QUANTIFYING NEURONAL SYNAPSES IN 5XFAD MICE UTILIZING IMMUNOLABELING AND TISSUE CLEARING

by

Sarah C. Nagel

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Project Approved:

Michael Chumley, Ph.D.
Department of Biology
(Supervising Professor)

Gary Boehm, Ph.D.
Department of Psychology

Giri Akkaraju, Ph.D.
Department of Biology

ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder that affects over 5 million Americans, and there is currently no cure. AD is characterized by Aβ plaques, neurofibrillary tangles, inflammation, and neuronal/ synapse loss in the brain. Oligomeric Aβ has been hypothesized to bind to receptors in neuronal synapses, thus disrupting synapse signaling. Neuroscientists are trying to determine if the synapse disruption caused by Aβ leads to the cognitive dysfunction seen in AD. The present studies goal is to determine if Aβ is responsible for the decrease in synaptic density in the CA1 region of the hippocampus in mice. To quantify synaptic density the present study will count the number of dendritic spines on neurons. The study utilized a tissue clearing technique, which removed lipids from the brain and eliminated light scatter due to these fatty acids. I expect the tissue clearing technique to result in enhanced resolution of the images of the neurons. This study will also determine if $5xFAD^{+}$, an AD transgenic mouse model, mice have fewer dendritic spines in comparison to Wild Type (WT) mice. I expect there to be a decrease in the number of observed dendritic spines in the $5xFAD^{+}$ mice compared to the control group.

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choose to attend will be lucky to have you. Thanks for staying by my side throughout my time at TCU. I have indeed found a true and incredible friend in you.

INTRODUCTION

Alzheimer`s Disease (AD) is a progressive degenerative brain disease resulting in dementia and memory impairment in patients (1). This disease affects more than 40 million people worldwide and is most prevalent in the elderly. Research is immensely important as there are currently no treatments available to slow, prevent, or cure the damage inflicted upon individuals (2). Scientists have studied the postmortem brains of people with AD and described the pathological changes to try to understand how to prevent them from occurring. These changes include inflammation, amyloid-beta (Aβ) protein plaques, neurofibrillary tangles, neuron loss, and synapse loss (1).

The elevation of the protein Aβ in AD patients plays a critical role in causing the disease. Aβ accumulates intracellularly as a soluble form and extracellularly as plaques inside the brain. The pathogenic form of this protein is formed by the cleavage of amyloid precursor protein (APP) by enzymes, γ-secretase and β-secretase (1). According to the amyloid-beta hypothesis, the overproduction of Aβ and inability of the body to clear this highly hydrophobic peptide from the brain causes the progression of AD (3). This hypothesis emphasizes strategies to enhance clearance of this neurotoxin to prevent or slow the damage inflicted to individuals (2). Research has shown the hippocampal region, primarily the CA1 region, is affected early on in AD patients and mice, resulting in episodic and spatial memory impairment (4). Early in the disease state, hippocampal synapse density is reduced. Synapses have been correlated with memory impairment (5). To treat AD, it is imperative to apprehend the mechanism behind how synapse disruption occurs.

Our lab previously showed that by injecting lipopolysaccharide (LPS) over seven days in mice induced inflammation and caused the formation of central Aβ. Furthermore, the

Aβ accumulation resulted in cognitive dysfunction (6). In a follow up study, our lab demonstrated the Aβ accounted for approximately 30% of the cognitive dysfunction (7). The Aβ inflammatory cascade pathway is of critical importance in determining how neuronal death and synapse loss occurs inside the brain of an AD patient (8). A synapse is associated with information transfer inside the brain; composed of pre-synaptic neurons, post-synaptic neurons, and additional supportive cells including glial cells and astrocytes (2). Neurotransmission is achieved through the flooding of calcium ions in the pre-synaptic neuron, which in turn releases neurotransmitters into the neuronal synapse (9,10). These chemical messengers then activate the post-synaptic neuron, triggering an electrical impulse which continues to carry the neural signal. This whole process results in signal propagation through the brain.

It has been thought either oligomeric Aβ binds to neuronal receptors or Aβ fibrils and oligomers accumulate inside the synapse, thus interfering with signaling (11). Some studies postulate Aβ binds to NMDA receptor subunits on the post-synaptic neuron, which would result in Aβ physically blocking the synapse (12). This synapse interference, results in the inability of the pre- and post-synaptic neurons to communicate with each other. No longer able to send electrical and chemical messages throughout the central nervous system, these neurons slowly die off (2).

Most synapses in the brain occur on dendritic spines, and a typical mature spine has a synapse located at its head. The literature states that the presence of a dendritic spine on a neuron indicates there is one of three possibilities: a live functioning synapse, a future synapse, or a recent a past synapse. Thus, one can quantify synapses by using dendritic spines to represent a functioning synapse. Dendritic spines, however, are heterogeneous in shape and size. Spines are also dynamic, constantly altering their appearance in response to brain activity. Therefore,

dendritic spine number and shape has been thought to directly relate to synaptic transmission and plasticity (16). Some previous studies have been able to show through calculating dendritic spines on neurons that the number of these dendritic spines indeed decreases when Aβ is elevated inside the brain (12).

Neuroscientists are trying to determine if the synapse disruption due to Aβ leads to the cognitive dysfunction seen in AD. However, first scientists must see if Aβ results in the destabilization of neurons and synapses in mice. The problem is how difficult it is to visualize individual neurons inside the hippocampus. To study neuronal synapses, the present study will use an immunolabeling and tissue clearing technique in an AD transgenic mouse model. These transgenic mice have been genetically modified to produce the pathophysiology of AD. The $5xFAD^{+}$ mice overexpress Aβ and resemble the cognitive deficits seen in AD. The 5xFAD mouse transgene has 5-point mutations in a human gene that forces the mouse to overproduce Aβ (13). The present research will also use the Thy-1-EGFP (Enhanced Green Fluoresce Protein) transgene. GFP is a protein that fluoresces green when excited by the correct wavelength of light. In these transgenic mice, GFP is found in a small percentage of neurons, which then allows them to be tagged by antibodies and visualized under a microscope (14). Antibodies will label GFP and Aβ.

Tissue clearing techniques are currently being used to study the brains of mice in more detail; however, these techniques can be complex and expensive. The goal of this project is to develop a simpler technique which combines immunolabeling, solvent-based clearing, and microscopy in 50 μm hippocampal sections. The tissue clearing technique will clear lipids from the brain, thus hopefully allowing enhanced visualization of neurons and axon guidance defects in the brain (15). This study will compare the images of brain sections that have undergone tissue

clearing to conventional staining sections. I hypothesize that the tissue clearing will provide better resolution of neuronal synapses in comparison to conventional staining techniques. The study will also see if the increased amount of Aβ in the hippocampus of GFP^+/FAD^+ mice results in a change in the number of dendritic spines compared to GFP^+/FAD^- mice. It is hypothesized that in comparison, the FAD+ mice will have fewer dendritic spines corresponding to the elevated levels of Aβ in their hippocampus.

MATERIALS AND METHODS

Subjects and Housing

All experiments employed the use of 3–5-month-old male and female $GFP^+/5xFAD^+$ and $GFP^+/5xFAD^-$ mice from the Texas Christian University vivarium. Animals were treated and housed following the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and in agreement with standards approved by the Institutional Animal Care and Use Committee (IACUC) of Texas Christian University. Animals were housed in groups of three or four in standard cages sized 12.5cm x 15cm x 25 cm. All the experimental groups were housed under the same conditions. Lights were on only from 7:00 AM to 7:00 PM daily and food and water were constantly available for mice to consume as they desired.

Tissue preparations

Mice were anesthetized using ketamine (100mg/kg) and xylazine (5mg/kg). After proper sedation, mice were perfused transcardially with 1X phosphate buffered saline for 5 minutes followed by 4% paraformaldehyde (PFA; pH7.4) solution for 7 minutes for fixation of the brain. Brains were embedded into 3% agarose to prepare for slicing. Next, the brains were cut into 50-

micron sections using a VT1000S vibratome (Leica Biosystems, Buffalo Grove, IL) and placed inside well plates filled with 1 % PFA and 0.03%azide solution.

Tissue Clearing / Immunolabeling Technique

The tissue clearing technique and immunolabeling followed the IDISCO+ Protocol (17). This procedure was utilized to eliminate lipids and control for light scatter. We followed the IDISCO+ protocol with the following changes. The methanol dehydration and rehydration steps were carried out in 20-minute steps. The incubation in dichloromethane and bleaching in 5% H2O2 in methanol steps were carried out in one-hour increments. We incubated the sections in permeabilization solution for 1 hour, blocking solution for 2 hours, primary antibody solution overnight, and secondary antibody solution for 2 hours. The primary antibodies used in the solution were chicken anti-GFP (Aves labs, Davis, CA) and mouse anti-Aβ (Thermo Fisher Scientific, Waltham, MA). The secondary antibodies used were donkey anti-chick-Alexa Fluor 488 and donkey anti-mouse-Cy3 both from Jackson ImmunoResearch Laboratories Inc. (Westgrove, PA).

Conventional Immunolabeling Technique

Three different 50 μm brain sections from each of the eight mice were washed in PBST (0.1 % Tween 20/1x Phosphate Buffer Solution) 4 times for 10 minutes each time. Sections were permeabilized for approximately 2 hours in a solution consisting of PTx.2 (100mL PBS 10X/ 2mL TritonX-100), Glycine, and Dimethyl Sulfoxide (DMSO). Then, sections were blocked for 1 hour in 3% donkey serum/1x PBS. Primary antibodies were added with 3% donkey serum/1x PBS and left overnight at 4°C. Primary antibodies included chicken anti-GFP and mouse anti-Aβ as previously stated. The next day sections were washed 6 times for 10 minutes each time in PBST. Secondary antibodies included donkey anti-chick-Alexa Fluor 488 and donkey anti-

mouse-Cy3 as previously stated. These secondary antibodies were added with 1000 μL PBST and the sections were covered with foil to avoid photo bleaching. After 2 hours, sections were washed 6 times for ten minutes each time in PBST. Sections were mounted with aqua-poly/mount to prepare for imaging.

Confirmation of 5xFAD Transgene

Mice were confirmed positive for the 5xFAD transgene by completing a thioflavin stain. The hemispheres fixed in PFA were washed with PBS, embedded in 3% agarose and cut into 40 μm sagittal sections with a Leica VT1000 S Vibratome (Leica Biosystems, BuffaloGrove, IL). Sections were then stored in a 1% PFA and 0.03% azide solution. Hippocampal sections were washed 3 times for 10 minutes in Millipore water, stained with Thioflavin-T for 5 minutes, washed for 2 minutes in 70% ethanol, washed twice for 2 minutes in 50% ethanol, and then washed twice for 2 minutes in Millipore water. The sections were then mounted and examined utilizing confocal microscopy on a Zeiss LSM710 (Carl Zeiss MicroImaging, Inc., Thornwood, NY) to observe plaques in the hippocampus.

Imaging

All images were collected on a laser-scanning confocal microscope Zeiss LSM710 (Carl Zeiss) using Zen2009 software (Zeiss). A 3D surface rendering was employed to obtain 3D representation of neurons before counting the dendritic spines. Five neurons were chosen from each section in the CA1 region of the hippocampus. Images were collected using the 63x objective and digitally zoomed to access an axon of approximately 30 microns in length.

Dendritic Spine Counting

For each section, dendritic spines were counted on a 20 μm length of an axon of 5 separate neurons in the CA1 region of the hippocampus. Three different volunteers who were blind to the

genotype of the mice were then employed to count dendritic spines. The average from the three counters was taken for each neuron. Then, all the FAD^+ neuron spine counts were averaged and all the FAD^- neuron spines counts were averaged. A Student's t-test was then conducted to determine statistical significance between FAD^- and FAD^+ groups.

RESULTS

Appearance of Brain Sections Following Tissue Clearing

The tissue clearing technique eliminated lipids from the brain sections. As seen in Figure 1, the cleared sections are translucent compared to the opaque white color of the non-cleared sections.

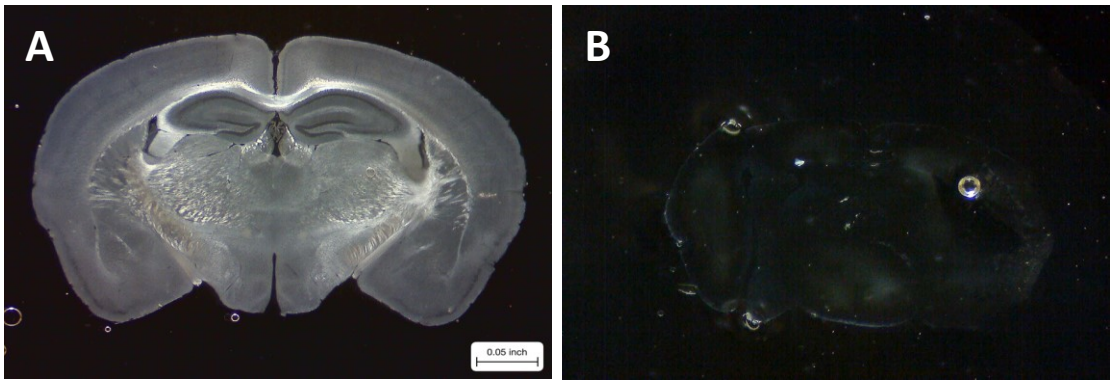

Figure 1. Comparison of cleared brain section to a non-cleared section. Panel A indicates the non-cleared hippocampal brain section. Panel B shows a cleared brain section.

Aβ Plaque Confirmation in 5xFAD Mice

The results show FAD^+ mice indeed have an overproduction of Aβ and Aβ plaques in their hippocampus. The FAD^- mice do not have a presence of Aβ and protein plaques in their hippocampus. The plaques are seen in the dentate gyrus and are clustered near the beginning of the CA1 region in the hippocampus. Figure 2 confirms the presence of Aβ plaques present in a FAD^+ mouse.

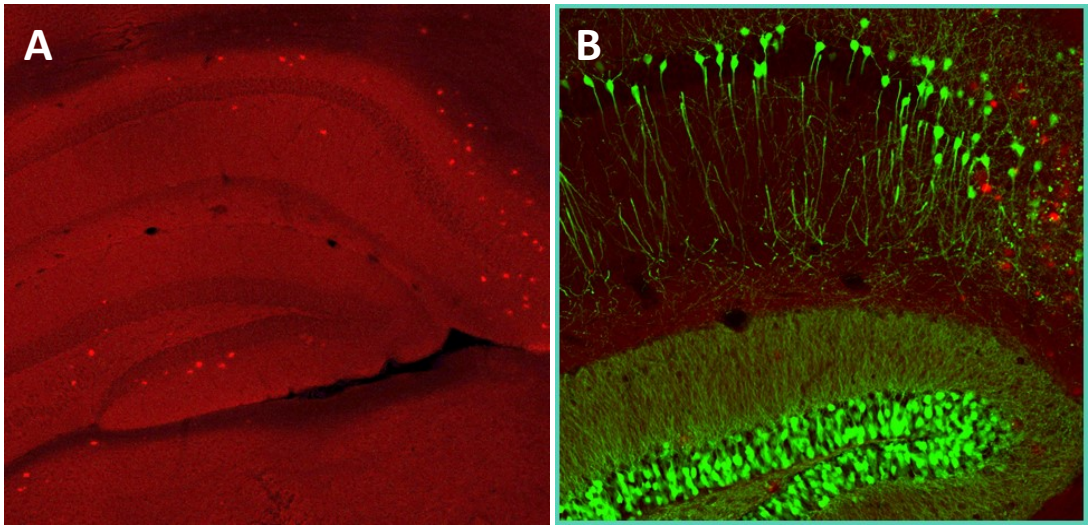

Figure 2. Amyloid Beta in a FAD$^+$ Mouse Hippocampus. Red indicates the presence of amyloid beta. Bright red spots signify amyloid beta plaques. Green indicates the presence of GFP. Panel B shows amyloid beta plaques in the CA1 region of the hippocampus.

Appearance of Microscopy Images Following Tissue Clearing

The tissue clearing technique results in microscope images which are difficult to see. The dendritic spines on the neurons are also not apparent, thus resulting in images which are difficult to count the dendritic spines. At this point in the project, I decided the tissue clearing technique was not worth continuing to use for quantifying synapses. The conventional technique however resulted in bright, clear images. As seen in Figure 3, the conventional technique gave a brighter resolution during imaging. For the rest of the project the conventional immunolabeling method was employed for counting dendritic spines. Figure 4 highlights one of the non-cleared sections, which was utilized for counting dendritic spines.

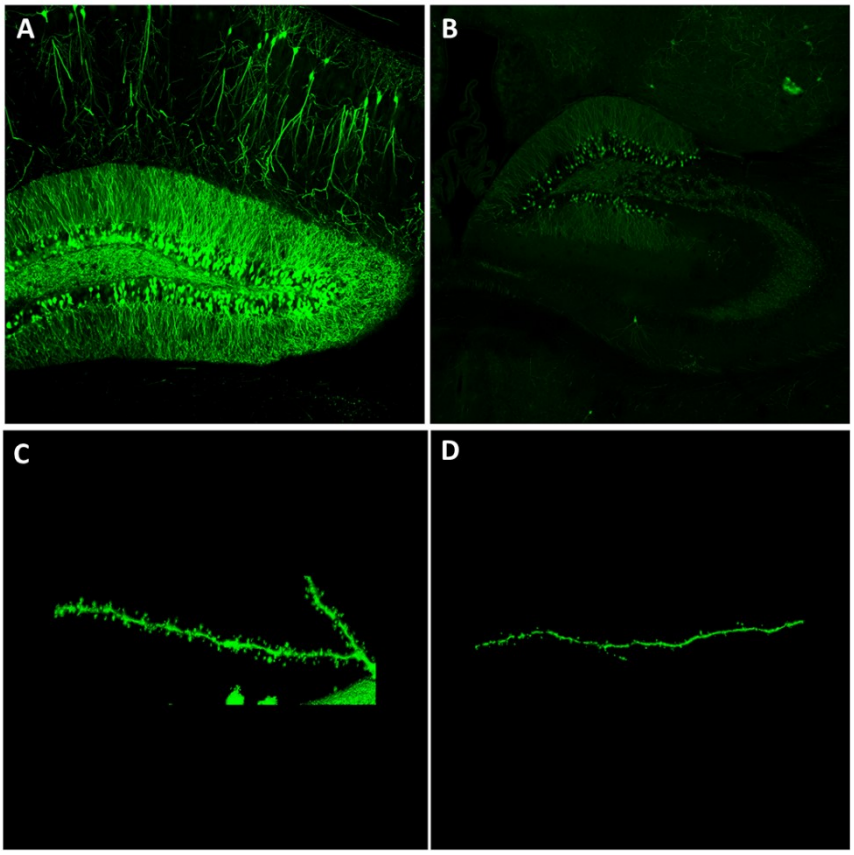

Figure 3. Comparison of Tissue Clearing Images to Conventional Staining Images of neurons. Hippocampal neurons from the conventional staining technique are shown in green in panel A, and panel B shows the same hippocampal section but from the tissue clearing technique. Panel C shows a dendrite with dendritic spines from the conventional staining method, with pnael D showing the same dendrite from the tissue clearing technique.

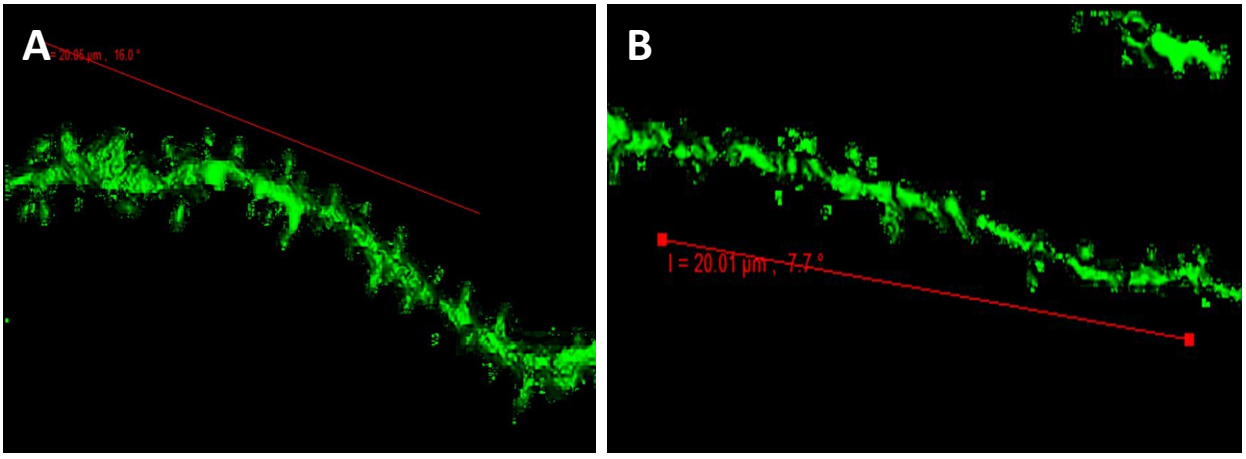

Figure 4. Comparison of Neurons in FAD- and FAD+ Mice. Panel A shows a neuron of a FAD- mouse. Panel B shows a neuron of a FAD^{+} mouse.

Aβ Effects on Dendritic Spine Counts

On average the FAD^+ mice have 15 dendritic spines over a 20 μm section of a neuron, as the FAD^- mice had an average of 21 dendritic spines per neuron. A Student's t-test confirms the FAD^+ mice have a statistically significant decrease in the number of dendritic spines compared to the control group, FAD^- mice. As seen in Figure 5, the FAD^+ mice exhibit a decrease in the number of dendritic spines. The p-value from the Student's t-test was <.005 meaning there is a very low probability the results from the test are due to chance.

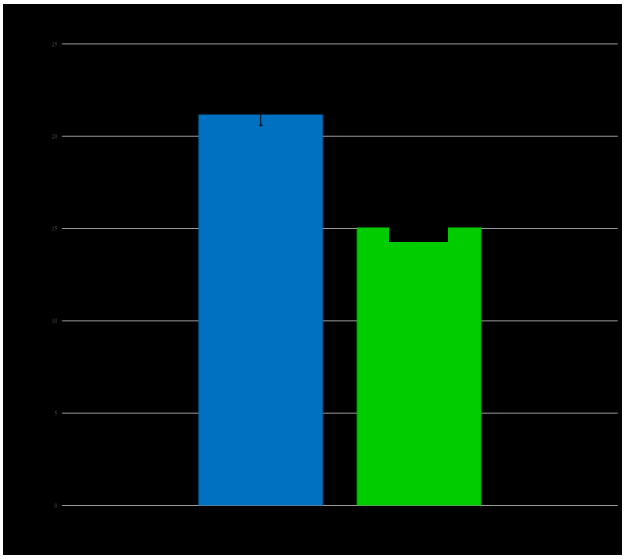

Figure 5. **The Effect of Amyloid Beta on Dendritic Spine Number**. FAD positive mice exhibited a significant decrease in dendritic spine number compared to the non-transgenic control. * represents a statistically significant difference at $p < .005$. Bars represent mean ± SEM.

DISCUSSION

The goal of this research project was 2-fold: first to see if tissue clearing provided enhanced images of neurons and second to determine if mice that overproduce Aβ have a decrease in the number of dendritic spines in the hippocampus region of the brain.

The first part of my hypothesis was not supported by the data. I had expected the tissue clearing technique to provide better resolution of neuronal synapses in comparison to conventional staining techniques. However, the results indicated tissue clearing did not provide

an advantage in imaging resolution. The tissue clearing technique was not beneficial as it was more difficult and expensive than the traditional method. The adaptation from the IDISCO$^+$ protocol intended for whole brain samples or whole organs to our 50 μm brain sections was not achieved. A challenge in utilizing the tissue clearing technique is how the treatment can alter the brain section's topography. The MeOH treatment used in the tissue clearing procedure might have altered the epitope in such a way that the antibodies had a poor binding affinity for their target antigen. With less primary antibodies bound to their target, the secondary luminescent antibodies are also decreased in number. This procedure thus diminishes the intensity of the fluorescent protein and causes the GFP antibodies to give off a poor signal. However, the conventional immunolabeling technique provided an efficient method in order to count dendritic spines in the CA1 region of the hippocampus.

The second part of my hypothesis was supported by my data. I expected in comparison, the FAD$^+$ mice would have fewer dendritic spines corresponding to the elevated levels of Aβ in their hippocampus. The FAD$^+$ mice have a statistically significant decrease in the number of dendritic spines compared to the control group, FAD$^-$ mice. Since the FAD$^+$ mice show a decrease in the number of dendritic spines, this likely means that Aβ is responsible for the destabilization of the neuronal synapses. This data supports the theory that Aβ oligomers bind to neuron receptors in the synapse and can cause synapse disruption and ultimately neuronal death.

The confocal microscope used in this project can only differentiate 2 separate objects which are 250 nm apart; however, neuronal synapses are only 20 nm wide. This means the technology utilized is unable to see inside neuronal synapses and cannot differentiate the post-synaptic neuron from the pre-synaptic neuron. Therefore, I used dendritic spines to represent a synapse. According to the literature, a dendritic spine on a neuron means one of three

possibilities: there is a live functioning synapse present, there will be a synapse in the near future, or there was a functioning synapse in the past (16). Future studies could utilize Electron Microscopes to look inside neuronal synapses and count the number of live functioning neurons.

For future studies we could also determine if the fewer synapses due to Aβ overproduction is the cause of cognitive deficits. This would provide a link as to why Aβ causes the learning and memory deficiencies seen in AD. Our lab has previously shown by injecting WT mice with LPS over 7 days that Aβ production can be induced. The increased levels of Aβ then leads to cognitive dysfunction in the mice (6). In the future, studies could see if these mice that do not possess the 5xFAD transgene but do make their own Aβ would also show a decrease in dendritic spines. By examining neurons in mice treated with LPS we would see if their overproduction of Aβ would also correlate with a decrease in synaptic density. This would provide a link as to why we see the cognitive dysfunction in mice. We could confirm whether the reduction of synapses in the hippocampus causes a role in cognitive dysfunction.

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