# Study of the effect of α-Synuclein on SNARE-mediated membrane fusion

By

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## ABSTRACT

Neurotransmitter release is a precisely orchestrated process in terms of time and space in neuron. SNAREs have been identified to function as the basic machinery mediating membrane fusion during neutotransmitter release. Many forms of neurodegeneration initiate presynaptically, but few of their molecular mechanisms have been revealed clearly. α-Synuclein (α-Syn) is a highly conserved synaptic vesicle-associated protein. Aggregation of α-Syn is a major component of the Lewy bodies, which is characteristic of Parkinson's disease (PD). We studied the effect of α-Syn on SNARE-mediated membrane fusion using fluorescent methods. Bulk lipid mixing assay shows that α-Syn has a role of inhibition in fusion and this effect requires phosphatidylserine (PS) on the vesicles. Disease related α-Syn mutants, A30P and E46K, shows higher inhibition effect on the lipid mixing than wild type. Synaptotagmin-1 (Syt-1) is a $Ca^{2+}$ sensor localized to synaptic vesicles and regulates neuronal exocytosis. C2AB, a soluble model of Syt-1 that lacks the transmembrane region, is shown here to accelerate the FRET significantly. This acceleration effect of lipid mixing also needs PS on the vesicles. α-Syn can inhibit C2AB's stimulatory effect to a large extent. Thus, α-Syn can inhibit SNARE-mediated membrane fusion event.

# CHAPTER 1: GENERAL INTRODUCTION

## Introduction

## Membrane fusion and SNARE proteins

A series of membrane fusion happens in cell to maintain its basic function. Membrane fusion, a process of two separate lipid bilayers merging to become one, is a universal reaction that varies vastly in space and time. One of the most studied membrane fusion is exocytosis. At the synapse, exocytosis is very important to ensure the efficient delivery of chemical signals. Synaptic vesicle fusion is mediated by a central fusion machinery SNAREs (soluble N-ethylmaleimide-sensitive factor attachment protein receptors), while it is also controlled by various regulators.

SNAREs vary widely in size and structure[1]. They are recognized by sharing a SNARE motif, which contains eight heptad repeats of 60-70 amino acids (Fig 1). The SNARE core complex is basically a parallel four helix bundle intertwined with each other between these SNARE motifs (Fig 2). SNAREs can be divided into two broad categories, t-SNARE in target plasma membranes and v-SNAREs in transport vesicles. The synaptic SNARE proteins are one of the best characterized and studied paradigms. In this system, syntaxin-1 and SNAP-25 on the plasma membrane is t-SNARE; Synaptobrevin/VAMP2 on the vesicle is v-SNARE. Most SNAREs contain a single, C-terminal transmembrane domain adjacent to the SNARE motif. SNARE motifs spontaneously assemble into a four-helix bundle between membranes to drive fusion[2].

Vesicle's docking and fusion process is mediated via SNARE assembly, during which SNARE motifs of t-SNARE and v-SNARE zipper from their membrane-distal N-terminus to membrane-proximal C-terminus and form a tight four helix bundle. According to the predominant fusion model, there are three concerted steps involved[3, 4]. Firstly, by forming a tight ternary complex, the SNARE motifs bring two opposing membranes together. Secondly, the outer leaflets of membranes contact with each other and merge into a hemifusion state[5, 6, 7], in which the outer leaflets merge together but the inner leaflets not. Thirdly, fusion pore is formed after hemifusion and expands to enable content mixing. The ternary complex which resides on two membranes is called trans-SNARE and it transits into cis-SNARE after membranes merge together (Fig 3). The ternary core complex was found to be highly stable and resistant to denaturation by SDS[8, 9].

Multiple studies have suggested that additional regulatory proteins are essential for the fast neurotransmitter release process[10]. Several proteins have been identified to play important roles in SNARE assembly, such as Munc-18, Synaptotagmin (Syt), complexin, and etc. Some of these regulatory proteins can be dispensed with in vitro at high SNARE concentrations.

**α-Syn**

α-Syn is a cytosolic protein that is enriched and highly conserved in mature nerve terminals[11]. More evidence has emerged that implicates its involvement in neurodegenerative disease[12, 13]. Aggregation into amyloid fibrils of α-Syn is a major component of the Lewy Body deposits, which is the pathological hallmark of Pakinson's Disease (PD) (Fig 4). Duplication, triplication of the wild-type α-Syn gene and several missense mutations are proposed to be linked with rare familial forms of early-onset PD. It has been shown that excess accumulation of α-Syn leads to cellular toxicity when α-Syn, or PD-related α-Syn mutants, is overexpressed in mouse, rat and even yeast. Despite intense studies, the exact function of α-Syn is still unclear.

A small protein of 140-143 amino acids as it is, α-Syn is natively unfolded in solution. According to circular dichroism measurements, the conformation of α-Syn in solution is quite random. However, in the presence of lipid vesicles, α-Syn adopts a highly helical structure in its N-terminal region and remains an unstructured C-terminal tail[14].

The extremely well conserved α-Syn sequence in evolution implies functional constraints on its three-dimensional structure. The presence of seven imperfect 11-mer repeats in the sequence has a high resemblance to 11-mer repeats of apolipoproteins, suggesting a lipid interaction role of α-Syn. The recurring 11-residue periodicity enables α-Syn to have a capacity to fold into an amphipathic α-helix. A helical wheel model has been proposed to represent α-Syn. In this model, several of the α-Syn 11-mers display a distinctive distribution of polar and nonpolar residues to opposite faces of the helix[11]. The structural observation of α-Syn led us to hypothesize that α-Syn would have an ability to interact with phospholipid membranes and this interaction would be dependent on the α-helical secondary structure.

Like other intrinsically unstructured proteins, α-Syn is proposed to have an interaction with one or more protein partners. The unstructured C-terminal tail is likely to function in protein-protein interaction.

**Syt-1**

Secretion of neurotransmitters is a fundamental activity of neurons. It is achieved by vesicular exocytosis, fusion of secretary vesicles with the plasma membrane. Synaptic vesicles dock at the active zone, electron-dense sites on the presynaptic plasma membrane, and are primed for exocytosis with the influx of $Ca^{2+}$[15, 16].

Syt family is involved in membrane trafficking and characterized by an N-terminal transmembrane region, a variable linker, and two C-terminal C2 domains-C2A and C2B[17] (Fig 5). Among the 15 members in the Syt family, only eight of them are able to bind $Ca^{2+}$, which are Syt-1, 2, 3, 5, 6, 7, 9, and 10. Syt-1 is the first one in the family to show a capacity to bind $Ca^{2+}$ with its C2 domains. In an atomic structure analysis, C2 domains of Syt-1 are shown to have a stable eight-stranded β-sandwich structure, with flexible loops emerging from the top and bottom. Nuclear magnetic resonance (NMR) studies have shown that only the top loops have the binding pockets for $Ca^{2+}$. Five conserved aspartate residues are involved: D172, D178, D230, D232, S235 and D238 of C2A, and D303, D309, D363, 365 and D371 of C2B[18, 19].

Syt-1 is proposed to function in early synaptic docking to the presynaptic membrane and later calcium evoked synaptic vesicle fusion[20-24]. However, its precise roles are still in debate. How does it interact with SNARE proteins and the membrane respectively? Does it require the negatively charged lipids on both membranes?

**Functional Study by ensemble lipid mixing assay**

To better study the process of membrane fusion, SNAREs were reconstituted into separate liposomes *in vitro* and the kinetics was examined by ensemble lipid mixing assay. Rothman's group has already demonstrated the effectiveness of fluorescence dequenching strategy in studying lipid mixing *in vitro*[25].

**Thesis Organization**

Chapter 1 provides a general background for the thesis. An introduction of SNARE proteins, membrane fusion process, and proteins that can affect the kinetics of fusion would help to build an overall picture of fusion in terms of considerations that should be taken into. Two key regulators are highlighted and focused for discussion in this thesis, which is α-Syn and Syt-1. Chapter 2 talks in detail about the α-Syn's structural property and studied its effect on SNARE-mediated membrane fusion by ensemble fluorescence lipid mixing assay. Utilizing mutagenesis, further studies the potential residues for α-Syn's inhibition function, giving some insights of the mechanism. Chapter 3 investigates the function of Syt-1 and its interaction with negatively charged lipids on the vesicles. By combining Syt-1 and α-Syn in lipid mixing assay, it shows their interaction and helps to find out more about α-Syn. Chapter 4 gives a summarization based on these results and some directions further studies might go.

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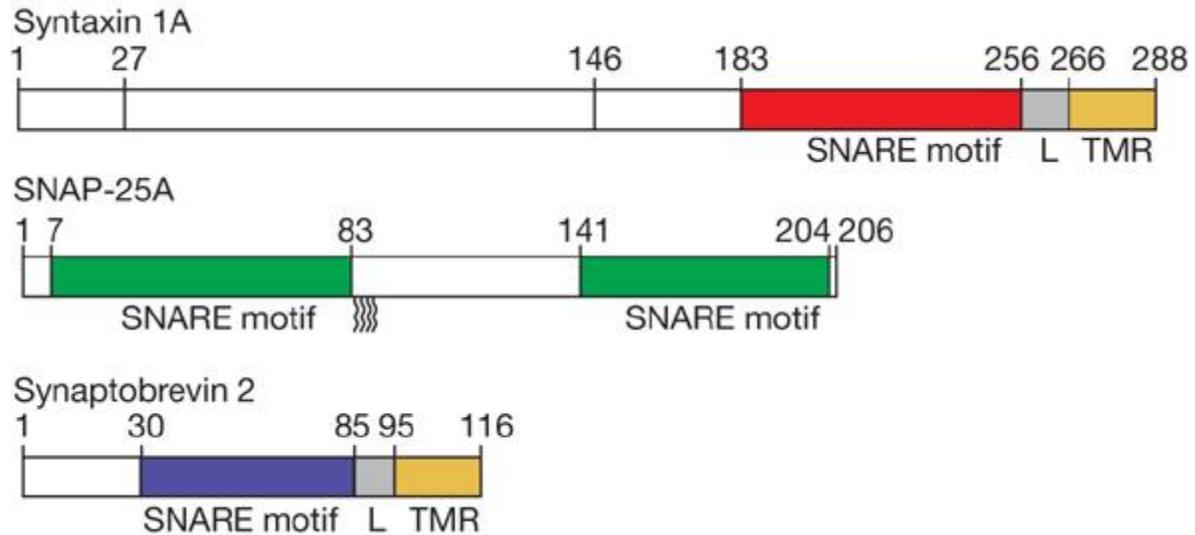
**Figures And Captions**



**Fig 1.  Primary structure diagram of neuronal SNARE proteins TMR, transmembrane region is located at the C-terminal of syntaxin 1A and VAMP2. The SNARE motifs are defined through the 16 layers as found in the crystal structure of the neuronal SNARE core complex. The N-terminal domain of syntaxin 1A is named Habc which can bind with the SNARE core domain or Munc18. SNAP-25A contributes two SNARE motifs to the core complex, which are SN1 and SN2, the four palmitoylation sites (cysteine 85, 88, 90 and 92) are indicated by lines. L, linker region is located between SNARE motif and transmembrane region.**
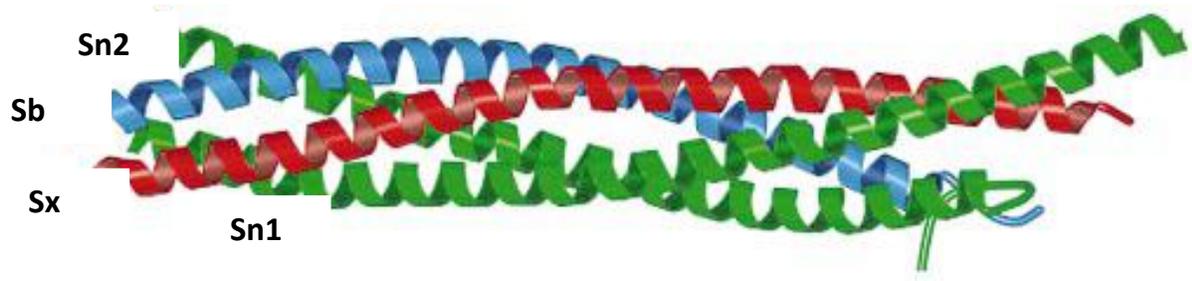
[26]Stein A et al. Helical extension of the neuronal SNARE complex into the membrane. *Nature*. Jul 23; 460(7254):525-8 (*2009)*

**Fig 2. Crystal structure of neuronal SNARE complex. The four-helix bundle. Sn1 and Sn2 are two 'SNARE motifs' of SNAP-25. Sx is Syntaxin and Sb is Synaptobrevin.**

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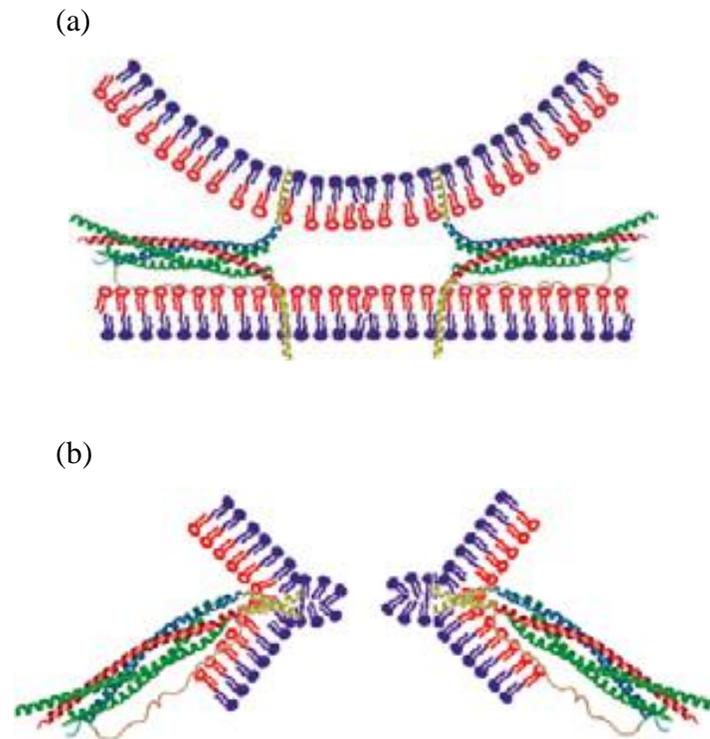
(a)

(b)

**Fig 3. Trans- and Cis-SNARE complex (a) Partially assembled trans-SNARE Structure (b) Fully assembled cis-SNARE complex.**

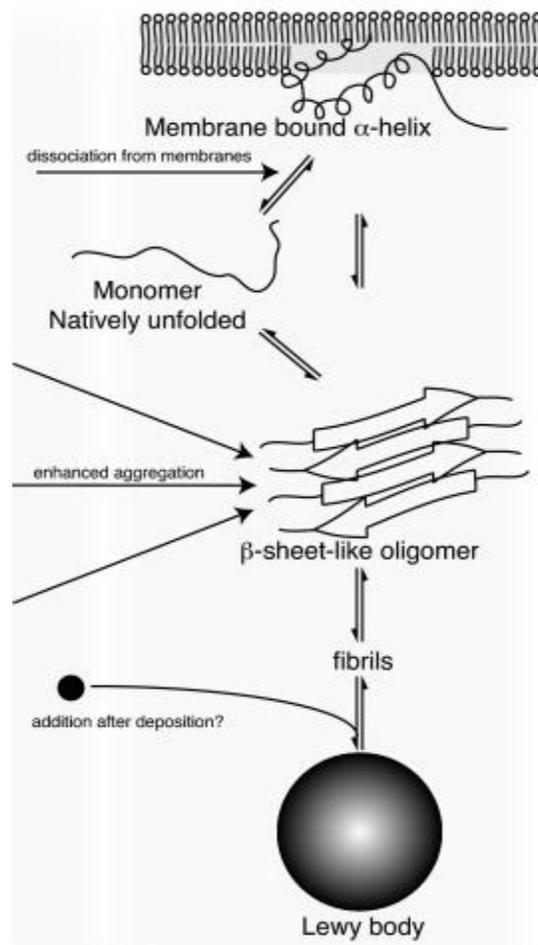[28] Brunger, AT. (2005). Structure and function of SNARE and SNARE-interacting proteins. *Q Rev Biophys*.38, 1-47.

**Fig 4. α-Syn aggregation pathway. Monomeric α-Syn is natively unfolded in solution. Upon binding to membranes, it adopts an α-helical structure in the N-terminal region. The unfolded monomer can also aggregate first into small oligomeric species that is stabilized by β-sheet-like interactions. Fibrils and further Lewy body can be formed after further aggregation into higher molecular weight.**
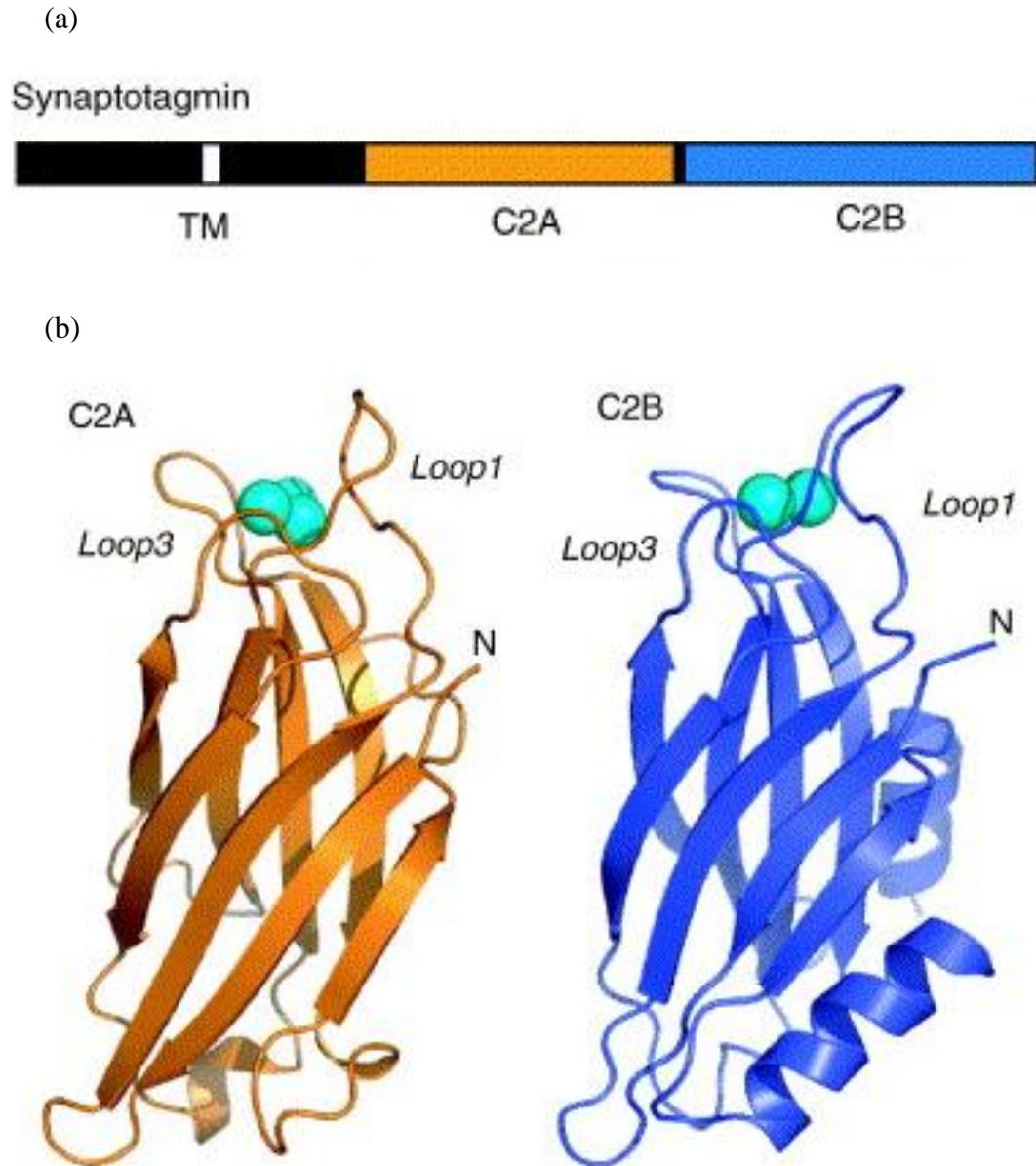
(a)



(b)



**Fig 5 Structure of Syt I (a) The primary domains structure of Syt 1, of which a single transmembrane domain(TM) near its N terminus helps anchor to the vesicle membrane (b) The NMR structure of the C2 domains, C2A and C2B. Blue spheres represent multiple calcium ions bound to loops 1 and 3.**

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# CHAPTER 2: α-SYN'S INHIBITION EFFECT ON SNARE-MEDIATED MEMBRANE FUSION

**ABSTRACT**

**α-Syn is a major constituent of Lewy Bodies, protein clumps that are the pathological hallmark of PD. Overexpression of the gene and mutations of the normal protein can cause an inherited form of PD[1-3]. Using a defined *in vitro* lipid mixing assay, we want to investigate the mechanism of α-Syn's interaction with SNAREs and the membrane.**

**We found out that α-Syn can inhibit SNARE-mediated lipid mixing. This inhibition effect requires the negatively charged lipid phosphatidylserine (PS) to be on the vesicle. Furthermore, we examined the effect of two mutations linked to familiar PD α-Syn A30P and E46K. Both of them show a higher inhibition role on lipid mixing than wild-type α-Syn and E46K is the strongest one. We give some novel insight of using *in vitro* lipid mixing assays to study the mechanisms of α-Syn on SNARE-mediated membrane fusion.**

**Introduction**

α-Syn is a soluble protein predominantly expressed in neural tissue, making up to 1% of all the proteins in the cytosol. It is found to be membrane bound in dopaminergic neurons and implicated in the regulation of dopamine release and transport. α-Syn is a small protein with 140 aa (Fig 1). The primary structure is usually divided in three distinct domains: (1): An amphipathic N-terminal region (residues 1-60) dominated by four 11-residue repeats including the consensus sequence KTKEGV[1]. This sequence has a structural propensity to be α-helical,

similar to apolipoproteins-binding domains[2]. (2) A central hydrophobic region (residues 61-95) which includes the non-amyloid component (NAC) region, involved in protein aggregation[3]. (3) A highly acidic and proline-rich C-terminal region (residues 96-140) which is basically unstructured. A helical pinwheel model has plot the first 94 residues by researchers (Fig 2). α-Syn can form filamentous aggregates that are the major non amyloid component of intracellular inclusions in several neurodegenerative diseases, including Parkinson's Disease (PD)[4].

Recent studies have highlighted the importance of α-Syn in vesicle trafficking. Overexpression of α-Syn gene in yeast and Drosophila has shown an inhibition effect on the vesicular transport between endoplasmic reticulum and the Golgi complex[5, 6]. In Chromaffin cells and in mouse neurons, evidence has been found that overexpression of α-Syn inhibits release of neurotransmitters[7]. α-Syn is found to co-localize with synaptic vesicles histologically[8]. Substantial evidence has shown that α-Syn interacts with phospholipid membranes and is a critical regulator of vesicle dynamics at the synapse. *In vivo*, it can bind to rat brain vesicles via the imperfect 11-mer repeats and inhibit the neurotransmitter release[9]. *In vitro*, in the presence of phospholipid membranes, it can adopt a secondary α-helical structure and attach to the membranes[10]. However, not all cellular α-Syn binds to membrane since it can be purified from the cytosolic part of the cells.

Mutations in the gene for α-syn, including the A30P and E46K missense mutations, are sufficient to cause PD as well as other Synopathies like dementia with LBs[11-15]. Studies have shown that α-syn A30P can generate nigrostriatal deficiency in mice. The E46K mutation in α-syn also increases amyloid fibril formation.

α-Syn is known to be able to associate with the negatively charged surfaces of phospholipids. Phosphatidylserine (PS) or 1, 2-diacyl-sn-glycero-3-phospho-L-serine is a negatively charged phospholipid component that might comprise 10 to 20 mol% of the total phospholipid of plasma membranes in cell[16]. It has three ionizable groups, the phosphate moiety, the amino group and the carboxyl function (Fig 3). Like other acidic lipids, in nature it exists in salt form; while it also has a high propensity to chelate to calcium. The conformation of the polar head group changed after $Ca^{2+}$ binding via the charged oxygen atoms of both the carboxyl and phosphate moieties.

Neuronal SNAREs is one of the core protein familes involved in the fusion process during neurotransmitter release. ATPase N-ethylmaleimide-sensitive factor (NSF), soluble NSF attachment proteins (SNAPs), and synaptotagmin family are the central machinery[17-25]. In a more defined *in vitro* system, we want to investigate α-Syn's direct effect on SNARE-mediated lipid mixing. Also, α-Syn A30P and E46K mutants are tested by the lipid mixing assay.

**Results And Discussion**

Parkinson's disease is a well-known neuronal disordered disease because it bears α-syn pathological inclusions known as Lewy bodies (LBs). Ultra structural analysis had revealed that LBs are amyloid like fibrils consisting of misfolded α-Syn proteins. (Spillantini et. al., 1998) α-Syn is a soluble protein expressed principally in the brain, shown to be involved in the functioning of the neuronal vesicle trafficking.

In aqueous solution, α-Syn is in the form of an extended random coil structure without a hydrophobic core. In the presence of small vesicles, α-Syn adopts an α-helical secondary structure at its N-terminus, that is suited for lipid binding; while the negatively charged C-terminus remains unstructured and is proposed to have an interaction with other proteins. Most recent studies have discovered the lipid-binding domain of α-Syn and propose its roles in the regulation of dopamine transporter activity. Sudhof's study shows that α-Syn interacts with VAMP2.

Many studies have already shown that α-Syn aggregation in cells interferes with the exocytotic pathway[4-7]. But the molecular mechanism by which α-Syn blocks the exocytosis has not been known. In this work, we use recombinant SNARE proteins (the purity was checked by SDS-PAGE gel Fig 4) to conduct the lipid mixing assay, mimicking the in vivo SNARE fusion process and studying α-Syn's effect upon it. Our data shows that α-Syn inhibits SNARE-dependent membrane fusion.

**α-Syn inhibits SNARE-mediated lipid mixing**

The fusion of liposomes induced by neuronal SNAREs was investigated by our well characterized lipid mixing assay. It is formerly shown that SNAREs can be well reconstituted into liposomes and the size of liposomes is ~80-100nm. The t-SNARE binary complex, formed by syntaxin 1A and SNAP-25, was reconstituted into t-vesicles made of POPC/DOPS/CHO/DiI (43:15:40:2). The v-SNARE VAMP2 was reconstituted into v-vesicles made of POPC/DOPS/CHO/DiD (43:15:40:2). When the t-SNARE and v-SNARE liposomes were mixed together at 35 °C at 1:1 ratio in the assay, an increase of the acceptor DiD signal was observed because of Fluorescence resonance energy transfer (FRET). It indicated that fusion happened. As a control, if SNAP-25 was missing from the binary complex, fusion would not happen and the increase of the acceptor DiD signal would not be observed (Fig 5a).

In vivo studies have already shown that overexpression of α-Syn inhibits neurotransmitter release[7]. Our *in vitro* lipid mixing assay also shows similar effect. Upon addition of α-Syn, signal of the acceptor dye DiD on the v-vesicle decreased. With the increasing of α-Syn's concentration, it shows higher inhibition effect (Fig 5 b).

**α-Syn inhibition effect on SNARE-mediated lipid mixing requires negatively charged lipids on the vesicles**

It is proposed that the interaction of α-Syn with lipid membrane might be very important to conduct its function. We wanted to investigate whether this interaction requires the negatively charged surfaces of phospholipids. Two kinds of lipid vesicles were introduced in our assay. One is normal vesicles with the composition mimic the physiological membrane, which has 43 mol% POPC, 15 mol% DOPS and 40 mol% CHO. The other one is neutral vesicles without DOPS, instead using POPC, which is 58mol% POPC and 40mol% CHO.

To study the PS effect on the SNARE-mediated membrane fusion, we reconstituted the t- and v-SNAREs to the normal vesicles and neutral vesicles respectively. After adding α-Syn, the group with normal vesicles still show decreased acceptor signal, which means α-Syn can inhibit the lipid mixing. But the group with neutral vesicles, we can hardly see any inhibition effect (Fig 6).

**α-Syn pathotype mutants shows stronger inhibition effect on SNARE-mediated lipid mixing assay than wild-type α-Syn**

Extensive studies have been done on disease related α-Syn mutants. α-Syn mutations promote the formation of transient protofibrils (prefibrillar oligomers), suggesting that protofibrils are linked to cytotoxicity. Sara Herrera and his colleague showed that α-Syn E46K mutant is more toxic to yeast than other familiar α-Syn mutants.

α-Syn is considered to be one of the most important proteins associated with inherited PD. Its A30P and E46K mutations result in an early-onset phenotype. These mutants are produced by site-directed mutagenesis and expressed with GST tags in *E.coli*. With same set up of the basic SNARE-mediated lipid mixing assay, we tested the effect of α-Syn wild-type, α-Syn A30P and E46K mutants. We found out that both mutants show higher inhibition effect than wild-type protein (Fig 7).

**Materials And Methods**

**Plasmids and site-directed mutagenesis.** DNA sequences encoding syntaxin 1A (amino acids 1-288 with three cysteines replaced by alanines), SNAP-25 (amino acids 1-206 with four native cysteines replaced by alanines), VAMP2 (amino acids 1–116 with C103 replaced by alanines), and α-Syn (amino acids 1-140) were inserted into the pGEX-KG vector between SmaI and XhoI as N-terminal glutathione S-transferase (GST) fusion proteins. A Quick Change site-directed mutagenesis kit (Stratagene) was used to generate all cysteine mutants, as well as α-Syn A30P and α-Syn E46K. DNA sequences were confirmed by the Iowa State University DNA Sequencing Facility.

**Protein expression and purification**

Expression of recombinant GST fusion proteins was conducted in Escherichia coli Rosetta (DE3) pLysS (Novagene). Cells were grown at 37 °C in LB medium with glucose (2 g/liter), ampicillin

(100 ug/ml) and chloramphenicol (34 ug/ml) until the absorbance at 600nm reached 0.6-0.8. Isopropyl-Dthiogalactopyranoside was added at a final concentration of 0.5mM. Cells were further grown at 16°C overnight. Cell pellets were harvested by centrifugation at 6,000rpm for 10 min and then stored at -80°C.

Purification of GST fusion proteins was conducted with affinity chromatography using glutathione-agarose beads (Sigma). Frozen cell pellets were resuspended in 10 ml PBS buffer(phosphate-buffered saline, containing 0.2% (v/v) Triton X-100, with final concentrations of 2mM 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), 2mM DTT, pH 7.4). Cells were broken by sonication in an ice bath and centrifuged at 15,000g for 30 min at 4 °C. The supernatant was mixed with 2 ml glutathione-agarose beads in PBS and nutated at 4 °C for 2 h. The protein-bound beads were washed with an excess volume of washing buffer (phosphate-buffered saline, pH 7.4) for at least 5 runs. When washing, 0.2% (v/v) Triton X-100 was added to syntaxin 1A, VAMP2, whereas no detergent was added to SNAP-25, α-Syn wild type and α-Syn mutants. The proteins were then cleaved by thrombin in cleavage buffer (50 mM Tris HCl, 150 mM NaCl, pH 8.0) with 0.8% g/ml n-octyl-D-glucopyranoside (OG) at room temperature for 1 hour. Purified proteins were stored at -80°C with 10% glycerol and examined with 13% SDS-PAGE. The purity was at least 85% for all proteins.

**Membrane reconstitution**

The mixture of POPC (1-palmitoyl-2-dioleoyl-sn-glycero-3-phosphatidylcholine), DOPS (1,2-dioleoyl-sn-glycero-3-phosphatidylserine), Cholesterol and DiI (t-vesicles) or DiD (v-vesicles) (molar ratio of 43:15:40:2) in chloroform was dried in a vacuum and was resuspended in a buffer (25mM HEPES/KOH and 100mM KCl [pH 7.4]) to make the total lipid concentration of about 5mM. Protein-free large unilamellar vesicles (~100 nm in diameter) were prepared by extrusion through polycarbonate filters (Avanti Polar Lipids). For net neutral charge lipid mixing, 15mol% DOPS was replaced by equimolar quantity of POPC. Syntaxin and SNAP-25 were mixed at room temperature at a molar ratio of 1:1.5 for 1 hour to allow the formation of binary t-SNARE complex. The binary t-SNARE and VAMP2 were then mixed respectively with t- and v-vesicles at 4°C for 30 min with 0.8% g/ml OG, as the protein lipid ratio is 200:1. The liposome/protein mixture was diluted two times before dialyzed against 2 liters of dialysis buffer at 4°C overnight.

## Lipid mixing assay

Reconstituted t-vesicle and v-vesicle were mixed at a ratio of 1:1. The total lipid concentration in the reaction is 0.1mM. The fluorescence intensity was monitored in two channels with the excitation wavelength of 530 nm and emission wavelengths of 570 and 670 nm for DiI and DiD dye pairs, respectively. Fluorescence changes were recorded with the same Varian fluorimeter. All measurements were performed at 35°C. The initial rate was calculated by analyzing the slope value within the beginning 150 sec. And the initial rate of control group was normalized to 1.

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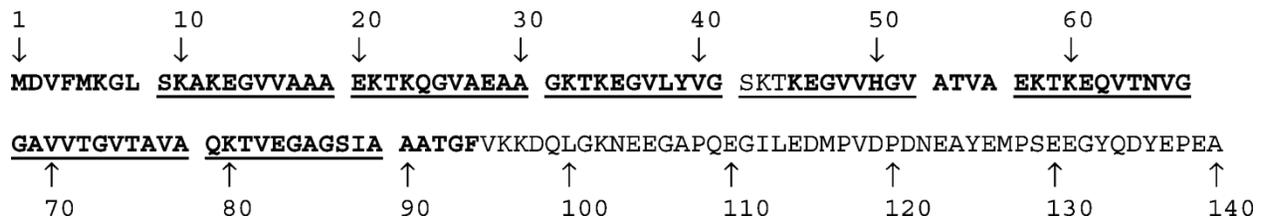
**Figures And Captions**

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↓            ↓            ↓            ↓            ↓            ↓            ↓
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    70           80           90          100          110          120          130          140
```

**Fig 1. The sequence of human α-Syn. Upon binding to membranes, the two helical regions of α-Syn are indicated in bold. The underlined region shows the imperfect 11-mer repeats.**

[1] Bussell R Jr, Eliezer D. A structural and functional role for 11-mer repeats in alpha-Synuclein and other exchangeable lipid binding proteins. *J Mol Biol* 329(4):763-78. (2003)

**Fig 2. (a) Helical pinwheel plot and (b) space filling representation of the micelle-bound region of αS (residues 1–94) as an ideal α-helix. Hydrophobic residues are in black, positively charged residues in red, negatively charged residues in blue and polar residues in yellow. Basic residues are in blue and acidic residues are in red.**

[1] Bussell R Jr, Eliezer D. A structural and functional role for 11-mer repeats in alpha-Synuclein and other exchangeable lipid binding proteins. *J Mol Biol* 329(4):763-78. (2003)
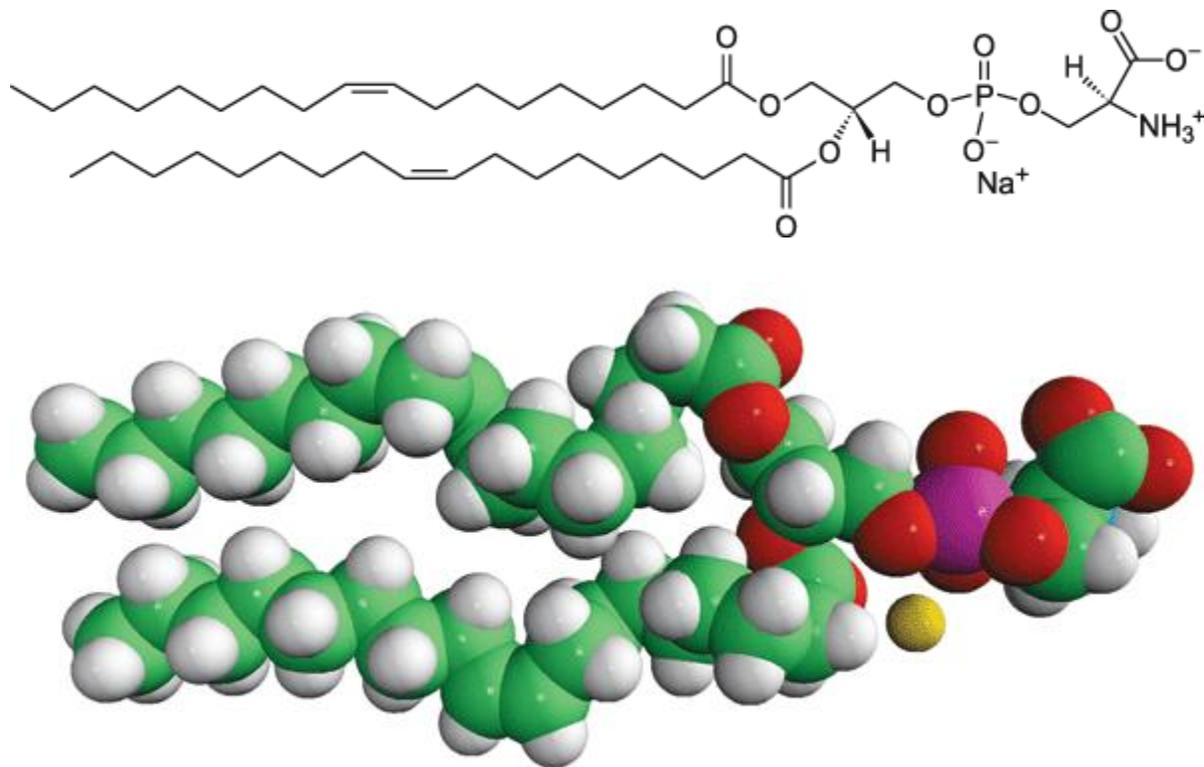
**Fig 3. Structure of DOPS. Phosphatidylserine (PS) is the most abundant negatively charged phospholipid in eukaryotic membranes, which has three ionizable groups.**

Avanti Polar Lipids, Inc.

α-Syn, syntaxin 1A, VAMP2, SNAP-25, C2AB,     α-Syn A30P, α-Syn E46K
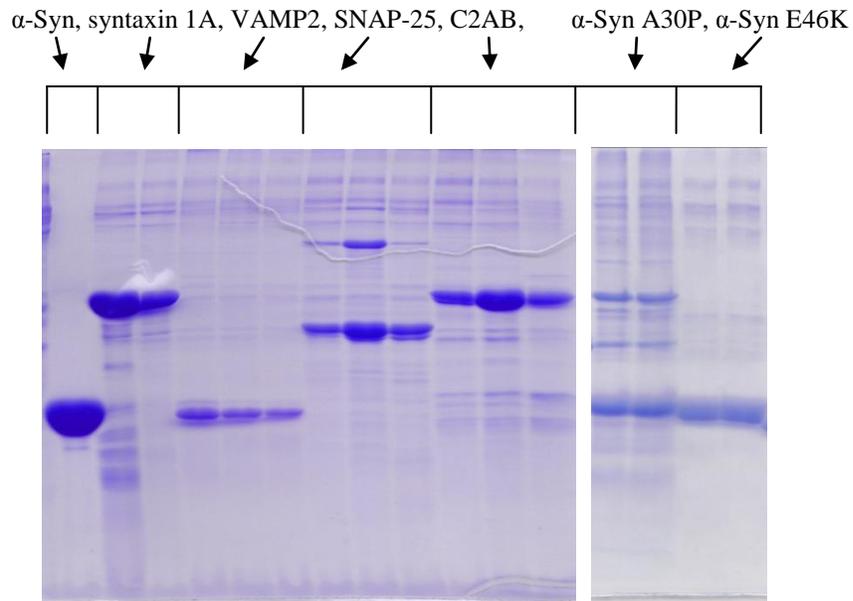


**Fig 4. SDS gel of purified recombinant proteins. From left to right: α-Syn, syntaxin 1A(two lanes), VAMP2(three lanes), SNAP-25(three lanes), C2AB(three lanes), α-Syn A30P and E46K mutants(two lanes each).**

(a)
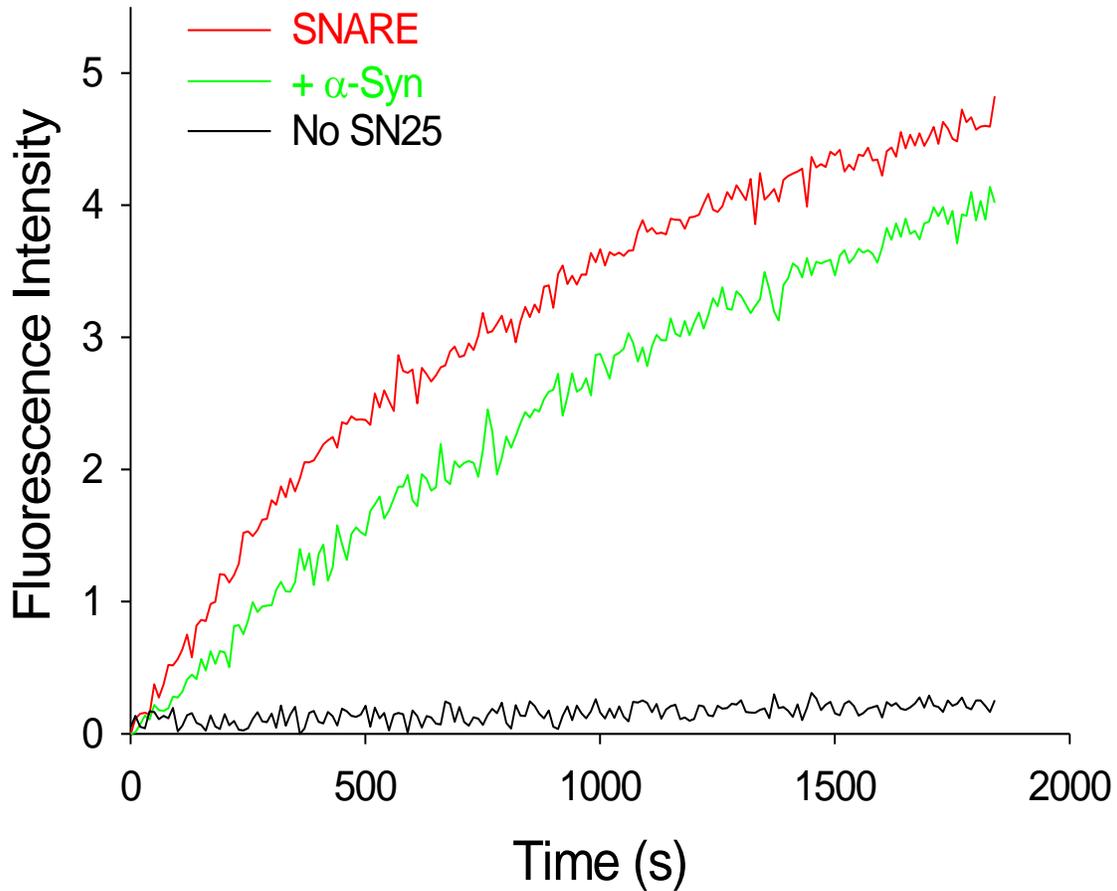


**Fig 5. α-Syn inhibits SNARE-mediated lipid mixing. It shows the change of DiD signal strength. The change of fluorescence intensity is due to t- and v- vesicle lipid mixing. (a)The red line is the fusion kinectics of lipid mixing between t-vesicle reconstituted with Syntaxin 1A/SNAP-25 and v-vesicle reconstituted with VAMP2. The green line is lipid mixing with 20uM α-Syn added to the assay. The black line is a control, which is in the absence of SNAP-25 on t-vesicle.**

(b)
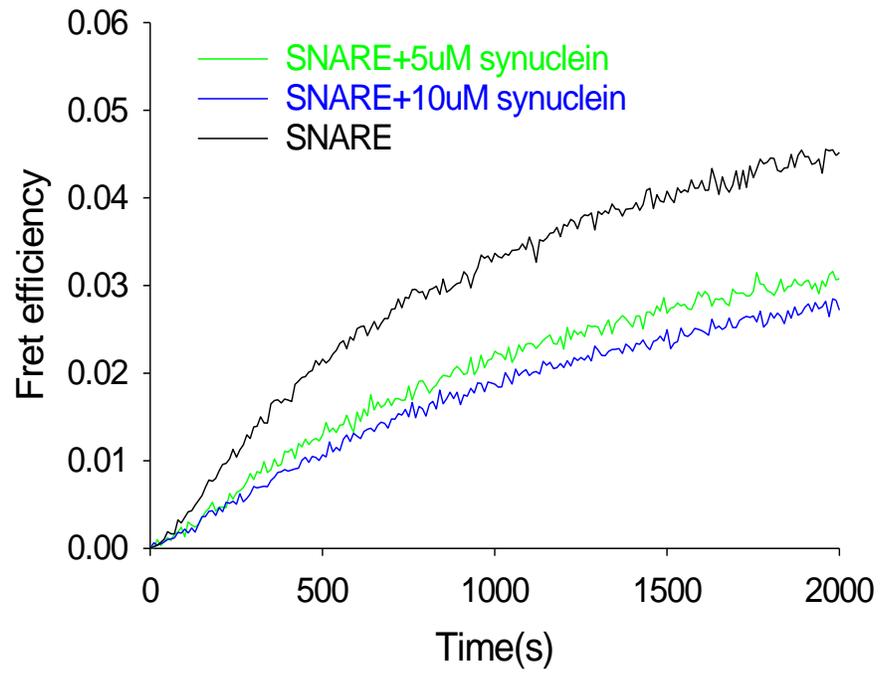


**(b) With the increasing concentration of α-Syn, the inhibition effect is stronger.**
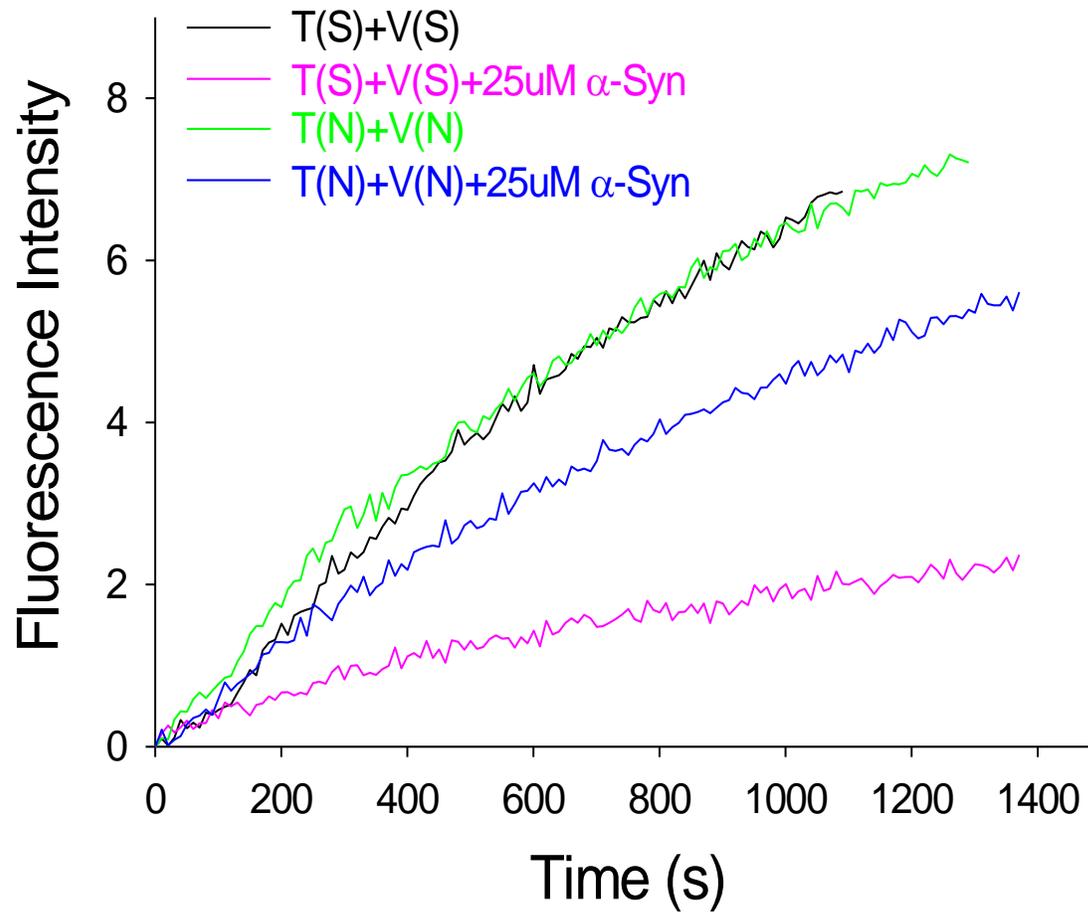
**Fig 6. α-Syn's effect on lipid mixing with neutral and PS lipids. T(S) and V(S) are vesicles with PS. T(N) and V(N) are vesicles without PS. Pink line and blue line are lipid mixing with 25uM α-Syn. The green line and black line are SNARE-only mediated lipid mixing. It shows that without PS on the vesicles, α-Syn's inhibition effect was attenuated.**
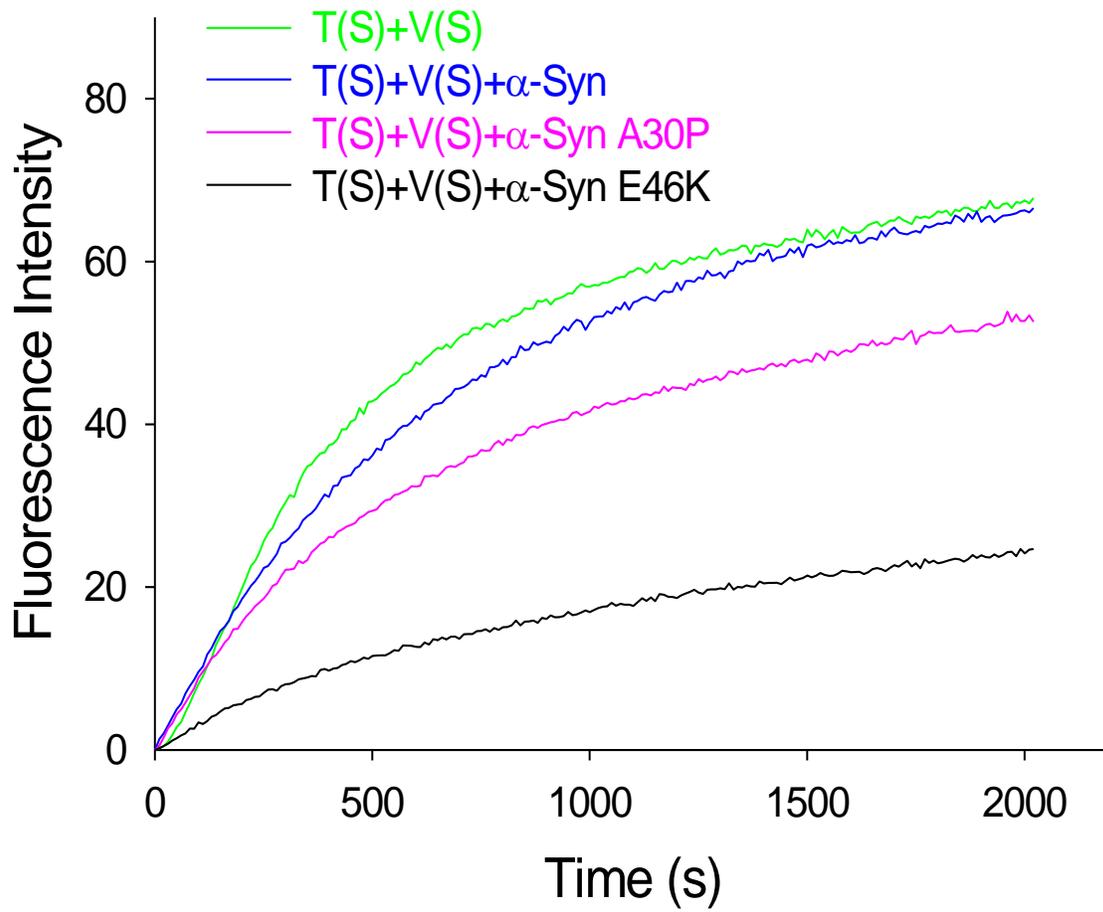
**Fig 7. α-Syn mutants A30P, E46K associated with PD exhibit stronger inhibition effect on SNARE-mediated lipid mixing than wild-type**

# CHAPTER 3: C2AB AND Ca2+ STIMULATE SNARE-MEDIATED LIPID MIXING WHILE α-SYN CAN INHIBIT THIS STIMULATORY FUNCTION

**ABSTRACT**

**For the fusion process, two membranes must be opposed with each other prior to the secretion event. Lipid membrane binding domains are importantly involved. Syt family is one of those protein families that affects vesicle trafficking. As the master switch responsible for allowing the human brain to release neurotransmitters, Syt-1 senses $Ca^{2+}$ concentrations and subsequently signals the SNARE complex to open fusion pores[1]. Extensive research has been done to show the interaction between Syt-1 and SNAREs[2-5], but few studies shed light on how Syt-1 interact with the lipid membrane during fusion. In this study, we use a soluble version of Syt-1, C2AB, which lacks the transmembrane domain, to further study the mechanism of Syt-1's function. Our data shows that with the presence of $Ca^{2+}$, Syt-1 stimulates the SNARE-mediated membrane fusion largely and this stimulation effect requires PS on the vesicles. An interplay between α-Syn and Syt-1 during lipid mixing assay was also investigated and results show that α-Syn can inhibit Syt-1's stimulatory effect.**

## Introduction

Neuronal transmitter release is precisely controlled at the synapses to ensure an effective communication between neurons. SNAREs are the core machinery to mediate fusion between vesicles and the plasma membrane[6]. However, SNAREs alone could not have a regulatory function to switch the fusion event on/off temporarily[7-9]. It is widely believed that a protein family Syts contributes highly to the temporal control of synaptic vesicle excocytosis.

Syts is a family of proteins composed of a single transmembrane domain, a variable linker region, and two C2 domains[11] (the C2A and C2B domains). There are 15 members in the mammalian Syt family, among which Syt1 is the best characterized isoform. Syt1 is localized to synaptic vesicles, which functions as $Ca^{2+}$ sensor for fast exocytosis. The influx of $Ca^{2+}$ resulted from an action potential triggers membrane fusion for neurotransmitter release. The synaptic membrane fusion is mediated by vesicle-associated v-SNARE VAMP2 and its corresponding partners on the target vesicle syntaxin and SNAP-25.

SNAREs have been demonstrated to have an ability to act as a fusogen *in vitro*. The coiled coil motifs offered by v- and t- SNARE associate with each other and form a parallel four-stranded helix bundle. This bundle is highly stable and tight. It is proposed that the formation of this bundle can provide enough energy to pull two membranes into close proximity to drive a fusion process[10, 11]. But this SNAREs only mediated fusion has slow kinetics, which could not meet the demand of fast neurotransmitter release *in vivo*.

It has been shown that Syt 1 can interact with both SNAREs and the plasma membrane in a $Ca^{2+}$-dependent manner. Plasma membrane binding ability has been shown in former *in vivo* studies by introducing several different Syt 1 mutants[12-14]. Syt 1's interaction with the plasma membrane especially relies on the negatively charged lipids[15,16], such as phosphatidylserine (PS), phosphatidylinositol 4, 5-bisphophate (PIP2). In our system, PS is introduced to the lipid vesicles as an acidic lipid.

Syt-1 is a key regulator *in vitro* for SNARE-dependent synaptic vesicle fusion process. We use the cytosolic domain of Syt-1(C2AB) as an alternative model in the *in vitro* fusion assay. C2AB has been shown to be an effective model to study the function of Syt-1[17-19]. To further study α-Syn's effect on SNARE-mediated membrane fusion, we want to incorporate more regulators of

this event and better mimic the *in vivo* situation. Thus we combine Syt-1 and α-Syn in our system to investigate whether they have an interaction.

## Results And Discussion

For lipid mixing assay, t-SNARE is the binary complex formed by incubating syntaxin-1A and SNAP-25 together. v-SNARE is VAMP2. The fluorescence detection of lipid mixing is conducted by incorporating DiI and DiD (each of 2 mol%) into the t- and v-vesicles respectively. C2AB, $Ca^{2+}$ and α-Syn are added to the mixture for a final volume of 100ul when the assay starts.

## C2AB and Ca2+ can stimulate the SNARE-mediated lipid mixing

Syt-1 has a single membrane-spanning helix and two soluble tandem C2 domains. The transmembrane domain anchors Syt to the vesicle membrane. The two C2 domains, C2A and C2B, bind two and three $Ca^{2+}$ ions respectively[20.21]. In flux of $Ca^{2+}$, both C2 domains of Syt-1 partially penetrate into lipid bilayers that contain anionic phospholipids such as phosphatidylserine[22] (PS). C2AB is a recombinant variable form of Syt-1 lacking the transmembrane region. It has been effectively used as an alternative model of Syt-1 in former studies.

Both C2 domains are composed of a structure of eight-stranded anti-parallel β-sandwich. C2A and C2B domain can bind to three and two $Ca^{2+}$ respectively. Assembling the binary t-SNARE complex and v-SNARE VAMP2 to the corresponding t- and v- vesicles, we conduct the normal SNARE-mediated fusion assay. After adding 1uM C2AB only to the mixture, we could not see an enhancement of the acceptor signal. Besides of C2AB, if we also add $Ca^{2+}$, a very obvious enhancement of the acceptor signal can be observed. With the increase of $Ca^{2+}$ concentration (from 50uM to 100uM), the enhancement is at a higher level (Fig 1). All of this indicates that with the presence of $Ca^{2+}$, C2AB can stimulate the SNARE-mediated lipid mixing.

**The stimulatory role of C2AB and Ca2+ on lipid mixing requires PS on the lipid vesicles**

Syt-1 has shown an interaction with both membrane lipids and SNARE proteins in former studies. To determine how strong the composition of membrane lipids will affect Syt-1's function on lipid mixing, we carried out our lipid mixing assay utilizing different vesicles. Normal and neutral vesicles are also used here. Our data shows that without the 15mol% PS on the t- and v-vesicles, C2AB do not have a stimulatory function on the fusion process (Fig 2).

**α-Syn can inhibit C2AB's stimulatory effect on membrane fusion**

α-Syn and Syt-1 can both affect the kinetics of SNARE-mediated lipid mixing[25,26]. Syt-1 is an indispensable regulator for $Ca^{2+}$ triggered release *in vivo*. Upon the rise of the $Ca^{2+}$, Syt-1 displaces complexin, another key regulator for fusion process, from the SNARE complex and allows complete SNARE assembly to a fusion competent SNAREpin[27-29]. α-Syn, as an important protein relates to the inhibition of vesicle trafficking in many organisms, can interact with both lipid vesicles and SNARE proteins[30]. We are curious whether α-Syn can inhibit C2AB's promotion effect on lipid mixing. C2AB can greatly promote the FRET efficiency with $Ca^{2+}$. But in the presence of α-Syn, this promotion effect can be attenuated (Fig 3). With the increasing concentration of α-Syn, the attenuation effect is enhanced (Fig 4a). We also compared the relative initial rate of each lipid mixing assay. And we found out that with 50uM α-Syn, C2AB's promotion effect can be attenuated by 80% (Fig 4b). Therefore, our data shows that α-Syn can inhibit the promotion of C2AB and $Ca^{2+}$ on SNARE-mediated lipid mixing.

**Materials And Methods**

**Plasmid construction**

Neuronal SNAREs, VAMP2 (amino acids 1–116), syntaxin-1 A(amino acids 1–288) and SNAP-25 (amino acids 1–206) were all inserted into pGEX-KG (between EcoRI and XhoI sites) as

glutathione S-transferase (GST) fusion proteins. Four native cysteines of SNAP-25 were replaced with alanines.

## Protein expression and purification

Recombinant proteins were expressed in Escherichia coli Rosetta (DE3) pLysS (Novagen). GST fusion proteins were purified by affinity chromatography using glutathione-agarose beads (Sigma). The proteins were cleaved by bovine thrombin (CALBIOCHEM) in a cleavage buffer (50mM Tris–HCl, 150mM NaCl, pH 8.0). Syntaxin-1A and VAMP2 contained 1% n-octyl-D-glucopyranoside (OG). Purified proteins were examined with 13% SDS–PAGE, and the purity was at least 90% for all proteins.

To purify C2AB, cell pellet was resuspended in 10 mL PBS buffer (Phosphate-buffered saline, PH 7.4, with 0.5% (v/v) TritonX-100) with the final concentrations of 2 mM AEBSF, 1 mM EGTA, and 5 mM DTT. The cell was broken by sonication on the ice bath and centrifuged at 15 000 × g for 25 min at 4 °C. The supernatant was then mixed with glutathione-agrose beads in PBS buffer and nutated in cold room for 2 h. After nutation, the beads were washed for five times with a high salt buffer (50 mM Hepes, 1 M NaCl, PH 7.4), in which 1 mM MgCl2, DNase (20 μg/ml) and RNase (4 μg/ml) were added. The mixture was incubated for 6 h at 4 °C. After washing by high salt buffer twice and low salt buffer (50 mM Hepes, 0.1 M NaCl, PH 7.4) five times, the protein was cleaved from GST beads by thrombin in low salt buffer.

## Membrane reconstitution

The mixture of POPC (1-palmitoyl-2-dioleoyl-sn-glycero-3-phosphatidylcholine), DOPS (1,2-dioleoyl-sn-glycero-3-phosphatidylserine), Cholesterol and DiI (t-vesicles) or DiD (v-vesicles) (molar ratio of 43:15:40:2) in chloroform was dried in a vacuum and was resuspended in a buffer (25mM HEPES/KOH and 100mM KCl [pH 7.4]) to make the total lipid concentration of about 5mM. Protein-free large unilamellar vesicles (~100 nm in diameter) were prepared by extrusion through polycarbonate filters (Avanti Polar Lipids). For net neutral charge lipid mixing, 15mol% DOPS was replaced by equimolar quantity of POPC. syntaxin and SNAP-25 were mixed at room temperature at a molar ratio of 1:1.5 for 1 hour to allow the formation of binary t-SNARE complex. The binary t-SNARE and VAMP2 were then mixed respectively with t- and v-vesicles

at 4°C for 30 min with 0.8% g/ml OG, as the protein lipid ratio is 200:1. The liposome/protein mixture was diluted two times before dialyzed against 2 liters of dialysis buffer at 4°C overnight.

**Ensemble fluorescence lipid mixing assay**

To measure the lipid mixing, v-SNARE liposomes were mixed with t-SNAREs liposomes in the ratio of 1:1. The final set of the solution for each reaction contained about 1 mM lipids in Hepes buffer (25 mM Hepes, 100 mM KCl, PH 7.4) with a total volume of 100µl. Fluorescence intensity was monitored with the excitation and emission wavelengths of 570 and 670 nm, respectively. The fluorescence signal was recorded by a Varian Cary Eclipse model fluorescence spectrophotometer using a quartz cell of 100µl with a 2-mm path length. All lipid mixing assays was carried out at 35°C.

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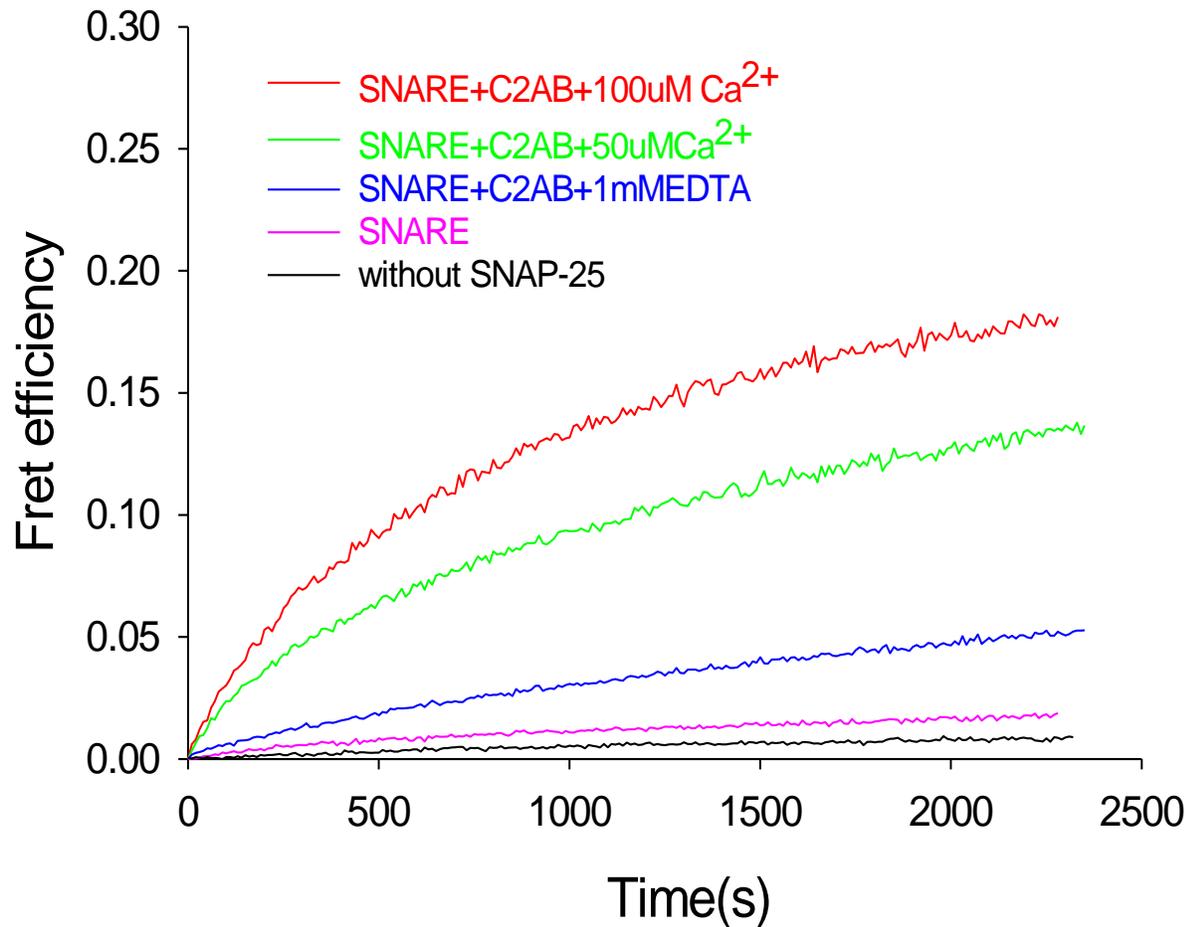
**Figures And Captions**



Fig 1. C2AB and Ca$^{2+}$ can stimulate the SNARE-mediated lipid mixing. 1uM C2AB was added to all the assays. With the increasing concentration of Ca$^{2+}$, from 0 (blue line) to 50uM (green line) to 100uM (red line), C2AB's shows higher stimulatory effect on the SNARE-mediated lipid mixing.
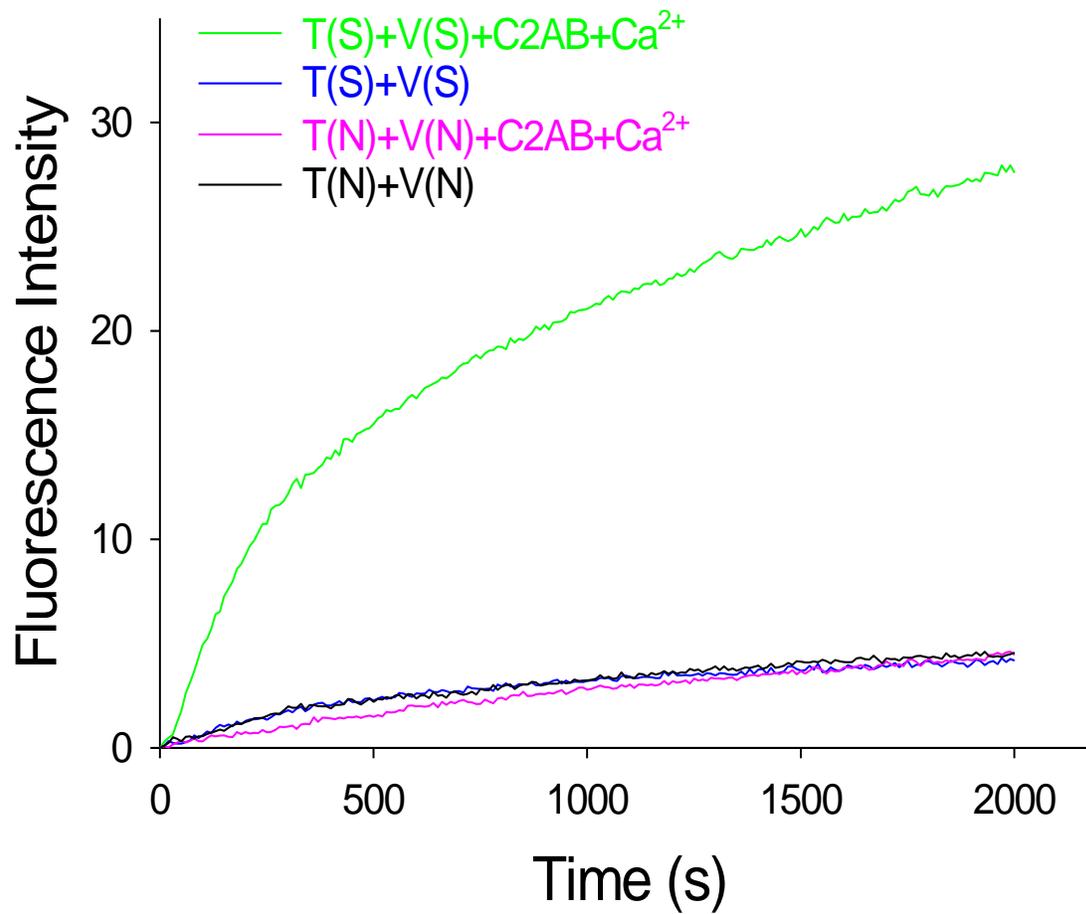
**Fig 2. C2AB and Ca$^{2+}$'s stimulatory effect on SNARE-mediated membrane fusion requires PS on the vesicles. Green and blue lines were assays with vesicles containing PS. Pink and black lines were assays with neutral vesicles. With the presence of PS, C2AB and Ca$^{2+}$ shows a much higher stimulatory effect on the lipid mixing.**
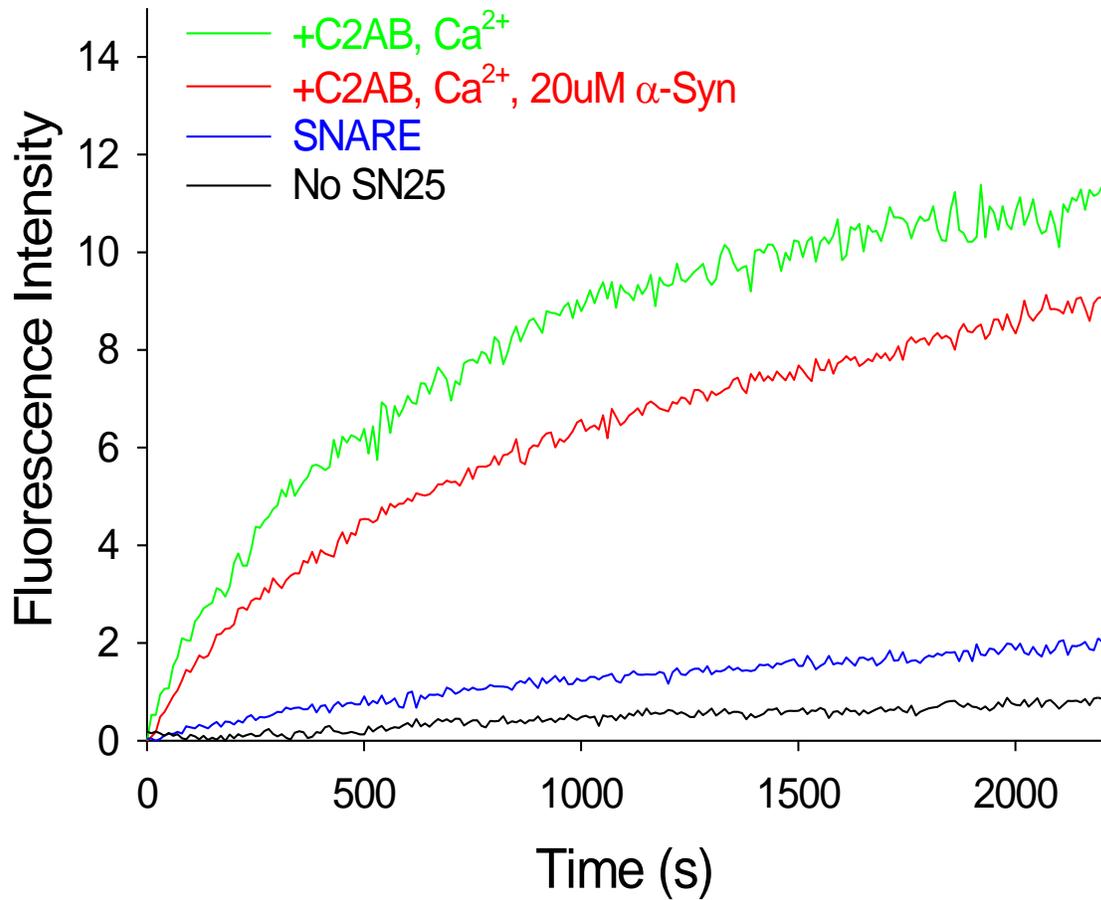
**Fig 3. α-Syn inhibits C2AB and Ca$^{2+}$'s promotion effect on SNARE-mediated lipid mixing. 1uM C2AB and 100uM Ca$^{2+}$ were added. Green line shows the stimulatory effect of C2AB and Ca$^{2+}$. The red line shows a decreased lipid mixing rate inhibited by α-Syn.**
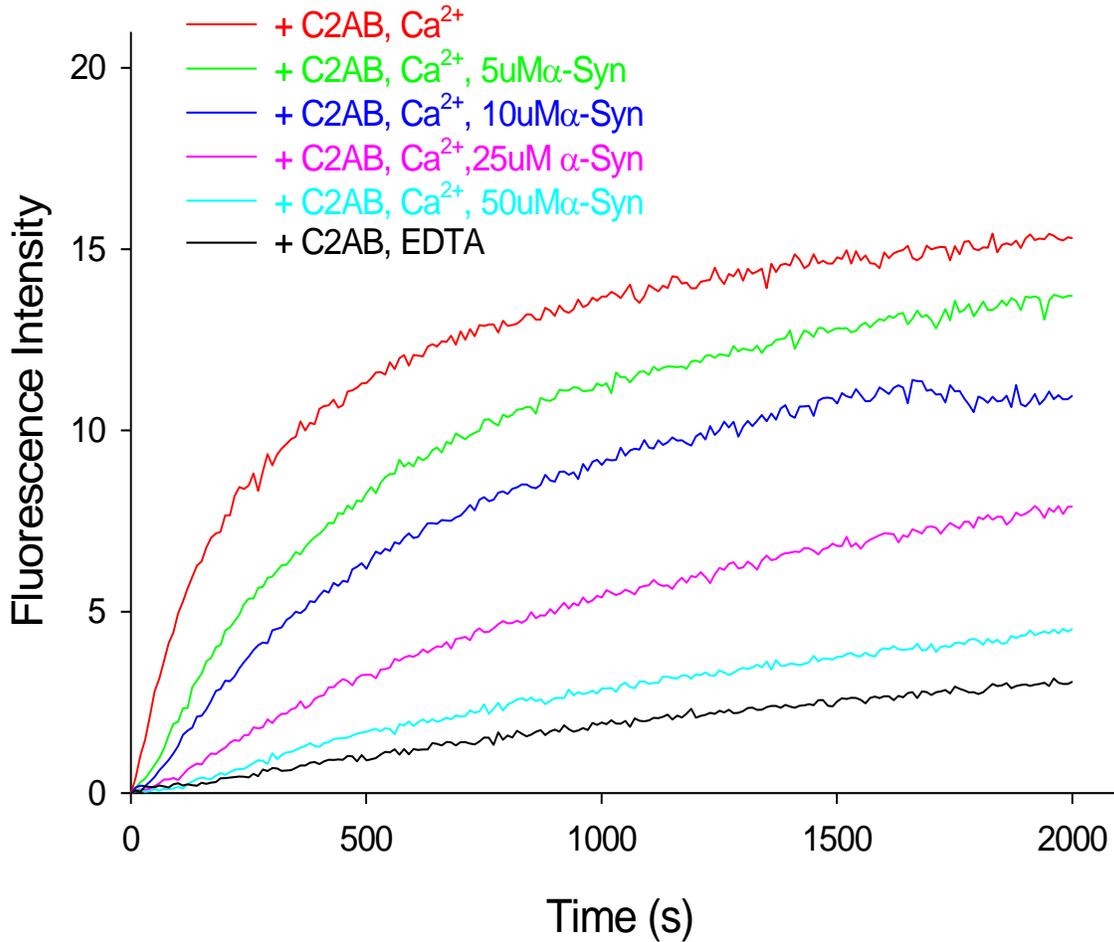
**Fig 4. (a) α-Syn inhibits C2AB and Ca$^{2+}$'s promotion effect on SNARE-mediated lipid mixing. The increase of fluorescence intensity reflects lipid mixing. The red line is lipid mixing with 1uM C2AB, 100uM Ca$^{2+}$. The green line is lipid mixing with 1uM C2AB, 100uM Ca$^{2+}$, and 5uM α-Syn, the blue line (10uM α-Syn), the pink line (25uM α-Syn), and the cyan line (50uM α-Syn). The black line is the control, which is lipid mixing with 1uM C2AB, and 1mM EDTA.**
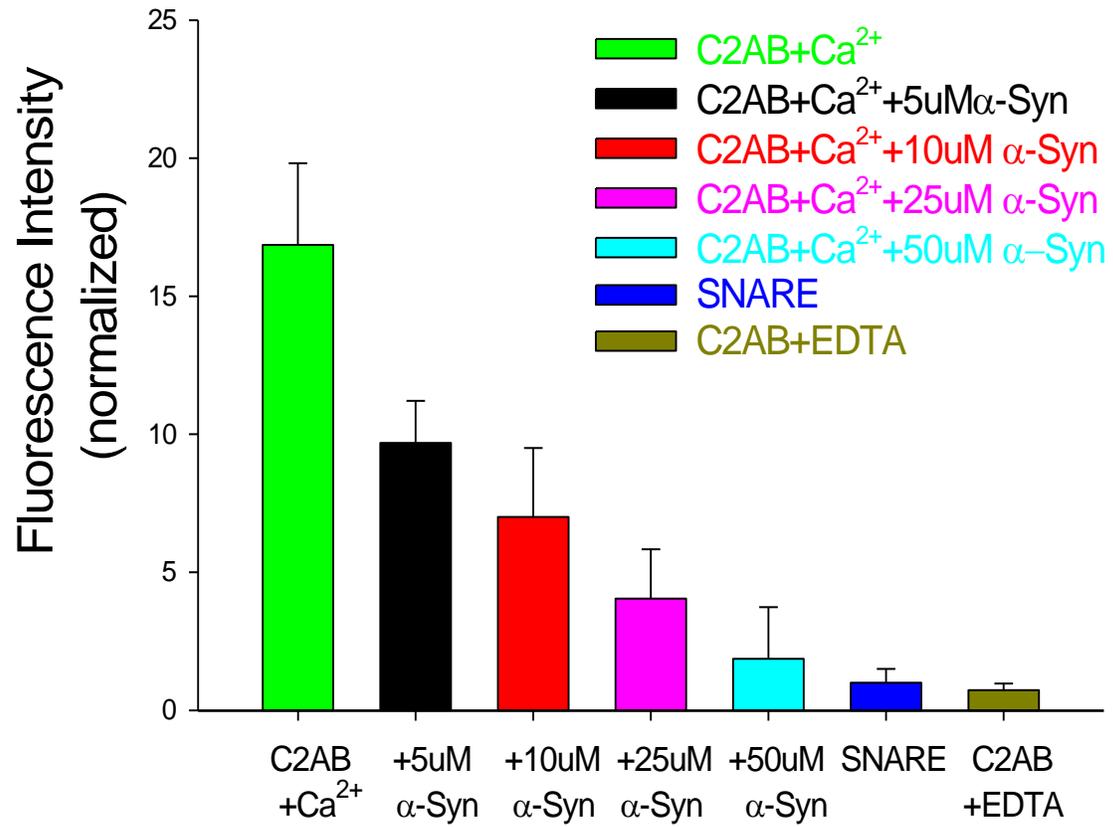
**(b) Normalized initial rates of the lipid mixing assays. Error bars were obtained from measurements of 3 independent assays.**

# CHAPTER 4: CONCLUDING REMARKS

**Conclusions**

In vitro reconstitution experiments has demonstrated that SNAREs are sufficient to mediate membrane fusion, in which t-vesicles harbor syntaxin-SNAP-25 binary complex and v-vesicles harbor synaptobrevin[1-3]. It is an effective system to recapitulate the function of cellular machines and directly ascertain the function of each component[4].

In recent years great progress has been made in the characterization of proteins involved in the process of neurotransmitter release, but the mechanism is still unclear. In mammals, $Ca^{2+}$ triggers exocytosis by binding to $Ca^{2+}$ sensor which mediates fast synchronous release[5-7]. Syt-1 is a sensor of this kind. Studies have suggested that Syt-1 interacts with $Ca^{2+}$ channels to mediate tethering of vesicles in close proximity to sites of $Ca^{2+}$ entry to enhance exocytosis[8]. Apart from the role as a $Ca^{2+}$ sensor, Syt-1 is presumably to modulate the synaptic vesicle docking step and fusion pore expansion dynamics, thus functions in the process of synaptic vesicle exocytosis itself [9,10].

α-Syn is a protein predominantly expressed in neuronal cells. Ueda et al. reported that a fragment of α-Syn is a major component of the amyloid plaques deposit in patients' brains with Alzheimer's disease[11]. In this dissertation, the kinetics of membrane fusion mediated by neuronal SNAREs syntaxin -1A, SNAP-25, and VAMP2. α-Syn and Syt-1's functions on the SNARE-mediated membrane fusion was studied by *in vitro* lipid mixing assay. PS on the vesicles plays an important role in their function. Without PS, both proteins' effect was decreased. α-Syn can inhibit SNARE-only mediated and C2AB and $Ca^{2+}$ stimulated lipid mixing assay, which might indicates that α-Syn might have several mechanisms to impact neuronal transmitter release *in vivo.*

Further aspects of protein interactions between α-Syn, Syt-1 and SNAREs might be something interesting to study. Characterization of whether and how these proteins interact with each other will help to build a bigger picture of SNARE-mediated fusion process and elucidate its regulatory mechanism. Single molecular techniques will be essential to achieve this goal.

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