

EXERCISE AND MICROGLIAL CELL ACTIVATION

by

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Exercise and Microglial Cell Activation

The recent ballooning of the elderly population in the United States is poised to bankrupt several government funding agencies, including Medicaid. One of the largest problems the elderly face is the astronomical prevalence of Alzheimer's disease (AD) and other dementias; every 67 seconds another person is diagnosed with the disease in this country alone. As of 2014, AD accounts for roughly 60–80% of all dementia cases, and an estimated 5.2 million Americans are currently living with the disease (Alz Facts 2014). AD was so named because, in 1906, Alois Alzheimer was introduced to a 51-year old woman who had been diagnosed with pre-senile dementia 4 ½ years earlier. Immediately preceding her death, she had been bedridden and unable to care for herself (Alzheimer, Stelzmann, Schnitzlein, & Murtagh, 1995; Miklossy, 2008). Upon post-mortem analysis, Alzheimer noticed that the woman's brain was severely atrophied, and was littered throughout with two categories of lesions: plaques and tangles (Miklossy, 2008). It was later discovered that these senile plaques were made of aggregated A β fibrils and the intracellular tangles were comprised of hyperphosphorylated tau, a microtubule-associated protein (Lee et al., 2008). It was revealed over the years that the widespread anomalies present in Alzheimer's original patient were specific to humans; other primates, namely baboons and rhesus monkeys, only show intracellular tangles within the hippocampal formation, and not within the cortex (Delacourte, 2006).

At present, AD is described as a progressive and irreversible process marked by cognitive decline, memory loss, and changes in personality (Huang & Mucke, 2012). One of the first symptoms of AD is the inability to remember new information; this happens because some of the first neurons to malfunction are those associated with

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learning and memory, e.g., areas of the hippocampal formation (Harris et al., 2010; Huang & Mucke, 2012). The reality of AD is that the biological effects of the disease can manifest well before the onset of clinical symptoms. Until recently, the only way to diagnose AD was in a post-mortem autopsy, but thanks to the efforts of the scientific community, there have been many advances in the diagnostic process. Specifically, the progressive loss of cortical tissue thickness can be observed using magnetic resonance imaging (MRIs), the changes in neural patterns of patients at risk for the development of AD can be seen using functional MRIs (fMRIs), and positron emission tomography (PET) scans can be used to determine the presence of neurotoxic markers in the brain (Huang & Mucke, 2012). While these tests may not, in of themselves, be wholly definitive, they have been shown to correlate with the cognitive decay, and have even been used to predict the conversion from mild cognitive impairment (MIC) to AD (Frisoni et al., 2010).

As noted by Alois Alzheimer, those diagnosed with AD show severe tissue volume loss in post mortem autopsies; specifically, the greatest loss occurs in the first region of the *cornu Ammonis* (CA1) of the hippocampus (Harris et al., 2010; Huang & Mucke, 2012). In addition to the loss of tissue, it appears that it is not simply the damage of neurons that is associated with the cognitive dysfunction present in AD, but more precisely it is the synaptodendritic rarefaction, or the diminished density of synapses on dendritic spines, that is most predictive of this disorder (Huang & Mucke, 2012; Rosenblum, 2014; Snyder et al., 2005). This type of damage is thought to be a byproduct of the plaques and tangles Alzheimer noted in his original autopsy (Alzheimer, Stelzmann, Schnitzlein, & Murtagh, 1995), and the idea that the destruction of neurons,

their synapses, and the resultant cognitive dysfunction is caused by these anomalies is strongly supported in the literature, via the use of familial Alzheimer's models in mice (Glennner & Wong, 1984; Masters & Selkoe, 2012; Mucke & Selkoe, 2012; Spires-Jones & Hyman, 2014).

1.1 The role of amyloid-beta in AD

Glennner and Wong discovered the composition of these senile plaques in 1984 and they found that the plaques were made of an aggregation of a hydrophobic polypeptide of amino acids that had a strong tendency to form stable dimers, trimers, oligomers, and eventually the insoluble plaques seen in AD brains (Delacourte, 2006; Masters & Selkoe, 2012). This protein is now referred to as amyloid- β ($A\beta$) and is derived through enzymatic breakdown of amyloid precursor protein (APP). When α -secretase fails to cleave APP into its neuroprotective sAPP α form, the enzymatic complexes beta-site APP cleaving enzyme 1 (BACE1 also known as β -secretase) and γ -secretase make cuts at their respective cleaving sites (McGeer & McGeer, 2001). The successive cleavage of APP by β -secretase and then γ -secretase into its aberrant, 42-amino-acid long peptide ($A\beta_{1-42}$), causes it to be released from the cell membrane and then capable of aggregating (Haass & Selkoe, 1993; Snyder et al., 2005). These enzymes are responsible for the amount of $A\beta$ that is produced, but BACE1 activity is the rate-limiting factor (Zhang, Thompson, Zhang, & Xu, 2011). BACE protein expression levels and enzymatic activity are elevated in the brains of patients with sporadic AD (R. Li et al., 2004), who also show elevated levels of soluble $A\beta$ fibrils and non-soluble plaques. Furthermore, in a BACE knockout mouse model, aggregated $A\beta$ plaques never form (Huang & Mucke, 2012). On the other hand, there is research to indicate that the

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improper cleavage of APP may come from mutations to *presenilins* (PS)1 or 2, which, along with nicastrin, APH-1 (anterior pharynx-defective 1), and PEN-2 (presenilin enhancer 2), form γ -secretase. These mutations cause increased γ -secretase activity and up-regulated production of A β (Harris et al., 2010; Huang & Mucke, 2012; R. Li et al., 2004). Patients with mutations to PS1 have been shown to have the largest amount of neurodegeneration, increased production and/or aggregation of A β_{1-42} , and more severe tissue loss (Snyder et al., 2005; Sokolova et al., 2009). *Presenilin* mutations are not the only genetic modifications that can cause increased AD symptomology. For example, in a mouse model of Alzheimer's disease in which the animals overexpress mutant APP, there is increased expression of A β plaque deposition throughout the hippocampus and cortex (Harris et al., 2010). The idea that AD may arise from genetic mutations was further supported when it was determined that APP is encoded on the 21st chromosome in humans, the same chromosome that, when tripled, produces Down syndrome (i.e., Trisomy 21). Down Syndrome produces remarkably similar neurological pathologies to those found in AD, i.e., the accumulation of A β (Haass & Selkoe, 1993; Masters & Selkoe, 2012; Selkoe, 1991). Since these findings, genetic models of AD constitute the majority of research efforts in the field.

Notwithstanding the years of research following the identification and characterization of these proteins, the typical physiological functions for them remain largely unknown, though there is speculation that both APP and its derivative, A β , have an important evolutionary past (Zhang et al., 2011). For example, there is some evidence to indicate that APP plays a role in protein trafficking within neurons. Specifically, it may interact with kinesin-I to mediate the axonal transport of BACE, γ -secretase and PS1, to

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name a few (Zhang et al., 2011). In addition, there is work to suggest that A β could act as a positive regulator of vesicle release at the presynaptic level, and may, at intermediate expression levels, actually maintain efficient excitatory neurotransmitter transmission across the synapse through calcium (Ca²⁺)-mediated fusion of vesicles to the membrane (Abramov et al., 2009). Despite the possible adaptive roles for these proteins, the production and aggregation of aberrant A β in modern humans results in catastrophic consequences for the communication networks of the central nervous system (Mucke & Selkoe, 2012).

1.2 Neurodegenerative effects of amyloid-beta

Communication from one neuron to the next occurs at the synapse, a minute space, roughly 20 ± 2.8 nm, between the pre- and post-synaptic terminals, where chemical messengers relay information; it is in these spaces that A β fibrils congregate to alter synaptic structure and function (Harris et al., 2010; Ribault, Sekimoto, & Triller, 2011). In fact, the presence of A β dimers, trimers, or oligomers at the synapse is more neurotoxic than the presence of insoluble A β plaques (Huang & Mucke, 2012). Research shows that, in the presence of A β oligomers, there is a 60% loss of excitatory synapses (Mucke & Selkoe, 2012; Rosenblum, 2014), with synapses that contain acetylcholine, glutamate, and serotonin in the neocortex and the hippocampus being the hardest hit (Grutzendler & Morris, 2001). More precisely, these neurotoxic effects are often seen near NMDA-type glutamate receptors, where the presence of A β causes endocytosis of the receptor and decreased signaling through cAMP response element binding protein (CREB), a transcription factor crucial to neuronal survival (Ittner & Götz, 2011; Snyder et al., 2005). There is evidence to suggest that these neurotoxic effects can be mitigated

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when neutralizing antibodies to the N terminus of A β are utilized in the rat hippocampus (Rosenblum, 2014; Shankar et al., 2008).

The consequences of synaptodendritic rarefaction and neuronal death are widespread but impact learning and memory most dramatically. Learning deficits have been seen long before the formation of plaques, when there is only diffuse, soluble A β accumulation (Lee et al., 2008; Rosenblum, 2014). One of the consequences of A β aggregation is the effect it has on long-term potentiation (LTP). LTP is a form of synaptic plasticity that is considered a molecular and electrophysiological correlate for learning and memory and is often studied in the hippocampal formation (Snyder et al., 2005). Specifically, LTP occurs when there is a brief, high frequency train of action potentials in the same axon that causes physiological changes to the pre- and post-synaptic neuron, and leads to the strengthening of the existing synapse and/or the creation of new ones. At the molecular level, when a neuron is simultaneously depolarized and has glutamate bound to the N-methyl-D-aspartate (NMDA) receptor, calcium ions (Ca²⁺) flood into the cell and activate calcium-calmodulin-dependent kinase II (CaMKII) which both phosphorylates existing AMPA channels, making them more permeable to sodium ions (Na⁺), and causes the insertion of new ones into the membrane (Mucke & Selkoe, 2012). The overall effect of LTP is to make the neuron more sensitive to subsequent release of neurotransmitters. Long-term depression (LTD) is the functional opposite of LTP, and leads to the weakening or loss of synapses. Research has shown LTP is severely inhibited by the presence of A β oligomers, while LTD and synaptic loss are induced (Ittner & Götz, 2011; Mucke & Selkoe, 2012; Rosenblum, 2014; Snyder et al., 2005; Wang et al., 2002).

1.3 Mouse models of AD

Over the years, several different mouse models of AD have emerged. The reason transgenic mouse models abound is due, in part, to the differences seen between human and murine A β , murine A β being much less likely to oligomerize and clump together making it difficult to study the progression of the disease that is only observed in humans. Unfortunately, the vast majority of these animal models mimic familial Alzheimer's disease, which results from genetic mutations and/or susceptibilities but only accounts for a very small proportion of all dementia cases. For instance, the Tg2576 mouse strain expresses human APP at a five times higher level than endogenous mouse APP, produces elevated A β ₁₋₄₀ and A β ₁₋₄₂ levels and plaque deposition in an age-dependent fashion, and demonstrates impaired cognition by 9–10 months of age (Elder, Sosa, & Gasperi, 2010). There are also mouse strains that have targeted PS1 mutations; in general, within a single mutation model. Although these mice do not develop plaques per se, they still demonstrate elevated A β ₁₋₄₂ production and cognitive deficits (Elder et al., 2010). When these mutations are combined, as in the 5xFAD mouse strain that contains 3 APP and 2 PS1 familial AD linked (FAD) mutations, severe neuronal loss is seen in conjunction with the development of plaques and high A β ₁₋₄₂ production (Elder et al., 2010). Outside of the roughly 10% of cases that are caused by genetic mutations, including but not limited to mutations on APP, PS1, PS2, or BACE, or specific inherited alleles like Apolipoprotein E-e4 (APOE4), are cases diagnosed as sporadic Alzheimer's disease that have no apparent genetic correlates. How sporadic AD develops remains unknown, but there is evidence to suggest that chronic inflammation plays a role. Chronic inflammation

occurs when the immune system fails to eliminate the initiating pathogens or pathogens are repeatedly introduced in the organism and the immune system becomes persistently activated (McGeer & McGeer, 2001). It is this chronic inflammation that we, and others, consider a major contributing factor in neurodegeneration, and one potential cause for sporadic AD (Sokolova et al., 2009).

1.4 Inflammation and AD

For decades, the brain was considered an immune-privileged site, because many assumed that the blood-brain barrier (BBB) would prevent inflammatory mediators from entering the brain parenchyma and wreaking havoc. This belief is simply untrue. In fact, the immune system and central nervous system (CNS) share a bidirectional relationship, and although immune responses in the brain are usually muted, they do occur.

Inflammatory markers affect CNS function well after the onset of peripheral markers in the serum; this is because peripheral inflammation sets off various signaling pathways that trigger immune responses in the CNS (Erickson & Banks, 2011; Konsman, Luheshi, Bluthé, & Dantzer, 2000). Cytokines are some of the inflammatory mediators between the immune system and the CNS, and, are released by cells including but not limited to: resident macrophages, T-lymphocytes, and B-lymphocytes. For example, following exposure to a bacterial mimetic, LPS, pro-inflammatory cytokines, e.g. IL-1 β , IL-6, TNF- α , are up-regulated centrally (Konsman et al., 2000). Cytokines can impact the CNS in various ways, but due to their size and hydrophilic nature, cytokines cannot readily diffuse across the blood-brain barrier, or BBB (Konsman et al., 2000). Instead, there are two putative mechanisms behind their entrance into the CNS. The first means of ingress is referred to as the neuronal pathway, whereby cytokines and/or pathogens trigger

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afferents in the vagus nerve that project to the CNS. This causes activation of the paraventricular nucleus, secretion of ACTH, de novo IL-1 β production, and immune activation within the CNS to combat the pathogen (Eskandari, Webster, & Sternberg, 2003). The second path is more direct. Cytokines can enter the brain via circumventricular organs (CVO's) or through active transport across the BBB (Dantzer, Konsman, Bluthé, & Kelley, 2000; Konsman et al., 2000).

There have been numerous studies that have shown that chronic peripheral infections can accelerate the onset and progression of AD (Nee & Lippa, 1999). As previously discussed, peripheral infections can cause inflammatory cascades in the CNS. One prevalent form of chronic peripheral inflammation comes from atopic disorders. Atopy is a hereditary tendency towards a hypersensitive reaction to common allergens, i.e., colloquially known as 'allergies', and affects nearly 20% of the adult population (Eriksson, Gatz, Dickman, Fratiglioni, & Pedersen, 2008; Kamer et al., 2008; Sarlus et al., 2012). Sarlus and colleagues (2012) showed that allergy-induced inflammation increased the phosphorylation of tau. However, they did not find any change in APP expression, and no attempts were made to determine whether there were changes in hippocampal A β levels. In a twin study by Eriksson and colleagues (2008) a history of atopy was shown to be associated with a modest increase in the risk for AD, and that a specific history of asthma is linked to a poorer survival prognosis following a diagnosis of AD. Other longitudinal identical twin studies have shown that a history of severe peripheral infections resulted in an accelerated onset of AD (Engelhart et al., 2004; Kamer et al., 2008; Sarlus et al., 2012). The use of a twin design in these studies allows

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for the conclusion that the connection between peripheral inflammation and AD is not mediated by genetic predispositions, but the chronic inflammation itself.

Within the peripheral nervous system (PNS) and CNS of patients with both sub-clinical dementia and full-blown AD, there are a multitude of inflammatory markers. Specifically, there are elevated levels of pro-inflammatory markers in the plasma of patients with subclinical AD and Mild Cognitive Impairment (MCI) (Engelhart et al., 2004; Sarlus et al., 2012). Within the CNS itself, cytokines IL-1 α , IL-1 β , IL-6, and TNF- α , are all up regulated in the brains of AD patients (Kamer et al., 2008; McGeer & McGeer, 2001; Sokolova et al., 2008). There are also indicators of an inflammatory process within and around the plaques themselves, i.e., the presence of alpha₁-antichymotrypsin (ACT), serum amyloid-P, activated microglia, cytokines, chemokines, and even proteases (Miklossy, 2008; Selkoe, 1991). In addition, levels of MCP-1 have been shown to be a reliable predictor of AD, via linear regression analysis of post-mortem autopsied human brain tissue (Sokolova et al., 2008). MCP-1 is a chemokine that promotes the infiltration of macrophages into tumors, and plays an important role in tissue repair and regeneration (Sokolova et al., 2008). The elevations of MCP-1 in AD may represent toxic levels; in which, under normal conditions, MCP-1 is restorative, but when chronically up-regulated promotes neurodegeneration. Many of these findings have been replicated in multiple mouse models of AD. For example, models that overexpress human APP, e.g. Tg2567, also show elevated levels of pro-inflammatory cytokines such as IL-1 α , IL-1 β , IFN γ , COX-2, and TNF- α (Birch, Katsouri, & Sastre, 2014). Furthermore, when the production of inflammatory mediators is altered in these FAD models, there are often resultant decreases in the amount of A β production and a myriad

of other effects. The combination of these results supports the view that inflammation plays a critical role in Alzheimer's disease pathology.

The neurotoxic effects of A β ₁₋₄₂ have already been discussed, but the role inflammation plays in A β production may prove considerable. It is known that activation of the innate immune system up-regulates pro-inflammatory cytokines, and it is these cytokines that have been shown to transcriptionally up-regulate BACE mRNA, protein, and enzymatic activity, and thus increase the cleavage of APP into A β (Lee et al., 2008). In addition, A β and microglia enter into a cycle whereby the net result is further inflammation and neurodegeneration. Various A β species activate microglia which then produce pro-inflammatory proteins such as TNF- α , and toxic free radicals like NO₂ (Meda et al., 1995).

Having established the relationship between chronic inflammation and amyloid beta pathology, it is important to discuss ways to create inflammatory models in mice. Lipopolysaccharide (LPS) is a fragment of a gram-negative bacterial cell wall that activates toll-like receptor-4 (TLR4). TLRs are some of the most studied pathogen-detection systems, with their ligands, downstream effects, and functional relevance fairly well understood (Nahid, Satoh, & Chan, 2011). LPS acts as a bacterial mimetic by initializing an innate immune response in both the PNS and CNS, and activating various transcription factors that cause the release of several pro-inflammatory cytokines and chemokines from macrophages and microglia (Kamer et al., 2008; Kranjac et al., 2012). Following a single injection of LPS, levels of IL-1 β , IL-6, and TNF- α , (MCP)-1 and macrophage inflammatory protein (MIP)- 1 α peak in the serum of animals roughly 4 hours post-injection, and return to basal levels around 24 hours post-injection (Kahn et

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al., 2012; Kranjac et al., 2012). Similarly, elevated levels of central cytokines can be detected 4 hours following a single injection of LPS (Nahid, Satoh, & Chan, 2011). The levels and timing of pro-inflammatory mediators are slightly different following multiple LPS injections. Erickson and Bank (2011) found that 24 hours following a three-injection protocol for LPS resulted in prolonged elevation of various cytokines and chemokines in the serum. When cytokines are overproduced, septic shock can occur in both humans and animals; the pathological symptoms of sepsis have been induced in animal models via very high doses of LPS (Nahid, Satoh, & Chan, 2011). It is important to note that neutrophils and monocytes from septic patients have decreased inflammatory responses to further stimulation with LPS; this phenomenon is referred to as *endotoxin tolerance*, and is believed to be an immune system adaptation designed to prevent the host from going into septic shock (for review, see Biswas & Lopez-Collazo, 2009). Our laboratory has previously demonstrated behavioral correlates to this phenomenon in mice, and found that the levels of central and peripheral cytokines are no longer significantly elevated 24 hours after a 7-day LPS injection protocol (Kahn et al., 2012).

There is an abundance of literature that shows that LPS can reliably produce sickness behaviors in mice (for a review, see Dantzer & Kelley, 2007 or McCusker & Kelley, 2013). ‘Sickness behaviors’ are considered adaptive changes in motivation in response to stimulation of the immune system (Cunningham & Sanderson, 2008; Dantzer & Kelley, 2007). In general, these behaviors include but are not limited to, anhedonia, diminished social contact, decreased appetite, decreased locomotion and exploratory behaviors, hypothermia, and decreased grooming behaviors (Dantzer & Kelley, 2007; McLinden et al., 2012). Besides simply cataloging these behaviors following LPS

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exposure, a burrowing task is often used to assess sickness behaviors. Burrowing is a species-typical, hippocampus-dependent task, for which healthy mice use a coordinated hind- and forelimb movement to kick the pellets outside the burrowing container; on average 60 g of food is burrowed out of an enclosed tube within two hours, with most, if not all, of the original 200 g being burrowed out of the tube within twenty-four hours (Deacon, 2006). Following hippocampal dysfunction or immune challenge, this behavior is disrupted, and mice tend to burrow little, if any, food from the container in the allotted time period (Cunningham et al., 2009; McLinden et al., 2012). When endotoxin tolerance has been reached, which our laboratory has shown occurs after 7 consecutive days of LPS injections, mice no longer display any of the markers of sickness discussed above and perform as well in a motor paradigm as saline controls (Kahn et al., 2012). It should be noted, that endotoxin tolerance most likely occurs before the 7 day marker described above, but this study did not specifically look at days 5 and 6 to determine if tolerance occurred.

Peripheral immune challenges do not only disrupt daily behaviors involving motivation and activity levels, but can affect cognitive processes as well. Copious literature has detailed the impact LPS has on learning and memory, and for the sake of brevity, only a sampling of studies will be discussed here. One of the major areas studied is the impact endotoxins have on spatial learning, which relies on the functional integrity of the hippocampus. A peripheral immune challenge has been shown to negatively affect hippocampus-dependent memory processes (Pugh, Fleshner, Watkins, Maier, & Rudy, 2001). One way to evaluate spatial learning is via the use of a water maze. For nearly two decades the Morris Water Maze (MWM) has been used when evaluating anything from

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hippocampal lesions to various drugs aimed at altering learning and/or memory in rodent models. For example, allopregnanolone, a progesterone metabolite that enhances the inhibitory effects of GABA functioning at the GABA_A receptor impairs learning in the MWM, as indicated by an increase escape latency (Johansson et al., 2002). The classic MWM has been adapted in a myriad of ways over the years, and other spatial mazes have been modified to work in water as well, because escape from water is a strong motivator in rodent behavior (and animals given LPS often are not motivated by appetitive reinforcers). In addition, the MWM is a hippocampus-dependent task; lesioned animals show deficits in spatial learning, as they can no longer locate the hidden platform using extramaze cues, but they are still able to find a visible platform (Morris, Garrud, Rawlins, & O'Keefe, 1982).

LPS and other endotoxins disrupt learning in visual-spatial paradigms. For example, LPS-induced memory impairments have been found, using a water Y-maze paradigm (Cunningham et al., 2009), and a hidden and visual platform MWM procedure (Barrientos et al., 2006; Sparkman, Kohman, Scott, & Boehm, 2005; Sparkman, Martin, Calvert, & Boehm, 2005; Yirmiya, Winocur, & Goshen, 2002). Another task commonly utilized in the literature is fear conditioning. Fear conditioning has many varieties, e.g. auditory, contextual, or delayed, that can assess different learning processes, but of specific interest for the current proposal, is contextual fear conditioning, another form of spatial learning that is hippocampus-dependent (Barrientos et al., 2002). As previously discussed, the hippocampus is one of the hardest hit brain areas in AD, and, therefore, an ideal area to study disease progression and characterization. In addition, fear conditioning paradigms are especially useful in assessment of cognitive deficits following immune

insults, because the learned response is freezing and animals that are sick should freeze more, but the opposite holds following LPS injections, animals that are given LPS freeze less than saline controls (Cunningham & Sanderson, 2008). LPS has been shown to detrimentally effect contextual learning in various experiments (Barrientos et al., 2006; Bilbo et al., 2008; Pugh et al., 1998; Thomson & Sutherland, 2005) and even reconsolidation of the fear memory (Kranjac et al., 2012). LPS also disrupts learning in passive avoidance conditioning (Jain, Patil, Kulkarni, & Singh, 2002) and two-way active avoidance tasks (Sparkman, Kohman, Garcia, & Boehm, 2005). In addition to these behavioral examples of LPS-induced cognitive deficits, electrophysiological studies have demonstrated that LTP, considered by some to be an important neural correlate of learning, is also impaired by LPS treatment (Cunningham et al., 1996).

Our laboratory has previously shown that seven days of repeated i.p. injections of LPS results in elevated levels of A β in the hippocampus for at least 15 days after the last injection (Kahn et al., 2012). In addition, the significant elevation of hippocampal A β found after 7 d of immune activation has been correlated with cognitive deficits, as measured via freezing behavior in a CFC paradigm (Kahn et al., 2012). Further research demonstrated that as soluble A β in the hippocampus increased, freezing behavior decreased; this relationship was found to account for roughly 30% of the total variance found in freezing behavior following 7 days of immune activation (Weintraub et al., 2014).

1.5 Targeted drug therapy for AD

There have been multiple attempts to treat and/or prevent the development of AD via inhibition of β -secretase or γ -secretase in order to decrease the formation of A β

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(Huang & Mucke, 2012). One way to decrease the activity of γ -secretase is through the use of imatinib methanesulfonate salt (IM). IM was designed to act as an Abl-specific tyrosine kinase inhibitor, but it has other known effects as well, one of which is to interfere with γ -secretase activity and thereby decrease the synthesis of A β from APP. Our laboratory has previously shown the IM can prevent the increase of hippocampal A β that would typically accumulate following 7 days of LPS exposure (Weintraub et al., 2013). In addition, co-administration of IM with LPS ameliorates the cognitive impairment found following chronic LPS administration (Weintraub et al., 2013). The problem with these secretase inhibitors is that they can affect other systems negatively, e.g., they can result in decreased cleavage of notch (Huang & Mucke, 2012). For example, in 2011, a phase III clinical trial of semagacestat, a γ -secretase inhibitor, was suspended because it was demonstrated that the drug was mildly impairing cognition (Schor, 2011); this effect was interpreted as evidence that Semagacestat was not decreasing A β production (Hillmann et al., 2012). In addition, non-steroidal anti-inflammatory drugs (NSAID) have been investigated as a possible drug treatment for AD due to their anti-inflammatory properties. Research indicates that NSAIDs act to inhibit cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) activity, which then decreases the production of pro-inflammatory mediators and rescues cognitive impairments induced by inflammation (Jain et al., 2002). There is some evidence to suggest that NSAIDs can modulate γ -secretase activity, thus decreasing A β production. Longitudinal studies have also revealed that regular use of NSAIDs is linked with a reduced incidence of developing AD (Eriksson et al., 2008; McGeer & McGeer, 1996). Unfortunately, despite research indicating the positive effects of NSAIDs, their

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usefulness in treating AD has been called into question in recent years. For example, Hillmann and colleagues (2012) found that 3 months of ibuprofen treatment in a 5xFAD mouse model of AD was not adequate to decrease overall A β ₁₋₄₂ and plaque load, and ibuprofen treatment led to worse performance on motor coordination tasks and caused impairments in working memory, as measured on a cross-maze alternation task.

The loss of excitatory synapses has been discussed in depth above, but a subsection of these synapses are also the target of a class of drugs called cholinesterase inhibitors. AD results in reduced levels of acetylcholine (ACh) in the synapse that can lead to cognitive deficits, and one way to increase the efficacy of the remaining ACh and improve cognition and mood is through the inhibition of acetylcholinesterase (AChE), the enzyme that degrades ACh in the synaptic cleft (Grutzendler & Morris, 2001). This method has been used in various studies with small, but significant positive effects in patients with AD. However, it appears that the effectiveness varies considerably from patient to patient and may be more appropriate for patients that have anxiety, attention, and concentration deficits (Lamstra, Richard, & Gool, 2007).

Despite the modest symptomatic benefits of drugs like cholinesterase inhibitors and NSAIDs, there remains no real treatment for AD (Hall & Roberson, 2012). Pharmacological interventions are extremely complicated and often have unforeseen consequences. For example, treatments aimed at the A β plaques already present may have the unintentional side effect of releasing the toxic soluble A β oligomers from the plaques into the extracellular space (Rosenblum, 2014). Based on these complications, and the results from multiple clinical and animals trials throughout the years, it is now thought that simply targeting the reduction of soluble A β may not be the best way to

block disease progression. To this end, we propose that there is a need for a more holistic approach to reduce AD symptomology.

1.6 Exercise as a treatment modality for AD

Despite the common understanding that exercise improves our health, the precise mechanisms through which this occurs remain to be fully understood. Over the years, the role that exercise plays in modulating inflammation has received considerable attention. Studies in humans indicate that acute bouts of high intensity exercise can lead to decreased TLR4 expression on monocytes, which the authors argue may explain exercise-induced immunosuppression, but they also found that this effect is relatively short lived with expression levels returning to baseline within four hours (Oliveira & Gleeson, 2010). Exercise, both prolonged moderate-intensity and intermittent high-intensity training will also result in the release of IL-6 from skeletal muscle independent of the pro-inflammatory cytokine TNF- α , demonstrating how exercise-induced inflammation is unique. Furthermore, a brief elevation of IL-6 inhibits the production of TNF- α and IL-1 β while inducing the release of cortisol and IL-10 which can have potent anti-inflammatory properties (for review, see Nimmo, Leggate, Viana, & King, 2013). While prolonged moderate activity and intermittent high intensity exercise seem to convey benefits, research tends to agree that a combined weight-resistance and aerobic training will have the most favorable impact on systemic inflammation by decreasing circulating pro-inflammatory cytokines like C- Reactive Protein (CRP), IL-1 β , INF- γ and TNF- α and also decreasing the inflammatory monocytes capable of releasing these inflammatory mediators (Olsen, Dengel, Leon, & Schmitz, 2007; Timmerman, Flynn, Coen, Markofski, & Pence, 2008; Balducci et al., 2010).

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Exercise not only effects the functioning of the immune system, but also improves cognition and can delay the onset of AD (Rovio et al., 2005). A meta-analysis revealed that older adults who perform exercise training show significantly greater improvements in cognitive performance than control subjects in a variety of cognitive tasks (Colcombe, Kramer, McAuley, Erickson, & Scalf, 2004). This phenomenon has been replicated in various animal models. For example, voluntary wheel running protected aged rats from *E. coli*-induced cognitive impairments in an immediate shock fear-conditioning paradigm through the blunted production of IL-1 β and TNF- α , which are generally significantly elevated following stimulation with *E. coli* (Barrientos et al., 2011). In addition, treadmill exercise in mice attenuates LPS-induced learning and memory deficits as measured through the Morris Water Maze (Wu et al., 2007). Voluntary wheel running has also been shown to enhance cognitive performance on a modified contextual fear-conditioning paradigm in mice (Kohman, Clark, DeYoung, Bhattacharya, Venghaus, & Rhodes, 2012). Relevant to AD, 12 days of voluntary wheel running moderated A β_{25-35} induced memory deficits in a Y-maze paradigm (Wang et al., 2013). Parachikova, Nichol & Cotman (2008) demonstrated improved spatial learning in a radial arm water maze following 3 weeks of voluntary exercise in a Tg2576 transgenic mouse model of AD. In the same model, 16 weeks of voluntary running resulted in increased exploration of a novel object, compared to a sedentary group and a forced-treadmill exercise group, indicating that voluntary exercise improves object recognition memory (Yuede et al., 2009). As Yuede and colleagues (2009) demonstrated, forced exercise does not always confer the same positive outcomes as voluntary exercise. Furthermore, forced exercise leads to elevated levels of corticosterone (Hoffman-Goetz, Spagnuolo, & Guan, 2008), a

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stress hormone released from the adrenal cortex that can, in large amounts, cause damage to pre-synaptic terminals and decrease overall synaptic functioning (Wuwongse et al., 2013). For these reasons, only voluntary exercise was used throughout these experiments. Furthermore, the benefits conferred to animals engaging in voluntary exercise is particularly important for the consolidation phase of learning, indicating the importance of investigation during this initial timeframe (Falls, Fox, & MacAulay, 2010).

In addition to the cognitive benefits of exercise, research shows that exercise improves overall brain health and function. For example, structural MRI studies have found a positive correlation between a person's VO_2^{peak} and total brain volume whereby AD patients with higher VO_2^{peak} levels, or higher fitness levels, show increased white and grey matter volume over AD patients with lower fitness levels (Burns et al., 2008). The decreased atrophy brought on by exercise was also shown to preserve performance on specific cognitive tasks, e.g., the delayed memory task from the Wechsler Memory Scale-Revised (WMS-R) and the digit symbol tasks on the WMS III (Burns et al., 2008). Furthermore, exercise increases adult neurogenesis by promoting the proliferation and differentiation of stem cells into neuronal progenitor cells (Farmer et al., 2004; Praag, Kempermann, & Gage, 1999; Wu et al., 2007; Vukovic, Colditz, Blackmore, Ruitenberg, & Bartlett, 2012). Praag, Kempermann, & Gage (1999) found that cell proliferation was only increased in the exercised mice, and that the surviving newborn neurons increased in the dentate gyrus of both runners and environmental enrichment groups. LTP is also increased in animals that exercise; exercise was found to lower the induction threshold for LTP in the dentate gyrus of the hippocampus (Farmer et al., 2004).

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Exercise also modulates a number of neuroprotective proteins in the brain. For example, following LPS stimulation brain-derived neurotrophic factor, or BDNF, is decreased, but exercise has been found to be protective against this effect (Farmer et al., 2004; Martin et al., 2013; Neeper, Gomezpinilla, Choi, & Cotman, 1995; Wu et al., 2007). BDNF is a neurotrophin that works primarily in the hippocampus and the cortex through a variety of mechanisms to promote synaptic plasticity, increase neuronal excitability, and induce dendritic branching (Kranjac et al., 2012; Martin, Dantzer, Kelley, & Woods, 2014; Wu et al., 2011). Following 28 days of voluntary wheel running in rats, BDNF levels remained significantly elevated for one week after exercise had ceased (Berchtold, Chinn, Chou, Kessler, & Cotman, 2005). Further, evidence indicates that three or six weeks of forced treadmill running in rats can significantly increase the number of glial fibrillary acidic protein (GFAP)-positive cells, which are mostly astrocytes, in the cortex and striatum (Li et al., 2005). In addition to BDNF, insulin-like growth factor-I (IGF-1) is a trophic factor that regulates the proliferation and differentiation of various cells throughout the body (for review, see Cohick & Clemmons, 1993). IGF-1 has been shown to increase in the serum following exercise, and that treadmill exercise can cause the serum IGF-1 to infiltrate into the brain and be taken up by neurons (Carro, Nuñez, Busiguina, & Torres-Aleman, 2000). Furthermore, in the Tg2576 mouse model of AD, 3 weeks of voluntary wheel running resulted in a significant decrease in soluble A β ₁₋₄₀, IL-1 β , and TNF- α (Nichol et al., 2008). In this same transgenic model, three weeks of running increases the levels of C-X-C motif ligand 1 (CXCL1) and CXCL12, two chemokines that are associated with neuron/glia communication (Parachikova, Nichol, & Cotman, 2008). In particular, the presence of

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CXCL12 increases glutamate release from astrocytes and can regulate neuronal excitability. In the TgCRND8 mouse model of AD, five months of voluntary exercise resulted in decreased plaque load and decreased levels of A β ₁₋₄₀ and A β ₁₋₄₂ centrally; the authors explained this reduction as being due to changes in the proteolysis of APP, as evidenced by the decrease in C-terminal fragments (CFTs, both α -CTFs and β -CTFs), which are the cleavage products of APP following secretase activity (Adlard, Perreau, Pop, & Cotman, 2005).

Finally, our laboratory has recently demonstrated that two weeks of voluntary wheel running, following seven days of LPS exposure, can decrease the amount of A β ₁₋₄₂ found in the hippocampus of mice (Weintraub, 2014; see Figure 1). Indeed, this finding is the impetus of this thesis, and the next step is to evaluate whether the LPS-induced cognitive deficits found by Kahn et al. (2012) are still present after two weeks of sedentary recovery, and whether voluntary exercise can ameliorate this effect. Furthermore, given the finding that A β ₁₋₄₂ levels are significantly reduced following two weeks of voluntary wheel running, the mechanisms behind this clearance remain unknown.

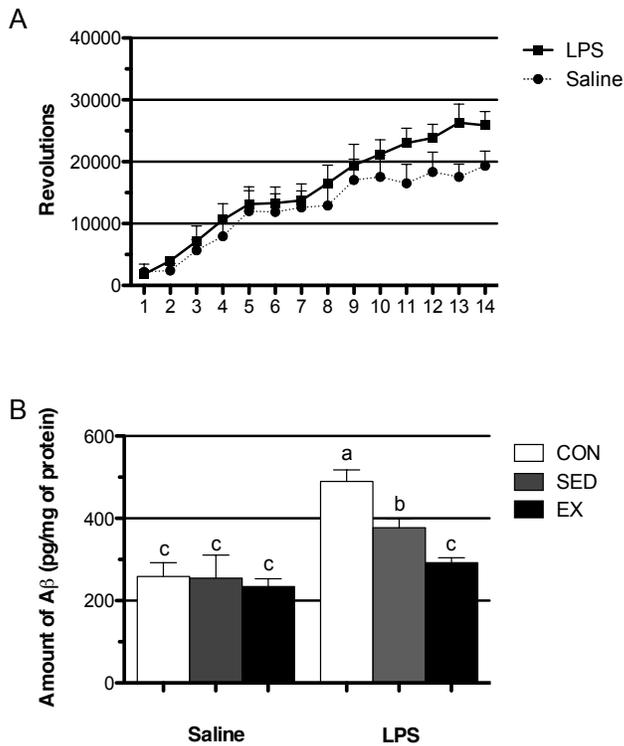


Figure 1. Two weeks of exercise significantly reduces Aβ following LPS-administration. (A) Repeated measures ANOVA revealed no significant differences in nightly wheel revolutions following administration of LPS or saline. (B) Two weeks of voluntary exercise, following LPS-administration, significantly decreased the level of hippocampal-Aβ as compared to matched SED mice. Interestingly, there were no significant differences in hippocampal-Aβ between LPS-administered EX mice and saline-administered EX and SED mice. Bars represent mean ± SEM. Letters that are different (a,b,c) represent significant differences ($p < 0.05$). CON = control mice EX = exercise recovery, SED = sedentary recovery, LPS = lipopolysaccharide

1.7 The role of microglia in AD pathology

Research on microglial cells and their role in AD pathology is an area of growing interest in the Alzheimer’s field. Microglial cells are born from uncommitted c-Kit⁺ stem cells that are found in the yolk sac; they eventually become CD45⁺/c-Kit⁻ cells and migrate to the CNS to become an autonomous population, which only make up roughly 5–10% of all the cells within the CNS (Elmore et al., 2014). Microglia are often called the resident macrophage of the CNS. In other words, it is the job of microglia to monitor the microenvironment of the CNS, initiate innate immune responses, clear cell debris, and a host of other stabilizing actions (Yang et al., 2011). For example, like macrophages, when LPS or Aβ oligomers bind to receptors on microglia, they become activated, take on a specific gene profile, and release pro-inflammatory cytokines (Wu et

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al., 2007); it has been shown that this LPS-induced activation can persist for up to 28 days (Kondo et al., 2011). Microglia are important mediators in inflammation, and have been linked to AD. For example, in APP/PS1 mice, microglia near plaques show inflammasome activation while other cells within the CNS do not. The inflammasome is a molecular complex involving the R(NLRP3), and it is known to initiate caspase-1 cleavage and the eventual production of IL-1 β (Goldman, Tay, & Prinz, 2013). Heneka and colleagues (2013) demonstrated that APP/PS1 mice deficient in either NLRP3 or caspase-1 show improved cognition and reduced A β -burden, indicating a role for microglia activation in A β pathology. Besides mediating inflammation, microglia are capable of taking up A β via A β -binding receptors, e.g., scavenger receptor A (SRA), scavenger receptor B1 (SRB1), scavenger receptor CD36, the receptor for advanced-glycosylation end products (RAGE), and macrophage receptor with collagenous structure (MARCO), and then degrading the peptide (Yang et al., 2011). There are also reports that CD14, TLR4 and TLR2 interact to bind A β fibrils, and are required for microglial activation and phagocytosis of the peptide (Reed-Geaghan et al., 2009). It is generally held that this function becomes less efficacious over time, and that microglial cells cannot keep up with the progression of the disease. Illuminating this point, cultured microglial cells become engorged with undigested A β after continuous incubation in an A β -rich milieu for four days, suggesting that there is a slow rate of A β degradation occurring within the microglia cells (Fu et al., 2014). Furthermore, the A β -binding receptors on microglia cells become down-regulated in aged PS1-APP transgenic mice leading to decreased phagocytic capabilities in the presence of ever increasing levels of pro-inflammatory mediators, e.g., TNF- α (Hickman, Allison, & Khoury, 2008). This suggests

that as AD progresses, deficits in microglia functioning combined with their decreased ability to phagocytose and degrade the A β further promotes the progression of the disease. Finding ways to maintain or enhance the A β degradation properties of microglia may prove especially important in the treatment or prevention of AD.

1.8 Microglia phenotype diversity

Recently, it has become established that microglia can adopt various phenotypes that are associated with either the release of pro-inflammatory cytokines and other neurotoxic proteins or a cytokine profile that is considered immunosuppressive and more associated with repair and regeneration (Bardou et al., 2014); the nomenclature for these different phenotypes varies, but for the purposes of the current thesis, the first will be referred to as a classical activation phenotype and the latter an alternate activation state (Gertig & Hanisch, 2014). Unfortunately, this dichotomy may be overly simplified, as it appears there are subpopulations of these broad activation states that have functionally different characteristics (Chhor et al., 2013). Although still debated, there are four activation states: classically activated (M1) microglia with cytotoxic properties, alternatively activated (M2a) microglia which are involved in repair and regeneration, immunoregulatory (M2b) microglia which can also produce pro-inflammatory mediators, and finally an acquired deactivating (M2c) classification for microglia that inhibit the production of pro-inflammatory cytokines and may be involved in debris clearance (Colton & Wilcock, 2010; Chhor et al., 2013; Madore et al., 2013; Mosser & Edwards, 2008).

There is still considerable debate about how to identify these different phenotypes; simple morphology has been one of the methods used to determine

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activation states; e.g., resting microglia display a ramified morphology which is then transformed into an amoeboid morphology upon activation (Hanisch, 2002; Madore et al., 2013; Vukovic et al., 2012). This focus on morphology may be too simplistic. Even in healthy brains, microglia are highly dynamic cells that are constantly surveying the microenvironment of the CNS, so it may be more important to focus on specific cellular markers rather than morphology alone, as evidence suggests that functionality and morphology do not always correlate in microglia (Krabbe et al., 2013; Madore et al., 2013). The need to focus on cellular markers is further supported by work showing that CD11b⁺ cells, an established activation marker for microglia and macrophages, show various morphologies depending on location within and around ischemic cores, signifying that activation does not always lead to an amoeboid form (Perego, Fumagalli, & Simoni, 2011).

While distinctions based on morphology await further investigations, what is clear about microglia functioning is that these phenotypes are not permanent, and appear to depend on the nature of the interstitial fluid and any further, secondary challenges (Schwartz, Butovsky, Brück, & Hanisch, 2006). For example, *in vitro* M1 or M2b activation can reduce, but not eliminate, the ability of microglial cells to acquire an M2a activation status (though the authors did not find the same phenomenon in the other direction, Chhor et al., 2013). Other *in vitro* studies have shown M1 activated cells that are treated with an anti-inflammatory cytokines, e.g. IL-4, can alter their phenotype commitment towards more neuroprotective functions (Schwartz et al., 2006). In addition, there are reports that M1 activated microglia exposed to anti-inflammatory cytokines have enhanced phagocytic properties (Fu et al., 2014). While the issue of microglial

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plasticity remains unresolved, it is clear that phenotype commitments are transient, as M1 cells revert to a surveying state once the immune stimulus is resolved (Fenn, Henry, Huang, Dugan, Godbout, 2011). This transition is brought on by the regulation of a number of anti-inflammatory mediators, which promote M2a activation status and finally the resolved surveying state.

These different activation phenotypes are based on specific markers, both those on the cell surface, and those expressed internally. Classical activation of microglia is brought on through stimulation by pathogens or other ‘danger signals’ in the CNS, and mediates the inflammatory response within the brain (for review, see Ransohoff & Perry, 2009). LPS, pro-inflammatory cytokines like TNF- α , large amounts of INF- γ and A β fragments have all been found to induce increases in M1 phenotype markers (Colton et al., 2006; Luo & Chen, 2012; Olah, Biber, Vinet, & Boddeke, 2011). For example, LPS treatment increases in gene expression for Cox-2, iNOS, CD68 and CD86, and also increases the release of proteins like IL-6, TNF- α , and IL-1 β (Chhor et al., 2013; Fenn et al., 2011). BV2 cells that are treated with INF- γ will also display increased mRNA expression of nitric oxide synthase 2 (NOS2) and TNF- α (Colton et al., 2006).

Furthermore, treatment with LPS suppresses M2a-associated repair and regeneration markers, and decreases the antigen presentation capabilities of microglia (Butovsky et al., 2006; Chhor et al., 2013; Olah et al., 2011). LPS decreases microglia motility and process extension, thereby decreasing the surveying capabilities of the cell (Madore et al., 2013).

The microenvironment caused by M1 activation can serve to propagate inflammation further and cause neuronal damage in vulnerable brain regions (for review, see Hanisch,

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2002). For these reasons, M1 activation status is pro-inflammatory (Goerdts & Orfanos, 1999).

At the opposite end of the spectrum is alternative (M2a) activation, which is often considered neuroprotective, and includes roles in neurogenesis and phagocytosis (Shwartz et al., 2006). This neuroprotective phenotype is marked by increased expression levels for a variety of markers, including, but not limited to, arginase-1 (Arg-1), mannose receptor (CD206 or MRC1), FIZZ1 (found in the inflammatory zone), CX₃CR1 (fractalkine receptor), and Ym1 (Chitinase 3-like 3), in addition to the increased production of IGF-1, transforming growth factor- β (TGF- β), and BDNF (Chhor et al., 2013; Kohman, 2012; Zhou, Spittau, & Kriegelstein, 2012). M2a activation in macrophages is induced via IL-4 stimulation (Raes et al., 2002); *in vitro* IL-4 treatment of microglia cells induces M2a activation demonstrated by a similar gene profile to that which occurs in macrophages (Chhor et al., 2013). In a review on antigen-presenting cells, Goerdts and Orfanos (1999) discuss how alternative activation of macrophages following stimulation by IL-4 or glucocorticoids leads to the enhanced expression levels of major histocompatibility complex class II molecules (MHC-II), indicating effective antigen presentation, and an enhanced capacity for phagocytosis, evident in the up-regulation of innate immunity receptors, e.g. scavenger receptors and macrophage mannose receptors. The effect of IL-4, an anti-inflammatory cytokine derived from T helper 2 (Th2) cells, has also been studied in microglia. IL-4 has been shown to cause the up-regulation of insulin-like growth factor 1 (IGF-1), BDNF, and MHC-II molecules, and IL-4 is thought to be involved in the process of microglia phenotype switching (Butovsky, Talpalar, Ben-Yaakov, & Schwartz, 2005; Ziv et al., 2006; Derecki et al.,

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2009). For example, Fenn and colleagues (2011) showed that mice given intraperitoneal injections of LPS up-regulate the IL-4R α on the surface of microglial cells, which they argue may be a compensatory mechanism for the LPS-induced decrease in IL-4. Cell culture studies in microglia have shown that IL-4 treatment leads to increased uptake and degradation of A β ₁₋₄₂ via the up-regulation of CD36, a membrane bound protein that binds A β fibrils and initiates phagocytosis (Fu et al., 2014; Shimizu, Kawahara, Kajizono, Sawada, & Nakayama, 2008).

The effect of IL-4 treatment on macrophages has been thoroughly documented elsewhere, and its ability to increase antigen presentation is well established, but though some controversy in the literature remains regarding how IL-4 effects antigen presentation in microglial cells (for review, see Martinez, Helming, & Gordon, 2009). For example, Townsend and colleagues (2005) argue that there are two distinct patterns of activity, that of phagocytosis and that of antigen-presentation. They found that, in culture, there is increased co-localization and interaction of MHC-II molecules and A β following CD40 ligation in the presence of exogenous A β , indicating that the A β is being redirected from clearance mechanisms to presentation by the MHC-II molecules which then activate T cells and increase the release of pro-inflammatory cytokines from microglia (Townsend et al., 2005). Furthermore, normal brain aging has been shown to increase the expression of MHC-II and multiple other pro-inflammatory mediators, e.g. interferon- γ (IFN- γ) (Frank et al., 2006). The authors claim that these results support the idea that aging shifts the microenvironment of the CNS to be more neurodegenerative, a commonly accepted theory in the field (Frank et al., 2006; Kohman, 2012). Given the varying reports within the literature, the presence of MHC-II molecules may be more

reasonably considered a marker of activation that can be present on multiple microglia phenotypes depending on the type and duration of the stimulus present.

Evidence in peripheral macrophages demonstrates that when phagocytosis of apoptotic cells occurs, the macrophage suppresses the expression on IL-1 β and TNF- α , implying that M1 and M2a markers may work antagonistically, with one suppressing the other (Fadok et al., 1998). This is further demonstrated in relation to the metabolism of arginase and iNOS, which have been used to discriminate between classically and alternatively activated macrophages in mice (Raes et al., 2002). Arginase 1 (Arg-1) is an enzyme that competes with nitric oxide (NO) synthases for L-arginine as a substrate and has been shown as the only marker to positively discriminate an M2a from an M1/M2b microglia phenotype at a variety of time points (Chhor et al., 2013). It is for this reason that Arg-1 has been chosen to serve as a marker for alternative activation of microglia in the following experiments.

1.9 The effect of exercise on microglial function

Of interest to this thesis is the interaction between exercise and microglia phenotype; our laboratory has shown that the LPS-induced increase in hippocampal A β is significantly reduced after engagement in two weeks of voluntary wheel running, but this reduction does not coincide with a reduction in the number of activated, Iba-1+, microglial cells (Weintraub, 2014; see Figure 2).

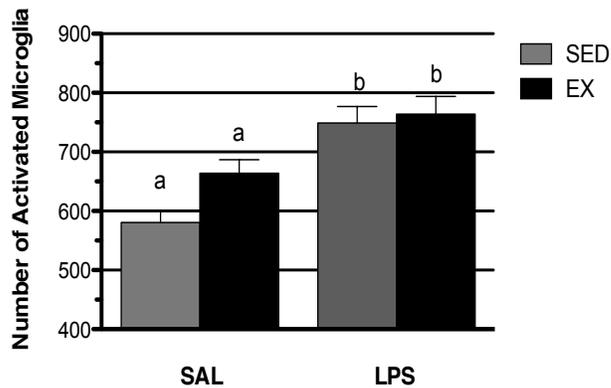


Figure 2. LPS-administration, followed by 2 weeks of exercise or sedentary recovery, leads to an increase in activated microglia. LPS-administration, regardless of recovery condition, significantly increased the number of Iba-1 positive microglia ($p < 0.0001$). However, there was no significant effect of exercise on Iba-1 positive microglia, though it approached significance ($p = 0.07$) in saline-injected animals. Bars represent mean \pm SEM. Letters that are different (a & b) represent significant differences ($p < 0.05$).

We now believe that exercise may not function to decrease activation per se, but alter the phenotype of the microglial cells that are activated. There are several lines of research that seem to indicate that exercise can increase various biological markers related to microglia M2a activation. A study involving elderly men found that, following a 12-week resistance-training program a bout of isokinetic exercise leads to a significant increase in IL-4 expression. This increase was associated with enhanced myogenesis and muscle regeneration (Della Gatta, Garnham, Peake, & Cameron-Smith, 2014). In addition, adults who engaged in a combined aerobic and resistance exercise program for twelve months expressed increased levels of IL-4 compared to adults in sedentary or high-intensity aerobic conditions (Balducci et al., 2010). Considering that IL-4 is most often used in cell culture work to stimulate the M2a phenotype and the increase in IL-4 following exercise, it would follow that there would be an increase in the expression of M2a phenotypic markers following exercise. For instance, IGF-1 production is increased by alternatively activated microglia and are thus acting in a neuroprotective role. Voluntary wheel running in aged mice has been shown to significantly increase the number of microglia cells that co-label with Iba-1, a microglia activation marker, and IGF-1, compared to sedentary controls, suggesting that eight weeks of voluntary wheel running in aged mice

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can lead to an increase in the expression of alternatively activated microglial cells within the hippocampus (Kohman, DeYoung, Bhattacharya, Peterson, & Rhodes, 2012). Soluble CX₃CR1 protein levels, which were previously discussed as a marker of M2 activation, have also been shown to increase following 14 days of wheel running in mice (Vukovic et al., 2012). Finally, Kohman and colleagues (2013), when using flow-cytometry methods, found that voluntary exercise had no effect on the percentage of microglia that were CD206-positive. It should be noted that the authors argued that there may be other M2 markers better suitable to assess phenotypic changes and suggested future research using the Arg-1 marker.

Exercise appears to also decrease many M1 activation markers. For instance, 10 weeks of voluntary wheel running significantly decreased the percent of CD86-positive microglia, a marker for M1 activation, in the hippocampus of aged male mice, but in adult mice of both sexes, there were minimal, non-significant increases in the percent of CD86 positive microglia (Kohman, Bhattacharya, Wojcik, & Rhodes, 2013). In a chronic restraint stress paradigm, mice who had access to a running wheel were protected from the restraint stress-induced rise in COX-2 (an M1 activation marker) positive microglia in the hippocampus, as compared to stressed sedentary controls (Gerecke, Kolobova, Allen, & Fawer, 2013). In addition to the decrease in CD86- and COX-2-positive microglia following exercise, microglia isolated from mice that had access to a running wheel for six weeks showed a blunted response to LPS in culture, as evidenced by meaningfully reduced levels of TNF- α and IL-1 β (Barrientos et al., 2011).

The phagocytic activity of peritoneal macrophages is also modified by exercise (Ferreira et al., 2010). For example, following forced treadmill running, without an

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electrical shock stimulus, for 12 weeks of five days/week, phagocytic activity was significantly enhanced in exercise mice compared to sedentary controls (Sugiura et al., 2001). In addition, a forced swim paradigm found that exercise enhanced the chemotaxis and phagocytic abilities of the macrophages (Ferrandez & Fuente, 1999), and an acute exercise model, a 60-minute bout of swimming, also increased phagocytic activity in macrophages (Silveira et al., 2007).

1.10 Hypotheses

As formerly discussed, our laboratory has shown that repeated i.p. injections of LPS results in elevated levels of A β in the hippocampus and cognitive deficits in a contextual fear-conditioning paradigm. If this inflammatory insult is followed by two weeks of voluntary exercise, then there is a significant decrease in the level of A β in exercised mice compared to sedentary controls. It was hypothesized that the reduced hippocampal A β would lead to rescued cognition in a fear-conditioning paradigm. More specifically, animals administered LPS and then allowed two weeks of exercise recovery were expected to show freezing levels comparable to the saline-injected animals, and higher than LPS sedentary animals. Also, we predicted that the improved cognitive performance would coincide with elevations in IL-4 and BDNF for animals that were in the exercise recovery condition.

In addition, this laboratory recently demonstrated that two weeks of voluntary wheel running leads to a general increase in the number of activated microglial cells within the hippocampus compared to sedentary controls. The immunohistochemistry (IHC) done in this experiment used Iba-1 as a marker of activation and did not examine phenotype. One purpose of the following experiments was to determine the effect of

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exercise on microglial phenotype. We believed that the decrease in A β found following two weeks of voluntary wheel running is a result of an increase in the population of M2 activated microglia within the hippocampus. It was hypothesized that within the LPS-stimulated group, two weeks of voluntary wheel running would cause an up-regulation in the amount of microglia that are alternatively activated.

2.0 Methods

2.1 Subjects

In all experiments, animals were male C57BL/6J mice between the ages of 4–6 months, bred in the TCU vivarium. All animals were housed and treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996), and in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of Texas Christian University. All subjects were housed in groups of three or four in standard polycarbonate cages (12.5cm x 15cm x 25cm). Food and water were available *ad libitum* and maintained on a consistent light/dark schedule, with lights on at 0700 and lights off at 1800.

2.2 Treatment conditions

In all experiments, animals were given once-daily i.p. injections for seven consecutive days of either 250 μ g/kg LPS (*Escherichia coli*, serotype 055:B5 Sigma-Aldrich, St. Louis, Missouri) or sterile saline, followed by a two week exercise/sedentary paradigm before all behavior testing or biological assays. Following the seven-day LPS injection protocol, animals were assigned to either the exercise or sedentary condition. All animals were group-housed during their light cycle (0700h-1800h), then moved into individual cages with or without a running wheel present for the entirety of their dark,

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active cycle (1800h-0730h), for a total of 14 nights. This configuration allowed individual monitoring of wheel rotations for each mouse, to ensure all mice ran equivalent distances, while simultaneously minimizing the potential for isolation stress. Overall, the four treatment groups were as follows: saline-sedentary (SAL-SED), saline-exercise (SAL-EX), LPS-sedentary (LPS-SED), and LPS-exercise (LPS-EX).

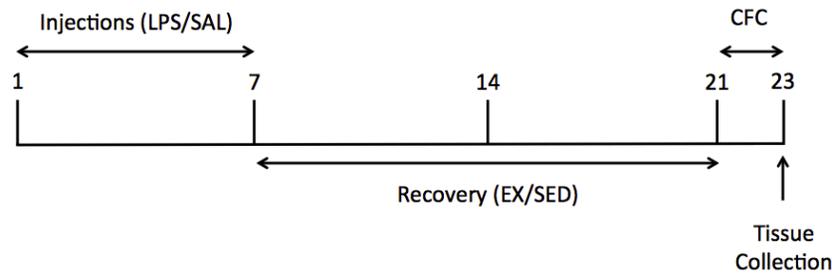


Figure 3. Experimental timeline. Animals were administered 14 consecutive days of once-daily LPS (250 μ g/kg) or saline. After the last injection, the running protocol was enacted for 14 nights. Animals were either in the wheel condition (EX) or the no wheel condition (SED). CFC training began after the last night of recovery, followed 24h later by testing. Tissue collection occurred immediately following CFC testing. Cytokine levels were analyzed via qRT-PCR in Experiment 1, and immunofluorescence in Experiment 2 was used to assess microglial phenotype.

2.3 Behavioral Paradigms

2.3.a Experiment 1A: Contextual fear conditioning protocol

This protocol began on the morning after the last night of exercise or sedentary recovery, and included a training session (day 1) and a testing session (day 2) 24 h later. Contextual fear conditioning (CFC) was conducted in testing chambers (Coulbourn Instruments, Whitehall, PA, 7W x 7D x 12H) equipped with an electrified grid floor, through which the mild foot shock (US) was delivered. The training session was a total of 182 s broken down into a 120 s acclimation period, followed by a 2 s shock at 0.7mA. Animals remained in the chamber for an additional 60 s following the conclusion of the aversive stimulus. On day 2, animals were placed back into the chamber and freezing was measured for 90 s; no shocks were delivered on testing day. The delivery of the stimulus

and the resulting freezing behavior was monitored and calculated using FreezeFrame™ software (ActiMetrics Software, Wilmette, IL).

Previous experience from our laboratory has shown that the incorporation of an olfactory cue (peppermint oil 1:10 in water) and a highly salient wall design (black polka dots) into the context increases the percent of time the mouse freezes. This increased freezing behavior has been interpreted as an increase in the learning of the context-shock pairing (Kahn et al., 2012; Kranjac et al., 2012). The time freezing was analyzed using analysis of variance (ANOVA) procedures (SPSS 22.0, IBM, Armonk, NY), in which Group (EX/SED) and Treatment (LPS/Saline) were used as independent variables. All statistical analyses were conducted with $p \leq .05$ to determine significant group differences, followed by Fisher's PLSD post-hoc comparisons.

2.3.b Experiment 1B: Contextual fear conditioning protocol

During the course of this thesis, it became apparent that the above-mentioned CFC protocol was not sensitive enough to detect learning differences between animals that exercised and those that were sedentary for two weeks following LPS or Saline administration. In an attempt to find a more appropriate protocol, a protocol with slightly altered parameters was used in the second attempt to understand the relationship between exercise and cognition following a short bout of inflammation. Following the last night of recovery, training occurred. The training session was a total of 182 s, but it was broken down into a 120 s acclimation period followed by a 2 s shock at 0.7mA, a 30 s inter-shock interval, and another 2 s shock at 0.7mA. Animals remained in the chamber for an additional 28s following the conclusion of the aversive stimulus, and were then returned to their home cage until testing. 48 hrs later, testing occurred as described above. These

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time points were chosen based on research showing that hippocampal BDNF levels remain significantly elevated for up to seven days following a twenty-eight day exercise protocol (Berchtold et al., 2005).

2.4 Tissue preparation and qRT-PCR

2.4.a Tissue extraction

Animals were rapidly euthanized by CO₂ inhalation. Tissue was collected by placing the whole brain into a RNase free stainless steel matrix and taking 1 mm slices of the hippocampus. Samples of the dorsal hippocampus were removed from the overlying parietal cortex and stored in RNAlater (Applied Biosystems, Foster City, CA). This process was conducted on a chilled glass plate to decrease the chance for RNA degradation. Samples were then stored at -20 °C until processing.

2.4.b RNA isolation and qRT-PCR

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR) is a fully quantitative method that is sensitive to relatively low levels of central cytokine expression. This assay was used in this study to assess the levels of IL-4 and BDNF present in the dorsal hippocampus. Total RNA was first isolated using RNeasy Micro Kits (Qiagen, Valencia, CA), and then quantified and assessed for purity, using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). The amount of specific mRNA present was quantified with a TaqMan™ probe and primer chemistry kit (Applied Biosystems, Foster City, CA) using a 7500 Real-Time PCR Thermal Cycling System (Applied Biosystems, Foster City, CA), and this amount was then compared to the levels of the endogenous control gene, beta-actin. The hippocampal

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samples collected from a single animal were run as triplicates for each gene of interest.

Target gene expression was then averaged and compared across the treatment conditions.

2.5 Tissue preparation and immunofluorescence

2.5.a Tissue collection

Following behavioral testing, animals were anesthetized with a mixture of ketamine (100mg/kg) and xylazine (5mg/kg). Once properly sedated, animals were given heparin and then transcardially perfused with 1X phosphate-buffered saline for 5 minutes (PBS; pH 7.4) followed by 7 minutes of a 4% paraformaldehyde solution (PFA; pH 7.4) to fix the brain tissue. Tissue was harvested and placed in a 4% PFA solution and kept at 2–8 degrees Celsius, until it was sectioned using a vibratome (Leica Biosystems, Buffalo Grove, IL).

2.5.b Immunofluorescence for analysis of microglial cell phenotype

Forty micrometer sections were placed in a 48-well plate containing 0.03% sodium azide and 1% PFA mixture. Before staining began, all sections were washed in deionized water (DI water) for a total of 30 min with three water changes to remove traces of sodium azide and PFA. Following the third wash, 1mL of sodium hydrobromide was added to each well and allowed to incubate at room temperature for 30 min. The sodium hydrobromide was then discarded and each sample was washed with 1X PBS, 3x 10min. A 1.5% donkey serum in PBST was used to block the sections overnight at 4°C. The next day, the primary antibodies, rabbit anti-Iba-1 (1:4000, Abcam, Cambridge, UK) and goat anti-Arg-1 (1:250, Santa Cruz Biotechnology, Santa Cruz, CA), were mixed with PBST and 500µl of the solution was added to each well and incubated overnight at 4°C. The primary antibodies were removed and discarded 24 h later and the sections were

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washed in PBST, 3 x 10min. Following the last wash, 500µl of peroxidase-conjugated AffiniPure Donkey anti-rabbit and Donkey anti-goat secondary antibodies (1:1000 in PBS) were added to each well and incubated overnight at 4°C. Donkey serum and secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). After 24 h the samples were washed in PBS for 45 min with multiple PBS changes and then mounted on slides. Iba-1 and Arg-1 positive cells were visualized with a Zeiss LSM 710 confocal microscope (Jena, Germany). Preliminary analysis revealed dense staining in the hilar region of the dorsal hippocampus; all microglial cells were then sampled from this area. From a 40x magnification, an 8.2x digital zoom was used to target individual microglial cells and then an image was taken. From the selected image, a mean fluorescence score was created using ZEN Digital Imaging for Light Microscopy (Zeiss, Jena, Germany) for both Iba-1 and Arg-1-positive cells. All cells examined were from the hilar region of the hippocampus. For each slice, a control fluorescence score was also created, and used to normalize all positive staining across animals. For each animal, twenty-four to thirty-two cells were used to create an overall mean fluorescence score per animal, which was then compared across treatment groups.

3.0 Results

3.1 Experiment 1: qRT-PCR

Two weeks of voluntary exercise does not alter IL-4 expression in the dorsal hippocampus following inflammation

A two-way analysis of variance (ANOVA) was used to assess whether two weeks of voluntary wheel running increased the amount of IL-4 present in the dorsal hippocampus. Analyses revealed no significant main effects for Condition (Exercise or

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Sedentary; $F(1, 29) = 1.150$, *NS*), Treatment (LPS or Saline; $F(1, 29) = 0.262$, *NS*), or the interaction of Condition X Treatment ($F(1, 29) = 0.369$, *NS*) (Figure 4). Overall, two weeks of exercise did not appear to alter the production of IL-4 regardless of treatment.

Two weeks of voluntary exercise has a modest impact on the level of BDNF in the dorsal hippocampus following inflammation

Using a two-way ANOVA to assess expression levels of BDNF in the hippocampus, no significant interaction effect between Condition (Exercise or Sedentary) and Treatment (LPS or Saline; $F(1, 29) = 0.502$, *NS*; Figure 4) was found following the two week recovery period. While there was no interaction effect, a marginally significant main effect for Condition was found ($F(1, 29) = 3.425$, $p = .07$), in which animals that were exposed to two weeks of voluntary exercise expressed significantly more BDNF than animals that had a sedentary recovery, regardless of Treatment (LPS or Saline). Additionally, no main effect for Treatment (LPS or Saline; $F(1, 29) = 0.721$, *NS*) was found, meaning that there was no significant difference in BDNF expression between animals given LPS or saline regardless of recovery condition.

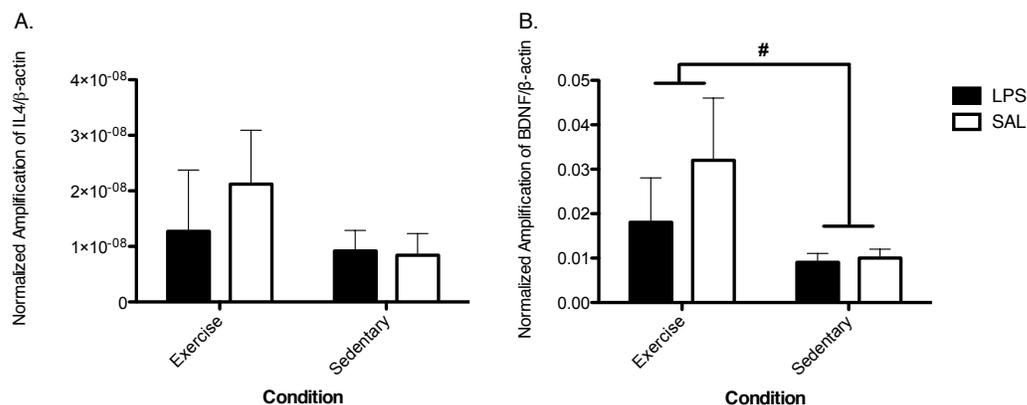


Figure 4. Two weeks of voluntary exercise results in no changes in IL-4 expression, but moderately elevated levels of BDNF expression within the dorsal hippocampus. (A) There were no statistical differences between groups in the expression level of IL-4 within the hippocampus. (B) Animals that had two weeks of exercise recovery show a marginally significant increase in BDNF expression compared to animals that had sedentary recovery ($p = .07$). Bars represent mean \pm SEM. SAL = Saline, LPS = Lipopolysaccharide.

3.2 Experiment 1A: Exercise and Cognition

No differences in the exercise volume between treatment groups after two weeks

To ensure that there were no differences in total exercise between animals administered LPS or Saline, a repeated measures ANOVA on total number of revolutions from individual wheels collected over a two week time period revealed no main effect of Treatment (LPS or Saline; $F(1, 16) = 0.944$, *NS*). A main effect for the number of revolutions by day was found ($F(13, 208) = 13.668$, $p < .0001$), suggesting that animals ran significantly more each successive night. No interaction effect between Treatment (LPS or Saline) and Day ($F(13, 208) = 0.429$, *NS*) was found indicating that administration of LPS did not alter the running patterns of the animals as compared to those of saline controls (Figure 5).

Two weeks of voluntary wheel running does not block LPS-induced cognitive deficits in a contextual fear paradigm

In order to determine whether two weeks of voluntary wheel running could restore cognitive function following 7 consecutive days of LPS exposure, freezing behavior in a CFC paradigm was assessed. A two-way ANOVA revealed that there were no significant differences in main effects for Condition (Exercise or Sedentary; $F(1, 31) = 0.001$, *NS*), Treatment (LPS or Saline; $F(1,31) = 2.188$, *NS*), or the interaction of Condition X Treatment ($F(1, 31) = 0.8$, *NS*) on the training day. Contrary to the proposed hypothesis, there were no significant differences in freezing behavior found for Condition (Exercise or Sedentary; $F(1, 31) = 0.034$, *NS*), Treatment (LPS or Saline; $F(1, 31) = 0.712$, *NS*), or the interaction between Condition and Treatment ($F(1, 31) = 0.193$, *NS*) on the

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testing day (Figure 5). Overall, all groups froze, suggesting that all groups learned the association between the context and aversive stimulus.

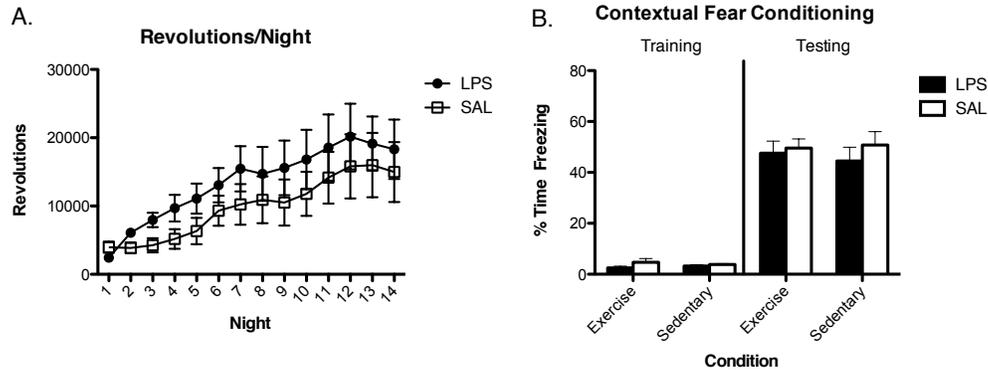


Figure 5. Two weeks of voluntary exercise following 7 consecutive days of LPS administration does not rescue cognition. (A) Repeated measures ANOVA revealed no significant difference in wheel rotations between animals that were administered Saline vs. LPS. (B) There were no significant differences in freezing behavior across all four groups. Bars represent mean \pm SEM. SAL = Saline, LPS = Lipopolysaccharide.

3.3 Experiment 1B: Exercise and Cognition

No differences in exercise volume between treatment groups after two weeks

As in Experiment 1A, a repeated measures ANOVA on total revolutions from individual wheels collected over a two week time period was conducted to ensure that there were no differences in the total amount of exercise between animals administered LPS or Saline prior to the start of cognitive testing. The analysis revealed no main effect of Treatment (LPS or Saline; $F(1, 18) = 0.027$, *NS*). As seen previously, a main effect for rotations by day was found ($F(13, 234) = 28.389$, $p < .0001$) and there was no interaction effect between Treatment and Day ($F(13, 234) = 0.485$, *NS*; Figure 6).

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Two weeks of voluntary wheel running enhances cognition in a varied contextual fear paradigm

Following the inconclusive results from Experiment 1A, a second attempt to understand the relationship between exercise and cognition following inflammation was conducted. Freezing behavior in a slightly varied CFC paradigm was assessed to examine for the final time whether two weeks of voluntary wheel running could rescue LPS-induced cognitive dysfunction. As in Experiment 1A, There were no difference between groups for Condition (Exercise or Sedentary; $F(1, 37) = 0.194, NS$), Treatment (LPS or Saline; $F(1, 37) = 0.809, NS$), or the interaction of Condition and Treatment ($F(1, 31) = 0.645, NS$) on the training day. Furthermore, The interaction effect between Condition (Exercise or Sedentary) and Treatment (LPS or Saline) on the day of testing was not significant ($F(1, 31) = 2.023, NS$). In contrast to the previous protocol, a significant main effect for Condition was found (Exercise or Sedentary; $F(1, 37) = 4.670, p = .03$), in which animals that exercised froze significantly more than sedentary animals regardless of previous exposure to either LPS or Saline. Additionally, there was not a main effect for Treatment (LPS or Saline; $F(1, 37) = 0.438, NS$; Figure 6). Overall, analyses show that exercise significantly increases freezing behavior, but the fact that LPS did not disrupt freezing behavior in the sedentary condition means that, contrary to the hypothesis, cognitive deficits do not persist 14 days after LPS exposure.

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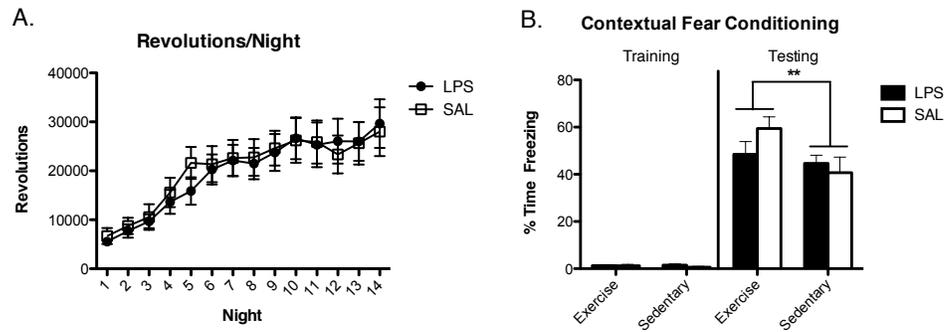


Figure 6. Two weeks of voluntary exercise following 7 consecutive days of LPS administration increases freezing in a Contextual Fear-Conditioning paradigm (A) Repeated measures ANOVA revealed no significant difference in wheel rotations between animals that were administered Saline vs. LPS. (B) Animals that exercised froze significantly more than animals that had two weeks of sedentary recovery. Bars represent mean \pm SEM. SAL = Saline, LPS = Lipopolysaccharide.

3.4 Experiment 2: Immunofluorescence

Two-weeks of voluntary exercise following immune challenge does not alter Iba-1 expression within microglial cells found in the hilar region of the hippocampus.

In order to understand the effects that LPS-administration and exercise have on microglial activation within the hilus of the hippocampus, the mean fluorescence scores for Iba-1 were analyzed, using a two-way analysis of covariance (ANCOVA). Treatment (LPS or Saline) and Condition (Exercise or Sedentary) were used as factors, with the mean fluorescence of the background staining used as a covariate. As seen in Figure 7, no significant differences were found for Condition (Exercise or Sedentary; $F(1, 12) = 1.96$, NS), Treatment (LPS or Saline; $F(1, 12) = 1.03$, NS), or the interaction of Condition and Treatment ($F(1, 12) = 0.619$, NS). As expected, neither exercise nor LPS-administration altered Iba-1 fluorescence.

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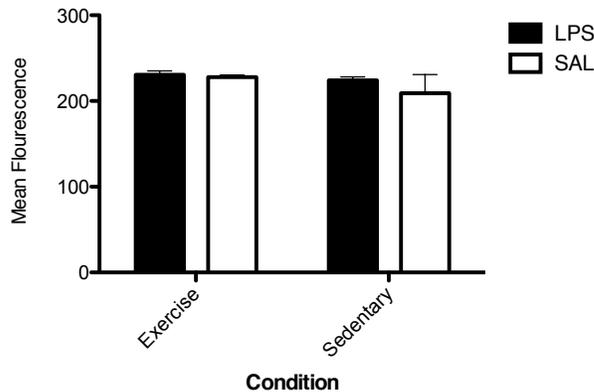


Figure 7. Two weeks of voluntary exercise following 7 consecutive days of LPS administration does not alter Iba-1 staining of microglial cells within the hilus. ANCOVA revealed no significant differences in the mean fluorescence of Iba-1 positive microglial cells across all four groups. Bars represent mean \pm SEM. SAL = Saline, LPS = Lipopolysaccharide.

Seven consecutive days of LPS administration followed by two-weeks of recovery leads to significant elevations in arginase expression within activated microglial cells found in the hilus of the hippocampus.

To investigate the roles that both exercise and LPS administration have on microglial phenotype, the mean fluorescence scores for Arg-1 were analyzed using a two-way analysis of covariance (ANCOVA). Treatment (LPS or Saline) and Condition (Exercise or Sedentary) were used as factors, with the mean fluorescence of the background staining used as a covariate. Unexpectedly, there was no interaction between Condition and Treatment ($F(1, 12) = 0.138$, NS), and no significant differences were found for Condition alone (Exercise or Sedentary; $F(1, 12) = 0.684$, NS). Although, analysis revealed a significant main effect for Treatment (LPS or Saline; $F(1, 12) = 9.366$, $p = .01$; Figure 8), in which animals that were administered seven consecutive days of LPS had significantly elevated fluorescence levels of Arg-1 compared to animals only administered Saline, regardless of Condition (Exercise or Sedentary). This is visualized in Figure 9.

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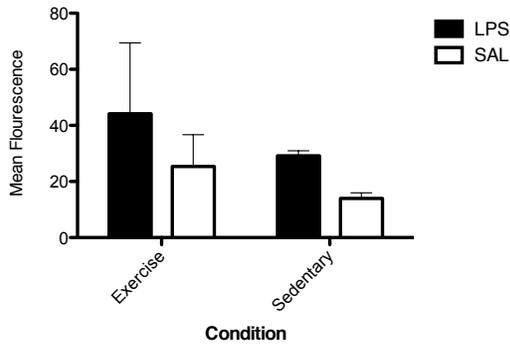


Figure 8. Two weeks of recovery following 7 consecutive days of LPS administration increases Arg-1 staining of microglial cells within the hilus. ANCOVA revealed a significant main effect of Treatment in the mean fluorescence of Iba-1 positive microglial cells across Conditions where animals administered LPS had increased Arginase expression ($p = .01$). Bars represent mean \pm SEM. SAL = Saline, LPS = Lipopolysaccharide.

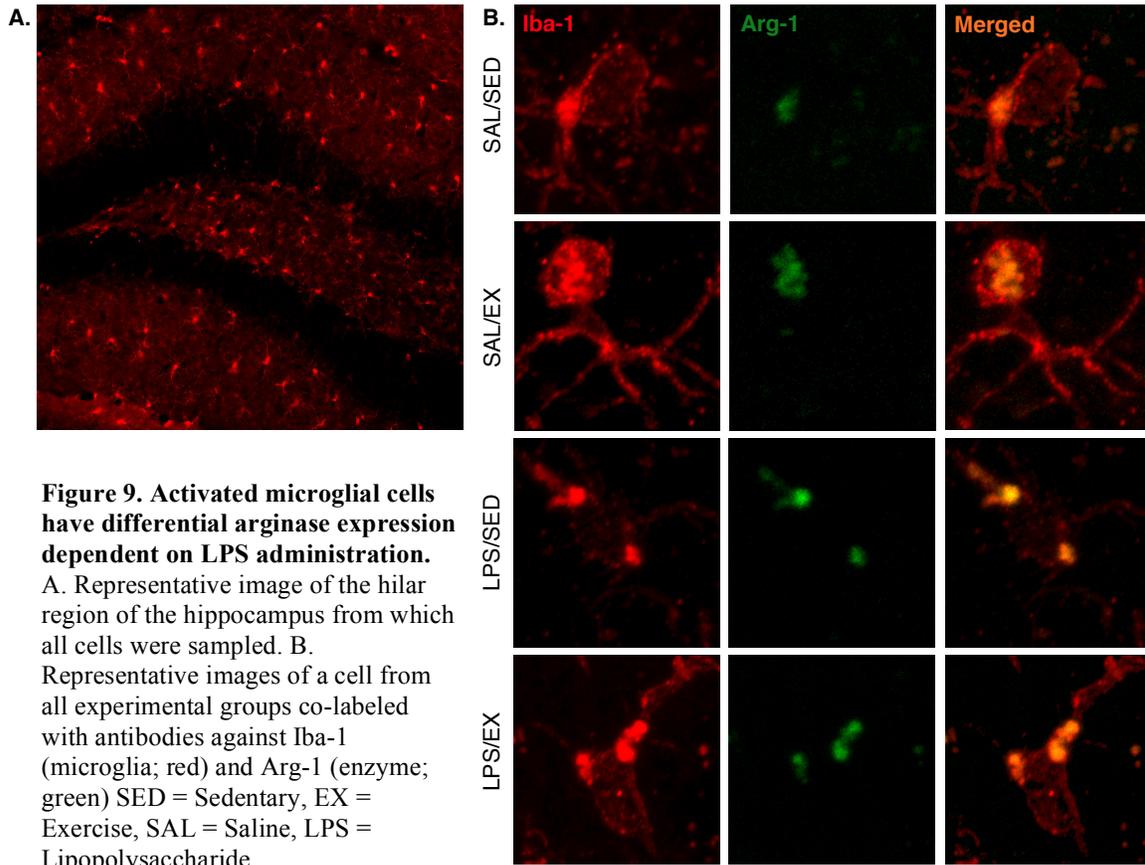


Figure 9. Activated microglial cells have differential arginase expression dependent on LPS administration. A. Representative image of the hilar region of the hippocampus from which all cells were sampled. B. Representative images of a cell from all experimental groups co-labeled with antibodies against Iba-1 (microglia; red) and Arg-1 (enzyme; green) SED = Sedentary, EX = Exercise, SAL = Saline, LPS = Lipopolysaccharide.

4.0 Discussion

The present studies tested the hypothesis that two weeks of voluntary exercise following inflammation would promote the differentiation of microglial cells towards a protective, M2a phenotype and attenuate LPS-induced cognitive deficits. We also hypothesized that this phenotypic switch would result from exercised-induced increases in IL-4 expression and the increased cognitive performance would be accompanied by increases in BDNF within the hippocampus of exercise animals. Our hypotheses were not fully supported. These studies demonstrated that, while exercise does lead to increased learning in a CFC paradigm, no cognitive deficits persist at the two-week post-final injection time point for exercise to attenuate. Furthermore, as expected, behavioral gains were accompanied by modest increases in BDNF, whereas we were unable to demonstrate exercise-dependent increases in IL-4 expression. Finally, the analysis of phenotypic markers for microglial cells did not confirm our hypothesis, as exercise had no effect on the fluorescence levels of arginase, while LPS-administration appeared to ubiquitously lead to increases in its expression over saline controls.

Exercise has a multilevel effect on the body. Although it can lead to initial immune suppression, exercise has been shown to inhibit the production of pro-inflammatory mediators, decrease soluble A β levels in AD transgenic animals, maintain cortical grey matter of patients with AD, increase neurogenesis, and improve overall cognition (Olsen et al., 2007; Nichol et al., 2008; Timmerman et al., 2008; Balducci et al., 2010; Colcombe et al., 2004; Kohman et al., 2012; Yuede et al., 2009; Burns et al., 2008; Farmer et al., 2004; Praag, Kempermann, & Gage, 1999).

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Barrientos and colleagues (2011) demonstrated that voluntary wheel running attenuates *E. coli*-induced cognitive deficits in aged rats, and thus provided support for the use of fear conditioning in this thesis. While our initial attempt to demonstrate rescued cognition proved futile as all animals learned the paradigm (Figure 5B), we hypothesized that the task was not sensitive enough to detect small learning differences between animals that exercised and those that were sedentary for two weeks following LPS or Saline administration. To rectify this, we developed a more difficult CFC paradigm. Following the last night of recovery, training occurred as previously prescribed. Unlike the original paradigm, however, testing occurred 48hrs later. It was hypothesized that this increased interval would more clearly define which animals demonstrated superior learning of the context, as previous research has shown that hippocampal BDNF levels remain significantly elevated for up to seven days following an extended exercise paradigm (Berchtold et al., 2005). Given this maintained availability of BDNF following exercise, and the importance that it plays in the consolidation of fear memories, we posited that the lengthy interval between testing would not impinge on the consolidation of training for exercised animals, but may highlight any consolidation deficits in animals given sedentary recovery. Our follow-up study revealed interesting results. In line with previous research and our expectations, animals that exercised froze significantly more than sedentary controls (Figure 6B). While our results show greater freezing behavior in exercise animals 48 hours after training, Kohman and colleagues (2012) demonstrated enhanced freezing behavior in a CFC paradigm with testing occurring 24 hours after training, but not 7 or 21 days post-training. The varying results between these two studies may be due to different paradigms. Kohman and colleagues

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(2012) instituted training following 30 days of wheel access or sedentary housing compared to the 14 days utilized here. Furthermore, their training protocol was enacted during the animal's dark phase, and consisted of 5 un-signalized footshocks (2 s, 0.70 mA) spaced 1 min apart. It is possible that had these procedures been employed in our study, an exercise effect may have been found 24 hours following cessation of wheel access. To our knowledge, this is the first time that the cognitive benefits of a moderately short duration of exercise have been extended beyond a typical 24 h consolidation interval.

Surprisingly, we were unable to demonstrate the presence of cognitive deficits within the sedentary condition despite previous research from this lab demonstrating elevated levels of A β two weeks following the end of LPS administration (Weintraub, 2014; Figure 1B). While this study was ongoing, our laboratory was able to demonstrate that 7 consecutive daily injections of LPS followed by two weeks of group-housed sedentary recovery leads to significantly elevated levels of A β with no corresponding deficits in our usual CFC paradigm (Figure 10). Given the results from both rounds of CFC presented here, in addition to work shown in Figure 10, it appears that the two-week time point used in this study was not an appropriate parameter at which to investigate whether exercise can overcome A β -induced cognitive impairments.

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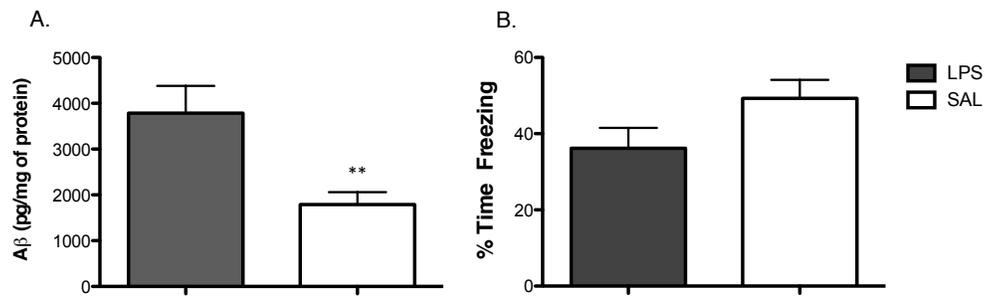


Figure 10. Two weeks of recovery following 7 consecutive days of LPS administration reveals elevated A β levels but no cognitive deficits in freezing in a CFC paradigm (A) ANOVA procedures revealed a significant difference in A β levels between animals that were administered SAL vs. LPS ($p = .01$). (B) No significant differences were found in freezing behavior between LPS or SAL animals. Bars represent mean \pm SEM. SAL = Saline, LPS = Lipopolysaccharide.

BDNF has repeatedly been shown to significantly increase following exercise (Berchtold et al., 2005; Wu et al., 2011; Barrientos et al., 2001). While we do demonstrate that the enhanced freezing behavior of exercise animals was accompanied by moderate increases in BDNF, these increases only reached marginal significance (Figure 4B). This inability to fully replicate documented increases in BDNF following exercise may be due to the exercise paradigm used here. For example, our animals were only given access to wheels during their dark phase for fourteen nights, while the Sprague-Dawley rats utilized by Berchtold and colleagues (2005) that showed elevations in BDNF protein levels, not mRNA as in our study, had continuous access to a wheel for the same fourteen day time period. Furthermore, Barrientos and colleagues (2011) demonstrated that *E. coli*-induced BDNF mRNA reductions are rescued in aged rats, but this was after a 6-week continuous access paradigm compared to our 14-night paradigm. Overall, it appears there is a necessary exercise threshold needed to engender consistent and stable increases in BDNF expression within the hippocampus that was not fully reached in these studies.

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In addition to the inquiry over whether exercise could attenuate the cognitive deficits seen following 7 days of LPS administration, we were interested in exploring whether the benefits seen following a two-week exercise paradigm could be attributed to changes in microglial phenotype. As previously mentioned, microglial cells are often divided into two broad categories, classical (M1; pro-inflammatory) and alternative activation (M2; repair) states, and Chhor and colleagues (2013) found LPS and IL-4 to be the best polarizing stimuli for the M1 and M2 states respectively. Research in adult male mice has shown that a single peripheral LPS injection can cause decreased mRNA expression of IL-4, which returns to baseline within 24 hours (Fenn, Henry, Huang, Dugan, & Godbout, 2012). This study failed to test how multiple LPS injections would effect IL-4 mRNA expression. Furthermore, they found that LPS administration leads to increased surface expression of IL-4R α and increased sensitivity to subsequent IL-4 stimulation. With these findings in mind, and given the link between exercise and IL-4 expression in human literature (Balducci et al., 2010; Della Gatta et al., 2014), in conjunction with the literature showing increased M2 phenotype markers in aged mice following exercise (Kohman et al., 2012), we hypothesized that the two-week recovery paradigm following 7 LPS injections would cause elevated IL-4 mRNA levels and altered microglial phenotype status for animals that exercised. Unfortunately, here we were unable to demonstrate the predicted significant increase in mRNA expression for IL-4 in the dorsal hippocampus following 7 days of LPS or Saline administration and a two-week exercise or sedentary recovery period (Figure 4A). It should be noted that samples were taken more than 24 hours after the cessation of exercise. Furthermore, expression levels of IL-4 were extremely low, and therefore came up in later cycles of the PCR

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quantification step. Either of these factors alone or in combination may attribute to the high variability seen in the exercise condition. Samples taken throughout or directly after the conclusion of the exercise paradigm may have provided a more accurate picture of how exercise was effecting central IL-4 expression. Additionally, it is possible that any exercised-induced production of IL-4 could have occurred in the periphery, as Balducci and colleagues (2010) only investigated IL-4 protein levels in plasma. To address this issue, serum samples from exercised animals, not administered LPS, could have been analyzed via ELISA for IL-4 protein levels.

We next sought to determine if exercise would impact the M2 microglial phenotype marker Arg-1 within cells found in the hilus. The hilus is one of the five major subdivisions of the hippocampus that hosts a number of subclasses of interneurons that play important roles in governing the excitability of granule cell neurons of the dentate (Mody, Otis, Bragin, Hsu, & Buzsaki, 1995). This area has also been shown to suffer significant neuron loss in a canine model of normal aging that can be rescued by a behavioral enrichment program that includes exercise and socialization (Siwak-Tapp, Head, Muggenburg, Milgram, & Cotman, 2008). Furthermore, research on CNS damage via ischemia and traumatic brain injury shows that hilar neurons are the first damaged, and large increases of activated microglial cells are found in this region following injury (Mody et al., 1995; Lowenstein, Thomas, Smith, & McIntosh, 1992). What the authors did not address was the microglial phenotype, which we argue are likely M2, Arg-1+ cells attempting to clean and repair the area. For these reasons, we limited our analysis of arginase expression to microglial cells found in the hilar region. We found that LPS administration followed by a two-week recovery period, regardless of exercise or

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sedentary, resulted in significantly elevated levels of Arg-1 expression, via mean fluorescence measurements, within Iba-1 positive microglial cells found in the hilus. At first glance, this would seem to be in opposition to the notion that LPS stimulation is associated with M1, inflammatory states, but Fenn and colleagues (2014) demonstrated that IL-4-dependent arginase induction was reliant on the microglial IL-4R α upregulation on microglial cells that occurs following inflammatory spinal cord injury in mice.

Although we did not show significant increases in central mRNA for IL-4, we cannot rule out the possibility that IL-4 proteins were produced peripherally, entered the CNS, and bound the IL-4R α on microglial cells as, IL-4 is produced by a range of cells in the periphery including, but not limited to, T cells, basophils, mast cells, eosinophils, and macrophages (Nelms et al., 1999). Specifically, cultured macrophages stimulated with LPS show rapid induction of TNF- α mRNA within as early as 1hr, with significant protein expression occurring after 4hrs of stimulation. Interestingly when stimulated with LPS for 48hours, the macrophages produced significantly elevated levels of both IL-4 proteins and mRNA (Mukherjee et al., 2009). LPS administered intranasally, utilizing ELISA procedures performed on broncho-aveolar lavage (BAL) fluid 1, 7, and 14 days later, also revealed that IL-4 protein levels were significantly elevated at the 7 and 14 day time points (Mukherjee et al., 2009). Furthermore, arginase activity has been linked to LPS stimulation as well. In a study conducted by Zhang and colleagues (2009), mice given peripheral injections of LPS showed increases in Arg-1 mRNA in the retina at 12, 16, and 24 hours post injection. These data, along with the data garnered in this thesis, support the idea that an immune challenge pushes both macrophages and microglia towards an M1 inflammatory response that is eventually resolved via self-induction of the M2

pathway (van Loon et al., 2013). Though the current findings in regard to arginase expression lend support to the existing literature on how microglial cells resolve LPS-induced inflammation (Orihuela, McPherson, & Harry, 2015), it remains possible that the timing of collection and length of exercise recovery were not optimal parameters with which to investigate how exercise effects M2 polarization status.

Increased inflammation and decreased phagocytic capabilities of microglial are a hallmark of Alzheimer like pathologies (Krabbe et al., 2013; Hickmn, Allison, & Houry, 2008). While oligomeric A β is a strong M1 producing stimulus in microglial cells (Michelucci et al., 2009), IL-4 treatment has been shown to increase A β uptake and degradation by M2 cells (Shimizu et al., 2008). Given that we know our LPS administration paradigm leads to elevated A β levels (Kahn et al., 2012), it is reasonable to assume that animals in the LPS condition did in fact demonstrate a M1 profile, but this could have been verified through sample analyses immediately after the last LPS injection. Furthermore, it is still plausible that exercise could have led to increased circulating IL-4 that mediated the central A β decrease seen previously in our laboratory after two weeks of voluntary exercise. To address this issue in the future, it will be important to quantify protein levels of IL-4 in the brain via ELISA, both immediately after the injection paradigm and the cessation of either exercise or sedentary recovery.

In summary, we have replicated findings that voluntary exercise increases cognitive capabilities over a more sedentary animals and that BDNF levels coincide these gains. Moreover, we provide evidence that microglial cells may be mediating the exercise-induced reduction in central A β following a bout of peripheral inflammation. However, this relationship between exercise and M2 microglia cannot be verified without

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the additional analysis of both serum and central tissue samples for IL-4 and arginase.

This work reinforces the need to explore how normal inflammatory processes have gone awry in the aging body and ways to target and dampen these actions in order to treat or even prevent neurodegenerative diseases like Alzheimer's. Without such interventions, Alzheimer's disease will continue on as one of the leading causes of death in America, without any hope to comfort those afflicted.

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EDUCATION

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EXPERIENCE

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ABSTRACT

EXERCISE AND MICROGLIAL CELL ACTIVATION

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Alzheimer's disease (AD) is characterized by the formation of amyloid-beta ($A\beta$) plaques and neurofibrillary tangles; inflammation has been implicated in this process. Our previous work resulted in an animal model of AD-like pathology using the bacterial endotoxin lipopolysaccharide (LPS). Following 7 consecutive injections of LPS, mice have significantly elevated levels of $A\beta$ with deficits in spatial cognition, but $A\beta$ levels are decreased following 2 weeks of voluntary exercise. We explored whether exercise could rescue cognitive function and alter microglial phenotypes to decrease $A\beta$ load. We found that at 2 weeks, there were no cognitive deficits present, but that exercise could enhance performance regardless of previous LPS or saline treatment. These gains were accompanied by modest increases in BDNF, whereas increases in IL-4 mRNA expression were not found. Exercise had no effect on the fluorescence of microglial phenotype marker, arginase, while LPS-administration appeared to increase its expression over saline controls.