EXERCISE AND INFLAMMATION CAUSE MICROGLIAL CELL

ACTIVATION: A DICHOTOMY FOR ALZHEIMER'S

DISEASE

by

Jessica Mussatto

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Project Approved:

Supervising Professor: Michael Chumley, PhD.

Department of Biology

Gary Boehm, PhD.

Department of Psychology

Marlo Jeffries, PhD.

Department of Biology

<u>ABSTRACT</u>

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder characterized by memory loss and cognitive decline. AD pathologies include neurofibrillary tangles, amyloid-β plaques (Aβ), and neuronal cell death. Our lab has previously shown that systemic inflammation in mice, resulting from 7 days of LPS injections, led to cognitive deficits as well as increased Aβ levels in the CNS. Interestingly, exercise was shown by our lab to decrease Aβ levels in mice treated with LPS to levels equal to those of control mice. This identified exercise as a possible therapeutic treatment for AD, but the mechanism for this effect is currently unknown. We identified microglia as one possible cell responsible for the decrease in Aβ. Microglia are the immune cells of the brain, with functions including secretion of pro-inflammatory cytokines, release of growth factors, and phagocytosis of debris. Microglia are activated by both inflammation and exercise. We hypothesized that exercise would increase the number of activated microglia in the CNS. We found that following LPS-administration animals in both the sedentary and exercise groups had increased levels of activated microglia, compared to saline-administered animals, but did not differ from each other. Interestingly, animals that had been injected with saline, followed by two weeks of voluntary exercise, displayed more activated microglia than those in the sedentary recovery group ($p = 0.07$). This suggests that exercise may play a role in activating microglia if not previously primed by inflammation. Activated microglia can be oriented either towards a neuroprotective or a neurodegenerative phenotype, and we suggest further studies should be performed, identifying whether exercise alters these phenotypes.

Understanding the role microglia and exercise play in the removal of Aβ from the CNS, may lead to changes in AD treatments.

ACKNOWLEDGEMENTS

There are many people to whom I owe gratitude for their support in this project, but certainly none more than Dr. Michael Chumley and Marielle Weintraub. I cannot express enough my thanks to you both. Your support and guidance have been indispensible to me and to my research goals. Thank you for putting forth so much time and effort on my behalf and for being so patient and open to assist me. I would also like to extend a huge thanks to the other members of the Chumley lab, who were always there to extend a helping hand, and to make my research experience at Texas Christian University such a pleasure.

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To Marielle Weintraub, my lab mom, I also want to express my thanks. You not only have a strong drive to succeed, but also to help with the success of those around you. I have greatly benefited from your time and selfless support. Much of what gets done in the lab is due to your hard work and the accountability you demand from yourself and from others. The lab will definitely be missing your presence next year. Working with you is always a pleasure and I will greatly miss you in the future!

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INTRODUCTION

Alzheimer's Disease (AD), first described by Alois Alzheimer in 1906, was given

its official name in 1910 by Emil Kraepelin (Alzheimer's facts and figures). It is the most

common form of dementia, accounting for 50 to 80 percent of all dementia cases (Terry,

2006). AD is a progressive neurodegenerative brain disorder characterized most

commonly by memory loss and cognitive decline (Alzheimer's facts and figures). The

greatest risk factor for AD is increasing age, especially in those over the age of 65

(Alzheimer's facts and figures).

Alzheimer's disease is currently the 6th leading cause of death in the United States

overall, and the 5th leading cause of death for people age 65 and older (Alzheimer's facts

and figures). Approximately 5.2 million Americans suffer from AD today, including

200,000 individuals under the age of 65 (Alzheimer's facts and figures). This number is

expected to rise with the growing population of seniors as life expectancy rises. The

estimated number of those infected by AD over 65 years old in 2025 is 7.1 million

(Alzheimer's facts and figures). Currently there is no cure for AD. Those individuals with

AD will either die with it or of it, and 1 in 3 seniors dies with AD or another form of

dementia (Alzheimer's facts and figures).

The financial burden caused by AD is staggering and it is also on the rise. The

direct costs of caring for AD patients to the American society are estimated at $203

billion. That figure includes $142 billion in costs to Medicare and Medicaid (Alzheimer's

facts and figures). The cost of care for AD patients is assessed to reach $1.2 trillion at the

current dollar value by 2050 (Alzheimer's facts and figures). Life expectancy is on the

rise, and as our society ages the financial burden of AD will continue to weigh on our society.

The figures previously listed do not include the personal burden placed on caregivers and families of patients. Family and friends supplied 17.5 billion hours of unpaid care to patients of AD and other dementias in 2012. The cost of this care is the equivalent to $216.4 billion (Alzheimer's facts and figures). In addition to the time and financial burden, many caregivers experience a large emotional burden. Over 60 percent of caregivers for patients suffering from AD and dementia rate their emotional stress as high or very high (Alzheimer's facts and figures). Caregiver suffering often mirrors patient suffering; the patient experiences a loss of memory, independence, and the ability to recognize their loved ones. Over one-third of caregivers report symptoms of depression caused by the stress of caring for AD and dementia patients (Alzheimer's facts and figures).

The clinical symptomology of AD described briefly is a progressive cognitive decline. Early on in the disease, individuals may have difficulty remembering newly learned information (Alzheimer's facts and figures). This occurs because the hippocampus, responsible for learning, is often where pathology begins (Alzheimer's facts and figures). Mild memory deficits progress into increasingly more severe symptoms affecting multiple spheres of brain function (Heneka & O'Banion, 2007). Late stage symptoms include disorientation, mood and behavior changes, suspicions about family, friends, and medical caregivers, and confusion about events, times, and places (Alzheimer's facts and figures). Additional neurological symptoms can occur in the

advanced stages and include slowed movements, difficulty speaking, and impeded motor coordination (Heneka & O'Banion, 2007).

One of the physical pathologies in AD is the formation of plaques made of β-amyloid (Aβ). Aβ promotes neurodegeneration by damaging synapses and by parallel mechanisms including the activation of glial cells (Heneka & O'Banion, 2007). Aβ is produced in the body when amyloid precursor protein (APP) is cleaved by proteases β-secretase and γ-secretase (Heneka & O'Banion, 2007). An altered proteolysis of APP leads to an increased production of Aβ-42. Aβ-42 builds up in the CNS as plaques and triggers an inflammatory response (Selkoe, 2001). Aβ plaques build up in the brain and disrupt neuronal function. They are often surrounded by activated microglia and astrocytes (Selkoe, 2001).

Other classic physical pathologies of AD are tau tangles and neuron and synapse degeneration. These neurofibrillary tangles are aggregates of abnormal fibers composed of microtubule associated protein tau (Selkoe, 2001). Tangles are shown to be associated with affected neurons in AD. Tau tangles are especially prominent in the entorhinal and hippocampus regions. These are the regions of the brain largely responsible for learning and memory acquisition (Terry, 2006). AD brains are often shriveled and have a lower volume than a normal brain. The reduction in brain volume is due to the significant degeneration of neurons and synapses (Mattson, 2004).

There are some genetic components that have been identified as risk factors for AD. As reviewed by Selkoe (2001), early onset AD has been linked to missense mutations in the presenilin genes, including presenilin 1 and 2. Mutations in these genes increase production of Aβ -42 and accelerate AD pathology (Selkoe, 2001). The 4 allele

of apolipoprotein E (ApoE) has been shown as a risk factor for classical late onset AD (Selkoe, 2001). ApoE is found within Aβ plaques and binds to Aβ -42 with high avidity. The 4 allele of ApoE has been shown to heighten the risk of AD, and lead to an earlier onset as compared to the other variations of the gene (Strittmatter et al., 1993). Other genes have been identified as possible risks in AD progression, but much is still unknown about the underlying causes of AD.

It has been shown in several studies that inflammation may have an impact in the pathogenesis of AD. Cunningham (2013) found that repeated systemic inflammation could also produce neuroinflammation. There is increasing evidence that inflammation significantly contributes to the development of AD. Recent studies have shown that inflammation may stimulate APP processing by the up regulation of β-secretase, which further stimulates the inflammatory response in a cyclical pattern (Heneka & O'Banion, 2007). Kahn et al. (2012) has demonstrated that systemic inflammation in mice resulting from 7 days of LPS injections led to cognitive declines in memory, a common symptom of AD. This inflammation also led to an increase in Aβ in the CNS (Kahn et al., 2012).

As the immune cells of the brain, microglia play a very important role in the inflammatory response. Microglial activation produces morphological changes in the cell, including a thickening of ramifications and cell bodies leading to a rounded amoeboid shape (Perego, Fumagalli, & De Simoni, 2011). Microglia are activated by inflammation and remain active after the initial inflammation (Bian, X. Zhao, Li, Zeng, & B. Zhao, 2013). Activated microglia release pro-inflammatory cytokines, clear debris, and work to maintain or restore CNS homeostasis (Perego et al., 2011; Hickman, Allison, & El Khoury, 2008; London, Cohen, & Schwartz, 2013).

Microglia perform many neuroprotective functions in the CNS such as clearing infection and commencing the inflammatory response (Heneka & O'Banion, 2007). The neuroprotective properties of microglia include phagocytosis of Aβ and the secretion of neurotrophic and growth factors, such as Glial cell-derived neurotrophic factor (GDNF), Brain-derived neurotrophic factor (BDNF), and insulin-like growth factor (IGF) (Polazzi & Monti, 2010). However recently, many studies have found some neurodegenerative effects of activated microglia in the CNS (Heneka & O'Banion, 2007). Proinflammatory mediators released by microglial cells include cytokines, complement factors, reactive oxygen species, neurotoxic secretory products, free radical species, and nitric oxide (NO). All of these mediators can contribute to neuronal dysfunction and cell death (Griffin et al., 1998).

In neurodegenerative diseases such as AD, it is thought that microglia are contributing to neurodegeneration rather than neuroprotection (Hickman et al., 2008; Heneka & O'Banion, 2007; Cunningham, 2013). Early on in AD, microglia activation promotes Aβ clearance; however, as the disease progresses, proinflammatory cytokines produced by the microglia in response to the Aβ deposition down regulates the genes involved in Aβ clearance which promotes Aβ accumulation (Hickman et al., 2008; Krabbe et al., 2013). Imai & Kohsaka (2002) found that directed process motility and phagocytic activity were weakened in mice with AD-like pathology compared to non-transgenic mice. In a study by Hickman et al. (2008), PS1-APP transgenic mice were compared with wild type control mice to study the effects of AD progression on the capacity of microglia to clear Aβ plaques. PS1-APP transgenic mice carry two human genes with mutations found in early-onset AD. One gene has a mutation in the APP gene

and the other in the presenilin-1 gene, both of which create an AD-like pathology in the mice. The authors found that at 1.5 months of age, expression of Aβ binding receptors on microglia were comparable between transgenic and wild type mice. However by 8 months of age, microglia from the PS1-APP mice showed significantly decreased levels of Aβ binding receptors compared to age-matched wild-type mice (Hickman et al., 2008). This study suggests the ability of microglia to clear Aβ may decrease with age and/or with the progression of AD. Hickman et al. (2008) also found that microglia in aged transgenic mice maintained their ability to produce proinflammatory cytokines, including TNF-α and IL-1β. These proinflammatory cytokines have actually been linked to an increase Aβ load in the CNS. TNF-α along with interferon-γ have been shown to up regulate β-secretase, while both TNF-α and IL-1β were found to stimulate the γ-secretase-mediated cleavage of APP (Yamamoto et al., 2007; Liao, Wang, Cheng, Kuo, & Wolfe, 2004). The proinflammatory cytokines released by aged microglia in response to Aβ may have actually been contributing to more accumulation of Aβ in the CNS, rather than aiding in its removal (Hickman et al., 2008).

A therapeutic strategy that maintained microglial neuroprotective capacity could be an effective method to reduce the Aβ load in the CNS and lessen the severity of the AD pathology in an affected patient. Perego et al. (2011) state that microglia "differentiate towards a multitude of phenotypes depending on the surrounding microenvironmental signals that can change over time". The authors suggest that strategies should be developed to guide the microglia cells towards a protective phenotype. Rather than perpetuating the inflammatory cycle by releasing proinflammatory cytokines, the protective phenotype facilitates degradation of Aβ and

promotes neurogenesis (Perego et al., 2011) (Figure 1). Hickman et al. (2008) also

suggest that an anti-inflammatory therapy that promotes the ability of microglial cells to

clear Aβ, while decreasing their ability to produce proinflammatory cytokines could be

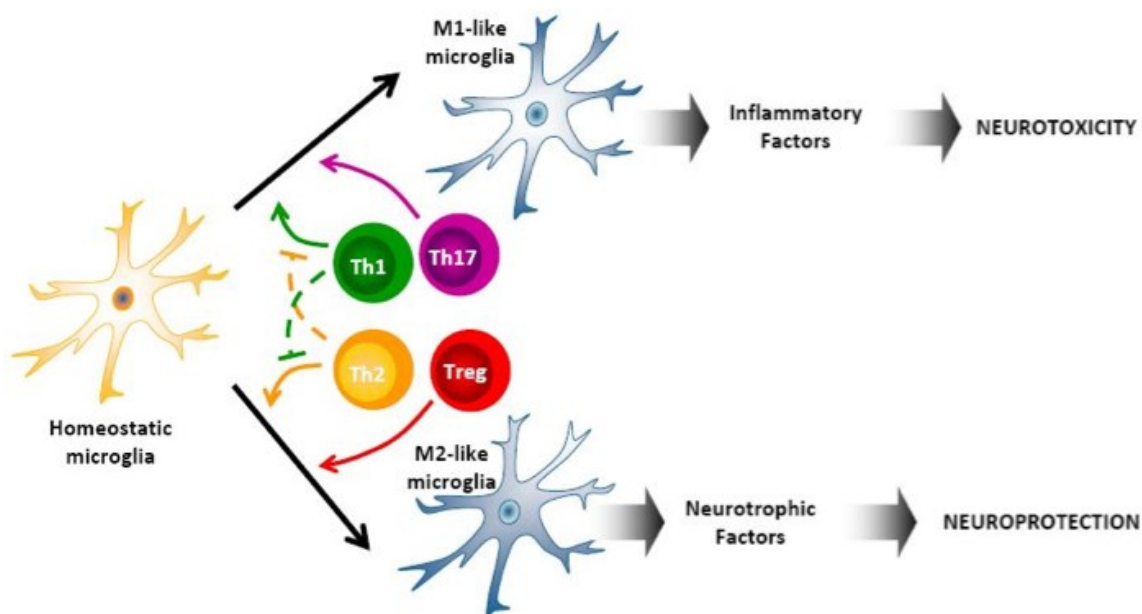very beneficial in delaying or arresting the progression of AD.

Figure 1: Two possible paths of activation in microglial cells. M1-like microglia produce pro-inflammatory mediators leading to neurotoxicity. By contrast, M2-like microglia secrete neurotrophic factors like GDFN, BDFN, and IGF. This category of microglia aid in neuroprotection. From: *Cell Interaction*- Chap 6: "Cells, Molecules and Mechanisms Involved in the Neuro-Immune Interaction" (Pacheco, Contreras, & Prado, 2012)

Exercise has been noted repeatedly as a way to reduce inflammation and increase

cognitive function in transgenic mice with AD-like pathology. In a study by Parachikova

et al. (2008), Tg2576 transgenic mouse model was used to study the effects of voluntary

exercise on AD. Tg2576 mice over express a human mutant form of APP, and develop

Aβ plaques and progressive cognitive deficits. Tg2576 mice that participated in voluntary

wheel running performed significantly better on the radial arm Morris water maze

(RAWM) than sedentary transgenic mice (Parachikova, Nichol, & Cotman, 2008). The

RAWM is a behavioral test that is widely used to measure hippocampus-dependent

learning and memory, thus indicating that exercised animals saw a rescue of hippocampus function from learning deficits of AD. Exercise not only positively affects behavior, but it also has been shown to change the molecular mechanisms in the CNS during an inflammatory response. In a study by Nichol et al. (2008), proinflammatory cytokines IL-1β and TNF-α were reduced to non-transgenic levels in Tg2576 mice after three weeks of exercise. This decrease in pro-inflammatory cytokines is correlated with improved ability to solve a water maze task, again indicating a beneficial effect on learning and memory (Nichol et al., 2008). Not only were pro-inflammatory cytokines reduced, but also anti-inflammatory makers IFN-γ, CD40, MHC II, CD11c, and MIP-1α were increased (Nichol et al., 2008). These inflammatory markers shift the immune response to a neuroprotective function. Recent findings suggest that physical exercise could arrest or decelerate the molecular mechanisms underlying neurodegeneration in AD (Stranahan, Martin, & Maudsley, 2012; Nichol et al., 2008).

Numerous human studies have also shown cognitive benefits resulting from moderate exercise, especially in aging populations. Several studies have found physical fitness to be associated with lower risks of cognitive decline in older individuals (Hillman, Weiss, Hragberg, & Hatfield, 2003; Laurin, Verreault, Lindsay, MacPherson, & Rockwood, 2001; Colcombe et al., 2004). A study by Laurin et al. (2001), notes that compared to no exercise, individuals who participated in physical activity had significantly lower risks of AD and general dementia. In a human study by Lautenschlager et al. (2008), participants that engaged in 20 minutes of physical activity per day had cognitive improvements, and these positive effects were maintained up to 12 months after stopping treatment. Colcombe et al. (2004) demonstrated that persons

participating in cardiovascular fitness showed greater task-related activity in regions of the brain involved in spatial selection and inhibitory functioning. These results suggest that increased cardiovascular fitness can help to improve neuroplasticity, and to reduce cognitive decline in the aging human brain.

The shift to a neuroprotective immune function in exercised mice is thought to be due to a shift in the phenotype of microglia cells from pro-inflammatory to neuroprotective (Parachikova et al., 2008). Exercise has been shown to increase IFN-γ, along with other inflammatory markers that are indicative of the neuroprotective phenotype of activated microglia (Nichol et al., 2008). Running has increased the number of microglia expressing IGF, a neuroprotective cytokine. It has also enhanced the survival of new neurons (Kohman, DeYoung, Bhattacharya, Peterson, & Rhodes, 2012). These findings suggest that microglia are a good candidate for the cognitive improvement and the reduction of neurodegenerative inflammation seen with exercise.

In an unpublished study by the Chumley Lab, two weeks of exercise following peripheral inflammation was found to significantly decrease the Aβ load in the CNS to levels similar to saline treated control animals (Figure 2). However, the mechanism by which the Aβ was removed is currently unknown. We sought to further investigate whether exercise may be activating microglia in a positive way, leading to a decrease in the Aβ load. Based on a protocol previously used in our lab and established by Kahn et al. (2012), mice were given 7 days of LPS injections to induce inflammation. Next the test group was subjected to 2 weeks of voluntary wheel running. After 2 weeks of exercise, the brains were removed and sectioned. Immunohistochemistry was performed on the tissue sections, using an antibody for Iba-1, a calcium-binding protein that is

expressed at high levels in activated microglia cells (Imai & Kohsaka, 2002). The stained

microglia in the hippocampus were quantified. Our hypothesis was that those animals

treated with LPS would have a higher number of activated microglia in the hippocampus

than those in the saline treatment group. We also hypothesized that those animals in the

exercised condition would have a higher number of activated microglia than those in the
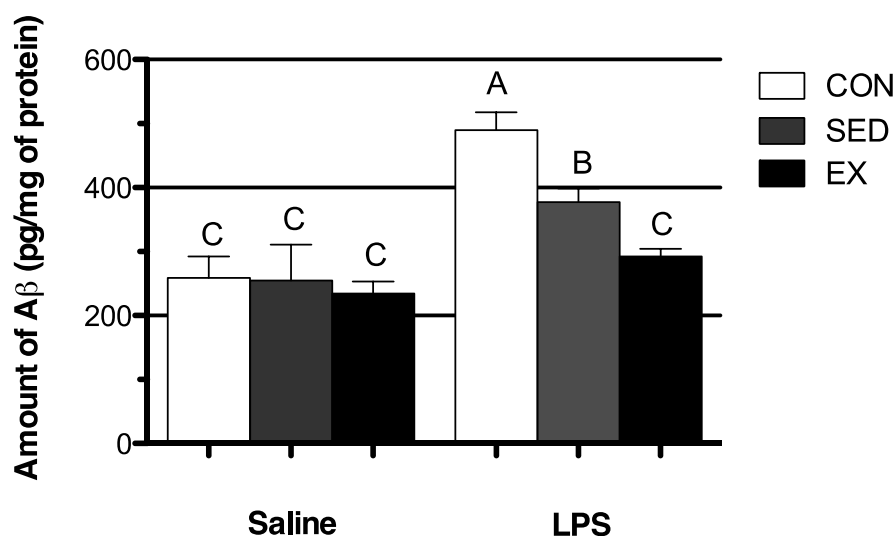
sedentary condition.

Figure 2: 2 Weeks of Voluntary Exercise or Sedentary Recovery. There were no significant differences in running revolutions following administration of LPS or saline. As expected, there was a significant increase in the level of hippocampal-AB in LPS-injected CON mice, mice that received only 7 injections followed by immediate tissue removal, as compared to saline-injected CON mice. Two weeks of voluntary exercise, following LPS-administration, significantly decreased the level of hippocampal-AB as compared to matched SED mice. Interestingly, there were also no significant differences in hippocampal-AB between LPS-administered EX mice and saline administered EX and SED mice. Bars represent mean ±SEM. Letters that are different (a,b,c) represents significant differences (p<0.05). CON= control mice, EX= exercise mice, SED= sedentary recovery. (Weintraub, M. K., Unpublished data)

<center>MATERIALS AND METHODS</center>

Subjects:

Male C57BL/6J mice (4-6 months old) were used in this experiment. They were

bred in the TCU vivarium from an original stock in Jackson Laboratory (Bar Harbor,

ME). All animals were cared for and housed in accordance with the Guide for the Care

and Use of Laboratory Animals (National Research Council, 2010), and in accordance with the guidelines sanctioned by the Institutional Animal Care and Use Committee (IACUC) of TCU.

Housing:

All subjects were housed in standard cages (12.5cm x 15cm x 25cm). Food and water were available *ad libitum*, and all experimental and control groups were on the same 12 hour light/dark schedule (lights on at 0700 and lights off at 1800). During the exercise portion of the study, animals were group housed during the day, and then moved to private cages from 1800h until 0700h at night. During this nocturnal cycle, animals were either given access to a running wheel for the exercise condition, or no running wheel for the sedentary condition. Solitary housing at night allowed us to track each individual's exercise volume, while avoiding depressive-like states by also providing group housing during the day

Treatment Conditions:

Injections of 250 μg/kg of LPS (Escherichia coli serotype: 055:B5; Sigma-Aldrich, St Louis, MO), or volume equivalent saline, were administered once a day for seven consecutive days. Animals were placed in one of four groups, exercise-LPS (EX-LPS), exercise-saline (EX-SAL), sedentary-LPS (SED-LPS), and sedentary- saline (SED-SAL). Animals in EX-LPS and EX-SAL had running wheel access in their private cages for 14 days following the 7th injection of LPS or SAL. Animals in the SED-LPS and SED-SAL groups had no wheels in their private cages. After the completion of the exercise regimen, the animals were transcardally perfused and their brains were collected for immunohistochemistry.

Immunohistochemistry:

After completing the exercise treatments, mice were anesthetized with a mixture of ketamine (120 µg/kg) and xylene (15 µg/kg) in sterile saline. They were then transcardally perfused, first by exsanguination with cold phosphate buffered saline (PBS; pH 7.4) for 5 minutes, followed by fixation with cold 4% paraformaldehyde (PFA; pH 7.4) for 7 minutes. Brains were next extracted and stored in tubes containing cold 4% PFA. Brains were later sectioned into 40 um slices using a vibratome (Leica Biosystems), and sections were stored in a 24 well plate containing .03% sodium azide in PBS to prevent fungal growth. Prior to staining, free-floating sections were rinsed with DI water (10 minutes with 3 changes) to remove any trace PFA. 1 mL of Sodium hydrobromide solution (0.5% in PBS) was added to each section and incubated at room temperature rocking for 30 minutes. Sections were then washed with PBS for 30 minutes with three changes. 500 µL of 0.3% H_2O_2 and 10% MeOH in PBS was added to each section and incubated at room temperature for 30 minutes rocking. Sections were then washed with PBST for 30 minutes with 3 changes. Sections were blocked overnight at 4° C rocking with a goat serum solution, provided in the Rabbit IgG ABC Kit (Vectastain, Vector Laboratories, Inc. Burlingame CA). The next day, 500 µL of the primary antibody Rabbit-anti-Iba1 (Wako USA, Richmond, VA) diluted 1:4000 was added to each section and incubated overnight at 4° C. The following day, sections were washed for 35 minutes in PBST with several changes. Then 500 µL of secondary antibody (anti-rabbit) was added using the aforementioned Vectastain kit, per manufacturer instructions, and incubated overnight at 4°C. The final day, the sections were washed for 35 minutes in PBST with several changes. Using the Vectastain kit, 500 µL of signal amplification

substrate, following kit instructions, was added to the sections and incubated at room temperature rocking for 30 minutes. The sections were then washed for 35 minutes in PBS with several changes. A liquid diaminobenzidine (DAB)-plus substrate kit (Invitrogen, Camarillo, CA) was used following manufacturers instructions to detect a chromogenic reaction under a microscope. Sections were mounted onto slides. To quantify Iba-1 positive cells in the hippocampus, every second most rostral section, including the dentate gyrus of the hippocampus, was used. Iba-1 positive cells were imaged using a light microscope (Zeiss AxioImager, Carl Zeiss, Jena, Germany) with a camera (AxioCam MRc, Carl Zeiss, Jena, Germany) attached. Cells were counted using Image J software (National Institute of Health).

Statistical Analysis:

All data were analyzed using Statview 5.0.1 software (SAS Institute Inc., Cary, NC). A two-way Analysis of Variance (ANOVA) was conducted in order to assess the differences between Treatment (SAL or LPS) and Condition (SED or EX). This led to four experimental groups for Iba-1 positive microglia assessment: EX-SAL, EX-LPS, SED-SAL, SED-LPS. All data figures are shown as mean ±SEM. Significance was determined using an alpha level of 0.05, followed by Fisher's PLSD post hoc tests for significant main effects.

RESULTS

Previous work in the Chumley lab has found that the elevation in hippocampal Aβ that results from 7 days of LPS treatment can be eliminated by allowing animals to exercise on running wheels for two weeks following the inflammation. We sought to determine if the mechanism for the Aβ removal might be exercise-induced enhancement

of microglial cell activation or cell numbers. We found that there was a significant increase in the number of activated microglia in animals treated with LPS [$F_{(1,16)}$= 27.47, $p<0.0001$] regardless of whether the animals were provided two weeks of exercise or sedentary recovery (Figure 3). Exercise alone did not produce a significant increase in activated microglia, however the numbers are trending towards exercised mice having higher numbers of activated microglia as compared to those in the sedentary condition [$F_{(1,16)}$= 3.695, $p=0.07$] (Figure 4).

Figure 3: **Iba-1 immunohistochemistry of murine hippocampus**. The four experimental groups of SED-SAL, EX-SAL, SED-LPS, and EX-LPS are represented clockwise from top-right. Images are taken at 5x magnification and 20x magnification (inset).
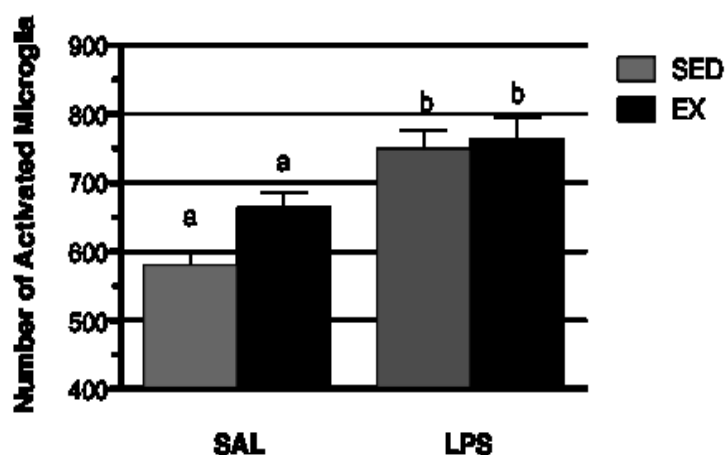
Figure 4: LPS-administration, followed by 2 weeks of exercise or sedentary recovery, leads to an increase in activated microglia. 7 days of LPS administration significantly increases Iba-1 positive microglia irrespective of exercise or sedentary recovery ($p < 0.0001$). While exercise alone does not significantly affect Iba-1 labeled microglia in saline treated animals, there was moderate effect ($p = 0.07$) when compared to sedentary animals. Bars represent mean ±SEM. Letters that are different (a,b) represents significant differences ($p < 0.05$). EX= exercise mice, SED= sedentary recovery.

DISCUSSION

Although it is still unclear whether inflammation is the cause of AD or simply a secondary response to neurodegeneration, studies have shown that inflammatory mediators may induce APP processing to produce Aβ in the CNS (Heneka & O'Banion, 2007). We do know that Aβ plaques are a physical structure associated with AD, and that increased levels of Aβ are present in the brain following inflammation (Selkoe, 2001; Heneka & O'Banion, 2007; Kahn et al., 2012). Kahn et al. (2012) has shown that injections of LPS for 7 days produced both inflammation and an increase in Aβ in the CNS. Further, the Chumley lab has shown that this Aβ can be decreased by two weeks of voluntary exercise (unpublished). Other studies have also highlighted exercise as an effective treatment to arrest or decelerate the molecular mechanisms of AD and to improve behavioral deficits in AD (Parachikova et al., 2008; Stranahan et al., 2012; Lautenschlager et al., 2008). The mechanism

behind the positive effects of exercise on AD cognition is not currently understood. One possible mechanism suggested by this study was the increased clearance of Aβ due to increased activation of microglia cells.

Similar to macrophages of the periphery, microglia are proposed to have two possible phenotypes of activation. The M1 phenotype is characterized by a high production of proinflammatory cytokines, nitric oxide (NO), and reactive oxygen species (ROS) (Perego et al., 2011). This phenotype is labeled neurodegenerative, because of its inability to degrade Aβ deposits in the brain, and the neurotoxic effects of NO and ROS secretions (Hickman et al., 2008; Streit, 2002). The inflammatory cytokines released also increase the inflammatory response and are detrimental to tissue recovery. By contrast, the M2 phenotype promotes tissue remodeling and repair along with uptake of debris, and is thought to be a neuroprotective phenotype of microglia (Perego et al., 2011).

Our data shows that overall, the exercised condition did not significantly increase the number of activated microglia in the hippocampus, but the exercised condition did seemed to have a much more pronounced effect on the saline animals. Due to the lack of microglia priming by LPS-inflammation in these saline-treated animals, it could be proposed that the increase in activated microglia observed in exercised animals could be of the M2 phenotype. The LPS-treated mice displayed an increase in microglia activation when compared to the saline treated animals. LPS is known to push microglia into an M1 phenotype (Gordon & Taylor, 2005; Nikodemova & Watters, 2012). If mice are first treated with LPS, it is reasonable to suggest that they had an increase in microglia activation, and that this may have

been M1-type activation. The animals that then underwent voluntary wheel running did not see a further increase in activated microglia when compare to LPS-sedentary mice. However, exercise has been shown to activate microglia (Barientos et al., 2011), and previous data from the Chumley lab reports that exercise led to a decrease in CNS Aβ levels and positive cognitive affects. This data suggests that perhaps exercise promotes a shift in the phenotype from the M1 induced by a week of LPS treatment to the M2 phenotype (Figure 4). This would not increase the number of activated microglia, but rather shift the phenotype of the activated microglia in the brain to one that is neuroprotective and capable of removing central Aβ.
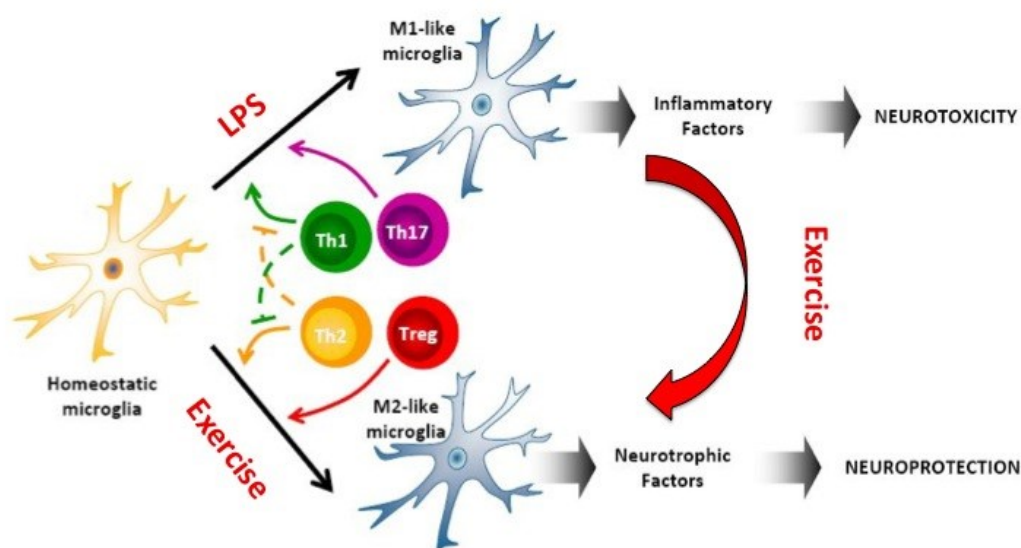

Figure 5: Hypothetical Model in which LPS and exercise induce different activated microglia phenotypes. LPS is thought to stimulate M1-like activation, while exercise could be guiding microglia towards an M2-like phenotype. After injection with LPS, we hypothesize that microglia could be converted by exercise to an M2-like, protective phenotype. Modified from: *Cell Interaction*-Chap 6: "Cells, Molecules and Mechanisms Involved in the Neuro-Immune Interaction" (Pacheco et al., 2012)

An alternative mechanism of Aβ clearance following exercise could be via an increase in phagocytic activity of astrocytes. Similar to microglia, astrocytes also serve both a neurodegenerative and neuroprotective role (Polazzi & Monti, 2010). Astrocytes are shown to produce glial-derived neurotrophic factor (GDNF) following inflammation with LPS (Bian et al., 2013). GDNF is neuroprotective and would stimulate recovery. Astrocytes are also able to phagocytose Aβ in the CNS (Buckwalter & Wyss-Coray, 2004; Koistinaho et al., 2004). It is possible that phagocytic and neuroprotective functions of astrocytes are being promoted by voluntary exercise, and this results in a decrease in the overall Aβ load in the CNS.

It is also possible that the clearance of Aβ from the CNS is being facilitated not by altered CNS function, but rather by a mechanism within the periphery. Much of the Aβ produced in response to inflammation caused by LPS injections is made in the periphery and then transported across the blood brain barrier (BBB) into the CNS (Weintraub et al., 2013). If exercise were potentially causing a decrease in the peripheral Aβ levels, then to restore equilibrium between the two sides of the BBB, the Aβ levels of the CNS would also decrease. Studies have shown that exercise results in an altered phenotype of macrophages in the periphery (Woods, Vieira, & Keylock, 2009). A change in macrophages to a phenotype capable of phagocytosing Aβ and reducing chronic inflammation could be responsible for a decrease in peripheral Aβ. This reduction of peripheral reduction of Aβ would further lead to a reduction in CNS Aβ levels.

RAGE (receptor for advanced glycation end products) is responsible for transport of Aβ into the brain (Deane et al., 2003), while LRP-1 (low density

lipoprotein receptor-regulated protein-1) is responsible for transport of Aβ out of

the brain (Banks, Robinson, Verma, & Morley, 2003; Bell et al., 2007). Jaeger et al.

(2009), has shown that LPS induced inflammation enhances the influx of Aβ into the

brain and decreases efflux of Aβ out of the brain. Voluntary exercise could possibly

be influencing the in/out ratio of Aβ to promote clearance from the CNS. Since influx

and efflux are regulated by separate mechanisms, exercise could either be

promoting efflux, or inhibiting increased influx previously noted with LPS

treatment. A beneficial continuation of this study would be to measure the

peripheral levels of Aβ in the serum and compare animals in sedentary and

exercised conditions.

Another possible exit point for Aβ out of the brain is through a recently

discovered CNS drainage system labeled the glymphatics system. The glymphatics

allows fluid to travel through the parenchyma via paravascular spaces and allows

exchange between the cerebrospinal fluid (CSF) and the interstitial fluid (ISF) (Iliff

et al., 2013). Iliff et al. (2013) have also shown that Aβ can exit the CNS through the

glymphatics. Therefore a possible explanation for the decrease in Aβ in the brain

following exercise could be that the Aβ is moving from the ISF to the CSF through

the glymphatics.

To broaden the scope of understanding of this study, and the possible role

that microglia may play in clearance of Aβ, additional studies of voluntary exercise

need to be performed to complement some of the limitations of our study. Due to

the hyperactivity of the exercised mice, no behavioral data could be collected using

an exercise or freezing task such as Morris water maze or Contextual Fear

Conditioning. Therefore, there is no data to report whether cognitive ability was improved in LPS-treated mice following exercise. A possible alternative behavioral test, such as the novel object recognition paradigm, may allow us to determine if exercise leads to a recovery of cognitive disruption following LPS administration.

Another limitation was the method used for quantifying activated microglial cells in the hippocampus. Microglia were quantified by human counters using Image J on a 5x-magnified image. Using stereology would allow less counter bias and thus reduce potential error. Stereology also allows for higher magnification to allow easier counting and thus potentially limit error.

Finally, antibodies for Iba-1 label all activated microglia, regardless of phenotype. Therefore we were unable to distinguish the phenotype of the microglia cells imaged. The M2 phenotype is shown to aid in the degradation of Aβ (Perego et al., 2011), whereas the M1 phenotype is shown to perpetuate the inflammatory response and be detrimental to Aβ clearance (Perego et al., 2011). Staining for markers specific to the microglia phenotypes, rather than the general microglia label Iba-1, would be a way to analyze the phenotypic ratio of the activated microglia. Being able to differentiate between these two different subcategories of activation could allow further insight into changes occurring to microglia as a result of inflammation or exercise.

Numerous studies, including those discussed previously, have shown exercise to be beneficial in decreasing Aβ loads and in restoring cognitive function. This suggests that exercise could be a very favorable treatment to arrest or slow the progression of AD. Based on our data, it is possible that microglia could be one of the

factors contributing to the benefits seen following exercise. Further research needs

to be conducted to better understand the complexities of CNS Aβ clearance

following exercise. Understanding the underlying mechanisms could allow more

effective exercise treatments, or treatments that target similar cellular mechanisms.

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