THE ROLE OF EXERCISE IN THE ALLEVIATION OF CENTRAL ACCUMULATION OF AMYLOID-BETA AND PREVENTION OF COGNITIVE DYSFUNCTION FOLLOWING PERIPHERAL INFLAMMATION

by

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1. Introduction

Alzheimer's disease (AD) is a ubiquitous malady that affects all aging populations and defies effective treatments despite being defined clinicopathologically more than 100 years prior. As the most frequently diagnosed form of dementia, AD affects upwards of one billion people globally (Bergen 2008) and thus, places a prodigious emotional and economic burden on patients, care-givers and society (Ferri et al., 2005). Alzheimer's disease is characterized clinically by progressive memory loss and cognitive decline, and neuropathologically by intracellular neurofibrillary tangles (misfolded/truncated and/or hyperphosphorylated Tau protein) and the age-dependent extracellular deposition of β-amyloid (Aβ) protein, known as plaques (Terry 2006). Despite years of interdisciplinary efforts, the exact mechanism leading to AD has yet to be firmly elucidated. Significantly, however, Aβ plays a recurring role in most cascade hypotheses proposed to lead to this debilitating neurodegenerative disorder (Fodero-Tevoletti et al., 2011; Jakob-Roetne et al., 2009; Verdile et al., 2004; Haass and Selkoe 2007; Shankar et al., 2008).

Amyloid plaques are composed predominantly of the Aβ peptide, a 40 or 42 amino acid peptide generated by a sequential cleavage of the amyloid precursor protein (APP) by processing enzymes β- and γ-secretase (Hoe et al., 2012). While this process normally occurs at a controllable rate and is balanced by natural clearance mechanisms in the brain, the physiological role of APP is not well understood. In AD patients, an imbalance in this process occurs resulting in the pathophysiological hallmarks of the disorder that are purported to lead to

oxidative stress and ultimately neuronal death. Causality between the Aβ aggregation pathways and neurotoxic effects has been studied extensively, with concrete links to one another and the pathologies observed remaining elusive. There is, however, a correlation between increases in plaques and Aβ levels and decreases in cognitive function (Chen et al., 2000; Oddo et al., 2003; Rodriguez et al., 2008), and Increases in Aβ lead to reduced synaptic density, neuronal cell death, and cognitive decline (Fiali, Lalonde, and Rivest, 2009).

Recent research on neurodegenerative diseases, such as AD, has suggested a role for inflammatory processes in the etiology, progression (Minghetti 2005) and clinical manifestation of these diseases (Eikelenbloom et al., 1998). Chronic CNS inflammation is a common component of Parkinson's disease (PD) and AD. Many inflammatory mediators are centrally elevated in AD, including cytokines such as IL-1β, TNF-α and IL-6, all of which are seen in increased numbers shortly after an insult to the immune system and are indicative of ongoing inflammation (Engelhart et al., 2004). Cytokines are secreted by the immune system in response to an insult to the immune system, such as an infection (Dinarello 2000). Cytokines can be proinflammatory (IL-1β & TNF-α), anti-inflammatory (IL-10 & IL-4) or both (IL-6). They can be increased centrally a couple of ways: First, through production and secretion centrally by neural cells such as microglial cells. Alternatively, they can enter from the periphery through circumventricular organs (CVOs), which lack the normal blood-brain barrier (BBB), and/or active transport across the BBB (Konsman, Parnet, and Danzer, 2002).

The presence of Aβ peptide, which can be found in the insoluble extracellular plaque formations associated with AD (Heneka and O'Banion 2007), can induce the production of pro-inflammatory cytokines and chemokines by microglia (Schwab and McGeer 2008). Once inside the CNS, the cytokines lead to central Aβ production and increased BBB permeability, and, therefore, increased central inflammation allows molecules such as Aβ and additional inflammatory mediators to cross the BBB easier from the periphery (Banks, Kastin, and Broadwell, 1995). Thus, Aβ peptide, microglia, and pro-inflammatory cytokines can all lead to increased central inflammation that induces further production of Aβ creating a self-perpetuating cascade.

Animal models have been created to examine the relationship between inflammation and AD (Akiyama et al., 2000; Jaeger et al., 2009; Kahn et al., 2012) and the behavioral effects of the inflammation (Kahn et al., 2012; Lee et al., 2008; Thirumangalakudi et al., 2008). Jager et al., 2009 show that peripherally administered lipopolysaccharude (LPS) leads to elevated Aβ in the brain and that with these inflammatory alterations you get a decreased clearance of the Aβ from the CNS and an increased influx of Aβ from the periphery to the brain. Due to these findings, we have conjectured that the self-perpetuating central cascade may be a result of peripheral inflammatory inputs to the central nervous system (CNS). Previous work in our lab has resulted in a non-transgenic murine model of acute inflammation that produces Aβ peptide at detectable levels centrally. In this model an increase in the amount of Aβ in the hippocampus and cognitive deficits on hippocampus-dependent tasks have been

demonstrated following administration of the endotoxin lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria that reliably produces an immunological response (Kahn et al., 2012). LPS stimulates toll-like receptor 4 (TLR-4) and increases the production of inflammatory cytokines. LPS through its mechanism of action induces sickness behaviors in animals including loss of appetite and impaired learning and memory (Kahn et al., 2012).

At present, we have determined that seven consecutive days of a single, daily peripheral injection of LPS, produces an increase in central Aβ and leads to significant disruptions in hippocampus-dependent learning. In our attempts to link inflammation with AD-like pathology data was acquired that provides the impetus for this study. More specifically, in animals that received LPS and swam the Morris Water Maze (MWM) behavioral paradigm we saw a significantly reduced concentration of hippocampal Aβ compared to LPS treated control animals that did not swim the maze (Fig.1). This data suggested a possible role for exercise in the reduction of central Aβ, possibly through enhanced clearance, and reversal of cognitive decline in our acute inflammatory model. Interestingly, we do know that most of the central Aβ seen in our model comes from the periphery (Weintraub et al., 2013) so this may be an issue of enhanced clearance, but it also could involve decreased influx or possibly both.

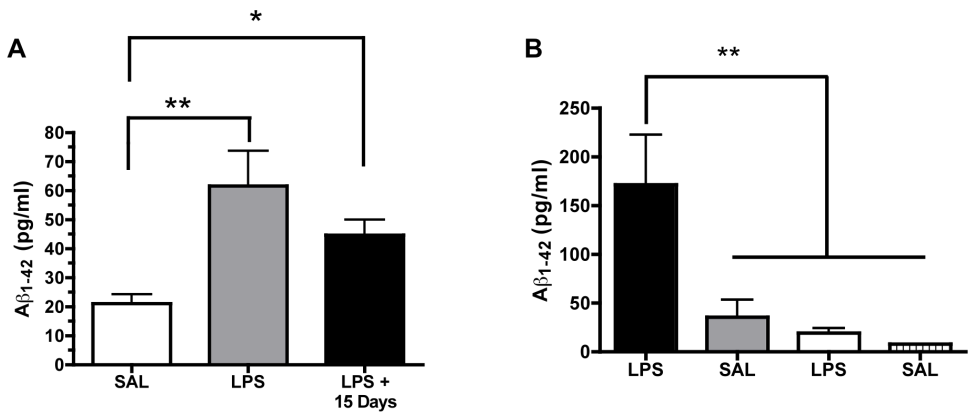

Figure 1. Previous work providing impetus for present study. **A 15 days of sedentary recovery after 7 injections.** 7 consecutive days of a single LPS injection significantly elevates $A\beta_{1-42}$ in the mouse hippocampus. 15 days following the last injection, $A\beta_{1-42}$ remains elevated but is significant to the $p<0.05$ level (not $p<0.01$). ** compared to saline control, $p<0.01$. * compared to saline control, $p<0.05$ (Kahn et al., 2012). **B Levels of $A\beta_{1-42}$ after participation in Morris water maze (MWM)**. After 7 days of LPS administration, we see a main effect of treatment on level of $A\beta_{1-42}$ in the hippocampus ($p<0.01$). We determined that 7 days of LPS administration resulted in significantly higher levels of Aβ as compared to saline controls. However, following participation in 9 days of MWM, Aβ levels returned to baseline .** compared to saline control, $p<0.01$ (unpublished data). Bars represent mean ± SEM.

The beneficial effects of exercise on human health are well documented (Blumenthal et al., 1999; Colcombe and Kramer, 2003; Dishman et al., 2006). However, epidemiological investigations of exercise in patients with neurodegenerative dementing disorders, including AD, have been contradictory. Laurin et al. (2001) and Rovio et al. (2005) reported an inverse association between physical activity and cognitive decline, while other researchers failed to find a relationship (Broe et al., 1998; Verghese et al., 2003). More recently, voluntary exercise has been demonstrated to reduce cognitive decline and CNS plaque load in a transgenic mouse model of AD (Adlard et al., 2005), but not in another (Wolf et al., 2006). In two studies, utilizing the Tg2576 transgenic AD mouse model, exercise was used in an attempt to alleviate AD-like pathology (Nichol et al., 2007; Yuede et al., 2009). Yeude et al. (2009) investigated whether 16 weeks of forced exercise (treadmill running) or voluntary exercise (running

wheels) differentially affected Aβ plaque load, hippocampal volume, and behavior. While, both voluntary and forced exercise resulted in larger hippocampal tissue volumes when compared to sedentary controls, only animals in the voluntary running group showed a reduction in soluble Aβ levels in the brain tissue and improved performance in the novel object behavioral paradigm. The authors propose that forced exercise may limit the positive outcomes from the physical activity, possibly due to stress. Lending credence to this conclusion is a study demonstrating that forced exercise elevates corticosterone levels (Hoffman-Goetz et al., 2008), which can lead to neuronal death in the brain, most prevalently in the hippocampus (McEwen et al., 1968). In a study of voluntary running, Nichol et al. (2007), found that three weeks of running rescues water radial arm maze behavioral deficits in aged Tg2576 mice. Interestingly, the Tg2576 mice given the opportunity to voluntarily exercise performed as well as the wild-type animals in a water radial arm maze. Due to the inconsistent results seen to date, there is interest in determining whether physical activity can slow progression of neurodegenerative diseases, such as AD.

Additionally, regular physical exercise has been shown to decrease inflammation without pharmaceutical intervention (Cho et al., 2003; Cotman and Berchtold, 2002; Cotman et al., 2007).

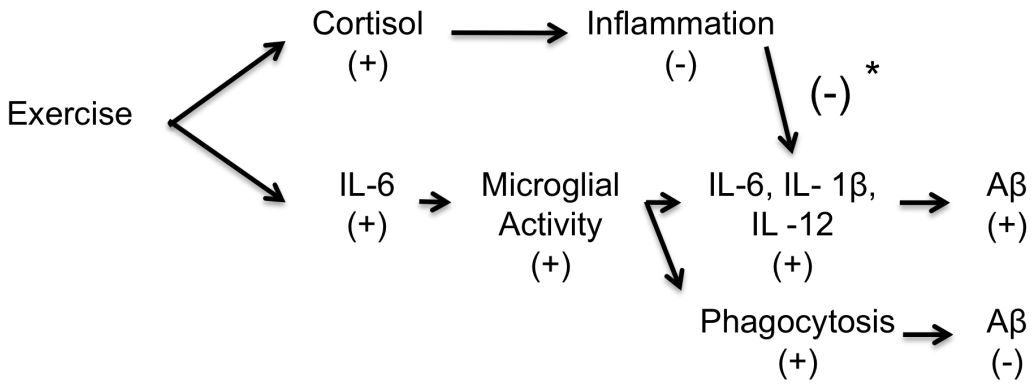

Figure 2. Flow chart detailing a proposed mechanism of exercises beneficial effects on Aβ production. * indicates point that cortisol reduces the inflammatory response by reducing the effectiveness of cytokines, thus increasing the threshold required for them to have an effect leading to decreased Aβ production and increased clearance.

Previous studies have shown increased performance on behavioral tasks such as radial arm maze (Schweitzer et al., 2006) and MWM (Vaynman et al., 2004) when animals were allowed the opportunity for regular exercise after induced inflammation. The change in performance has been proposed to be due to exercise-induced long-term potentiation (LTP) in the dentate gyrus of the hippocampus (Famern et al., 2004). Others speculate that exercise may enhance the availability of neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) (Berchtold et al., 2005) and insulin-like growth factor-1 (IGF-1) (Trejo et al., 2001), which serve to enhance plasticity within the brain. Furthermore, previously conducted rodent studies have shown that increases in synapse density and hippocampal neurogenesis are observed post-physical exercise (Kohman et al., 2011; Wu et al., 2008; Wu et al., 2007).

In a study of the effects of peripheral inflammation on hippocampal neurogenesis and learning, Wu et al. (2007), found that following 5 weeks of treadmill running, murine subjects displayed an increase in neural precursor cell

proliferation in addition to increased behavioral performance. Intriguingly, Wu et al. (2007) further uncovered an increase in BDNF and it's receptor, tyrosine receptor kinase B (TrkB), after physical exercise, both of which are normally reduced after LPS administration. BDNF has been implicated in multiple neurophysiological processes, including neurogenesis, synaptogenesis, and cell survival (Mattson et al., 2004). Additionally, in a study examining sickness and physical exercise interaction, six weeks of voluntary running reduced the memory impairments from *E. coli* infection in rats. Interestingly, hippocampal BDNF levels were significantly increased with exercise as compared to sedentary (locked wheel) animals (Barrientos et al., 2011). Analogous findings are reported in human studies. For instance, BDNF expression was significantly decreased in donated hippocampus samples from postmortem AD patients, as compared to control samples (Connor et al., 1997). Interestingly, some studies have shown that the presence of Aβ, even without the prototypical plaque formation seen in AD, can lead to alterations at the cellular level, including a reduction in brain-derived neurotrophic factor (BDNF) signaling in neuronal cultures (Tong et al., 2006), inflammation that leads to reduced BDNF expression (Tanaka et al., 2006), and deficiencies in learning and hippocampal neurogenesis (Shaftel et al., 2007). In fact, a single injection of Aβ into the hippocampus led to decreased BDNF expression, lower 5-HT_{2A} receptor levels, and cognitive impairment (Christensen et al., 2007). Thus, induction of BDNF expression may be a possible mechanism to attenuate cognitive deficits related to AD-like pathology.

Another possible mechanism by which exercise may exert positive effects

on AD-like pathology involves the enhanced production of the cytokine interleuken-6 (IL-6) that results. APP transgenic mice display decreased amyloid production and rescued cognitive function when forced overexpression of IL-6 is induced (Chakrabarty et al., 2010). Intriguingly, the increased levels of IL-6 were accompanied by an enhancement of microglia-mediated phagocytosis of Aβ peptide, suggesting that IL-6 may actually be beneficial early in AD disease progression, by enhancing clearance of Aβ. Exercise induces production of IL-6 by working muscle tissue naturally, without forced overexpression (Pedersen 2011; Woods et al., 2009). In a human study that probed the effect of exercise on inflammatory mediators, researchers determined that participants in the exercise group, along with the IL-6 treated group, displayed significantly less tumor necrosis factor alpha (TNF-α) than the sedentary control group, following an injection of LPS (Starkie et al., 2003). Thus, a feasible benefit of exercise is a transient increase in IL-6 that may act to suppress the release of proinflammatory mediators such as TNF- α and aid in the clearance of Aβ through microglial activation.

Although Alzheimer's disease (AD) has been established as a serious neurodegenerative disorder since 1906, the need for an effective therapeutic intervention still remains as currently there are no disease-ameliorating drugs, and existing therapies only offer unpredictable, short-term symptomatic relief (Drew and Barnham 2011). The purpose of this study was to determine the effects of voluntary exercise on the AD-like symptomology of our non-transgenic inflammatory model of AD. We hypothesized that voluntary physical exercise will

decrease the accumulation of Aβ within the hippocampus and attenuate LPS-induced cognitive deficits. We also hypothesized that voluntary exercise will lead to increased microglial activation and increased BDNF levels.

2. Methods

2.1 Subjects and Care

Male 3 – 6 month old C57BL/6J mice were utilized in all experiments. 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 two or three in standard cages (12.5cm x 15cm x 25cm). All experimental groups and control groups were on the same light schedule, lights on at 0600 and lights off at 1800, and both food and water were available *ad libitum*.

2.2 Lipopolysaccharide (LPS)

Peripheral injections of LPS were used to increase Aβ and induce cognitive deficits in the C57BL/6J mouse, as observed in previous experiments (Kahn et al., 2012; Lee et al., 2008). A single injection of LPS or saline was given daily for seven consecutive days prior to exercise or sedentary conditions. The LPS strain used was derived from *Escherichia Coli* (Serotype: 055:B5; Sigma-

Aldrich, St. Louis, MO). In all experiments, LPS was injected intraperitoneally (i.p.) at a dose of 250 µg/kg.

2.3 Tissue preparation

Upon completion of study parameters, mice were euthanized in accordance with IACUC-approved methods, and hippocampal tissue was extracted and prepared for protein assay and ELISA procedures, after being placed in a -80°C freezer for 24h. The tissues were subsequently homogenized with a protein extraction solution (PRO-PREP, Boca Scientific, Boca Raton, FL.) containing protease inhibitors, and were then further lysed for 30 minutes on ice. The lysate was centrifuged at 15,000 rpm for 30 minutes and the clear lysate was removed for *DC* Protein Assay (Bio-Rad Laboratories, Hercules, CA.).

2.4 DC protein assay

The DC protein assay utilizes a working reagent that is used with detergent-based buffers. To prepare the protein standard curve, dilutions ranging from 0.2 mg/ml – 1.5 mg/ml of gamma-globulin were used, and made in the same buffer as in the lysates. Standards (5µl) and samples (5µl) were pipetted into the 96 well plate with 25µl of reagent A' and 200 µl of reagent B. After 15 minutes, the plate was placed in the plate reader (BMG LabTech FLUOstar Omega, Cary, NC) and the optical density of each sample was read at 750nm. The protein assay results were used to normalize protein content for the ELISA.

2.5 Aβ ELISA procedure

The BetaMark $A\beta_{x-42}$ ELISA (Covance Research Products, Dedham, MA) is a 2-day procedure utilizing a 96-well antibody coated plate. Both experimental samples (soluble) and standards of known concentrations are placed into the wells to be measured. The protocol for this ELISA requires a preparation of working incubation buffer, 1X wash buffer and standard intermediates be made prior to the start of the assay. Next, the Standard Diluent is used to reconstitute the Aβ standard and to prepare the standard curve. The samples were then diluted 2:1 with working incubation buffer, which includes the HRP-labeled detection antibody. Each dilution of the standard curve (100 µl) was pipetted in duplicate, and 100 µl of each unknown sample in duplicate was added to the plate and incubated overnight at 2–8° C. On the subsequent day, the wells were washed 5 times with the 1X wash buffer. After the washes, 200 µl of TMB, a substrate for the HRP enzyme, was added to each well. The plate was then incubated in the dark for 45 minutes at room temperature and read at the optical density of 620nm in the plate reader (BMG LabTech FLUOstar Omega, Cary, NC).

2.6 Statistical analysis Aβ levels

For all experiments, Aβ levels from the ELISA were analyzed using analysis of variance (ANOVA). In these experiments Fisher's PLSD post-hocs were used to determine differences if a significant omnibus F value was found.

2.7 Contextual fear conditioning (CFC) protocol

Mouse freezing behavior during the CFC behavioral paradigm was monitored using the FreezeFrame™ software (ActiMetrics Software, Wilmette, IL). The system (Coulbourn Instruments, Whitehall, PA, 7Wx7Dx12H) utilizes an electrified grid floor through which the aversive stimulus was delivered (Fig. 2).

Figure 3. Contextual fear conditioning (CFC) unit. Visual cues (black dots) on wall and olfactory cue (peppermint) container seen under bars.

Our protocol began 24 hours after the 7th day of the exercise or sedentary condition, and consisted of a training session (day 1) and a testing session 24 hours later (day 2). The training session had a 120-second acclimation period, followed by a single 2-second 0.7mA shock. Animals then remained in the apparatus for 60 seconds. On the testing day, no shocks were delivered, though the system recorded the movement of the animal for 90 seconds. Freezing behavior was monitored, and total freezing time (sec) was collected as the dependent variable.

Previous experiments conducted at TCU have revealed that the incorporation of an olfactory contextual cue (peppermint oil 1:10 in water) and a wall design (black polka dots) increases the freezing time suggesting an increase in the learning of the context-shock pairing (Fig.2)(Kahn et al., 2012; Kranjac et

al., 2012). The time freezing was analyzed using analysis of variance (ANOVA) procedures (Statview 5.0, SAS, Cary, NC), in which Group (LPS/SAL) and Treatment (EX/SED) were independent variables. The alpha level used for all statistical analyses was 0.05. Any significant main effects and interactions were further analyzed using Fisher's PLSD post-hocs.

2.8 Novel object protocol

For the novel object behavioral paradigm a three arm maze or Y maze was utilized. Maze walls were 0.16 m high and constructed of 1.6 cm-thick wood panels, painted white and sealed, and the entire apparatus was placed on a table 0.67 m high. Each arm was identical in length (0.32 m).

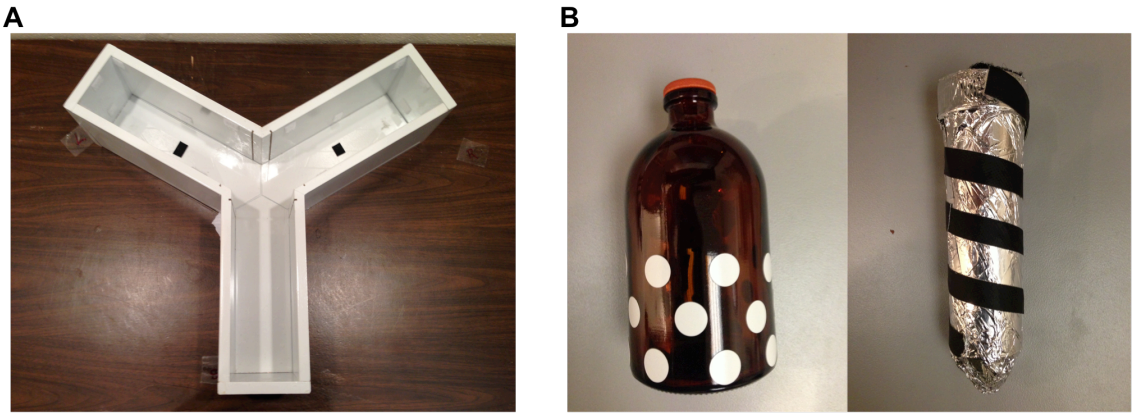

Figure 4. Novel object apparatus and objects. **A Three arm maze. B Objects.** 100 mL bottle and foil wrapped and stripped 50 mL conical tube.

The starting arm remained the same for all subjects. The walls of the two remaining arms were covered in black vertical stripes or small black dots (both on white background) to provide visual cues. These visual cues were alternated between animals such that half received one orientation and half received the

opposite wall orientation. Mice were first allowed to explore the environment (A three arm maze) for 5 min on day 1 with no objects present to acclimate to the apparatus. On day 2, animals were placed in the Y maze with two identical objects (familiar object) for 3 min, for training, and then they were returned to their home cage. Four hours later animals were placed back into the Y maze with one familiar object (from training) and one novel object (target object) for the novel object recognition test. The fraction of time spent exploring the novel object of total time spent exploring both objects was used for statistical analysis. Two objects used were a foil wrapped and stripped 50 mL conical tube and a 100 mL brown glass bottle with large white dots (Fig 3). The objects were alternated such that the novel object for half of the animals was the conical tube and the bottle for half of the animals.

2.9 Voluntary Exercise: Wheel Running

Animals were housed in pairs to allow for maximum running wheel accessibility. Wireless running wheels (ENV-044), wireless running wheel USB interface hub (DIG-804) and wheel manager software (SOF-860) were all obtained from med associates inc. Injections of 250μg/kg LPS (*Escherichia coli* serotype: 055:B5; Sigma-Aldrich, St. Louis, MO) or saline were administered once a day for seven consecutive days. Animals were placed in one of four different groups (Figure 1), exercise groups that received LPS or saline (LPS EX, saline EX), and sedentary groups that received LPS or saline (LPS SED, saline SED). Animals in LPS EX and saline EX groups had low profile running wheels

placed into their home cages for 7 days following the 7th injection of LPS or saline. Animals in the LPS SED and saline SED groups remained in the cages but no running wheel was accessible. After the condition (exercise/sedentary) week, animals completed the CFC paradigm, or in a subsequent identical study Novel object paradigm, followed by hippocampus removal, protein assay and ELISA procedures, to determine effects of exercise on Aβ accumulation. In an additional follow up and identically designed experiment, after completion of the injection and exercise portions, animals either had their brains fixed by transcardial perfusion and used to analyze microglial activation state (future studies). A second group of animals were euthanized via CO_2 inhalation and their brains were placed into a stainless steel matrix. Sections, 1 mm thick, were obtained and flushed with nuclease-free PBS, and, using an RNase-free sample corer, hippocampal tissue punches were obtained to determine BDNF mRNA expression by qtPCR.

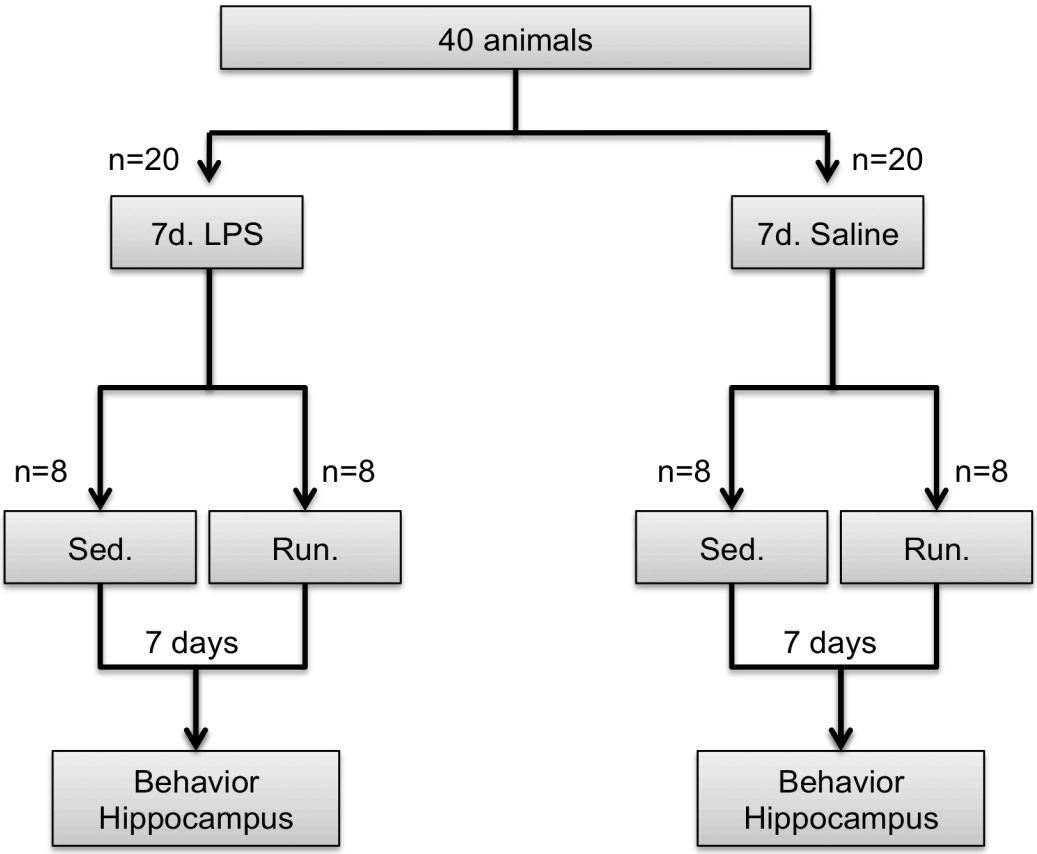

Figure 5. Organizational flow chart for the exercise study; methodology and treatment.

2.10 RNAi and qtPCR

Mice were rapidly euthanized via CO_2 inhalation, and the brains were extracted and placed into a stainless steel matrix. Coronal sections (1 mm) were cut and bathed with nuclease-free PBS. Utilizing an RNase-free sample corer, brain tissue punches were obtained, rinsed, placed in a nuclease-free tube containing RNAlater® (Ambion, Austin, TX), and kept at 4°C until use.

Hippocampal RNA was isolated (RNeasy Micro kits, Qiagen, Valencia, CA), and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, NanoDrop products, Wilmington, DE). Quantitative reverse transcription polymerase chain reaction (qRT-PCR) was utilized, 7500 Real-Time

PCR Thermal Cycling System (Applied Biosystems, Foster City, CA), to assess the BDNF mRNA concentration present in the hippocampus at the time of tissue collection. Initial mRNA concentration was determined by using PrimeTime™ qPCR primers (Integrated DNA Technologies, Coralville, IA), and this amount was normalized to β-actin, an endogenous control gene. To avoid amplification of genomic DNA, the PrimeTime™ primers used were designed to span the intron splice junction between BDNF exon locations 2–5 and β-actin exon locations 5–6. Each hippocampal sample collected was run as separate triplicates for each gene. For the qRT-PCR data, gene expression was calculated by normalizing the amplification rate of the target gene's expression against the amplification rate of the endogenous control gene, β-actin, using the DART-PCR method (Pierson et al., 2003). As in our previous study (Kahn et al., 2012), we chose to use DART over other commonly used methods of analysis, because DART does not assume a perfect amplification efficiency for all cycles. Instead, it calculates the actual amplification efficiency from cycle to cycle using the amplification kinetics of each sample. Additionally, Pierson et al. (2003) have compared the accuracy of DART to the more commonly used $2^{-\Delta\Delta CT}$, and found DART to be superior.

3. Results

3.1 Appearance and weight loss following LPS administration

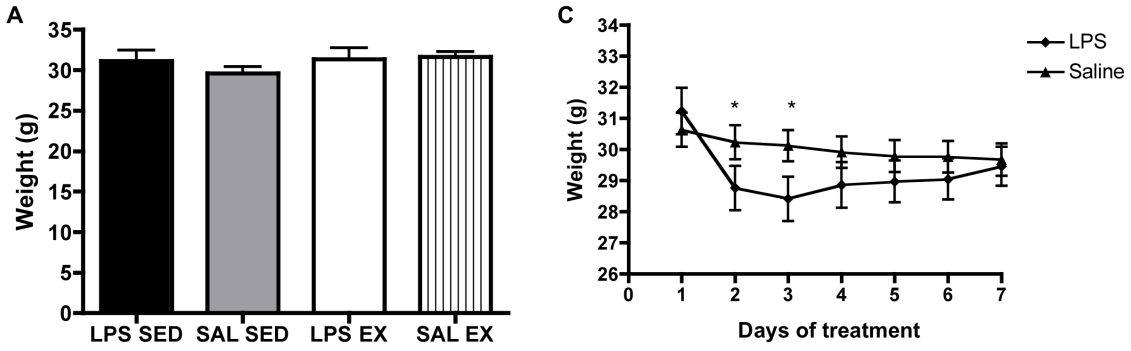

Figure 6. Weights and weight loss. **A Initial animal weights**. No difference in initial weight was observed on day 1 of injections ($p>0.05$). **B Overall weight loss between treatment groups.** As expected, a significant main effect of treatment ($p < .05$) was found, showing a small but significant drop in body weight for LPS-injected animals, as compared to saline-injected animals. **C Repeated measures ANOVA**. On day 2 a significant difference in animal weight is seen ($p<0.05$) and again on day 3 ($p<0.05$), indicating that animals receiving LPS are sick. Beginning on day 4, LPS animals are no longer losing weight and are starting to gain weight. The lack of continued weight loss while still receiving LPS indicates that animals receiving LPS have developed endotoxin tolerance because they are no longer displaying sickness behaviors, such as abstaining from food, and they are recovering initial weight loss. Bars represent mean ± SEM.

Mouse weights were measured daily during IP administration of treatment (LPS or sterile saline). Mice that received seven consecutive daily injections of LPS displayed sickness-related symptoms such as weight loss, lethargy, piloerection and reduced grooming that continued until day 4, after which weight loss abated and reversed. On day 1, saline and LPS groups showed no significant difference in starting weight ($F_{(1,36)}=0.427$, ns). A significant main effect of treatment ($F_{(1,36)}=5.111$, $p < .05$) was found, showing a small but significant drop in body weight for LPS-injected animals, as compared to saline-injected animals (LPS: 1.775 ± 0.233 g; saline: 0.960 ± 0.280 g) overall after 7

days. Animals receiving LPS should be gaining weight back toward the end of the injection protocol (Kahn et al., 2012), suggesting that the animals are becoming tolerant to the endotoxin and are no longer sick by the 7th day of injections. We can confirm endotoxin tolerance on day 4 when there no longer exists a significant difference in weight between LPS and saline treatment groups ($F_{(1,36)}$=0.887, ns).

3.2 Aβ volumes after voluntary exercise

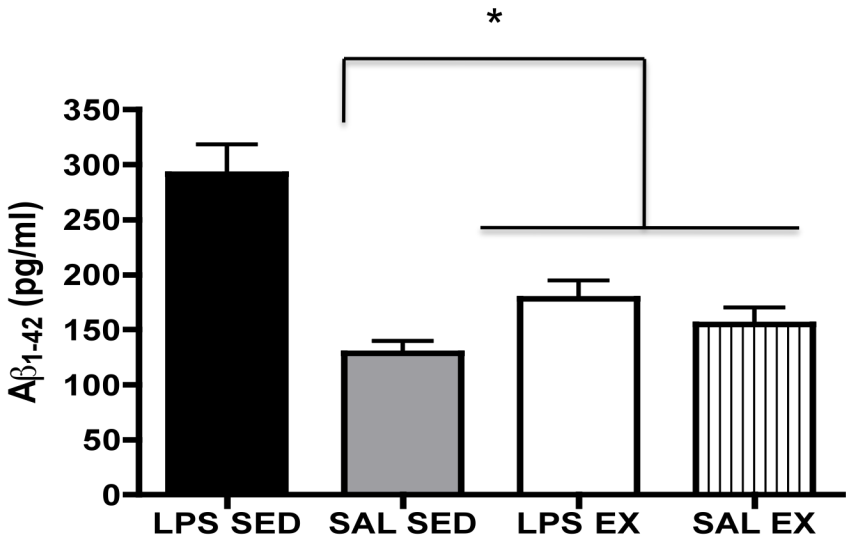

Figure 7. LPS-induced $A\beta_{(1-42)}$ production and exercise induced clearance. Previous work has shown that 7 consecutive days of LPS injection significantly elevates $A\beta_{(1-42)}$ in the mouse hippocampus and that this elevation decreases, but remains significant after 15 days (Kahn et al., 2012). Animals allowed access to a running wheel for 7 days after receiving LPS (LPS EX) have increased $A\beta_{(1-42)}$ clearance and measured levels were not different from that of saline control. Mice without a running wheel and given LPS (LPS SED) have an $A\beta_{(1-42)}$ level that remained significantly elevated. * compared to all other groups, $p<0.05$. Bars represent mean ±SEM.

Four groups of animals were administered (i.p.) a single dose of LPS or saline for 7 consecutive days. Subsequently, half of the subjects were given

access to a running wheel (exercise groups) and half were left unenriched in their home cage (sedentary groups) for an additional 7 consecutive days. Twelve hours after the final day (day 7) of wheel running, the mice were euthanized via CO_2 inhalation, and hippocampal tissue samples were extracted and lysed for Aβ concentration measurement. Our results indicated that mice receiving LPS but not given access to exercise had significantly more Aβ in the hippocampus as compared to all other groups ($F_{(1,33)}$ = 14.915, p = 0.0005). Importantly, no difference was discerned between the remaining three groups.

3.3 Running wheel counts

Table 1. Wheel Running. Descriptive statistics on the daily average of revolutions and Aβ for each group. Pearson correlation and significance values showing the correlation between the amount of Aβ and amount exercised (revolutions).

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	Mean Revolutions ± Std. Dev. (N)	Pearson Correlation (Sig. 2-tailed)	Aβ ± Std. Dev. (N)
Saline	12163.93 ± 7141.6 (9)	-0.791 (p=0.011)	136.4985 ± 35.22526 (18)
LPS	9349.11 ± 5230.51 (10)	0.492 (p=0.148)	237.5151 ± 85.46540 (19)

Revolutions were recorded to see if a relationship exists between the Aβ level in the hippocampus and the number of revolutions (amount exercised). No difference in wheel revolutions (exercising) between LPS and saline exercise groups was discerned ($F_{(1,36)}$=2.482, ns) (Table 1). Interestingly, however, we found a significant inverse correlation between Aβ and revolutions with saline animals, r(7) = -0.791, p = 0.011, but not with LPS animals r(8) = 0.491, ns.

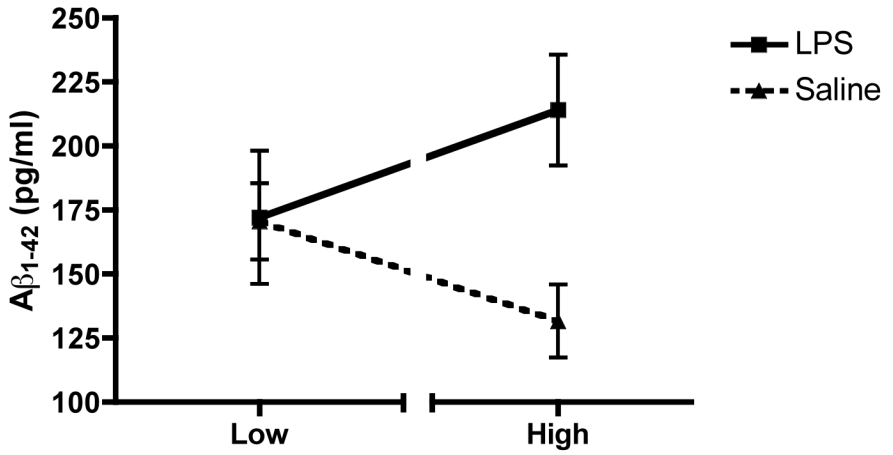

Figure 8. Median split ANOVA. Data split by median revolutions into low and high revolution groups. Animals receiving LPS or saline and exercising at a lower rate show no difference in Aβ. Animals receiving LPS and exercising more than the median revolutions (10,040 rev/24h) show a non-significant trend toward higher Aβ. Animals receiving saline and exercising more than the median revolutions show a non-significant trend toward lower Aβ. Bars represent mean ± SEM.

A median split ANOVA splits the subjects within their treatment group (LPS or saline) based on whether the mice exercised more or less than the statistical median number of wheel revolutions per 24 hours (10,040.13 rev/24h). For example, animals receiving LPS and registering a wheel revolution count per 24 hours below the median of 10,040.13 rev/24h were placed in the LPS-low revolution subgroup for additional analysis. When performing a median split ANOVA (low v. high revolutions), some trends began to emerge. First, there appears to be a trending difference in Aβ level between LPS and saline animals ($F_{(1,18)}$=3.471, p=0.082). Second, when looking at the split, there appears to be no difference in Aβ concentration between groups in the low revolution group (animals in cages that registered less than the median revolutions, 10,040.13 wheel revolutions/ 24h), while a trend surfaces when looking at the high revolution split. LPS animals with high revolutions are trending towards higher

levels of Aβ, where as saline animals with high revolutions are trending towards lower levels of Aβ ($F_{(1,18)}$=3.214, p=0.093).

3.4 BDNF

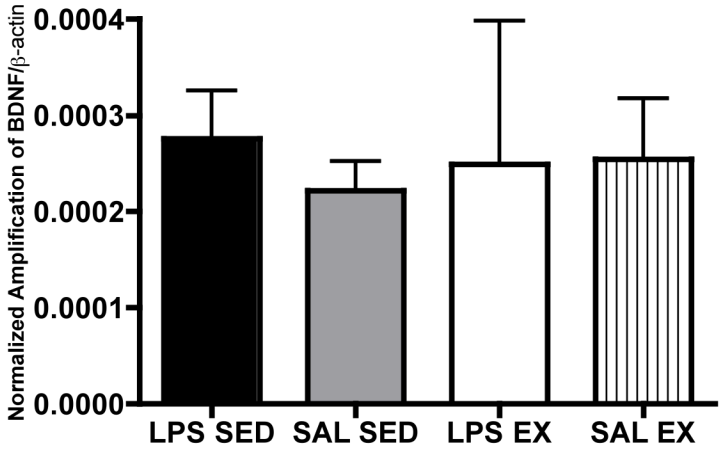

Figure 9. BDNF mRNA levels. Peripheral LPS administration followed by 7 days of exercise or sedentary conditions show no effect on hippocampal BDNF mRNA levels compared to saline controls (p>0.05) . Bars represent mean ± SEM.

We had originally hypothesized that exercise would eliminate the build-up of Aβ in the hippocampus, and that this decrease in Aβ could lead to the recovery of cognition as we have previously shown (Weintraub et al., 2013). Therefore, we assessed the level of BDNF in the hippocampus following the completion of the study to account for the possibility that exercise might elevate BDNF, which might also account for recovery of cognitive function. However, administration of LPS or saline followed by either the exercise or sedentary condition demonstrated no significant differences in BDNF mRNA in the hippocampus ($F_{(1,10)}$ = 0.134, ns).

3.5 CFC

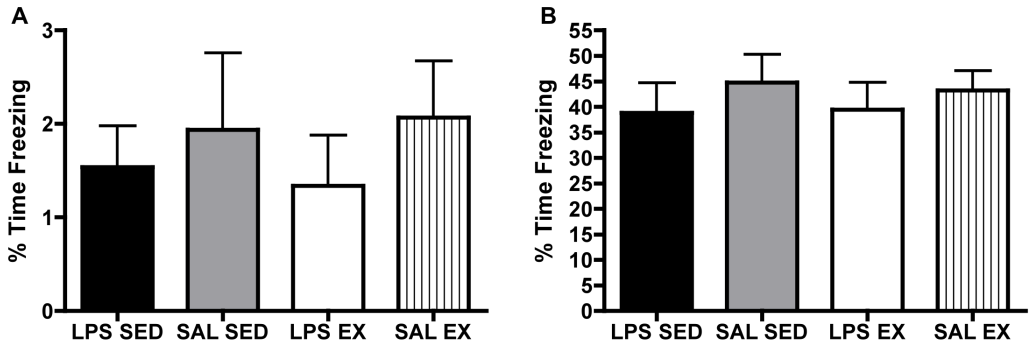

Figure 10. Contextual fear conditioning (CFC). **A CFC training**. No difference between groups was seen when data from initial training was analyzed using an ANOVA ($p>0.05$). **B CFC Testing.** Analyzed testing data revealed no cognitive differences between groups. All groups showed ability to learn the task, freezing >30% of the time ($p>0.05$). Bars represent mean ± SEM.

In order to assess whether cognitive differences exist between treatment and condition groups in a hippocampus-dependent task, we used a contextual fear condition behavioral paradigm. As expected, no significant differences in percent time freezing were found on training day between our groups for treatment (LPS or saline)($F_{(1,42)} = .840$, ns), condition (exercise or sedentary)($F_{(1,42)} = 0.004$, ns), or the interaction (treatment x condition)($F_{(1,42)} = 0.073$, ns), suggesting that animals treated peripherally with 7 consecutive days of LPS no longer displayed altered motor behaviors as compared to saline control animals, and importantly, were not displaying sickness behaviors after LPS injections. Surprisingly, however, we found no differences in percent time freezing between any groups including control groups (neither exercise nor sedentary) for treatment (LPS or saline)($F_{(1,42)} = 0.895$ ns), condition (exercise or sedentary)($F_{(1,42)} = 0.009$, ns), or the interaction (treatment x condition)($F_{(1,42)} = 0.048$, ns) indicating that there was no difference in hippocampus-dependent learning using this paradigm.

3.6 Novel object

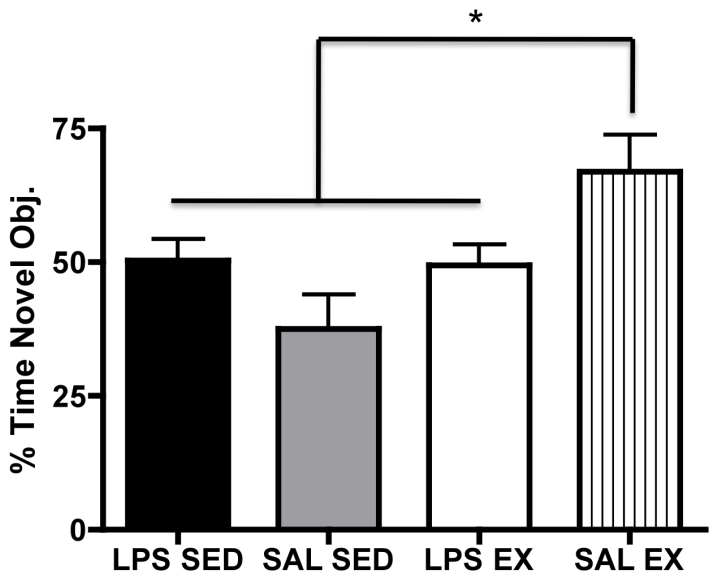

Figure 11. Novel object testing showing significant difference in learning. Saline exercise animals showing a significant preference for the novel object compared to LPS (Exercise and Sedentary) and Saline (Sedentary) groups. * compared to all other groups, $p<0.05$. No differences exist between other groups. Bars represent mean ± SEM.

In a separate experiment, a variation of the novel object paradigm was used to assess if cognitive differences were apparent between groups following the conclusion of exercise. As expected, no differences in percent time spent with familiar objects were found during training (data not shown). While there was no main effect of treatment ($F_{(1,31)} = 0.190$, ns), we did uncover a main effect of group ($F_{(1,31)} = 6.78$, $p=0.014$) and a significant interaction effect ($F_{(1,31)} = 7.67$, $p=0.009$). Unexpectedly, upon testing 4 hours later, the saline exercise group showed a significant preference for the novel object over the remaining groups ($p<0.05$).

4. Discussion

Physical activity is being increasingly recognized as an environmental factor that can affect the development and progression of sporadic AD (Podewiils et al., 2004; Larson et al., 2006). In rodents, studies examining the effects of exercise on the cognitive and pathological features of Alzheimer's disease have yielded equivocal results. Jankowsky et al. (2005) found increased plaque load in an environmental enrichment paradigm while Lazarov et al. (2005) demonstrated reduced Aβ levels in APPSW/PS1 transgenic mice. Still other studies report improved cognition but unchanged amyloid plaque deposition with exercise (Wolf et al., 2006; Arendash et al., 2004). These studies accentuate the importance of environmental factors, such as exercise, in modifying aspects of AD and suggest that the idea of environmental enrichment is a multi-faceted one.

Yuede et al. (2009) reported a reduced brain plaque load with voluntary exercise compared to sedentary controls in Tg2576 transgenic mice, a finding that is consistent with results of exercise in previous studies of mouse models of AD (Adlard et al., 2005; Nichol et al., 2007; Nichol et al., 2008; Parachikova et al., 2008). Of note however, is that these studies were carried out using the Tg2576 transgenic model of AD. The purpose of this study was to determine whether voluntary exercise reduced hippocampal Aβ and rescued cognitive deficits after 7 consecutive days of peripheral LPS injections. We found that 7 days of voluntary exercise following 7 days of peripheral administration of LPS reduced hippocampal Aβ to an equivalent level with the saline control groups; a finding consistent with results seen in transgenic mice (Yuede et al., 2009).

In order to determine whether the reduction of Aβ seen in the LPS exercise group correlated to a rescue of cognitive deficits the behavioral paradigms contextual fear conditioning (CFC) and Novel Y-maze were utilized. We hypothesized that voluntary exercise would also attenuate the cognitive deficits seen in our inflammatory AD model as seen in other studies using different animal models. Unfortunately, the CFC results were inconclusive and therefore based on these findings we cannot make a definitive statement as to whether there is an effect of exercise on our model behaviorally. Interestingly, however, the novel Y maze result indicated that the saline exercise control group performed the task significantly better than the other groups. This could indicate that animals that were previously sick (LPS exercise) could not produce the same results with exercise because their systems were still recovering even though previous evidence shows they are no longer exhibiting sickness behaviors (Kahn et al., 2012). Additionally, it may be that exercise increases brain function, possibly through upregulation of BDNF or BDNF receptor (Trk3) transcription. Our BDNF results do not reflect this, however, suggesting another neurotrophic factor or possibly enhanced brain glucose utilization over sedentary animals as potential explanations. It is also possible that BDNF does play a role, but it is more transient. We took tissue for BDNF mRNA expression measurement 24h after cessation of exercise. BDNF is at its highest exercise induced levels soon after exercise and then falls off, but Trk3 expression could be higher during behavioral training, thus enhancing learning even though BDNF is no longer elevated. The study should be repeated and tissue should be taken

closer to the end of the exercise period to examine BDNF, but also to examine Trk3 levels at separate time points.

Some possible explanations for why the CFC behavior did not provide data in support of our hypothesis include the ease of the task to learn. The CFC protocol used requires that the animals learn the task from a single shock. The single shock combined with both visual and olfactory cues could be too salient given that our results indicate that all groups leaned the task. The 7 days post-injection of LPS may have given the animals enough time to eliminate just enough Aβ from their hippocampus (specifically, the LPS SED group) that no deficit was seen. The test itself could be lacking the sensitivity to pick up on what may be minor cognitive deficits if any exist. It is likely this study will need to be repeated to definitively answer the question of exercise and its effect on cognitive deficits seen in our model.

It is worth noting that while the novel object behavior yielded some interesting data, it too did not produce results as anticipated. In fact, while saline exercise animals were significantly different the other three groups were not, indicating that the task may also be too easy and that the difference in cognitive deficits between groups were too small to be detected using an easy behavioral task.

We sought to determine if the inconclusive behavioral data was due entirely to failed behavior or an artifact of our study design. To do this we examined hippocampal BDNF levels. BDNF has been shown to be transiently increased after exercise, so it is possible that exercise prior to training and

testing resulted in an increase in BDNF in exercising animals, possibly offsetting any effect of Aβ in the hippocampus. Additionally, exercising animals may be hyperactive causing differences in training between exercising groups and sedentary groups. As mentioned previously, BDNF has been implicated in many neurophysiological processes including neurogenesis, synaptogenesis, and cell survival (Mattson et al., 2004) and after six weeks of voluntary running BDNF levels were significantly increased in the hippocampi of E. coli infected rats, as compared to sedentary (locked wheel) animals (Barrientos et al., 2011). The involvement of BDNF in these vital neurophysiological processes suggests that the cells of exercising animals may be more robust and may be able to tolerate a larger Aβ volume than could sedentary animals. These more hardy cells may be more able to clear Aβ from the CNS and thus be a mechanism by which exercise exerts its enhanced clearance. However, we found that BDNF was not significantly different across groups 24 hours after one week of exercise ceased. This is consistent with the many studies that report increases in BDNF as short-term or transient. It may be that a more chronic exercise period of up to six weeks is needed to lead to elevations in BDNF as was the case in Barrientos et al. (2011).

Previous studies in our lab have determined that after 7 consecutive days of LPS injections subjects have significantly higher levels of hippocampal Aβ and that these significant differences are maintained for 15 days after injections cease (Kahn et al., 2012). Thus, in the present experiment we can reasonably conclude that the significant reduction of hippocampal Aβ is likely due to exercise

and not simply normal clearance over time. Interestingly, we were also able to show that in saline treated animals the amount of wheel revolutions correlated inversely with the amount of hippocampal Aβ (Table 1). Additionally, using a median split ANOVA (low v. high revolutions) there is a trend that animals receiving LPS and having a high number of revolutions (> 10040.13 revolutions/24h) see an smaller decrease in Aβ, suggesting that too much exercise might be bad. This makes sense as in human studies, large training volumes have a negative affect on the immune system (Cotman et al., 2007). Thus, animals that have just been sick and then get a larger amount of training might not fully recover from their inflammation and thus Aβ does not decrease as rapidly as in the low training group. These findings warrant further investigation to see if a larger sample size will yield a strong correlation and if there is a plateau effect, or rather a point at which point at which exercise no longer has the beneficial effect of eliminating Aβ.

We can only speculate how the build up of Aβ is cleared from the brain or how exercise induces this clearance. This aspect warrants further investigation to determine the mechanism in which exercise exerts it positive effects. It is possible that exercise increases activation of microglia, as mentioned previously, in the brain through induction of IL-6 production and thus may increase the rate of phagocytosis and clearance of hippocampal Aβ. Another possibility may be that exercise and inflammation alter the ratio of the transporter protein that shuffles peripheral Aβ into the brain (RAGE) and the transporter protein that moves Aβ out of the brain (LRP-1)(Jaeger et al., 2009). If the RAGE:LRP-1 ratio

were to be shifted to increase transcription of LRP-1, net movement of Aβ across the blood brain barrier (BBB) would be shifted toward clearance from the brain. Lastly, most of the Aβ found in the CNS in our model was produced in the periphery (Weintraub et al., 2013). Because of this we cannot rule out that exercise may increase peripheral clearance of Aβ, leading to a decrease in the Aβ pool, and a subsequent reduction by mass action of Aβ from the brain.

In summary, we have demonstrated that exercise increases the clearance of central Aβ following our acute inflammatory model. While we were unable to show conclusively that this enhanced clearance ameliorated the cognitive deficits typically induced in our animal system and we speculate that in order to detect cognitive benefits, a more sensitive behavioral paradigm might be needed. Additionally, the relationship between the wheel revolutions and amount of hippocampal Aβ yielded interesting results and we feel that by increasing the power of the study with more animals the relationship could prove to be significant. Ultimately, this work reinforces the idea that exercising has positive impact on your health and that it extends to a model of inflammation that mimics some of the Alzheimer's disease pathological hallmarks. However, more work needs to be done to build on the results of this study to provide a more definitive outlook for exercise as a true preventative measure or mode of treatment for Alzheimer's disease.

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ABSTRACT

THE ROLE OF EXERCISE IN THE ALLEVIATION OF CENTRAL ACCUMULATION OF AMYLOID-BETA AND PREVENTION OF COGNITIVE DYSFUNCTION FOLLOWING PERIPHERAL INFLAMMATION

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Emerging research on neurodegenerative diseases, such as Alzheimer's disease (AD), has tentatively implicated inflammatory processes in the progression of the disease and its clinical manifestations. Previously our lab has explored this link between AD and inflammation. We established a non-transgenic animal model of acute inflammation that produces Aβ peptide in a purportedly similar fashion to early sporadic AD, showing that peripheral administration of the endotoxin lipopolysaccharide (LPS), a component of the cell wall of gram-negative bacteria, leads to elevated hippocampal Aβ and cognitive deficits. Interestingly, while investigating these peripheral inflammatory connections to AD, data suggested that animals that swam as part of a 9-day behavioral paradigm have reduced hippocampal Aβ levels. In the present study we sought to explore the impact of exercise on the cognitive deficits and Aβ peptide load seen in our inflammatory model. We hypothesized that voluntary exercise would reduce Aβ protein volume in the hippocampus and reduce cognitive impairment after administration of LPS. Our results indicate that mice allowed voluntary exercise (running wheel) for seven days following 7

consecutive days of a single LPS injection displayed reduced hippocampal Aβ levels compared to sedentary controls with no differences in behavior between groups.