor fluorophore (29). This assay was modified by using single cysteine mutants of Synaptotagmin labelled with Alexa-488 dye, as the donor fluorophore and Texas Red-PE in liposomes as the acceptor fluorophore. The advantage of this current approach over the previous tryptophan-based approach is that since artificial fluorophores are used (i.e. not tryptophans), SNARE proteins could be introduced in the assay to test the effect of SNAREs on the lipid binding of Synaptotagmin. Additionally, the FRET signal changes are larger and more consistent thereby providing more reliable data. Initially, several different Synaptotagmin single cysteine mutants, labelled with Alexa-488 were tested. Eventually, Synaptotagmin labelled at position 342 was selected for further experiments since it showed the largest and most consistent FRET-dependent signal change.

Before the assay could be used to study the effect of lipid composition on Synaptotagmin - membrane interaction, it had to be tested if it could reliably measure

binding of Synaptotagmin to lipid membrane. Since the two main requirement of Synaptotagmin – lipid membrane interaction is the presence of calcium and the anionic phospholipid, PS, the assay was done using liposomes with either no PS (0%) or high amounts of PS (25%). In the absence of PS, no changes in the FRET signal could be observed. On the contrary, liposomes containing 25% PS showed a dramatic reduction in the donor signal and a corresponding increase in the acceptor, typical to a FRET-dependent signal change. This FRET change could be reversed using EGTA, which removes calcium from the solution, demonstrating the calcium dependence of the binding (Figure R5).

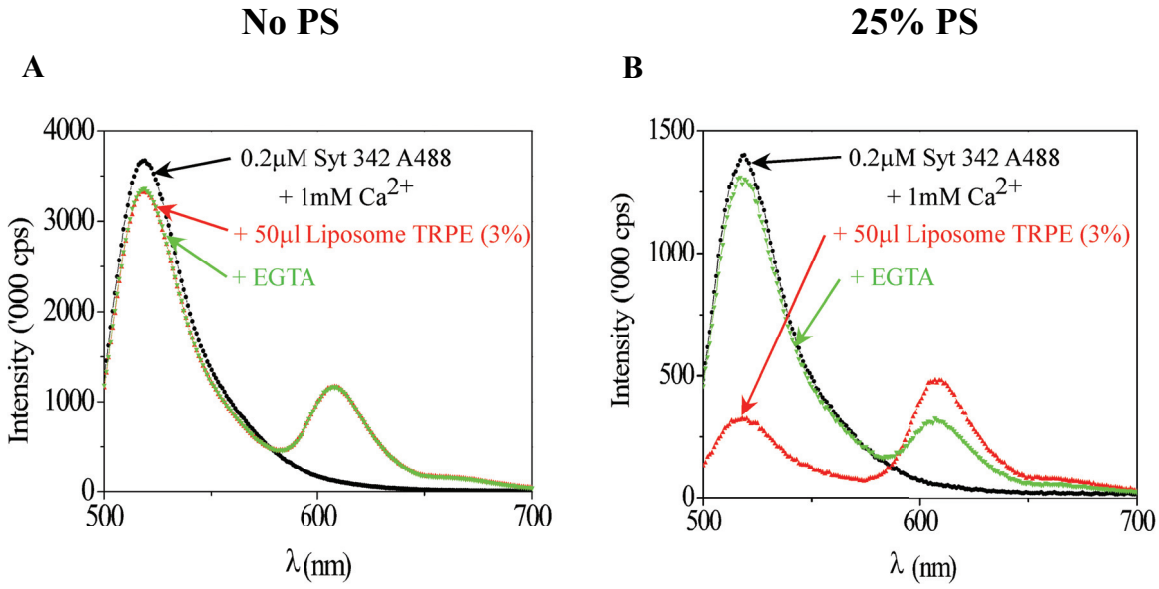


Figure R5: FRET signal is only observed when PS and calcium is present. 50 µl of Texas Red labelled liposomes (acceptor) is added to 0.2 µM Synaptotagmin labelled with Alexa-488 (donor) in the presence of 1 mM calcium. The addition of liposomes without PS does not induce the FRET-dependent quenching of the donor signal (λ = 520 nm) **(A)**. However, when PS is present in the liposome, the donor signal is quenched, indicating a FRET dependent signal change and can be reversed using EGTA **(B)** showing the calcium dependence of this interaction.

3.2.1 Influence of lipid composition on the binding of Synaptotagmin to the liposome membrane

In order to study the influence of lipid composition on the membrane binding of Synaptotagmin, liposomes containing different amounts of PS in its membrane were titrated to a cuvette containing Synaptotagmin. Lipid stock solutions containing either no PS or 30% PS (in addition to other phospholipids; PC, PE and cholesterol and Texas Red labelled PE) were prepared and diluted in appropriate volumes to obtain lipid mixes, and subsequently liposomes containing 5, 10, 15, 20, 25 and 30% PS. The increasing concentration of PS in the liposome membrane can be correlated to an increase in the density of PS in the liposomes since the liposomes have a similar surface area. The labelled liposomes (acceptor) were titrated into 0.2 μM of labelled Synaptotagmin (aa. 342 Alexa 488) (donor) in the presence of 1 mM calcium. The emitted fluorescence intensity of the donor (λ = 520 nm) was measured (F) and normalised (F_0/F) to the intensity before the addition of labelled liposomes (F_0). The emission intensity of the donor (Synaptotagmin) is quenched in the presence of the acceptor (liposomes). The degree of quenching is directly dependent on the amount of acceptor that is bound to the donor. Therefore, when all the donor molecules are bound to the acceptor, the signal reaches saturation and no further changes can be observed. Since Synaptotagmin is the limiting factor in this experiment, saturation would indicate that all Synaptotagmin molecules are bound to the lipid membrane at signal saturation. The normalised donor intensity counts were then plotted against either the volume of liposomes used or the concentration of PS in the liposome. Additionally, to study the effect of PIP_2 on the binding of Synaptotagmin to the membrane, the titration was repeated in the presence of 1% PIP_2 in the lipid mix.

When the data was plotted against the volume of liposomes used, it seems that when the amount of PS in the membrane is increased, less liposomes are needed to saturate the signal (Figure R6). In liposomes with higher amounts of PS in its membrane, it would be logical to assume that since more PS molecule is present per surface area, the K_d of the binding between Synaptotagmin and PS is achieved sooner, i.e. with less volume. If this was indeed the case, then plotting the data against the PS concentration would provide identical titration curves with similar EC_{50} values. Interestingly, when the data was plotted against the PS concentration (Figure R7), the affinity of Synaptotagmin to PS was increased as the density of PS in the liposome membrane was increased (EC_{50} = ~ 25 μM

to ~5 µM). The addition of PIP_2 to the membrane seemed to also affect the affinity of Synaptotagmin to PS. At lower PS densities (5 and 10%) the addition of PIP_2 seems to increase the affinity of Synaptotagmin to PS (EC_{50} = ~25 µM (- PIP_2) and 12 µM (+ PIP_2)). However, at higher PS densities (15% and higher), the addition of PIP_2 does not seem to increase the EC_{50} value as dramatically (EC_{50} = ~5 µM (- PIP_2) and 4 µM (+ PIP_2)) (Figure R7C). This suggests that the binding of Synaptotagmin to the lipid membrane is not only dependent on the PS concentration. Perhaps other factors, such as cooperativity between PIP_2 and PS as well as formation of membrane microdomains might play a role in the modulation of the Synaptotagmin – lipid membrane interaction.

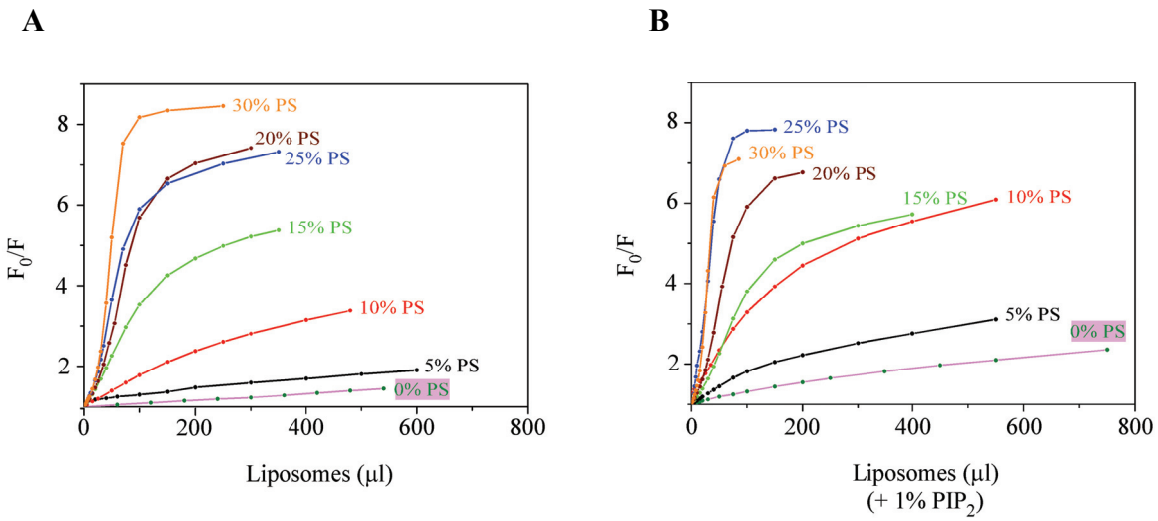


Figure R6: Binding of Synaptotagmin to the membrane is dependent on the lipid density and composition of the membrane. Liposomes were titrated into 0.2 µM Synaptotagmin in the presence of 1 mM calcium. The normalised Synaptotagmin (donor) fluorescence was then plotted against the volume of liposomes used. Increasing the amount of PS in the membrane seems to decrease the volume of liposomes needed to reach saturation. In the absence of PS, no changes in the donor fluorescence were observed.

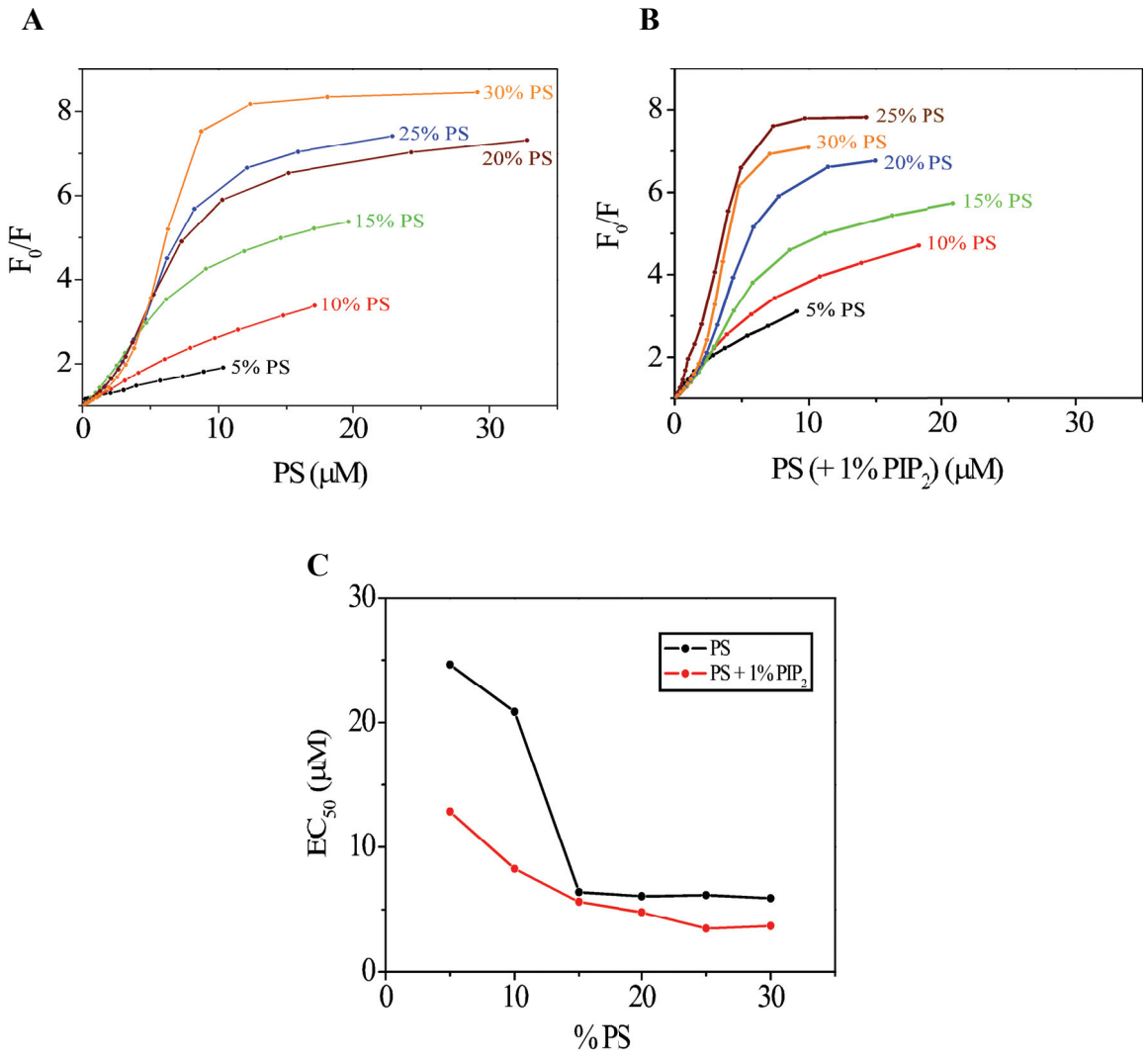


Figure R7: Increasing the PS density in the liposome reduced the amount of PS needed to saturate the binding of the lipid membrane to Synaptotagmin. Titration of liposomes labelled with Texas Red conjugated PE to Synaptotagmin labelled with Synaptotagmin Alexa-488 was done for liposomes containing different amounts of PS. Both in the absence **(A)** and presence **(B)** of PIP_2, increasing the amount of PS seems to reduce the amount of liposomes needed to saturate the signal. Interestingly, when the EC_{50} of the titration curves were plotted against the amount of PS (%), it seems that PIP_2 induces a significant affinity increase when the amount of PS is under 15% **(C)**. At higher PS concentrations, the EC_{50} of both experiments are very similar.

3.3 Synaptotagmin – SNARE Interaction

3.3.1 Binding of Synaptotagmin to the SNARE

3.3.1.1 Synaptotagmin – SNARE interaction: A gel-based approach

Binding of Synaptotagmin to the single isolated Q-SNAREs, Syntaxin and SNAP 25, as well as the binary and ternary complex have been described before (see section 1.5.1). It might be possible that Synaptotagmin binds to SNAREs in all stages of exocytosis but highly improbable since the early stages of docking and priming are not influenced in the Synaptotagmin deletion mutants (17) (16) (15). It has been difficult to prove the link between Synaptotagmin and the SNARE complex *in vivo* due to the dynamic nature of neuronal secretion. However, the interaction between the SNARE complex and Synaptotagmin is an important implication since this interaction would be the crucial link between the membrane fusion machinery and calcium-dependent membrane mixing, which is the basis of synaptic transmission. Therefore, the main aim of this section of the work was to characterize the binding of Synaptotagmin to the SNARE complex.

Initially, in order to test the binding of Synaptotagmin to the SNARE complex qualitatively, a simple assay that would enable the visualization of the binding of unmodified Synaptotagmin and SNARE complex was needed. This was accomplished using a method known as native PAGE. Native PAGE separates proteins based on their net charge. This net charge, when measured at pH 7, is known as the isoelectric point (pI) of a protein. In this experiment, equal amounts of the purified proteins (5 μg) are incubated with or without excess calcium (5 mM), overnight on ice. This incubated mix is then run on the native gel and visualised using Coomassie-blue-blue staining. The binding of Synaptotagmin to either the isolated single Q-SNAREs, binary and ternary complexes, without the transmembrane domains, were tested using this method. The ternary complex used here was pre-purified on the ion exchange column to remove the excess single SNAREs. The binary complex was not pre-purified but assembled from the Q-SNARE monomers. Isolated Synaptotagmin C2 domains as well as the calcium mutants from the ITC experiments were also tested for binding to the SNARE ternary complex.

The binding of the different Synaptotagmin constructs to the SNARE ternary complex is shown in Figures R8 & R9. The addition of Synaptotagmin to the pre-

purified ternary SNARE complex promoted the formation of a new band (denoted as χ) both in the absence and presence of calcium. This new band represents the SNARE – Synaptotagmin complex. The formation of this protein complex occurs for the isolated C2B as well as the tandem C2AB construct. However, when the isolated C2A domain was used, no new band was formed indicating the isolated C2A domain is unable to form a complex with the SNAREs. In order to study if the calcium binding sites are important for the SNARE complex interaction, the calcium binding mutants used in the ITC experiments were incubated with the SNARE complex overnight in the presence and absence of calcium. Binding to the SNARE complex was seen in both the constructs with the inactive single domain or the construct with the tandem domain inactivated indicating that the calcium binding site in not crucial for the Synaptotagmin – SNARE interaction.

In previous studies, binding of Synaptotagmin to the binary SNARE complex (46) as well as the isolated Syntaxin (43) and SNAP 25 (44) were described. Using a similar approach, both these interactions were tested. The binary complex was formed by incubating equal amounts (5 μg) of Syntaxin and SNAP 25 with Synaptotagmin in the presence or absence of 5 mM calcium. Similarly, the isolated Syntaxin or SNAP 25 was incubated with Synaptotagmin. Binding was seen for the binary SNARE complex both in the absence and presence of calcium (Figure R10) but not for the isolated Q-SNAREs, Syntaxin (Figure R11) and SNAP 25 (Figure R12). As in the case of the ternary complex, the binding to the binary complex required the Synaptotagmin C2B domain to be present. When only the isolated C2A domain of Synaptotagmin was used, no complex formation was observed. Based on the results above, it seems that Synaptotagmin is able to bind only to the binary and ternary complex but not to the isolated Q-SNAREs. This binding is mediated via its C2B domain and occurs both in the absence and presence of calcium. Additionally, the calcium binding site of the C2 domains seemed not to be crucial for the SNARE binding since inactivating these domains by mutating the calcium coordinating aspartates to alanines seemed to still promote binding to the SNARE ternary complex. An important question that arises from this study is whether the complex formed in the absence and presence of calcium is similar. It could be that the addition of calcium could cause a conformational change or increase the binding affinity of Synaptotagmin to the SNARE complex. To address these questions, more sophisticated biochemical assays utilizing environmentally sensitive dyes as well as fluorescence techniques like anisotropy was used.

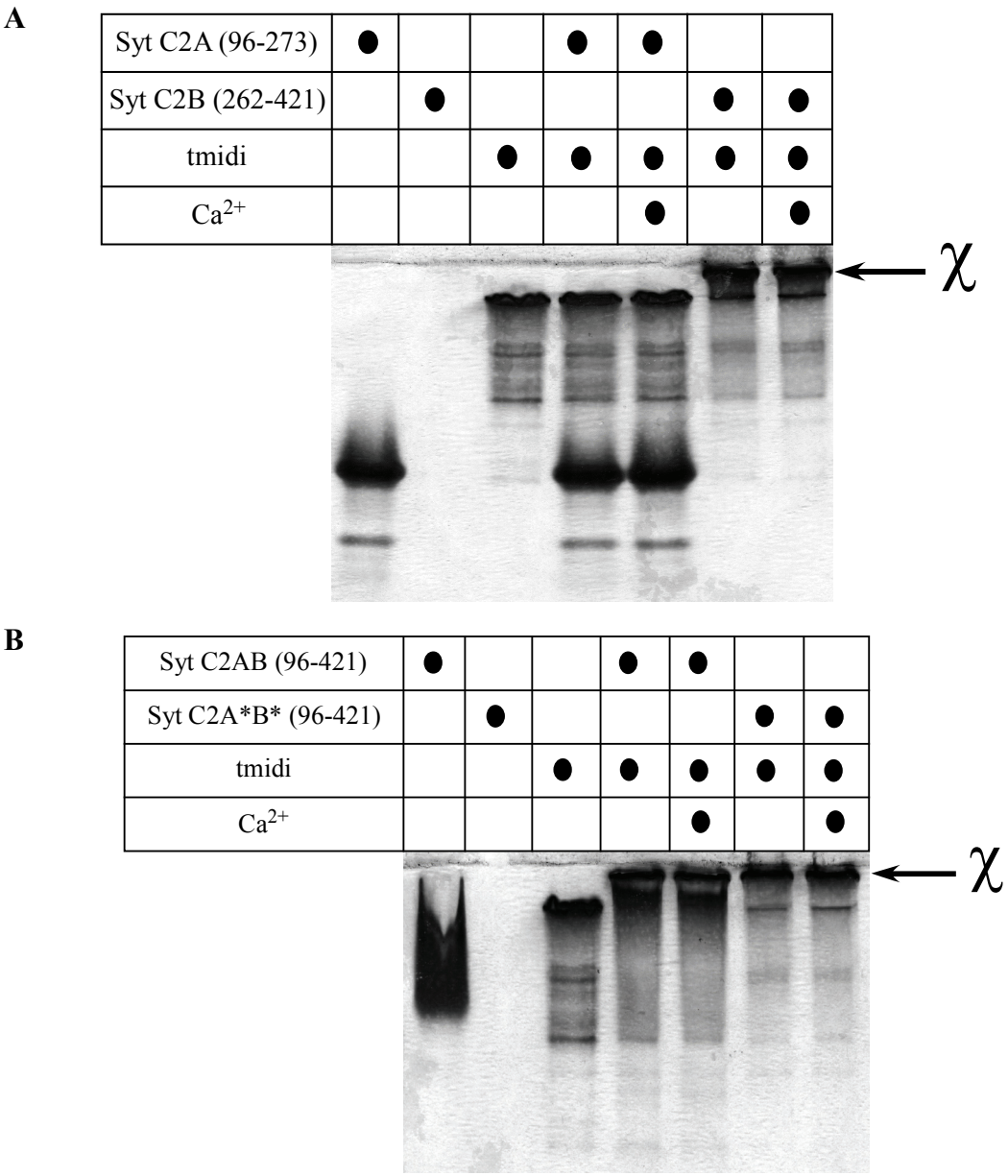


Figure R8: Binding of Synaptotagmin to the ternary SNARE complex (1). The binding of the isolated C2A and C2B domains **(A)** as well as the wild type tandem C2AB and the double domain mutant C2A*B* **(B)** were tested in the native PAGE assay to determine SNARE binding. The addition of Synaptotagmin to the SNARE complex both in the presence or absence of calcium showed the presence of a new band on the gel indicating complex formation (marked as χ). All the Synaptotagmin tested here bound the SNARE ternary complex except for the isolated C2A domain **(A)**.

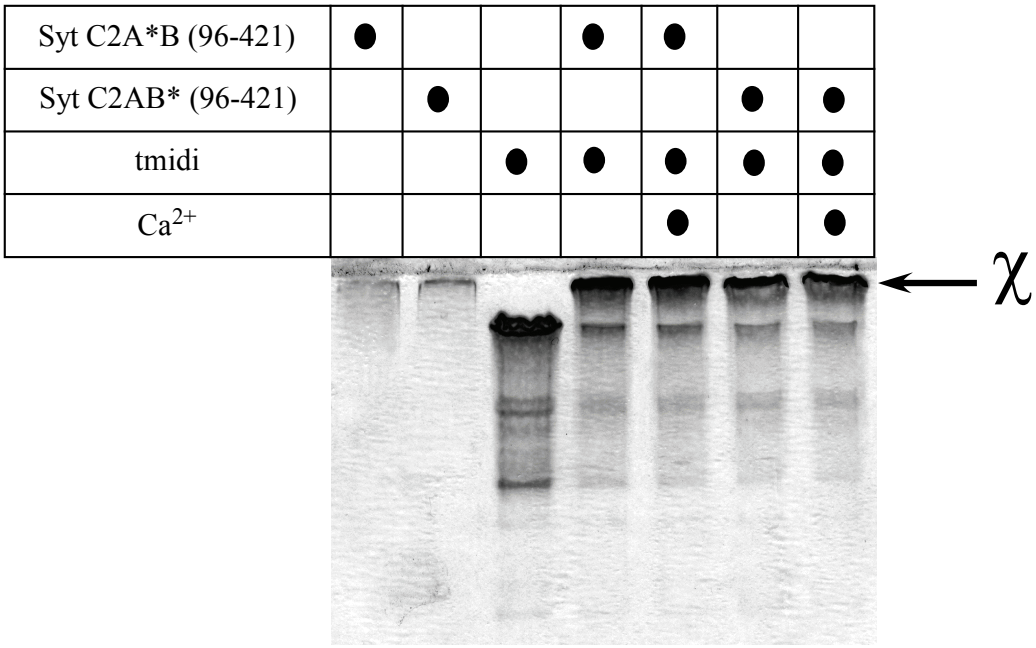


Figure R9: Binding of Synaptotagmin to the ternary SNARE complex (2). The binding of the tandem domain with either the C2A or the C2B domain mutated to disable calcium binding, were tested in the native PAGE assay to determine SNARE binding. In both cases, the addition of these Synaptotagmin mutants to the SNARE complex both in the presence or absence of calcium showed the presence of a new band on the gel indicating complex formation (marked as χ).

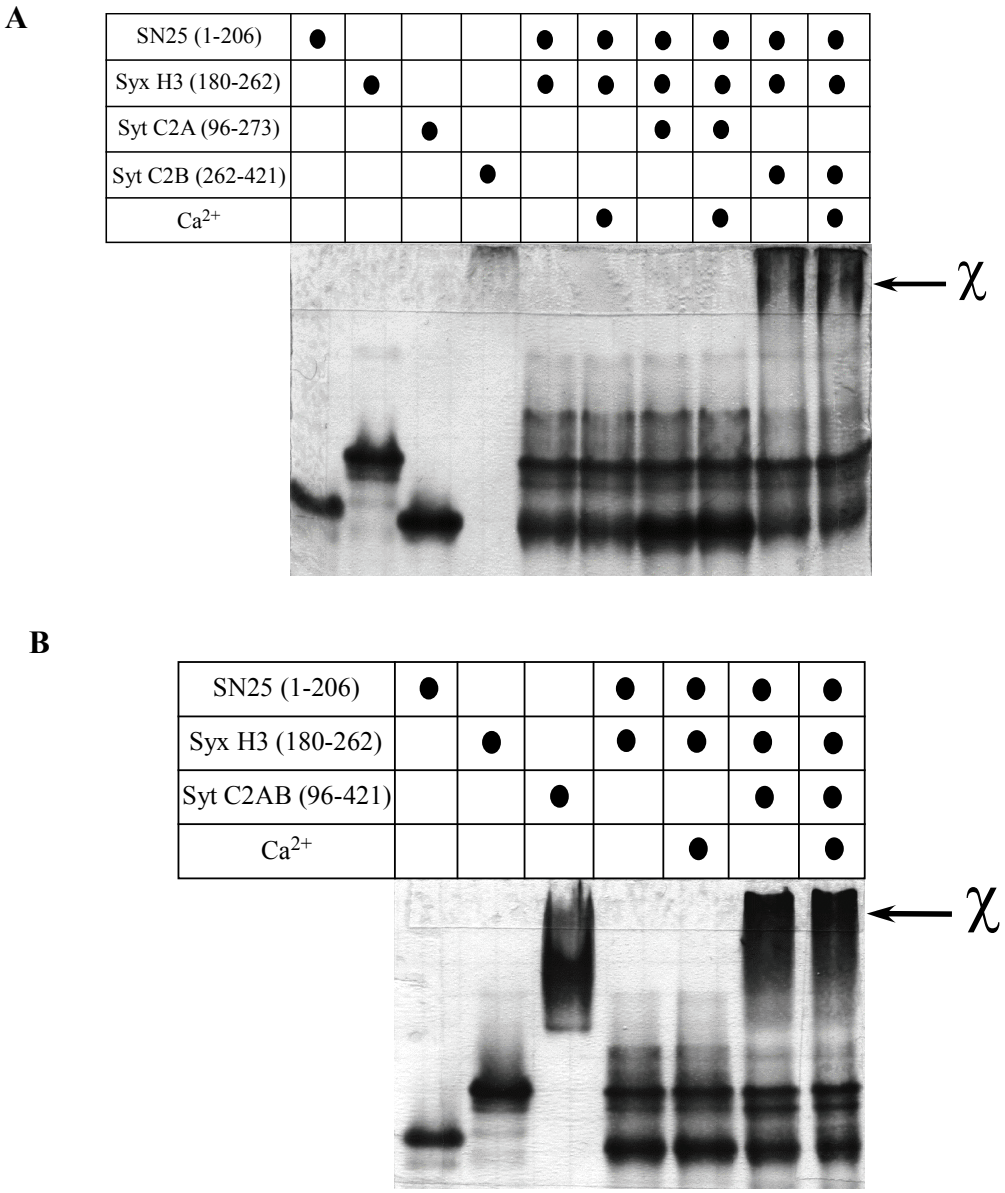
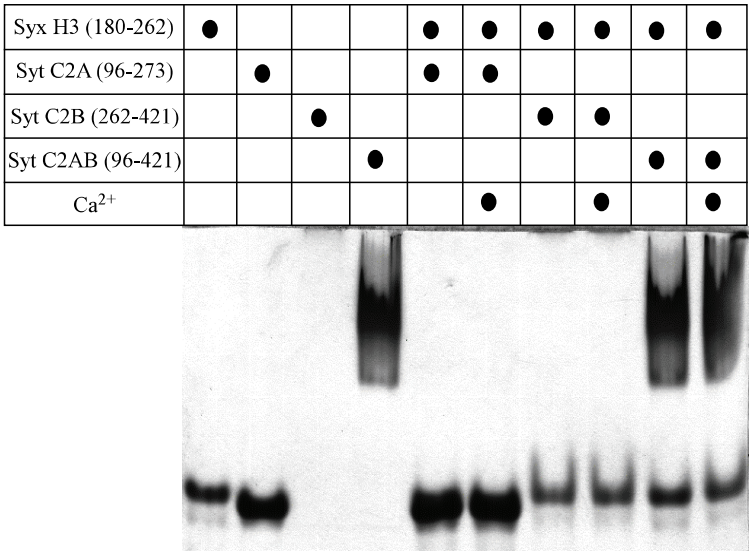


Figure R10: Binding of Synaptotagmin to the SNARE Binary Complex. The isolated Q-SNAREs were incubated in equal amounts (40 μM) in the presence or absence of Synaptotagmin and calcium to study the binding of Synaptotagmin to the binary complex. The binary SNARE complex - Synaptotagmin complex, which is represented by the presence of a new band on the gel (denoted as χ), was only observed when the isolated C2B domain **(A)** and the tandem C2AB domain construct **(B)** of Synaptotagmin was incubated with the binary complex. The isolated C2A domain did not show any binding to the binary complex **(A)**.

A



B

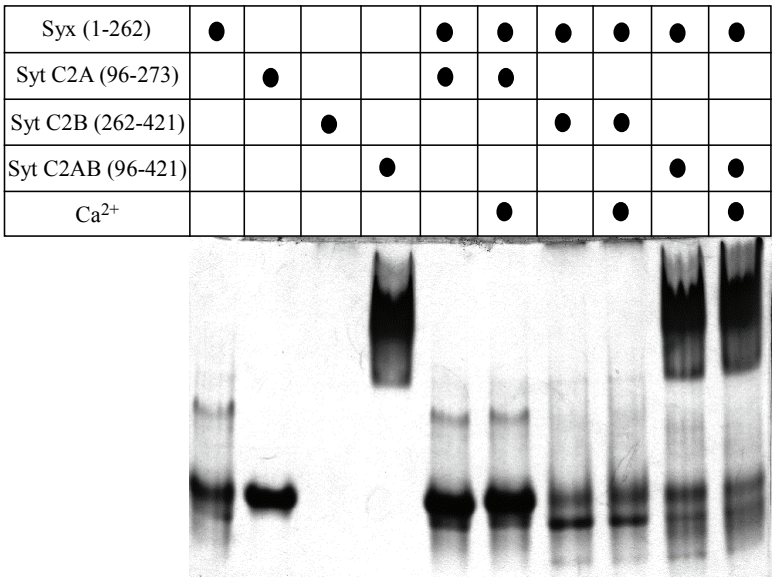


Figure R11: Binding of Synaptotagmin to Syntaxin. Purified Synaptotagmin C2A, C2B and tandem C2AB domains were incubated, in equal amounts (40 µM), overnight on ice, with either Syntaxin H3 (180-262) (**A**) or Syntaxin (1-262) (**B**) and visualized on a native gel. This was done both in the absence and presence of 5mM calcium. No detectable complex formation between the proteins could be observed.

SN25 (1-206)	●				●	●	●	●	●	●
Syt C2A (96-273)		●			●	●				
Syt C2B (262-421)			●				●	●		
Syt C2AB (96-421)				●					●	●
Ca^{2+}						●		●		●

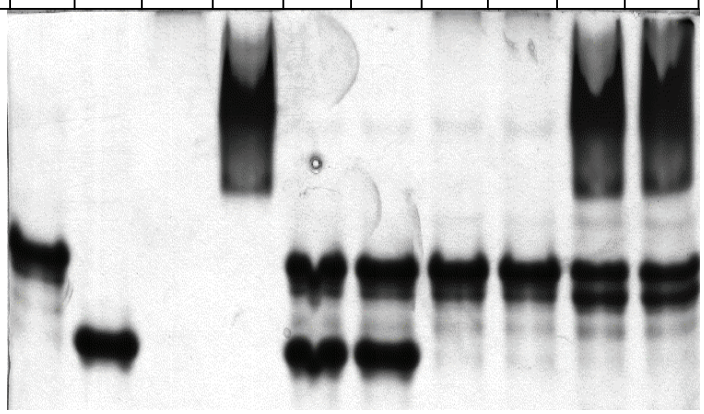


Figure R12: Binding of Synaptotagmin to SNAP25. Purified Synaptotagmin C2A, C2B and tandem C2AB domains were incubated in equal amounts, overnight on ice, with SNAP 25 and visualized using native PAGE. This was done both in the absence and presence of 5 mM calcium. No detectable complex formation between the proteins could be observed.

3.3.1.2 Synaptotagmin – SNARE interaction: A spectroscopic approach

3.3.1.2.1 Interaction of Synaptotagmin with SNARE complex labelled with IAANS

2-(4'-(iodoacetamido)anilino)naphthalene-6-sulfonic acid (IAANS) (λ_{ex} = 320nm; λ_{em} = 460 nm) is a fluorescence label that is able to detect change in its local environment. The fluorescence intensity of the dye increases when the environment around the dye becomes more hydrophobic (78). Based on the results obtained in the native PAGE assay, Synaptotagmin binds to the SNARE binary and ternary complex. IAANS was used to study the binding of Synaptotagmin to the ternary complex. The dye was coupled to SNAP 25 via a single cysteine that was introduced using site-directed mutagenesis. The labelled SNAP 25 was then incubated at a ratio of 2 : 1 : 2 (Syntaxin H3 (180-262) : labelled SNAP 25 (1-206) : soluble Synaptobrevin (1-96)) overnight on ice to form the SNARE ternary complex. Synaptotagmin, calcium and EGTA were added sequentially to 10 µl of the pre-incubated, labelled ternary SNARE complex in 2.5 ml buffer. After screening a number of different SNAP 25 labelled positions, it was determined that IAANS coupled to position 48, close to the 0-layer of the SNARE complex, showed the most significant change upon the addition of Synaptotagmin and calcium (Figure R13).

This observed signal intensity change occurred only when the ternary complex was present since the addition of Synaptotagmin and calcium to isolated SNAP 25 did not show any significant signal changes. The addition of calcium in the presence of Synaptotagmin and the ternary SNARE complex caused a large increase in the intensity of the emitted light. The calcium effect was fully reversible when the added calcium was removed with the chelating agent, EGTA. This strong effect was seen with calcium and to some degree, strontium. Approximately four times more strontium (~2 mM as compared to 0.5 mM calcium) is needed to evoke a signal change. Furthermore, the magnitude of signal change is not as large as the calcium evoked signal change. Magnesium is unable to show any detectable signal change when added to this system (Figure R15). Based on these results, it seems that the addition of calcium is “changing” the Synaptotagmin – SNARE complex. This change is only induced efficiently by calcium. It is still uncertain whether the change observed is due to an increase in affinity or protein conformation using this assay. The next step was to determine the apparent affinity of calcium for the

Synaptotagmin – SNARE complex as well as the affinity of Synaptotagmin for the SNARE complex in the presence of calcium.

This was determined by titrating either calcium or Synaptotagmin into the assay. The titration of Synaptotagmin was done with in the presence of the labelled SNARE complex and excess calcium (1 mM). Synaptotagmin was added in gradual steps until saturation was reached. The emission intensity at 460 nm wavelength was measured for every titration step, normalised to the intensity value obtained in the absence of Synaptotagmin (F_0) and plotted against Synaptotagmin concentration. The half-maximal binding (EC_{50}) of Synaptotagmin to the SNARE complex in the presence of calcium was determined to be approximately 200 nM. Calcium titration was done in the presence of 10 mM DPTA, a calcium chelator used to calibrate the amount of free calcium in the solution to obtain accurate calcium concentrations. The total amount of calcium needed to obtain specific amounts of free calcium was calculated using the IGOR software (with assistance from Jacob Sorenson). The amount of Synaptotagmin present was in excess (2 μM) to ensure that Synaptotagmin is not the limiting factor. The titration of calcium showed a sigmoidal curve indicating a cooperative reaction. The half-maximal binding was determined to be approximately 50 μM (Figure R14).

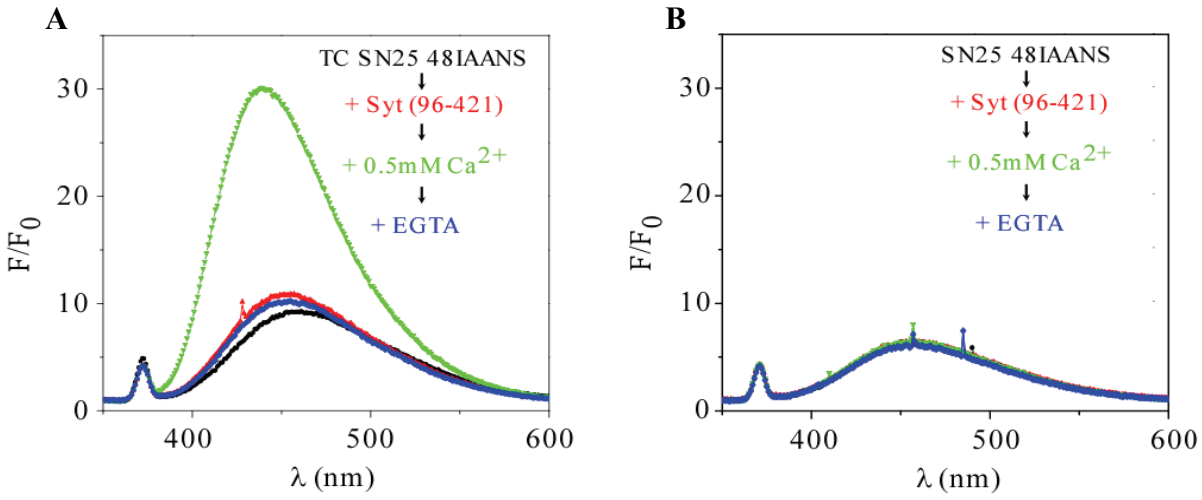


Figure R13: SNAP 25 48IAANS binding assay. The addition of Synaptotagmin and calcium to the SNARE ternary complex labelled on SNAP 25 (48IAANS) induces a large signal change **(A)**. This signal change is not detectable when only isolated SNAP 25 (48IAANS) is present supporting the idea that Synaptotagmin does not bind to the isolated Q-SNARE, SNAP 25 **(B)**.

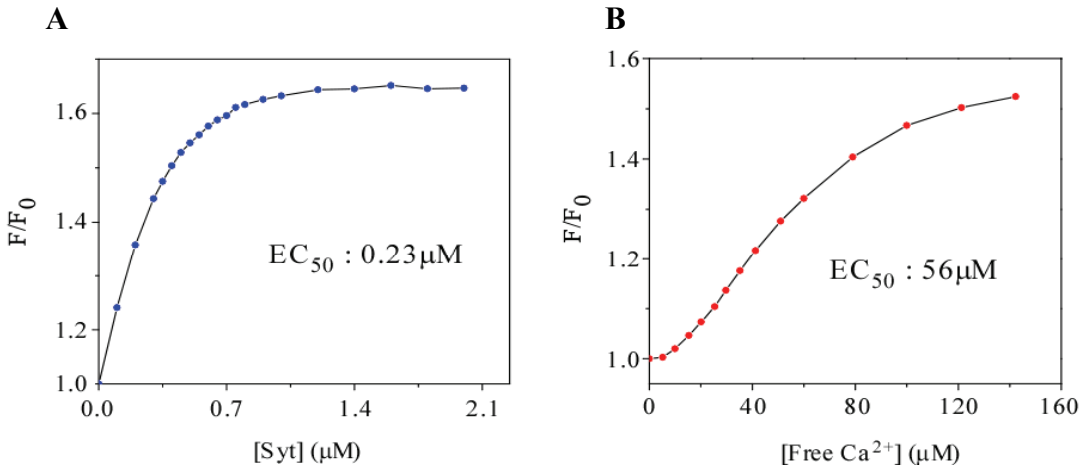


Figure R14: Titration of Synaptotagmin or calcium to TC labelled with SNAP 25 (48IAANS). Synaptotagmin was titrated in the presence of calcium (1 mM) to the labelled SNARE ternary complex **(A)**. The half-maximal binding of Synaptotagmin to the SNARE ternary complex in the presence of calcium is approximately 200 nM. The half maximal binding of calcium in this interaction was determined to be approximately 50 mM by titrating calcium to the labelled SNARE complex in the presence of excess Synaptotagmin **(B)**.

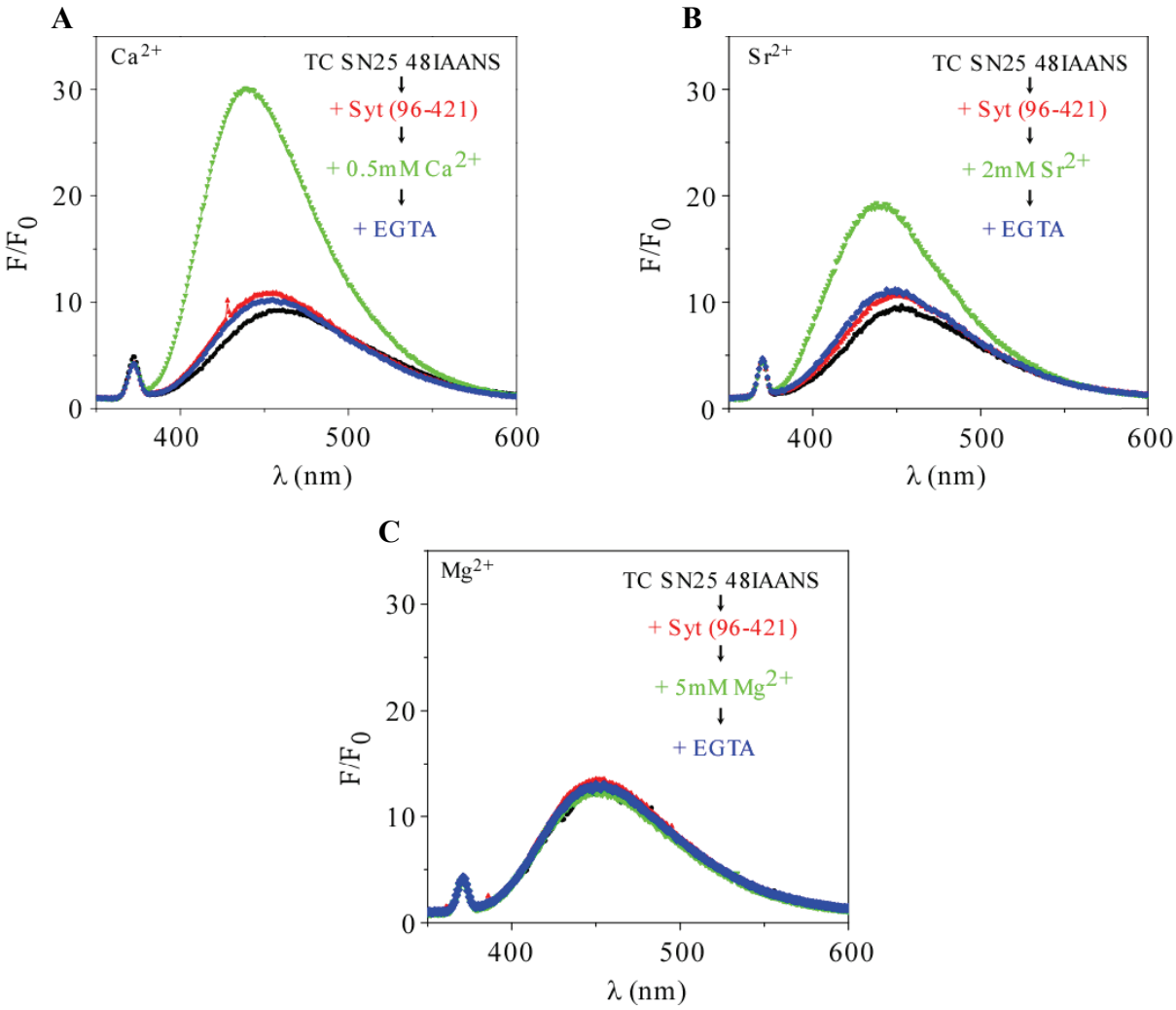


Figure R15: Effect of different divalent ions in Synaptotagmin binding to TC labelled with SNAP 25 (48IAANS). The addition of calcium induces a large change in the fluorescence emission intensity, which might indicate binding of Synaptotagmin to the SNARE **(A)**. Strontium is able to induce an intensity increase as well. However, the increase in the intensity has a lower magnitude and requires four times more strontium as compared to calcium **(B)**. Magnesium shows no detectable signal change **(C)**.

In a previous study, the binding site of Synaptotagmin was mapped to the C-terminal portion of Syntaxin using the GST-pulldown assay (53). Since the SNAP 25 molecule is labelled in this assay, by incubating this isolated SNARE protein with Syntaxin of differing lengths and soluble Synaptobrevin, the effect of shortening the Syntaxin molecule on the calcium effect observed in the IAANS assay could be studied. For this purpose, two different Syntaxin constructs; the BoNT-C fragment (183-253) and the shortened H3 domain (183-240) of Syntaxin were used. Both these constructs have been shown before to be able to form the SNARE ternary complex (79). Synaptotagmin, calcium and EGTA were added sequentially and the spectrum was recorded upon each addition. The large signal change observed upon calcium addition to Synaptotagmin and the labelled TC seemed to be reduced in magnitude when the BoNT-C fragment was used until complete abolishment in the case of the shortened H3 domain (Figure R16). These results indicate that Syntaxin, which does not bind Synaptotagmin directly, is important in forming the binding surface for the Synaptotagmin – SNARE ternary complex interaction. When the Syntaxin molecule is gradually shortened, this binding site might be destabilised causing a gradual loss of the observed calcium effect with the addition of Synaptotagmin and calcium. Another possibility would be that the binding between Synaptotagmin and the SNARE complex is still occurring but the conformational change induced by calcium and detected by the IAANS label is no longer present in the shortened Syntaxin complex.

3.3.2.1.2 Anisotropy measurements of Synaptotagmin – SNARE complex interaction

Based on the labelling position of SNAP 25 in the previous assay, an amino acid position close to this region on Syntaxin was chosen. The region, at which, the assays were based on is a negatively charged region on the SNARE ternary complex bundle, which was proposed to be the binding site for Synaptotagmin (3) (Figure I6A). Initially, Syntaxin was labelled at position 225 with IAANS. However, experiments with this dye showed small intensity changes, which were difficult to interpret. Therefore, a different approach was chosen. Anisotropy is method that uses polarized light to measure the rotational movement of a fluorescence dye. The increase in anisotropic values indicates a restriction in the rotational movement of the dye and can be used to track protein-protein interactions. The dye used in anisotropy measurements varies. Here, the dye Alexa-488

was chosen because of the high quantum yield of the dye, which makes it more resistant to quenching as compared to IAANS. Additionally, this approach also allows the monitoring of binding with only one of the binding partners fluorescently labelled. The anisotropy of this dye coupled to the SNARE ternary complex via Syntaxin was measured after each sequential addition of Synaptotagmin, calcium and EGTA. Initially, the SNARE ternary complex without the transmembrane domains was used (as in the previous experiment with IAANS). Based on the initial screening experiment, the dye coupled close to the 0-layer showed the most calcium independent and dependent changes. The N-terminal (position 197) and C-terminal (position 259) showed no significant changes upon Synaptotagmin addition (Figure R17A). Therefore, future experiments were done using the Syntaxin 225 Alexa 488 labelling position. In a separate experiment, when the SNARE complex with the transmembrane domains was used, the observed anisotropy changes were far more dramatic indicating that the transmembrane domains of the SNARE proteins were important in Synaptotagmin binding. It seemed that when the SNARE complex was inserted into liposomes, the changes observed was the most dramatic indicating that part of the signal change might be due to lipid binding of Synaptotagmin hinting that perhaps both lipid and SNARE binding occur simultaneously (Figure R17B). Since the transmembrane domain of the SNARE proteins increased the anisotropy changes observed, the next experiments were done using the SNARE complex with the transmembrane domain. The experiments were done in detergent (15 mM CHAPS) since the concentration of the SNARE complex was far too low to be inserted into liposomes.

The addition of Synaptotagmin C2AB to the ternary complex labelled with Alexa 488 (Syntaxin 225) led to an increase in the anisotropy. This increase was further enhanced when calcium was added to this reaction and was fully reversible upon removing the calcium with EGTA (Figure R16 (2)). Initially, since Synaptotagmin have been reported to bind to isolated Syntaxin, Synaptotagmin, calcium and EGTA were added to the labelled Syntaxin (225A488) to investigate this binding. Weak calcium dependent and independent signal changes were observed, which was extremely small when compared to the signal changes when the ternary complex is present (Figure R16 (1)) indicating a weak binding of Synaptotagmin to the isolated Syntaxin. When strontium was substituted for calcium in the experiment, the characteristic signal change was also observed. However, the amount of strontium needed was three times more than calcium (1.5 mM of strontium as compared to 0.5 mM calcium) for a similar magnitude of anisotropic change (Figure

R16 (2 & 3)). The final experiment was to study if the SNARE complex preferentially bound to either the isolated C2A or C2B domain of Synaptotagmin. The addition of the isolated C2A domain did not induce any detectable changes in the anisotropic measurements, which might indicate that this domain alone is unable to bind to the SNARE complex. On the other hand, the addition of the isolated C2B domain induced an anisotropic change that was similar to the tandem C2AB domain (Figure R16 (2, 4 & 5)). However, the size of the calcium dependent increase in the anisotropy value was lower when only the isolated C2B domain was present as compared to the tandem domain protein indicating that although the C2A domain is not binding the SNARE complex, it might still have a role in assisting the C2B domain in its function.

These results indicate that the transmembrane domains are important for the binding of Synaptotagmin to the SNARE complex. These experiments have also confirmed further the results shown previously in both the IAANS as well as the native PAGE assay. It seems that the ternary complex is needed for the interaction of Synaptotagmin to the SNAREs. The isolated Q-SNAREs are not able to bind to Synaptotagmin. Additionally, there seems to be a difference in the presence and absence of calcium indicating further that there might be a change in the conformation or affinity in the Synaptotagmin – SNARE complex interaction when calcium is added.

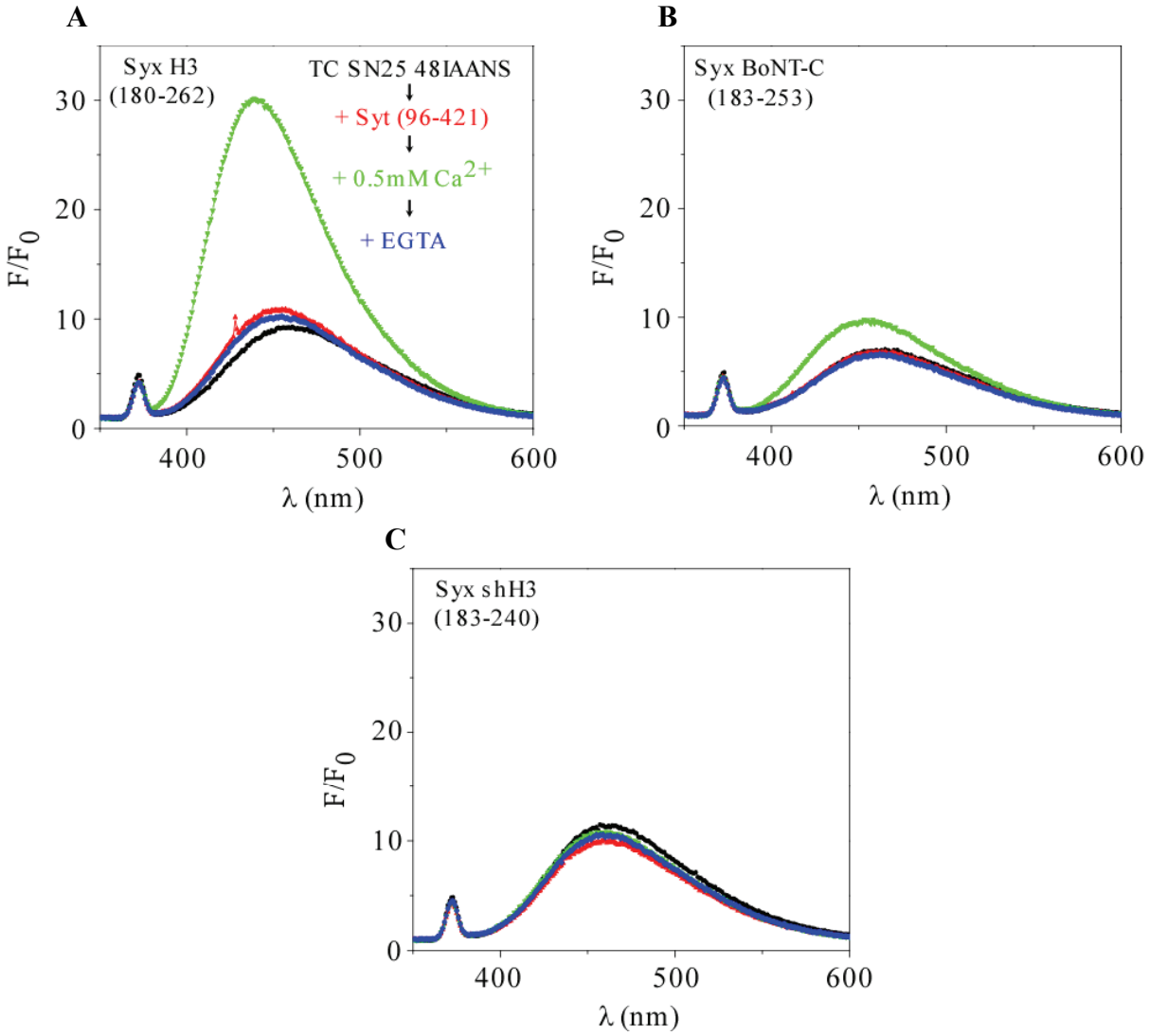


Figure R16: Effect of shortening the Syntaxin molecule on the binding of Synaptotagmin to the SNARE ternary complex labelled with IAANS. The binding of Synaptotagmin to the SNARE ternary complex induces a large signal change when calcium is added in the presence of the proteins **(A)**. When the Syntaxin molecule is shortened to either the BoNT-C fragment **(B)** or the shortened H3 domain **(C)**, the large increase induced by calcium addition to Synaptotagmin and the SNARE complex is reduced and eventually abolished.

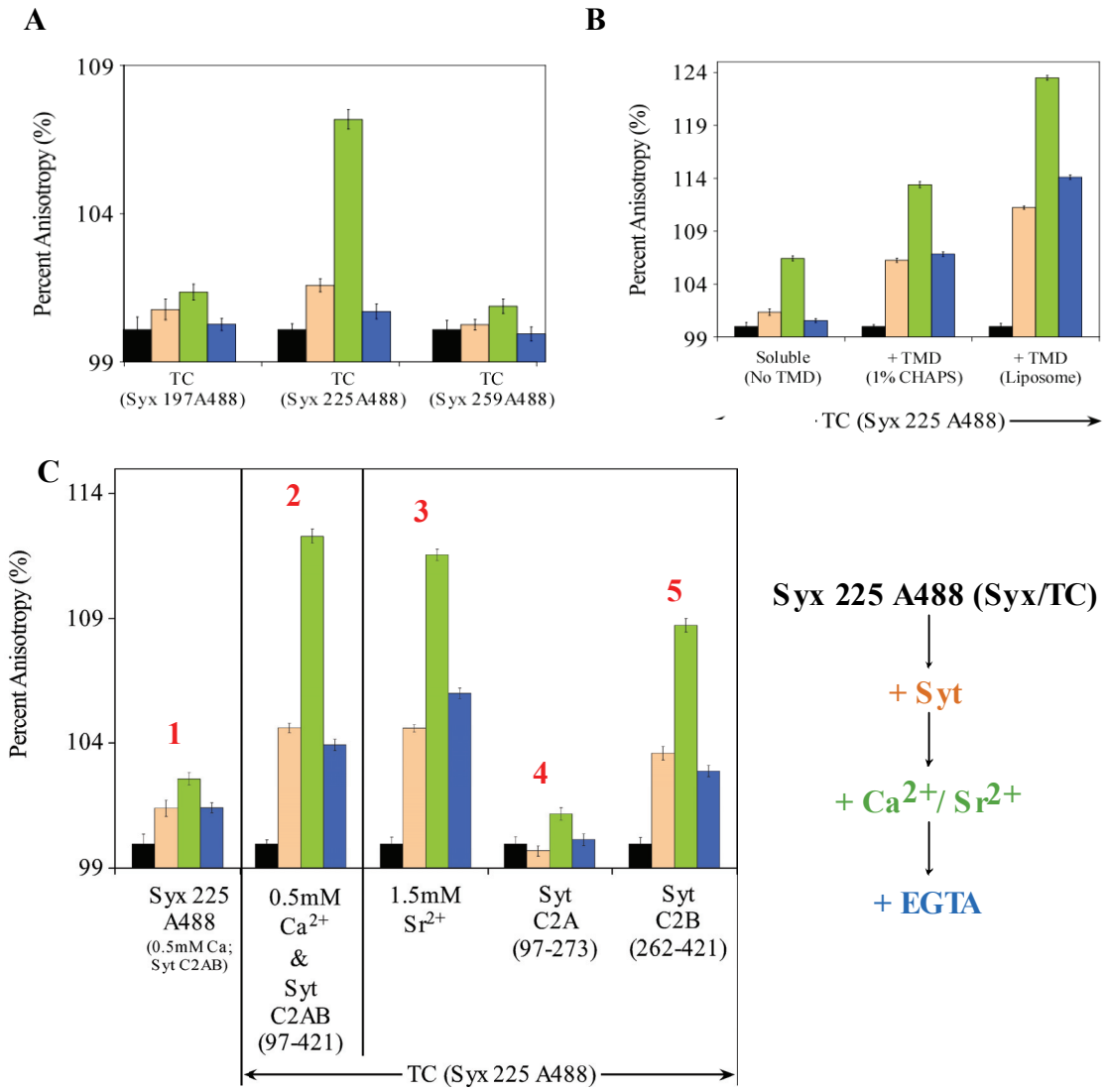


Figure R17: Alexa 488 anisotropy measurements to monitor the Synaptotagmin – SNARE complex interaction. The anisotropy of 0.2µM SNARE ternary complex labelled with Alexa 488 upon the addition of Synaptotagmin (0.5 µM), calcium (1 mM) and EGTA was monitored (C2). Anisotropy changes were only observed for the labelling position close to the 0-layer (Syntaxin 225C) and not for N-terminal (Syntaxin 197C) and C-terminal (Syntaxin 259C) positions on the SNARE ternary complex **(A)**. The anisotropy changes observed upon Synaptotagmin and calcium addition to the SNARE ternary complex labelled at position 225 of the Syntaxin is more pronounced when the transmembrane domains of the SNARE complex is included **(B)**. Syntaxin alone did not show a significant signal change (C1) and 3-fold more strontium is needed as compared to calcium (C3). The isolated C2A domain of Synaptotagmin is unable to induce an anisotropy change indicating the lack of binding (C4). Conversely, the C2B domain is able to bind the SNARE complex (C5) although the amplitude of the anisotropy is lower than when both domains are present (C6). The error bars indicate the standard error of mean (SEM) as a measure of the signal fluctuation.

3.3.2 Functional Characterization of Synaptotagmin-SNARE Interaction

3.3.2.1 Influence of Synaptotagmin on the kinetics of SNARE complex assembly

Since Synaptotagmin binding to both the SNARE binary and ternary complex but not to the individual SNARE proteins was observed and these two entities represent the sequential stages in the formation of the SNARE complex, it might be that Synaptotagmin influences the SNARE complex formation. There are two alternatives that could be inferred from the previous experiments. The first is that Synaptotagmin is increasing the speed of the binary complex formation and the second is that Synaptotagmin is increases the speed of the Synaptobrevin binding to the binary complex to form the ternary complex. The kinetics of the SNARE complex assembly was measured using fluorescence anisotropy measurements on isolated SNARE proteins. The SNARE proteins used here did not contain the transmembrane domain but experiments done with proteins containing the transmembrane domain provided a similar result (Per. Comm. Alexander Stein). The rate of binary complex formation was measured using SNAP 25 labelled with Texas Red at Cys 130. Approximately 0.2 μM of the labelled protein was added into a cuvette containing buffer. After measuring the baseline of the anisotropy signal, unlabelled Syntaxin (H3 domain; 183-262) was added, in excess (0.7 μM) and the anisotropy signal is measured. Due to the binary complex formation, the anisotropy signal increases until saturation, indicating that the complex is fully assembled. When either calcium (1 mM) or unlabelled Synaptotagmin (1.5 μM) or both were added to the reaction, no detectable change in the rate of the assembly was observed (Figure R18A).

The ternary complex formation was studied using two separate assays. Both utilized Synaptobrevin labelled with Texas Red either at aa. 28 of the soluble Synaptobrevin (1-96) construct or a C-terminal label at aa. 88 for the shortened Synaptobrevin construct (1-87). In both cases, unlabelled SNAP 25 and the labelled Synaptobrevin were added to a cuvette containing buffer. Upon addition of unlabelled Syntaxin H3, the rate of the ternary complex formation was monitored using the anisotropy signal. In all cases, the rate of the ternary complex formation was not significantly altered when the reaction was measured in the presence of calcium alone or in the presence of Synaptotagmin and calcium, indicating that Synaptotagmin does not influence the rate of SNARE complex assembly (Figure R18B & C).

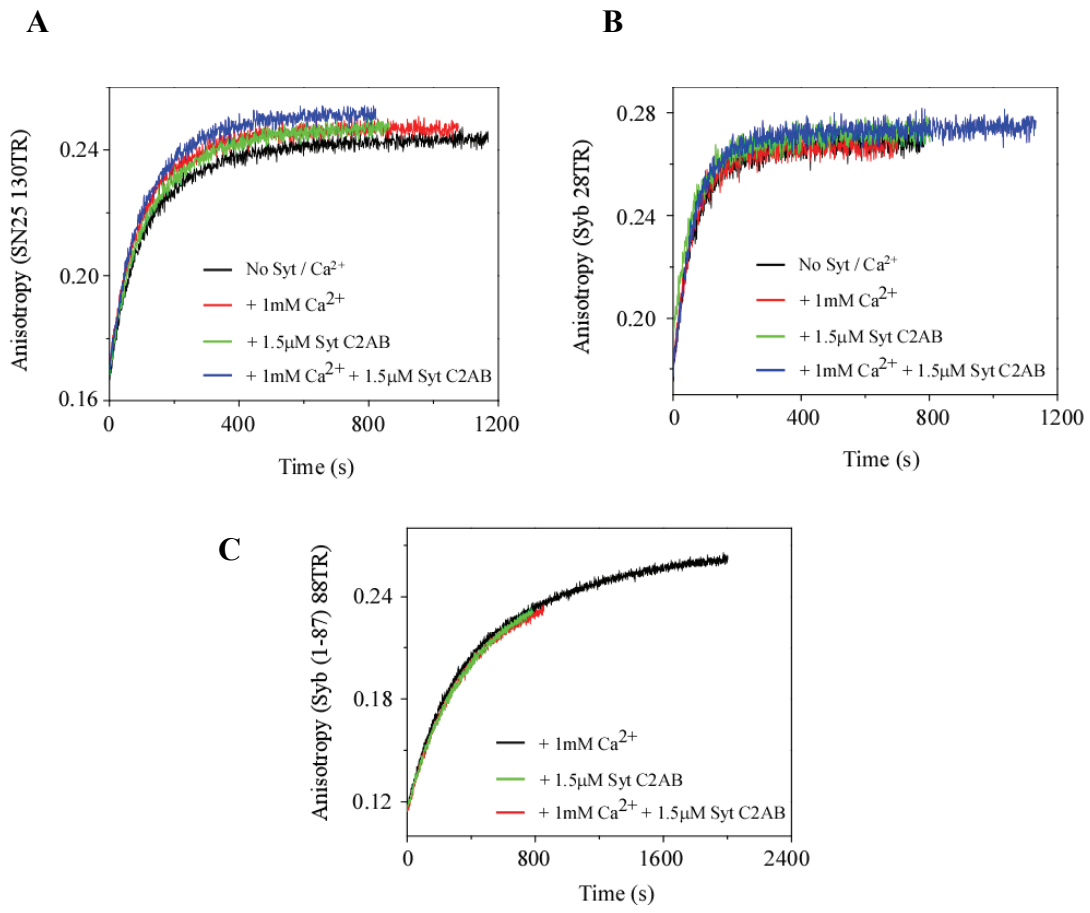


Figure R18: SNARE complex assembly kinetics in not modulated by Synaptotagmin both in the absence and presence of calcium. SNARE complex assembly was monitored using Texas Red anisotropy measurements. Binary complex assembly was monitored using labelled SNAP 25 (aa. 130TR). After baseline measurement, Syntaxin H3 (180-262) was added in excess, in the presence of Synaptotagmin, calcium or both and the kinetic of the binary complex formation was measured **(A)**. The ternary complex formation was done in a similar way. In this case two separate assays were used to measure the kinetics of the complex formation. In both cases, Synaptobrevin was labelled with Texas Red (aa. 28 **(B)** or aa. 88 **(C)** (Synaptobrevin 1-87; C-terminal labelling). Baseline measurement was done for labelled Synaptobrevin in the presence of SNAP 25 after which Syntaxin H3, in excess, was added to this mix in the presence of wither Synaptotagmin, calcium or both. No detectable enhancement of the complex assembly kinetic could be seen for both the binary and ternary complex formation.

3.3.2.2 Synaptotagmin – SNARE complex interaction in liposome membranes

The influence of the SNARE complex on the interaction of Synaptotagmin with the lipid membrane was studied using the FRET assay between Synaptotagmin (342 A488) as the donor and Texas Red labelled liposomes as the acceptor. To do so, the unlabelled individual SNARE protein and the SNARE complexes with the transmembrane domain was purified and inserted into liposomes containing Texas Red labelled PE (protein to lipid ratio of 1:270). These labelled liposomes also contained either 10 or 25% PS in the presence or absence of 1% PIP_2. The proteo-liposomes were then titrated to 0.2 μM of labelled Synaptotagmin (342 A488) in a cuvette containing buffer with 1 mM calcium. The fluorescence emission intensity of the labelled Synaptotagmin was recorded and normalised to the intensity before the addition of the liposomes (F/F_0). This was then plotted against the concentration of PS in the lipid membrane. The different concentrations of PS were chosen to represent low and high PS densities in the membrane respectively. In the previous section, PIP_2 seemed to influence the affinity of Synaptotagmin binding to lipid membranes only in the low density PS environment. Therefore, by reconstituting the SNARE proteins in liposomes containing PIP_2 in both the low and high PS densities, the influence of SNAREs in the binding of Synaptotagmin to the membrane can be better understood. The titration is shown in Figure R19. As described above, increasing the amount of PS from 10 to 25% in the lipid mix causes an increase in the density of PS in the liposome membrane and reduces the amount of liposomes needed to saturate the signal indicating an increase in affinity of Synaptotagmin binding to the lipid membrane. This increase is further augmented with the addition of 1% PIP_2 in the membrane. In the presence of the SNARE proteins, the affinity of Synaptotagmin binding to the liposomes was not significantly affected. This experiment illustrates that the binding of Synaptotagmin to the lipid membrane is independent of the SNARE proteins.

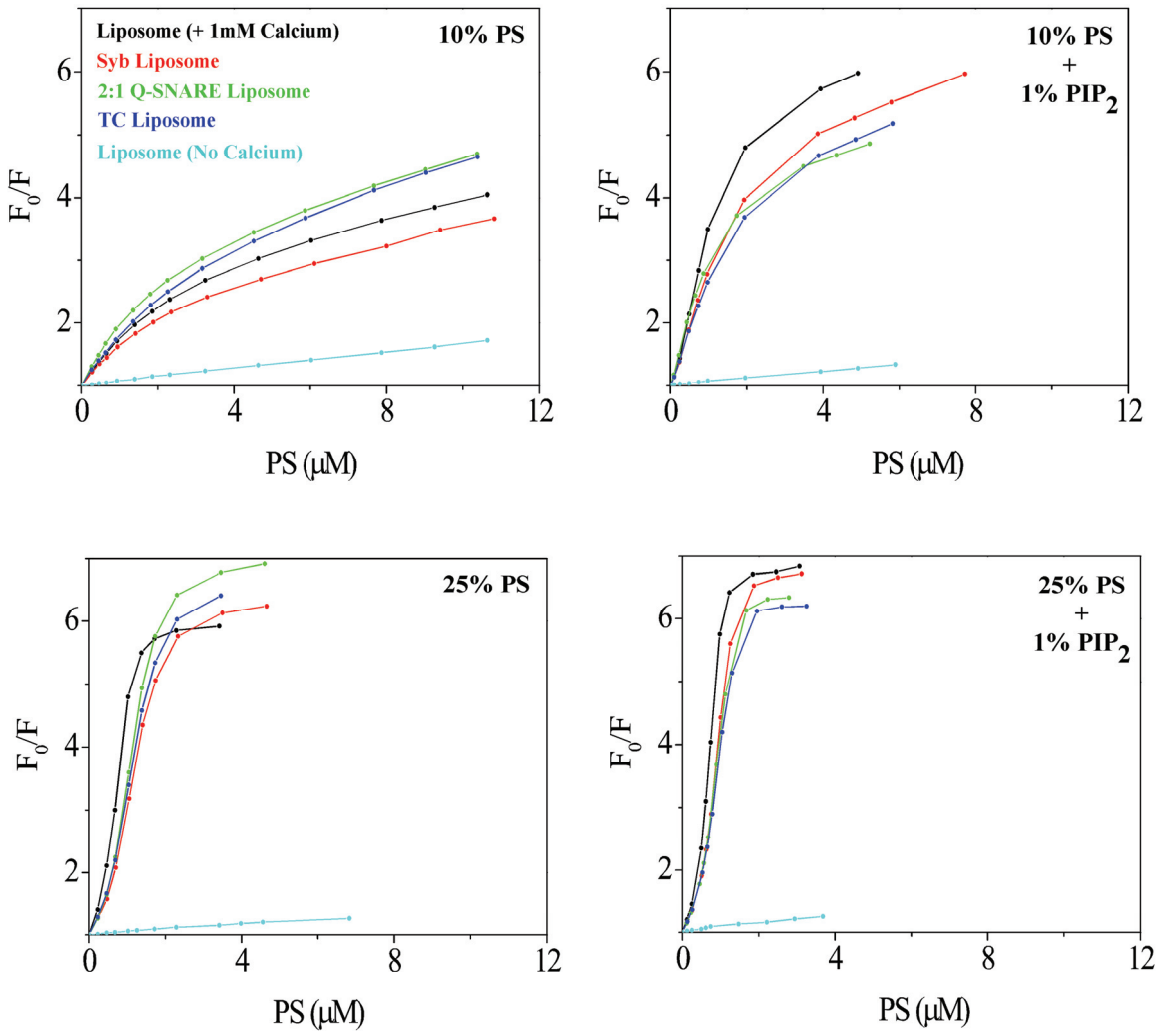


Figure R19: Influence of SNARE protein in the binding of Synaptotagmin to the lipid membrane. SNARE proteins were reconstituted into liposomes containing either 10 or 25% PS in the presence or absence of 1% PIP_2 (Protein : Lipid = 1 : 270). The SNARE used were the full length Synaptobrevin (1-116), the binary complex consisting of Syntaxin (180-288) and SNAP 25 (1-206) and the ternary complex containing all the SNARE proteins with TMD in a ratio of 1:1:2 (Syntaxin : SNAP 25 : Synaptobrevin) incubated overnight and purified over an ion exchange column to remove the excess single SNARE proteins. Increasing the amount of PS in the membrane, causes a reduction in the amount of liposomes needed to saturate the signal indicating an increase in affinity. However, the addition of the SNAREs to the liposomes, did not exhibit a significant change in the affinity of Synaptotagmin to the liposomes in all the conditions tested.

3.3.3 Synaptotagmin – SNARE interaction: A FRET-based approach

Recent studies have illustrated the importance in integrating the different components involved in the final stages of calcium dependent exocytosis (63) (64). These components include the SNARE complex, Synaptotagmin, Complexin, calcium and the lipid membrane. Initially, this was attempted using the SNAP 25 48IAANS assay (see above). The labelled protein was assembled into a SNARE ternary complex with Syntaxin and Synaptobrevin containing their TMD. However, when the assay was performed in the presence of detergents, for unknown reasons, no detectable change in the signal was observed (data not shown). Due to this reason and both the IAANS and the anisotropy assays only measure changes in one of the two interacting proteins, i.e. the SNARE complex, upon addition of unlabeled Synaptotagmin and calcium, the next step was to develop a FRET-based assay to monitor changes in both Synaptotagmin and the SNARE complex upon binding. Since the anisotropy of the Alexa-488 label on Syntaxin at position 225 in the SNARE ternary complex seems to be altered in the presence of calcium and Synaptotagmin, this label on the SNARE complex was utilised to screen for a possible FRET partner in Synaptotagmin. Single cysteine mutants of Synaptotagmin were cloned, expressed and purified and labelled with Texas Red, as the acceptor FRET molecule. These labelled mutants were then screened with the SNARE complex containing labelled Syntaxin as the donor FRET molecule. The first set of sccrening was done using the SNARE complex without the transmembrane region. No FRET-dependent signal changes were observed in this case (Figure R20B). When the SNARE complex containing the transmembrane domain was used, FRET-dependent signal intensity changes was clearly seen in both the presence and absence of calcium (Figure R20A). After the initial recording of the emission spectra of the labelled SNARE complex, the other components were added sequentially and the spectrum for each sequential addition was measured. Of all the mutants tested, Synaptotagmin labelled at position 217 gave the most consistent FRET signals and was used for further experiments.

The FRETting between Synaptotagmin and the SNARE complex increased upon the addition of calcium. This change could indicate an increase in the affinity or perhaps a conformational shift. The calcium dependent signal could be completely reversed using the calcium chelator, EGTA to remove calcium from the solution. Other studies have reported that Complexin is able to displace Synaptotagmin from the SNARE

complex (63) (64). Therefore, in order to reverse the calcium independent signal, Complexin was tested in this assay. The FRET signal between the SNARE complex with the transmembrane domain and Synaptotagmin could be reversed completely using Complexin. For the soluble SNAREs, the addition of Complexin seems to cause the signal intensity of the Alexa-488 label attached to the SNARE complex to increase dramatically. The reason for this is currently unknown but could be related to the dequenching of the fluorescence dye due to the monomerization of the oligomeric SNARE complexes. In all the subsequent experiments, Syntaxin was pre-labelled with Alexa 488 before incubating it with the other SNARE components to form a ternary complex to avoid any non-specific labelling of other SNARE components. Additionally, the ion exchange purification was excluded since the ternary complex with the labelled Syntaxin precipitated on the ion exchange column. For the future experiments, the maximum signal intensity for the donor signal (SNARE ternary complex labelled with Alexa 488) was determined and normalised to the baseline without the addition of the acceptor (Synaptotagmin 217TR) (F_0/F) and plotted as a bar diagram.

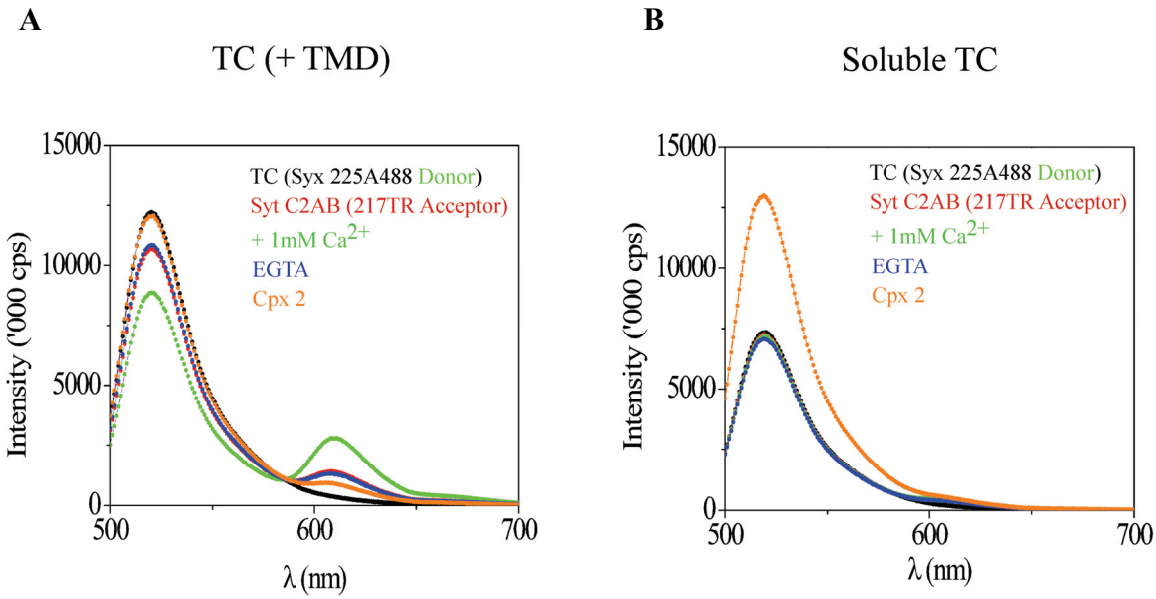


Figure R20: FRET is only observed when the TMD of the SNARE complex is present. The addition of 0.5µM Synaptotagmin 217TR to the SNARE complex labelled with Alexa 488 (Syntaxin aa. 225) (0.2µM) caused a FRET signal change only when the TMD of Syntaxin and Synaptobrevin were present **(A)**. The absence of the TMD (soluble SNARE complex) did not show any detectable signal intensity change **(B)**. Puzzlingly, the addition of Complexin to the soluble SNARE complex and not the SNARE complex with the TMD caused the signal intensity to increase dramatically (**Orange curve, B**).

The SNARE complex containing labelled Syntaxin was reconstituted into liposomes with either no PS or 10% PS in the presence or absence of 1% PIP_2 (Protein : Lipid = 1 : 270). Three different experiments were done, each differing in the sequential addition of calcium, Complexin and EGTA (Figure R20, 21 & 22). The schemes of the sequential additions are shown above the bar diagram.

Upon addition of labelled Synaptotagmin to the SNARE complex in the absence of PS or PIP_2 ((Figure R21 (1)), a reduction in the donor signal (SNARE TC) occurred indicating that the acceptor (Synaptotagmin) was bound to the SNARE complex. This drop in the donor intensity increased when calcium was added indicating a change in conformation or affinity in the Synaptotagmin – SNARE complex interaction. Based on these results, it seems that there exist two phases, calcium dependent and independent, in the interaction between Synaptotagmin and the SNARE complex. In order to prove the specificity of the interaction, these separate phases need to be fully reversible. The calcium dependent phase was reversed using the calcium chelator, EGTA and since previous publications have shown that Complexin is able to displace Synaptotagmin off the SNARE complex (63), this method was used here to reverse the calcium independent binding of Synaptotagmin to the SNARE complex. Since Complexin seemed to reverse the binding of Synaptotagmin to the SNARE complex, an important question is whether this Complexin-mediated displacement of Synaptotagmin is calcium dependent.

This was tested in the next sets of experiments by changing the organization in the sequential addition of Complexin, EGTA and calcium. The addition of Complexin after calcium seems to reverse the signal only partially indicating that Complexin only influences the calcium independent binding phase. Calcium removal using EGTA is still needed to completely reverse the binding to the baseline (Figure R22 (5)). This is further confirmed in the third set of experiments (Figure R23 (9)). In these experiments, both the calcium independent and dependent phases of the Synaptotagmin – SNARE complex interaction is resolved clearly. Upon addition of Complexin in the absence of calcium, the FRET signal observed due to the addition of Synaptotagmin was reversed. Subsequently, the addition of calcium caused the donor signal to be reduced indicating an interaction between Synaptotagmin and the SNARE complex, in the presence of Complexin. The addition of EGTA causes the signal to be reversed to the baseline. In summary, these experiments indicate that there exist two separate binding phases (calcium dependent and independent) in the interaction between Synaptotagmin and the SNARE complex. The

addition of Complexin is able to displace Synaptotagmin from the SNARE complex only in the absence of calcium but when calcium is present, Synaptotagmin is still able to bind the SNARE complex. The changes described above were based on the experiments with liposomes containing neither PS nor PIP_2 in the liposome membrane. However, a similar result was also observed in the presence of 10% PS, 1% PIP_2 or both lipids in the liposome membrane (Figure R21 (2-4), Figure R22 (6-8) & Figure R23 (10-12)). An interesting observation is that when the anionic lipids PS and PIP_2 are present in the membrane, the addition of Complexin and EGTA is unable to reverse the signal completely to the baseline, as was observed in the experiment without any anionic phospholipids ((Figure R20 (1), Figure R21 (5) & Figure R22 (9)).

Are these two proteins (Synaptotagmin and Complexin) binding the SNARE complex at the same time? In previous studies, it was suggested that upon the addition of calcium, Complexin leaves the SNARE complex and allows Synaptotagmin to bind (64). In order to study whether these proteins bind the SNARE complex at the same time, a previously described assay with labelled Complexin (aa. 39 Oregon Green) was used (70). Upon SNARE ternary complex binding, the anisotropy signal of the label on Complexin increases. It was previously used to study the kinetics of this protein-protein interaction. In this case, the anisotropy of the labelled Complexin was measured in the presence of calcium after which, the pre-purified unlabeled ternary complex (with or without the TMD) and Synaptotagmin (C2AB; 97-421) were added sequentially (Figure R24). It seems that both in the absence and presence of the TMD, no significant anisotropy signal change occurs upon Synaptotagmin addition indicating that Complexin is still bound to the SNARE ternary complex. If indeed Complexin leaves the SNARE complex upon Synaptotagmin addition, the anisotropy values would be reversed when Synaptotagmin was added in the presence of calcium.

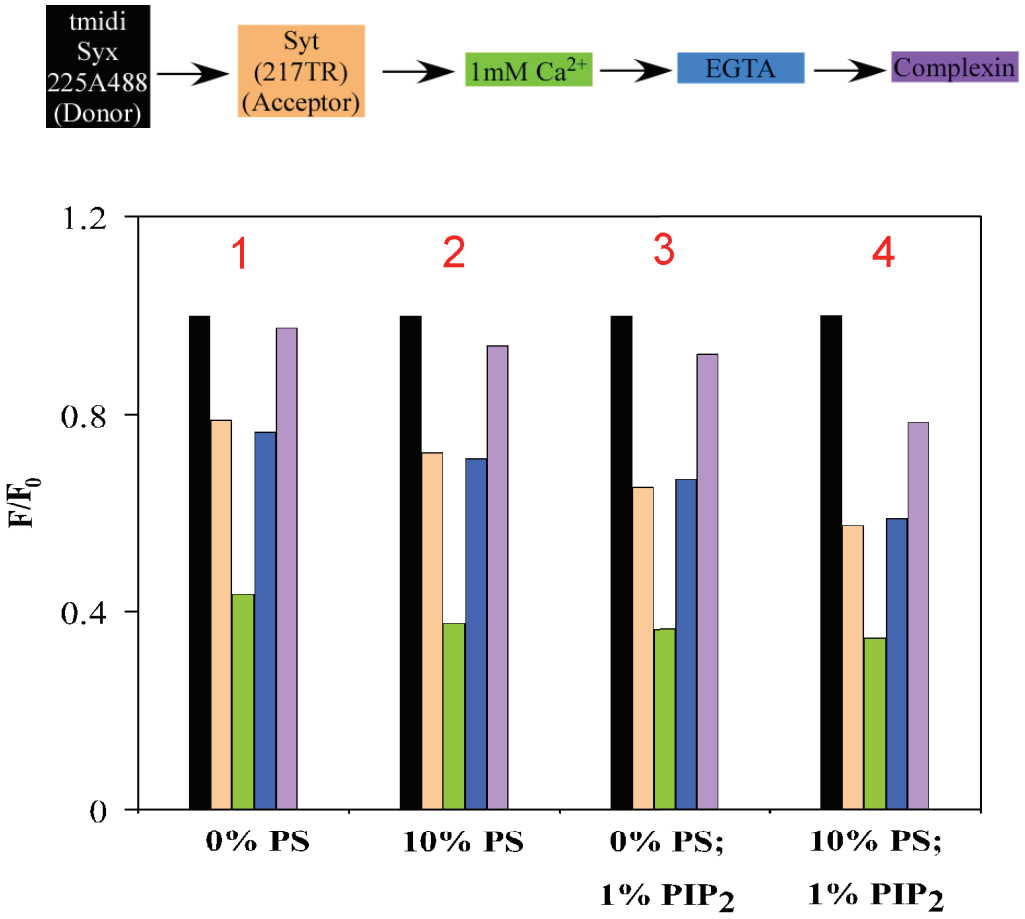


Figure R21: The interaction of Synaptotagmin to the SNARE complex was monitored using the FRET assay between these proteins (1). The spectra of 0.2 µM labelled SNARE complex (Syntaxin 225Alexa 488) was first recorded prior to the addition of the other components. For simplicity, only the normalised peak signal intensity of the donor signal is shown here as a bar diagram. Upon addition of Synaptotagmin (labelled at position 342 with Texas Red), the signal intensity of the SNARE complex was quenched in a FRET dependent manner. Addition of calcium caused an increased quenching. In order to prove the specificity of this FRET-dependent signal change, EGTA was added to remove calcium. The addition of EGTA was accompanied by an increase of the donor signal. Complexin was shown to before to be able to remove Synaptotagmin off the SNARE complex (see text). When Complexin was added to the reaction, the signal returned to baseline (1). The addition of anionic phospholipids showed similar FRET dependent changes with the exception that upon addition of Complexin the signal is not reversed completely (2 – 4).

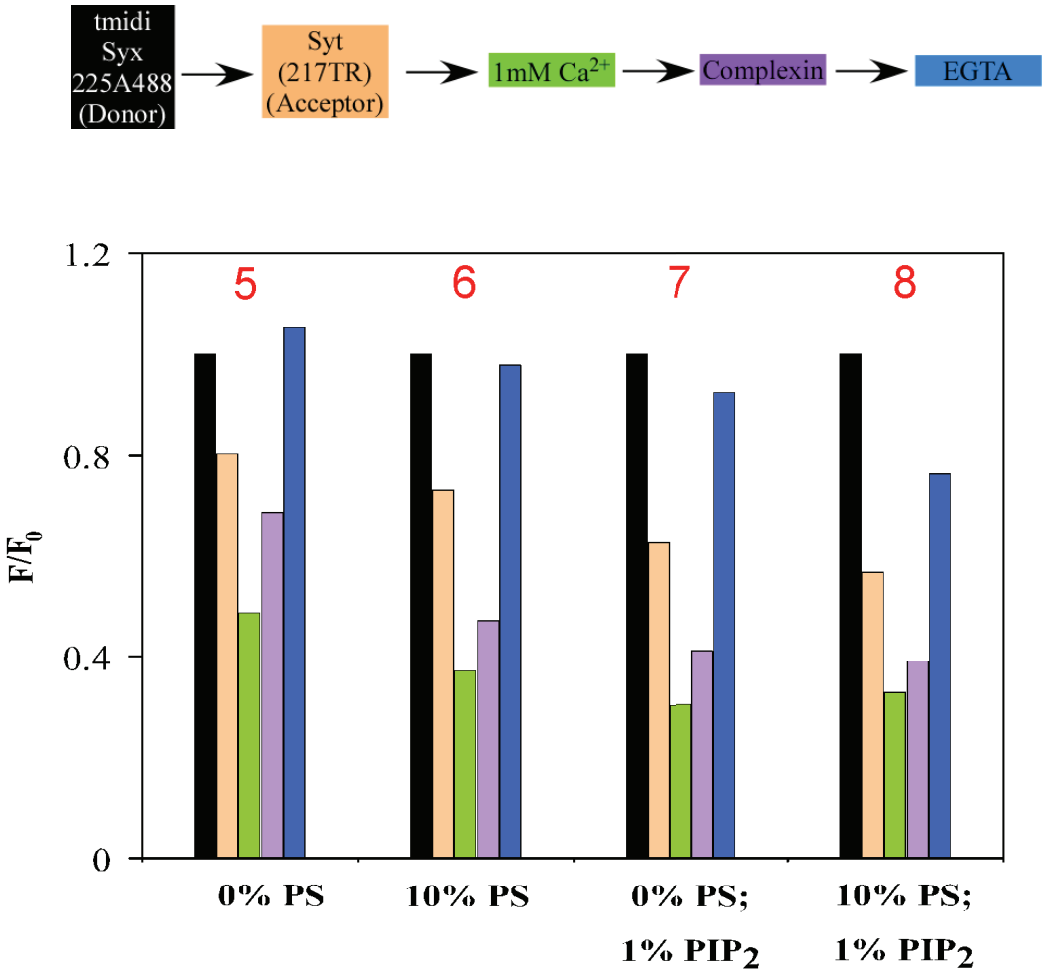


Figure R22: The interaction of Synaptotagmin to the SNARE complex was monitored using the FRET assay between these proteins (2). In order to confirm the specificity of the effect of Complexin on reversing the FRET signal, the order at which Complxin and EGTA was added to the reaction was altered. Complexin seems to only reverse the calcium independent signal since EGTA was still needed to reverse the signal completely to the baseline (5). Similar to the previous experiment, inclusion of anionic phospholipids tend to prevent the signal from completely reversing to the baseline (6 - 8).

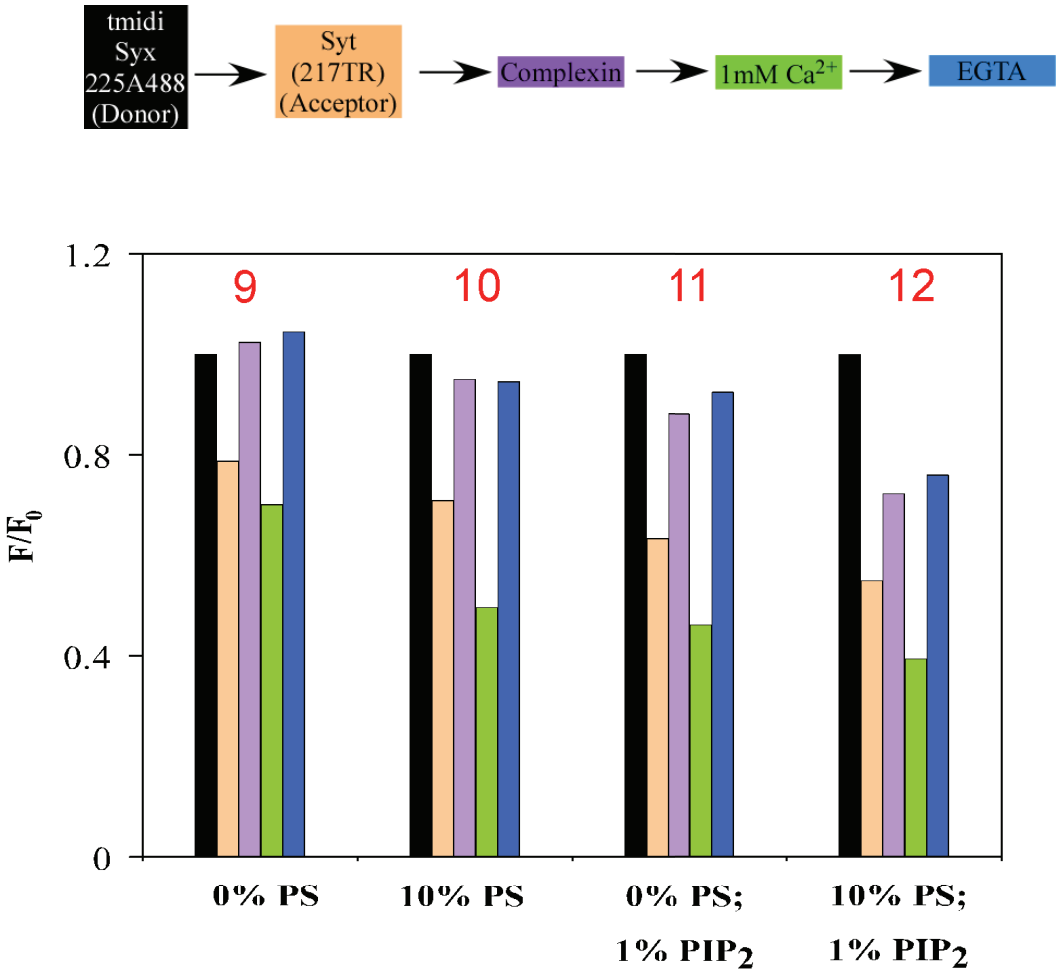


Figure R23: The interaction of Synaptotagmin to the SNARE complex was monitored using the FRET assay between these proteins (3). The two separate phases, calcium independent and dependent, in the binding of Synaptotagmin and the SNARE complex, was confirmed by adding Complexin before the addition of calcium and EGTA. Upon the addition of Complexin, the quenching induced by the addition of labelled Synaptotagmin was completely reversed to the baseline. However, the addition of calcium caused the signal to be quenched and EGTA, to remove calcium allowed the reversal of the signal (9). In the presence of anionic phospholipids in the liposome membrane, the signal was not reversed completely indicating that perhaps Synaptotagmin remains in close proximity to the SNARE complex (10 – 12).

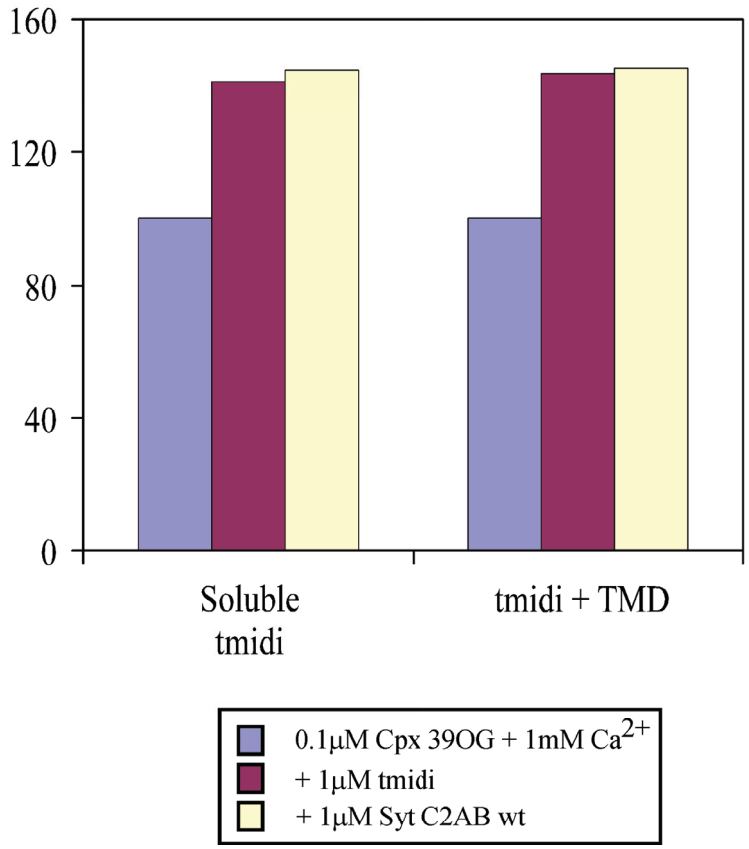


Figure R24: Anisotropy of Complexin (aa. 39) labelled with Oregon Green to monitor SNARE ternary complex binding. 0.1 μM labelled Complexin was added to a cuvette in the presence of 1 mM calcium. Purified SNARE ternary complex with or without the TMD and Synaptotagmin C2AB was added sequentially to determine if in the presence of Synaptotagmin and calcium, the anisotropy signal would be reversed to before the ternary complex addition indicating that the binding of Complexin to the SNARE complex is abolished. In all cases, this was not observed confirming that Complexin is still bound to the SNARE ternary complex after addition of Synaptotagmin and calcium.

4. Discussion

4.1Calcium Binding

Previously, calcium binding to the isolated C2 domains of Synaptotagmin was studied structurally to a high resolution using NMR (5) (6). However, NMR was not a suitable method for obtaining the binding isotherms and also due to size limitations intrinsic to the NMR method, the tandem C2 domains could not be studied. Isothermal Titration Calorimetry (ITC), a method used before to characterize the binding of calcium to PKC C2 domains (75) and the C2 domains from phospholipase Dα and β isoforms (76) was used to study the binding of calcium to the Synaptotagmin C2 domains.

4.1.1 Limitations of ITC

ITC experiments are limited by the affinity of the system studied. For lower affinity systems, the titration is usually done with high concentrations of macromolecule. In order to determine the concentration of protein that is needed for the experiment, the Wiseman constant is used (80). The Wiseman constant, c, is the product of the affinity of the system and the macromolecule concentration (c = [Mt]K). When the value of the constant is between 1 and 1000, accurate estimation of the binding affinity (K) can be determined. In the case where the c value is below 1 and above 1000, the affinity value becomes increasingly inaccurate but the ΔH and stoichiometry (n) values can be determined accurately. However, at lower c values, in order to obtain accurate values for the affinity (K), the stoichiometry (n) has to be fixed (81). By fixing the n value, only two variables needs to be considered in the fitting process thereby making the fitting of the ITC data considerably easier. In the case of the ITC measurements of calcium binding to the Synaptotagmin C2 domains, the c value is 0.1 for the titrations done at 25μM protein concentration and 0.2 for the 50μM. The c value can be increased further by increasing the concentration of protein in the sample cell, for example, 250μM for the C2A domain to achieve c = 1, which is impractical since at higher concentration, the protein is known to precipitate. Since the number of calcium ions bound to each of the C2 domains of

Synaptotagmin has already been described in other studies (see Introduction), it is therefore possible to determine the ΔH and K values from the ITC experiment by fixing the n value.

Another important consideration is which model is used in the fitting of the integrated data. In this case, the one-site binding model, which assumes that all binding sites have equal affinity, was used. Although, the NMR data suggests that at least in the C2A domain, three calcium ions bind to the calcium binding site with very different affinities, attempts at fitting the data using other binding models caused large error margins for the thermodynamic parameters. Therefore, the one-site model which considers that all the binding sites bind the ligand with a similar affinity could provide the overall affinity of the C2 domains for calcium. Furthermore, the determination of the thermodynamic parameters of binding, such as enthalpy, entropy and free energy, using ITC is far more important to obtain insights on the mechanism by which the C2 domains of Synaptotagmin bind calcium. The advantage of the method also lies in the fact that the tandem domain, which is too large for NMR, can be used for the titration experiments and the amount of protein used is lower as compared to NMR experiments.

4.1.2 Calcium Binding mechanism of the Synaptotagmin C2 domains

C2 domains are modular structural component that exists in a wide variety of proteins. The basic function of this domain seems to be calcium-mediated membrane binding. Structurally, these domains are very similar. The 3D alignment of C2 domains from classical protein kinase C isoforms, phospholipase A2, rabphilin and Synaptotagmin are shown in Figure D1. This alignment clearly shows that the domains have a highly conserved structure. However, calcium titration to C2 domains studied using ITC, as reported previously in classical protein kinase C (75) and phospholipase D isoforms (76) suggests that the C2 domains, although structurally similar have different mechanisms for calcium binding. These remarkable differences in the calcium titration profiles of the C2 domains prompted this study on the investigation of calcium binding to C2 domains of Synaptotagmin. The two C2 domains of Synaptotagmin are very similar in its 3D structure. However, the C2B domain has been reported to contain two helical motifs which are not found in the C2A domain (6). These motifs were initially thought to be important for the

function of the protein although no direct evidence for this have been shown (6). Calcium titration to the isolated C2 domains exhibit very different thermodynamic properties upon calcium binding. The titration of calcium to the isolated C2A domain (97-273) showed an endothermic reaction (ΔH = 2100 cal/mole) as opposed to the isolated C2B domain (262-421), which was exothermic (ΔH = -2600 cal/mole). The difference in the enthalpies for calcium binding in these two domains indicates a divergent calcium binding mechanism for the domains. When the tandem wild type C2AB construct was used, the titration profile obtained was the sum of the two C2 domains, indicating that the domains bind calcium independent of each other. The independence of the domains was further supported when the tandem domain calcium binding mutant constructs were used. In these mutants, either the C2A or the C2B domains were rendered inactive using site-directed mutagenesis to mutate the calcium coordinating aspartates to alanines. Calcium titration to the C2A domain mutant (C2A*B) exhibited a titration profile which was very similar to the isolated C2B mutant and vice versa for the C2B domain mutant (C2AB*).

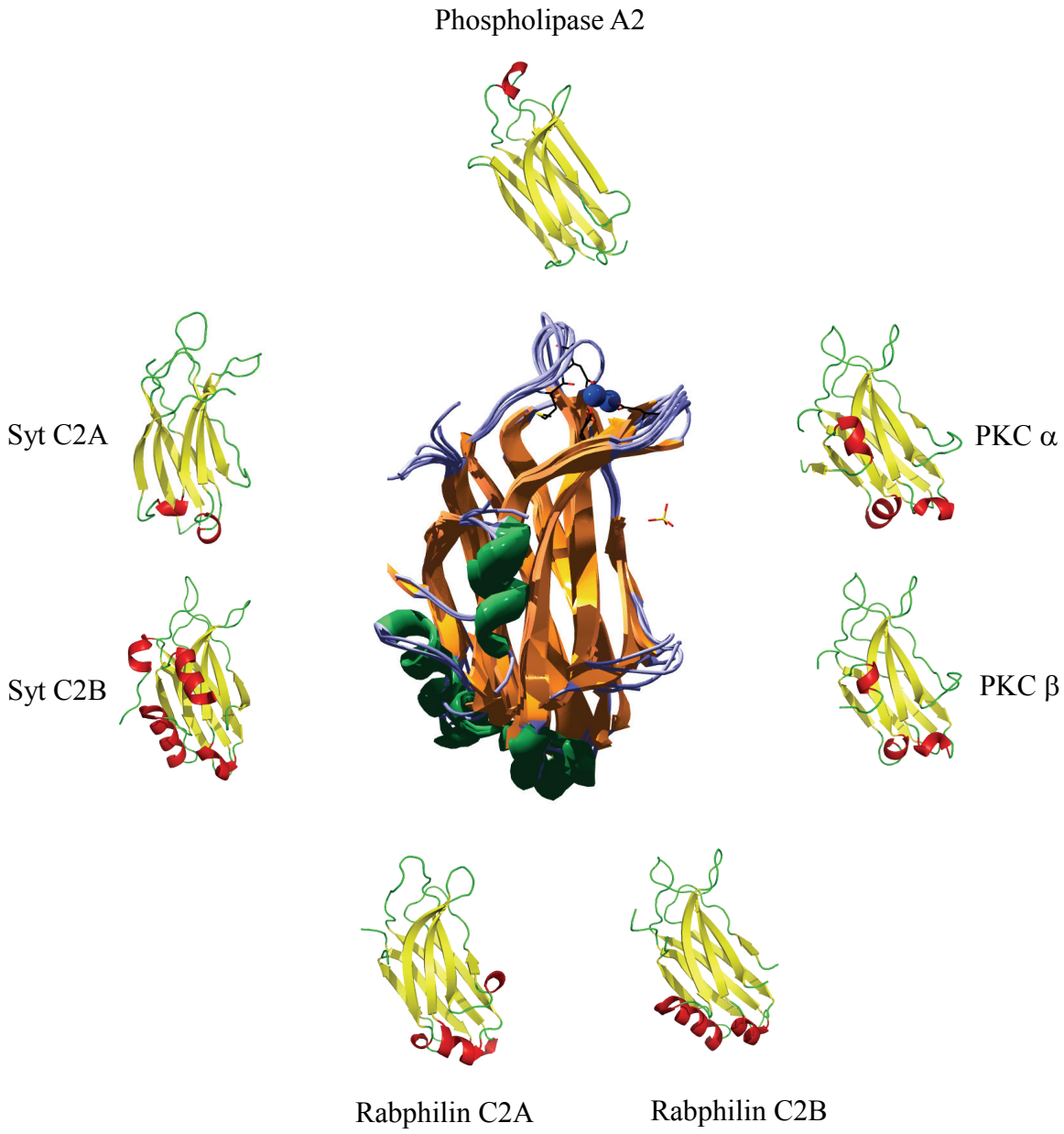


Figure D1: Structural alignment of C2 domains from Synaptotagmin, Phospholipase A2, Protein Kinase C (PKC) and Rabphilin. The C2 domains from a diverse set of proteins with different functions exhibit a similar 3D structure (PDB ID: Phospholipase A2 (1BCI), Protein Kinase C-α (PKC-α) (1DSY), Protein Kinase C-β (PKC-β) (1A25), Rabphilin 3A C2A (2CHD), Rabphilin 3A C2B (2CM5), Synaptotagmin C2A (1BYN), Synaptotagmin C2B (1UOW).

4.1.2.1 Enthalpy – Entropy Compensation

The free energy required for calcium binding to the two C2 domains are very similar (ΔG = -5 kcal). However, the titration of calcium to the two domains indicates very different thermodynamic behaviour. The isolated C2A domain exhibits an endothermic reaction (ΔH = 2400 cal/mole) and the isolated C2B domain exhibits an exothermic reaction (ΔH = -2600 cal/mole). The values for these domain in the tandem protein, determined from the experiment with the calcium mutants is ΔH = 1900 and -1700 cal/mole for the C2A and C2B domains respectively. The entropy of the domains is approximately 20 and 10 cal for the C2A and C2B domains respectively. Changes in the enthalpy value usually reflect the changes in the non-covalent bond energy during a chemical reaction. These non-covalent bonds could include van der Waals interaction, hydrogen bonding, salt bridges and solvent reorganization near protein surfaces (82). The enthalpy value obtained from an ITC experiment is the total heat change occurring upon the titration of the ligand. Therefore, in addition to changes of the non-covalent bonds, bulk hydration and protonation effects are also factored into the measured enthalpy value.

The entropy value on the other hand, represents all other positive and negative driving forces that contribute to the free energy of a chemical reaction. The total entropy change calculated from the ITC experiment usually consists of the sum between the entropy of the hydration effect as well as the rotational degrees of freedom around the protein torsion angles. The entropy value also accounts for the reduction in the number of particles in the solution (82). Based on the equation describing Gibbs free energy ($\Delta G = \Delta H - T\Delta S$), if the free energy and temperature remains constant, a linear relationship between the enthalpy and entropy can be described. This linear relationship is called the enthalpy – entropy compensation effect (82). This relationship states that any small changes in the enthalpy of a system causes an opposite change in the entropy of the system thereby allowing the stabilization of the system and only small changes in the binding affinity. For example, increased bonding in a binding process results in a more negative enthalpy and will be compensated by an increased order leading to more negative entropy value.

In the case of the two C2 domains, the C2A domain seems to have a positive enthalpy value (ΔH = 2400 cal/mole) and a high entropy value (ΔS = 24.3 cal) and

the isolated C2B domain has a negative enthalpy value (ΔH = -2600 cal/mole) and a low entropy value (ΔS = 6.61 cal). Since the free energy in both systems is similar, the C2A domain seems to be influenced more by its entropy and the C2B domain seems to be influenced by its enthalpy. These differently influenced chemical reactions of the two C2 domains could indicate a divergent mechanism in calcium binding to these domains. In the C2A domain, since entropy values indicate mainly hydration effects, perhaps upon calcium binding, the water molecules bound to the calcium binding aspartates are displaced causing the loss in the surface area exposed to the solvent. Another explanation is that upon calcium binding, the calcium binding loops might become more rigid (reduction in the rotational degrees of freedom around the protein's torsion angle also contributes to a more positive ΔS). Calcium binding to the C2B domain has been suggested not to cause a conformational shift (27). Therefore, since the reaction is enthalpically driven, calcium binding might cause the breaking and forming of hydrogen bonds to stabilize the C2 domain. Apart from that, local structural rearrangements at the binding site can also lead to an enthalpically driven chemical reaction.

4.1.2.2 Low affinity calcium binding

The affinity measured using ITC for calcium binding to the C2 domains is rather low. The C2A domain has an affinity of approximately 250μM. Interestingly, the affinity of the isolated C2B domain for calcium binding seem to increase from 470μM to 270μM when the domain is attached to the C2A domain. Due to these low affinities, the requirement for the activation of these domains is high local calcium concentration. The existence of high calcium transients close to the calcium channels have been shown for the first time in the squid presynaptic terminal using a high speed camera and a modified low-affinity calcium binding dye (aequorin), to record the movement of calcium through the channel (83). The influx of calcium through the channel causes a high local calcium concentration (~400μM) region known as the calcium nanodomain (reviewed in (84)). This high concentration would be sufficient to overcome the low affinity of Synaptotagmin C2 domains suggesting that perhaps Synaptotagmin might be localized close to these nanodomains to carry out its function. A previous study has suggested that Synaptotagmin might bind the synprint peptide of the voltage gated calcium channel (85). Alternatively,

Syntaxin, which is a Synaptotagmin binding partner, has been shown to bind the calcium channel, thus localizing the SNARE complex close to the channel (86). However, these protein-protein interactions with the calcium channel still need to be confirmed. On the other hand, synapses, such as the calyx of Held, have shown that lower amount of calcium (~25μM) is enough to saturate the release mechanism (87). These variations in the calcium requirement for fast exocytosis could be due to a number of factors, such as the organization of calcium channels on the membrane, the presence of other Synaptotagmin-interacting partners and the contribution of the different Synaptotagmin isoforms.

4.2 Membrane Organization and Lateral Diffusion

Early work into studying membrane organization suggests that the membrane components are distributed homogenously and can diffuse freely. This membrane organization hypothesis was known as the fluid mosaic model (88). However, in recent years, the existence of specialized areas known as membrane microdomains is thought to play an important role in signal transduction and the secretory pathway (89). A number of different types of microdomains have been proposed which include caveolae and lipid rafts. Caveolae are membrane pits that consist of the protein caveolin that are implicated in endocytosis, lipid trafficking and signal transduction (reviewed in (90)). Lipid rafts were first described as detergent resistant membranes (DRM) owing to the fact that under low temperatures (4ºC), these rafts are resistant to solubilization by the detergent Triton X-100 (91). This insolubility of the lipid raft is due to high amounts of cholesterol and sphingolipids which render the membrane rigid. This rigid physical state of the membrane also known as the liquid ordered state (l_o), a quasi two-dimensional crystalline solid, as opposed to the non-raft portion of the plasma membrane that exist in a quasi two-dimensional liquid, known as the liquid disordered state (l_d) (reviewed in (92)). The existence of rafts is still highly controversial and due to its dynamic state as well as the minute size, it has yet to be visualised. However, increasing evidence for the existence of these rafts, based on experiments measuring lateral diffusion on model membranes and also erythrocyte membranes, has accumulated over the years (reviewed in (89)).

The involvement of membrane microdomain in exocytosis is an appealing idea since the release site for neurotransmitters are localized to a very specialized region known as the active zone (10). However, attempts at studying whether the SNARE complex is localized to membrane domains have yielded contradictory results. SNARE-localized microdomains that are sensitive to methy-β-cyclodextrin, an agent that is able to deplete cholesterol from the membrane thus disrupting the microdomains, have been described in PC12 cells (93). Additionally, with the aid of STED microscopy that allows the visualization of particles in the lower nanometer range, Syntaxin have been shown to cluster in microdomains (71). However, studies in giant unilamellar vesicles (GUV) have shown that SNAREs preferentially localize outside the raft region (94). Therefore, the functional role of the SNARE microdomains is still unclear.

4.2.1 Membrane microdomains and Synaptotagmin

In this study by utilizing a FRET assay between Synaptotagmin and the lipid membrane, the influence of the anionic lipid composition and PS density in the membrane was studied. This was done by making liposomes with increasing amounts of PS in the lipid mix, in the absence or presence of PIP_2. Since the size distribution of the different liposome samples are uniform (i.e. 40-100 nm diameter), the surface area of the liposomes are similar. Therefore, increasing the amount of PS in the lipid mix tends to increase the density of PS in the membrane. Since PS is an anionic phospholipid, the higher density of PS increases the negative charge in the membrane. The binding of Synaptotagmin to the membrane is mediated by an electrostatic interaction between the calcium ion bound to the C2 domain of Synaptotagmin and the anionic phospholipids. In order to test if microdomains influence the binding of Synaptotagmin to the lipid membrane, liposomes with increasing PS densities, in the absence or presence of PIP_2 were titrated into Synaptotagmin. Based on results from these titration experiments, increasing the amount of PS in the membrane tends to reduce the amount of liposome needed to saturate the signal indicating an increase in affinity. Furthermore, it seems that the addition of PIP_2 to the liposome membrane, which contributes to an increase in the negative charge of the membrane, causes an increase in the affinity of Synaptotagmin to the membrane only at lower PS concentration (5% and 10% PS). This effect of PIP_2 was not present when the PS concentration was increased to more than 15%.

It seems that a threshold amount of negative charge is needed for Synaptotagmin to efficiently interact with the membrane. At lower PS concentrations, this threshold is reached when PIP_2 is added to the liposome. This is unnecessary at higher PS concentrations since PS by itself could fulfil the requirement. A similar hypothesis was proposed in the lateral organization of membrane lipids into superlattices, which is highly dependent on the concentration of lipids. Above this critical concentration, the anionic phospholipids tend to form lattices which are mediated by long range columbic interactions (95). In the liposome experiment, this critical concentration is mimicked by increasing the PS density in the membrane. At lower PS densities, the PS molecules are not organised and therefore the negative charge on the membrane is dispersed. In the presence of PIP_2, which causes an increase in the negativity of the membrane, the effect of this dispersion might be overcome due to the long-range columbic interaction between the PS and PIP_2 molecules

thereby forming highly organized structures and causing an increase in the affinity of Synaptotagmin to the membrane. When the PS density in the membrane is increased, due to the strong negative charge present in the liposome membrane, the lipids are laterally organised and able to bind Synaptotagmin efficiently (Figure D2). Although this model for Synaptotagmin binding to lipid membrane is interesting, it must be noted that more work is necessary to confirm this mechanism. The idea hinges on the fact that at lower PS densities, the affinity of Synaptotagmin for the lipid membrane is heavily modulated by PIP_2. However, the titration curves are incomplete since saturation of these curves is not reached. Therefore the vales obtained for the EC_{50} of the curves are not completely accurate. Nevertheless, it can be clearly seen when the titrations done in the presence and absence of PIP_2 are compared, the titration curves for the lower PS density liposomes (5% and 10%) tend to be steeper in the presence of PIP_2 indicating an increase in the affinity. This effect is not seen at higher PS concentrations (>15%) confirming that this modulation of PIP_2 at lower PS densities is not just an artefact.

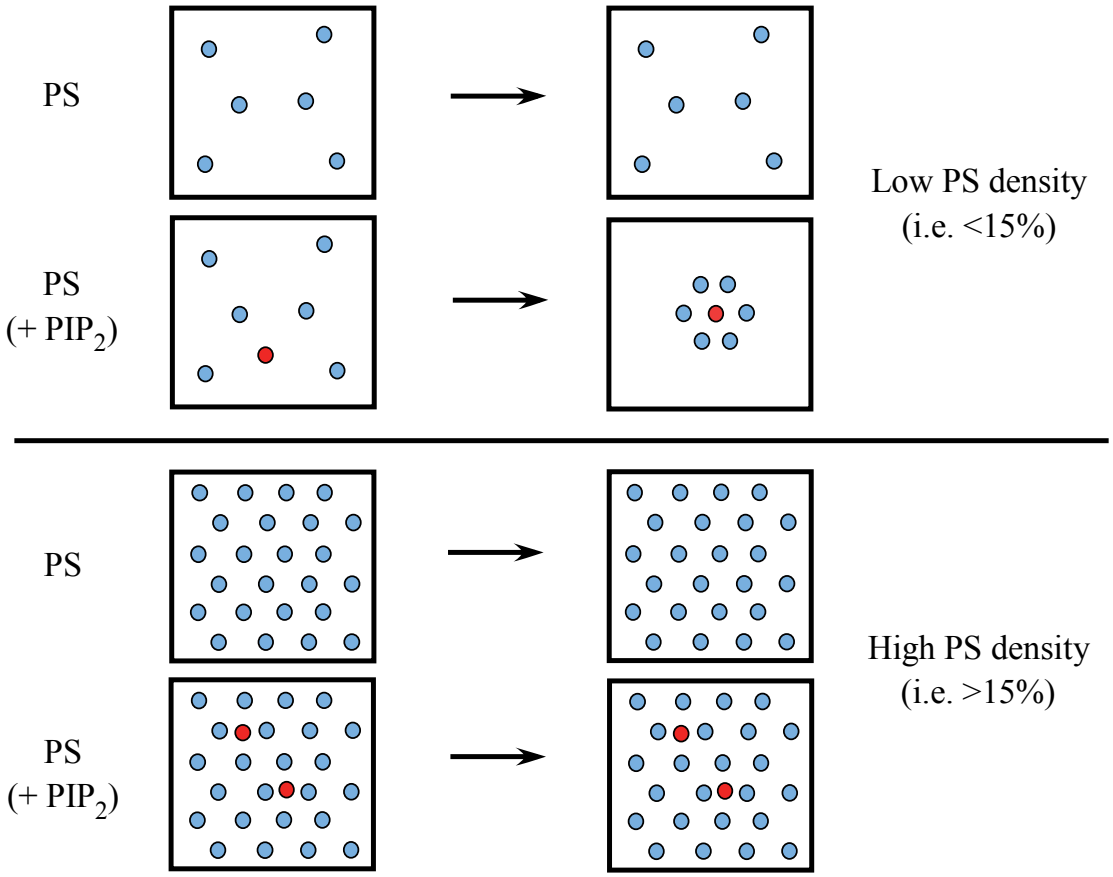


Figure D2: Lateral organization of negatively charged phospholipids in the membrane. At lower PS (Blue dots) concentration, the addition of PIP_2 (red dots) causes the dispersed PS molecules to form organised domains that would cause a local negative charge increase and allow for the efficient binding of Synaptotagmin. At higher PS concentration, due to the high density of PS, the negative charge already existing on the membrane leads to an efficient binding of Synaptotagmin to the membrane without the assistance of PIP_2.

4.3 Binding of Synaptotagmin to the SNARE complex

The interaction between Synaptotagmin and the SNARE complex have been extensively studied in the past decade. Initially the binding of these proteins was discovered by immunoprecipitation using anti-Syntaxin antibodies, which managed to pull down Synaptotagmin as one of the binding partners (43). Since then, Synaptotagmin have been shown to bind to isolated SNAP 25 (44) (48), the binary (44) (46) as well as the ternary (47) (51) SNARE complex. Additionally, SNARE proteins have also been shown to bind to both the isolated C2A domain (56) as well as the isolated C2B domain. These studies employed the use of co-precipitation methods using either immobilized antibodies or protein with artificial tags introduced using cloning, such GST or His6, which are then incubated with brain extracts to "fish" for interacting partners. The advantage of this assay is its sensitivity in "pulling-out" interacting partners from a relatively impure mix, such as the brain detergent extract. However, the weakness of this method is that, due to its sensitivity, it can also generate false positive results. In addition, the intermediate states of an interaction are usually impossible to visualize since the result only provides an impression of the end-point of a reaction. Therefore, the results obtained showing binding to the SNARE proteins at all its assembly stages needs to be carefully evaluated. Co-precipitation methods are also insensitive to milder phenotypic changes due to point mutations, which might affect the affinity or kinetic of the interaction. Due to these reasons, the main aim of the study was to develop better methods that would assist in probing the interaction of Synaptotagmin to the SNARE complex to resolve the controversy surrounding this protein-protein interaction.

Two main approaches were used in probing the interaction of Synaptotagmin to the SNARE complex. The first, a gel-based assay, utilized the intrinsic charge of the protein, which usually changes when the protein forms a complex, thereby allowing the separation of individual proteins and the proteins in a complex. The advantage of this assay was that very small amounts of protein were needed, the proteins were unmodified and no label was necessary for the proteins since visualization is through Coomassie-blue staining. The second approach was using fluorescence-based assays. Although the gel-based assay allowed the visualization of complex formation, the resolution of the assay was too low to obtain any real time information. This apparent "information vacuum" was filled with the fluorescence-based assays. These assays allowed

the measurement of affinity and kinetics of interaction. However, the main disadvantage of these assays was that the protein used here had to contain a single cysteine that was introduced through site-directed mutagenesis for the attachment of the labels to the protein.

4.3.1 Synaptotagmin – SNARE interaction: Binding Site

The main controversy surrounding the Synaptotagmin – SNARE interaction is the specific point in the SNARE assembly pathway where Synaptotagmin is functioning. Studies have suggested that Synaptotagmin is bound to single isolated SNAREs, binary and ternary complexes. Here in this study, binding to both the binary and ternary complex, in the absence and presence of calcium was seen. The single isolated Q-SNAREs were unable to interact and form a complex with Synaptotagmin. Previously, when the interaction of Synaptotagmin to Syntaxin was studied using immunoprecipitation (52), Synaptobrevin bands were present suggesting that perhaps Synaptotagmin was bound to the ternary complex and not the isolated Syntaxin. However, the presence of the ternary complex was never determined in this study. Also, the binding between the isolated Syntaxin tagged with GST and recombinant Synaptotagmin was used to demonstrate the interaction of the two proteins. Later studies from the same group also showed that the binding of the isolated C2 domains to SNAP 25 is influenced by the GST moiety, since in the absence of GST; no binding was observed (59). Taken together, it is clear that the binding of Synaptotagmin to the isolated Syntaxin and SNAP 25 is purely an artifact due to the presence of GST and when highly pure proteins without tags are used, this binding is no longer occurring.

On the Synaptotagmin molecule, earlier work has suggested that the C2A domain is more important in the interaction to the SNARE proteins (55) (56). However, in many recent investigations, the C2B domain has been shown to interact with the SNARE complex (58) (51) (46). The results presented here showed that using only the C2B domain an interaction to the SNARE ternary complex was detected using the native gel as well as the anisotropy measurement of Syntaxin labeled with Alexa 488 at position 225. The binding of Synaptotagmin to the SNARE complex seems to have both a calcium dependent and independent phase. Upon addition of calcium (or strontium), an increase in the fluorescence signal was observed indicating a change in either the binding affinity of the

Synaptotagmin to the SNARE complex or the conformational change of the Synaptotagmin bound to the SNARE complex. These different possibilities are difficult to distinguish in these assays. However, based on the amplitude of the change from the anisotropy measurement, which might reflect either affinity or conformational difference upon calcium addition, the C2B domain is not as effective as the tandem C2AB domain indicating that the C2A domain might play a role in influencing the C2B domain but is not directly binding to the SNARE complex. Mutating the calcium binding sites of Synaptotagmin still showed a complex formation between the SNARE complex and Synaptotagmin in the native PAGE assay. However, whether this complex with the calcium mutants has a lower affinity or slower rate of formation is not yet determined.

4.3.2 Synaptotagmin – SNARE interaction: Functional Relevance

The function of Synaptotagmin is mediated via its two C2 domains. The binding of these C2 domains to the SNARE complex and the lipid membrane have been suggested to be distinctive events that occur in separate binding site (45). Previously, it has been suggested that the SNARE complex might increase the affinity of Synaptotagmin to the lipid membrane or that Synaptotagmin assists the SNARE complex in its assembly (reviewed in (1)). Here, it has been shown that both these interpretations of the literature on Synaptotagmin do not occur. The rate of the binary and ternary complex assembly is unaltered in the presence of Synaptotagmin with or without calcium.

In order to probe the binding between Synaptotagmin and the SNARE complex, a FRET assay, which could monitor the state of both binding partners, was developed. Since a stronger effect of the addition of Synaptotagmin and calcium to the anisotropy measurements on the SNARE ternary complex with a transmembrane domain, labeled with Alexa 488 was observed, this SNARE complex was tested with labeled Synaptotagmin to obtain a FRET pair. It seems that FRET was only observed with this SNARE complex with its transmembrane domain and not with the soluble SNARE complex. This might be due to an oligomeric state of the SNARE complex with the transmembrane domain that allowed a FRET dependent signal change with Synaptotagmin. Since only Synaptotagmin and the SNARE complex is “visible” in the FRET assay, other factors such as Complexin and the lipid membrane can be incorporated

to understand the effects of these factors on the binding of Synaptotagmin to the SNARE complex. The most important observations in this FRET assay were:

i. Synaptotagmin binding to the SNARE complex occurs in both the absence and presence of calcium and these binding phases are distinctive.

ii. The addition of Complexin is able to reverse the binding of Synaptotagmin to the SNARE complex only in the absence of calcium.

iii. The presence of anionic phospholipids in the liposome membrane prevents the donor signal of the SNARE ternary complex to be reversed to the baseline before the addition of Synaptotagmin.

These observations indicate that Complexin plays an important role in the regulation of the Synaptotagmin binding to the SNARE complex. Since Complexin have been shown before to bind only to the ternary complex (4) and Synaptotagmin, based on this study, binds to the binary and ternary complex, an important question to elucidate the mechanism of calcium dependent exocytosis would be the state of the SNARE complex prior to the arrival of the calcium signal. The binding site of Synaptotagmin to the SNARE complex was proposed to be a highly negative cluster close to the 0-layer (arrow Figure D3) (3). If the SNARE complex is in the form of an acceptor binary complex, then Complexin is unable to bind to the SNARE complex before membrane fusion and release occur. In this case, Complexin can only bind after exocytosis occurs and would be responsible to block the binding of Synaptotagmin to the SNARE complex after exocytosis. Alternatively, if a ternary complex in its *trans* configuration (i.e. a partially assembled ternary complex without membrane mixing), stabilized by some other factor, forms prior to the arrival of calcium, then two separate events could occur depending on whether the Complexin binding site is formed on the SNARE complex. If the binding site of Complexin does not exist on the partially formed ternary complex, then the process would occur similar to when the binary complex is present. However, if this binding site already exists, then Complexin would most likely bind to the SNARE complex and prevent the binding of Synaptotagmin by blocking the binding site of Synaptotagmin to the SNARE complex. Upon the arrival of calcium, the binding site of Synaptotagmin is

exposed thereby allowing the Synaptotagmin – SNARE interaction and subsequent calcium dependent release (Figure D4A). At the C-terminal end of the Complexin molecule, a stretch of eight negatively charged amino acids were found (Figure D4B). This region could be important for this finely tuned regulation by Complexin of the Synaptotagmin – SNARE interaction. This model assumes that both Complexin and Synaptotagmin bind to the SNARE complex at the same time. This was cofirmed in this study using the anisotropy measurements of labeled Complexin, when the addition of Synaptotagmin to the SNARE complex bound to Complexin was not reversed in the presence of calcium. This result is in direct contradiction to the previously published work stating that Complexin leaves the SNARE complex upon Synaptotagmin binding (64).

Previous studies have also suggested that Synaptotagmin might bind to PIP_2 in a calcium-independent manner (96). However, based on the titration of labeled liposomes containing SNARE proteins to Synaptotagmin (Figure R19), in the absence of calcium, no FRET dependent signal change was observed for the liposomes with PIP_2. In the FRET assay between the SNARE complex and Synaptotagmin, the presence of anionic phospholipids, tend to prevent the full reversal of the FRET signal indicating residual FRETting between these proteins. This could be due to either the proteins are still bound to each other due to an effect of the anionic phospholipids or that Synaptotagmin is bound to the membrane containing PIP_2 in a calcium independent manner and is in a close enough proximity to induce a FRET dependent change. More experiments are needed to further understand this anomaly.

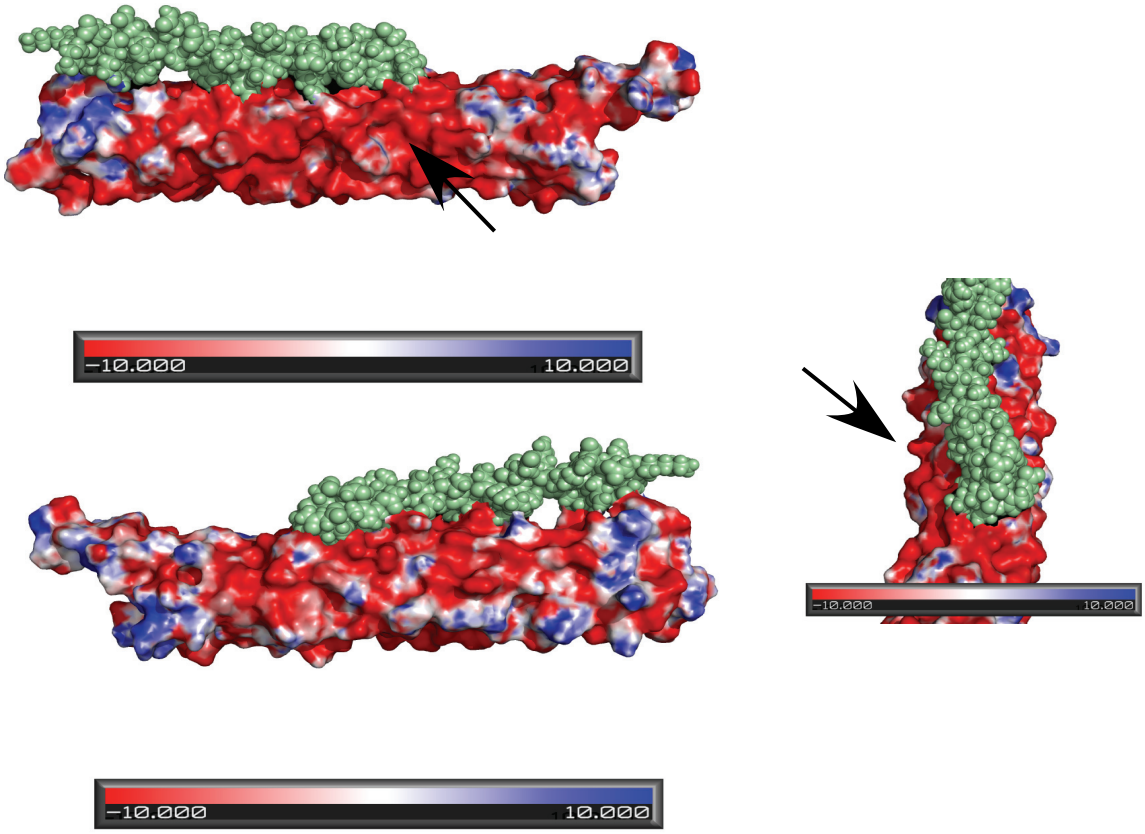


Figure D3: Electrostatic potential of the SNARE complex. The electrostatic potential of the SNARE complex (with Complexin) was calculated using the Adaptive Poisson-Boltzmann Solver (APBS) plugin in the Pymol (DeLano Scientific) software. The red and blue regions represent -10 and +10 kT/e electrostatic potentials. The arrow shows the proposed binding site as described in Rickman *et. al.* (3).

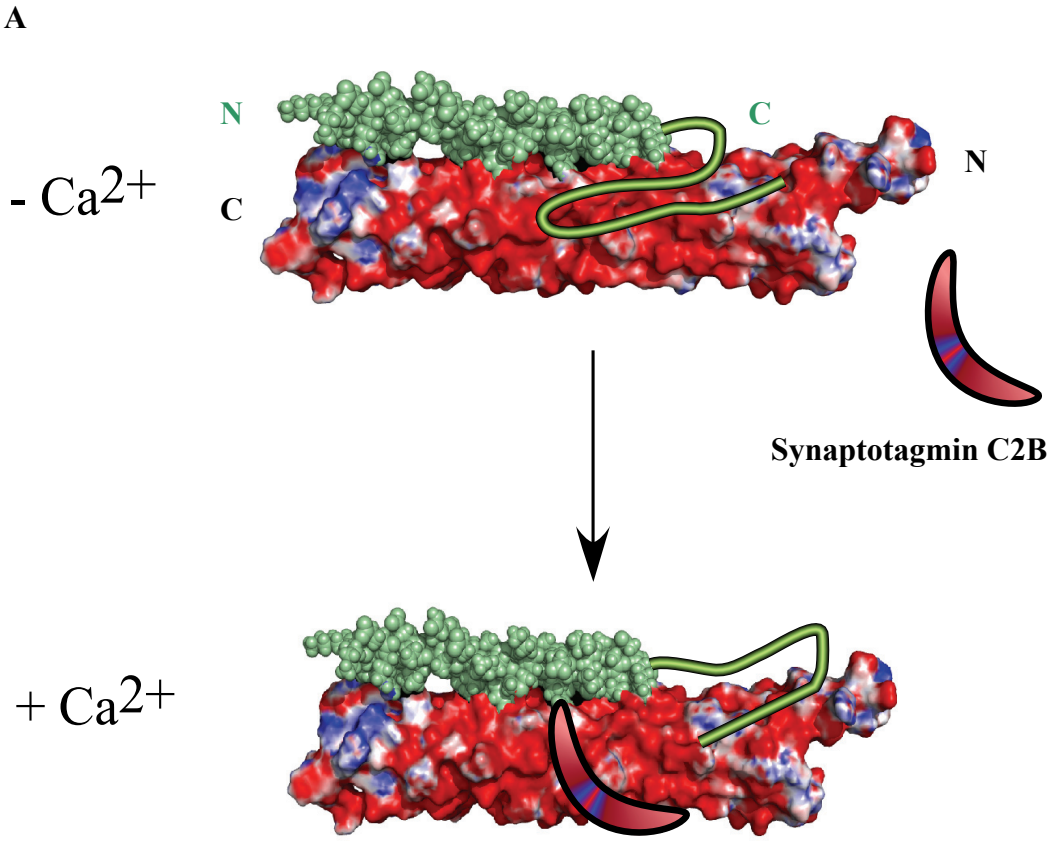


Figure D4: Regulation of Synaptotagmin – SNARE complex interaction by Complexin. Complexin might serve to regulate the interaction of Synaptotagmin to the SNARE complex. In the absence of calcium the C-terminal end of Complexin might occlude the Synaptotagmin binding site. This inhibition is alleviated upon calcium influx **(A)**. The C-terminal end of Complexin consists of an eight acidic amino acid residues which might be important for this regulation.

5. Conclusion & Outlook

The three sections of this study is an attempt at dissecting the function of Synaptotagmin. Current knowledge in this field is highly ambiguous due to the many controversies surrounding the protein's mechanism. Therefore, in order to clarify some of the open issues, a number of quantitative biochemical assays were developed to probe the protein's function. The first section attempted to study the calcium binding property of the C2 domains of Synaptotagmin using ITC. The C2A domain exhibited an endothermic reaction and conversely the C2B domain exhibited an exothermic reaction in calcium binding. This divergence in the domains may indicate a general difference in the mechanism of calcium binding to the C2 domains. Even so, the thermodynamic parameters obtained from the experiment suggest that the domains are stabilized upon calcium binding. This needs to be further studied using a thermal melt and circular dichroism (CD) measurement. This method allows the determination of the melting temperature (T_m), which is the unfolding temperature of the secondary structures in the protein. This can be done both in the absence and presence of calcium to monitor if calcium would increase the T_m thereby showing that more energy for unfolding is needed due to an increase in the structural stability upon the addition of calcium.

The second section dealt with the binding of Synaptotagmin to lipid membranes. Here, the main aim was to observe the effect of lipid membrane composition on the Synaptotagmin – membrane interaction. Increasing the amount of PS in the membrane, mimics the increase in the density of PS since the surface area of the liposomes is similar. Additionally, the experiment was also done in the presence or absence of PIP_2. The results showed that only at lower PS concentrations (i.e. low PS densities in the membrane), the addition of PIP_2 increased the affinity of Synaptotagmin to the lipid membrane. At higher PS concentrations, this effect was completely abolished. This indicates that a certain amount of charge is needed for Synaptotagmin to interact with the membrane and in the native plasma membrane, the enrichment of a high local negative charge could be mediated through the formation of lipid rafts or microdomains which would act as the preferential release site. This hypothesis needs to be tested further by modulating the amount of PIP2 in the membrane to study whether PIP_2 densities might play a role in the binding of Synaptotagmin to the membrane.

The final section of the thesis deals with the interaction of Synaptotagmin to the SNARE complex. Since Synaptotagmin is believed to be the calcium sensor and the SNARE proteins the membrane fusion machinery, the interaction between these proteins serves as the single most important molecular event in the presynaptic calcium dependent exocytosis. In order to systematically address the issue, assays that could qualitatively examine the interaction was developed. This gel-based native PAGE assay managed to show that Synaptotagmin only binds to the SNARE binary and ternary complex via its C2B domains. To understand the mechanism further, fluorescence-based assays were developed. Based on these assays, two phases of this protein – protein interaction, calcium independent and dependent, was observed. Whether the addition of calcium induced an affinity or conformation change is still unknown. Shortening the Syntaxin in the SNARE complex caused a gradual loss of the calcium dependent signal suggesting the destabilization of the binding site. Using the FRET based- assay, Complexin was observed to be able to reverse the binding of Synaptotagmin to the SNARE complex only in the absence of calcium. When calcium was present, binding between Synaptotagmin and the SNARE complex was still detected. Additionally, both Complexin and Synaptotagmin were seen to bind to the SNARE complex simultaneously. These data suggest that Complexin might be responsible in regulating the Synaptotagmin – SNARE complex interaction. The binding of Complexin in the absence of calcium occludes the binding site of Synaptotagmin thus preventing the interaction of Synaptotagmin to the SNARE complex. However, upon calcium entry, the binding site is exposed causing Synaptotagmin to bind to the SNARE complex. In order to truly confirm the details of this regulation, future work will focus on using fragments of Complexin in the FRET assay. Currently, a number of fragments of Complexin are in the process of being expressed and purified. These include 1-69, 70-134, 21-97. 21-107 and 21-117.Using this fragment, the importance of the C-terminal part of Complexin can be determined.

The work presented here is a starting point to studying the function of Synaptotagmin in calcium induced exocytosis. The main aim of the work was to develop the assays needed to study this highly dynamic and fast event. Using these tools, insights into the process can be obtained to develop a model explaining calcium dependent exocytosis.

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Appendix 1: Isothermal Titration Calorimetry (ITC)

Isothermal titration Calorimetry (ITC) is a powerful tool that allows the determination of thermodynamic binding parameters of a chemical reaction. The calorimeter (Figure A6A) consists of an adiabatic jacket containing two coin-shaped containers called the sample and reference cell. The adiabatic jacket ensures that there is no net energy gain or loss in this system and therefore any fluctuation of energy measured is from the chemical reaction. The reference cell contains water and the sample cell contains the macromolecule. A computer-controlled, auto-titrating syringe which doubles as a stirrer is inserted into the sample cell and the ligand solution, which is usually 4 – 20 times more concentrated as the macromolecule solution (depending on the affinity of the reactants), is titrated into the sample cell.

During the titration of the ligand into the macromolecule solution in the sample cell, the temperature difference between the sample and reference cell is measured and the amount of electrical power input (μcal/sec) needed to keep the sample cell temperature equal to the reference cell is calculated and plotted (upper panel figure A6B). This electrical power input is known as the differential power (DP) or feedback energy. In endothermic reactions, when a chemical reaction absorbs energy, the DP is increased until the temperature in the sample cell is equal to the reference cell. This increase in the DP is recorded as the raw data. In an exothermic reaction, the heat evolved chemically from the reaction replaces the DP provided to the cell. This change in the amount of electrical power input that constitutes the raw data is dependent on the macromolecule concentration. When the binding of the ligand to the macromolecule is saturated, no heat is absorbed or released due to the chemical reaction. At this point, the peaks represent the heat change due to the injection.

To obtain the thermodynamic parameters of this chemical reaction, a time integral is calculated from the peaks produced by the DP measurements (lower panel, Figure A6B). This time integral signifies the amount of heat evolved at a specific titration point and is plotted against the molar ratio between the macromolecule and ligand. This can then be fitted using non-linear least squares fitting with binding models that reflect the binding of the ligand to the macromolecule. The most common binding model used in this study was the one-site binding model. This model assumes that all the binding sites of a

given ligand have the same affinity for the ligand. The total heat evolved (Q) in a given volume, V_0 for this binding model is described as:

$$Q = \frac{nM_t\Delta HV_0}{2}\left[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} - \sqrt{1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} - \frac{4X_t}{nM_t}}\right] \quad \textbf{(1)}$$

Where; n = no. of binding sites

M_t = Total macromolecule concentration

X_t = Total ligand concentration

ΔH = Molar heat of ligand binding

K = Binding constant

However, the volume in the sample cell is not constant. At the end of the titration, the volume is altered (V_i) and this difference in the volume contributes to the heat evolved. Therefore, the correct expression for the heat evolved ($\Delta Q(i)$) at the i^{th} injection is:

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_0}\left[\frac{Q(i) + Q(i-1)}{2}\right] - Q(i - 1) \quad \textbf{(2)}$$

These equations clearly shows that the stoichiometry (n), binding constant (K) and molar heat of ligand binding (ΔH) can be determined by initially determining the amount of heat released in each injection and iteratively improving the initial values of n, ΔH and K through standard Marquardt methods. After obtaining these values, a third equation can be used to determine the entropy (ΔS) and Gibbs free energy (ΔG) of the chemical reaction:

$$\Delta G = -RT\ln K = \Delta H - T\Delta S \quad \textbf{(3)}$$

Where; R = Gas constant ($1.987 calK^{-1}mol^{-1}$)

T = Temperature (in K) (25ºC = 298K)

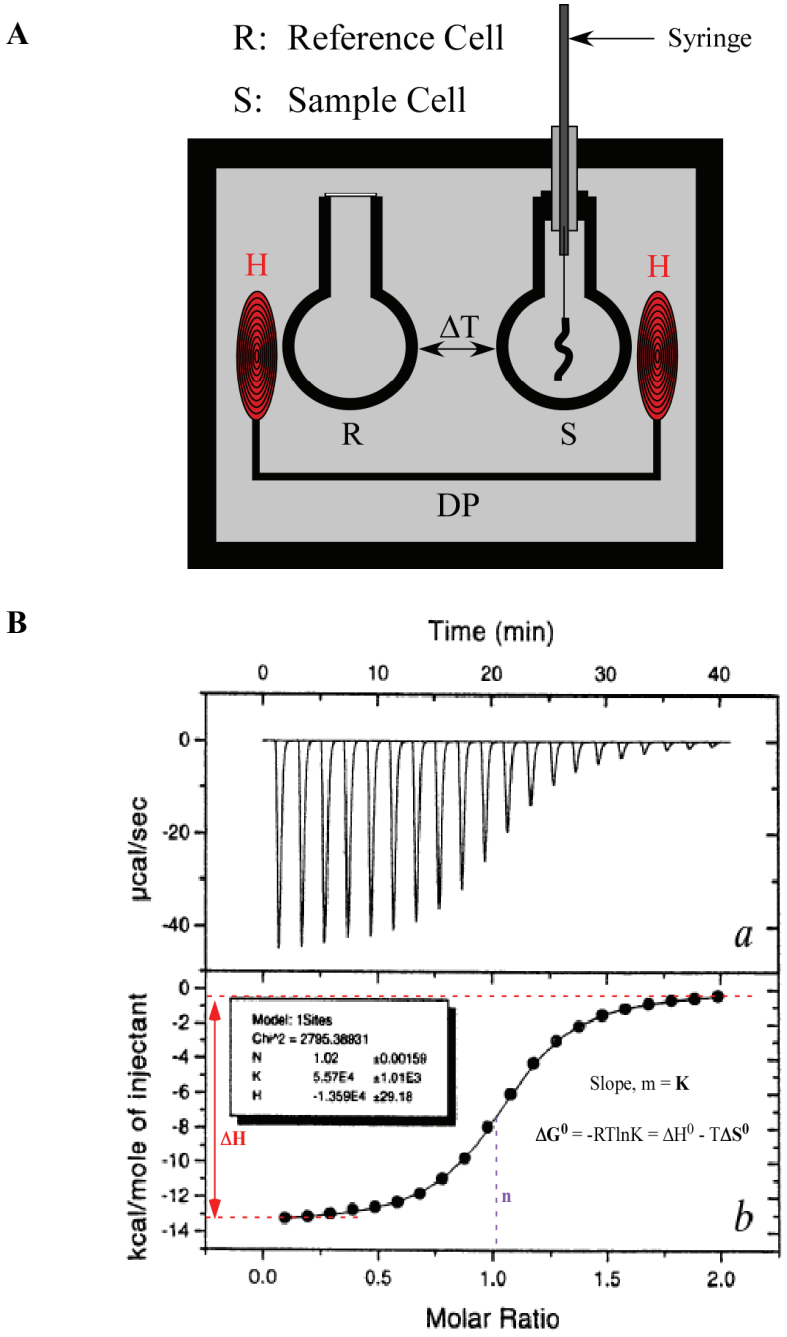

Figure A6: Isothermal Titration Calorimetry (ITC). ITC is done in a calorimeter where the difference in the temperature of the sample and reference cell (**A**) is measured and the amount of electrical power input (μcal/sec) (differential power) needed to adjust the temperature in the sample cell to match the reference cell is calculated and plotted against time (**B, upper panel**). This raw data is then, integrated to obtain the thermodynamic parameters for a chemical reaction (**B, lower panel**).

Appendix 2: A typical protein purification experiment

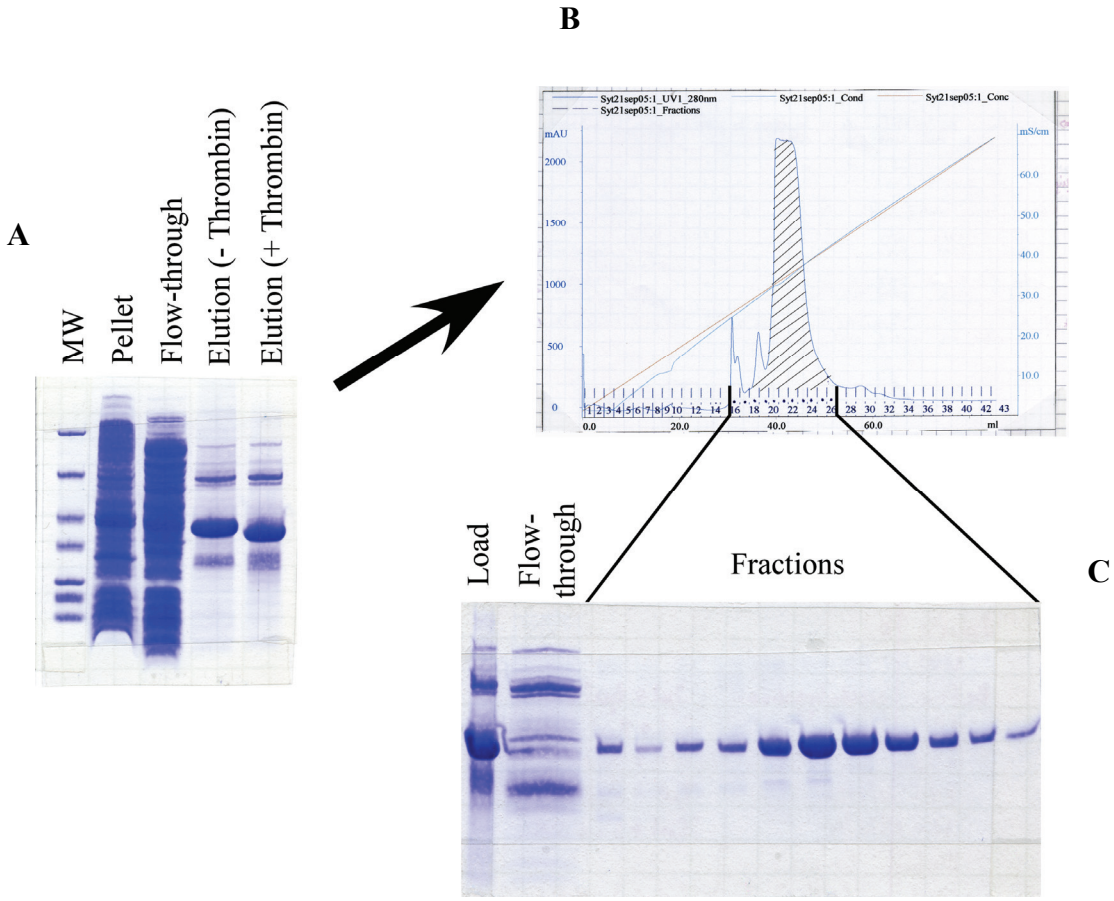

Figure B1: Purification of Synaptotagmin C2AB (96-421). After the lysis of the bacterial pellet, the expressed protein is captured using the Ni-NTA agarose. The eluate from this capture step is then incubated overnight with thrombin to cleave the His6 tag **(A)**. The eluate is then purified using an ion exchange column with a salt gradient **(B)**. The peak fractions are resolved using SDS PAGE and the pure proteins are pooled and stored in the -80ºC freezer **(C)**.

Curriculum Vitae

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May 1999 – Mar. 2000 : ***B.Sc. (Hons.)*** **Biochemistry & Toxicology**,
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Oct. 2001 – Mar. 2003 : ***M.Sc.*** **(Molecular Biology)**,
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Project : Purification of native Synaptotagmin I from rat brain.
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List of Publication

1. The PIP_2 binding mode of the C2 domains of Rabphilin 3A.
   Montaville P., Coudevylle N., Radhakrishnan A., Becker S.
   (Manuscript in preparation)

2. *In vitro* studies of Synaptotagmin – SNARE interaction.
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   (Manuscript in preparation)

3. Effect of Synaptotagmin on the liposome fusion assay.
   Stein, A., Radhakrishnan, A., Fasshauer, D., Jahn, R.
   (Manuscript in preparation)