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Extent of Cysteine Modification of SNAP-25 *In Vitro*

Alex McGregor DaBell

A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Master of Science

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ABSTRACT

Extent of Cysteine Modification of SNAP-25 *In Vitro*

Alex McGregor DaBell

Department of Physiology and Developmental Biology, BYU
Master of Science

Exocytosis, the fusion of a vesicle to a cellular membrane, involves a protein named SNAP-25. This protein, containing two alpha helices connected with a linker region, is localized to the cell membrane via palmitic acids attached to the cysteine residues of its linker region in a process called palmitoylation. Are cysteine residues of the SNAP-25 linker region palmitoylated in an ordered manner and to a particular extent? The answer to this question may give insight into the regulated nature of exocytosis. While it is generally accepted that SNAP-25 must be palmitoylated in order to perform its exocytotic functions, the details surrounding this process are still being discovered, defined, and understood.

In these studies we replicate the oxidation, reduction, and palmitoylation of SNAP-25 *in vitro*. Palmitoylating SNAP-25 *in vitro*, a process which occurs regularly *in vivo*, allows us to determine the extent of palmitoylation. *In vitro* palmitoylation of SNAP-25 was studied both with and without a native palmitoyl acyl transferase (PAT), DHHC-17, the enzyme to attach palmitic acids to cysteines in the linker region of SNAP-25. These studies were done under a variety of conditions designed to identify (1) components necessary for optimal palmitoylation and (2) extent of palmitoylation with components that mimic native conditions.

Palmitoylation is a common modification for a variety of proteins, both soluble and membrane-bound. Like phosphorylation, palmitoylation is reversible and may play an important role in regulation of cellular processes. Specifically, the palmitoylation of SNAP-25 may play a critical role in the regulation of exocytosis and therefore learning further details about this important process may help us to better understand a variety of neurodegenerative diseases and states of decreased or compromised exocytosis.

Keywords: palmitoylation, palmitoyl acyl transferase, SNAP-25, DHHC-17.

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Finally, to my parents who have always been there for me and have always helped me believe that I can do anything- thank you.

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CHAPTER 1: Review of Literature

Synaptic Fusion

Synaptic fusion (exocytosis of synaptic vesicles at nerve terminals) is required for nerve cells to communicate. Neurons communicate via neurosecretion and synaptic vesicle fusion is believed to be the primary process responsible for this event. Since the late 1980's, the conserved mechanism of regulated, as opposed to constitutive, exocytosis has been thoroughly investigated (1). Perhaps surprisingly, the membrane fusion dogma established over 20 years ago has remained consistent.

In order that synaptic fusion can be rapidly repeated and tightly regulated there must be (a) pre-docking of the synaptic vesicles at the active zone (site of membrane fusion), (b) a general low probability that any specific synaptic vesicle will exocytose in response to a Ca^{2+} trigger, and (c) synaptic vesicles are endocytosed rapidly following and close to the site of exocytosis. This synaptic vesicle cycle is understood to be completed in a minute or less (2), (3).

Early insights into synaptic fusion lead to the development of the SNARE hypothesis, wherein associating SNARE proteins “zipper” together to bring apposing membranes together allowing for synaptic fusion (4). This same process occurs for cellular organelles to move from one compartment to another (e.g., Golgi to endoplasmic reticulum) using variants of these same SNARE proteins. Though this description of membrane fusion may appear simplistic, elucidating the details of proteins involved, their actual physiological behavior, and the regulation of this critical process continues to occupy the attention of many researchers around the world.

Exocytosis and movement of intracellular products between cellular compartments is necessary for the cell to function properly and sustain growth, and is also required for extracellular communication. One of the fundamental principles of eukaryotic cells is that cellular organelles are compartmentalized by means of membranes. The challenge of this arrangement is that cellular components, and individual cells, cannot exist in isolation; rather, they must be able to transport macromolecules and other cellular products across membranes, without compromising the integrity of the membrane that encompasses them. The process of membrane fusion (e.g., exocytosis) overcomes this necessary challenge (5).

Although many proteins have been determined to participate in synaptic fusion, there are a specific few that satisfy the minimum requirement to model neurotransmission *in vitro*. SNARE proteins on the surface of both vesicular and target membranes have been shown to be the minimal requirements for membrane fusion (6, 7). Furthermore, the SNARE family of proteins has been deemed irreplaceable in most, if not all, intracellular membrane trafficking events, in addition to a central role in intercellular communication (8).

SNARE Proteins

The key protein components of regulated exocytosis have proven to be highly conserved throughout evolution. Among such components are SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptor proteins). The SNARE proteins are divided in two general classifications: v- (vesicular) and t- (target membrane) SNAREs (9).

The first SNARE proteins were identified, although their function was not understood, using *in vitro* trafficking assays developed in the early 1980s. SNAP (Synaptosomal-associated protein) proteins were used to affinity-purify their membrane receptors (a complex of SNAP receptor proteins) from brain tissue. A separate classification system based on SNARE structure

also exists, designating those proteins with a key arginine residue as R-SNAREs and those with a key glutamine residue as Q-SNAREs (10). Syntaxin (t-SNARE or Q-SNARE) and synaptobrevin (v-SNARE or R-SNARE) are two SNARE proteins that perform key roles in the formation of the SNARE complex and are required for regulated exocytosis.

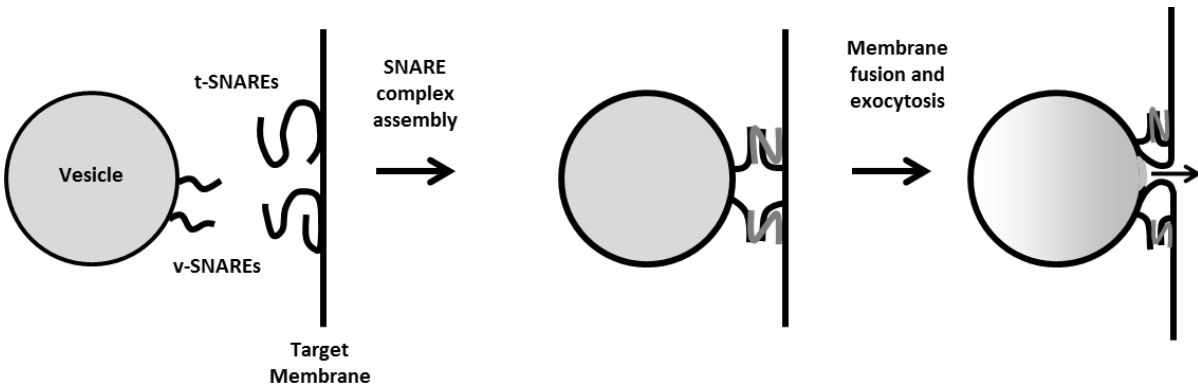


Figure 1.1: SNARE Fusion Cartoon.

SNARE proteins are characterized by the presence of a SNARE motif (11). These cytoplasmic, amphipathic, alpha-helical motifs are required for the formation of the coiled-coil SNARE complex that drives membrane fusion (12, 13). The neuronal SNARE complex is composed of four motifs from three proteins. SNAP-25 is unique when compared to the other SNARE proteins, synaptobrevin and syntaxin, in two important ways: SNAP-25 has two, not one, SNARE motifs and it lacks the transmembrane domain found in the other SNAREs (10).

SNAP-25

SNAP-25 plays an important role in the formation of the SNARE complex and synaptic fusion. SNAP-25 and syntaxin (both t-SNAREs) form a receptor complex to which synaptobrevin binds. This general theme is also seen in yeast where the homolog of SNAP-25

and the yeast protein Sec9p (which is required for fusion of secretory vesicles from the Golgi with the plasma membrane) performs a synonymous function (1).

Alternative RNA splicing gives rise to two isoforms of the SNAP-25 SNARE protein: SNAP-25a and SNAP-25b. With the exception of nine amino acids (of 207), these isoforms share identical sequences (9). Interestingly, although both isoforms have a cysteine-rich region containing four cysteines, one of the cysteines moves to a different position of the linker region (4 amino acids away) between the two isoforms. The expression of these splice variants is regulated developmentally. SNAP-25a is expressed early in the development of neurons and SNAP-25b is expressed later during synaptogenesis and functions in synaptic plasticity and axon growth, and in mature neurons (14). Additionally, SNAP-23, which shares much sequence homology with SNAP-25, also differs in positioning and number of cysteines in the linker region: SNAP-23 contains five cysteines, three at the common locations found in both SNAP-25s and two more at the two individual cysteine locations. These differences in expression of SNAP-25 isoforms make it reasonable to infer that the differences in cysteine location are physiologically significant.

As mentioned above, SNAP-25 differs from other SNAREs in that it is anchored to the plasma membrane differently from the other SNAREs. Instead of possessing a trans-membrane domain, SNAP-25 is anchored to the cell membrane via the fatty acid, palmitic acid, attached to cysteines in the middle of the protein. In addition, SNAP-25 alone contributes two alpha helices to the SNARE complex while other contributors, syntaxin and synaptobrevin, contribute only one each (14, 15).

Palmitoylation and Palmitoyl Acyl Transferases

Palmitoyl acyl transferase (PAT) enzymes attach a palmitic acid group to the cysteine residue of proteins. Specifically, for SNAP-25, they attach palmitic acid to the cysteines of the linker region (16). We show that this process does in fact occur in a spontaneous manner with relatively high doses of Palmitoyl-CoA ($EC_{50} \sim 30\mu\text{M}$). At this dose, palmitoylation is achieved in both the presence and absence of a PAT. *In vivo*, this process is reversible; palmitic acid is attached by a PAT while it is detached by a palmitoyl protein thioesterase (PPT), as depicted (Figure 1.2).

DHHC-17 is a membrane-bound protein with active sites on the cytosolic side of the cell (17). Therefore, we needed to use detergent to disrupt the cell membrane in order to isolate the protein and detect it with an antibody. However, since DHHC-17 is membrane-bound, disrupting the cell membrane could also disrupt the structure and functionality of this protein. Since we isolated a membrane-bound protein, it is possible that our experimental design to disrupt the cell membrane and expose the DHHC-17 may have actually “hid” the active site of the enzyme (even though we positively detected the epitope using the DHHC-17 antibody, as shown below). Detecting DHHC-17 in multiple fractions could indicate a bimodal distribution of this PAT throughout the cell. This too would be an interesting discovery.

Bovine Adrenal Medulla

Since Grant et al. described the differential expression of SNAP-25 in the bovine adrenal gland (18) I reasoned that I should be able to locate associated palmitoyl acyl transferase enzymes in the bovine adrenal gland as well. Fukata et al. (19) and others have established that SNAP-25 requires palmitoylation for full functionality, and this modification is accomplished by PAT's. Hence, where native SNAP-25 is, we should also be able to find native PAT's. In

particular, Fukata et al. showed that DHHC-17 is associated with palmitoylation of SNAP-25. Therefore, we used Western blotting to definitively determine the presence of DHHC-17 in the bovine adrenal gland.

Kotani et al. (20) demonstrated a method for partially purifying the medulla from the bovine adrenal gland. Major steps from his studies were implemented in the resection, homogenization, and centrifugation to achieve partial purification of DHHC-17 from the bovine medulla. Western blotting results and associated enzyme-containing study results are included in greater detail in chapter 2 and 3.

Significance of Study

It is generally accepted that palmitoylation of SNAP-25 is critical because it helps localize SNAP-25 to the cell membrane. Since SNAP-25 lacks a transmembrane domain, palmitoylation is required to anchor this otherwise soluble protein (21, 22). Studies have shown that palmitoylation of SNAP-25 changes its location within the cell (23, 24). It is conceivable that palmitoylation of SNAP-25 could also aid in transmission of tension produced when SNARE proteins form the coiled-coil complex and drive fusion of vesicle and cell membranes. Without the more rigid localization afforded by palmitoylation, the coiling of SNARE proteins may not be sufficient to overcome the repulsion of opposing vesicle and cell membranes.

It has also been demonstrated using molecular modeling simulations that oxidation of SNAP-25b at the same cysteine residues hinders SNARE complex formation and therefore, palmitoylation of SNAP-25b can have a negative-inhibitory effect (25).

In addition, palmitoylation may serve as a type of regulatory mechanism, much like phosphorylation (26). Since palmitoylation of SNAP-25 is required to drive membrane fusion (27), the palmitoylated or non-palmitoylated state of SNAP-25 could potentially serve as an

on/off switch for regulated exocytosis. Additionally, if all four cysteines can be palmitoylated, then this could increase the fidelity of such an “on/off” switch. The dynamic state of protein palmitoylation is made possible by the intracellular presence of both PATs and PPTs – PPT’s being responsible for removal of palmitic acid from the sulfurs of cysteine residues by cleaving the thioester bond (Figure 1.2).

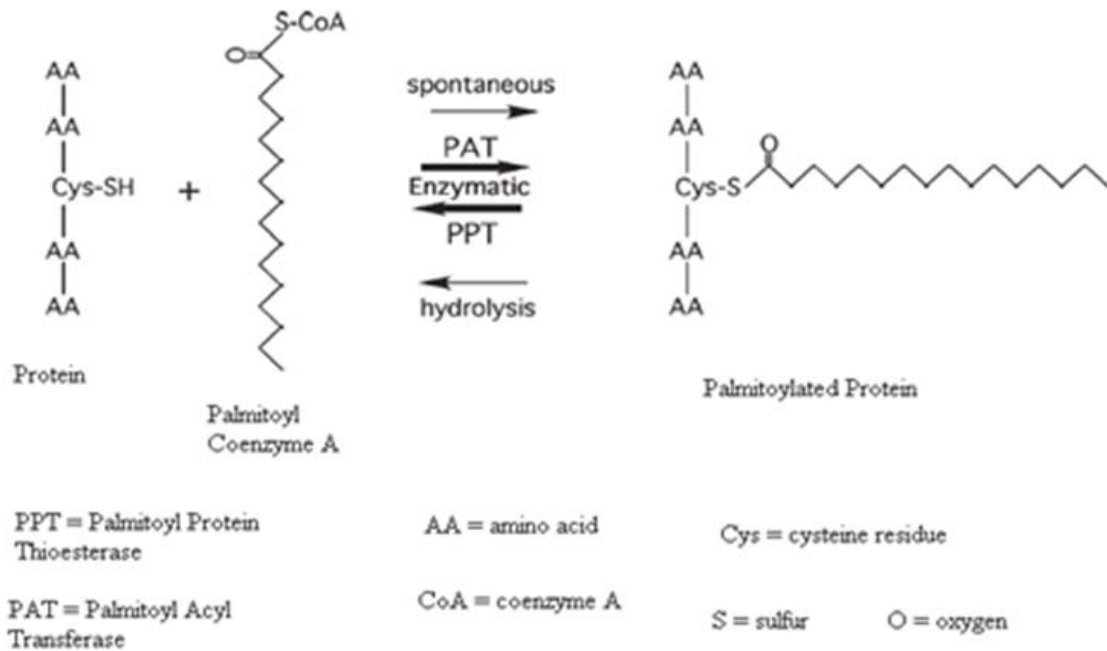


Figure 1.2: Palmitoylation Reaction and Regulation.

Others have shown that palmitoylation of SNAP-25 occurs *in vivo* in an enzyme-catalyzed fashion (22), but to an unknown extent. Enzyme catalysis allows for lower substrate concentrations, which is a significant advantage when considering such things as cellular resource allocation, enzyme kinetics, substrate concentration thresholds, and substrate-enzyme proximity. As mentioned above, PATs are the enzymes responsible for palmitoylation.

There is a large family of predicted PATs. Among these, 23 DHHC proteins have been found in mammalian genomes that have confirmed PAT activity (17). DHHC refers to the conserved amino acid sequence (aspartic acid – histidine – histidine – cysteine) of a particular

class of PATs. Others have shown that DHHC 3, 7, and 17 palmitoylate SNAP-25 at its cysteine residues (17).

While it has been established that SNAP-25 is palmitoylated *in vivo*, the extent of palmitoylation has heretofore remained unknown. Is just one cysteine or all four palmitoylated? Does the palmitoylation (and de-palmitoylation) of a variable number of cysteines add fine regulation to the functioning of this SNARE protein (other SNARE proteins have also been shown to be palmitoylated, but at only 1-2 sites)? As shown below, we have investigated the extent of palmitoylation, both spontaneous and enzymatically-catalyzed, to learn whether or not any of the cysteine residues of SNAP-25's linker region are preferentially palmitoylated. As previously mentioned, the bovine adrenal gland is a readily available tissue containing PAT's. The adrenal medulla is an endocrine organ that secretes a number of hormones and neurotransmitters in a highly regulated manner. Removing the bovine adrenal gland and isolating it from other tissues is a relatively simple process. It has been shown that SNAP-25 is abundant in the bovine adrenal medulla (but not the adrenal cortex) (18). Therefore, it was expected that the required PAT enzymes would also be located in the adrenal medulla. We obtained an antibody against the PAT DHHC-17. We used it to confirm the presence of the enzyme in bovine adrenal tissue and to aid in its partial purification. Since PATs are membrane-bound proteins, it is assumed that complete purification away from membranes would render the enzyme inactive. For the purpose of this study, only a functional native PAT was required; thus, we only needed to purify the PAT sufficiently to separate it from any confounding components (e.g., substrates, enzymes, and regulators of the reaction).

I acquired adrenal glands from a local abattoir and dissected the adrenal medulla from the adrenal cortex (as trained by Dr. Allan Judd, BYU). The dissected tissue was homogenized and

re-suspended in buffer as described (20). Differential centrifugation was used to partially purify and isolate the membrane-bound PAT enzymes from the other cellular components. I blotted different fractions from the centrifugation steps on PVDF membranes and conducted a Western Blot with the anti-DHHC-17 antibody (specific to a PAT known to modify SNAP-25) to determine which fraction(s) most richly contained PATs.

Specifically, three centrifugation steps were performed to produce four fractions (P1, P2, P3, and S3) expected to contain plasma membranes, large cellular organelles (e.g. nuclei), small organelles (e.g. recycling endosomes) and soluble proteins, respectively (Figure 1.3). The first centrifugation step consisted of spinning the homogenized adrenal medulla tissue at 1,000 g for 5 minutes at 4°C to yield S1 and P1. The second centrifugation step involved spinning the supernatant (S1) at 40,000 g for 30 minutes at 4°C to yield S2 and P2. The third and final centrifugation step yielded S3 and P3 by spinning the S2 supernatant at 150,000 g for 60 minutes at 4°C.

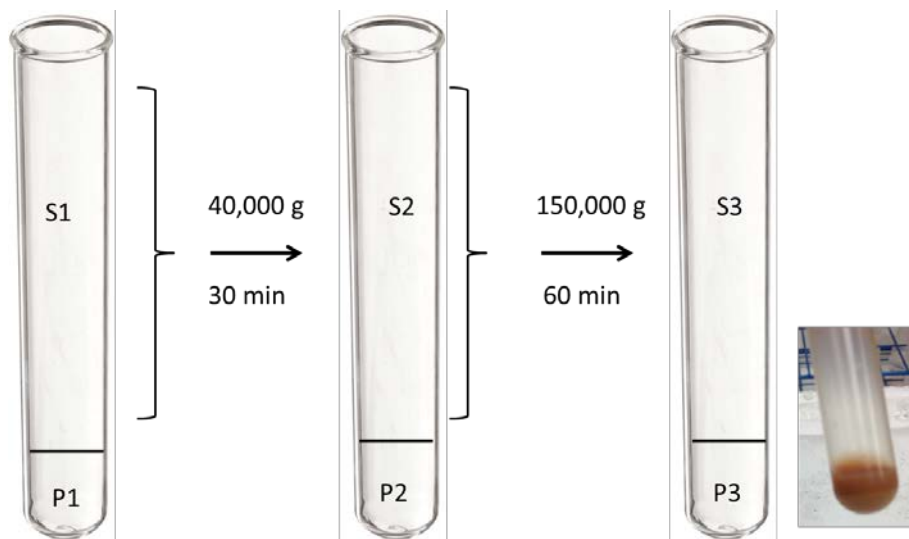


Figure 1.3: Tissue Fractionation. As described in the text, bovine adrenal medulla tissue was homogenized and separated into 6 fractions by differential centrifugation. Three pellet fractions, P1-P3, and three supernatant fractions, S1-S3, were obtained and tested for the PAT, DHHC-17.

PATs were expected to fractionate with P3, recycling endosomes (28), and the procedure to isolate endosomes was as described (20, 29). As shown below, DHHC-17 was detected in several of the fractions, but mostly in S3 and P3. The detection in multiple fractions was likely due to the partial centrifugation and extraction techniques not being able to completely separate the PAT from other cellular components.

Objectives

In vivo studies have shown which particular PATs specifically recognize and palmitoylate SNAP-25 (17, 22, 30). Similarly, I studied the ability of DHHC-17 to recognize and palmitoylate SNAP-25 *in vitro*. Enzyme-catalyzed palmitoylation studies included: the minimum Palmitoyl-CoA concentration, the time and temperature required, and the reaction buffers and pH levels that allowed enzyme-catalyzed palmitoylation of SNAP-25 to occur optimally *in vitro*.

Another objective of my studies was to show to what extent (i.e. how many) of the four cysteines of SNAP-25 were palmitoylated *in vitro*. The biotinylation chemiluminescence assay allowed us to determine how many of the four cysteine residues of each SNAP-25 had been palmitoylated. Using different time, temperature, and concentration levels for these enzyme-catalyzed *in vitro* palmitoylation experiments allowed us to determine which parameters allow for maximal palmitoylation and which are the most limiting.

We incubated SNAP-25, Palmitoyl-CoA, and DHHC-17-containing fractions together in a reaction chamber and determined the extent of palmitoylation of SNAP-25's cysteine residues. Protein palmitoylation with a lower concentration of palmitoyl-CoA, than would be required for *in vitro* palmitoylation, would be positive proof of enzymatic activity in the fraction tested.

Studying the palmitoylation of SNAP-25 involved controlling a variety of physiological conditions including temperature, pH, and palmitoyl coenzyme A (PCoA) concentrations.

Physiological conditions mimicked were 37°C, pH 7.4, and a variety of PCoA concentrations (0.01 – 1.0 mM).

As described in chapter 3, the initial protocol for modification of SNAP-25 did not allow us to clearly distinguish between oxidized or palmitoylated cysteine residues. Therefore, to ensure that the results obtained substantiated our claims of in vitro palmitoylation, we ran careful controls and used reducing agents to prevent oxidation of SNAP-25, thereby decreasing the likelihood of obtaining a false positive.

CHAPTER 2: Materials and Methods

Many of the methods used in my research were established previously in my lab (see Figure 2.1). However, they were adapted for my particular studies. Adaptations include changes to treatment temperature, treatment time, reducing agents, oxidizing agents, palmitoyl Co Enzyme A (PCoA) substrate concentrations, and palmitoyl acyl transferase enzymes. Further details of the nano assay are given in (31).

The isolation and partial purification steps were primarily novel to my studies (see Figure 3). Gross resection of the adrenal gland and separation of the medulla from the cortex were techniques shared from Dr. Allan Judd. The recommended centrifugation steps and homogenization buffer were from (20).

Western Blotting

Partially-purified fractions containing bovine medulla tissue were transferred to polyvinyl difluoride (PVDF) membranes and developed using chemiluminescence West-Femto procedure (Pierce, Rockford, IL). The antibodies were obtained from Abcam (Cambridge, MA). The images of the PVDF membranes containing the DHHC-17 enzyme were captured with the FluorChem 8900 system (Alpha Innotech, San Leandro, CA).

Materials used in the Western blotting included: Non-Fat Dry Milk in TBS with 0.05% tween-20, Primary antibody, Secondary antibody, and Femto reagent. All reagents were obtained through Thermo Fisher Scientific (Hanover Park, IL), unless otherwise noted.

Antibody

Polyclonal anti-ZDHHC-17 antibody from Rabbit designed to react with the DHHC-17 palmitoyl acyl transferase from human, mouse, rat, chicken, cow, dog, and zebra fish and were ordered from Abcam (ab55882)

Reduction of the Cysteines of SNAP-25

Reduction of SNAP-25 was accomplished using the following reducing agents, at concentrations described: L-Cysteine, N-Acetyl Cysteine, Cysteine HCl, Cystamine, and TCEP.

Oxidation of the Cysteines of SNAP-25

Oxidation of SNAP-25's cysteine residues was accomplished by diluting from a stock 200 mM CuCl₂ in solution. Oxidized SNAP-25 appeared in the data sets as a reduced signal or reduced chemiluminescence in the assay.

Palmitoylation of the Cysteines of SNAP-25

Palmitoyl CoA Solutions

Palmitoyl CoA solutions were diluted from a 3 mg/mL stock solution using double-deionized water as diluent. The stock was near the saturation point.

PT7 Buffer Stock

The PT7 buffer stock solution was 150mM NaCl, 50 mM phosphate buffer pH 7.0, 5 mM Tris, and 1.5% Triton X-100 as described in (31).

Nano-Assay Protocol

As depicted in Figure 2.1 and described in (31), the nano assay protocol included Biotin-PEO11 stock solution, STV-HRP, and Pico/Femto developing solution.

SNARE Synthesis and Purification

SNARE proteins were synthesized and purified using the methods found in (31), which was modified from (6).

Palmitoyl Acyl Transferase Isolation

We confirmed the presence of the palmitoyl acyl transferase DHHC-17 in bovine adrenal medulla tissue through a series of centrifugation steps and western blotting to verify enzyme presence. The Western Blotting was done using materials described above and according to the following outline:

1. Harvest adrenal gland from abattoir
2. Dissect medulla from cortex and cut into small pieces (see Figure 2.2)
3. Homogenize in H-buffer solution
4. Centrifugation steps (varied from published protocols, see Figure 1.3)
5. Anti-body – Western blot

Homogenization Buffer (H-Buffer)

H Buffer Contains:	Molar Mass (grams / mole)	To make 7.0 mL (7,000 μ L), for 3.5 g tissue
0.1 M Pipes pH 6.8 (pwd)	302.37	0.2117 g
0.9 M glycerol (liq)	92.09	0.5802 g
2 mM EGTA (pwd)	380.35	5.3249 mg
1 mM MgCl ₂ (pwd)	95.211 (anhyd)	0.6665 mg
1 mM AEBSF (pwd)	239.69	1.6778 mg

*Transfer medulla to 0.5 vol of a solution of H-Buffer

*Mix into ddH₂O to make 7.0 mL total

Chemiluminescence Biotinylation Assay

The protocol for the chemiluminescence biotinylation assay which was implemented for these studies was developed in my lab and is a procedure with which I have become proficient (31). I also have several undergraduate students who I have helped to train and who helped in running these experiments.

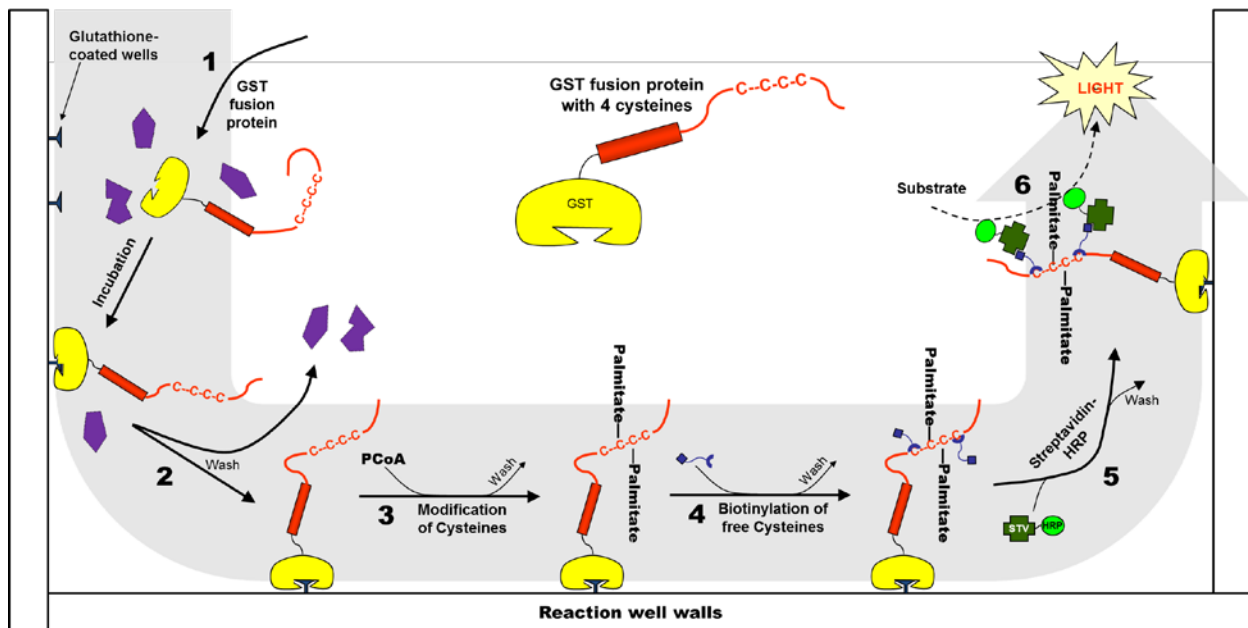


Figure 2.1: Protocol Cartoon. Figure adapted from (31). 1. Incubation of the SNARE protein to the well. 2. Reduction of the cysteine residues. 3. Treatment step. 4. Post-treatment reduction and biotinylation of cysteine residues. 5. Streptavidin HRP incubation with the biotin-linked maleimide. 6. Development of the HRP with substrate.

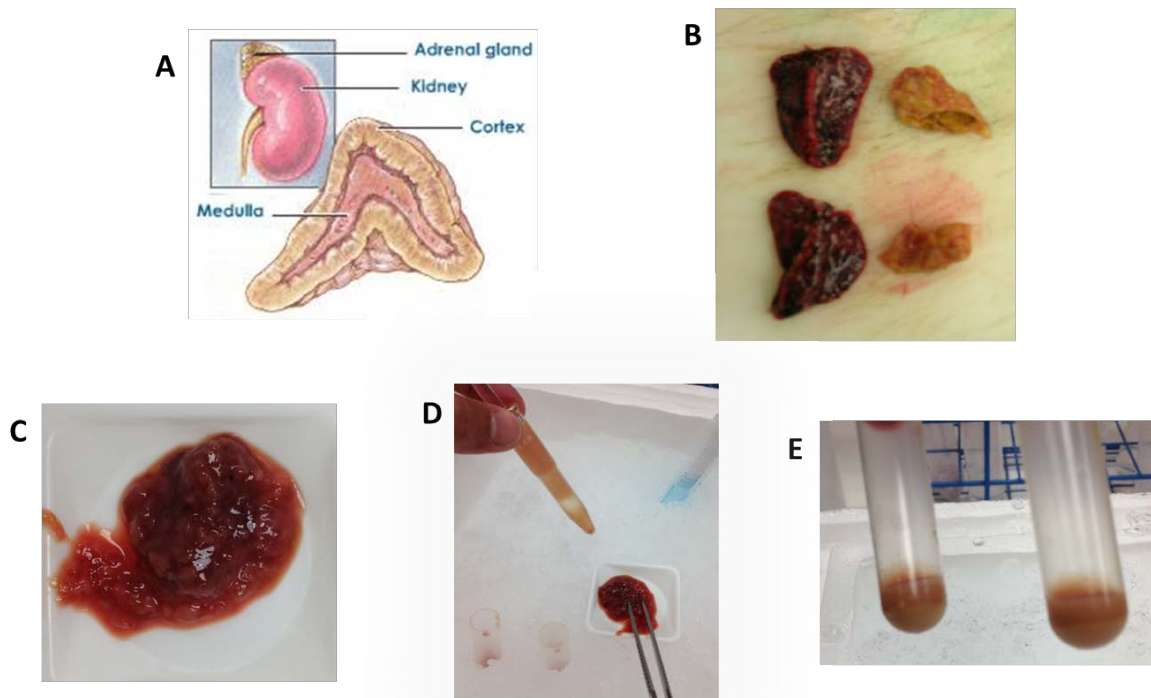


Figure 2.2: Bovine Adrenal Gland. Adrenal gland partial purification. (A) Cartoon of adrenal gland cortex and medulla (B) Bovine adrenal gland, prior to dissection (C) Bovine adrenal medulla cut into fine pieces (D) Bovine homogenization (E) S3 and P3 after partial purification

CHAPTER 3: Discussion, Major Conclusions, and Future Directions

Discussion

In this chapter I will present data describing the extent of modification of the cysteines of SNAP-25. The outline of each major conclusion will begin with discussion, followed next by the data, and concluding with a summary and possible future direction for the lab's studies. As discussed in chapter 1, palmitoylation is important in cells. For example, Koticha et al. have shown that palmitoylation is significant and necessary, and that depalmitoylation results in a cyclical on/off mechanism capable of regulation. Despite its importance in the exocytosis process, little data exists to describe the extent of palmitoylation in proteins that control this process. We chose to work on addressing this question in an *in vitro* system. Though measuring spontaneous palmitoylation *in vitro* is relatively easy, it is much more difficult to experiment with native enzymes to measure enzymatically-catalyzed palmitoylation *in vitro*. We demonstrate the ability to measure SNAP-25 palmitoylation, a key protein in exocytosis.

Though palmitoylation is important *in vivo*, it has been difficult to quantitate the extent of palmitoylation. Some of the reasons for these difficulties include: minimal access to intracellular environment where SNAP-25 modification occurs, shortage of quantification methods to determine the extent of palmitoylation, and disruption of intracellular processes from experimental intervention.

Determining the physiological reasons regarding the extent of modification, and the mechanisms regulating these modifications, has proven to be much more difficult to define than simply proving whether or not SNAP-25 modification is necessary.

Dose, Time, and Temperature Dependence of Palmitoylation

In order to study enzymatic palmitoylation, we first wanted to look at spontaneous palmitoylation. We found that incubating SNAP-25 for 1 hour in the presence of high concentrations of palmitoyl Co-Enzyme A (PCoA) resulted in spontaneous palmitoylation, as shown in Figure 3.1. The response to palmitoyl CoA was dose dependent (we didn't go higher than 0.5 mM because of solubility constraints). Surprisingly, just 0.15% of the detergent Triton X-100 disrupted spontaneous palmitoylation. This was unexpected since detergent should help keep the amphipathic molecule PCoA fully soluble in solution and enhance delivery of single molecules to the protein. Complete block of spontaneous palmitoylation by detergent suggests that the detergent blocks or weakens palmitoyl CoA's interaction with the protein.

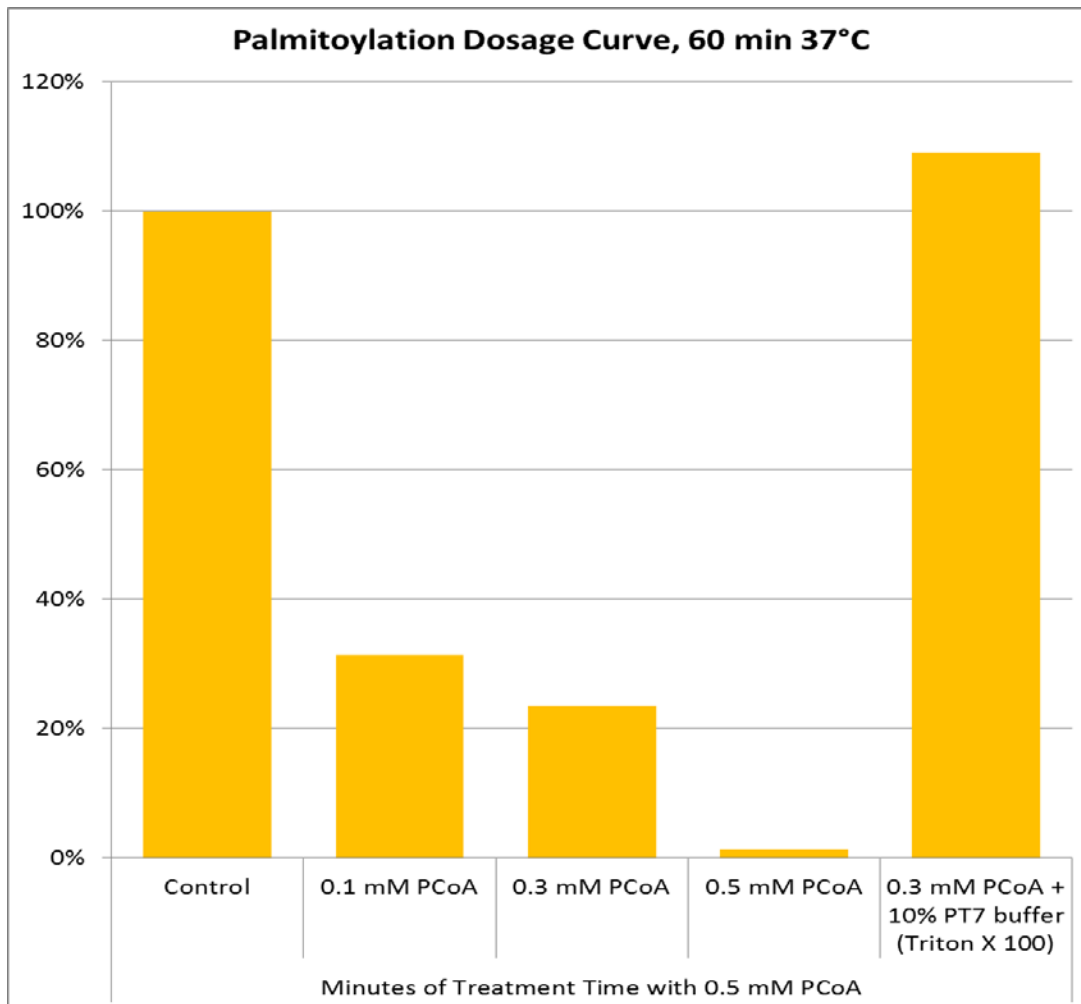


Figure 3.1: Palmitoylation Dosage Curve for SNAP-25. These data demonstrate that increasing dosages of palmitoyl CoA decrease free sulfhydryl (reduced cysteines) indicating more extensive palmitoylation. Note that detergent completely interferes with palmitoylation.

My studies have shown that the extent of spontaneous palmitoylation is not surprisingly both time and temperature dependent. Whereas some reactions occur within milliseconds, others take much longer, minutes or even hours, to occur. With such a wide variation in time requirements for various biological processes, I set out to determine the time and temperature requirements for both spontaneous and enzyme-catalyzed palmitoylation of SNAP-25.

Figure 3.2 shows time courses for spontaneous palmitoylation of SNAP-25 at 37° C. These data demonstrate that half of SNAP-25 is palmitoylated within the first 10 minutes. However, continued incubation (60 min) leads to near maximal palmitoylation.

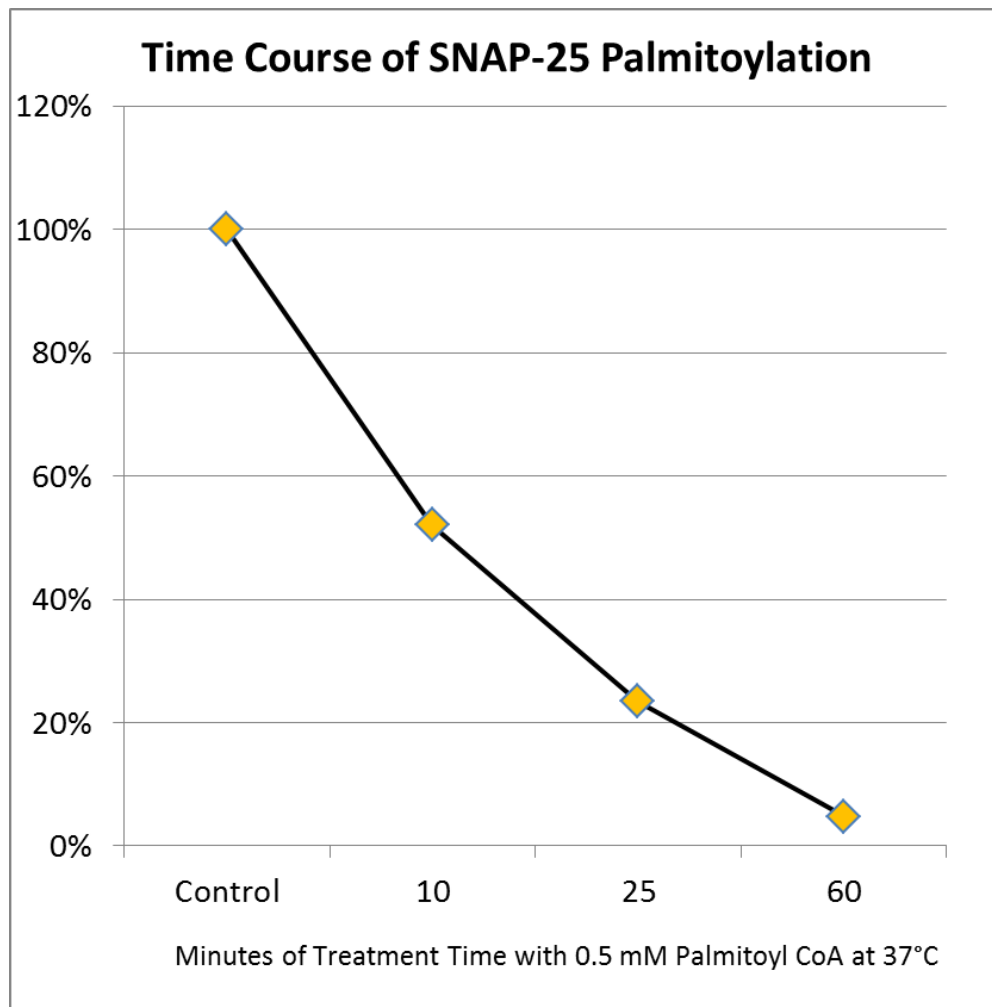


Figure 3.2: Spontaneous Palmitoylation Time Course. One hour is needed to achieve maximal palmitoylation. Proteins were treated at 37°C with 0.5 mM palmitoyl CoA for 10, 25, and 60 minutes. Following treatment, the proteins were reduced using 10 mM L-cysteine for 10 minutes (see Methods).

In the above two data sets (Figure 3.1 and Figure 3.2) the incubation temperature for palmitoylation was 37°C. *In vitro* palmitoylation was also tested at 22°C and 4°C. As expected, at these lower temperatures palmitoylation was much slower (data not shown).

Figure 3.2 shows a palmitoylation level that suggests near-maximal palmitoylation after 60 minutes. However, these early data were obtained using methods which did not control for concurrent oxidation during the palmitoylation step (although we had determined that oxidation occurred on a slower time scale). Since palmitoylation and oxidation both result in decreased signal, the above data do not clearly distinguish between palmitoylation and oxidation. Ideally, we needed to block oxidation without interfering with palmitoylation. This was not possible using most reducing reagents since they all contained a sulfhydryl and could be palmitoylated themselves. Through a careful literature search we identified a possible exception. TCEP (tris (2-carboxyethyl) phosphine) is a reducing agent that contains no sulfhydryls. Subsequent palmitoylation studies were performed in the presence of TCEP, which effectively kept non-palmitoylated cysteine residues in a reduced state so as to not confound the palmitoylation results (see Figure 3.3 and Figure 3.11 below). TCEP did not block palmitoylation.

Using these initial data on time and temperature (1 hour at 37°C) for spontaneous palmitoylation, we did a careful statistical analysis of palmitoylation as a function of palmitoyl CoA concentration.

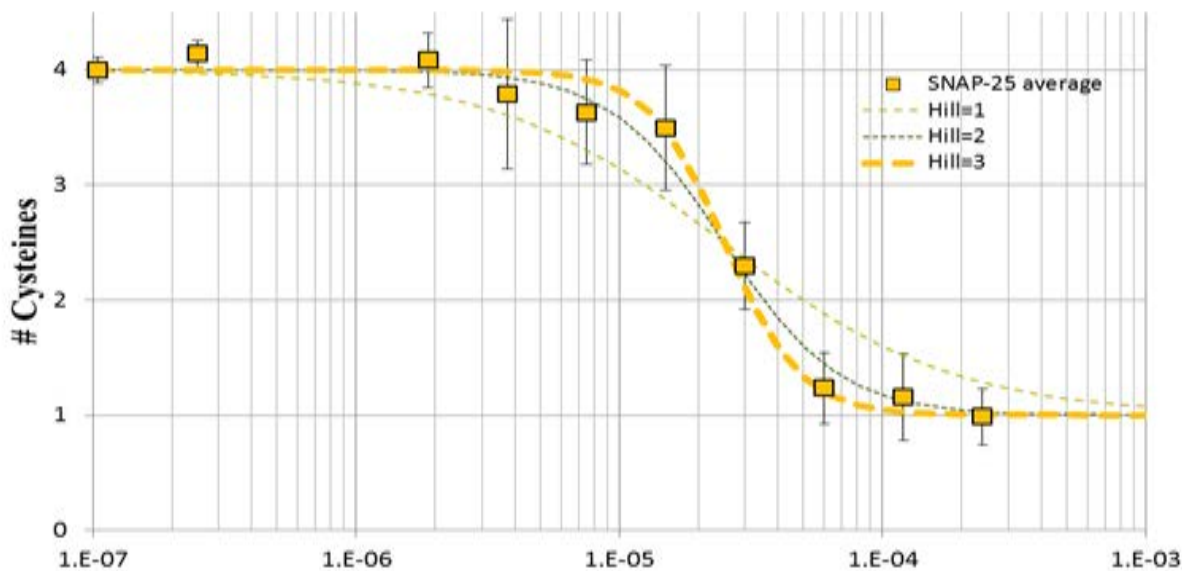


Figure 3.3: SNAP-25 Spontaneous Palmitoylation in the Presence of a Reducing Agent (TCEP). Error bars are 95% confidence intervals. The heavy-dashed line shows a binding curve with an EC_{50} of 25 μM and a Hill Coefficient of 3. The thinner dashed lines are for smaller Hill Coefficient's as noted.

Figure 3.3 shows a full dosage curve for palmitoylation of the cysteines of SNAP-25. Of interest is the fact the palmitoylation reaction seems to be nearly complete by 60 μM and that even at 4x high concentrations (250 μM) of PCoA, palmitoylation is still only 75% complete. This suggests that even with cooperative binding, there is an apparent maximal palmitoylation of three of four available cysteines of the SNAP-25 linker region. Such a restriction may be due to steric hindrance of three closely-spaced palmitoylated cysteines blocking access of PCoA to the fourth cysteine. This observation is in contrast to my initial results shown in Figure 3.1 and Figure 3.2. In early experiments such as Figure 3.2, we recognized that oxidation of cysteines would confound our results and were very careful to minimize oxidation by using deoxygenated buffers, but as mentioned above, we could not use standard reducing agents because they would react with the palmitoyl CoA. As illustrated in Figure 1.2, the palmitoylation reaction requires

reduced cysteine residues to be able to react with. By using TCEP, we discovered that oxidation was blocked, but not palmitoylation. This made it possible to accurately measure the extent of palmitoylation, without the complication of simultaneous oxidation.

The methods, described previously, include incubating surface-bound SNAP-25 with palmitoyl coenzyme A (PCoA), in the presence of TCEP, at 37° C for 1 hour. Averaged data (squares) from over 10 independent experiments are shown in Figure 3.3. The lines show theoretical fits to the data using the Hill Equation. The half-maximal dose for PCoA is 25 μ M and the best fit curve (heavy dashed line) has a Hill Coefficient of 3 for palmitoylation (see equation below). Although the data are also consistent with a Hill Coefficient of 2, they are inconsistent with a Hill Coefficient of 1, suggesting that cooperative binding of palmitoyl CoA occurs with SNAP-25. Though determining which interactions occur between SNAP-25 and palmitoyl CoA to support a Hill Coefficient of 3 is outside the scope of my studies, one hypothesis is that the hydrophobic nature of PCoA results in a micellular aggregation and the delivery of multiple PCoA molecules at one time to each SNAP-25 protein.

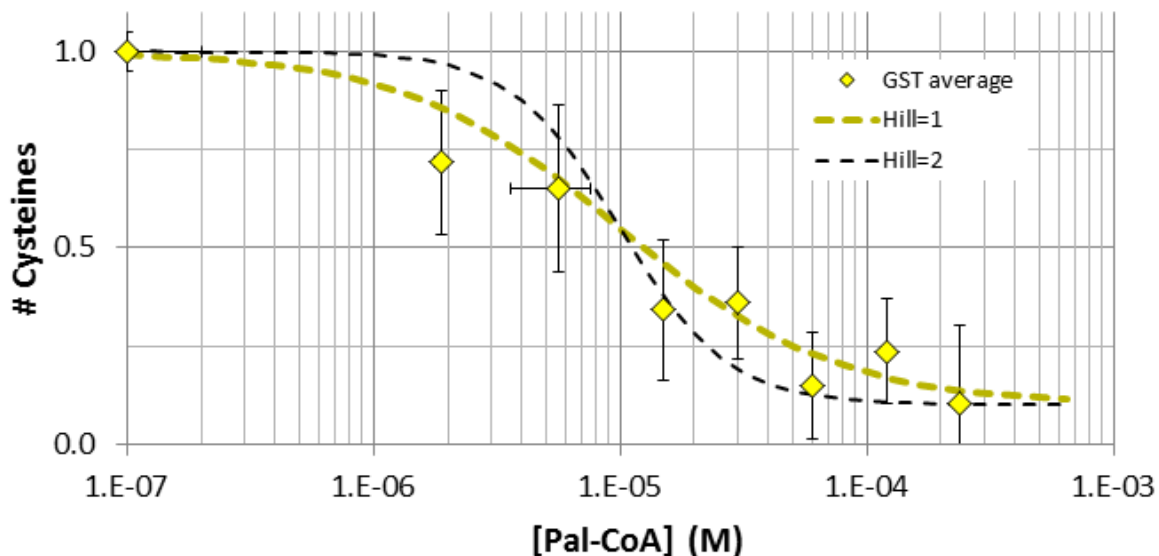


Figure 3.4: Spontaneous Palmitoylation of GST Protein. GST palmitoylation (up to 85%) *in vitro* by free palmitoyl CoA has an EC_{50} of 10 μ M and a Hill Coefficient of 1.

Since SNAP-25 is fused to GST, all palmitoylation reactions on SNAP-25 included the possibility of GST palmitoylation. As a control, I also palmitoylated glutathione S-transferase (GST) on its own (Figure 3.4). As shown, GST is nearly completely reacted *in vitro* by free palmitoyl CoA, with an EC_{50} of 10 μ M. Furthermore, a Hill Coefficient of 1 most closely fits the data. The data is not well fit with a Hill Coefficient of 2. These control data were subtracted from raw SNAP-25-GST data to obtain the resulting SNAP-25 palmitoylation data shown in Figure 3.3.

Hill Equation

The Hill Equation describes a standard reaction or binding interaction between two molecules and is defined as:

Hill Equation:

$$\theta = \frac{[L]^n}{(K_A)^n + [L]^n}$$

θ - Occupied sites where the ligand can bind to the binding site of the receptor protein.

[L] - Free ligand concentration

K_A - dissociation constant derived from the law of mass action

K_A - half-occupation ligand concentration

n - Hill coefficient, describing cooperativity

The value of the Hill coefficient can describe the cooperativity of ligand binding in this way:

- $n > 1$: Positively cooperative binding: Once one ligand molecule is bound to the enzyme, its affinity for other ligand molecules increases.
- $n < 1$: Negatively cooperative binding: Once one ligand molecule is bound to the enzyme, its affinity for other ligand molecules decreases.
- $n = 1$: Non-cooperative binding: The affinity of the enzyme for a ligand molecule is not dependent on whether or not other ligand molecules are already bound.

Oxidation/reduction of SNAP-25

Most changes resulting in the loss of hydrogen from the sulfhydryl group of the cysteine is an example of oxidation. If it is reversible, which it often is, then controlling the oxidation level could serve as an important step in regulating the palmitoylation of SNAP-25, which is required for regulated exocytosis.

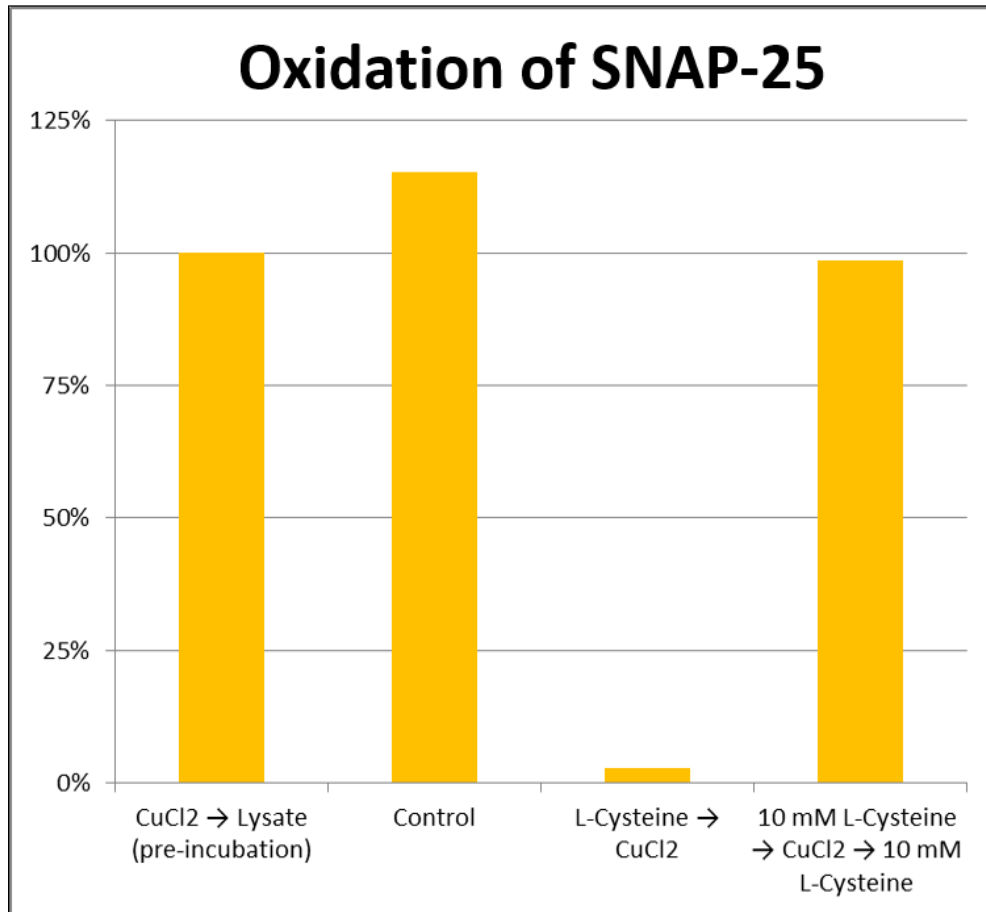


Figure 3.5: Oxidation of SNAP-25. The oxidizing reagent CuCl₂ (100 μM) effectively oxidizes SNAP-25 cysteine residues as show by their inability to be reacted further in the biotinylation chemiluminescent assay.

In the simplest case, oxidation of SNAP-25 is when two cysteine residues of the cysteine-rich domain each lose a hydrogen molecule and form a disulfide bond. This reaction can be driven or prevented experimentally by incubating bound SNAP-25 with oxidizing agents or reducing agents. Figure 3.5 shows that oxidation of SNAP-25 can be tightly controlled in our *in vitro* experiments. In the presence of copper chloride, SNAP-25 is oxidized. However, when copper chloride-oxidized SNAP-25 is treated with excess L-Cysteine, SNAP-25 is reduced and the cysteine residues can be otherwise modified as described in Figure 2.1.

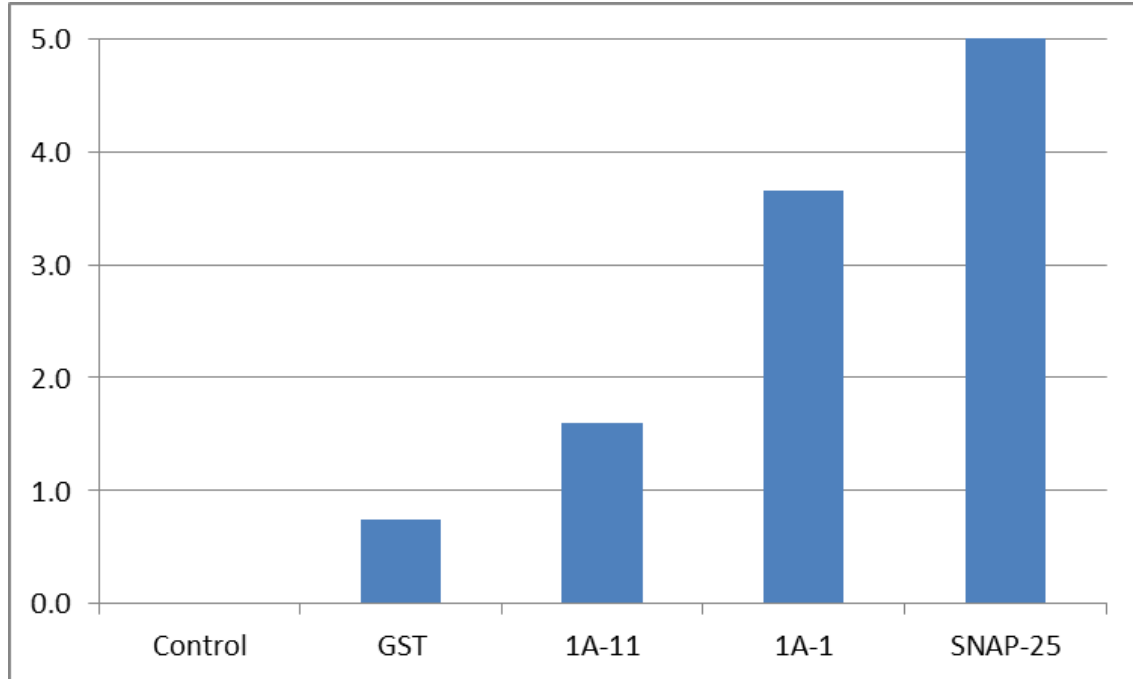


Figure 3.6: Chemiluminescent Cysteine Count for Different SNARE Proteins. Different numbers of cysteines of various SNARE proteins are indicated by relative chemiluminescence.

In order to create a standard curve and baseline measurement for the analysis of reacted SNARE proteins, I incubated GST, 1A-11, 1A-1, and SNAP-25 according to the standard protocol as shown in Figure 2.1. The relative intensity of the chemiluminescent signal was standardized to the baseline control. As shown in Figure 3.6, GST has 1 reactive cysteine residue (of 4 in the full length protein), 1A-11 (a truncated mutant of syntaxin with 1 cysteine) has 1 reactive cysteine residue plus a GST tag, 1A-1 (full length syntaxin with 3 cysteines) has 3 reactive cysteine residues plus a GST tag, and SNAP-25 has 4 reactive cysteine residues plus a GST tag. The standard curve shown in Figure 3.6 (average of duplicates) is representative of multiple such experiments and is consistent with the standard curve previously published (31) which served as a baseline against which all chemiluminescent data sets could be compared.

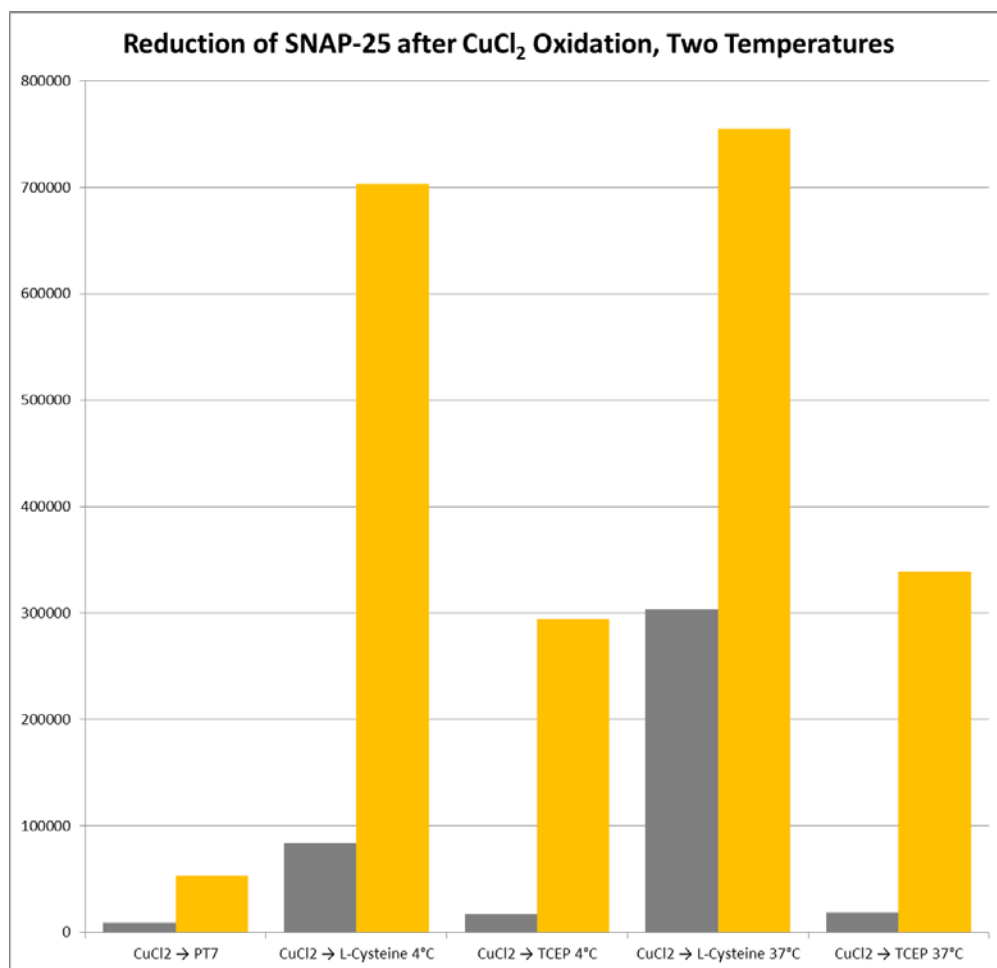


Figure 3.7: Reduction of SNAP-25 at Two Temperatures. Reduction of SNAP-25 by different reducing agents, at two different temperatures, after oxidation with CuCl₂.

We were interested in measuring the effect that temperature has on the reduction of SNAP-25 after oxidation with copper chloride. In order to compare both L-Cysteine and TCEP's effective rate of reduction at both 4°C and 37°C, we compared them to a buffer control (PT7) at room temperature, 25°C. As shown in Figure 3.7, both L-Cysteine and TCEP are more effective at higher temperature at reducing SNAP-25, after CuCl₂ oxidation. While this result is unsurprising, it had yet to be confirmed until these experiments were performed. In addition, determining the effect of temperature on these types of reactions was critical for our studies since

we had supposed that physiologically-relevant temperatures would not only improve reduction of SNAP-25, but also oxidation and palmitoylation of SNAP-25. Therefore, a broad understanding of the effect of temperature on our proteins, reagents, and assay was critical to interpreting our results.

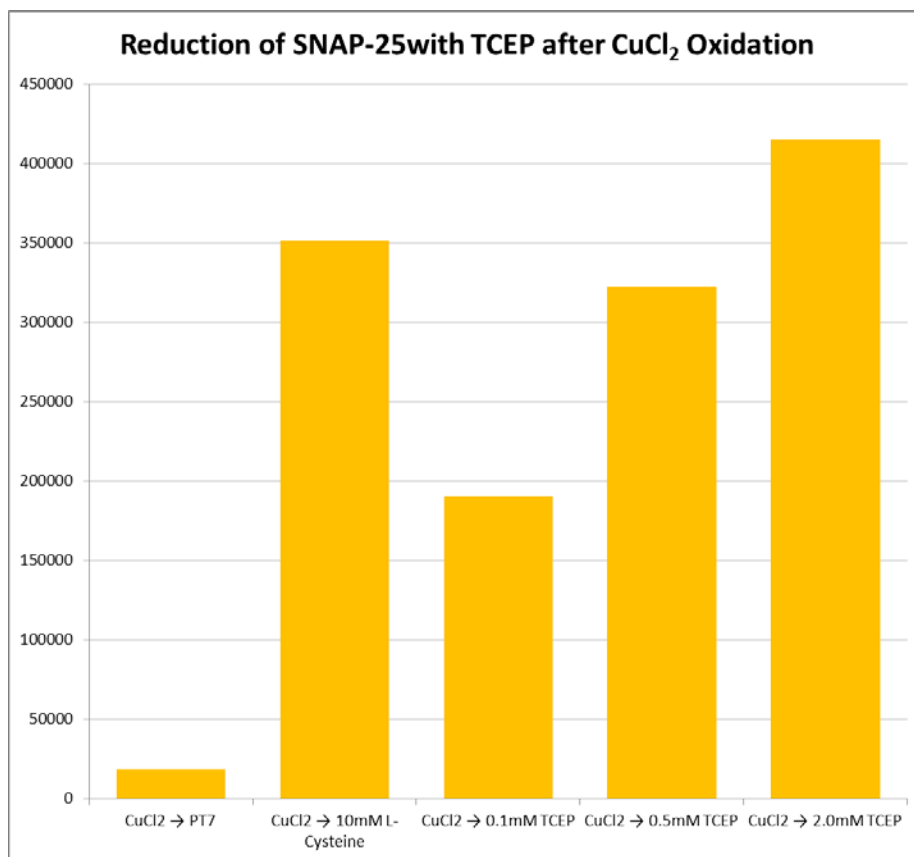


Figure 3.8: Reduction of SNAP-25 by TCEP at 3 Concentrations After CuCl₂ Oxidation. Signal recovery from copper chloride by L-Cysteine and 3 concentrations of TCEP. TCEP (2.0 mM) is effective at reducing oxidized SNAP-25 whereas 10 mM Cysteine is only ~85% effective.

Though we had used L-Cysteine extensively as a reducing agent during our early experimentation, we later discovered its inability to be used as a reducing agent during palmitoylation. This is due to the L-Cysteine binding up and reacting with the PCoA necessary for palmitoylation. Therefore, as discussed earlier, we determined to investigate TCEP, a non-sulfur-containing reducing agent, as a possibility for keeping SNAP-25's cysteine residues

reduced during palmitoylation with PCoA. To investigate the most effective concentration for reducing SNAP-25 after being oxidized by copper chloride, we treated oxidized SNAP-25 with L-Cysteine, and 3 concentrations of TCEP (0.1, 0.5, and 2.0 mM). As shown in Figure 3.8, 2.0 mM TCEP was most effective at reducing SNAP-25 after oxidation with CuCl₂.

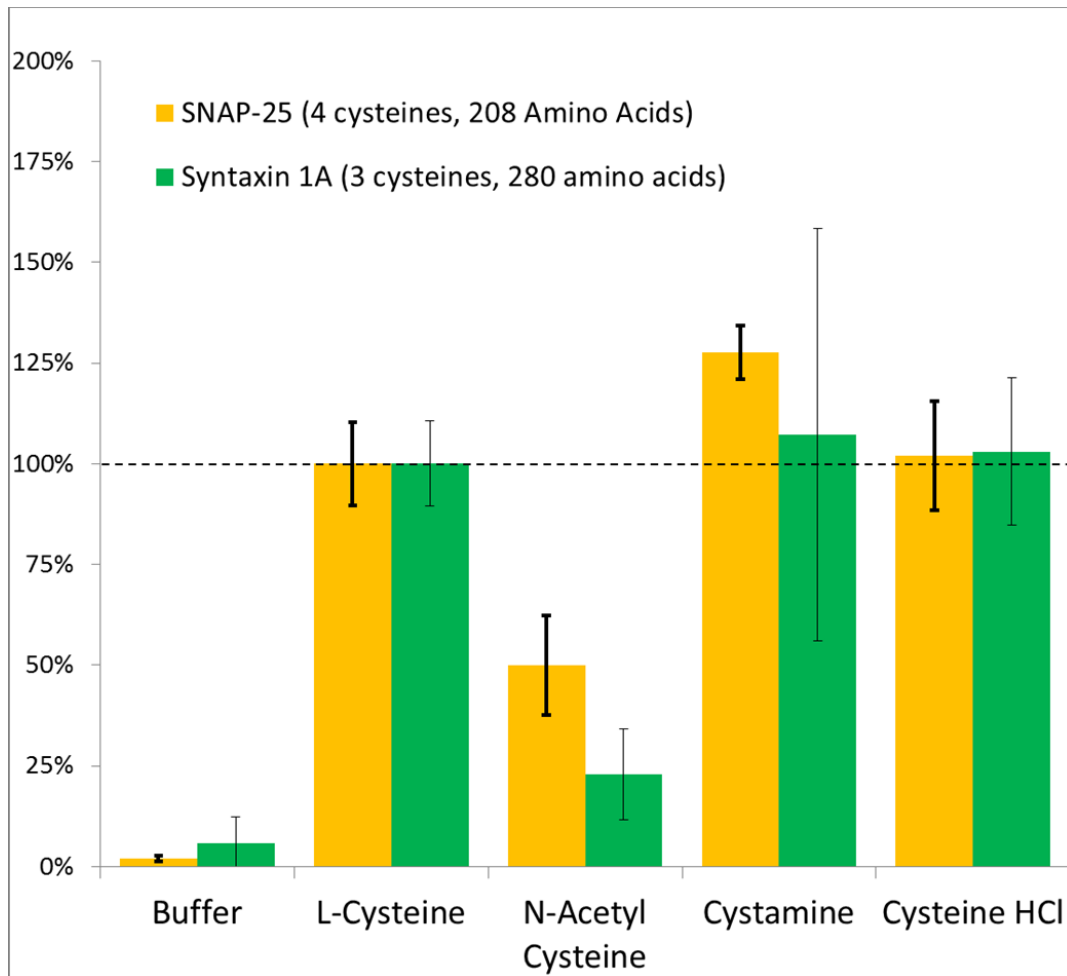


Figure 3.9: Efficacy of Different Reducing Agents After CuCl₂ Oxidation of SNAP-25. Various cysteine-containing reagents are effective at reducing oxidized SNAP-25.

In addition to comparing the effectiveness of L-Cysteine and TCEP at reducing SNAP-25, we also investigated the effectiveness of N-acetyl cysteine, Cystamine and Cysteine HCl at reducing SNAP-25. (Studies also included Syntaxin 1A-1 for comparison, see Figure 3.9). In

order to compare the effectiveness of these reducing agents at reducing cysteine residues, we first had to oxidize the cysteines. This was done by treating them with copper chloride, as has been explained previously. Though the L-Cysteine, N-acetyl cysteine, and Cysteine HCl showed similar ability to reduce the cysteine residues, the L-Cysteine proved to be more consistent and convenient for our studies. Furthermore, L-Cysteine is the reducing agent that had been used most extensively in the lab, by far, so continuing with its use made sense to be able to standardize results from previous experiments using L-Cysteine as the reducing agent.

To continue my studies on the oxidation and reduction of SNAP-25, I wanted to investigate how oxidation with 100 μM CuCl_2 compared to oxidation with 10 μM CuCl_2 . As Figure 3.10 shows, both concentrations of copper chloride effectively oxidize the cysteine residues of SNAP-25. Although the 100 μM CuCl_2 appears to be more potent, the difference is not significant, and the lower concentration has proven to be just as effective at oxidizing the cysteine residues of SNARE proteins.

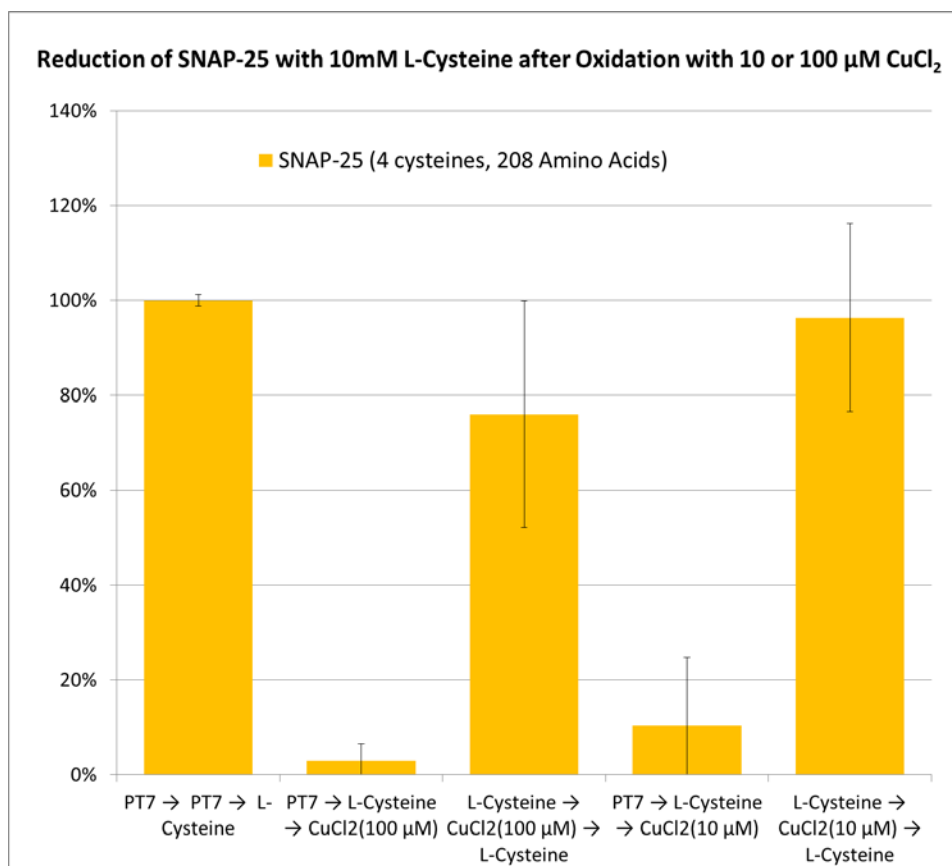


Figure 3.10: SNAP-25 Reduction with L-Cysteine After Oxidation. L-Cysteine (10 mM) is effective at reducing SNAP-25 which has been oxidized with 10 and 100 μM CuCl_2 .

In Vivo Palmitoylation of SNAP-25

Although *in vitro* studies allow for spontaneous palmitoylation of SNAP-25, this reaction is less-likely to occur *in vivo* under physiological palmitoyl CoA concentrations. The intracellular concentration of palmitoyl CoA has been reported in muscle to be approximately (5.9 pM palmitoyl CoA/10 mg muscle, or approximately 1 μM (32). This is $\sim 25\text{x}$ below the concentration needed for half maximal palmitoylation without the enzyme, showing that palmitoylation is unlikely to occur in the cell without a PAT.

Our studies show that enzyme-catalyzed palmitoylation of SNAP-25, under similar spontaneous-palmitoylation-concentrations, achieve 95% levels of palmitoylation after 60 minutes amount of time. We have shown (Figure 3.3), as have others, that palmitoylation of SNAP-25 occurs *in vitro* in the absence of PAT enzymes (21). However, this enzyme-free palmitoylation only occurs at physiologically-irrelevantly high Palmitoyl-CoA dosages (~25 μ M).

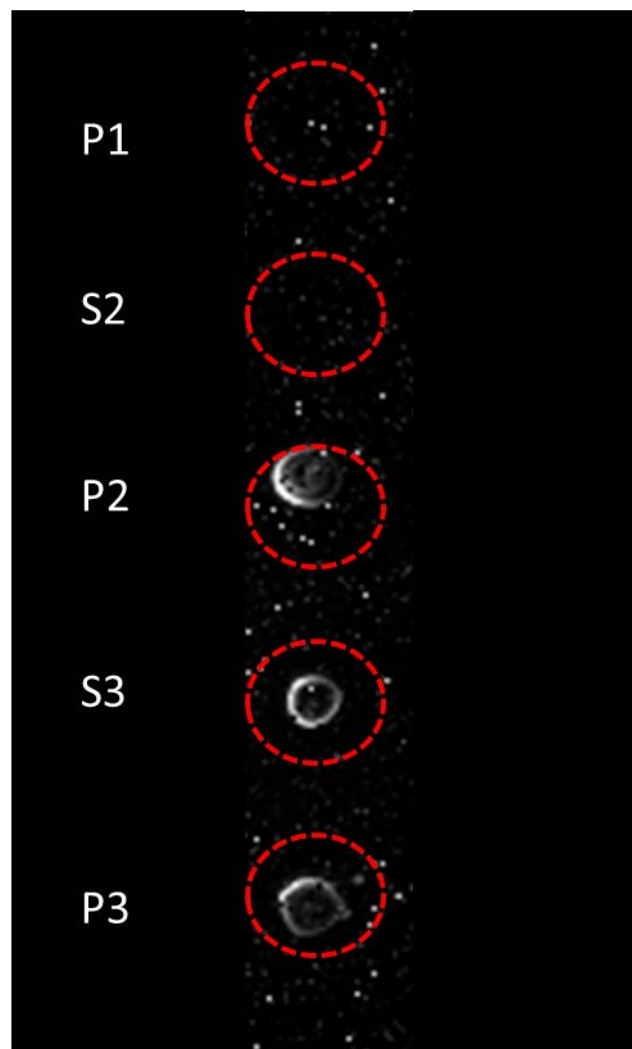


Figure 3.11: DHHC-17 Western Blot. Bovine adrenal medulla tissue was homogenized and blotted according to standard Western Blot technique, using an anti-DHHC-17 antibody, as described in methods. As shown, the PAT, DHHC-17 is found in P2, S3, and P3 fractions.

We wanted to obtain a native PAT and knew that adrenal medulla contained SNAP-25(18). Therefore, we hypothesized that adrenal medulla would also contain a native PAT for SNAP-25. We obtained and processed Bovine adrenal tissue (see chapters 1 and 2). As described in chapter 1, PATs are membrane proteins expected to be found in recycling endosomes. As shown in Figure 3.11, DHHC-17 was identified in fractions P2, S3, and P3.

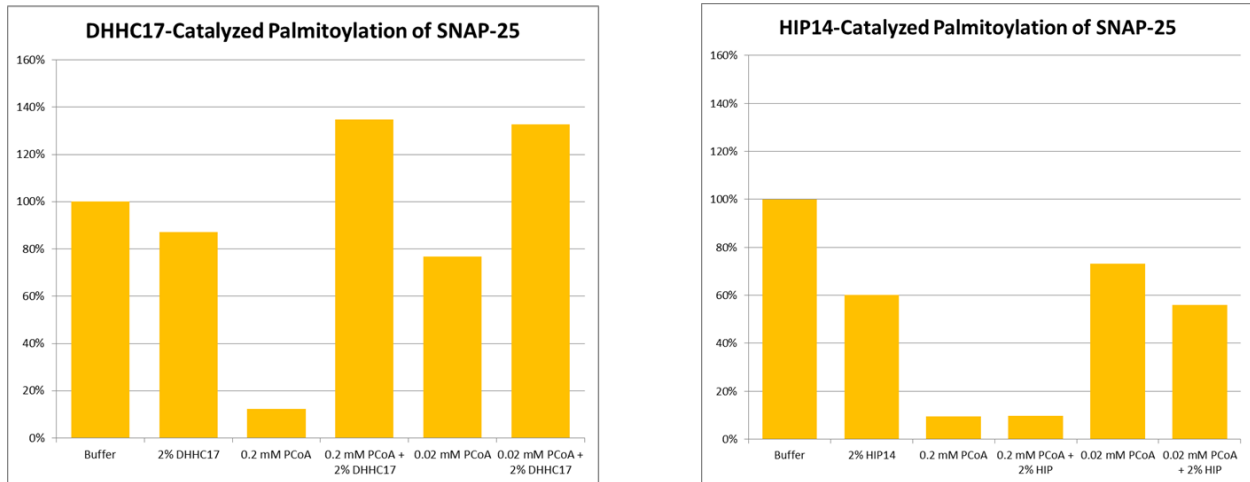


Figure 3.12: DHHC-17 vs HIP14 – Catalyzed Palmitoylation of SNAP-25. HIP14/DHHC-17-catalyzed palmitoylation of SNAP-25 at different PCoA dosages. DHHC-17 has demonstrated the ability to enhance the chemiluminescent signal of SNAP-25 during palmitoylation studies, which is the opposite expected effect.

We wanted to investigate and compare the ability of DHHC-17 and HIP-14, both palmitoyl acyl transferase (PAT) enzymes, to enzymatically catalyze the palmitoylation of SNAP-25 in the presence of free palmitoyl CoA. As shown in Figure 3.9, DHHC-17 demonstrated the ability to enhance the chemiluminescent signal of SNAP-25, which is the opposite of what we would expect. Though we do not know what was causing this result, we had a few hypotheses, which we tested. One of the possibilities is that something in the DHHC-17/H-buffer solution was interfering with the glutathione coated wells. After extensive testing of the wells with individual reagents and various combinations of reagents, this hypothesis was

ruled out. Another hypothesis, which we also tested, was that the DHHC-17 solution contained other proteins which were indiscriminately binding to the glutathione-coated wells, and subsequently binding biotin, streptavidin, and reacting with peroxide substrate to yield a higher chemiluminescent signal. We were able to test for this (data not shown) and determine that this hypothesis did not explain our data. We have yet to determine why the DHHC-17 is causing repeated, yet somewhat inconsistent, elevated chemiluminescent signals and leave to future workers the solution of this problem.

Future Directions

Although there are many possible future directions for the lab's studies, some of the following are logical, promising, and relevant. They include:

- Confirming the suspected presence of palmitoyl protein thioesterase (“depalmitoylating enzymes”) in bovine adrenal medulla tissue
- Hill coefficient studies with other SNARE proteins
- Further studies to determine whether there is true bimodal distribution of DHHC17 throughout the bovine adrenal medulla cell
- Using palmitoylated SNAP-25 in a functional, bilayer fusion assay – a close representation of *in vivo* exocytosis

CHAPTER 4: Appendix

This appendix is included to further demonstrate and document my studies of SNARE proteins and synaptic vesicle fusion. The scope of the following paper, written as part of my coursework, is broader than my experimentation and thesis, providing additional context for my more-focused graduate studies.

SNAREs and Synaptic Vesicle Fusion

Abstract

The SNARE family of proteins is one of the most studied elements of machinery involved in intracellular trafficking. These proteins form a complex which brings the vesicle and plasma membranes together to allow for synaptic vesicle fusion to occur. Syntaxin-1, synaptobrevin, and SNAP-25 combine to form a 4-helix bundle in a 1:1:2 helix arrangement. This SNARE complex is the center of synaptic fusion and has been the focus of exocytosis studies for over a decade. Other proteins involved in the regulation of this fusion event are less understood, but their involvement is accepted as critical for proper fusion.

Introduction

SNARE proteins were first identified in the late 1980s. The first discoveries were the localization of these proteins on either the synaptic vesicle or the target membrane, leading to the theory that two classes of SNARE proteins exist: vesicular SNAREs (v-SNAREs) or target-membrane SNAREs (t-SNAREs). Over the two decades following their discovery, much research was done to assign all members of the fusion machinery to a SNARE class; either as a v-SNARE, t-SNARE or both. There are now 24 identified SNARE proteins, with more than 50 associating proteins involved in exocytosis.

Vesicle-priming reactions serve to arrange required proteins in a semi-stable, energetically transitory state in order to prepare for fusion. This priming action organizes components of synaptic transmission into a type of assembly line which allows for rapid

accomplishment of the final fusion-exocytotic event. This preparation is not trivial because synaptic transmission is a vital part of regular brain activity, information processing, and memory formation. Indeed, the creation and utilization of the exocytotic components is virtually constitutive throughout all life forms including animals, plants, bacteria, and humans.

The diversity of proteins involved in and required for synaptic vesicle fusion is impressive. From length and intracellular localization mechanisms to secondary structure and function, the proteins of vesicle fusion comprise a sort of protein melting pot. The various proteins contain specific elements that enable precise assembly and tight regulation.

The carefully timed protein-zipper process of exocytosis is carried out with exactness, each protein domain interacting exactly with complement domains, allows for rapid transmission of neurotransmitters from one cell to another. The speed of synaptic vesicle fusion is facilitated by the preassembly and docking of fusion machinery, including soluble N-ethylmaleimide sensitive factor attachment receptor (SNARE) proteins, Sec1/Munc18 (SM) proteins, and a number of other accessory proteins. Although these proteins differ in size, shape, and function, all are required for the smooth process of synaptic vesicle fusion *in vivo*.

The process of neuronal communication is required for all eukaryotes. Neurons communicate by secreting vesicularized chemicals through the membrane of one neuron into an open space where these vesicles are then taken up by an adjacent neuron. The brain sends signals to various areas of the body to regulate homeostasis and facilitate cognition and voluntary actions, amongst other processes. Without this general mechanism of communication between adjacent and distant cells, life as we know it could not exist. The exocytosis, accomplished by synaptic vesicle fusion, is certainly one of the most important processes that allow for neuronal communication.

SNARE Proteins

Most types of intracellular membrane traffic are governed by SNARE proteins. Three proteins, synaptobrevin, syntaxin 1, and SNAP 25, form the basic SNARE complex (Figure 4.1) by associating directly with one another to form a parallel four-helix bundle (33).

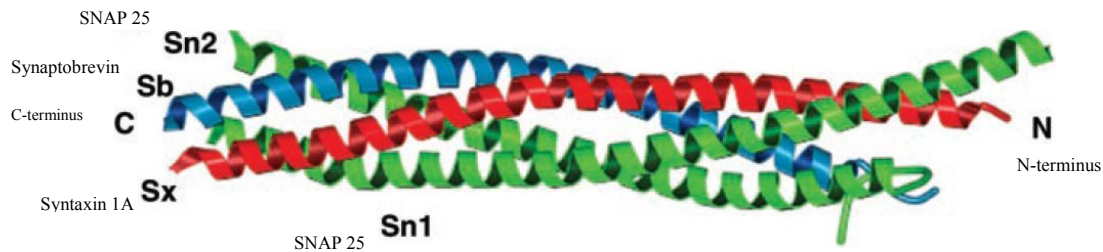


Figure 4.1: Four-helix Bundle.

Syntaxin 1A

Syntaxin 1A adopts a conformation typical of most SNAREs; it contains a transmembrane domain at its C terminus and an independently folded N-terminal domain. The N-terminal domain contains a three-helix bundle in antiparallel conformation and a flexible linker region facilitating SNARE motif interaction. Syntaxin 1A is highly abundant in neurons and neuroendocrine cells.

The role of syntaxin 1A as a t-SNARE is to participate in the four-helix bundle helping to bring opposing membranes together. Furthermore, evidence has been shown that syntaxin 1A interacts directly with and functionally regulates calcium channels (34). It has been hypothesized that syntaxin 1A inhibits calcium channel function directly by causing a shift in the current-voltage relationship of the calcium channels (35).

Synaptobrevin 2/VAMP 2

Synaptobrevin 2, like syntaxin 1A, contains a C-terminal transmembrane domain and a small linker region allowing SNARE motif interaction. Unlike syntaxin 1A, synaptobrevin 2 does not contain an independently folded N-terminal domain. It does, however, contain an N-terminal proline-rich extension. It is palmitoylated near its transmembrane domain which stabilizes the protein-membrane interaction.

The role of synaptobrevin 2 is to bind calmodulin, a calcium binding protein, in order to facilitate calcium-dependent exocytosis, which may serve as the key regulatory step in SNARE complex assembly (36). Further details of this regulatory mechanism are still being discovered.

SNAP-25

SNAP-25 deviates from the normal SNARE structure for two reasons: it lacks a transmembrane domain and it contains two SNARE motifs joined by a flexible linker region. This linker contains a cysteine-rich region of four cysteine residues that are palmitoylated to anchor SNAP-25 to the plasma membrane. While SNAP-25 is neuron-specific, there are other SNAP proteins (23, 29, 47) that are expressed ubiquitously(37).

SM PROTEINS (Sec1/Munc18-1)

Since the discovery that Munc18-1 binds to the target membrane SNARE syntaxin 1, it has been understood that SM proteins play a role in synaptic fusion. Their initial discovery came as a result of genetic screens in *C. elegans* and yeast for mutants exhibiting defects in membrane traffic and secretion (38). The role of Sec1 appears similar to that of Munc18; both associate with SNARE proteins in multiple ways, one of which is in a clasping configuration that allows for regulation. By binding the regions of SNARE proteins, SM proteins are able to help organize the SNARE complex in a meta-stable configuration that allows for quick zippering and eventual

fusion. They are thus referred to as SNARE pins. Their necessity is demonstrated since genetic deletion of SM proteins results in a block of the fusion reaction. However, their particular role has yet to be definitively confirmed since there appear to be multiple binding modes that can play both negative and positive roles (39, 40).

Rab Proteins

Rab proteins are peripheral membrane proteins, anchored to the membrane through a lipid group that forms a covalent bond with an amino acid. Specifically, Rabs are anchored via prenyl groups on two cysteines in the C-terminus. Because Rab proteins are anchored to the membrane through a flexible C-terminal region, they can be thought of as a 'balloon on a string'.

Like other GTPases, Rabs switch between two conformations, an inactive form bound to GDP (guanosine diphosphate), and an active form bound to GTP (guanosine triphosphate). In their active state Rabs bind a variety of effector proteins, including components of motor complexes involved in vesicle movement (41), vesicle cargo proteins, and GTP exchange factors for other Rab proteins (42, 43) a GDP/GTP exchange factor (GEF) catalyzes the conversion from GDP-bound to GTP-bound forms, and GTP hydrolysis to GDP is catalyzed by a GTPase-activating protein (GAP). REPs carry only the GDP-bound form of Rab, and Rab effectors, proteins with which Rab interacts and through which it functions, only bind the GTP-bound form of Rab. Rab effectors are very heterogeneous, and each Rab isoform has many effectors through which it carries out multiple functions.

After membrane fusion, Rab is recycled back to its membrane of origin. A GDP dissociation inhibitor (GDI) binds the prenyl groups of the inactive, GDP-bound form of Rab, inhibits the exchange of GDP for GTP (which would reactivate the Rab) and delivers Rab to its original membrane.

Rab proteins are involved in trafficking vesicles and other cellular membranes. They are localized according to their interactions with other proteins and lipids (44-46). Rab GTPases' regulation of membrane traffic, vesicle formation, vesicle movement along actin and tubulin networks, and membrane fusion is critical. These processes make up the route through which cell surface proteins are trafficked from the Golgi to the plasma membrane and are recycled.

Synaptotagmin and Complexin

Crucial components of the calcium-dependent triggering of synaptic fusion are synaptotagmin-1 and complexin (Figure 4.2). Calcium, which enters the cell as a result of depolarization, binds to synaptotagmin-1, a calcium sensor for fusion. Synaptotagmin associates with SNARE protein complexes and phospholipids which allows for regulation via calcium binding (47-49).

There have been many studies done that explore how synaptotagmin-1 and its association with SNARE proteins may induce the curving of membranes that precedes membrane pore formation and eventual fusion. Although the exact role that synaptotagmin plays has yet to be elucidated, it is known that it plays an important role in synaptic fusion since calcium-bound synaptotagmin-1 can enhance SNARE-mediated liposome fusion (50, 51).

Complexin molecules bind the SNARE complex tightly and form an alpha helix that associates with synaptobrevin and syntaxin-1 in an antiparallel manner (52, 53). Studies suggest that complexin acts as a fusion clamp to inhibit SNARE-induced liposomal fusion. Furthermore, this fusion clamp appears to release the SNARE complex via the actions of calcium-bound synaptotagmin-1 (54, 55). However, a dual role for complexin appears likely because complexin stabilizes the C terminus of the SNARE complex creating a semi-stable substrate for synaptotagmin-1 to trigger fast release (56).

Assembling and Disassembling the Complex

The process of vesicle fusion is highly bioenergetically unfavorable; hence the need for molecular machinery to overcome this energy barrier.

Early studies of vesicle fusion followed the simplicity approach wherein only controlled components were added to reconstituted systems in order to discover the minimal requirements for vesicular fusion (57). Later studies of *in vitro* SNARE complex assembly showed that SNARE proteins will spontaneously fuse with one another to form complex arrangements of heterogeneous proportions that are not found in nature. Specifically, syntaxin-1 and SNAP-25 tend to form 2:1 heterodimers where a second copy of syntaxin-1 is substituted for synaptobrevin in the four-helix SNARE complex (58). However, if you coexpress syntaxin-1 and SNAP-25 you can observe *in vitro* 1:1 heterodimers suggesting that coexpression produces a metastable state between these two SNARE complexes (59, 60).

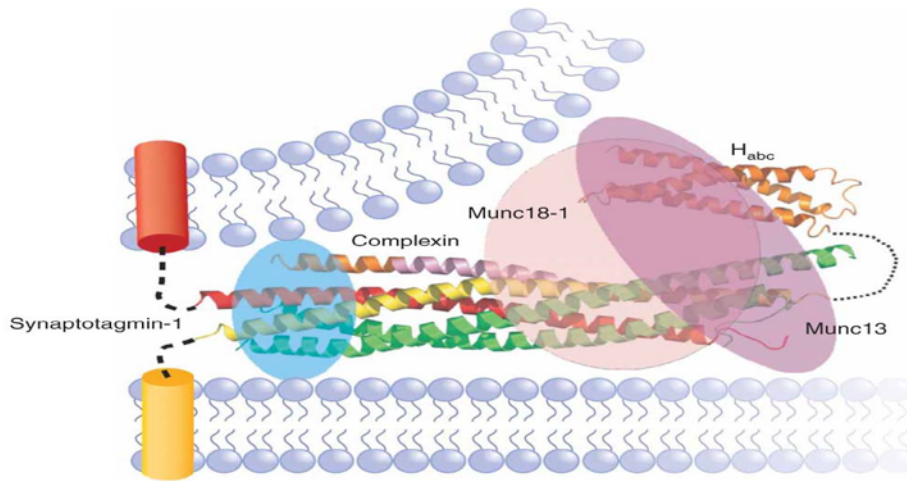


Figure 5 Diagram illustrating the notion that Munc18-1, Munc13, complexin and synaptotagmin-1 bind to the SNARE complex. The ribbon diagram shows the structure of the complexin-I–SNARE complex²⁶, which illustrates the only one among these interactions that has been revealed at atomic resolution. The binding site of synaptotagmin-1 on the four-helix bundle is inferred from mutagenesis⁹², whereas those of Munc18-1 and Munc13 are unknown. The figure intends to illustrate that all these SNARE complex interactions may be compatible, mutually exclusive, or partially competitive, and that resolving this issue as well as how these interactions occur during the steps that lead to exocytosis will be critical to understand the mechanism of release.

<http://www.nature.com/nsmb/journal/v15/n7/full/nsmb.1450.html>

Figure 4.2: SNARE Complex and Accessory Proteins at the Vesicle-membrane Junction.

- Before fusion

Prior to the fusion event, the proper fusion site must be determined. Accordingly, syntaxin proteins are concentrated in 200 nm large, cholesterol-dependent clusters where secretory vesicles preferentially dock and fuse (61). Also, the arrangement of SNARE proteins at the site of synaptic fusion is such that the “zippering” of proteins occurs from the vertex of associating proteins inward, rather than adjacent/touching to outward/non-touching (25).

The cytosolic calcium concentration has been shown to directly impact the rate of synaptic fusion. Calcium cooperativity with SNARE proteins is high and reduction of SNARE proteins via mutation has shown to reduce this cooperativity and the rate of fusion (62, 63).

-Maintenance of close membrane apposition, allow further fusions steps (64)

-Licensing of fusion (detection of SNARE complex by fusion machinery increases likelihood of fusion) (65)

- During fusion

The fusion process is one of careful timing and intricacy.

-Recruitment of fusion machinery into fusion machine (64, 66-70)

Before the fusion process can occur all of the key players must be in place. This involves many preparatory steps.

-Releasing proteins upon formation of the SNARE complex, which could inactivate regulatory proteins or activation proteins involved in fusion (33, 71)

In order for the fusion process to properly occur there must be a rearrangement of proteins.

-Binding of lipids, causing or facilitating their transition to a fusion state (36, 72)

- After fusion

-Imparting vectorality to fusion events that are not kiss and run (73)

When calcium binds synaptotagmin the complexin clamp is released and fusion is triggered by binding the SNARE protein complexes and the phospholipids.

The disassembly of the SNARE complex is not spontaneous. Due to the cis arrangement the complex is quite stable and requires energy to disassemble. The ATPase NSF (N-ethylmaleimide-sensitive factor) functions to disassemble cis-SNARE complexes into free SNAREs. This ATPase is a member of the ATPase associated with other activities (AAA) protein family that serve the general function of untangling protein aggregates and complexes (74).

Some Controversy

Differences of opinion exist regarding whether or not full fusion must occur with each round of exocytosis or if the docked vesicle can fuse, open a pore, release part of its contents, close the pore, and detach back to the cytosol for future availability. The difficulty in declaring that one type of fusion occurs at the exclusion of the other is that multiple groups have produced data to show that both fusion types occur.

The “full fusion” explanation is based on the premise that once docking and initial fusion events have occurred, the most energetically favorable direction is for complete fusion to take place. The “kiss and run” hypothesis centers on trying to make sense of data obtained through biophysical studies using bilayers and electrical recordings wherein a brief conductance spike is observed before vanishing, ostensibly because the fusion pore has closed off (75).

Since we are unable to view the fusion or exocytotic events microscopically at high resolutions, the only realistic way to elucidate more clearly which method occurs (predominantly or exclusively) is through data analysis and further discovery of all of the components involved in synaptic fusion, including their conformations, locations, and functions.

Perspective

Many of the processes involved in synaptic fusion and neurotransmitter release have been discovered through years of recent exploration. It is established that SNARE proteins are not only involved in, but required for fusion and exocytosis. Also, beyond the initial recognition that SNAREs are involved, many other accessory proteins, some of which are discussed above, also play critical roles.

The largest questions yet to be answered regarding synaptic transmission are those related to the roles of each accessory protein. While the basic docking, zippering, and fusion events are recognized, the more specific preparatory interactions of accessory proteins are less understood. For example, it still remains to be discovered exactly how accessory proteins such as SM proteins, Munc proteins, and complexin, amongst others, are able to induce conformational changes in the key SNARE proteins that ultimately leads to fusion. Even though we still need to elucidate specific mechanisms, we do know that were it not for the individual preparatory events preceding synaptic fusion, such fusion could never occur.

Future experimentation will no doubt help in the development of drugs and techniques related to the therapeutic intervention in regulating this crucial-to-life process.

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