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Localization of Synapses on Adipocytes and Stromal Vascular Fraction Cells in Adipose Tissue Using Electron and Immunofluorescent Microscopy

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LOCALIZATION OF SYNAPSES ON ADIPOCYTES AND STROMAL VASCULAR FRACTION CELLS IN ADIPOSE TISSUE USING ELECTRON AND IMMUNOFLUORESCENT MICROSCOPY

by

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ABSTRACT

Obesity, weight gain and the many metabolic disorders that can arise from being overweight are predominant health issues in America and in the State of Maine. The body's ability to balance energy intake and energy expenditure is what determines whether a person gains or loses body fat. Although there are many different factors that influence energy storage and expenditure, neural innervation of white and brown fat (or adipose) tissues is an important aspect of energy balance that is not well understood. The Townsend Lab focuses on brain-adipose communication and the role of adipose peripheral nerves in maintaining proper body weight and metabolic health. One of the major unanswered questions in the field is how the peripheral nervous system innervates individual adipocytes as well as cells in the stromal vascular fraction (SVF), or the immune and progenitor cells that reside adjacent to adipocytes in the adipose organ. I imaged synapses in inguinal adipose tissue using transmission electron microscopy (TEM), which provides ultra-structural detail of the neural connections in adipose tissue. Although I could not find synapses using EM, I was able to observe SVF immune cells and axons in the SVF. We are also imaging synapses in inguinal and axillary adipose tissue using immunofluorescence for synaptic markers at a magnification of 100X that will be a more appropriate scale for synaptic identification on specific cell types.

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INTRODUCTION

Energy balance and metabolism

The purpose of studying sympathetic innervation of adipose tissue is to better understand how the body controls energy balance. It is already well established that energy input occurs through food intake and nutrient absorption, while energy output occurs through exercise, thermogenesis and general activity of the body. There are many different factors that affect metabolic rate, whether it be daily food consumption, physical movement/exercise, or genetic influences that predispose the body to either expend or store energy. The body must maintain homeostatic regulation of energy intake and expenditure, because too much of a shift in either direction leads to decrease in health and quality of life. Too much energy output (or not enough energy input) can result in malnutrition, which has symptoms that include changes to behavior such as irritability, tiredness and difficulty focusing, and changes to the body, such loss of muscle and bone mass, decrease in immunity and longer healing time for wounds (1). These changes, if prolonged, can cause irreversible damage to the body and potentially lead to illness or death. On the other hand, too much energy input (or not enough output) can result in gaining excess fat tissue that leads to either being overweight or obese. Symptoms of being overweight include easily becoming fatigued, pain and inflammation of joints and back, sleep apnea, high blood pressure, and increased risk of disorders such as type II diabetes, heart disease, kidney disease, osteoarthritis and many others that can damage the body and can eventually lead to death (2). Maintaining energy balance is essential for keeping the body healthy and preventing dysfunction.

Prevalence of metabolic-related health issues

With the increasing number of foods containing high levels of carbohydrates, sugars and salts and with technologies such as computers and TVs enhancing the appeal of sitting down instead of moving around, it is no surprise that being overweight is one of the biggest health issues in America. Studies show that 39% of American adults (over one third of the adult population) are obese and almost one third of the global population is either overweight or obese (3,4). Obesity is indisputably a health disorder that can further reduce quality of life, damage the body, and increase chances of premature death. However, obesity is also a socio-economic issue since those who are impoverished have less access to fresh and healthy food and also have less time to exercise. This would explain why 33% of adults who earn less than $15,000 annually are obese (5). Obesity is also a cultural issue, since obesity prevalence varies depending on country (with America having the greatest percentage of adults who are overweight). Obesity has a large influence on many different societal issues, which is why the problem needs to be better understood through research so that its influence can be reduced. A lot of research has already been done on the causes of obesity and the impact it has on the body, but there is still minimal understanding of how different factors, such as different types of adipose tissue or the neural system's innervation of adipose tissue, affects a person's level of obesity.

Plasticity of adipose tissue

Researching innervation of adipose tissue is essential for better understanding of how the body maintains metabolic homeostasis. Sequestering triglycerides in adipose is one of the primary ways our bodies store energy, and the amount of energy adipose tissue

stores as fat is determined through communication with the brain through the peripheral nervous system. The evidence of neural plasticity and neuropathy within adipose tissue comes from adipose tissue having to cycle through different processes depending on what the metabolic needs of the body are at that time (6,7). Sensory feedback to the brain is needed to regulate sympathetic nerve activation of lipolysis or lipogenesis, the breakdown or formation of fat, respectively (7). Fat tissue can also go through hyperplasia, which is the proliferation of adipocytes, or it could be going through hypertrophy, where the cells enlarge instead of multiply (8). Changes in neural connections can even change the type of adipose tissue through transdifferentiation of white adipose tissue (WAT) to brown adipose tissue (BAT). When ligands are bound to beta-3 adrenergic receptors, this stimulates BAT to perform its thermogenic function (9). BAT contains mitochondria that uses uncoupling protein 1 for thermogenesis, which shifts adipose tissue function to favor energy output and thus leads to a decrease in weight gain and is often considered “good” fat (10). WAT, on the other hand, specializes in energy storage and gives fat its notorious reputation. Adipose is able to efficiently revert back and forth between these antagonistic processes due to its high innervation and vascularization (11). Researchers claim that cold temperatures stimulate sympathetic axon growth by inducing the expression of nerve growth factor TrkA (NGF-TrkA) in order to increase release of catecholamines, giving NGF-TrkaA an essential role in beiging of WAT (12). Adipose tissue is constantly receiving signals from peripheral nerves that alter its activity and composition depending on the body’s metabolic needs. A better understanding of the changes that occur to nerves and synaptic connections can

help us determine how to increase the likelihood that processes that combat obesity (such as lipolysis or browning of tissue) will occur.

Within adipose tissue, the vasculature and extracellular matrix have also been shown to have a plastic nature, with the ability to change in composition and abundancy. White adipose tissue is highly vascularized due to the high demand for oxygen and nutrients for adipocytes and other cells, as well as the need to transport immune cells (such as macrophages and lymphocytes) to adipose tissue (13). As the size or number of adipocytes changes, the morphology or abundancy of blood vessels has to be altered in order to adapt to the changing size of the tissue. Adipocytes release certain cytokines and hormones that regulate angiogenesis, or the creation of new blood vessels. Adipokines such as leptin will promote angiogenesis through a tyrosine kinase-dependent intracellular pathway, while adiponectin will inhibit it through a Rho kinase pathway (14,15). Adipokines control different processes that are meant to maintain vascular homeostasis and integrity. With obesity, though, release of adipokines and other signaling factors is thrown off and the rate of angiogenesis is unable to keep up with the increased metabolic needs of enlarged adipocytes, which can result in damage to the tissue. With the extracellular matrix, the amount of ECM is constantly fluctuating in order to keep up with the changes in cell size and number within adipose tissue. Certain enzymes, such as matrix metalloproteinases, are released to break down ECM proteins to create more space in adipose tissue during tissue hypertrophy or hyperplasia (16,17). Obesity leads to ECM remodeling becoming defective by causing excessive ECM accumulation, which in turn leads to fibrosis and damage to the tissue due to the increase in material found in the ECM, such as collagen (18). The increase in factors that are released by the ECM also

contributes to dysfunction by increasing certain processes, such as apoptosis of adipocytes and decreasing the amount of nutrients and oxygen the tissue receives through inhibiting angiogenesis (16). The plasticity of adipose tissue is very useful for maintaining metabolic homeostasis, since the amount of energy intake and output is never consistent and the body needs to be able to efficiently adapt based on its metabolic needs. However, this also means that factors maintaining balance (such as signaling proteins) can easily be thrown off, resulting in tissue dysfunction and damage that can be difficult to reverse.

Synapsing in adipose and other tissue types

Adipose tissue is continuously and efficiently changing both its morphology and composition in response to metabolic changes in the body. It is well known that endocrine hormones cause most of these changes, but the high amount of innervation in adipose also indicates that nerves communicate with the tissue through synapsing. The synapse is the part of the neuron that transmits a signal from the neuron to another cell (whether it be neural or non-neural) through the release of neurotransmitters. Synapses can be identified based on their 2 um average width and the round, neurotransmitter vesicles within the synapse (19,20). The greatest amount of evidence that we have that synapsing occurs in adipose tissue is from research done by Kosacka et al., where adipocytes were co-cultured with nerve cells from the dorsal root ganglion (19). When cultured together, there was evidence of synapsing in immunofluorescent microscopy through the fluorescence of PSD95 (marker for post-synaptic proteins) and synapses were also found using TEM, however, synapsing was occurring on other neurons instead of on

adipocytes (19). Also, all of this synapsing only occurred on cultured cells and synapsing on cells in adipose tissue *in vivo* has yet to be imaged. Most research done on synapsing and how it relates to communication between adipose tissue and the brain, focuses on the brain itself. When research is done on the neural communication between brain and adipose, images are taken of synapses of central nerves in the hippocampus or paraventricular nucleus, and research is done on central nerves affected by metabolic dysfunction instead of the peripheral nerves in adipose tissue (21,22). The focus is placed on the brain, though, because it is much easier to image neurons in nerve tissue that contains high innervation. There are other tissues with synapses that have been imaged extensively because of their high innervation. Muscle tissue is common for researching peripheral synapses because of the abundance of neuromuscular junctions needed to stimulate muscle contraction, as well as synapses for the many different types of receptors in the retina, such a photoreceptors or bipolar cells (20). There are also cases where synapsing is researched in a certain type of tissue, but it is still not imaged much. With the enteric nervous system, extensive research has been done on the synapses in the gastrointestinal tract, but the focus is on measuring postsynaptic potentials instead of taking images of the synapses themselves (23,24). With the images that are taken of synapsing in the GI tract, the synapses are those from the smooth muscle portion of the digestive tract (25). Imaging and research on synapses, though, is mainly focuses on nerves in the central nervous system due to their abundance. A substantially decreased amount of research exists for peripheral innervation of tissues, especially in the case of adipose tissue.

Role of Stromal Vascular Fraction in Adipose Tissue

Adipose is a heterogeneous tissue that contains a variety of cells that are part of the stromal vascular fraction (SVF). SVF is the interstitial space that surrounds adipocytes and contains cells such as mesenchymal progenitor/stem cells, preadipocytes, endothelial cells, pericytes, and immune cells such as lymphocytes and macrophages (26). Within the SVF also lies the extracellular matrix (ECM), which has numerous roles that are involved in metabolic regulation in adipose tissue. ECM can either contribute to beta-adrenergic stimulated lipolysis and increased insulin sensitivity in cells from non-diabetic tissue, or inhibit lipolysis and increase insulin resistance in cells from diabetic tissue (27). The proteins and enzymes within the ECM have such a profound and specific role in adipocyte metabolism, that when ECM from diabetic adipose tissue is transferred to non-diabetic adipose tissue, that tissue starts to develop the defects associated with the diabetic tissue, where lipolysis and glucose uptake is decreased (27). Proteins in ECM from diabetic cells most likely include proteins such as endotrophin or lysyl oxidase (LOX). Endotrophin has been associated with increases in inflammatory responses and decreases in lipolysis, while LOX is often found in obese tissue and is associated with decreases/dysfunction in glucose uptake (28,29). Proteins in ECM from non-diabetic cells most likely include micro-fibril glycoproteins such as MAGP1, which has been be found to promote thermogenesis and is usually abundant in lean fat tissue (30). MAGP1 increases browning/beiging of WAT by regulating transforming growth factor-beta (TGF-β). TGF-β contributes to obesity through inhibiting UCP1, which prevents conversion of WAT to BAT, but MAGP1 inactivates TGF-β by storing it in the ECM (30). In addition to playing a role in white adipocytes transdifferentiating into brown

adipocytes, the ECM is also capable of stimulating preadipocyte differentiation. Vascular endothelial cells in the SVF will secrete ECM components such as laminin and fibroblast growth factors that promote adipogenesis (31).

Almost all of the SVF cells have some role that influences energy balance within adipose tissue. The endothelial cells in the SVF line the blood vessels that supply oxygen to adipose tissue, and the pericytes localize around blood vessels in order to give them stability (32). Endothelial cells' involvement with vasculature includes increasing blood fluidity by releasing vasodilators and inhibitors of platelet adhesion, and also releasing factors that dissolve fibrin in blood clots (33). However, endothelial cells also have functions that are very specific to adipose tissue. They are capable of acting as a barrier against inflammatory macrophages entering adipose tissue in order to prevent damage to the tissue (34). Endothelial cells have also been found to differentiate into adipocytes, where in response to activation of the nuclear receptor PPARγ the cells lose their endothelial characteristics and gain those of adipocytes instead (35). However, there is other research that conflicts with this finding and claims endothelial cells do not have the ability to differentiate into other cell types (36).

There are either types of cells in the SVF, though, that are capable of differentiating into mature adipocytes. The multipotent stem cells in the SVF can be signaled to differentiate into pre-adipocytes, which then in turn can further differentiate into fully functional adipocytes (37). Although, usually the pre-adipocytes differentiate into adipocytes, it has also been found that pre-adipocytes can take on the role of macrophages. When macrophages come into contact with pre-adipocytes, the pre-adipocytes will express antigens found on macrophages, such as Mac-1 or CD80, and

they can perform immune functions such as phagocytosis (38). Usually pre-adipocytes are induced to become macrophages due to increased inflammation of adipose tissue. The inflammation that occurs due to higher levels of adipose tissue in obesity is linked to increased inflammatory cytokine release from macrophages and other immune cells in the SVF (26). The pre-adipocyte's ability to differentiate into multiple cell types indicates the plasticity of adipose tissue. This increase in inflammation, in addition to causing damage to nerves in the adipose, is believed to affect adult stem cells by decreasing their lipid accumulation and decreasing their ability to differentiate (26).

Fibroblasts are also capable of taking on a role as a progenitor cell by being able to differentiate into mature adipocytes. One protein that has been found to stimulate fibroblast differentiation is PPARγ2, and PPARγ proteins have already shown to have important roles in both adipocyte differentiation and lipid metabolism (39). There was also an experiment done where a mixture of epidermal growth factor (EGF), hepatocyte growth factor (HGF), dexamethasone (Dex) and insulin stimulated fibroblast differentiation, but all four hormone were needed for fibroblasts to convert to mature adipocytes (40). It was found that the STAT5 signaling pathway was activated, a pathway that is often associated with the growth and differentiation of mammalian cells (40). Fibroblasts also have another role that promotes adipogenesis in adipose tissue. $FSP1^{+}$ fibroblasts maintain preadipocytes and their ability to differentiate by secreting platelet-derived growth factor (PDGF), which means that they are abundant in obese tissue and are often linked to tissue defects (41).

Multiple immune cell types have displayed metabolic regulatory roles, in addition to the roles specific to fighting off pathogens. Lymphocytes are most well known for

their immunoregulatory roles; either as B cells that release antibodies with numerous functions including neutralization and opsonization, or as T cells that either act as helper T cells for recruiting other immune cells or cytotoxic t cells that release cytotoxins that induce apoptosis in infected cells (43,44). However, T lymphocytes specifically have been found to play roles in metabolic homeostasis in adipose tissue, including inhibition of beiging of white adipose tissue (45). Cytotoxic T lymphocytes with CD8 markers release IFN-γ, an interferon that in addition to causing inflammation in adipose tissue, also inhibits the expression of tyrosine hydroxylase (TH), an enzyme that catalyzes the rate-limiting step in catecholamine synthesis (45,46). Beige adipogenesis is sympathetically driven by the activity of catecholamines, which means that the decrease in catecholamine synthesis results in a decrease in beiging of WAT. Also, an increase in cytotoxic T cell numbers means a decrease in other cell types that could promote beige adipogenesis, such as eosinophils or other innate lymphoid cells (45).

Helper T lymphocytes with CD4 markers have also been found to release IFN-γ in adipose, however that discovery was made in visceral adipose tissue instead of subcutaneous (47). However, CD4+ and CD8+ T cells that released IFN-γ were upregulated in obese tissue, not only inhibiting the beiging white adipocytes, but also contributing to adipose tissue dysfunction through releasing inflammatory cytokines (45,47). CD4+ T cells are also capable of having alternative roles that protect against damage to adipose tissue. Specifically, a subtype of helper T cells labeled Th2 cells have demonstrated roles that not only decrease adipose inflammation but also increases insulin sensitivity (48). Th2 cells secrete interleukins such as IL-3, IL-4 and IL-10, which have roles of inhibiting inflammatory cytokines, which decrease insulin resistance (47,48).

Depending on the subtypes of these T lymphocytes, they can either have a protective role in defending against obesity-related dysfunction, or these immune cells can contribute to adipose tissue dysfunction.

Macrophages are immune cells that defend adipose tissue by phagocytosing microbes and harmful material. However, there are two subtypes of macrophages with drastically different roles, but are both involved in maintaining metabolic homeostasis. M1 macrophages release inflammatory factors such as IL-1, IL-6 and TNF- α, promotes insulin resistance and decreases levels of UCP1 (49,50). M2 macrophages, however, promote preadipocyte survival through the release of PDGF, secretes anti-inflammatory cytokines such as IL-10 and protectin, and increases levels of UCP1 (50,41). M1 macrophages contribute to the adipose tissue dysfunction and are found at higher levels in obese adipose tissue, where their release of inflammatory cytokines can result in damage to the cells and the defects in glucose uptake from insulin resistance can result in type II diabetes (41). M2 macrophages, on the other hand, protect adipocytes from defects often caused by metabolic imbalance (42,41). Macrophages have also shown to have numerous roles involving sympathetic nerves in adipose tissue. When it comes to the relationship between macrophages and sympathetic nerves, recent findings show that macrophages play a role in regulating innervation of brown adipose tissue. Macrophages in brown adipose tissue expresses PlexinA4, which is able to send a signal from Sema6A that causes the repulsion or pruning of sympathetic axons (51). Since brown fat expends energy through thermogenesis instead of storing energy as its main function, it cannot be said for certain that the role macrophages have in regulating innervation would be the same in white adipose tissue. However, the plasticity of white adipose tissue indicates

that innervation needs to be regulated by some method, and macrophages could fulfill that role as they do in brown adipose tissue. Macrophages will also regulate activity of neurons by metabolizing certain neurotransmitters, such as norepinephrine (NE). Macrophages will migrate towards varicosities on sympathetic axons and phagocytose NE that is released from those synaptic boutons (52). It is believed that this disposal of neurotransmitters prevents sympathetic activity from becoming too high. There has been some research that looks at the possibility that macrophages are adjacent to synapses/axonal varicosities because they are being activated through neurotransmitter release from synapses in addition to degrading neurotransmitters and synapses, but this research has been done on other types of tissue rather than adipose (25,53) .

It is already well known that communication occurs in the SVF through protein signaling. Cytokines such as IFN-gamma will activate macrophages to produce inflammatory proteins, while neuropeptide substance P will bind to NK-1R receptor on macrophages to induce immune activity (54). We are still uncertain, though, if there is communication between nerves and SVF in adipose tissue, which opens up the possibility that that communication could occur through peripheral nerve signaling.

Electron Microscopy and Immunofluorescent Microscopy

In order to take images of the adipose tissue at a cellular level and get the ultrastructure of the adipocytes and SVF cells, we needed to use a transmission electron microscope (TEM). Electron microscopes use a beam of electron instead of a beam of light to illuminate the sample (55). An electron beam travels at a much smaller wavelength than a light beam, which allows greater resolution of the sample and thus

greater magnification without losing detail (55). Because TEM requires thin specimens, tissues must be sectioned and so the images end up being two-dimensional cross-sections of adipocytes, SVF cells and synapses (55). Images are produced on a phosphorescent screen with crystals that show electrons that pass through the specimen and strike them. Denser parts of the specimen scatter electrons out of the imaging beam and so it appears darker on the image (56). Immunoelectron microscopy can be used to increase efficiency and accuracy of co-localizing synapsing on adipocytes and/or SVF cells by using gold nanoparticles conjugated to antibodies that bind to antigens of synaptic proteins. The gold nanoparticles appear black under the electron microscope due to electrons scattering from the greater thickness of the tissue. This allows for quick and easy pinpointing of where a synapse is since the gold nanoparticles will group where the synaptic proteins are. However, the method of processing these tissues that specifically maintains their antigenicity requires using a fixation protocol that does not preserve ultrastructure very well, meaning that it is harder to confirm identity of synapses and other structures based on morphology.

Immunofluorescent (IF) microscopy, on the other hand, is light microscopy that uses antibodies that are conjugated to fluorophores instead of gold nanoparticles. This allows the detection of antibodies because they fluoresce under the microscope, which aids in localization of synapses. Using a light microscope means sacrificing the ability to view the synapses themselves since the magnification only goes up to 1000X, but it gives the ability to find synapses more efficiently by looking for fluorescence over a wider area of tissue.

Objective

I used TEM and IF microscopy to investigate whether adipocytes and SVF cells have direct contact with axonal projections and directly receive neurotransmitters through a synaptic formation. Due to the small size of synapses, I used electron microscopy to achieve the degree of magnification and resolution needed to see a synapse and its ultrastructure. Two different electron microscopy techniques will allow us to determine which method is easier for finding synapses; finding synapses based on appearance or finding synapses based on labeling of synaptic proteins. Although IF microscopes are unable to magnify to as great of a scale as electron microscopes, we are still attempting to find synapsing at 1000X magnification by fluorescing synaptic proteins. Since immunofluorescent microscopy has been used many times before (both by the Townsend lab and by other labs), this method is more likely to yield us images that show synapsing.

METHODS AND MATERIALS

Mouse Models

For transmission and immunoelectron microscopy, TH-Rosa-eYFP reporter mice were used for collecting inguinal and axillary whole depot subcutaneous white adipose tissue (scWAT). TH reporter mice were used so that the eYFP knock-in gene for tyrosine hydroxylase would be expressed around neurotransmitters in synapses, however, this genetic model ended up not being necessary since immunofluorescence was not able to be combined with electron microscopy. All four mice were females that were at least 12 weeks old and they were all fed normal chow. They were then placed in 4 ^{o}C for seven days (in order to increase expression of eYFP), and then were used for collecting adipose tissue. Ipsilateral inguinal and axillary scWAT sections were fixed and stained for immunoelectron microscopy while contralateral inguinal and axillary scWAT sections were fixed and stained for transmission electron microscopy. For immunofluorescence, LysM-Cre-Rosa reporter mice were used for collecting inguinal and axillary whole depot scWAT. GFP is expressed on myeloid lineage cells of LysM-Cre-Rosa-eYFP mice, so that we can look for innervation in macrophages and other myeloid cells. These mice were also at least 12 weeks old and fed normal chow.

Regular Transmission Electron Microscopy

Contralateral inguinal and axillary tissue samples were collected as 1 mm^3 increments. They were fixed with a mixture of 2% glutaraldehyde-2% paraformaldehyde mixture for 2 hours and then washed overnight. The samples are then postfixed in 1%

OsO_4 in PB, dehydrated in an acetone series (50%, 70%, 95% and 100%) and embedded and polymerized at 60 ^oC in Epon resin. Once the tissue samples are in resin, an ultramicrotome is used to cut the tissues into sections that do not exceed 60-90 nm in thickness. Those samples are then collected directly on a 300-mesh nickel grid and stained with uranyl acetate and lead citrate. The samples were imaged using a CM10 transmission electron microscope at magnifications between 1450-25,000X.

Immunoelectron Microscopy

Ipsilateral inguinal and axillary tissue samples were collected in 1 mm^3 blocks. Once samples were collected, they were fixed in 4% paraformaldehyde overnight at 4 ^oC. After washing, samples were dehydrated in an EtOH series (50%, 70%, 95%, 100%). Samples were then infiltrated and polymerized in LR White resin overnight and then sectioned with an ultramicrotome into sections that do not exceed 60-90 nm thickness and placed on a 300-mesh nickel grid. The grids were placed on droplets of 10X phosphate buffered saline (PBS) containing 1% BSA and 1% Tween 20, then were placed on droplets of 3% normal serum (goat) in 1X PBS. These tissues were incubated in two types of antibodies and gold nanoparticles for immunostaining. First, the tissues were incubated in the primary antibody PSD95 (1:100) and then incubated in the secondary antibody goat anti-rabbit that was conjugated with 10 nm gold nanoparticles (1:50). The tissues were then incubated in the primary antibody SV2, which stains for synaptic vesicles (1:25) and the secondary antibody goat anti-mouse that was conjugated with 15 nm gold nanoparticles (1:50). After conjugation, the sections were stained with uranyl

acetate and lead citrate. The samples were imaged using a CM10 transmission electron microscope, taken at magnifications between 4600-130kX.

Immuno-fluorescent Microscopy

Both axillary and inguinal scWAT were collected from the LysM-Cre-Rosa eYFP reporter mice, since GFP is able to bind to eYFP on myeloid lineage cells. One inguinal depot (contralateral) was kept as a whole mount but the rest of the depots were cut into 100 um-thick slices. Once the tissues were fixed, one or two slices from each depot was put into blocking solution in order to block any antigen sites that antibodies might attach to non-specifically and to permeabilize the tissue. After sitting in blocking solution overnight, the tissues were washed and stained with True Black in order to reduce auto fluorescence and to increase contrast between the fluorophores and the tissue background. Then the tissues began their antibody staining regiment. The tissues were first stained with the primary antibody, PSD95 (ab18258 at 1:1000) for 48 hours. Then they were stained with goat anti-rabbit secondary antibody bound to AlexaFluor 647 (1:1000) overnight. Finally, they were stained with the primary antibody conjugated to GFP (ab6662 1:1000) for 72 hours. Some of the sections imaged were mounted, while others that were imaged were not squished. Tissues were either imaged using a Nikon Eclipse E400 microscope at 100X magnification, or they were imaged using a confocal microscope at 83X magnification. All tissues imaged with a confocal microscope were stained with DAPI in addition to PSD95, AlexaFluor 647 and GFP, in order to stain cell nuclei blue and confirm that myeloid lineage cells imaged were not artifacts.

RESULTS

Although sections of tissue were taken from both the inguinal and axillary fat depots, images were only taken of inguinal sections for electron microscopy since that is where we believe high levels of synapsing occurs, based on our previous studies localizing nerves in whole mounts. Furthermore, we focused on imaging sections taken near the lymph node (Fig. 1). The lymph node is sympathetically innervated for regulation of immune responses (transport, filtering, etc.), which increases the likelihood of innervation and synapsing in the surrounding area (57). There will also be a higher concentration of lymphocytes and macrophages surrounding the lymph node, giving more immune cells to view to find synapsing. When slicing one of the blocks of tissue, part of the lymph node was sectioned with the block and a cross-section of the very edge of the lymph node can be seen in figure 2. The lymph node was identified based on its shape, dark bordering and surrounding structures. In figure 2, there is extracellular material surrounding the lymph node, and that is most likely the fibrous capsule (58). The lymph node could not be identified based on its size, since it is very small due to cross-section coming from the very edge of the lymph node and is not indicative of the actual size of the structure. For all of our TEM and ImmunoEM images, cells and structures were cross-referenced with images from H. Jastrow's "Electron microscopic atlas of cells, tissues and organs" before confirming classification (20).

Using TEM, only one image was taken that potentially shows synapsing on an adipocyte. The synapse surrounds a blood vessel while also bordering the cell membrane of an adipocyte (Fig. 3). There is no way to confirm whether synapsing is occurring on

the adipocyte or the blood vessel since it is in close proximity of both. It most resembles a synapse due to the width being around 2 uM and the presence of structures in the synapse that resemble synaptic vesicles. However, the shape is unusual for a synapse, since synapses usually have a rounded end that is stout instead of one that is long and is adjacent to more than one cell. It also lacks a definitive cell membrane, and so there is still the likelihood that a synapse has not been found.

Although no definitive synaptic innervation was found on adipocytes, sympathetic nerves were still found in apposition to fat cells and other structures. Mature peripheral nerve axons were in the interstitial space on the surfaces of adipocytes, potentially adhering to the fat cells (Fig. 4). The peripheral nerve axons were identified based on their smooth, ovaloid shape. Although peripheral nerve axons from Figure 4A and 4B are unmyelinated, the peripheral nerve axon in Figure 4C has darker material surround the outer edge that could potentially be myelination. While the axons were only found around adipocyte, the neurites were found in close proximity to blood vessels as well as adipocytes (Fig. 5). It was rare to find neurites that were by themselves in the interstitial space. Although they were difficult to identify due to the images being taken with immunoelectron microscopy, neurites were also potentially found adjacent to groups of adipocytes, always neighboring the nucleus of an adipocyte (Fig. 6).

Although not a neural structure, blood vessels were also found in the interstitium of adipose tissue (Fig. 7). Capillaries were some of the easiest structures to locate, due to adipose tissue's high vascularization and the blood vessels' discernable characteristics. Capillaries were identified by the endothelial cells adhering to the inner wall of the vessel and by the erythrocytes in the blood vessels if they were present. Blood vessels were also

classified based the thickness of the vessel wall and the empty area inside the vessel if other identifying factors were not present (Fig. 7). Blood vessels in both WAT and BAT are found to have sympathetic innervation, which made the lack of nerves and axons around the blood vessels all the more peculiar (68, 69). The only evidence found of innervation of vasculature was neurites that were in close proximity of capillaries (Fig. 5).

With TEM, all of the cells found in the SVF were cell types that were already known to be in adipose tissue. One of the immune cell types found were macrophages, which were identified based on their globular shape and the presence of lysosomal vesicles in the cell (Fig. 8). The macrophages in the images are smaller than the average macrophage size, because most likely the cross-sections was taken on the edge of the macrophage instead of straight down the middle, where the image would show the full size of the cell. The macrophages contained other cellular components as well, such as mitochondria (Fig. 8A) One macrophage in particular was found bordering a blood vessel, and the entire cell was completely filled with lysosomal vesicles to the extent that no other structures could be seen (Fig. 8B). The other type of immune cell found in the SVF were lymphocytes, which were either nearby or directly adjacent to adipocytes, always surrounded by material from the ECM (Fig. 9). There was no way to differentiate B cells, T cells and natural killer cells from each other based on visual attributes. Fibroblasts were found somewhat near adipocytes and there were always bundles of collagen near them (Fig. 10). There were many other images taken using TEM, however these images did not contain any structures or cells that were identified and analyzed (Fig. 11).

The ImmunoEM approach was not as effective in producing quality EM images, and there was no success with finding synapsing due to contamination and many other potential issues. Through the first round of staining certain areas of tissue were contaminated with bacteria and, in general, binding of gold nanoparticles was non-specific and did not attach to any synaptic proteins (Fig. 12). With the second round of immunostaining with gold nanoparticles, there was greater contamination than with the first round of staining. The cloud patches and dark material scattered about the tissue is most likely an issue of not washing the grids properly throughout the staining process (Fig. 13 & 14). The only images that can be analyzed from immuno EM are the ones taken of potential neurites that are surrounded by adipocytes (Fig. 6). It is important to note, though, that these neurites were found without the help of gold nanoparticles and could have been found just as easily if the tissues were prepared for TEM instead of immuno EM.

With immunofluorescent microscopy, different myeloid lineage cells were found to have synapsing occurring. Post-synaptic density proteins (PSD95) show up as bright purple/red dots under the fluorescent microscope. Immune cells varied in size, shape and general appearance, but the majority of them had concentrated fluorescing in the center of the cell, which was most likely the nucleus (Fig. 15). With immunofluorescent microscopy, post-synaptic proteins were found either bordering the edge of the immune cells or they were directly on the surface of the cell (Fig. 15). Synapsing on immune cells and adipocytes can be seen with both images taken from a regular fluorescent microscope and from images taken using a confocal microscope, but synapsing is much clearer and more easily identifiable in confocal images due to the absence of background

fluorescence (Fig. 15 & 16). With confocal images, the myeloid lineage cells still fluoresce green, but PSD95 fluoresces in red instead of purple and DAPI is also included to fluoresce cells' nuclei as blue. Figures 15 A-C show synapsing on myeloid lineage cells, where Figures A and C only show post-synaptic proteins bordering the cell while Figure B shows that the myeloid cell is completely covered in post-synaptic proteins. Confocal microscopy also showed synapsing on blood vessels, where Figure 16D shows the blood vessel is completely covered in post-synaptic proteins while Figure 16E shows post-synaptic proteins only bordering the blood vessel. Figure 16F shows synapsing on a non-myeloid lineage cell that is not fluorescing green, but has a cell nuclei that is fluorescing blue and is surrounded by a high concentration of post-synaptic proteins. Although there is no way to confirm the identity of the cell, it is most likely a preadipocyte based on size.

DISCUSSION

The electron microscopy did not yield the results hoped for when finding synapses on adipocytes or SVF cells. Only Fig. 3 out of the multitude of figures shows potential synapsing on an adipocyte. The structure was identified based on the width of the potential synapse and the presence of vesicles within the synapse. No confirmation can be made about this being a synapse yet due to the odd shape and lack of cellular membrane, and there is still the potential that this structure is not actually a synapse. Most images taken with TEM showed the composition of the stromal vascular fraction, which included macrophages, fibroblasts, lymphocytes and blood vessels. All of these cells and structures are consistent with what is usually found in the SVF, although other cells that are usually found in the SVF, such as pericytes and pre-adipocytes, were not found using TEM (26). We were also able to find cross-sections of both peripheral nerve axons and neurites within the SVF, which shows how there is communication from the brain to adipose tissue using sympathetic nerve signaling (6).

Neurites were found in close proximity to blood vessels, which s. No varicosities were found projecting from the neurite to the blood vessel, but this could be because the cross-section imaged happened to be in an area of the neurite lacking any type of synaptic projection. It is difficult to confirm with only an image of the cross section of the neurite and the blood vessel instead of having an actual image of a connection between the two, but there is potential communication because of the plasticity of vasculature in adipose tissue. It is already well known that degree of vascularization is constantly fluctuating depending on the metabolic needs of the body. Different proteins and signaling factors

that are released by adipocytes and other cells in adipose tissue are able to control angiogenesis and integrity of blood vessels. Sympathetic nerves are also able to release factors that regulate the maintenance of vasculature. With BAT, sympathetic nerves release nerve growth factor (NGF) that will stimulate both adipocytes and endothelial cells of blood vessels (59). NGF causes endothelial cells to release vascular endothelial growth factor (VEGF), which in turn promotes angiogenesis (59). Although BAT rather than WAT was used to find this potential role for nerves, it would make sense that this process also occurred in our fat depots since vasculature is still highly maintained in WAT. This neural projection close to the blood vessel, whether it is through an actual synapse or through varicosity, could be regulating blood flow through inducing either vasoconstriction or vasodilation, or the neurite could be releasing neural growth factors that stimulate the endothelial cell on the vessel. Peripheral nerve axons were also found, but they were neighboring adipocytes instead of blood vessels. There is still the same issue with the neurites in apposition to blood vessels; since there was no evidence of varicosities coming off of the axons to innervate the adipocytes, it is still unclear if there is any functional purpose for peripheral nerve axons to be neighboring so close to adipocytes.

One of the more commonly found cells in the SVF were fibroblasts. Most likely the fibroblasts found in our images were releasing collagen in order to maintain structural integrity of the tissue, since all of the fibroblasts had collagen surrounding them. One of the main roles of fibroblasts is to help remedy degradation of adipocytes by releasing collagen and increasing the amount of connective tissue in adipose (60). Just through visual identification, there was no way to confirm if the fibroblasts were carrying out

roles for maintaining metabolic homeostasis. Since preadipocytes were never found during imaging, there was no way to confirm if some the fibroblasts assisted in preadipocyte maintenance and differentiation. There is also not enough evidence in the current literature on what morphological changes fibroblasts go through when differentiating into adipocytes, and so fibroblasts that are in the process of differentiating cannot be identified based on imaging alone.

The final type of cells that we found were lymphocytes and macrophages. The macrophages were very evident due to the presence of lysosomal vesicles, while lymphocytes were identified based on the dark bordering often found on lymphocytes imaged using TEM. The lymphocytes that were imaged could be B cells, helper T cells, cytotoxic T cells, or natural killer cells. There was no way to identify the types of lymphocytes based on their morphological characteristics. With the important role T lymphocytes play in lipid metabolism and adipogenesis, especially in obese fat tissue, it would have been interesting to see the ratio of cytotoxic T cells to helper T cells and analyze how that relates to the health of the tissue before it was collected. We also had the same issue with macrophages, where M1 and M2 macrophages cannot be differentiated from each other based on appearance. Knowing if those macrophages promote tissue inflammation and insulin resistance or if they promote inflammation resolution and insulin sensitivity could also be used to analyze how the identity of the cells relates to the health of the tissue, especially if we could identify the specific types of macrophages that are innervated. Since none of the macrophages localized were found near neural structures, so far there was no evidence that macrophages had synapsing from

sympathetic nerves. This meant that macrophages had to be imaged and analyzed further through immunofluorescent microscopy.

Even though we were able to find many different types of cells and structures in the SVF, we were unable to find any evidence of direct communication between adipocytes/SVF cells and sympathetic nerves through synapsing. We were also unable to find any evidence of synapsing with the immuno EM. There was a common, reoccurring structure that always appears in between neighboring adipocytes and is always adjacent to at least one adipocyte nucleus (Fig. 13). We believe that those structures are axonal cross sections of peripheral nerves axons, similar to those found using TEM. However, no synapses could be found and those peripheral nerves were the only structures found using immuno EM. There are many different explanations for our lack of success. One of the biggest issues was preventing contamination from occurring to the tissue samples. Most likely the contamination occurred during staining with antibodies, since this issue did not occur for TEM imaging. Although the tissues from the second round of staining was much more affected by contamination than the tissue from the first round of staining, both tissue sections were found to have bacteria and other types of contaminants on them. This is such a big issue because not only does it make it harder to see the tissue under the microscope, but it also decreases antigenicity and the likelihood that antibodies will bind to synaptic proteins or synaptic vesicles. Contamination most likely came from the diluents and tools used throughout the staining/washing process not being sterile. In the future, better care will have to be taken to properly sterilize equipment and to use sterile solutions.

Another likely reason for the lack of success was due to having to find synapses in such a small area of tissue at a high magnification. The likelihood that a synapse would actually be found was greatly reduced because we were focused on areas that are only 60-90 nm, and it would take too long to image all the 60 nm an entire fat depot (See Fig. 1). There are currently methods that can be used automate TEM imaging, which would greatly increase its efficiency. Automated Transmission-Mode Scanning Electron Microscopy (tSEM) is able to create images that are the same quality as TEM images, but the size of the image field is greater while maintaining the same pixel size because the ATLAS software is able to combine multiple grid sections that are scanned into one image (61). The Townsend Lab, however, would not be able to use the tSEM method due to lack of funding and proper equipment. We must also take into consideration that another potential issue could have been with the protocol for preparing tissues for electron microscopy. This protocol was created just recently by the Townsend Lab and was only used a couple of times, and so there have not been very many opportunities for troubleshooting due to time and money. So more edits to the protocol and more trial runs need to be accomplished before the procedure can yield us accurate results.

Immunofluorescent and confocal microscopy yielded better results by displaying potential synapsing on both adipocytes and myeloid lineage immune cells, such as macrophages. Synapsing was indicated by purple/red fluorescent dots (PSD95) either on top of or bordering the cell. The images taken with the confocal microscope are the most accurate, since the synapsing is easier to see due to eliminated background fluorescence and staining cell nuclei with DAPI confirms that we are looking at cells instead of artifacts (Fig. 15). The adipocytes and myeloid lineage cells all have synapsing that is

concentrated on the edges of the cells, with some that have synapsing evenly distributed over the entire cell (Fig. 14 & 15).

There is the potential that adipocytes are not innervated, but instead receive signals from neurotransmitters in passing (62). Slavin et al. claims that only 2-3% of adipocytes are innervated, which means that the rest of the adipocytes receive signals from neurotransmitters released into the interstitial area instead of through direct synapsing (63). However, researchers are still unsure of the actual degree of synapsing on adipocytes since very little research has been done on the subject. Since our research found multiple adipocytes with synapsing through the use of immunofluorescent microscopy, our research argues against claims of adipocyte synapsing being rare. Since neurites and axons of sympathetic nerves were found adjacent to adipocytes in EM images, the post synaptic proteins found on adipocytes are most likely due to synapsing through varicosities on the nerve axons

The cell that displayed the highest concentration of post-synaptic proteins is one that has blue fluorescence to indicate a cell nucleus, but does not fluoresce green and so cannot be myeloid lineage. There is no way to definitely determine the type of cell, but most likely it is a preadipocyte due to the size and round shape. A potential explanation for the high level of synapsing is that preadipocyte is in the process of differentiating into a mature adipocyte. However, there is very little research on neural communication with preadipocytes.

Although we have no way to definitively classify the specific cell type of each myeloid lineage cell, cells that were found to be 30 um or bigger were identified as macrophages (64). Macrophages, of all the myeloid lineage cells, are the most likely to

have synapsing. There is already evidence of macrophages having roles associated with peripheral nerves. In the gastrointestinal tract, macrophages were found to have tendrils and processes branching into and surrounding somas and axons of sympathetic nerves (25). Since some of the extensions of these macrophages contacts varicosities on sympathetic axons, it is believed that these macrophages are either being activated by the nerve or they are serving a phagocytic function for the nerve (25). There are many different potential roles that these macrophages could have that would lead to synapsing. One potential role could be that the macrophages are not actually being activated by the neurons, but are instead within the vicinity in order to metabolize certain neurotransmitters such as norepinephrine and other catecholamines (52,65). Certain types of macrophages have specialized machinery for NE uptake, and NE degradation associates these macrophages with obesity since catecholamines are known for promoting lipolysis and other processes that are considered protective (52). These macrophages are activated by signaling factors, such as insulin and IL-4, ligands with well-established roles that affect the activity of adipocytes as well as other SVF cells (65). However, if synapsing is occurring on macrophages in order to activate them, then most likely those synapses are releasing neuropeptide Y (NPY). Nerves that contain NPY have been known to innervate macrophages and activate an inflammatory response from macrophages (66,67). Since NPY is released from sympathetic nerves, that macrophages surrounded by PSD95 in our images could be synapses from neurons that release NPY. There were also instances where PSD95 fluoresced in a different location from a myeloid cell, which could indicate synapsing on the adipocytes. Although we have promising

results, further imaging needs to be done to confirm that we are seeing communication by direct synapsing on immune cells.

In summary, imaging using immunofluorescent microscopy has shown that there is potential synapsing on adipocytes, preadipocytes, and myeloid lineage immune cells such as macrophages. It was already known that catecholamines would influence adipose function and processes, such as lipolysis, fat mass growth and transdifferentiation of WAT to BAT. However, there is very little visual evidence that synapsing occurs on adipocytes. If further imaging confirms the presence of synapses on adipocytes, this information could be further used to look into the degree of synapsing on adipocytes, and how degree of synapsing changes based on the health of the tissue. There was also very little visual evidence of synapsing occurring on macrophages in adipose tissue. This type of research is unable to determine whether a macrophage's close proximity to a synapse is due to housekeeping or actual synapsing, but still shows the macrophages are a key part of neuro-immune interactions. Although much more research needs to be done to confirm our findings, it currently supports the idea that the sympathetic nervous system plays a key role in modulating metabolic processes in adipose tissue through directly interacting with both adipocytes and SVF cells.

Further Research

Since immunofluorescent microscopy at 100X showed us our most promising results, we will continue taking images of synaptic proteins and myeloid lineage SVF cells that are labeled with fluorescent markers. We are also open to the idea of staining the tissue with other markers besides PSD95 in order to help localize synapses on cells.

The tissues could be stained with a synaptic vesicle marker (SV2), which would be located around the same area as PSD95 since they both attach to synapses. The tissues could also be stained with a pan neuronal marker such as beta-3 tubulin or PGP9.5, so that fluorescing of synaptic proteins at the ends of these nerves could confirm that we are actually seeing synapses instead of artifacts or non-specific binding. At this point and time, we are not sure if we are going to continue with TEM imaging.

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APPENDICES

APPENDIX A

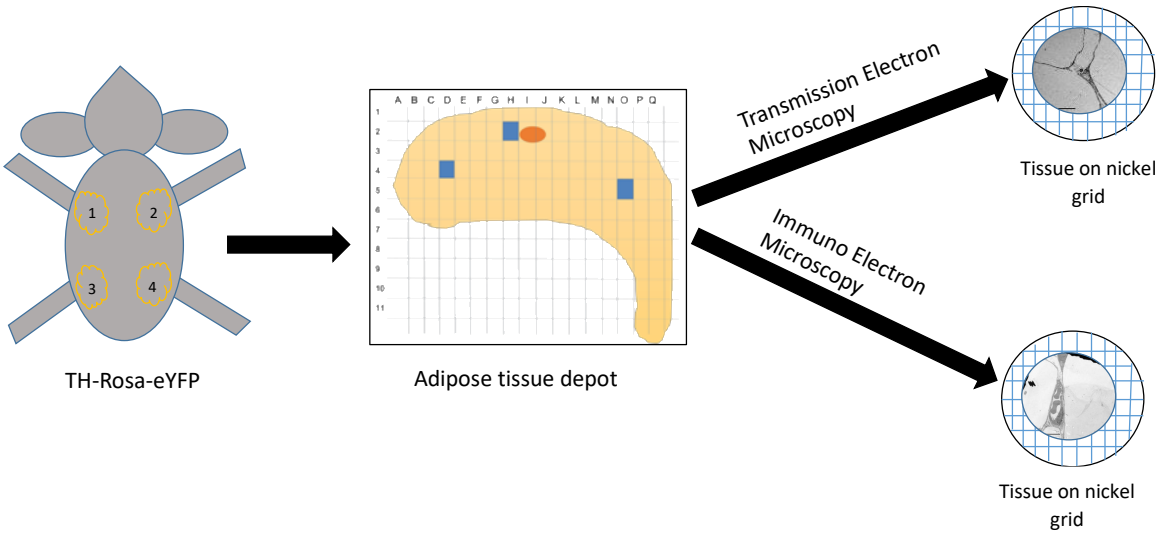

Figure 1: Workflow figure of tissue extraction for electron microscopy. Tissues from TH reporter mice inguinal and axillary depots. Depots 1 and 3 were used for transmission electron microscopy and depots 2 and 4 were used for immuno electron microscopy. Tissue sections were always taken from the three spots indicated on the tissue grid, and then further sectioned after processing so that they could fit onto 300-mesh nickel grids.

APPENDIX B

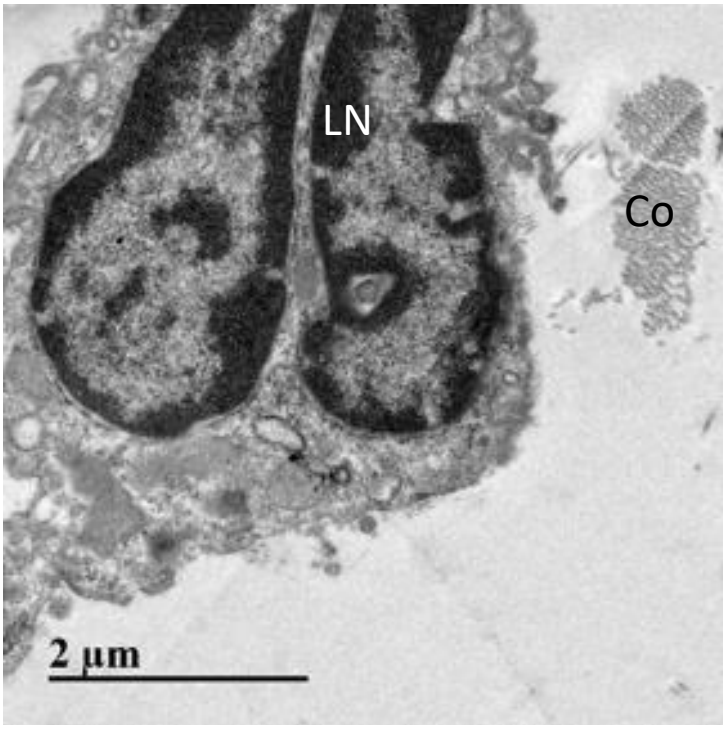

Figure 2: Lymph node in adipose tissue. This tissue section has the cross-section of the very edge of a lymph node (LN). The adipose tissue surrounding the lymph node is believed to have the greatest amount of innervation/synapsing. There were also multiple collections of collagen (Co) surrounding the lymph node. Magnification at 10500x.

APPENDIX C

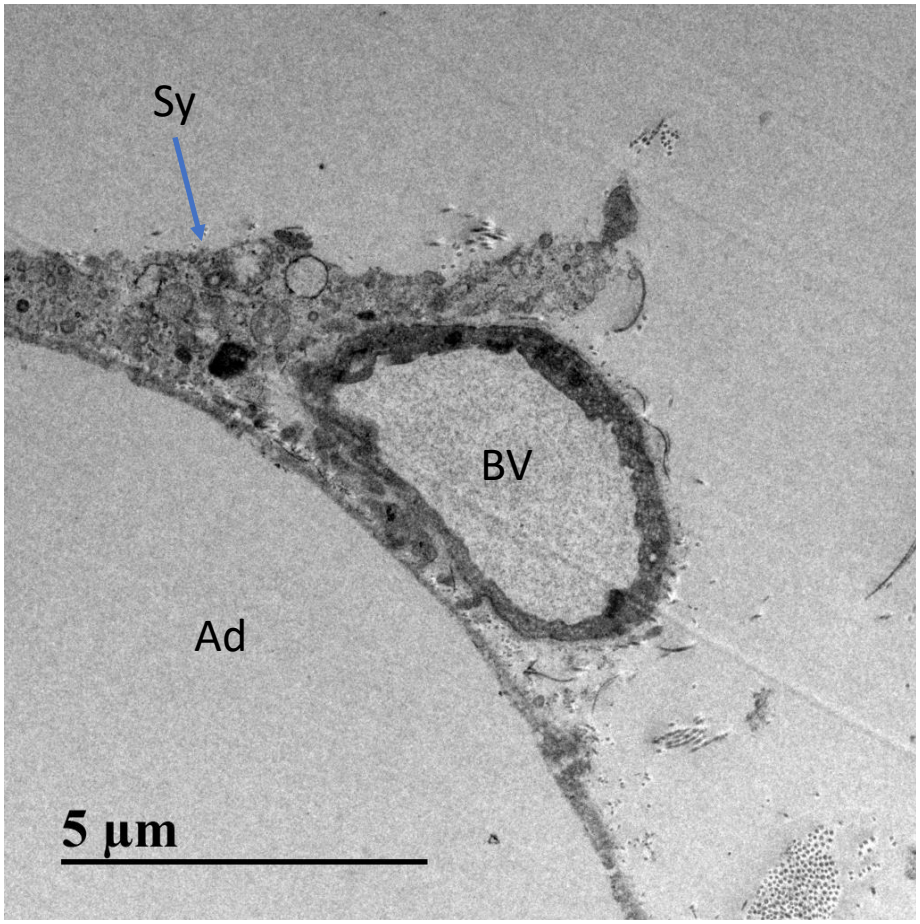

Figure 3: Potential synapsing on an adipocyte and a blood vessel. The potential synapse (Sy) is surrounding a blood vessel (BV) and is closely adjacent to an adipocyte (Ad). The synapse contains structures that look like vessels contain neurotransmitters. Magnification at 4600x.

APPENDIX D

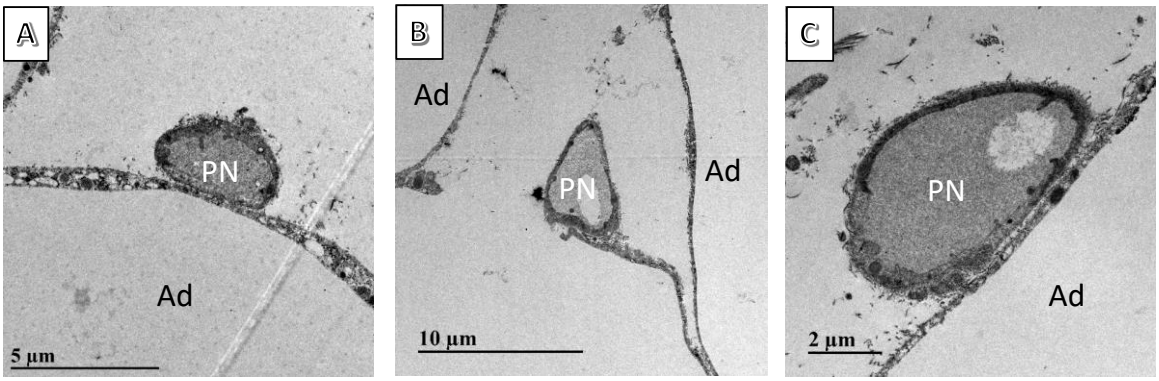

Figure 4: Peripheral nerve axons adhering to adipocytes. A) Non-myelinated peripheral nerve axon (PN) that is adhering to an adipocyte (Ad). Magnification at 4600x. B) A peripheral nerve axon that is lighter in color than most others and doesn't have the usual ovaloid shape. Magnification at 2600x. C) Peripheral nerve axon that has myelination. Magnification at 5800x.

APPENDIX E

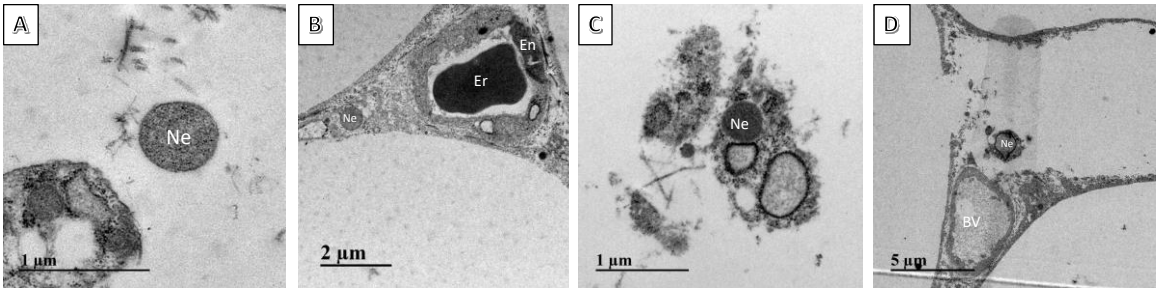

Figure 5: Neurite neighboring of capillary blood vessel. A) Zoomed in image of neurite at 25000x. B) Neurite is adjacent to both the capillary blood vessel and to two different adipocytes. The blood vessel contains a blood cell (Er) and has a large endothelial cell (En) within the blood vessel. C) Neurite not bordering any structures or cells. Magnification at 13500x. D) Another neurite found adjacent to a blood vessel (BV). Magnification of neurite at 3400x.

APPENDIX F

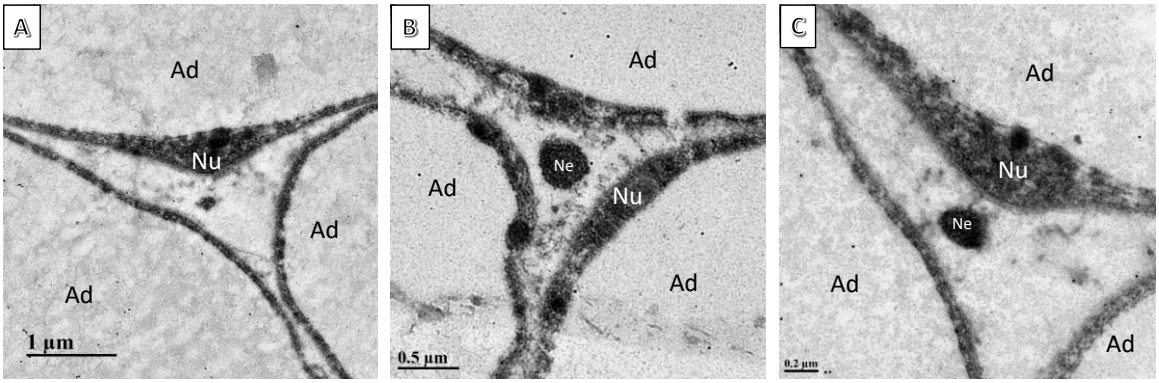

Figure 6: Potential neurites neighboring the nucleus of an adipocyte. A-C) The neurites (Ne) are always found between three different adipocytes (Ad) where one of them has a nucleus (Nu) that is right next to the neurite.

APPENDIX G

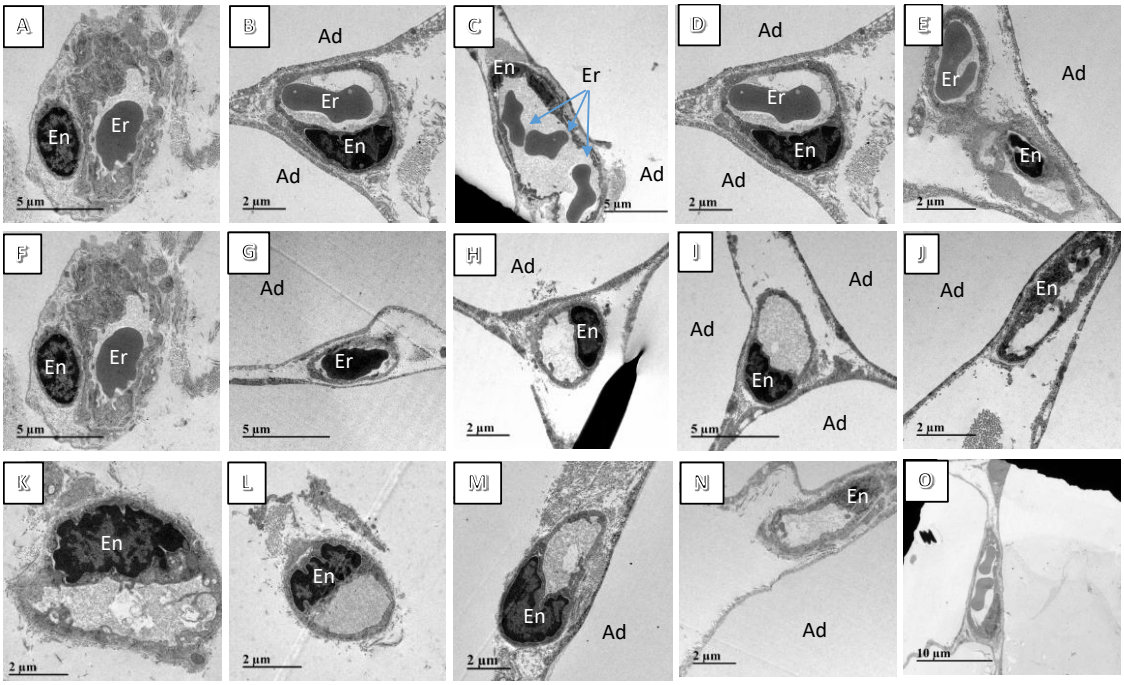

Figure 7: Capillary blood vessels in the SVF of adipose tissue. The range of magnification used was between 3400x-7900x. A-N) The blood vessel images were taken using TEM. Blood vessels identified based on the endothelial cells (En) within the walls of the blood vessel and based on the presence of erythrocytes (Er) within the blood vessel. Identified as capillaries due to their size since they have a small circumference and can only hold 1-3 erythrocytes. Usually found adjacent to adipocytes. O) This image of a blood vessel was taken on tissue that was prepared for immuno EM instead of TEM, hence the decreased detail in the image.

APPENDIX H

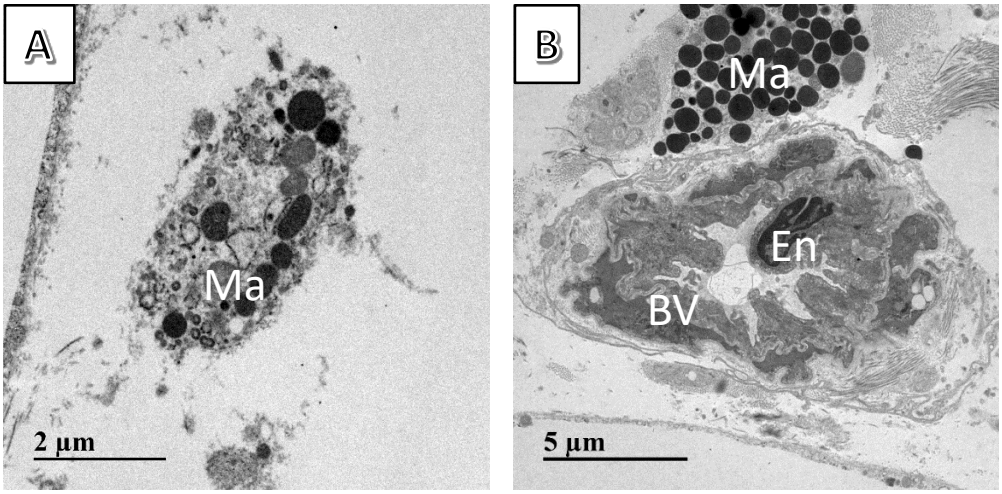

Figure 8: Macrophages neighboring both adipocytes and capillary blood vessels. A) Identified as a macrophage (Ma) based on globular shape and presence of lysosomic vesicles in the cell. Also contains mitochondria. Magnification at 7900x. B) This macrophage also has a globular shape, but contains many more lysosomic vesicles. Closely adjacent to a blood vessel. Magnification at 3400x.

APPENDIX I

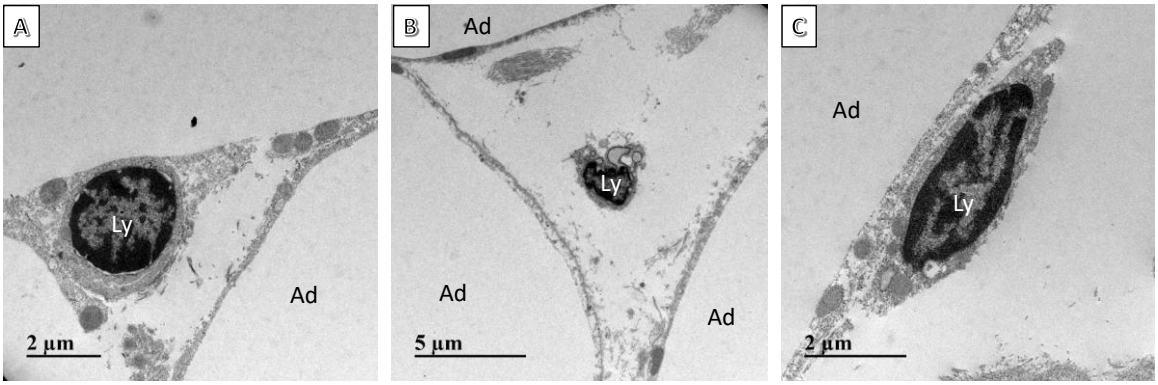

Figure 9: Lymphocytes adjacent or adhering to adipocytes. A-C) Lymphocytes identified based on dark bordering around the cells and dark patterns within the cells specific to lymphocytes. Magnification between 3400x-7900x.

APPENDIX J

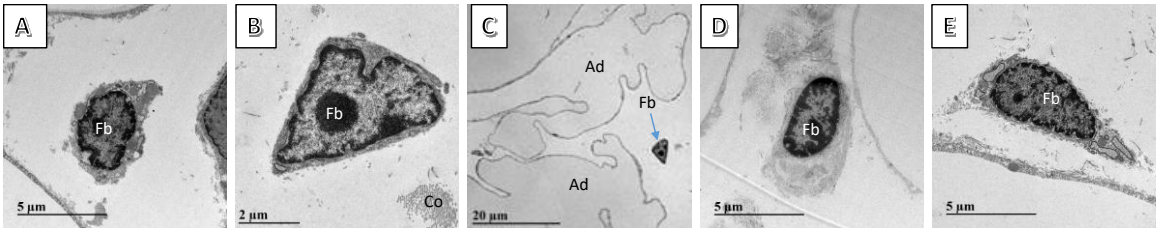

Figure 10: Fibroblasts in the SVF of adipose tissue. A-E) Magnifications ranged from 1100x-7900x. All fibroblast identified based on the very clear nucleus in the center and by

APPENDIX K

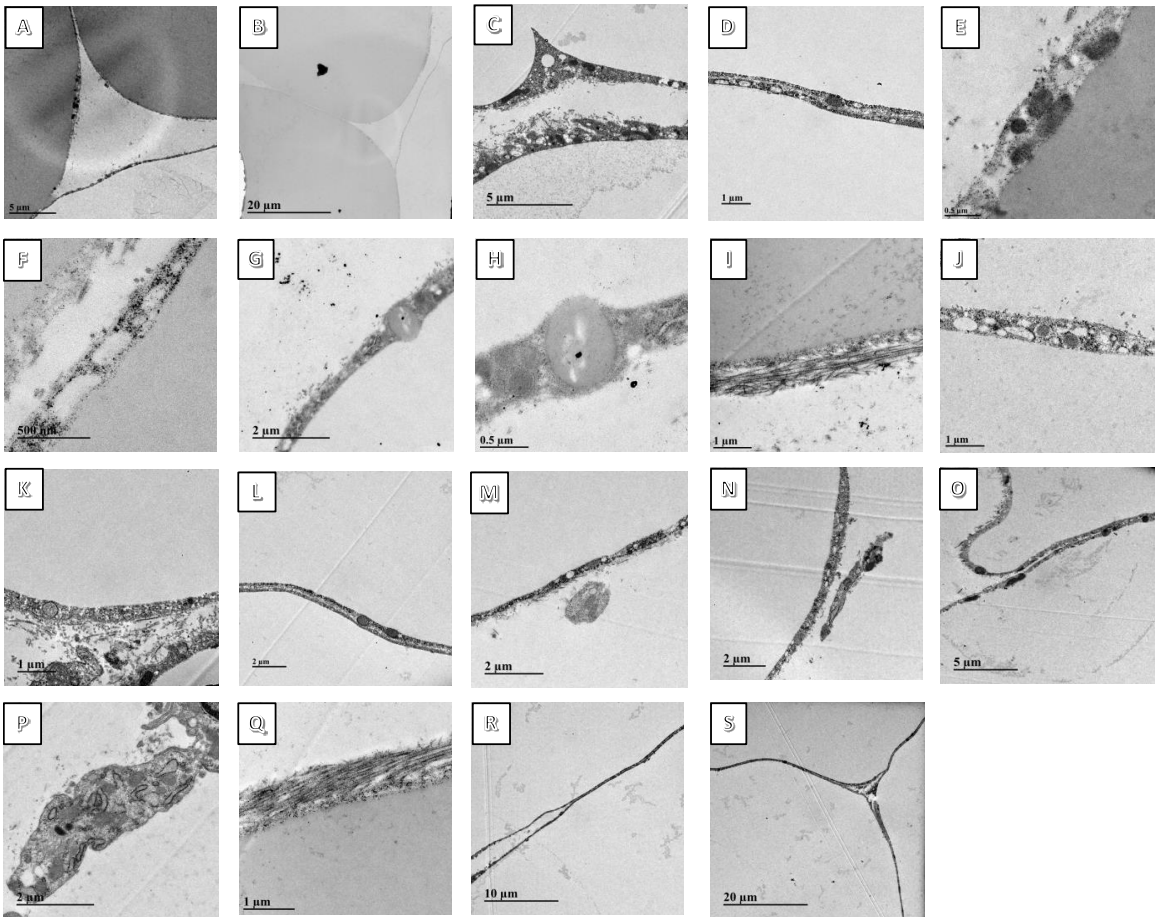

Figure 11: All other images taken using TEM. A-S) Magnifications ranged from 1100x-34000x for these images. These were all of the images taken that did not have any nerve structures (synapses, axons, etc.) nor did they have any SVF cells. These images were not

APPENDIX L

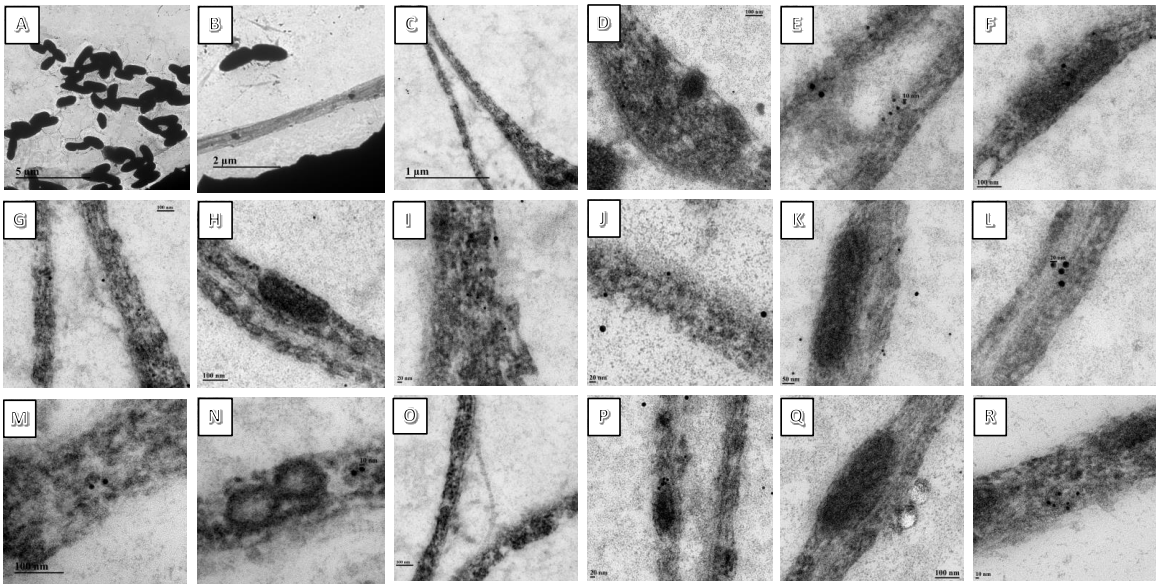

Figure 12: Initial images taken using immuno EM. A-B) Some parts of the tissue sections were contaminated with bacteria (black, elongated figures). C-R) There was a lot of non-specific binding of gold nanoparticles to adipose tissue, especially on and around adipocyte nuclei. None of these images were used for analysis.

APPENDIX M

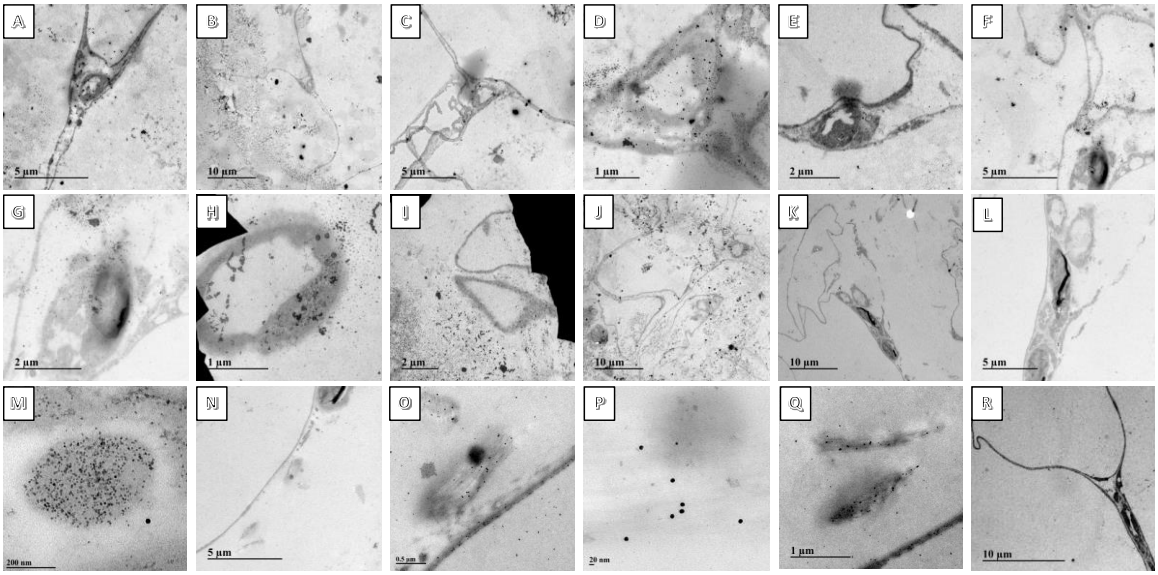

Figure 13: With the second round of preparing tissues for immuno EM, there was a lot more contamination of the tissues than with the previous round of staining. A-R) There were many areas that had cloudy portions or lots of dark material scattered around that made it very difficult to see the structures and cells in the tissue. There was non-specific binding of gold nanoparticles with these tissue sections as well. None of these images could be used for analysis.

APPENDIX N

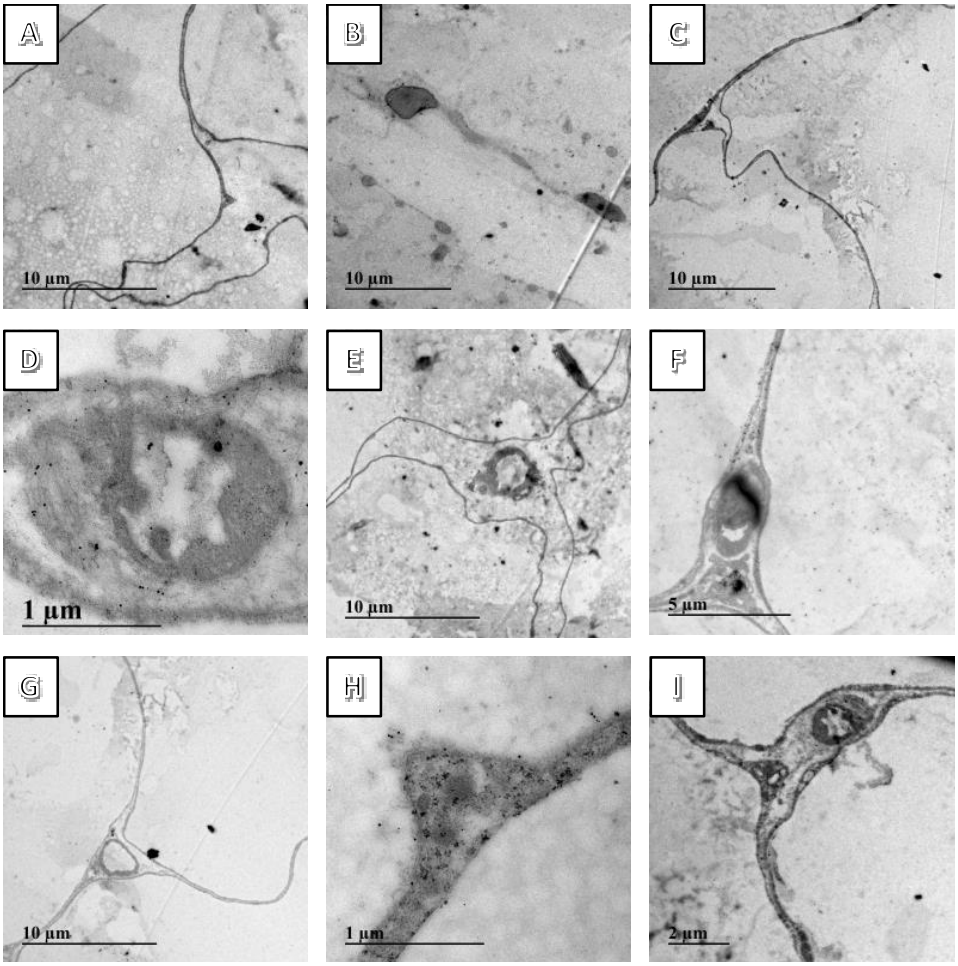

Figure 14: Images showing contamination of tissue sections during second round of staining. A-I) Due to issues with non-specific binding of gold nanoparticles and contamination ruining the quality of the images, these images were not analyzed.

APPENDIX O

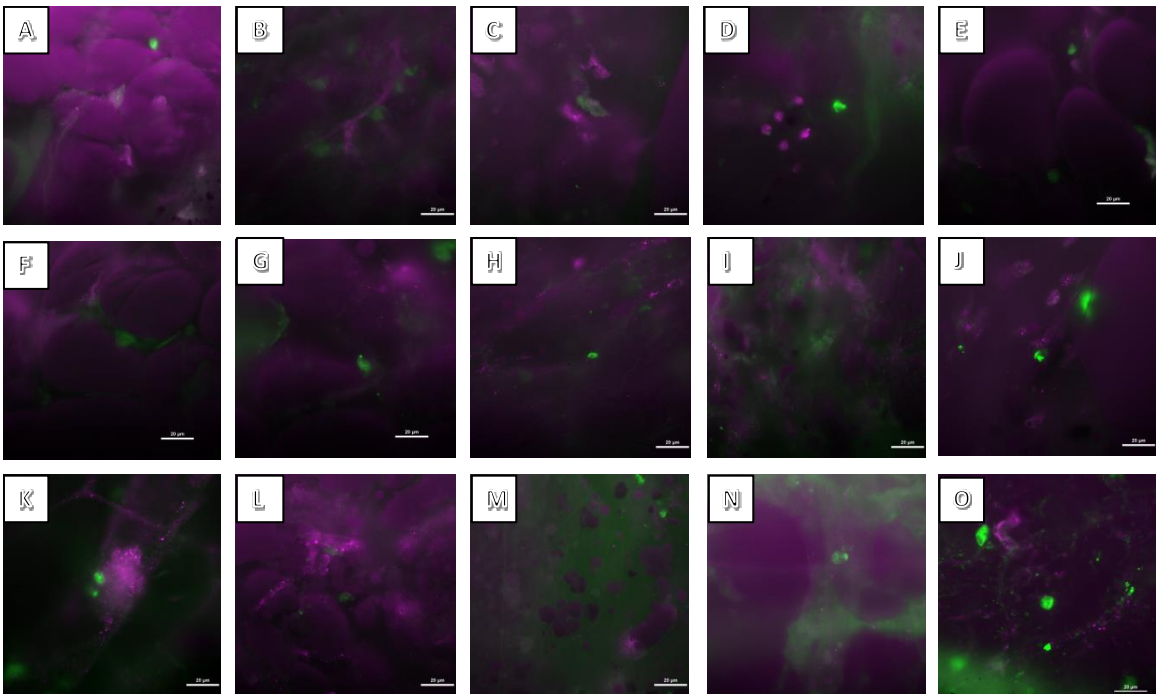

Figure 15: Sympathetic synapsing on adipocytes and myeloid lineage cells. Synaptic proteins were stained purple using PSD95 ab18258 (1:1000), and myeloid lineage cells were stained green with GFP ab6662 (1:1000). A) Myeloid lineage cell with a clear nucleus situated between three adipocytes, with PSD95 surrounding its outer edge. B) Myeloid lineage cells are adjacent to postsynaptic proteins. C) PSD95 was found on the surface of the myeloid lineage cell and also bordering a groove in the myeloid lineage cell.

APPENDIX P

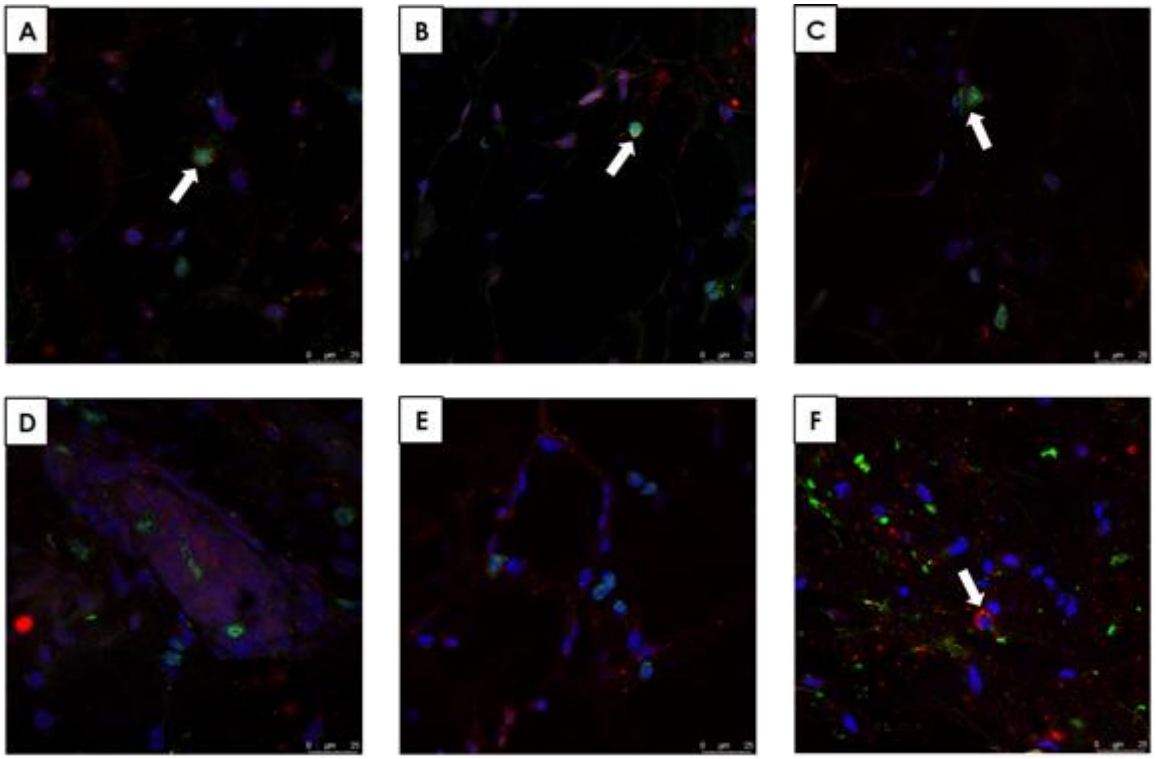

Figure 16: Sympathetic synapsing on macrophages and adipocytes, using confocal microscopy. Synaptic proteins were stained red using PSD95 ab18258 (1:1000), myeloid lineage cells were stained green with GFP ab6662 (1:1000), and cell nuclei were stained blue with DAPI. A-C) Myeloid lineage cells with clear cell nuclei stained in blue, with PSD95 either bordering the edge of the cell or distribute evenly across the cell. D-E) PSD95 found on blood vessels in adipose tissue. Figure 16D shows post-synaptic proteins evenly distributed across the blood vessel, while figure 16E shows post-synaptic proteins only bordering the edge of the vessel. The blue staining on the blood vessel in figure 16E could be the nuclei of endothelial cells lining the vessel. F) High concentration of PSD95 surrounding an unidentified cell, most likely a preadipocyte.

APPENDIX Q

EM Protocol for Adipose Synapses
EG Updated 1/23/2019

Mice Protocol:

Procedure:

1. Once the adipose tissue samples are collected, ipsilateral inguinal scWAT (whole depot) will be fixed and stained for immunoelectron microscopy and contralateral inguinal scWAT (whole depot) will be fixed and stained for transmission electron microscopy.
2. TH-Rosa-eYFP reporter (boosted with quantum dot direct conjugated antibody), plus antibodies against synaptic markers (PSD95 post synaptic protein, SV2 synaptic vesicles) attached to secondary antibodies that are conjugated to gold nanoparticles of different sizes.

Tissue Processing for EM (No AuNP):

Reagents:

- Fixative:
    - 2% glutaraldehyde
    - 2% paraformaldehyde
    - 0.1 M PB at pH 7.4 for diluent
- 1% OsO_4 with PB as diluent
- Acetone
- Toluidine Blue
- Uranyl Acetate
- Lead Citrate

Materials:

- Epon Resin
- Gelatin capsules
- Ultramicrotome
- Metal grid
- (Find out what type of TEM UMaine has)

Procedure:

1. Collect contralateral inguinal (or axillary if necessary) scWAT tissue (Cinti et. al, 2001).
2. Fix small fragments of tissue in 2% glutaraldehyde-2% paraformaldehyde in 0.1 M PB (NOT PBS), pH 7.4, for 4 hours at 4 ℃ (Cinti et. al, 2001). Then cut with a razorblade to fragments not exceeding 1 mm^3 once the tissue is out of the fixative (Kelly).
3. Wash overnight in the same buffer at 4 ℃ (Cinti et. al, 2001).
4. Postfix in 1% OsO_4 in PB. Have half the mixture be 2% OsO4 and half the mixture be 0.2 M PB so that you are left with 1% OsO4 in 0.1 M PB. Postfix in 1% OsO4 in PB for either 45 minutes, or with 14 second exposure in a microwave (Cinti et. al, 2001).
5. Wash the tissues three times using a 50% H2O and 50% 0.2 M PB mixture.
6. Dehydrate in upgraded acetone series: (Cinti et. al, 2001).
    a. Without Microwave:
        i. 50% 10 min. x 2
        ii. 70% 10 min. x 3

iii. 95% 10 min. x 2
iv. 100% 10 min. x 4

b. With Microwave:
i. 25% 14 second exposure in a microwave
ii. 50% 14 second exposure in a microwave
iii. 75% 14 second exposure in a microwave
iv. 95% 14 second exposure in a microwave
v. 100% 14 second exposure in a microwave

c. The concentrations of the acetone and the number of steps will most likely be changed to whatever concentrations the lab already has.

7. While the tissue is dehydrating, the resin should be made. See resin protocol for how to make resin.
8. Make a 50/50 mixture of resin and 100% acetone. Put the tissues in that mixture and leave them overnight with the caps to the bottles slightly open so that the acetone can evaporate out during the night (Kelly).
9. Put the tissues on a droplet of pure resin (usually using a microscope slide works for this part) and leave them in a vacuum chamber for 15 minutes in order to get all leftover acetone out.
10. Put the tissues on another droplet of pure resin and put them in a vacuum chamber for another 15 minutes.
11. While the tissues are in the vacuum chamber, the labels that will be embedded with the tissues can also be put in resin and in the vacuum chamber in order to get rid of all air bubbles that might be in the paper. When labeling, remember to use pencil on paper.
12. Embed in silicone rubber moulds. When embedding, first fill the moulds halfway with resin. Then put the tissues on the pointed end of the mould and the label on the other end of the mould. Fill the rest of the mould so that it is slightly overflowing. The resin will slightly shrink while curing/polymerizing. Have the tissues that are in moulds covered by something (usually a petri dish works) in order to keep dust out until they are ready to be polymerized (Cinti et. al, 2001).
13. Polymerize in 60 ℃ for 48 hours (Cinti et. al, 2001).
14. Stain with Toluidine blue for 2-5 min (Cinti et. al, 2001) and ("Routine Transmission Electron Microscopy").
15. Use an ultramicrotome to cut the tissues into segments that do not exceed 60-90 nm in thickness (Cinti et. al, 2001).
16. Stain the samples directly on the grid with uranyl acetate and lead citrate (Cinti et. al, 2001).

Tissue Processing for ImmunoEM:

Reagents:

- Fixative
  - 4% paraformaldehyde
  - Diluent is 0.1 M PB
- 1 X PBS
- EtOH
- LR white resin
- 0.1 M PBS containing 1% BSA and 1% Tween 20
- PBS with 3% normal serum
- Uranyl acetate
- Lead citrate

Instruments/Tools:

- 300-mesh nickel grid.
    - Use nickel since tween buffer will react with a copper grid.
- Fridge
- Ultramicrotome

Procedure:

1) Fix small fragments (2 mm^3) of ipsilateral inguinal scWAT tissue directly or after perfusion of 4% paraformaldehyde in 0.1 M PB, pH 7.4, overnight at 4 ℃ (Cinti et. al, 2001).
2) Wash in PB. (Cinti et. al, 2001).
3) Dehydrate in graded EtOH series:
    a) Without Microwave:
        i) 50% 10 min. x 2
        ii) 70% 10 min. x 3
        iii) 95% 10 min. x 2
        iv) 100% 10 min. x 4
    b) With Microwave:
        i) 25% 14 second exposure in a microwave
        ii) 50% 14 second exposure in a microwave
        iii) 75% 14 second exposure in a microwave
        iv) 95% 14 second exposure in a microwave
        v) 100% 14 second exposure in a microwave
4) Infiltrate with LR white resin at room temperature, have 2-3 changes with 1 hour in between each change, then leave overnight (Cinti et. al, 2001).
5) Embed in gelatin capsules and then thermal cure 50 ℃ overnight (Cinti et. al, 2001).
6) Resin blocks are sectioned with an ultramicrotome and made into ultrathin sections that are then placed on 300-mesh nickel grids.
7) Grids are placed on droplets of 0.1 (10X) PBS containing 1% BSA and 1% Tween 20, then left for 20 minutes at room temperature (Cinti et. al, 2001 and Cousin et. al, 1992).
    a) Having Tween as part of the BSA/PBS solution is an option, used by Cousin on AT immunostaining for EM. The purpose of Tween would be to increase permeabilization.
8) Sections are then placed on droplets of 3% normal serum (goat) in 1X PBS for 20 min at room temp (Cinti et. al, 2001).
9) Incubate the tissues with the first primary antibody PSD95, diluted in 1X PBS containing 1% BSA at 1:100 dilution, overnight at 4 ℃.
10) After several washes in 1X PBS (usually ten dunks into the 1X PBS, moved to another droplet and this is done 6 or 7 times), sections should be incubated with the first secondary antibody, goat anti-rabbit, conjugated with 10 nm gold particles diluted 1:50 in 1X PBS for 30 min at RT (Cinti et. al, 2001).
11) Wash in PBS and MilliQ H_2O (Cinti et. al, 2001)
12) Incubate the tissues with the second primary antibody SV2, diluted in 1X PBS containing 1% BSA at 1:25 dilution, overnight at 4 ℃.
13) After several washes in 1X PBS, sections should be incubated with the second secondary antibody, goat anti-mouse, conjugated with 15 nm gold particles diluted 1:50 in 1X PBS for 30 min. at RT.
    a) It is important to incubate with the smaller nanoparticles first (10 nm), and then the bigger ones (15 nm) (“TEM Immunogold”).
14) Stain with uranyl acetate for 15 min and lead citrate for 1 min (Nagase et. al, 1996).

AUTHOR'S BIOGRAPHY

Emma R. Garner was born in Beverly Massachusetts on December 11, 1996. She was raised in Sandown, New Hampshire and graduated from Pinkerton Academy in 2015. Emma is majoring in biology with a pre-med concentration and is minoring in psychology. She worked for the Townsend Neurobiology Lab, while also working as an Area Coordinator for the Tutor Program.

Upon graduation, Emma plans on applying to medical school to become a doctor in either psychiatry or women's health.