THE EFFECTS OF LIPOPOLYSACCHARIDE ON DENDRITIC SPINE DENSITY

by

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ABSTRACT

Alzheimer's Disease (AD) is a neurodegenerative disease that is characterized by deficits in learning and memory. In AD, Amyloid-Beta (Aβ) is over-produced and accumulates in the brain forming plaques. This extracellular deposition of Aβ interferes with normal neuronal synapse activity leading to neuronal dysfunction and degeneration. Lipopolysaccharide (LPS), a component of gram-negative bacteria, has previously been shown to induce an inflammatory response that increases Aβ found in the brain. Dendritic spines are protrusions on the dendrites of neurons that exhibit plasticity depending on outside stimuli. This plasticity is heavily correlated to learning and memory. Likewise, the quantity of these spines is correlated with the number of functioning synapses on the neuron. The purpose of this study was to investigate how LPS-induced inflammation would affect dendritic spine density of hippocampal neurons. LPS was administered and dendritic spine density was quantified. There was not a significant difference in dendritic spine density following LPS treatment when compared to saline controls. The loss of dendritic spines occurs early on in AD development making it an important target for early intervention in AD. Future studies should further investigate the relationships between inflammation, Aβ, and functional synapses.

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INTRODUCTION

There was an estimated 5.5 million Americans living with Alzheimer's Disease (AD) in 2017 ("Alzheimer's disease facts and figures," 2017). The incidence of AD increase with age; 10% of those 65 years and older suffer from AD ("Alzheimer's disease facts and figures," 2017). As the baby boomers age, a larger percentage of our population will enter into the 65 years and older population. With this increase in the older population, we will see an increase in AD. As the disease progresses, sufferers lose the ability to do everyday tasks, making them dependent on caregivers. The cost of care for those with AD impacts the individual, insurance companies and the American taxpayer. Medicare expenses for those with AD or other dementias were over three times as expensive compared to other Medicare beneficiaries in the same age group ("Alzheimer's disease facts and figures," 2017). Likewise, total Medicaid spending for people with Alzheimer's or other dementias was projected to be $44 billion in 2017 ("Alzheimer's disease facts and figures," 2017). While all other major causes of death have significantly decreased, between 2000 and 2014, deaths from AD rose 89% ("Alzheimer's disease facts and figures," 2017). With this increasing death toll and the large emotional and financial burden, current research aims to understand the mechanism of AD progression in hopes of potential treatment options.

In 1907, Alois Alzheimer was the first to report a case of "intellectual deterioration with histological findings" (Selkoe, 2001). One histological finding he described is referred to as "senile plaques", which is now used as criteria to diagnosis of AD (Murphy & LeVine, 2010). These extracellular plaques form from aggregates of Amyloid Beta (Aβ) protein (Serrano-Pozo, Frosch, Masliah, & Hyman, 2011). Aβ plaques disrupt neuronal signaling, and induce synaptic dysfunction, resulting in neuronal death and the cognitive dysfunction associated with AD

(Harris et al., 2010; Murphy & LeVine, 2010).

Elevated levels of Aβ have also been shown to reduce dendritic spine density of the neurons in the hippocampus. (Shrestha et al., 2006). Dendritic spines are protrusion on the neurons that can vary in quantity and morphology (Kasai, Matsuzaki, Noguchi, Yasumatsu, & Nakahara, 2003). Throughout development, dendritic spines change in response to outside stimuli and are believed to underlie the establishment of functional synaptic connections (Engert & Bonhoeffer, 1999; Maletic-Savatic, Malinow, & Svoboda, 1999; Zhou, Homma, & Poo, 2004). In the hippocampus, this synaptic plasticity has been associated with learning and memory (Bliss et al., 1999; Collingridge & Bliss, 1993; Engert & Bonhoeffer, 1999; Kasai, Matsuzaki, Ellis-Davies, & Honkura, 2004). The number of dendritic spines on a neuron is positively correlated with its number of synapses. Therefore, a reduction in spine density may reflect a loss of neuronal connectivity, accounting for the deficits in learning and memory that are associated with AD (Shrestha et al., 2006).

Chronic inflammation has been associated with multiple neurodegenerative disorders, including AD (McGeer & McGeer, 2004; Schwab & McGeer, 2008). Neuroinflammation leads to upregulated production, and decreased clearance, of Aβ protein, accelerating plaque formation (Cai, Hussain, & Yan, 2014). In addition, Aβ has been shown to induce an inflammatory reaction in the CNS, further exacerbating the damage (Schwab & McGeer, 2008). Our lab has previously shown 7 consecutive days of peripheral Lipopolysaccharide (LPS) injections administered to C57BL/6J mice produces an inflammatory response, followed by a significant increase in the production of Aβ in the hippocampus and impaired hippocampus-dependent learning (Kahn et al., 2012).

Using the relationship between Aβ and dendritic spines, this study investigates a potential mechanism for the learning deficits seen with LPS-induced inflammation. Knowing that LPS-induced inflammation leads to increases of Aβ, and that Aβ can reduce dendritic spine plasticity, we hypothesized that the animals receiving LPS injections will have significantly less dendritic spines than the control animals receiving saline injections.

METHODS

Subjects and Housing

Male C57BL/6J mice (4–6 month-old), bred in the TCU vivarium from a breeding stock acquired from The Jackson Laboratory (Bar Harbor, ME), were used in all experiments. All animals were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals and protocols approved by the Institutional Animal Care and Use Committee (IACUC) of TCU. Mice were housed in standard cages (12.5cm x 15cm x 25cm) and in groups of 3 to 4 animals with food and water available ad libitum on a 12-hour light/dark schedule.

Injections

Intraperitoneal (i.p.) injections of 250 mg/kg LPS (Lipopolysaccharides from Escherichia coli O55:B5: Sigma-Aldrich: St. Louis, MO) or volume-equivalent saline were administered once a day for 7 consecutive days.

Tissue Collection

4 hours after completion of all treatments, mice were euthanized by CO_2 inhalation, and brain tissue was removed. A sagittal cut divided the brain into two hemispheres. Each hemisphere was then placed into separate containers of Golgi-Cox staining solution, detailed below.

Golgi Stain

Brain tissue was stained using the procedure outlined in (Zaqout & Kaindl, 2016). In this process, the brain tissue was suspended in an impregnation solution for 7 days, at room temperature, in a dark fume hood. This impregnation solution was made by mixing 1.4% (w/v) K_2CrO_4, 1.4% by (w/v) $HgCl_2$, and 0.8% by (w/v) $K_2Cr_2O_7$ (Sigma Aldrich, St. Louis, MO) into MilliQ water. The tissue was then suspended for an additional 7 days at 4 degrees Celsius in a tissue protectant solution. This solution contained 30% (w/v) sucrose ($C_{12}H_{22}O_{11}$: Amresco, Solon, Ohio), 1% (w/v) polyvinylpyrrolidone (PVP40), 30% by volume ethylene glycol ($C_2H_6O_2$: Sigma Aldrich) and a phosphate buffer at pH 7.2. The phosphate buffer was made by dissolving 0.159% (w/v) $NaH_2PO_4\,H_2O$ (Fisher Scientific, Hampton, NH), 0.547% (w/v) Na_2HPO_4 (Sigma Aldrich, St. Louis, MO), and 9% (w/v) NaCl (Em Science, Gibbstown, NJ) in MilliQ water. After tissue protection, the stained brains were embedded into 5% agarose (Sigma Aldrich, St. Louis, MO), and sectioned sagittally using a vibratome (Leica VT1200, Leica Biosystems Inc., Buffalo Grove, IL). Sections were cut at 150 μm thickness at a vibration frequency of 60 hertz and speed of 0.55mm/s. These sections were then placed on gelatin coated slides and laid to dry in a dark dust free environment for two days. Slides were developed and dehydrated using a series of washes. Washes proceeded as follows: distilled water twice for 5 minutes each, 50% ethanol for 5 minutes, 25% ammonia (Acros, Hampton, NH) for 8 minutes,

an additional 2 distilled water washes for 5 minutes each, 5% thiosulfate (Sigma Aldrich, St. Louis, MO) for 10 minutes in the dark, 2 washes of distilled water for 1 minutes each, 3 washes consisting of 70%, 95% then 100% ethanol in order for 6 minutes and finally a xylene wash for 6 minutes each. To mount, the slides were removed from the xylene solution and laid out until they were semidry. A streak of Eukitt (mounting medium: Sigma Aldrich, St. Louis, MO) was added in a thin line at one end of the slide. One side of a number 1.5 cover slip was then slowly lowered over the slide using tweezers to avoid air bubbles. The slides were sealed using nail polish and dried in the dark for at least 48 hours prior to imaging.

Microscopy and Data Collection

Slides were examined under a Nikon 90i light microscope (Nikon Instruments Inc., Melville, NY). For each brain, Z-Stacks were taken of 5 sections containing the CA1 region of the hippocampus, using Nis Elements software (Nikon Instruments Inc., Melville, NY). For each brain section, 5 areas on the dendrite were selected to count dendritic spines. Each of these dendritic areas was at least 20 µm away from the cell body and 20 µm in length. In order to be counted as a dendritic spine, protrusions had to be at least 0.5 µm in length. Five individuals, without prior knowledge of condition, independently counted dendritic spines from the Z-Stacks. The number of dendritic spine reported by the 5 individuals were then averaged for each dendritic area. Following this, the number of dendritic spines from the 25 dendritic areas

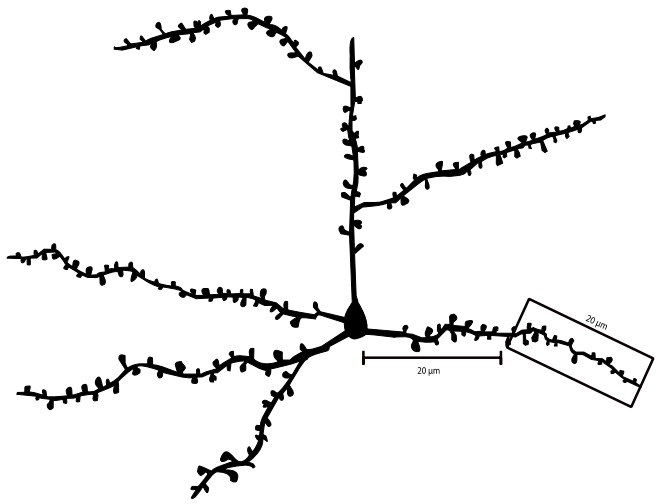

Fig. 1 An illustration of a neuron. The box indicates a representative area of interest in which dendritic spines were counted. These area of interest were 20 µm from the cell body and 20 µm long. Each slice had 5 areas of interest.

per mouse (5 dendritic areas per section, 5 sections per mouse) were averaged. These averages of dendritic spines per 20 μm per mouse were then statistically analyzed.

Statistics

The data obtained from counting dendritic spines was analyzed using Minitab software (Minitab, Inc, State College, PA). For this dendritic spines data, a one-way Analysis of Variance (ANOVA) was utilized to determine any significant main effects and interactions between the LPS and Saline treatments. All data in figures are shown as mean ± SEM. The alpha level used for all statistical analyses was 0.05.

RESULTS

Our lab has previously shown 7 consecutive days of peripheral LPS injections in C57BL/6J leads to a significant increase of Aβ in the hippocampus and impaired hippocampus-dependent learning (Kahn et al., 2012). This study investigated how acute inflammation from these repeated injections affected dendritic spine density. Individuals, without prior knowledge of condition, independently counted dendritic spines from 5 z-stacks, taken from each of the 12 mice. The number of dendritic spines per 20 μm section was averaged for the saline and LPS conditions. A 1-way ANOVA revealed no significant differences of dendritic density in the mice receiving LPS injections (Fig. 2).

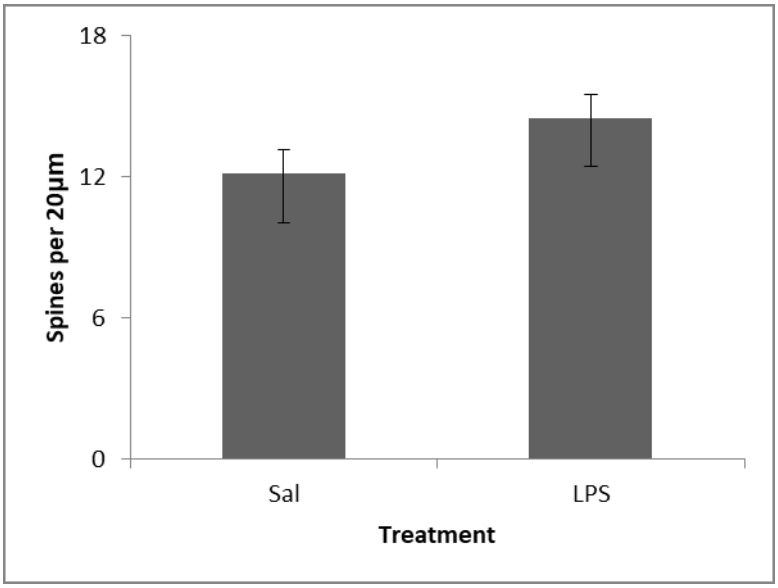

Fig. 2 Comparison of dendritic spines per 20 μm in mice that received either saline or LPS for 7 days. Mean spine density is not significantly different between groups (1-way analysis of variance [ANOVA]: $F_{1,10}=2.635$, $p=0.1355$). Error bars represent two times standard error.

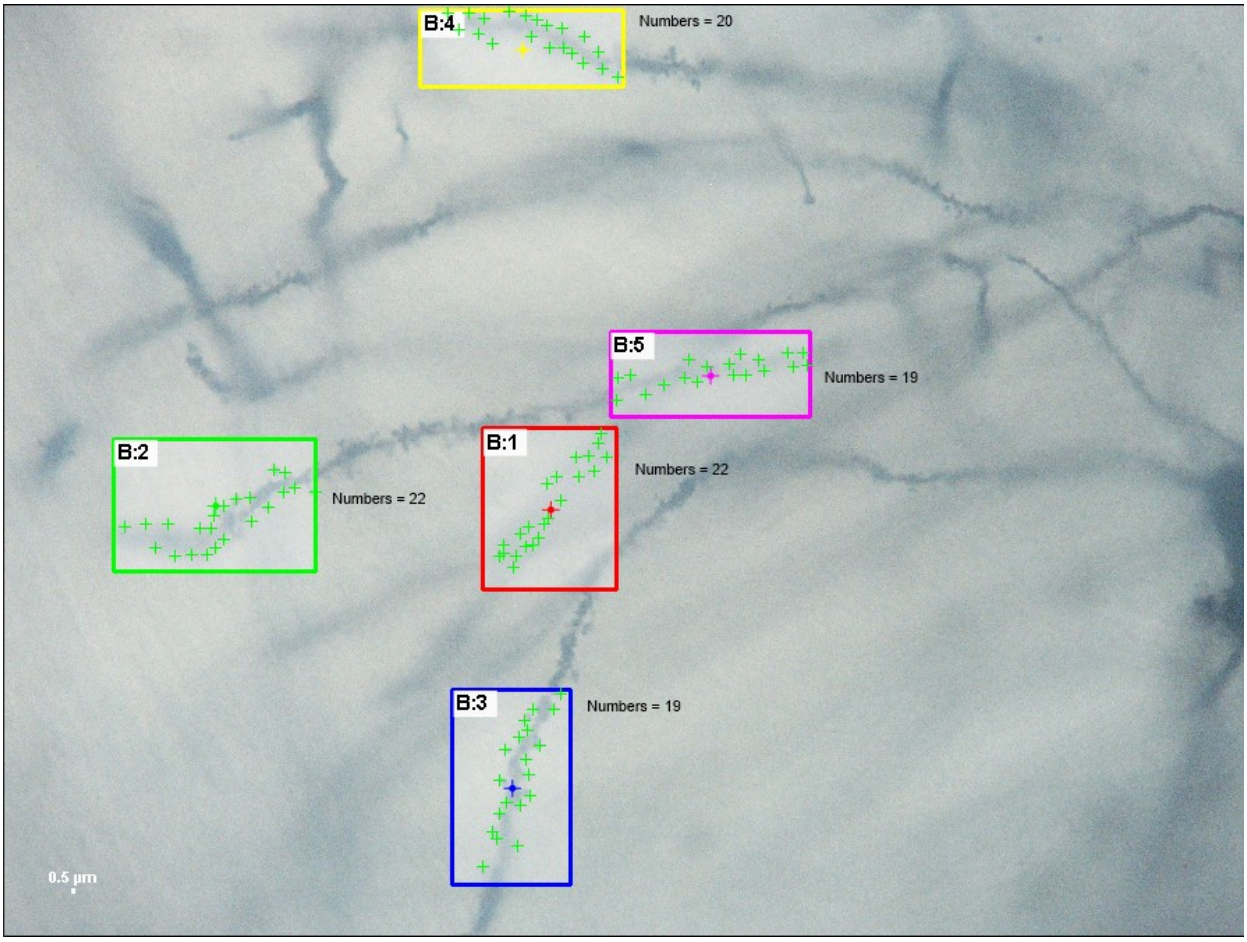

Fig. 3 A representative still image from a z-stack containing 5 regions where dendritic spines were counted.

DISCUSSION

In the progression of AD, one of the first pathological events is the loss of dendritic spines and synapses, making it an important mechanism to explore as a target, in order to slow AD development. (De Ruiter & Uylings, 1987; Shrestha et al., 2006). In the present study, we investigated how daily injections of LPS affected dendritic spine density. Previous studies have shown that these daily injections increase Aβ in the hippocampus (Kahn et al., 2012). In addition, Aβ has been correlated with decreased dendritic spines in hippocampal neurons (Shrestha et al., 2006). With this in mind, we hypothesized a decrease in synaptic spines would be seen in the group that receive daily i.p. injections of LPS. However, in the

present study, we observed no significant difference in mean spine number between the animals that received injections of LPS and the animals that received injections of saline.

In the future, if this experiment were to be repeated more animals (approximately 8-12) should be used in each group. This would ensure that our results are not an artifact of small sample sizes. There is also a possibility that there are significant changes in spine density in particular regions of the brain, even if there were not significant differences when averaging data from all hippocampal sections of the brain, as this study did. To determine if the medial or lateral sections of the hippocampus respond differently to LPS, future studies could make separate averages for the different locations of the brain.

There is some unreliability that comes with the Golgi-Cox staining method which can add uncertainty to data (Globus & Scheibel, 1966). In the future, our lab will use Thy-1 GFP transgenic mice to study dendritic spines. These transgenic mice express green fluorescent protein in their neuronal cells but not in any other cells (Feng et al., 2000). Specifically, our lab plans to utilize the M line of Thy-1 GFP which only expresses GFP in a few ganglion cells (Feng et al., 2000). Reducing the number of neurons expressing the fluorescent protein makes individual neurons distinguishable and dendritic spines can be counted.

These transgenic mice will not only eliminate many complications that come with the Golgi-Cox staining method, it will also allow for the use of antibodies to stain for specific proteins. While there may not be a change in the number of dendritic spines, there is a possibility that the number of functional synapses are changing. PSD95 is a protein that is found in clusters on the post synaptic domain of excitatory neurons in the hippocampus (Broadhead et al., 2016). Synaptophysin is a protein found exclusively and abundantly on synaptic vesicles and therefore often used as presynaptic terminal marker (Kwon & Chapman, 2011). If Synaptophysin and PSD95 are stained using antibodies with different colored fluorescent tags, when the proteins are located in close proximity the colors of their fluorescent

tags will appear to blend. The abundance of this blended color can be used to determine the number of functional synapses.

If staining for these synaptic markers reveals that there is a decrease in synaptic proteins even though there is not a decrease in dendritic spines, it could imply that even though a pre-synaptic neuronal connection has been eliminated, the dendritic spines remain. Another possible explanation for a decrease in synaptic proteins without a decrease in dendritic spines may be that dendritic spines are being lost, but the neuron is creating new dendritic spines as a compensatory mechanism. Staining for synaptic markers will reveal if the dendritic spines present have the capability of being involved in functional synapses. Previous research has indicated that compensatory growth of dendrites in the hippocampus is not sufficient in overcoming memory deficits in AD patients (Coleman & Flood, 1987). A compensatory mechanism which increases dendritic spines without increasing functional synapses would align with LPS-induced increase in dendritic spines paired with cognitive impairments.

Thy-1 GFP mice have never been used with our inflammation model (daily injections of LPS for 7 days). It would be important to check both Aβ and inflammatory cytokine (IL-1β and IL-6) levels in the transgenic animals after LPS injection, to ensure they are having the intended inflammation response.

In addition, if future studies find significant differences in the number of dendritic spines or in synaptic proteins in response to LPS injections, it will be important to determine if theses difference are a consequence of the inflammation or from the increase of Aβ that results from the bacterial mimetic. Gleevec (imatinib mesylate) is a selective tyrosine kinase inhibitor that inhibits gamma secretase and prevents the production of Aβ, without blocking the inflammation response (Netzer et al., 2003; Weintraub et al., 2013). If Gleevec is administered with LPS and the animals respond the same way as the control animals (just saline injections), it would imply that the LPS induced changes are a result of Aβ rather than inflammation. In summary, while there was no difference in dendritic spine density as a result of LPS injections in our initial experiments, we will use the same LPS-induced inflammation model in a

more sensitive model, the Thy-1 GFP mice, to measure the effects of dendritic spines and synaptic proteins in the future. If significant differences are found, the drug Gleevec will be used to determine if these differences are a result of Aβ or inflammation.

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