Synaptic Ultrastructure and the Recycling Vesicle Pool: Characterized using a Novel Ultrastructure - Function Approach

LIFE SCIENCES PROJECT

Submitted for the degree of:

Bachelor's in Neuroscience

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University of Sussex, 2021

Word Count: 4544

Abstract

Synapses are critical for information transmission and synaptic vesicle pools are likely to be key substrates for regulation; understanding principles of vesicle pool organization and their relationship to transmission is therefore important, but challenging. Here we exploit Focused Ion Beam Electron Microscopy (FIBSEM) to gain a high-resolution view of function and ultrastructure at unprecedented resolution, which will allow us to use depth of image to scan through hippocampal tissue at the nanoscale. Using this approach, we image the recycling pool using FM1-43 on recently recycled vesicles, which are then photoconverted to reveal fundamental relationships of the recycling pool between neighbouring synapses (those sharing a common axon) including their size, structure and other characteristics involving the axon they reside in. The ultrastructure of these synapses will be reconstructed making use of the high-resolution z-plane to highlight the positioning of the recycling pool both within the synapse and potentially along the axon (extra-synaptic vesicles). Our findings show a preferentially segregated recycling vesicle pool towards the active zone, along with key similarities in those sharing an axon. We suggest mitochondrial functions may be key regulators for vesicular function. Our approach also provides a new perspective on the extra-synaptic "superpool", capable of vesicular turnover between synapses for further vesicle fusion and neurotransmitter release. The heterogeneity in these parameters reflects the complex role of synapses as loci capable of setting synaptic weight in plasticity. Furthermore, these positions may possibly have a role as targets in treatment for the pathogenesis of neurological disorders such as Parkinson's Disease, recently linked to vesicular dysfunction.

Introduction

Synapses are small specialized structures that connect neurons together. These consist in a presynaptic terminal and a postsynaptic terminal. The presynaptic terminal contains vesicles filled with neurotransmitter that fuse with the active zone once voltage gated ion channels are activated and release calcium ions within the terminal (Südhof and Rizo 2011; Südhof 2012). There are functionally different sub-pools within vesicle pools to determine synaptic performance (Sudhof, 2004). The fusion between vesicle and membrane occurs through SNARE proteins which bind to the membrane and to the vesicle on two points. The snares will twist to push the two membranes together and release the embedded neurotransmitter (Rizo and Rosenmund, 2008). The first vesicles to be released are what are known as the readily releasable pool (RRP). Once the neurotransmitter is released in the synaptic cleft, it will bind to postsynaptic receptors on the other end to propagate a signal, in the form of action potentials. Once the RRP is depleted, continued release occurs through a secondary vesicle pool called the recycling pool (Stevens and Williams, 2007) which consists in vesicle formation through the neuronal membrane. The term "synaptic vesicle recycling" was coined by Heuser and Reese (1973) and is what we will focus on here. The presynaptic terminal has various structures for different functions. In this practical, hippocampal CA3-CA1 synapses will be studied through the analysis of vesicle pool properties. These properties determine the function of vesicles (recycling, resting, readily releasable, docked, spontaneous and superpools) and can

be studied in different synapses. The organization of vesicle pools is believed to be of importance in synaptic strength tuning (Alabi and Tsien, 2012). This is seen through the different states of vesicle organization: in the basal state, vesicles are organized randomly but are modulated when signalling demands change for, for example, learning or adaptive adjustments. These are also organized differently in a diseased state, showing that the organizational properties of synapses are linked to functional properties.

The study of vesicle organization is thus crucial to study synapse function, but presents several problems. Synapses can be imaged using fluorescent proteins/dyes (such as FM1-43) since their size makes it impossible to view with a microscope (Iwabuchi et al., 2014). However, image diffraction is limited (Huang et al., 2010); in other words, the resolving power of microscopes is limited by the wavelength of light and the numerical aperture of the objective; in most cases, a structure as big as 500nm would show little to no detail (a vesicle can be as little as 30nm). To tackle this problem, transmission electron microscopes (TEM), which use short-wavelength electron beams, can be used to get a resolution of 0.3nm (Franken et al., 2017). Once again, however, there are a few problems with this technique. First off, for TEM to work, the tissue must be fixed, dehydrated and embedded in resin before being viewed which will impact the study of functional events. Furthermore, this technique slices tissue in 70nm sections, which will cause most of the 3D structure to be lost within each section. To get around this problem, we use both functional readouts of synapses and automated 3D ultrastructure view. This is known as functional connectomics and once again uses FM1-43 dye to distinguish between recycling and resting vesicles. Once the tissue is prepared, a technique known as FIBSEM (Focused Ion-Beam Scanning Electron Microscopy) is used to collect tiny sections which will make it possible to scan up and down a tissue block and view various structures, and it will also maintain the X-Y plane in each section to make the reconstruction of a 3D structure possible (see Methods). The data can then be easily read out and functional vesicles can be characterized and validated. The image stacks will most likely show many presynaptic vesicle pools adjacent to a protein-rich and electron-dense active zone (Schikorski and Stevens 1997) making it easy to study various properties including vesicle positioning compared to the active zone, along with other synaptic vesicle properties such as total vesicle pool size, functional pool size, extra-synaptic vesicles (vesicles along the axon, also known as the superpool), etc.

Methods

FIBSEM is used to collect 6-9nm sections of a tissue block, to create an image stack perfectly aligned to make 3D structures available to view on a nanoscale. This technique uses FM1-43 dye. This dye is included in the extracellular solution of neurons which, once washed, will stick to the outer plasma membrane. When synapses are stimulated and vesicles are recycled from the neuronal membrane, they will take this dye up. After the samples are washed and fixed with PFA+GA, they can be photoconverted using illumination to drive oxidation of DAB (diamino benzine) to an electron-dense form. Only vesicles which took up dye will turn dark (electron-dense vesicles), meaning these dark vesicles will represent recycled vesicles. After

preparing the sample, we use FIBSEM (Focused Ion-Beam Scanning Electron Microscopy). This is a variation from a conventional electron microscope as an entire tissue block is placed on the vacuum of the electron microscope. Instead of generating different sections, we read out the signal from the surface of a sample.

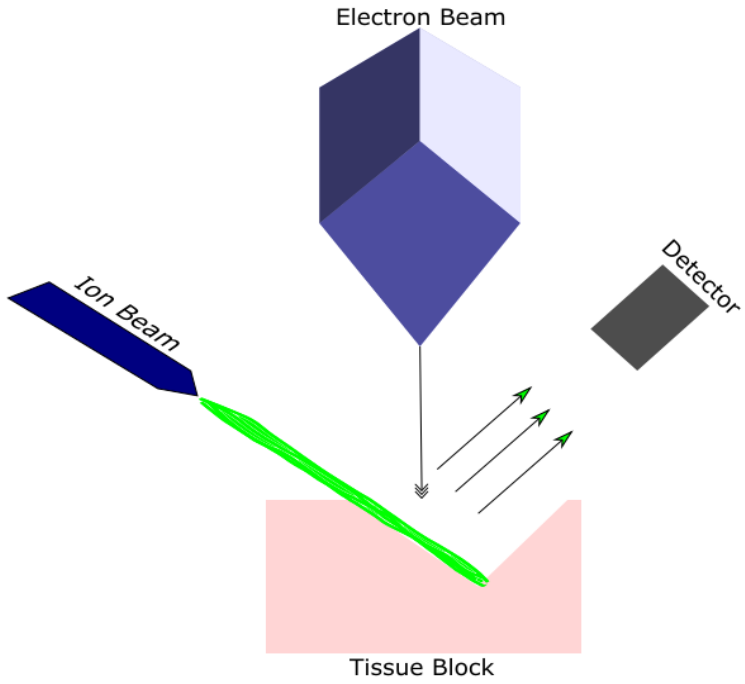

Figure 1: FIBSEM process: Electron firing (using electron beam)-> electrons backscattered detected (using detector) -> layer burned (through use of ion beam)-> repeat (Figure adapted from Staras Labs)

Electrons are fired using an electron beam; the detector collects the backscattered signal (green arrows), an ion beam burns a layer of tissue off (green beam) and the whole process is repeated until the tissue is completely scanned (figure 1). Each layer burned off by the ion beam is about 6-9nm thick, meaning the sections collected are very small. Furthermore, the sample will stay on a perfectly aligned X-Y plane, so that every section is aligned to the previous one to maintain an accurate 3D structure. The stack sections can be used to go through the tissue block using dedicated software which will allow us to see many different structures including synapses and pool size. Structural parameters will then be measured to quantify the vesicle pool and a 3D reconstruction can be formed, which is what we call functional connectomics.

After collecting all the Image stacks, FIJI software was used to analyse vesicle and synapse organization within the tissue block. Firstly, vesicles were characterized between dark (recycling) and empty (non-recycling) vesicles. Grey value intensity within FIJI was used to plot profiles for different vesicles. For each pixel, a value for grey intensity was calculated and the process was repeated for 5 vesicles in each category (dark and clear). Average values for each pixel were then calculated and plotted to show mean profiles of grey values though different vesicles. The lower the value, the darker the pixel. The clear difference between the plots would differentiate between empty and full (clear and functional/recycling) vesicles.

After the characterization was complete, functional vesicle pools were defined. This consisted in finding synaptic terminals with both functional and clear vesicles within the image stack which also included an active zone linking the presynaptic terminal to a postsynaptic structure. By scanning through the image stack, the axon connected to the synapse could be followed to find other synapses within the same axon. If one was present within the stack, then the axon

would qualify. Once found, the two synapses would then be compared using different parameters such as total vesicle number, dark/clear vesicle ratio, mitochondrial size/volume, etc. These parameters could be used to find conclusions on correlations between linked synapses.

The FIJI software was then used to recreate synapses within the stack and collect data from them. So, for each axon, three categories were formed: dark vesicles, clear vesicles and active zone. The software enables the user to add "balls" within the image at different sections in the stack. Each dark and empty vesicle was then recreated by placing these balls within the image stack and the middle of the active zone was also characterized by another ball. Once all of these were specified, the software was used to extract data on the positioning of each one of the vesicles within the stack. Moreover, once all balls were placed, FIJI enabled the 3D reconstruction of the synapse, giving a clearer view of a certain synapse to then be used to extract more information out of the organization of the vesicle pool. The same technique was used to recreate superpools throughout an axon and thus even made it possible to reconstruct an entire axon linking different synapses together.

RESULTS

Vesicle Characterization

To define the functional vesicle pool, along with confirming the quality of the experimental sample, vesicles were characterized between functional (electron-dense) and non-functional (clear). Using FIJI, electron dense vesicles will look dark and will represent the vesicles that have recently been recycled from the membrane. We expect these vesicles to thus have lower grey intensity towards the middle due to their dark nature, while the rest of the vesicle pool will look clear and thus have lower grey intensity on the outside (the membrane) compared to the middle.

Five clear vesicles were selected, and grey intensity values were measured. Averages for each pixel in these 5 vesicles were calculated and plotted, as seen in figure 2. The higher grey intensity values towards the middle of the vesicles represent a clear centre, thus meaning these are not electron dense and thus have not been recently recycled. Five darker vesicles were selected and the same process was repeated. Here, the grey intensity values drop towards the middle, representing a darker shade, meaning these are electron dense and thus have been recycled. The relative difference of grey intensity values towards the middle of the two classes of vesicles ($p < 0.005$, two-tailed paired t test, $n = 5$) helps us quantify and characterize the vesicle pool.

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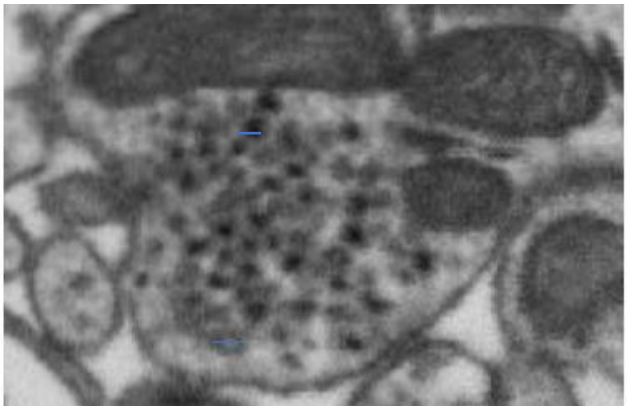

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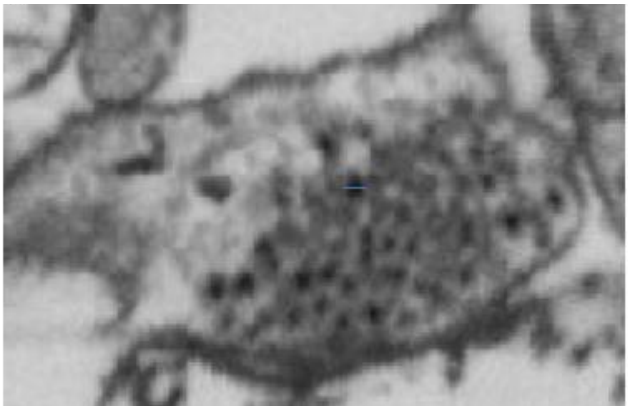

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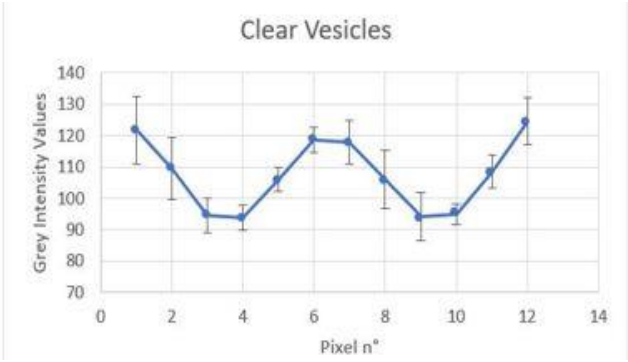

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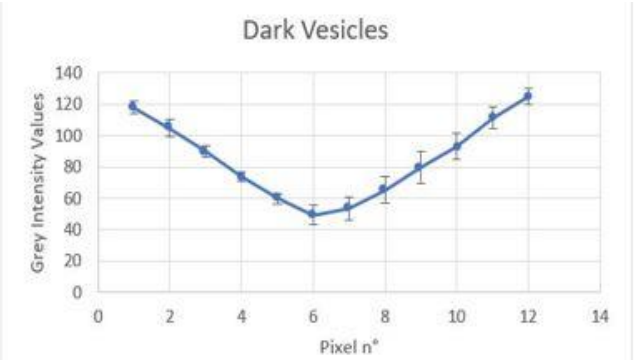


E

Clear Vesicle Pixel n°	Average Grey Intensity
0	121.776
1	109.4585
2	94.50028
3	93.77832
4	105.9505
5	118.5198
6	117.9979
7	106.1283
8	94.10676
9	94.98296
10	108.4993

F

Dark Vesicle Pixel n°	Average Grey Intensity
0	118.05
1	104.9086
2	89.93788
3	73.80756
4	59.81162
5	49.31716
6	53.31918
7	65.42072
8	79.54142
9	92.9451
10	111.2723

Figure 2: Grey Intensity Values for (A, C, E) clear and (B, D, F) dark vesicles. Blue line on figures A and B depict vesicle types to be quantified. Figures C and D show the grey intensity values along the horizontal axis (pixels along the blue line) of these vesicle types and (E and F) their respective raw data.

Defining the functional vesicle pool

Pyramidal cells contain multiple synaptic sites along their axon. These cells may define synaptic function and, therefore, structure; we believe this leads to these pools having similar characteristics (for example, a similar functional vesicle ratio). Mitochondria are also believed to play an important role in neurotransmission through synaptic form and function, but this relationship is poorly understood. We hope to find important relationships to uncover possible

mitochondrial functions within the synapse. Here, we analysed two synapses sharing a common axon to study parameters linked to the synaptic ultrastructure; this analysis was carried out using a representative middle section for each synapse, thus using 2D analysis and later repeated using 3D analysis. Three different axons were defined (total of 6 synapses) to give a good idea on synaptic relationships. Vesicles were individually counted, together with potential connections on to postsynaptic dendritic branches. The ratio of dark to clear vesicles for every synapse along with an average of the three axons for each value was measured and plotted. The results can be seen in figure 3 (A).

The 2D results here show a somewhat strong relationship between synapses sharing a common axon in their ratio of functional vesicles to non-functional vesicles. The mean gradient of the three axons from synapse 1 to synapse 2 was in fact close to zero (figure 3C), meaning there is little difference in the ratios when two synapses share an axon. Figure 3 (D and E) also shows results for total number of vesicles. Here, however, the relationship was not as concrete. The number of connections to postsynaptic dendrites were similar in neighbouring axons. Furthermore, the synapses with larger vesicle pools had more connections; in fact, axon 1 synapses were by far the largest by volume and had 3-4 connections. Synapse 1 in axon 3 was also defined by a large volume and had 3 connections

When 3d analysis was carried out while using the same 6 synapses, the results were somewhat different as the range of vesicles studied was much larger for each synapse. The data suggests that the ratios once again showed a relationship as axon 1 and 3 synapses all hovered around 40-50% functional vesicles, while axon 2 synapses both had around 30% functional vesicles. These results also confirm that there is no relationship in the total number of vesicles in neighbouring synapses as seen from the big fluctuations in these values and the large gradient in the mean values. In terms of mitochondria, only four out of six of the selected synaptic sites contained them where axon 1 synapses contained 3-4 mitochondria. Interestingly, axon 1 synapses had the largest recycling pools, while axon 2 synapses had the least average mitochondrial volume and in fact had the smallest fraction of functional vesicles. This may link mitochondrial functions to enhanced vesicle recycling.

Vesicle Pool Spatial Analysis (2D and 3D)

The recycling pool is needed for quick release once the RRP is depleted, so it's positioning must be of some importance in synaptic function. Spatial analysis of recycling/functional vesicles within their respective pools in hippocampal synapses was thus carried out. We expect the functional vesicle pool to be preferentially positioned near the active zone for quick release, and we believe this can be a marker for synaptic strength. We also expect the 2D analysis to be somewhat inaccurate as the three-dimensional structure of the synapses is lost. As mentioned in the "methods" section, we will have a very big dataset referencing the positioning of each vesicle, dark and clear, on the X-Y plane for 2D analysis and on the X-Y-Z plane for 3D analysis for 10 unique synapses. To determine the distance from the active zone of these vesicles, we will also need to extract the positioning of the centre of the active zone on the presynaptic side. By subtracting the position of the active zone from the positioning of each vesicle we will achieve the distances in the X, Y and Z direction. Using the Pythagorean theorem with the X and Y distances, we will achieve the diagonal distance on the X-Y plane

for each vesicle for 2D analysis. For 3D analysis, a double Pythagorean theorem was used to achieve a distance through the Z plane. These distances were then used to form cumulative frequency plots to assess the positioning of dark vesicles compared to clear vesicles. All cumulative frequency values were then averaged to give one plot representing the 10 synapses, both in 2D and 3D.

A

Axon n°	Axon 1				Axon 2				Axon 3			
Synapse n°	Synapse 1		Synapse 2		Synapse 1		Synapse 2		Synapse 1		Synapse 2	
Analysis (2D/3D)	2D	3D	2D	3D	2D	3D	2D	3D	2D	3D	2D	3D
Total Vesicles	61	256	56	395	40	369	55	187	43	201	57	398
Dark Vesicles	32	118	27	193	12	118	19	57	16	99	21	171
Ratio	.525	.460	.482	.448	.300	.320	.345	.305	.372	.493	.368	.430
Connections	4		3		1		1		2		2	
Mitochondria	3		4		1		0		0		1	

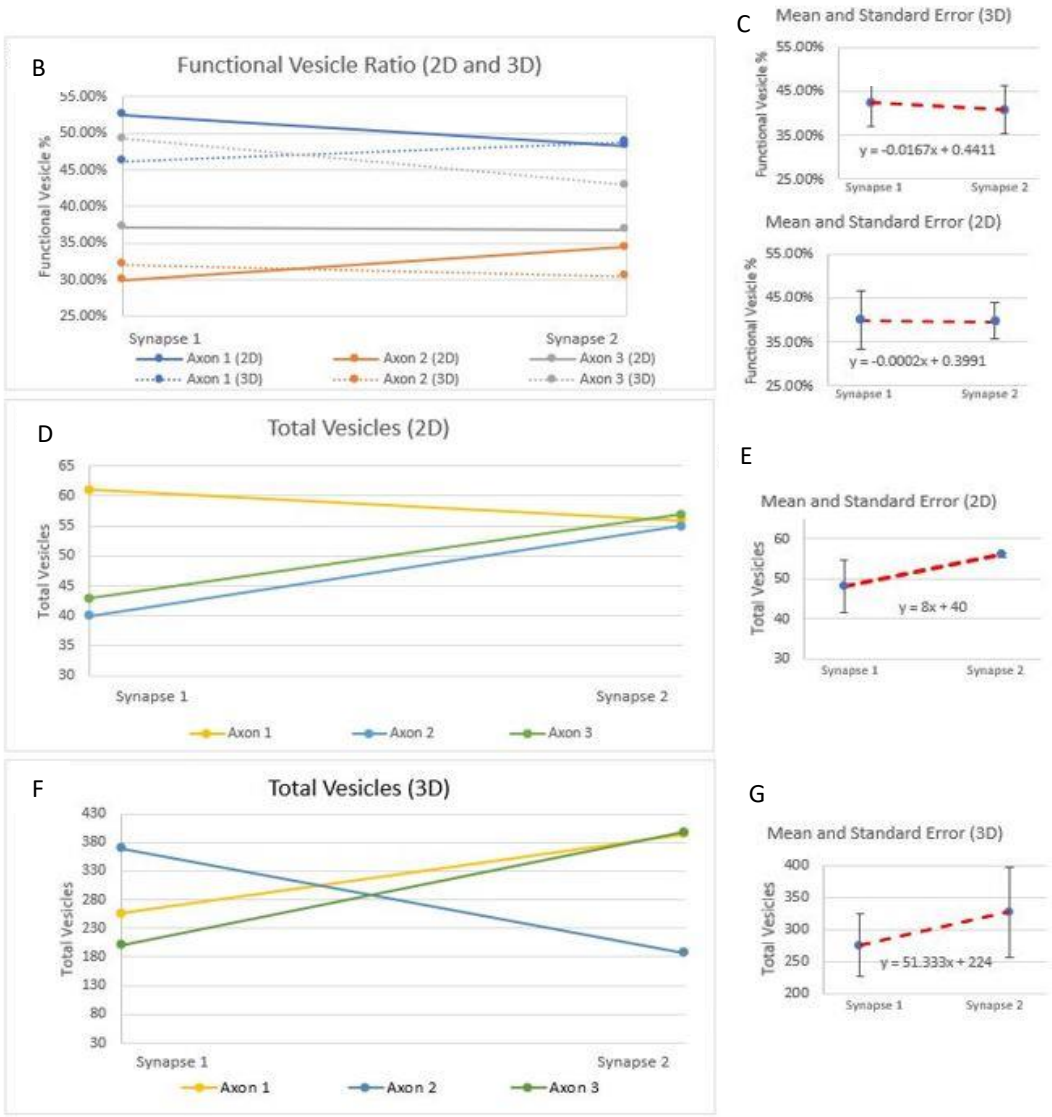
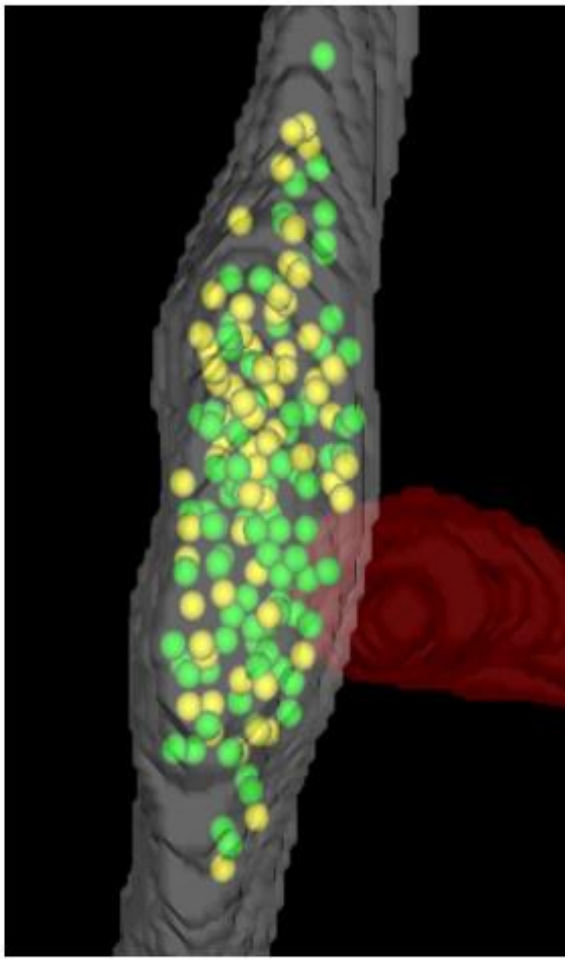


Figure 3: (A) Relationship between neighbouring synapses (those sharing a common axon) of specific parameters (Functional vesicle ratio, connections to postsynaptic dendritic branches, number of mitochondria, superpool size). (B) Functional vesicle ratio in 2D and 3D analysis for 3 synapses along with (C) the mean and standard error for both analyses. Vesicle pool sizes for three synapses in 2D (D) and 3D (F) analysis, along with means and standard error (E and G). (H) Depicts example of synapses at each end of an axon with their respective active zone (red structure) and vesicles (dark, clear and extra-synaptic).

Figure 4 shows synapse 5 and its vesicle pool. The left side (figure 4A) shows how the mid-section of the synapse would look in 2D while the right side (figure 4B) is the whole 3D reconstruction of the vesicle pool along with the dendritic branch to which it connects. From this image, it is clear to see that there is a lot of information missing from the 2D mid-section. A lot of vesicles towards the top of the synapse are lost as they were on a different z-plane. The same can be said about many other dark vesicles closer to the active zone. Furthermore, the 2D view may camouflage some dark vesicles as empty, such as the ones circled in the left image. This is due to the way that FIBSEM works as the X-Y resolution lacks where the Z-resolution shines. This may cause some disruption in the efficacy of using mid-sections for this study, thus making 3D analysis more compelling. Figure 4 also shows the average distributions of the 10 synapses in 2D analysis (4C and 4E). Here, we can see that the distributions are similar between dark and clear vesicles. There seems to be no preferential positioning for recently recycled (functional/dark) vesicles towards the AZ ($p = 0.658$, two-tailed paired t test, $n = 10$). However, the distribution of vesicles in 3D analysis (figure 4D and 4F) show a preferentially segregated recycling pool. The dark vesicle distribution (functional / recycling pool) clearly tends more towards the active zone (position 0 on the x-axis, red structure in figure 4B) while clear vesicles are further away ($p < 0.0001$, two-tailed paired t test, $n = 10$). These results are in line with those found in synapse 5 regarding the two-dimensional and three-dimensional nature of the analysis and confirm our hypotheses on preferential positioning.

A

B



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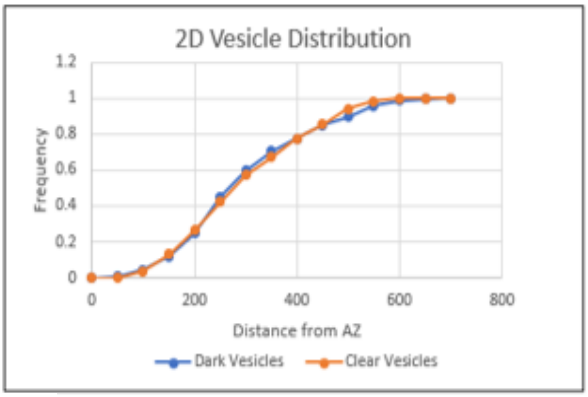

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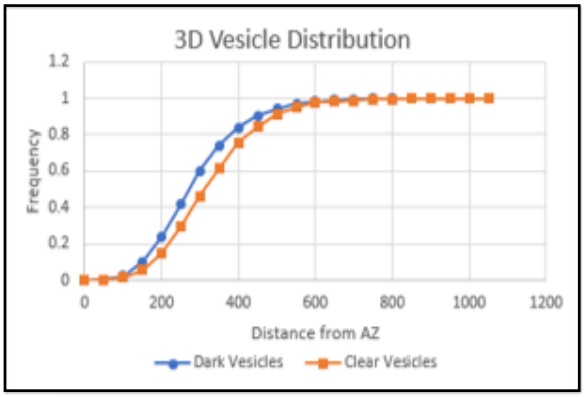

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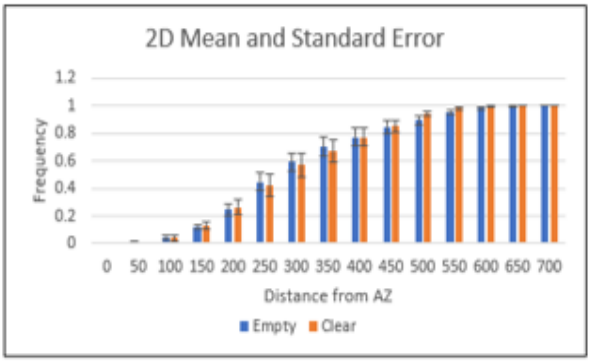

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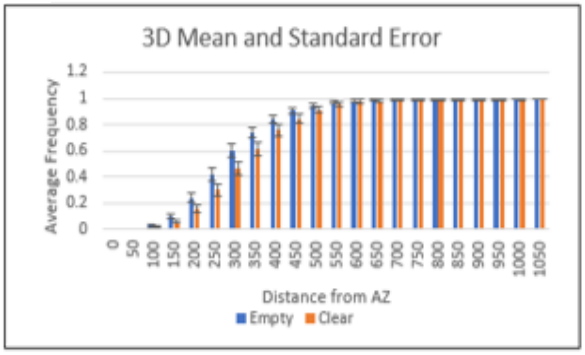

Figure 4: (A) FIBSEM output of a particular synaptic terminal. Red circles represent examples of dark vesicles camouflaged as empty due to the lacking 2D resolution. Blue circle represents the mid-section of the active zone. Represents example mid-section of a synapse used in 2D analysis. (B) 3D reconstruction of the synaptic terminal; green circles represent recycling vesicles; yellow circles represent clear / non-functional vesicles; red structure represents post-synaptic dendritic branch thus providing the centre of the active zone. Represents example structure used for 3D analysis. (C) Average vesicle distribution in 2D spatial analysis of 10 synapses with (E) their standard error. (D) Average vesicle distribution in 3D spatial analysis of the same 10 synapses with (F) their standard error.

Superpool Analysis

Extra-synaptic vesicles make up the superpool, and are believed to be crucial for effective vesicle turnover to possibly increase the recycling/readily-releasable pool for efficient vesicle release on demand. The size of these superpools may thus be important to tune synaptic strength. Furthermore, these vesicles are believed to undergo fusion at specialized sites outside the synapse and along the axon. This fusion may thus be paired with localized recycling of extra-synaptic vesicles. We expect the size of the superpools to be comparable to the size of the vesicle pools within the axon they reside in, as the average turnover rate of extra-synaptic vesicles is usually a somewhat fixed fraction. We also believe these vesicles will be scattered in clumps along the axon, meaning their release from the vesicle pool is non-random and there may be specialized locations along the axon for extra-synaptic vesicle fusion.

Superpools (extra-synaptic vesicles) were thus studied in 3 axons between two synapses containing a visible recycling pool. The size of the superpools had some correlation with the

size of the vesicle pools along their respective axon ($R^2 = 0.506$) but was not enough to confirm our hypothesis. More importantly, there was a strong relationship between the average sizes of vesicle pools (at each end of the superpool) and the recycling vesicle fraction within the superpool ($R^2 = 0.891$). Finally, the relationship of the distance between the two synapses and the superpool sizes was again fairly relevant ($R^2 = 0.702$). In all three axons, the superpool was mainly made up of recycling / functional vesicles (electron-dense) (figure 5A) and their positioning seemed to be non-random (vesicles seem to be positioned in clumps due to their uneven spread along the axon) which may suggest specialized superpool sites.

A

Axon n°	Axon Length	Average Vesicle Pool Size	Superpool Size	Functional Vesicle Fraction
1	4114.08	326	75	0.81
2	3287.41	278	28	0.61
3	6371.85	300	88	0.64

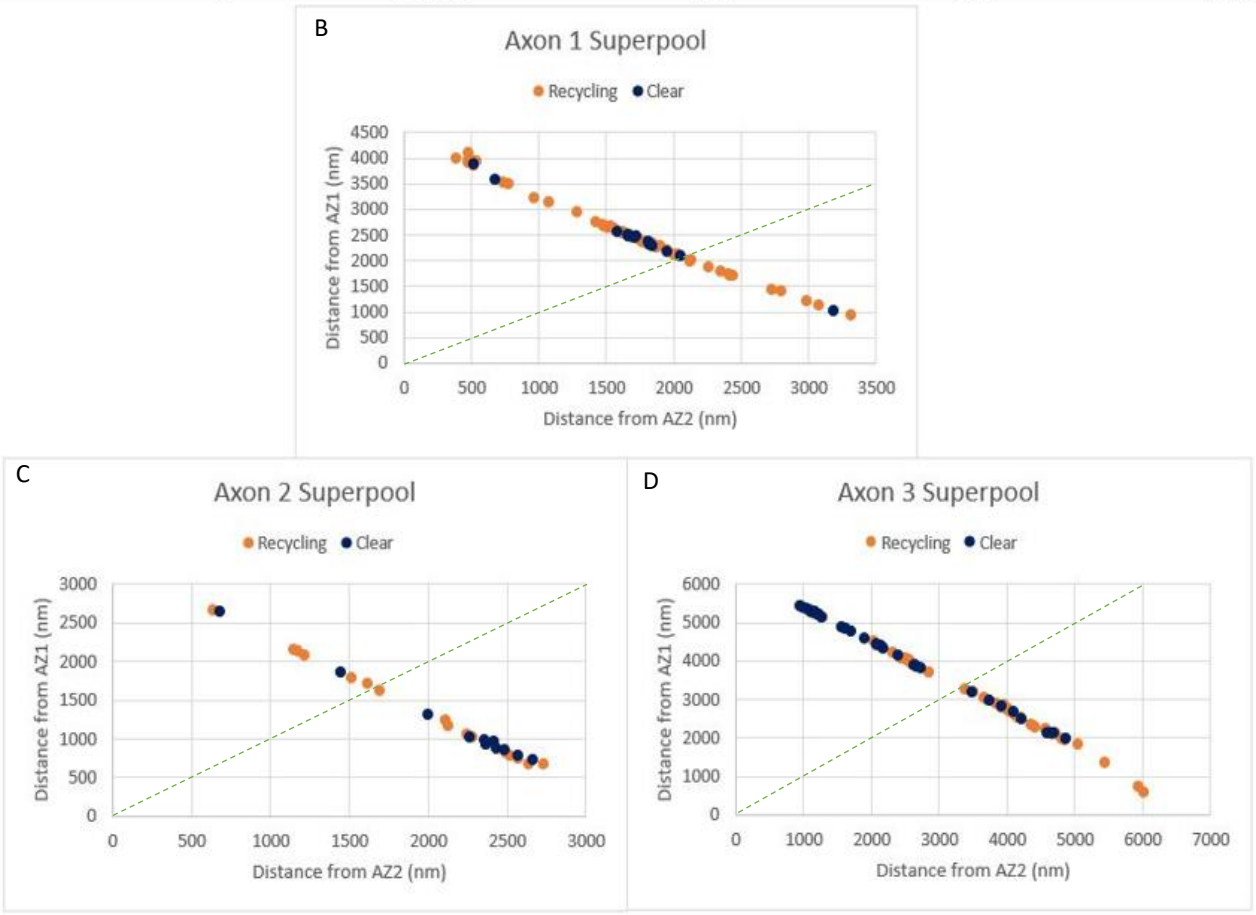

Figure 5: (A) Raw data to depict relationship between axonal / synaptic properties and extra-synaptic vesicles. (B, C, D) Axon 1, 2 and 3 superpool vesicle distributions (respectively). Y axis represents distances of each vesicle from synapse 1 active zone (AZ) while x axis represents distances of the same vesicle from synapse 2 AZ. Show density of extra-synaptic vesicles throughout each axon. Green dotted line represents mid-point of axon. Orange markers represent recycling vesicles while blue markers represent clear vesicles. Show possible positioning patterns for each vesicle type.

DISCUSSION

Here, we used a novel approach to provide unprecedented z-resolution in characterizing vesicle pools. This approach provides a three-dimensional readout of the functional pool. Our findings offer important insights into the relationship between functional pool size and synapse size. Spatial analysis of recycling vesicles reveals important aspects of the functional pool in relation to synaptic strength in neurotransmission, while our approach in superpool analysis offers a new insight in extra-synaptic vesicle recycling and fusion.

First, we focused on analysing certain parameters of synapses sharing a common axon. These results found that vesicle pools tend to contain around 30-50% functional vesicles. In physiological conditions, the recycling pool size has been widely discussed; however, this value usually consists in about 20% of the total pool (Rizzoli and Betz, 2005; Haratata et al., 2001; Marra et al., 2012); while other studies have found values directly comparable with ours (35%-40%) (Welzel et al., 2010). However, in these conditions, the tissue block is highly stimulated to visualise the organizational properties of these vesicles as clearly as possible, which may cause the neuronal membrane to over-activate and therefore over-produce recycling vesicles. Neighbouring synapses had a similar fractional organization of the functional pool, as well as a similar number of connections to postsynaptic dendritic branches and a somewhat similar number of total vesicles. These last two parameters are not related the magnitude of stimulation and thus may suggest that the neuronal axons have certain properties which may make them more prone to synaptic transmission, thus having more connections and better synaptic strength. In fact, some stronger synapses can have up to 50 times greater release probabilities than others (Orenstein, 2018), proving there must be something making certain connections stronger than others. Theories have been circulating on the role of postsynaptic targets in tuning presynaptic properties (Branco et al, 2008), which may explain the slight heterogeneity in neighbouring synapse parameters. Particular pyramidal cells (both pre-synaptic and post-synaptic) may thus be used as drug targets in neurological disorders affecting neurotransmitter release, to increase release probabilities.

Our results lead us to believe that mitochondria may be crucial in proper neurotransmission, as mitochondrial number and volume had a linear relationship with functional pool size. We believe that a larger recycling pool may lead to better neurotransmission due to enhanced neurotransmitter release. Mitochondrial functions, such as calcium handling and ATP production, are believed to be crucial steps in proper synaptic neurotransmission (Vos et al, 2010). Mitochondrial dysfunction is in fact linked to Alzheimer's Disease, Parkinson's Disease, amyotrophic lateral sclerosis and other psychiatric disorders (Johri and Beal, 2012). Efforts such as these in better understanding the mitochondrial function in synaptic transmission may therefore lead to novel therapeutic approaches to neurological disorder treatment.

The top 10% of synapses with the largest recycling pools account for 25% of vesicular release (Welzel et al., 2010) which suggests that the recycling pool may have a role in tuning synaptic strength. The size of the recycling pools in these results was much larger than previous reports

on recycling pools (Mean = 126 recycling vesicles). Studies found that the average recycling pool size is about 45 (Marra et al., 2012). This discrepancy is likely due to the 2D nature that most studies used before now. Although the use of different SEM techniques can make 2D analysis very compelling due to a great X-Y resolution, the lack of depth can cause many structures (such as individual vesicles) to be lost. This is one example of how FIBSEM can revolutionize the way we study structures such as these and can change our view on certain synaptic properties. This approach may improve the accuracy of pool sizes in synapse research to select synapses that account for most of the vesicular release, making them more compelling in drug targeting.

The spatial analysis part of this study mainly focused on finding patterns in the distribution of vesicles within a synapse. This again was repeated in 2D using a representative mid-section. As explained, the 2D results had some faults. This was seen by the lack of confirmation of our hypotheses, as most recycling pools studied showed no preferential positioning. These results were very improbable, hence why the process was repeated using 3D analysis. All 10 synapses using this approach show that the recycling pool is preferentially positioned towards the active zone, meaning vesicles that have undergone fusion tend to stay towards the release sites. Previous studies focusing on synaptic ultrastructure (such as Marra et al., 2012) found similar results. The use of FIBSEM and 3d analysis (thanks to the high z-plane resolution) makes these results very compelling and a good confirmation to these other studies. Studies focusing on the RRP found that these vesicles also position themselves towards the active zone (Schikorski and Stevens, 2001) which shows the importance in positioning when it comes to release probability. Further analysis in this aspect of the study may involve researching the mechanisms for which vesicles are preferentially positioned. Studies have found that actin may be a crucial scaffolding component to guide vesicles to their intended position (Sankaranarayanan et al., 2003; Shupliakov et al., 2002). Research in the cytoskeletal element actin and its role in positioning can thus be very compelling, as found in studies using jasplakinolide (Marra et al, 2012) where the compound was used to stabilize actin thus disrupting the preferential distribution of the recycling pool, proving actin's importance in remodelling the vesicle pool.

While most of the functional pool was positioned towards the active zone, some recycling vesicles stood far away. Recent studies have found that different functional vesicle classes have different molecular signatures (Hua et al., 2011) which may explain the discrepancy in their positioning. Other studies in the neuromuscular junction of a frog found that RRP vesicles, having preferential release, were mixed randomly within the vesicle pool (Denker et al., 2011; Rizzoli and Betz, 2004) meaning their position in the synapse did not dictate their release probability (Richards et al, 2000, 2003). A plausible hypothesis would be that the release probability of recycling pool vesicles in hippocampal synapses is dictated both by their position towards the active zone and by other cytoskeletal and molecular properties similar to those found in the neuromuscular junction and in other vesicular classes. Further studies may find this particular aspect of vesicle positioning compelling to extend the knowledge of vesicular release probabilities for better drug targeting (to increase or inhibit the release of neurotransmitter in the synaptic cleft).

To extend our knowledge of vesicle recycling, we attempted to understand the use of extra-synaptic vesicles, contained in what we call superpools, with an unprecedented z-resolution through the use of FIBSEM. The superpool sizes (number of vesicles between one synapse and another within an axon) had a strong correlation with the distance between the two vesicle pools. Therefore, it may be that with less space between synapses, there is a reduced probability of superpool formation within an axon. Spatial analysis of the superpool suggests that extra-synaptic vesicles are distributed in clumps, possibly meaning that vesicles are not released outside the synapse randomly but are located in organized patterns along the axon to form specialised areas dedicated to extra-synaptic fusion and recycling. This may confirm theories of extra-synaptic vesicle pools, believed to be spread in specialised locations through the axon, where vesicle fusion and recycling can occur outside of the synapse (Ratnayaka et al, 2011). Interestingly, about 4% of all total vesicles within a synapse go through a superpool every minute (Staras et al., 2010). Although this is an average value, it may explain the slight relationship between synapse sizes and superpool sizes, as a fixed fraction will mean more vesicles go through the axon when they are released by a bigger pool. All 3 axons contained a large percentage of recycling vesicles within these superpools. This may suggest that these vesicles had been recently recycled outside of the synapse, to be sent back to either synapse for better neurotransmission. Studies suggest there are specific proteins (such as synapsin) dedicated to the transport of vesicles between synapses (Orenbuch et al, 2012). Here, we support this theory, while extending the idea of extra-synaptic vesicle fusion to complement synaptic neurotransmission. These mobile recycling vesicle clusters can be very helpful in supporting ongoing signalling demands outside of the common synapse, and can thus be used as drug targets for enhanced neuronal signalling. Further analysis on this subject can make use of in vivo work, as these results do not show the movement of superpool vesicles, but rather show their spatial positioning at a certain timestamp. Since these vesicles are believed to be used as an extension to the classical recycling pool, their real-time movement within a living organism can be very compelling to focus on their true function.

Acknowledgments

I would like to thank Prof. Kevin Staras for his help and interest in my research project. I would also like to thank his lab and everyone working in it for granting me the possibility of working with a novel data set only achievable through rare and expensive equipment. Finally, I would like to extend my appreciation to those who those who could not be mentioned here but have supported my work throughout.

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