THE EFEFCT OF AMYLOID BETA ON SYNAPSES

by

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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disease that leads to cognitive deficits. The brain dysfunction in AD is marked by an increase in amyloid-beta, the protein responsible for plaque deposition in the brain. The severity of the cognitive deficits positively correlates with the load of Aβ. Prior research in animal models has pointed to soluble Aβ causing synaptic disruption. In the present study, the aim was to understand the effect that Aβ has on synapses. We used immunolabeling in an AD transgenic mouse model. The 5xFAD mouse model utilized in this study rapidly develops Aβ pathology. This model mimics the pathophysiology of AD in humans. The mouse model used was also knock-in transgenic for Green Fluorescent Protein (GFP) on mature CNS neurons. Using immunolabeling, GFP was tagged with antibodies, thus making neurons visible under the microscope. Antibodies were also used for Aβ in order to visualize the amount of Aβ protein and its location. Images of synapses were obtained in both FAD+/GFP+ and FAD-/GFP+ mice. Comparing these images, we were able to determine that Aβ accumulation affects the observed number of synapses in the hippocampus.

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INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disease that effects over 40 million people worldwide. The primary population affected by AD is people over the age of 65 (1). AD causes cognitive deficits and memory loss. There is currently no cure for AD, therefore research into this disease is extremely important (2). Studies have been conducted to understand the pathology and effects of AD. Studying post mortem brains has revealed that pathological changes, including the accumulation of amyloid beta protein, disruption of synapses, and loss of neurons, occur as a result of AD (1).

One of the main proteins linked to the progression of Alzheimer's disease is amyloid beta (Aβ). Aβ accumulates extracellularly as plaques inside the brain. Aβ oligomers have also been shown to form intracellularly. The precursor of Aβ is known as amyloid precursor protein (APP). APP is cleaved by β-secretase and γ-secretase to from Aβ. It is believed that an inability to clear Aβ, along with overproduction of the protein, causes the progression of AD (3). Studies have shown that when oligomers of Aβ form, it causes synapse disruption, which is linked to cognitive deficits (4). A synapse is the gap between two neurons in which neural impulses pass through, usually via a chemical signal known as a neurotransmitter. The neurotransmitter signals the next neuron to begin an electrical impulse so the signal can continue to be carried to the proper area of the brain or body. Disruption of a synapse leaves the pre-synaptic neuron unable to send a signal to the post-synaptic neuron. One of the main areas of the brain affected in early stages of AD is the CA1 region of the hippocampus (5). In order to further understand AD, more research into how synapse disruption occurs is necessary.

Previous studies from our lab have shown that lipopolysaccharide (LPS) induced inflammation leads to increased accumulation of Aβ (6). LPS is usually found on outer

membrane of gram-negative bacteria. When injected into mice it causes an immune response that leads to inflammation. This inflammation has been shown to increase amyloid beta accumulation in the periphery and in the brain (7). Aβ accumulation has been linked to cognitive disruption including spatial and episodic memory impairment (5).

Understanding how Aβ accumulation causes synapse disruption is vital to developing treatments for AD. It is believed that Aβ oligomers accumulate inside the synapse or that oligomeric Aβ may bind to neuron receptors resulting in signal interference (8). Multiple studies postulate that Aβ physically blocks the synapse by binding to the NMDA receptor subunits on post-synaptic neurons (9). This synapse disruption causes the neurons involved to slowly die (5). Previous studies have confirmed that elevated Aβ levels cause a decrease in the number of synapses observed in the brain (10).

Currently, a topic of interest in neuroscience is whether synapse disruption caused by Aβ accumulation is what leads to the cognitive deficits observed in AD. In order to determine this, scientists must confirm that Aβ does in fact cause a decrease in the number of observed synapses. One way to confirm this relationship is via immunofluorescent microscope images of amyloid beta blocking synaptic junctions. The present study will use immunolabeling in an AD transgenic mouse model. The 5xFAD mouse model utilized in this study rapidly develops Aβ pathology (11). This model mimics the pathophysiology of AD in humans. A thioflavin stain will be used to confirm the 5xFAD mice are producing Aβ. The mouse model used will be crossed with the Green Fluorescent Protein (GFP) transgenic mouse. GFP fluoresces green when exposed to ultraviolet light. In this transgenic model, GFP is found on some mature central nervous system neurons. The offspring of this cross will produce animals that express GFP in the neurons of mice that may or may not have the 5xFAD transgene. Using immunohistochemistry,

GFP can be tagged with antibodies, thus making neurons visible under the microscope (12). Antibodies will also be used for Aβ in order to visualize the amount of Aβ protein and its location. Images of synapses will be obtained in both FAD+/GFP+ and FAD-/GFP+ mice. To quantify synapses dendritic spines will be counted on the neurons of both FAD+ and FAD- mice. A dendritic spine represents either a forming synapse, a previously active synapse or a currently active synapse (13). Comparing these dendritic spine counts will determine if Aβ accumulation effects the observed number of synapses in the hippocampus. This study hypothesizes that the neurons of FAD+ mice will have fewer dendritic spines compared to the neurons of FAD- mice.

MATERIALS AND METHODS

Subjects and Housing

This study employed four 5xFAD+/GFP+ and four 5xFAD-/GFP+ mice ranging from 3-6 months old. Mice were bred in the TCU vivarium, originating from a breeding colony located in Jackson Laboratory in Bar Harbor, Maine. Animals were cared for according to the Guide for the Care and Use of Laboratory Animals (National Research Council, 2010) and in agreement with procedures outlined by the Institutional Animal Care and Use Committee (IACUC) of Texas Christian University. Mice were housed in groups of three or four, in cages measuring 12.5 cm x 15 cm x 25 cm. A 12-hour light-dark cycle was used with lights turned on at 7:00 AM and turned off at 7:00 PM. Food and water were available ad libitum.

Tissue Preparation

Mice were anesthetized using ketamine (100 mg/kg) and xylazine (5 mg/kg). After proper sedation, mice were perfused transcardially with 1X phosphate buffered saline (PBS; pH

7.4) for 5 minutes followed by 4% paraformaldehyde (PFA; pH 7.4) solution for 7 minutes for fixation of the brain. Tissue was stored in 4% PFA at 4°C until they were sectioned into 50 μm sections using a VT1000S vibratome (Leica Biosystems, Buffalo Grove, IL) and placed inside well plates filled with 1x PBS.

Confirming the Presence of Amyloid Beta

The presence of amyloid beta in the hippocampus was confirmed by completing a thioflavin stain. Hippocampal sections were washed 3 times for 10 minutes in Milli-Q 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 Milli-Q 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.

Immunohistochemistry

Three different 50 μm brain sections from each of the eight mice were washed in PBST (0.1 % Tween 20/1x PBS) 4 times for 10 minutes each time. Sections were permeabilized for approximately 2 hours in a solution consisting of PBS, 1% Donkey Serum. 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 (1:4000; Aves Lab, Davis, CA) and mouse anti-Aβ (1:1000; Thermo Fisher Scientific, Waltham, MA). The next day sections were washed 6 times for 10 minutes each time in PBST. Secondary antibodies which included donkey anti-chicken-Alexa Flour 488 (1:1000) and donkey anti-mouse-Cy3 (1:1000), both purchased from Jackson ImmunoResearch Laboratories, Inc., West

Grove, PA were added with 1000 uL PBST and the sections were covered with foil to avoid photo bleaching. After 2 hours, sections were washed 6 times for 10 minutes each time in PBST. Sections were mounted with aqua-poly/mount and imaged.

Imaging

Neurons were analyzed using a Zeiss LSM 710 laser-scanning confocal microscope (Thornwood, NY) at 63x optic. Image analysis was performed using Zen2009 software (Zeiss). Five neurons from the CA1 region of the hippocampus were imaged from each of the brain sections. A 3D surface projection render was employed to obtain a 3D representation of each neuron before counting dendritic spines.

Counting Dendritic Spines

Three different volunteers who were blind to the genotype of the mice were the employed to count dendritic spines. Dendritic spines were counted within a 20 µm area of each neuron. 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 t-test was conducted to determine if there was statistically significant difference between groups.

RESULTS

Amyloid beta was observed in high levels in the hippocampi of FAD+ mice. This included amyloid beta plaques (Figure 1). Amyloid beta was observed throughout the CA1 region of the FAD+ mice and was present in the same location as the neurons that were imaged in this study (Figure 2). No amyloid beta was observed in the hippocampi of FAD- mice.

Dendritic spine counts ranged from 15-40 spines per 20 μm in FAD- mice and 5-30 spines in FAD+ mice. On average FAD+ mice had 15 spines per 20 μm section of the neurons in their CA1 region while the average dendritic spine count for the FAD- mice was 22. (Figure 4). The results of the t-test indicated $p<0.005$, suggesting a statistically significant difference in the number of dendritic spines between the FAD+ and FAD- mice.

DISCUSSION

This study hypothesized that increased levels of Aβ in the FAD+ mice would correlate with a decrease in the number of dendritic spines. This hypothesis was supported. The FAD+ mice showed a statistically significant decrease in the number of dendritic spines compared to the FAD- mice. The presence of Aβ in the hippocampi of the FAD+ mice coupled with the decrease in dendritic spine number indicates that Aβ likely has a role in causing a decrease in synaptic density. This supports the theory that Aβ oligomers disrupt synapses by binding to receptors on the neuron. However, the technology to image amyloid-beta within a synapse is not currently used in our lab. The confocal microscope used can only distinguish two separate objects within 250 nm. A synapse is typically 20 nm wide. Using electron microscopy could confirm the presence of Aβ oligomers in synapses and would help support the data in this study.

In this study, we used dendritic spine counts as an estimate of the number of active synapses on each neuron. A dendritic spine could be an active synapse, a forming synapse or a previously functioning synapse. In order to get a more accurate idea of how many active synapses a neuron has, immunolabeling synaptophysin and PSD-95 would be helpful. Synaptophysin acts as a pre-synaptic marker and PSD-95 acts as a post-synaptic marker. By labeling these, future studies could possibly get a more accurate synapse count because the

presence of a pre- and post-synaptic neuron would indicate that dendritic spine is likely an active synapse.

Our lab has previously used an LPS model to induce amyloid beta production in WT mice. In these studies, 7 days of LPS injections induced amyloid beta production and cognitive deficits in the WT mice. Future studies could examine neurons in GFP+ mice treated with LPS to see if amyloid beta production and observed cognitive deficits correlate with a decrease in synaptic density. This research would help give us a better idea of what causes the memory impairment observed in AD patients.

Figure 1

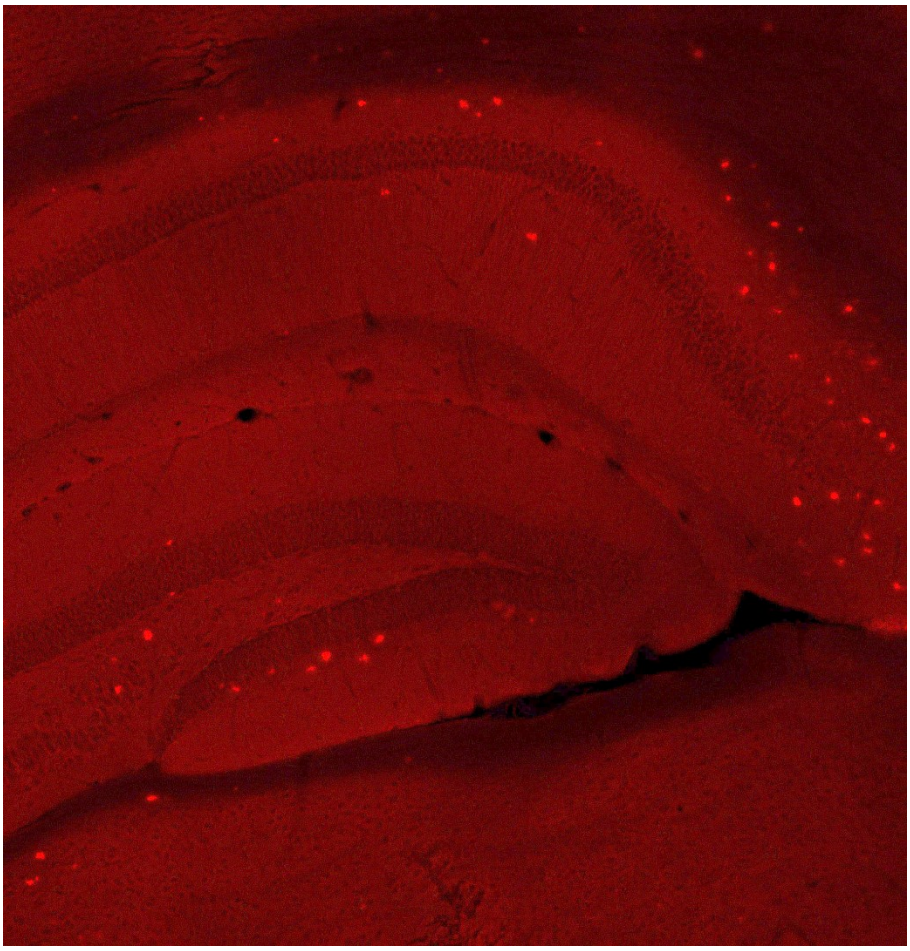

Figure 1. Amyloid Beta in a FAD+ Mouse Hippocampus. Red indicates the presence of amyloid beta. Bright red spots signify amyloid beta plaques. Amyloid beta is observed throughout the hippocampus in FAD+ mice.

Figure 2

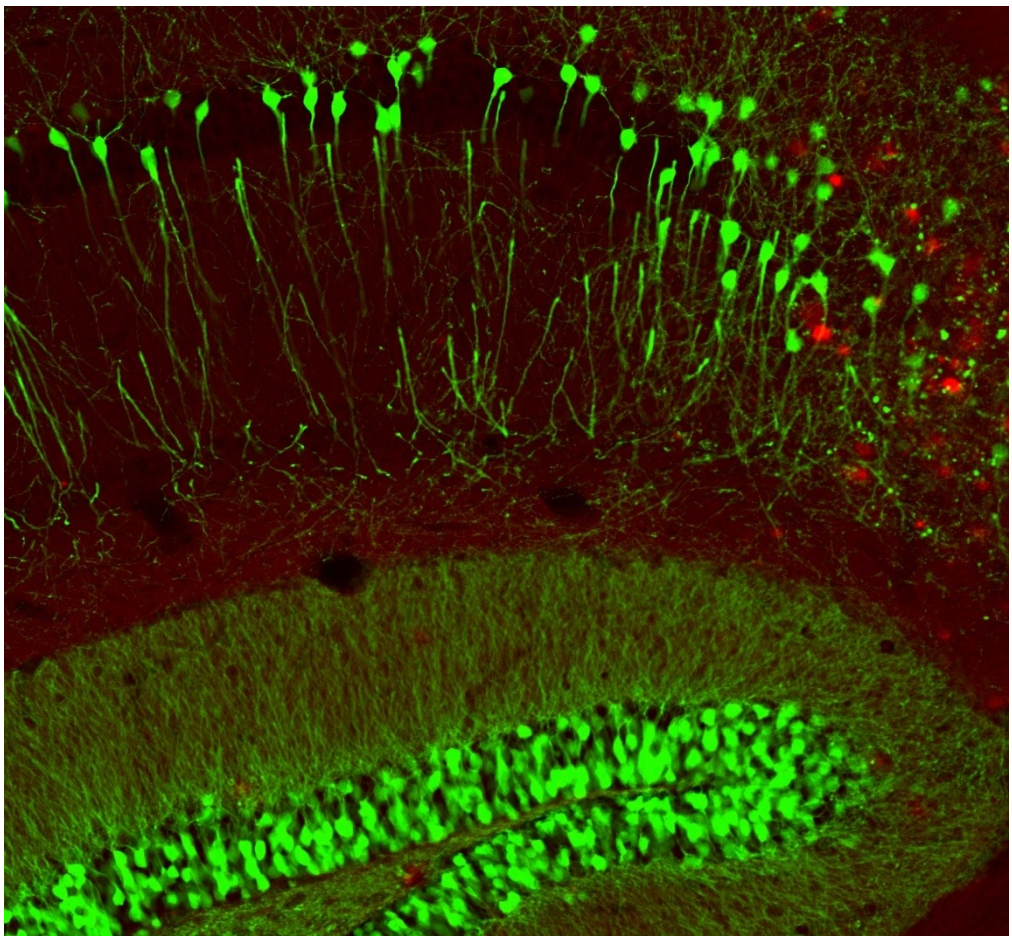

Figure 2. Amyloid Beta and GFP in a FAD+ Mouse Hippocampus. Red indicates the presence of amyloid beta. Bright red spots signify amyloid beta plaques. Green indicates the presence of GFP on neurons.

Figure 3

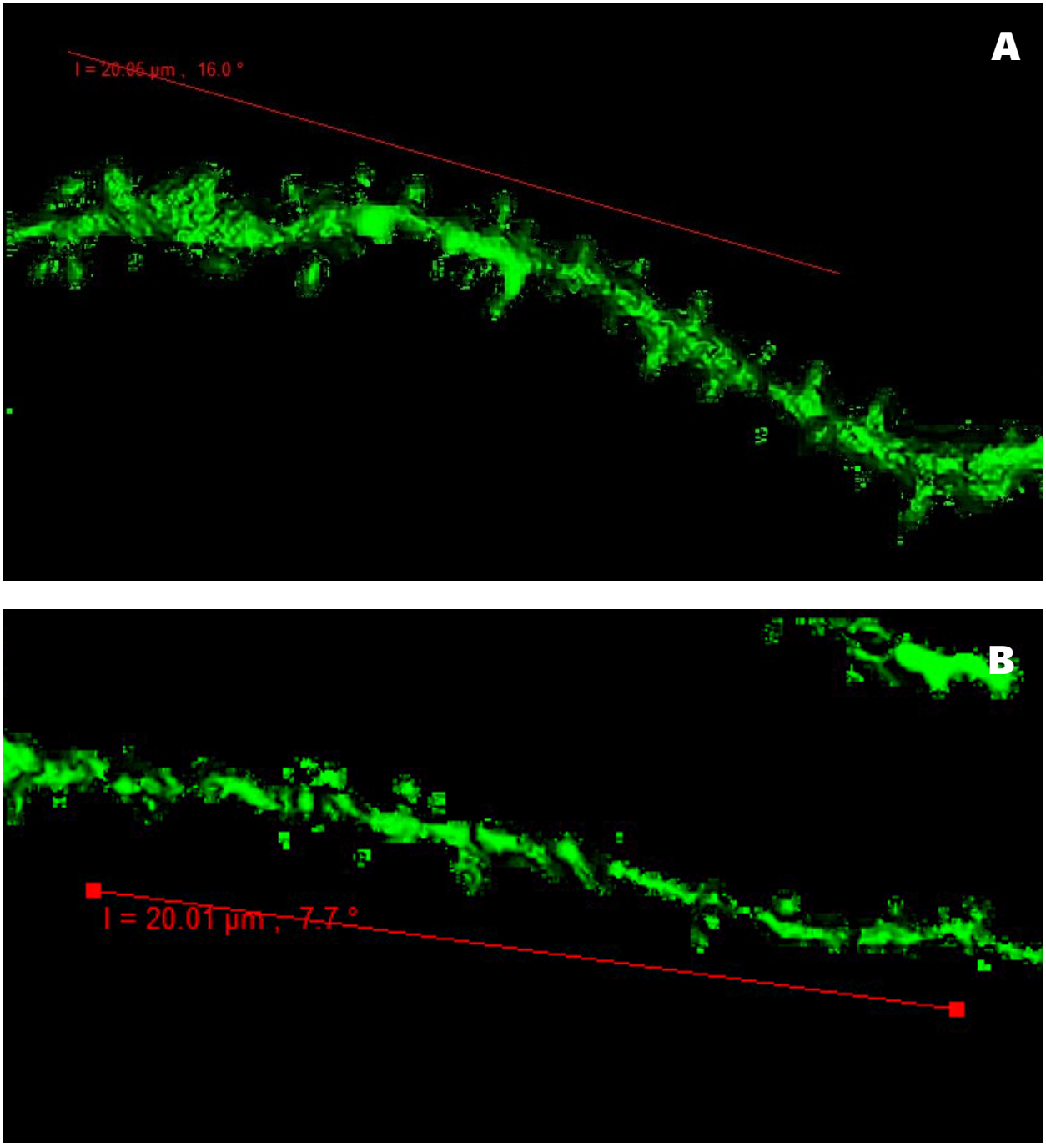

Figure 3. Comparison of Neurons in FAD- and FAD+ Mice. Panel A shows a neuron of a FAD- mouse. Panel B shows a neuron of a FAD+ mouse.

Figure 4

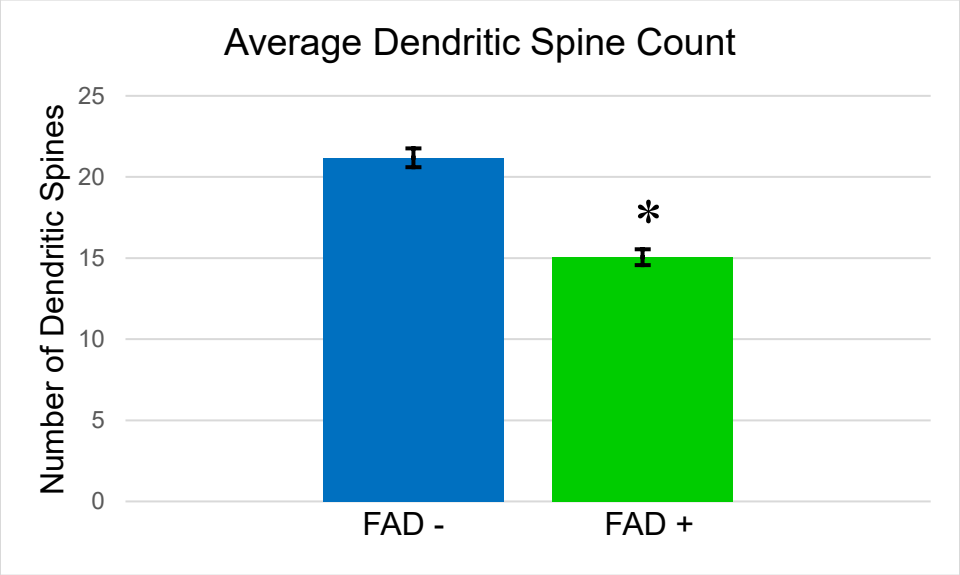

Figure 4. The Effect of Amyloid Beta on Dendritic Spine Number. FAD+ mice exhibited a significant decrease in dendritic spine number compared to the FAD- control. * represents a statistically significant difference at $p < .005$. Bars represent mean ± SEM.

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