SELECTIVE MOVEMENT OF SYNAPTIC VESICLES FROM REGIONS OF THE VESICLE CLOUD TOWARD THE ACTIVE ZONE FOLLOWING SYNAPTIC IMPULSE ACTIVITY

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ABSTRACT

Selective movement of synaptic vesicles from regions of the vesicle cloud toward the active zone following synaptic impulse activity. (May 2013)

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Clouds of synaptic vesicles, which contain neurotransmitter molecules, are distributed along the length of a neuron's axon terminal. A small number of synaptic vesicles are docked on (held at) specialized regions of the presynaptic plasma membrane known as active zones. When a nerve impulse arrives at the axon terminal, the membrane of a docked synaptic vesicle fuses with the presynaptic membrane and releases its neurotransmitter molecules into the synaptic cleft to mediate impulse transmission. The fused vesicle membrane then moves laterally and recycles into a fully functional synaptic vesicle to be used in synaptic transmission again, while a vesicle from the cloud moves to occupy the vacant docking site. I used electron tomography on tissue sections from simply arranged axon terminals at frog neuromuscular junctions fixed at rest and after synaptic activity. The aim of was to determine the spatial distribution of undocked vesicles in the synaptic vesicle cloud with a view to understanding how vesicles move from the cloud to the active zone during synaptic transmission. My results led to the conclusions that when synapses are active in vivo, vesicles in the region of the cloud just proximal to the active zone selectively move to occupy the docking sites on the presynaptic membrane vacated by former

docked vesicles that have fused with the membrane. They, in turn, are replaced by vesicles that move in small groups from an intermediate region in the cloud, and these intermediate region vesicles are replaced more slowly by newly recycled vesicles.

CHAPTER I

INTRODUCTION

Understanding synaptic signaling during neuronal communication is fundamental for deciphering how the brain functions and for developing treatments for neurological disorders and diseases. During neuronal communication, an electrical impulse is transmitted along a neuron's axon. Upon reaching the axon terminal, this impulse is translated into a chemical signal by the release of neurotransmitters at specialized regions on the terminal's plasma membrane called active zones. The transmitter then diffuses to the target cell where it triggers a response. Several methods including biochemical assays, electrophysiology studies, and microscopy have been used to identify chemicals involved in signaling, the magnitude and duration of impulses, and the arrangement of macromolecules at active zones (Nicholls, 2012). In the last century, advances in electron microscopy and particularly electron tomography have greatly improved the spatial resolution of cell structures in situ. Using electron tomography which provides nanometer scale resolution in 3 dimensions, I have imaged axon terminals which contain membranous structures called synaptic vesicles that hold neurotransmitters. Synaptic vesicles form a cloud surrounding the active zone, and with the aid of macromolecules, called active zone material, some dock on and fuse with the presynaptic membrane releasing their contents to mediate synaptic impulse transmission. I used electron tomography on resting and active axon terminals to learn how vesicles in the cloud are recruited to active zones to replace docked vesicles that vacate the active zone after fusing with the presynaptic membrane. From my analysis, I conclude that there are 3 functionally distinct regions of the cloud based on vesicle movement and that the synaptic vesicles move in small, ordered groups. Moreover, I have outlined the pathway synaptic vesicles

travel in the axon terminal as they are utilized in synaptic transmission. Detailed knowledge of how synaptic vesicles behave in axon terminals is a requisite to a comprehensive understanding of the mechanisms that regulate synaptic transmission.

Background

The nervous system is responsible for specified communication between the brain and the rest of the body. Neurons, the cells directly involved in such communication, use processes called axons to send messages to targets. These messages travel along an axon as electrical impulses; however, at axon terminals, messages are often translated into chemical signals (Dale et al., 1936). The highly regulated transmission of chemical signals occurs at a point of contact between an axon's terminal and its target called the *synapse* (Foster and Sherrington, 1897; Lopez-Munoz et al., 2006). My study examines the mechanism of chemical synaptic transmission at the frog *neuromuscular* (neuron to muscle) *junction* because it is a particularly convenient synapse for experimentation.

The axon terminals of a neuron receive electrical impulses from the axon, but they cannot pass this electrical signal on to the muscle fiber because there is a 50 nm gap between the axon terminal and the muscle fiber called the *synaptic cleft* (Alberts, 2010; Hall and Sanes, 1993). Therefore, in order for the signal to be passed on, the message must be converted into a chemical signal that can travel across the synaptic cleft (Dale et al., 1936). At chemical synapses like the neuromuscular junction, chemical signal transmission is initiated by the secretion of *neurotransmitters* at *active zones* (Dale et al., 1936; Ruiz et al., 2011).

Surrounding the active zone in a terminal is a cloud with tens to hundreds of *synaptic vesicles* (Sudhof, 2004). Synaptic vesicles are generally spherical and contain the chemical neurotransmitter molecules that can cross the synaptic cleft following their secretion at active zones (Sudhof, 2004). At neuromuscular junctions, *acetylcholine* is the neurotransmitter used to relay the signal during chemical synaptic transmission (Tansey, 2006). The influx of calcium ions into the axon terminal through *voltage-gated calcium channels* (triggered by the electrical impulse received from the axon) causes the membranes of some of the docked vesicles to fuse with the presynaptic membrane and release their neurotransmitter molecules into the synaptic cleft through the process of *exocytosis* (Katz, 1971).

The fusion of synaptic vesicles at active zones on the presynaptic membrane (the terminal membrane of the neuron) is mediated by a proteinaceous complex called the *active zone material* (Harlow et al., 2001; Szule et al., 2012). The 3 dimensional structure of the active zone material at a frog neuromuscular junction has been well established by prior research in the McMahan Lab (see Figure 1 taken from Szule et al.) (Harlow et al., 2001; Szule et al., 2012). Harlow identified the first individual components of the active zone material and called them the beams, ribs, and pegs (Harlow et al., 2001). Beams are long macromolecules that run parallel to a row of docked vesicles, while ribs are perpendicular to this axis and connect the docked synaptic vesicles to the beams (Harlow et al., 2001). Pegs are small macromolecules that connect ribs to the presynaptic membrane and likely attach to calcium channels (Harlow et al., 2001). Szule followed up Harlow's experiments and fully solved the structure of the active zone material. First, Szule discovered 3 other regions of active zone material which included 6 new active zone components: pins, steps, spars, masts, top masts, and booms (Szule et al., 2012). Pins are

structures that secure synaptic vesicles to the membrane on the side opposite the rest of the active zone material (Szule et al., 2012). Steps run on top of and parallel to the beam and have spars branching out similarly to how beams have ribs protruding outwards (Szule et al., 2012). Projecting upwards from the step is the mast which terminates in multiple top masts; top masts secure undocked synaptic vesicles (Szule et al., 2012). Finally, booms spread out orthogonally from the mast attaching to the middle of the docked synaptic vesicles (Szule et al., 2012). These macromolecules aid in the docking and fusion of synaptic vesicles upon the arrival of an electrical impulse (Szule et al., 2012).

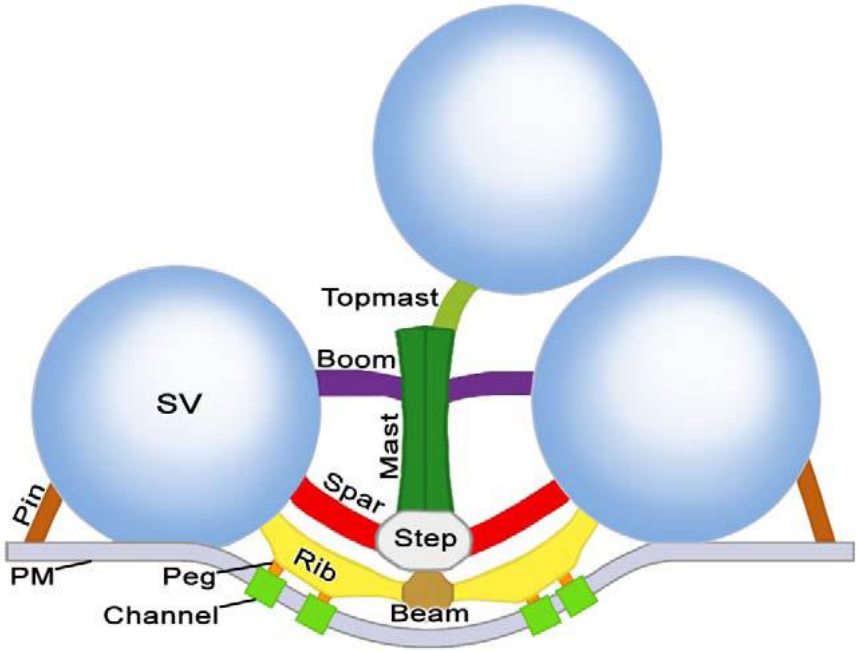

Figure 1. The organization of the frog's active zone material at the neuromuscular junction

Harlow (2001) and Szule et al. (2012) described the architecture of the frog's active zone material at neuromuscular junctions. Synaptic vesicles (SV) dock on the presynaptic membrane (PM) with the aid of the active zone material. Docking involves the successive movement from

topmasts to booms, spars, and then ribs and pins. This figure was taken with permission from Szule et al., 2012.

After vesicles have fused with the presynaptic membrane, they flatten out and move laterally (Heuser and Reese, 1973). They then undergo reuptake into the synaptic vesicle cloud where they become fully functioning vesicles once again (Heuser and Reese, 1973). There are 2 hypotheses regarding vesicle recycling: *full collapse fusion* and *kiss-and-run* (Ceccarelli et al., 1973; He and Wu, 2007; Heuser and Reese, 1973). Heuser and Reese found that vesicle membrane is endocytosed at a location away from the active zone and that a *clathrin* coating aids in the reuptake of the vesicles (full collapse fusion) (1973). Ceccarelli et al. found evidence that vesicles fuse and reform near the active zone without being fully incorporated into the presynaptic membrane (kiss-and-run) (1973). It has been established that the full collapse fusion process observed by Heuser and Reese is the predominate, if not the only, form of recycling in frog neuromuscular junctions (Heuser and Reese, 1973; Morgan et al., 2002).

The cloud of synaptic vesicles surrounding the active zone material is of particular interest for this study. It has been established that there are 3 functional pools of vesicles in axon terminals: the *readily releasable pool,* the *recycling pool*, and the *reserve pool* (Rizzoli and Betz, 2005). The vesicles in the readily releasable pool probably include those that are docked and most likely to fuse when an impulse arrives at the axon terminal (Ruiz et al., 2011). The vesicles in the recycling pool are those that replace the vesicles in the readily releasable pool after fusion, and, by definition, the recycling pool also includes vesicles that have been recently reformed by endocytosis (Rizzoli and Betz, 2005). Finally, the vesicles in the reserve pool are those that move towards the active zone for undergoing fusion upon extreme activation (Rizzoli and Betz, 2005).

No obvious morphological differences have been observed between vesicles in different pools prior to my study (Rizzoli and Betz, 2005).

The miniscule size of the structures located in neurons makes it difficult to study the finer details of synaptic transmission by light microscopy. Transmission electron microscopy on sections cut from fixed and stained tissue has been used for decades to examine sub-cellular structures, but it has limitations in providing information about 3-dimensional layout. In this study, electron tomography was used to observe structures in the axon terminals of frog neuromuscular junctions. Electron tomography involves taking a series of transmission electron micrographs at different tilt angles. These micrographs can then be aligned and reconstructed into a 3-dimensional volume using the computer program EM3D. From this reconstruction, virtual slices can be cut; these slices provide substantially more structural information than conventional electron microscopy. This is possible because the spatial resolution in a typical section examined by electron tomography is 2-3 nm for high contrast structures while there is no information in the depth axis in a section examined by electron microscopy (Ress et al., 1999).

The neuromuscular junction was chosen for examination because of its accessibility and relative simplicity (compared to other junctions in the nervous system). Neuromuscular junctions are easier to access than junctions in the central nervous system because they are not incased in bone. Also, neurons in the brain and spinal cord are often connected to hundreds or thousands of other neurons, but most muscle fibers are connected to only a single neuron (Nicholls, 2012). Furthermore, at neuromuscular junctions, acetylcholine is the only neurotransmitter released; however, multiple neurotransmitters are utilized at other synapses which can add complexity to

interpreting results (Nicholls, 2012). The cutaneous pectoris muscle of *Rana pipiens* (frog) was used for this study because the orderly layout of its neuromuscular junction makes it convenient for experimentation (Harlow et al., 2001). Also, the arrangement of docked vesicles along the active zone material in the frog has been established by previous studies in the McMahan Lab (Harlow et al., 2001; Szule et al., 2012).

The aim of my study was to determine how synaptic vesicles are recruited from the cloud to active zones during synaptic activity. I developed a procedure that used resting frog cutaneous pectoris muscles as controls, while stimulated muscles were used to observe changes in the synaptic vesicle pool population. Images were taken using electron tomography and analyzed with EM3D. I divided the synaptic vesicle pool into bins and analyzed the bins for distribution of synaptic vesicles. The advancement of electron tomography and image analysis techniques has allowed me to examine the synaptic vesicle cloud with a much a higher resolution than has ever been possible and to take measurements in 3 dimensions. From this, I identified 3 functionally distinct regions of the synaptic vesicle cloud and determined that vesicles move in small groups while progressing through the axon terminal. Furthermore, I have confirmed the conclusions of others that recycled vesicles are preferentially recruited to the active zone. Altogether, my findings reveal the pathway synaptic vesicles use in transporting neurotransmitter molecules to active zones.

CHAPTER II

MATERIALS AND METHODS

Sample Preparation

The pair of cutaneous pectoris muscles is located in the frog's chest, and each muscle is individually innervated by its own nerve. The cutaneous pectoris muscles are 2-3 muscle fibers thick which facilitates rapid and uniform fixation. Typically, both muscles of a pair, together with a 5 mm stretch of their nerve, were removed from the frog and pinned out in a Sylgard coated Petri dish. The frogs had been terminally anesthetized in tricaine methane sulfonate (MS-222), and the central nervous system had been pithed. Thus, the nerve to one pinned out muscle could be stimulated while the other remained at rest.

The pinned out nerve-muscle preparations were bathed in Ringer's solution containing 10^{-5} g/ml (+)-Tubocurarine chloride hydrate. The curare blocked acetylcholine receptors in the postsynaptic membrane of the muscle fibers preventing the muscle from contracting in response to the release of acetylcholine from the axon terminal following nerve stimulation. Afterwards, one of the nerves was drawn into a suction electrode and the curare-containing Ringer's solution was replaced with a Ringer's solution containing 0.8 % glutaraldehyde in phosphate buffer (220 mOsM, total); simultaneously, the nerve was stimulated at a frequency of 10 Hz with 10 µA of current for 2 minutes. Previous experiments show that after 2 minutes of stimulation, the synapses were fixed so they no longer transmitted signals. The other nerve-muscle preparation was not stimulated. Once stimulation was completed, the muscles remained in the fixative for 40 minutes. Then the resting and active nerve-muscle preparations were further processed for

electron tomography by fixing and staining them in 1% OsO_4 in phosphate buffer for 1 hour. Following this, the nerve-muscle preparations were rinsed in deionized water, bathed in saturated aqueous uranyl acetate, and then dehydrated in ethanol and propylene oxide. Finally, the nerve-muscle preparations were embedded in plastic (Eponate-12).

Transmission electron microscopy requires that samples are thin enough for electrons to penetrate through it. To account for the thickness requirements, approximately 100 nm thick tissue sections were cut from the muscle using a diamond ultra-microtome. 5 nm colloidal gold particles were deposited on the sections to act as fiducial markers for later analysis.

Data Collection

Electron tomography was used to observe the sections at x 53,000 calibrated magnification on a FEI TECNAI G2 F20 FE-TEM. The stage of the microscope was cooled with liquid nitrogen to reduce shrinkage of the specimen due to the electron beam. Images were taken at 1 degree tilt-intervals ±60 degrees along a single axis.

Data Analysis

Using EM3D, the 2-dimensional micrographs were semi-automatically aligned by a back-projection method using the gold fiducial markers as reference points. Once aligned, the images were reconstructed into a 3-dimensional volume. In this volume, the axon terminal was separated into virtual slices approximately 1 nm thick which is 100 x thinner than the original section.

The mid-slice of each synaptic vesicle was segmented from the volume in EM3D. These slices were then rendered in a surface model, and from this model, the x-, y-, and z- coordinates of the center of each vesicle were obtained. The beam, a portion of the active zone material that runs through the axon terminal between the docked vesicles in frogs, was selected as a reference point for each of the axon terminals. The beam was also segmented throughout the whole volume, and the x-, y-, and z- coordinates were calculated for each virtual slice. These beam coordinates were used to calculate the distance between the active zone material and each vesicle. Only vesicles between 0-630 nm from the beam were used in the analyses. This was done for 4 stimulated datasets, CP48U_1x2, CP48U_2x2, CP48U_3x2, and CP48E_15x2 and 5 resting datasets, CP28_76, CP28_77A, CP28_77B, CP28_86, and CP28_87. These coordinates were used in many of the statistical analyses which are seen in the Results and Discussion.

To determine the volume of each vesicle, the diameter distance in the x-, y-, and z- axes were measured in EM3D. Equation 1 was used to calculate the volumes:

$$\boldsymbol{V = \frac{4}{3}\pi\left[\left(\frac{diameter_{x-axis}}{2}\right)\left(\frac{diameter_{y-axis}}{2}\right)\left(\frac{diameter_{z-axis}}{2}\right)\right]}$$

Equation 1. Vesicle volume

The expected shape of a synaptic vesicle is spherical, and it was noted that the vesicles in the data sets were slightly oblong. Some stretching and squishing occurred during slide preparation, so a correction factor for each axis was applied for each dataset. Because the vesicles are expected to be spherical, it was assumed that radii in the x-, y-, and z- axes were supposed to be the same. The expected radius was calculated for each data set using an average of radii in the x-,

y-, and z- axes. A correction factor in each dimension was calculated for each data set by finding the ratio of this expected real radius to the actual average radius in each dimension (Equation 2 and Equation 3).

$$r_{real} = \left[(r_{x-axis})(r_{y-axis})(r_{z-axis})\right]^{\frac{1}{3}}$$

Equation 2. Expected real radius

$$x \text{ factor} = \frac{r_{real}}{r_{x-axis}}$$

Equation 3. Stretch factor

(comparable equations were used in y- and z- axes)

All of the coordinates were multiplied by their respective calculated correction factors (Figure 2).

	x factor	y factor	z factor
CP48_1x2	0.96163	0.95526	1.0886
CP48_2x2	0.94889	0.94595	1.11408
CP48_3x2	0.9245	0.9393	1.15256
CP48E_15x2	0.93435	0.90595	1.18136
CP28_76	0.92853	0.95776	1.124468
CP28_77A	0.95273	0.95463	1.0995
CP28_77B	0.95273	0.95463	1.0995
CP28_86	0.921572	0.910242	1.192103
CP28_87	0.9184	0.8877	1.2267

Figure 2. Correction factors in x-, y-, and z- axes

In the statistical analyses, the synaptic vesicle cloud was divided into bins. The 5 bins used were 0-150 nm, 150-270 nm, 270-390 nm, 390-510 nm, and 510-630 nm. They were selected after

preliminary observations. Due to slight irregularities in the shape of the presynaptic membrane, there were occasionally differences in the volumes of the bins for different axon terminals. In order to account for differences in bin sizes, the volume of each bin was calculated. To find the volume of a bin, it was segmented on a single slice of the volume using EM3D. This slice was then rendered and the surface area was calculated. The surface area was then multiplied by the thickness of the slide to obtain the volume for the bin.

From these bins, 3 regions of the synaptic vesicle cloud became apparent: the proximal, the intermediate, and the distal region. The proximal region is the innermost region and extends in a 150 nm radius from the beam. The intermediate region covers from 150-390 nm in the synaptic vesicle cloud. Finally, the distal region includes from 390-630 nm. Figure 3 shows these 3 regions in a 2-dimensional electron micrograph.

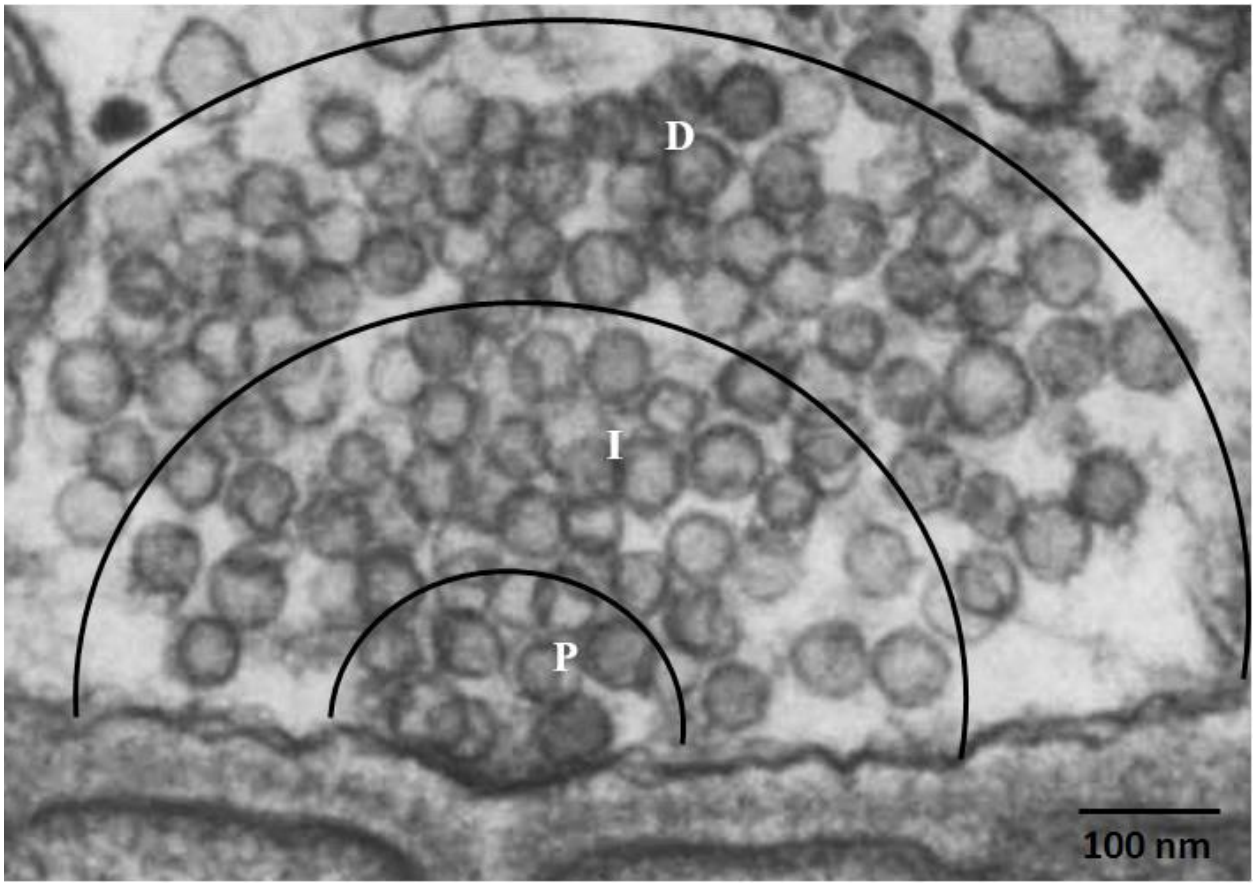

Figure 3. Regions of the synaptic vesicle cloud

This is a 100 nm thick 2-dimensional electron micrograph of a tissue section from the axon terminal of a frog neuromuscular junction showing the 3 regions in the synaptic vesicle cloud. The 3 regions of the synaptic vesicle cloud are the proximal (P) (0-150 nm), the intermediate (I) (150-390 nm), and the distal region (D) (390-630 nm).

Using the bin volumes, the vesicle number density in each bin was calculated. Vesicle number density was equal to the total number of vesicles in a bin divided by the volume of that bin. Finally, the distance between each vesicle and the closest neighboring vesicle (termed "nearest neighbor") was calculated using the coordinates already obtained from the virtual slices. These calculations are included in the Results and further addressed in the Discussion.

CHAPTER III

RESULTS

I made 3 main observations after examining tissue sections from stimulated and resting axon terminals of frog neuromuscular junctions. First, the number density of synaptic vesicles in the proximal region, containing docked vesicles and vesicles associated with docked vesicles, did not change after axon terminal stimulation. Similarly, the density of synaptic vesicle in the distal region also did not change after stimulation. However, the density of synaptic vesicles in the intermediate region declined by 40% after stimulation while the nearest neighbor distances for vesicles in this region remained the same as in resting axon terminals. Finally, vesicles in stimulated axon terminals have significantly larger volumes than those in resting terminals, and particularly, vesicles in the intermediate region are substantially more swollen.

Number Density

The proximal and distal regions show no significant change in density after axon terminal stimulation. However, in the intermediate region of the synaptic vesicle cloud, there is a 40% decline in the density of synaptic vesicles. Figure 4 shows the results for the density of synaptic vesicles throughout the synaptic vesicle cloud for both simulated and resting axon terminals, and Figure 5 shows this trend in virtual slices obtained from EM3D.

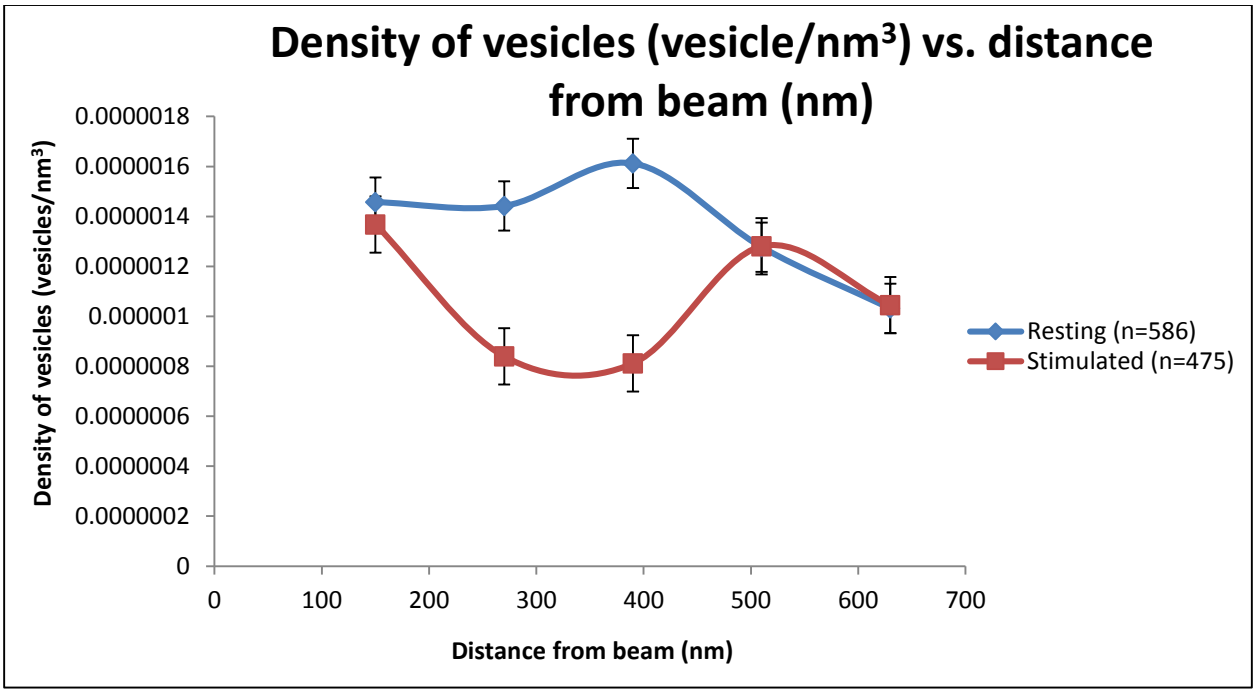

Figure 4. Number density of vesicles throughout the synaptic vesicle cloud in resting vs. nerve-stimulated frog axon terminals

The data have been organized into 5 bins according to distance from the active zone material's beam, and the vesicle density of that bin is shown on the y-axis. The first pair of points represents the density of vesicle in the proximal region of the cloud (within 150 nm) which is the same before and after nerve stimulation. On the right, the final 2 pairs of points show the density of vesicles in the distal region (390-630 nm) which is also comparable before and after stimulation. The second and third pairs of points represent the intermediate region (150-390 nm) which undergoes a 40% decline in density upon nerve stimulation. The bars represent standard error.

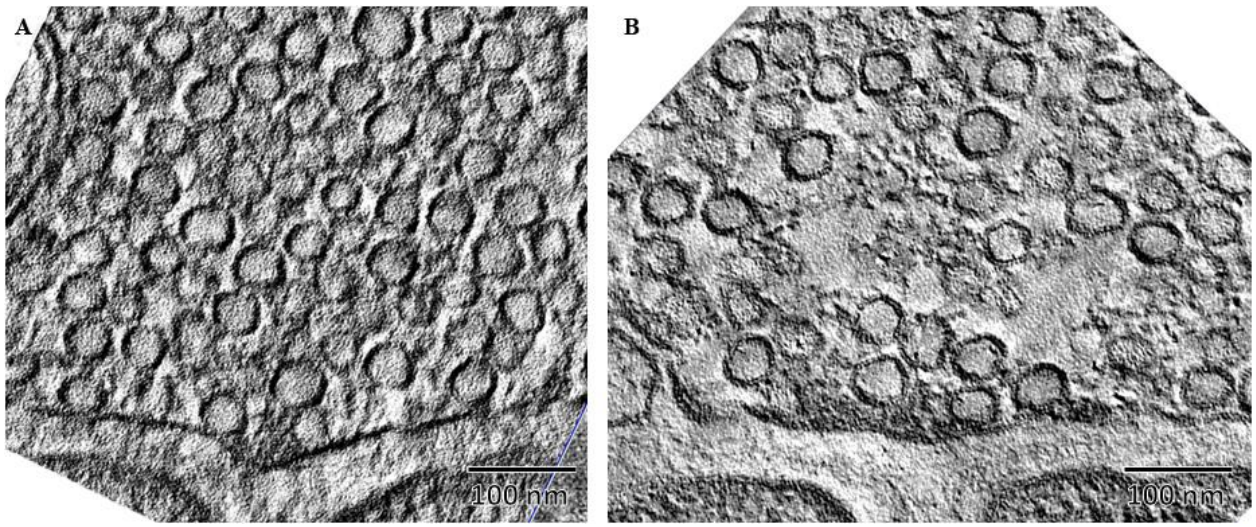

Figure 5. Virtual slices showing synaptic vesicle density in the vesicle cloud before and after axon terminal stimulation

The images are 1 nm thick virtual slices obtained from EM3D. In the resting axon terminal on the left (A), the density of synaptic vesicles in the cloud is constant throughout the axon terminal. In the nerve-stimulated axon terminal on the right (B), there is a marked decline in the synaptic vesicle density in the intermediate region of the cloud while the densities in the proximal and distal regions are comparable to their counterparts in resting axon terminals.

Nearest Neighbor Distance

Next, I found that the distance between a vesicle and its nearest neighbor is the same in resting and stimulated axon terminals. This distance was termed the nearest neighbor distance. Figure 6 shows the trend that the normalized nearest neighbor distances are statistically the same for resting and stimulated axon terminals.

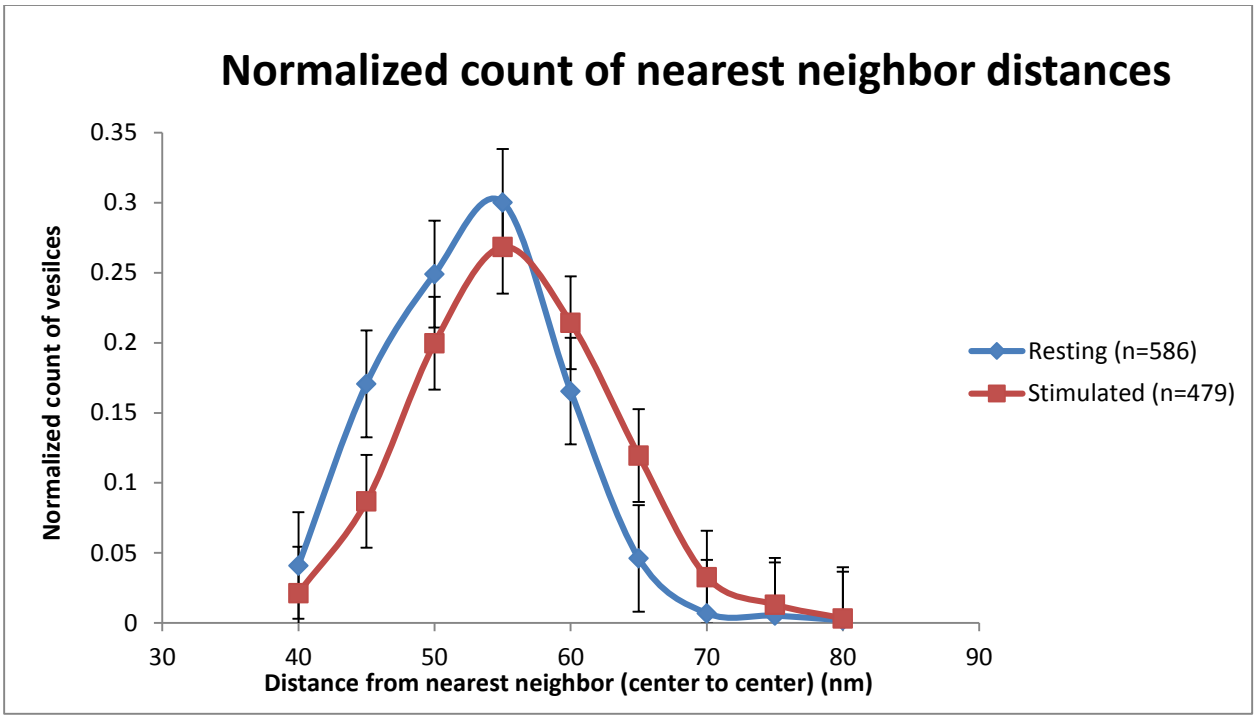

Figure 6. Normalized count of nearest neighbor distances

The figure shows that the normalized distribution of nearest neighbor distances is statistically the same for resting and stimulated axon terminals. The bars represent standard error.

I then analyzed these nearest neighbor distances as a function of distance from the beam and found that nearest neighbor distances are the same throughout the synaptic vesicle cloud in resting and stimulated axon terminals. Figure 7 shows this trend for resting axon terminals and Figure 8 shows the same for stimulated axon terminals. With both of the slopes being essentially 0, the graphs show that distance from the beam does not play a role in determining the proximity of the nearest neighbor. The y-intercepts from the 2 graphs appear different, but Figure 6 already indicated that the nearest neighbor distances are statistically the same.

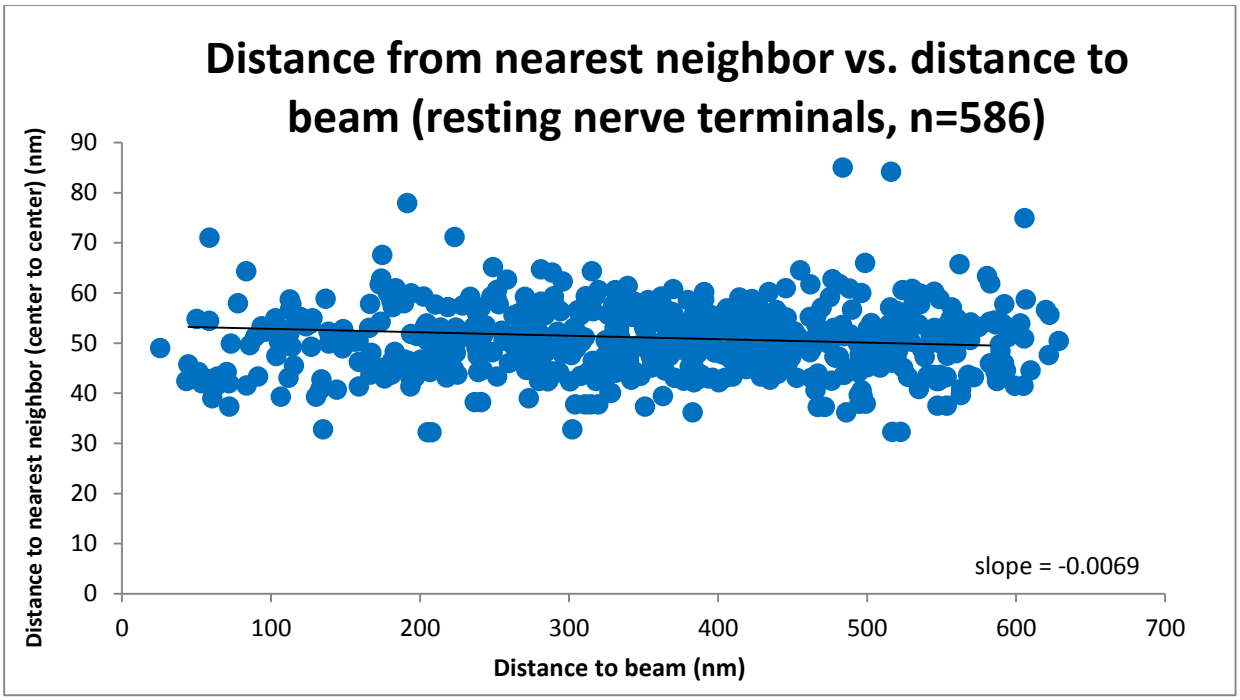

Figure 7. The distance of the nearest neighbor vs. distance to the beam in resting frog axon terminals

The figure shows that the nearest neighbor distance is not correlated with distance to the beam in resting frog axon terminals indicating that the nearest neighbor distances are the same throughout the synaptic vesicle cloud.

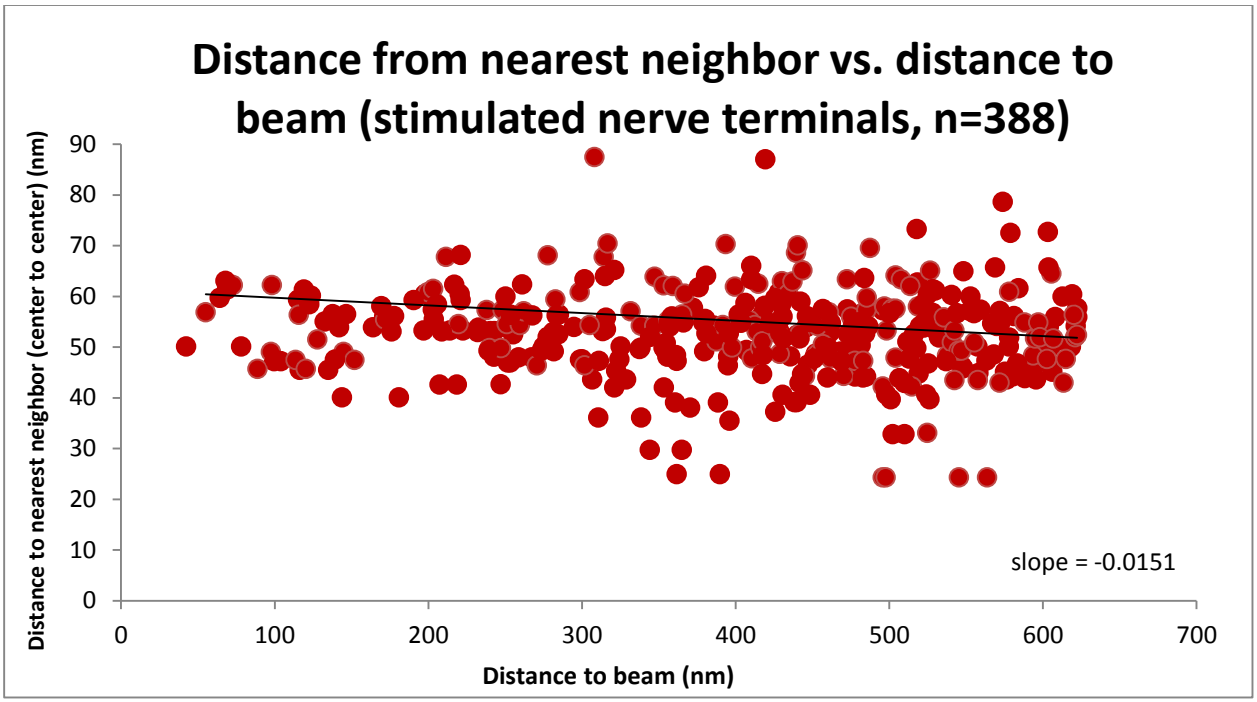

Figure 8. The distance of the nearest neighbor vs. distance to the beam in stimulated frog axon terminals

Similar to resting axon terminals, nearest neighbor distance does not relate to the distance from the beam in stimulated frog axon terminals.

Vesicle Volume

Finally, I measured the luminal volume of vesicles throughout the synaptic vesicle cloud and determined that synaptic vesicles are larger on average in stimulated axon terminals than in resting axon terminals (Figure 9). The average increase in volume was greatest in the intermediate region of the cloud (Figure 9). The overall increase in vesicle volume was due to some vesicles having the same volume as vesicles in the resting terminal while others had a much greater volume (Figure 10).

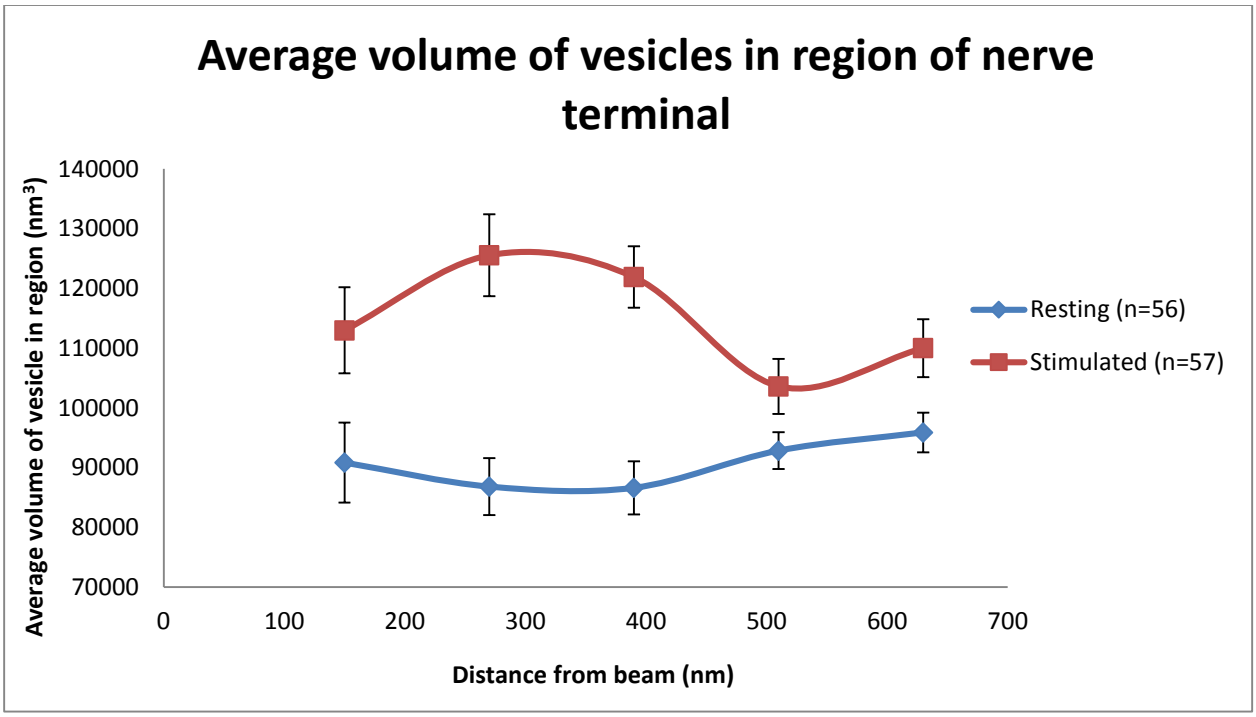

Figure 9. Average volumes of vesicles in regions of the synaptic vesicle cloud in resting and stimulated axon terminals

Synaptic vesicles in the vesicle cloud of stimulated axon terminals are significantly larger on average than those in resting terminals. In particular, vesicles in the intermediate region show a marked increase in volume. The bars represent standard error.

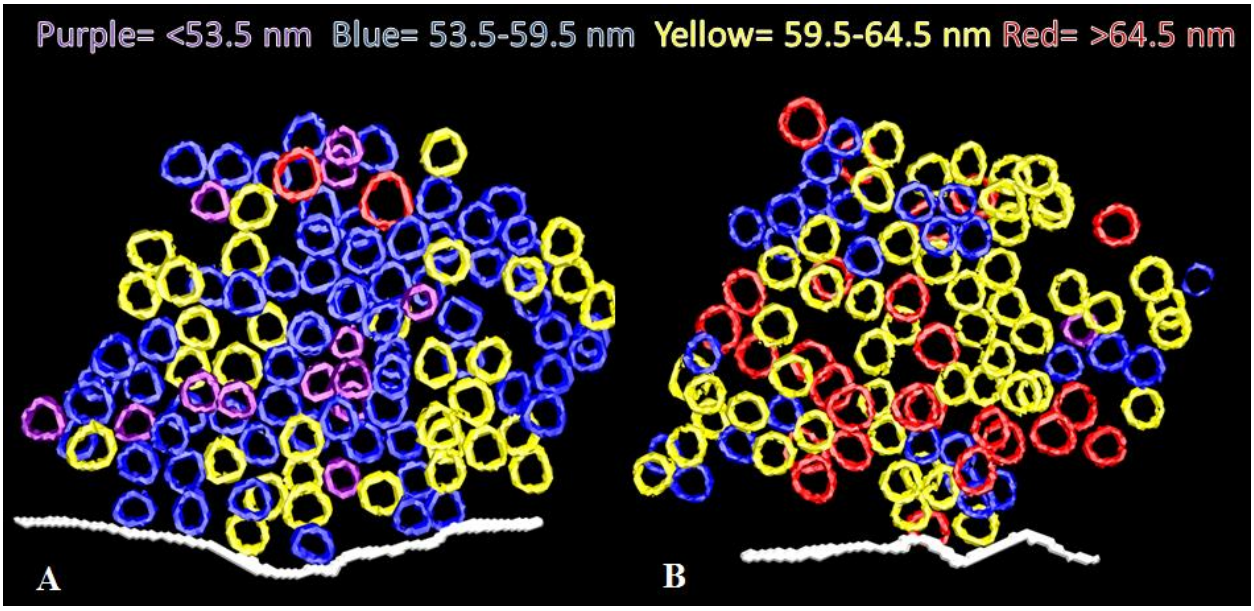

Figure 10. Diameter of synaptic vesicles in the synaptic vesicle cloud in resting and stimulated axon terminals

This figure shows two 100 nm thick tissue sections. On the left (A) is a resting axon terminal, and on the right (B), a stimulated axon terminal. The circles represent the mid-slice of synaptic vesicles in the cloud taken from virtual slices created in EM3D and the white line is the presynaptic membrane. The color of the circle designates the size of the vesicles. In resting axon terminals, there are more of the smaller vesicles represented by purple and blue circles. In stimulated axon terminals, there are significantly more of the larger vesicles which are represented by yellow and red circles. In particular, there are a substantial number of swollen vesicles in the intermediate region of the synaptic vesicle cloud in stimulated axon terminals.

To summarize, the data showed that there is a decline in the number density of synaptic vesicles in the intermediate region in stimulated frog axon terminals, yet there is no change in the proximity of the nearest neighbor anywhere in the axon terminal. Synaptic vesicles in the vesicle cloud of stimulated axon terminals have a significantly greater volume than those in resting axon

terminals with the most substantial difference seen in the intermediate region of the synaptic vesicle cloud.

CHAPTER IV

DISCUSSION

Prior research has explored the nature of the synaptic vesicle cloud. Rizzoli and Betz described 3 functionally distinct pools in the cloud based on electrophysiology studies (2005); however, they did not locate these pools in specific regions. My study explored regions of the synaptic vesicle cloud in the axon terminal of a frog neuromuscular junction by electron tomography. I made 3 main observations after stimulating frog axon terminals at a rate of 10 Hz for 2 minutes. First, the density of synaptic vesicles in the proximal and distal region of the synaptic vesicle of cloud was constant after stimulation. Second, despite the density of synaptic vesicles declining by 40% in the intermediate region after stimulation, the nearest neighbor distances are constant. Finally, many of the vesicles in stimulated terminals were larger than those typical of resting terminals and in particular in the intermediate region. From these observations, I conclude that the vesicles from the proximal region of the vesicle cloud replace vesicle that have fused with the membrane. Subsequently vesicles are selectively recruited from the intermediate region of the cloud to replace those removed from the proximal region. The density of the intermediate region is then slowly restored primarily, if not entirely, by recycled vesicles.

I first observed a decline in synaptic vesicle density in the intermediate region of the cloud after axon terminal stimulation while the density of the other regions remained constant. The functional advantage of such constancy is that the axon terminal has to remain poised for transmission; axon terminals still function even at levels of stimulation much more extreme than normal physiological conditions. Because the density of the proximal region is constant, vesicle

must be moving in from nearby. It is possible that newly recycled vesicles are sustaining the density of the proximal region; however, Heuser and Reese found the recycling occurs away from the active zone (1973). From observing this significant reduction in vesicle density in the intermediate region of the cloud, I concluded that vesicles are selectively recruited from this region of the vesicle cloud to the proximal region during synaptic transmission. My next focus became determining whether vesicles are removed uniformly or in groups from the intermediate region.

In order to determine how vesicles are removed from the cloud, I looked at vesicles and their nearest neighbor. I found that resting and stimulated axon terminals have the same nearest neighbor distances; therefore, the grouping of vesicles is constant after nerve stimulation. I then looked to see how nearest neighbor distances change as a function of distance from the beam, and I found that vesicle nearest neighbor distances were not dependent on distance from the beam. Therefore, the grouping of synaptic vesicles is the same throughout the vesicle cloud in both stimulated and resting axon terminals.

In stimulated axon terminals, it is easier to see the grouping of vesicles. The vesicles in resting axon terminals appear to be interconnected multiples times; however, due to the lower density in stimulated axon terminals, it is possible to see vesicles linked to only 1 or 2 other vesicles. One way that I often saw vesicles grouped in stimulated axon terminals is in chains. Figure 10 shows an example of a chain of 5 vesicles terminating at the presynaptic membrane linked together by 5-10 nm long filaments.

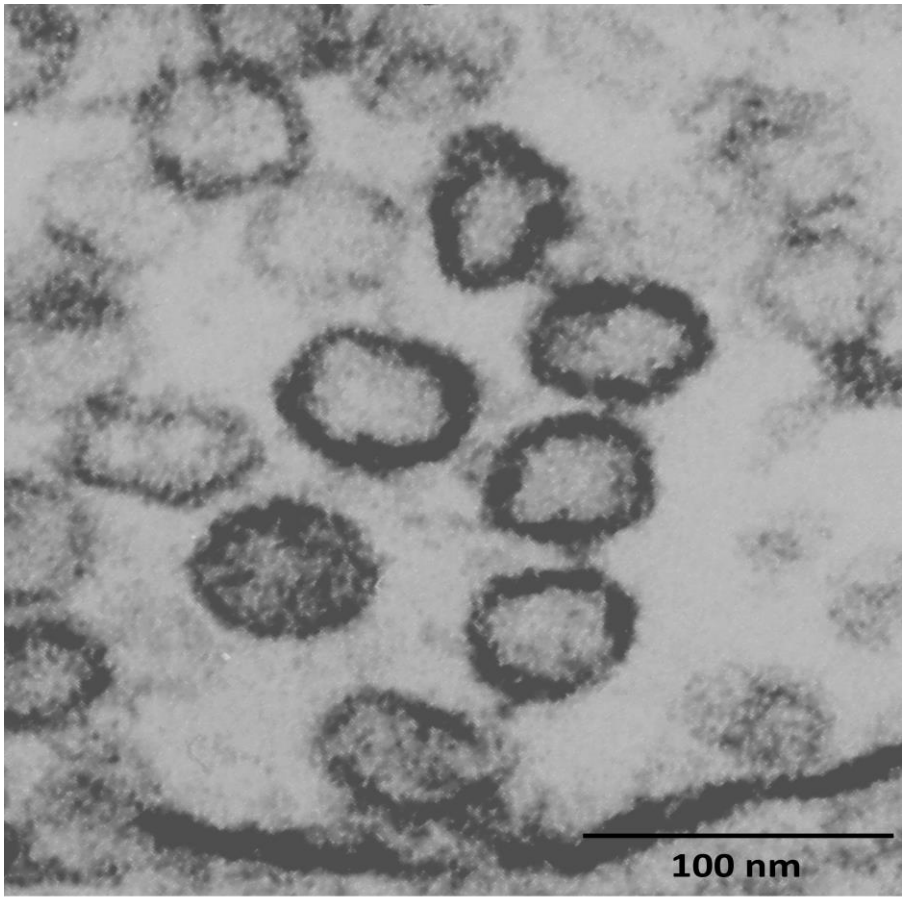

Figure 11. Chain of synaptic vesicles

The figure shows a vesicle chain in a tissue section through an axon terminal of a frog neuromuscular junction imaged by conventional 2-dimensional electron microscopy. The chain contains 5 synaptic vesicles and extends to the presynaptic membrane. There are 5-10 nm long filaments linking the vesicles together.

From this information, I concluded that vesicles move from the intermediate region of the synaptic vesicle cloud to the active zone in groups. After determining this, I sought another way to identify the intermediate region of the vesicle cloud. I found that throughout the synaptic vesicle cloud the volume of vesicles are larger in stimulated axon terminals; however, there is a

particular increase in volume in the intermediate region of the vesicle cloud. My hypothesis for why there are larger vesicles in this region is that the swelling of synaptic vesicles is a step in vesicles refilling with neurotransmitter molecules.

From these observations, I conclude that fused vesicles are immediately replaced by adjacent vesicles in the proximal region of the synaptic vesicle cloud. The vesicle density of the proximal region is maintained by the selective recruitment of synaptic vesicles in groups from the intermediate region of the vesicle cloud. The vesicle density of this region then recovers by the recycling of synaptic vesicles back into the cloud. However, there is a decline in vesicle density in this region because vesicles are not recycled as quickly as they fuse and vesicle membrane builds up in the presynaptic membrane.

Therefore, I have outlined 3 region of the synaptic vesicle cloud by describing anatomical differences. These 3 regions may correspond to the 3 pools from the electrophysiology studies done by Rizzoli and Betz (2005). The proximal region very likely relates to the readily releasable pool, the intermediate region to the recycling pool, and the distal region to the reserve pool. The closest region has the highest turnover rate with vesicles fusing at the active zone and releasing their contents. I found then that there is a 240 nm band where vesicle recruitment and recycling likely occurs. It is probable that the specific size of this intermediate region is related to my stimulus paradigm and that under different condition, this region shrinks or expands. Finally, in the distal region, there are few changes observed after stimulation which is consistent with how the reserve pool described by Rizzoli and Betz would behave (2005).

I have 2 aims for future research. I plan to test directly whether synaptic vesicles are recruited from the intermediate region of the cloud in chains, as I have proposed, by using electron microscope labeling techniques. I will use horseradish peroxidase to produce an electron dense pigment that can be endocytosed during vesicle recycling. This will allow me to see how such vesicles are grouped in the vesicle cloud. Second, I will determine whether the swollen vesicles that appear predominantly in the intermediate region of the vesicle cloud during recycling are undergoing neurotransmitter reloading by exposing the axon terminals to vesamicol which is known to block reloading. This will provide insight into the process of vesicle reloading.

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