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In the Absence of Synaptobrevin and Synaptosomal-Associated Protein 25, Syntaxin Seems to Hinder Vesicle Fusion

Physics 492R Capstone Report

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Abstract

Cellular exocytosis is driven by the formation of SNARE complexes between synaptobrevin (VAMP), SNAP25, and syntaxin on the plasma membrane. These SNARE proteins work together to drive fusion, but the individual effects of each of these proteins are still undetermined. We show that syntaxin can be reconstituted into the membranes of artificial vesicles for *in vitro* fusion experiments. The nystatin/ergosterol fusion assay was used to measure individual fusion events of artificial vesicles to a planar lipid bilayer formed on hole of a plastic cup. We found that reconstituted syntaxin has a possible hindering effect on fusion in the absence of other SNARE proteins, but further testing is necessary to confirm this hypothesis. Furthermore, we suggest that syntaxin keeps vesicles from sticking together, allowing the vesicles to diffuse more readily in solution.

Introduction

The nervous system of the human body is quite complex. Cells communicate with one another and the brain communicates rapidly with the rest of the body by means of electronic impulses passed from one neuron to the next. The nervous system consists of billions of neurons, which communicate with other cells by releasing chemicals called neurotransmitters. These neurotransmitters are originally packed in synaptic vesicles within nerve cells. After receiving the correct signals, neurotransmitters are released by exocytosis. Exocytosis is a process in which a synaptic vesicle containing neurotransmitter fuses to the phospholipid bilayer membrane of the presynaptic terminal and opens up, releasing its contents into the space between the neuron and the target cell, called the synapse. On the postsynaptic side of the synapse, receptors embedded in the membrane cause changes within the target cell as they bind a specific neurotransmitter. In this way, information travels long distances very quickly through the body.

The fusion process of a synaptic vesicle to a lipid membrane has recently become a topic of interest. It is known that SNARE (Soluble N-ethylmaleimide-sensitive factor Attachment Protein Receptor) proteins are involved (Lang and Jahn, 2008, Finley *et al.*, 2002, and Siddiqui *et al.*, 2007), but it is still uncertain exactly how they combine and interact with one another. Some of the major SNARE proteins of interest are SNAP-25, synaptobrevin (VAMP), and syntaxin. We worked with syntaxin, which has a trans membrane domain (TMD), and a regulatory domain. The TMD sticks into the plasma membrane of the cell while the regulatory domain is the section of the protein that actually interacts with other proteins. The

regulatory domain contains the Habc domain, which consists of 3 alpha helices. These alpha helices help regulate syntaxin's ability to bind other SNARE proteins during vesicle docking and fusion.

During vesicle fusion to a lipid membrane, these three proteins interact and bring the vesicle close to the bilayer so that it can fuse and release its contents to the other side of the membrane (Takamori *et al.*, 2006). We look at the effects of syntaxin in the absence of VAMP and SNAP25 to see whether it has an effect on fusion rates of vesicles to a planar lipid bilayer. We create a mock cell membrane by forming a membrane bilayer on the hole of a plastic cup. We then add artificial vesicles made up of different types of phospholipids in a ratio similar to that of vesicles found in nature. Because the lipid bilayer acts as an insulator, we can apply a voltage across it and thereby detect changes in membrane conductance by measuring current through the membrane. Nystatin and ergosterol form ion channels through membranes, so we add these to our vesicles so that when one vesicle fuses to the membrane, we see a spike in current as ions flow through the channels. The ability to see individual fusion events sets this research apart from others that look at thousands of vesicle-vesicle fusions by measuring properties of the population.

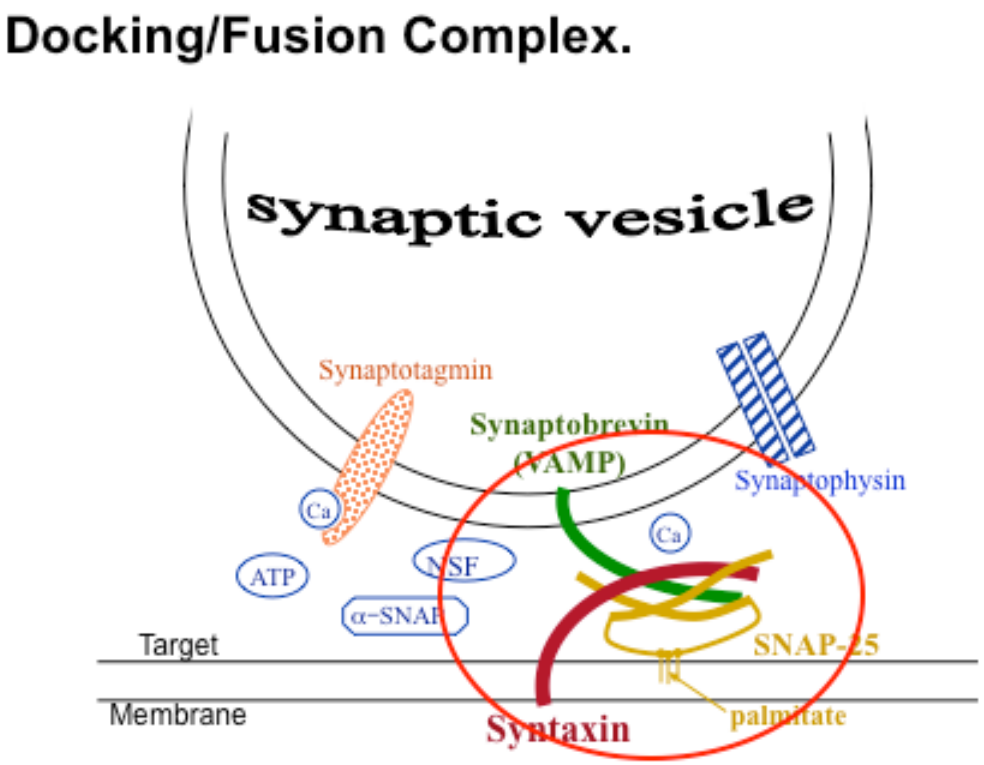


Figure 2: (Reproduce with permission from Dr. Dixon Woodbury, 2000) The machinery involved in the docking and fusion of a vesicle to a target membrane. The SNARE proteins VAMP, syntaxin, and SNAP25 are known to play especially important roles in this process.

This research is an important step towards understanding how membrane fusion works at a fundamental level. The work done here is a step, albeit a very small step, toward a greater understanding of the human body. Furthermore, if we can find out what each of the SNARE proteins does individually we will have a better model of the human nervous system and body.

Materials and Methods

Artificial Vesicle Preparation

Artificial vesicles were prepared with phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylserine (PS) lipids in a molar ratio of 7:3:3, respectively. For our fusion assay, we added 25 or 40 μL of Ergosterol (a sterol similar to cholesterol) and 9 or 18 μL of Nystatin for a single batch of 230 μL of vesicles (we began with the smaller volume of ergosterol and nystatin but found that higher volumes gave a more clear signal). Contents were dried with N2 gas until chloroform (lipids were stored in chloroform and stored in the freezer) was completely evaporated. 230 μL of 150 mM KCl buffer was then added with 29 μL of 10% glycerol in water or 29 μL syntaxin protein in its 10% glycerol buffer. Each mixture of vesicles was then snap frozen in liquid nitrogen, thawed, and sonicated for 15 seconds. This was repeated two more times with the last sonication just over 10 seconds. This recipe allowed us to achieve a consistent vesicle diameter of around 150 nm as measured by dynamic light scattering.

Sizing of Artificial Vesicles

Each batch of vesicles was sized by dynamic light scattering (DLS). The machine used was the 90 plus Particle Size Analyzer by Brookhaven Instruments Corporation. If vesicles were much larger than the desired 150nm diameter, we put them in a sonication bath for a few more seconds until the appropriate diameter was achieved. Sonicating vesicles decreases their size because shear forces off of an

oscillating micro-bubble, pulling off strands of vesicle. This eventually pinches off and forms a smaller vesicle (Woodbury, Richardson, *et al.*, 2006).

Vesicle Centrifugation

To show that the syntaxin protein was integrated into the membranes of the vesicles and not just floating in solution, we centrifuged100 μL of vesicles with a Beckman® Airfuge. The vesicles were spun in the airfuge for four hours at about 150,000xg. This deposited the vesicles and whatever was stuck in the vesicle membranes to the bottom of the centrifuge tube as a pellet. Anything that was not stuck in the vesicle membranes remained in the supernatant.

Western Blot

A Western Blot of the centrifuged contents was used to determine whether the syntaxin had become incorporated into the vesicle membranes. We spotted nine samples of the centrifuged artificial vesicles (AVs) on a membrane. The nine samples were the top, middle, and pellet of the test tube that had been in the airfuge, a ¼ dilution of the top, middle, and pellet, and a 1/10, 1/40, and 1/100 times dilution of the pure syntaxin (before adding to vesicles).

A polyvinylidene difluoride (PVDF) transfer membrane was blocked for two hours in 5% Non Fat Dry Milk to assure that the primary antibody did not stick to anything other than the syntaxin. We then incubated the membrane in the primary

antibody (against syntaxin) for 16 hours at 4° C. The membrane was then washed 3 times in TBS-Tween buffer at room temperature for 10 minutes each. We re-blocked the membrane by placing it in a 10% Non Fat Dry Milk solution for 10 minutes and then incubated it in the secondary antibody (made against the constant region of the primary antibody) for 30 minutes at room temperature. The membrane was again washed 3 times in TBS-Tween buffer for 10 minutes each in preparation for the addition of chemiluminescent reagents. The reagent used was provided by Thermo Scientific, SuperSignal® West Pico Chemiluminescent Substrate. The membrane was observed in a FluorChem™ fluorometer to determine the relative brightness of each spot, which is proportional to the amount of protein contained in each spot.

Proteins and Antibodies

Syntaxin was made using Dr. Dixon Woodbury's protocol (Woodbury and Rognlien, 2000). Ammonium sulfate precipitation was then performed to concentrate the protein. The primary antibody was anti-syntaxin antibody made by mouse and the secondary antibody was an anti-mouse made by rabbit.

Fusion Assay

Making Cups

To create an artificial cell membrane, we brushed a bubble of lipids dissolved in decane across the opening of a hole to form a planar lipid bilayer. The holes were created by pressing a conical wire heated with a soldering iron against the inside of

cups until the wire had nearly melted through the side of the cup. Cups were made of polystyrene and were about 1 cm in diameter and 1.5 cm tall. A heat gun was also used to help the soldering iron begin to press through to the outside of the cups. The cups were allowed to cool and holes were formed by shaving away the desired amount of material from the bulging area of the cup using a microtome blade. Holes were between 150 nm and 350 nm in diameter. It was important to know the diameter of the hole because a larger diameter hole produced a larger membrane, as measured by electrical capacitance of the membrane.

Setup

We used a chamber in which we placed the cups, with a separate opening in the chamber on the other side of the cup where buffer, osmotic gradient, and vesicles were added. Lipids were made from 100 μL PE and PC lipids in a 7:3 molar ratio. Lipids were stored in chloroform that was evaporated with N2 gas and 50 μL of decane was used to dissolve the lipids. Less than 1 μL of this solution was used to precoat the hole of a cup, which was then allowed to dry for 5 minutes. 1 mL of 150 mM KCl buffer was added to the front chamber and 800 μL was added to the back chamber such that the solution height was the same on both sides of the cup. Ag/AgCl electrodes were placed in the cup and in the front chamber to run current across the lipid membrane.

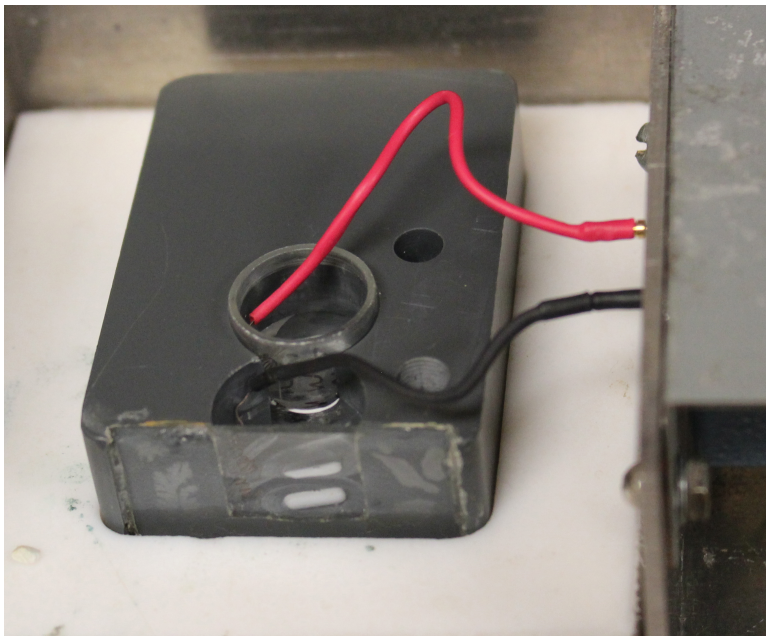


Figure 3: Experimental setup of the nystatin/ergosterol fusion assay. 150 mM KCl buffer was added to both the inside of the cup and the front chamber. Ag/AgCl electrodes are immersed in the solution in the front and back chambers. The front chamber contains a stir bar to mix the osmotic gradient and vesicles.

Experiment

A pipette tip was dipped into the lipid mixture and the contents were expelled out. To form a lipid bilayer on the hole of the cup, a bubble was produced and brushed across the opening of the hole. Lipid membranes act like capacitors so larger holes produced larger capacitances. Also, we could determine whether a membrane was too thick if the capacitance was lower than expected, because a low capacitance meant that the two lipid layers of the membrane were relatively far apart with a thick decane layer in between. To obtain appropriately large capacitances, we re-brushed a bubble across the hole to thin the membrane. Larger capacitances also meant higher chances for vesicles to fuse.

Nystatin and ergosterol form ion channels so we were able to see individual fusion events by measuring the current across the membrane (Woodbury, 1999). A voltage of -60 mV was placed across the membrane, a step necessary to visualize fusions. As a vesicle fuses, it becomes part of the membrane. As the lipids from both the vesicle and planar bilayer mix, ion channels also mix into the membrane, so that ions are able to flow through, producing a surge of current. This tiny current (several picoamps) is amplified and displayed as a spike on our computer screen. Spikes in current indicated the opening of nystatin/ergosterol channels and thus the fusion of a single vesicle.

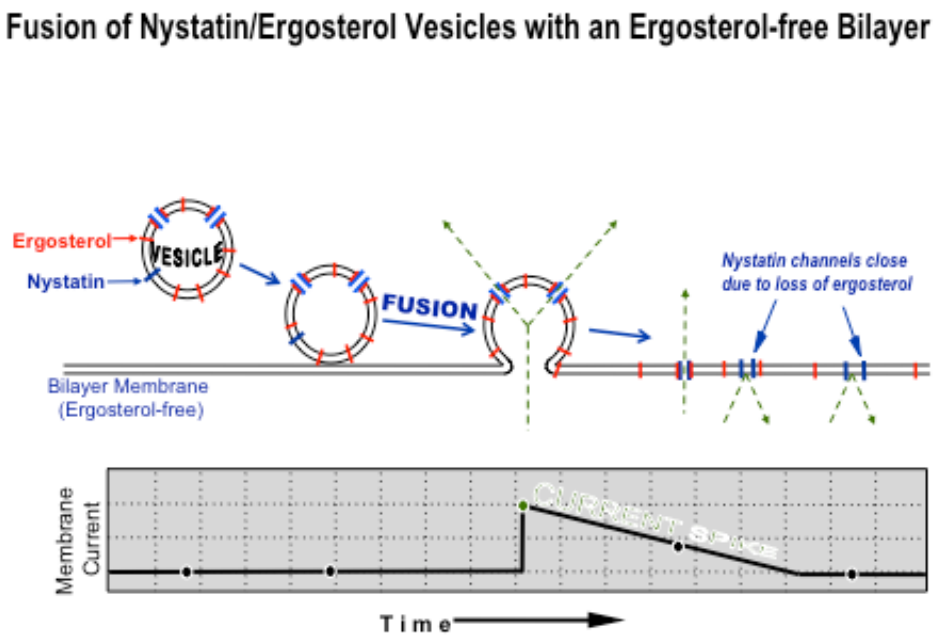


Figure 4: (Reproduced with permission from Dr. Dixon Woodbury, 2000) The fusion of a vesicle with nystatin and ergosterol to a membrane without nystatin or ergosterol. When the vesicle fuses (middle), the conductance of the membrane increases. If there is a voltage across the membrane, the current will spike. After the vesicle fuses, the nystatin channels will close because the bilayer membrane contains no ergosterol.

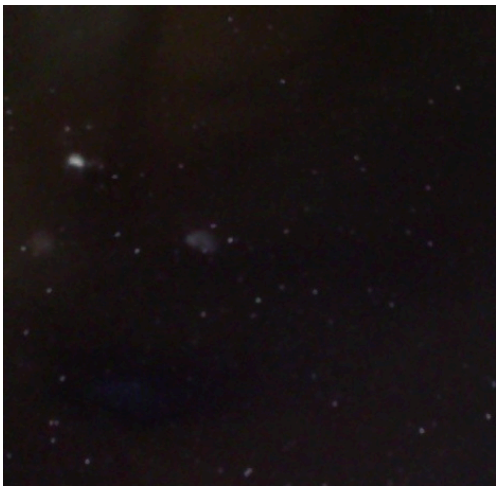
The leakiness of a membrane was determined by applying a voltage across it. If the current did not return back to near zero (low conductance), we would form a new membrane by brushing another bubble across the hole of the cup. Once a stable membrane was formed, we added 100 μL of 3M KCl buffer to the front chamber, let it mix for a few seconds, and removed 100 μL. This was done 3 times to create an osmotic gradient, which helped drive fusion. We then added 15 μL of vesicles to the chamber and allowed them to fuse for at least 6 minutes. When another type of vesicles was to be used, the front chamber was emptied, refilled with fresh solution (150 mM KCl buffer), and another membrane was formed. This allowed us to use a very similar membrane for different types of vesicles. All fusion events were counted, recorded, and that data was analyzed to determine fusion rates.

Results and Discussion

Syntaxin SNARE protein was successfully integrated into vesicle membranes

We hypothesized that by first adding syntaxin to our buffer, the protein would integrate itself into the membranes of the vesicles as the dried lipids also dissolved in the buffer. This was a fairly safe assumption to make because syntaxin has a trans membrane domain (TMD) that interacts with lipids. The TMD allowed syntaxin to mix in with the lipids of the vesicles. We performed a Western Blot on vesicles that had been separated from the solution by centrifugation. This deposited the vesicles as a pellet on the bottom of the centrifuge tube, while lighter molecules remained in the supernatant. While the top and middle portions of the sample

showed no sign of syntaxin, the pellet spot glowed brightly by Western analysis, proving that most or all of the protein had become incorporated into the vesicle membranes. This also suggested that syntaxin was oriented in the correct way. Our method of preparing syntaxin vesicles did not guarantee that the regulatory domain of syntaxin would stick out of the vesicle. However, if the regulatory domain stuck into the vesicles, it would not have been available for the antibodies of the western blot to attach. Thus, the results from our western blot also suggest that syntaxin is oriented in the desired fashion, sticking out from the vesicles.



- 1/10 syx
- 1/40 syx
- 1/100 syx

- pellet
- middle
- top
- ¼ pellet
- ¼ middle
- ¼ top

Figure 5: Results from a western blot against syntaxin of pure syntaxin and syntaxin vesicles. The brightest spot at the top left is a 1/10 dilution of pure syntaxin. Below that spot is a 1/40 dilution and below that spot is a 1/100 dilution, which was too dilute to see. The middle spot is the pellet from the centrifuged syntaxin vesicles. This spot proves that syntaxin was incorporated into the vesicles. Also note that no syntaxin showed up in the top or middle portions of the centrifuged sample, suggesting that a high percentage of the syntaxin added was incorporated.

Another interesting result from our western blot is that none of the top or middle spots showed any signs of syntaxin. This indicates that most, if not all of the syntaxin became integrated into the membranes of the vesicles.

These results are helpful because we now know how to add different proteins to vesicles. In the future we will be able to test the effects of different SNARE proteins by adding them to vesicles and performing *in vitro* experiments.

Syntaxin alone seems to have an impeding effect on vesicle fusion

It has been previously shown that SNARE proteins induce fusion *in vivo* (Finley, et. al., 2002 and Dennison *et al.*, 2006). But what are the effects of a single SNARE protein on vesicle fusion? We tried to answer this question by creating artificial vesicles with syntaxin incorporated into the membranes and measuring their fusion rates to a planar lipid bilayer by a nystatin/ergosterol fusion assay.

Syntaxin in vesicles could affect fusion rates in one of three ways: (1) Syntaxin could increase fusion rates. Perhaps because the protein is sticking out of the vesicles, it interacts with the lipids of the membrane bilayer and sticks to the membrane so that the vesicle becomes stuck to the bilayer. Once stuck, the vesicle might have a better chance of fusing simply because it is closer to the bilayer. (2) Syntaxin may decrease fusion rates. Maybe the strands of syntaxin embedded in the vesicle block it from coming into close enough contact with the membrane for fusion to occur. (3) Syntaxin may have no effect on vesicle fusion. This could simply mean that it really does not affect fusion unless it can interact with other SNARE proteins. VAMP and/or SNAP 25 might need to be present for syntaxin to hook onto and pull a vesicle close enough to a membrane to cause fusion.

We ran each experiment for at least six minutes, counting the number of fusions in each five-minute period. During the first minute, few vesicles have diffused to the membrane. We wanted to know the fusion rate once fusion had

begun, so we counted the fusions during the first one-minute of each experiment separately. For each stable planar lipid bilayer that formed, we alternated which type of vesicles we added first, those with or without syntaxin. This may have been a more important decision than we originally thought because in one set of experiments we added control vesicles, cleaned the chamber, added syntaxin vesicles, cleaned the chamber again, and added control vesicles again. Membrane size was similar in each experiment, but fusion rates changed quite drastically, increasing with each successive experiment. We do not have a legitimate explanation for this phenomenon but perhaps the vesicles were still cold during the first experiment, which caused them to excessively aggregate. They could have warmed by the time we began the later experiment and diffused more easily.

We averaged the fusions per minute for all of the data we obtained for both control vesicles and syntaxin vesicles. The overall average fusion rate for the control vesicles was 4.97, with a standard deviation of 5.35, while the average fusion rate of the syntaxin vesicles was 3.75, with a standard deviation of 2.85. While this data suggests that syntaxin decreases fusion rates by more than 1 fusion per minute, the data is not statistically significant due to the high standards of deviation. Fusion experiments vary a lot from day to day. Some cups allow for more fusions than others. Larger diameter holes in cups provide a larger surface area and more chances for vesicles to stick and fuse to the bilayer membrane. More experiments are necessary to prove that syntaxin by itself hinders vesicle fusion.

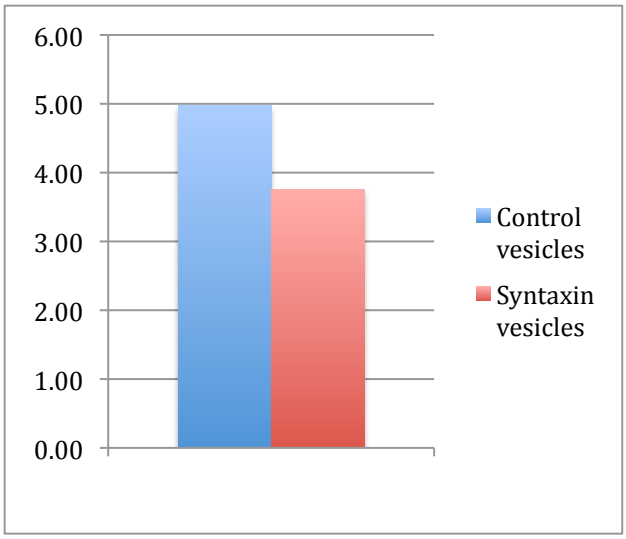


Figure 6: Bar graph of overall average of control and syntaxin vesicles after the first minute of fusion. Though the chart suggests that vesicles that did not contain syntaxin fused more rapidly, the data is not statistically significant.

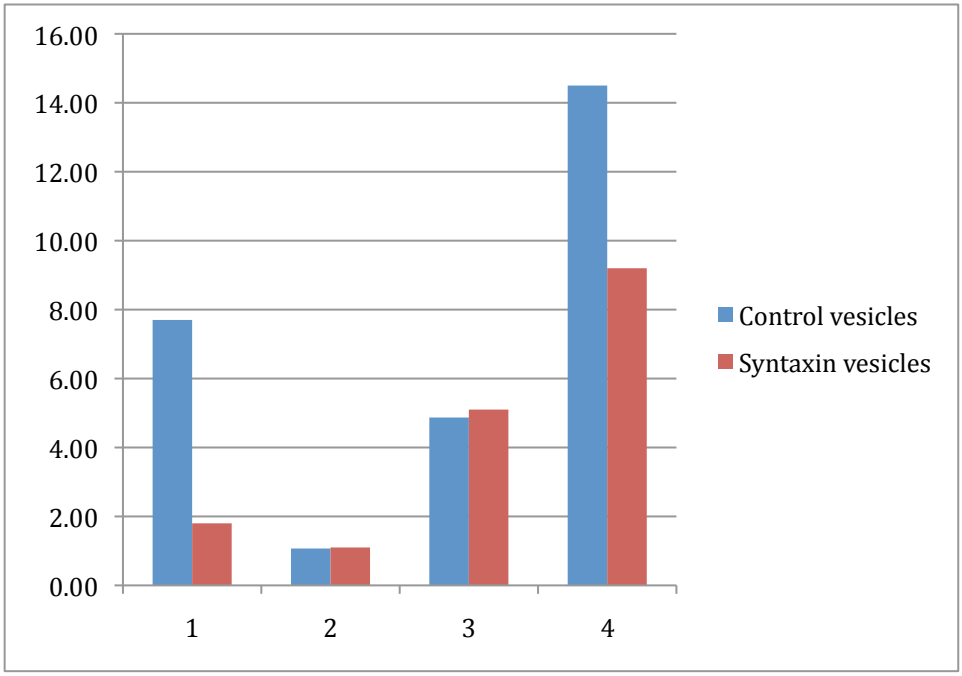


Figure 7: Chart showing average fusions per minute in a given day. The same cup was used for both control and syntaxin vesicles, providing a similar surface area for the vesicles to fuse to. On days 2 and 3, similar fusion rates were measured for both types of vesicles, though on day 2, the cup may have just formed a smaller membrane that did not allow for many fusions at all. Days 1 and 4 show how, despite a similar membrane size, the control vesicles fused much more rapidly after the first minute than the syntaxin vesicles.

Looking at each day's experiments (Figure 5), we see that syntaxin vesicles never fused much better than control vesicles. On two of the days, fusion rates were

both similar, though they were also somewhat low. Day 2 in particular shows a very low rate for both types of vesicles, suggesting that the cup used that day had a small hole and thus a small membrane formed. This would account for the small number of fusions, because a small diameter membrane would not allow many fusions. On days 1 and 4, control vesicles clearly fused more rapidly than syntaxin vesicles, which supports our hypothesis that syntaxin hinders vesicle fusion. However, more data is needed to confirm or disprove our hypothesis.

When analyzing the data from the first minute (Figure 6) of fusion experiments we noticed that for each membrane, fusions increased at similar rates for both types of vesicles. Interestingly, however, the syntaxin vesicles fused more readily during the first minute in every case. We had originally planned to disregard the data from the first minute because it takes time for the vesicles to diffuse into the solution and reach the membrane once they are added to the chamber. This diffusion time is due to the fact that vesicles stick together somewhat when suspended in solution. This is the reason we add PS lipids. PS lipids have a negatively charged head group, so the vesicles will repel each other to some extent when added to the front chamber. Despite the presence of PS lipids in both the control and syntaxin vesicles, it seems that the control vesicles still stuck together more than the syntaxin vesicles. We suggest that syntaxin keeps vesicles from adhering to each other, allowing them to reach the membrane more quickly (smaller particles diffuse faster). Syntaxin does stick to itself to a degree, but only in a parallel fashion. The trans membrane domain of syntaxin would cause it to be orientated in the same direction in every vesicle and this position would most likely

inhibit strands of syntaxin from different vesicles from clinging to one another. Thus it seems likely that syntaxin inhibits vesicles from sticking together. Syntaxin thus helps vesicles diffuse more rapidly and reach the membrane more quickly during the first minute of an experiment.

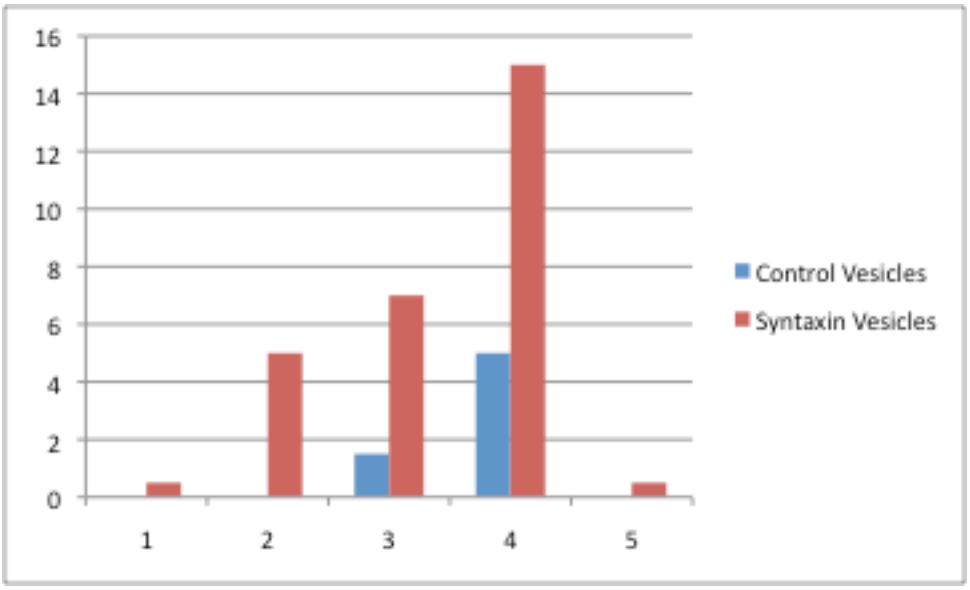
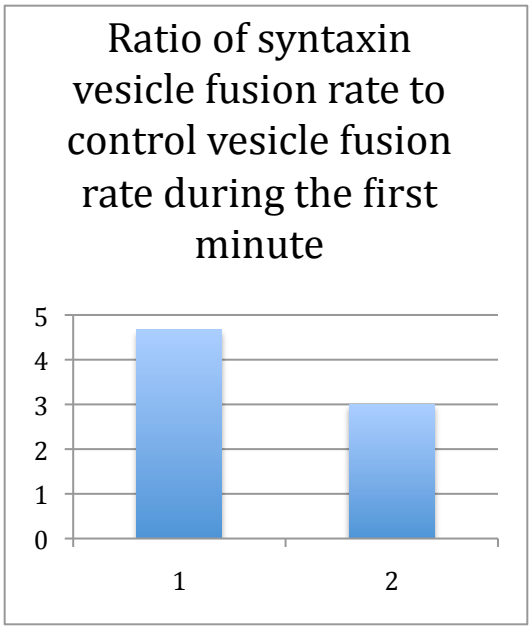


Figure 8: Numbers 1,2,3,4 and 5 represent experiments from different days, using the same cup on a given day for both types of vesicles. Syntaxin vesicles had a higher fusion rate in each experiment, suggesting that they diffused more rapidly after being added to the chamber. This indicates that syntaxin inhibits the aggregation of vesicles when added to solution.



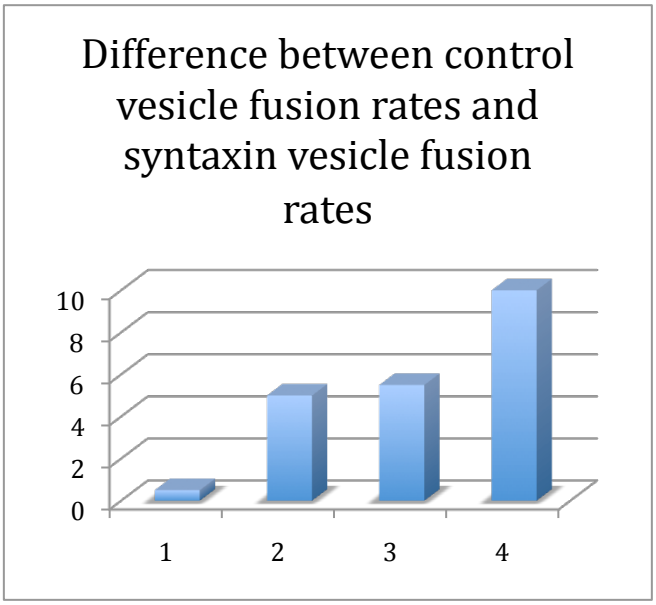


Figure 9 shows the ratio of fusion rates of control vesicles to syntaxin vesicles during the first minute. Column 1represents one day of experiments and indicates that the control vesicles fused at a rate 4.67 times the rate of syntaxin vesicles. Column 2 represents a different day, using a different cup and shows control vesicles fusing at a rate 3 times that of syntaxin vesicles. Figure 10 shows the difference in fusion rates between control and syntaxin vesicles during four days of experiments. Again, each column represents a set of experiments using a different cup. Column 1 shows a fusion rate difference of 0.5 fusions/minute. Column 2 shows a difference of 5 fusions/minute, column 3 represents 5.5 fusions/minute, and column 4 represents a difference of 10 fusions/minute. This data also indicates that control vesicles have a higher fusion rate than syntaxin vesicles.

Conclusion

We have shown that syntaxin can be integrated into artificial vesicles for *in vitro* experiments. This will be helpful in the future, as we will be able to add different SNARE proteins to vesicles to test their effects on fusion. We also acquired data that suggests that in the absence of other SNARE proteins, syntaxin impedes vesicle fusion. This could be due to the regulatory domain of syntaxin repelling the vesicles of the planar lipid bilayer. More experiments are necessary to prove the

hindering effect of syntaxin outright, but the data provided here is a good step forward.

Despite seemingly hindering the overall fusion rate, syntaxin seemed to increase the rate during the first minute of each experiment. This indicates that syntaxin helps vesicles diffuse when added to a solution. Vesicles with syntaxin may not stick to each other as much as they do without syntaxin so they are able to diffuse to the membrane more quickly. The reasons for increased diffusion are probably similar to the reasons that explain why syntaxin decreases overall fusion rates. The regulatory domain of syntaxin must not interact favorably with lipid heads of other membranes. The alpha helices of the Habc domain may contain a high number of polar amino acids that repel the lipid heads of a membrane. This would account for both the increased diffusion of vesicles during the first minute and the slower overall fusion rate after the first minute.

Obviously, there is a lot of work to do if we are to understand SNARE proteins and their functions. This paper has presented data on only one of the SNARE proteins, but we plan to test vesicles with VAMP as well. Eventually, we will try to form an *in vitro* SNARE complex with syntaxin, VAMP, and SNAP25. That data will give us great insights into the workings of the fusion process.

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