EFFECTS OF VOLUNTARY WHEEL RUNNING AND FORCED TREADMILL RUNNING ON INFLAMMATION INDUCED BEHAVIORAL ABNORMALITIES IN YOUNG AND OLD MICE

BY

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DISSERTATION

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ABSTRACT

Peripheral infection stimulates the innate immune system to produce pro-inflammatory cytokines, such as tumor-necrosis-factor-α, interleukin-1β, and interleukin-6, which signal through various communication pathways to induce a ‘mirror image’ of cytokine expression within the brain. Centrally, these pro-inflammatory cytokines act directly or indirectly on neurons and supporting cells to alter autonomic nervous system output, endocrine system output, and behavior to regulate the body’s response to infection. One host factor affecting the behavioral response to infection is aging. Due to microglia priming and exacerbated neuroinflammation, aged individuals exhibit prolonged sickness behavior and depressive-like behavior following activation of the immune system. Regular moderate intensity exercise has been show to exert neuroprotective effects that may protect against inappropriate neuroinflammation and behavioral disturbances in aged subjects. The purpose of these studies was to investigate the effects of voluntary wheel running and forced treadmill running on lipopolysaccharide (LPS) and Bacillus Calmette-Guérin (BCG) induced sickness behavior (food intake, fluid intake, body weight, locomotor activity), depressive-like behavior (tail suspension test, sucrose preference), and activation of brain cytokine signaling pathways in young adult and aged mice. We hypothesized voluntary wheel running prior to LPS administration would induce anti-inflammatory effects and attenuate LPS-induced sickness behavior and depressive-like behavior. In contrast, we hypothesized forced treadmill exercise following BCG inoculation would exacerbate BCG-induced sickness behavior.

Voluntary wheel running induced expected adaptations including body weight loss, fat loss, and improved forced exercise tolerance. However, voluntary wheel running did not protect
against LPS-induced sickness behavior, depressive-like behavior, or whole brain proinflammatory cytokine and indoleamine 2,3-dioxygenase gene expression in young adult and aged mice. Forced treadmill running following BCG inoculation caused protracted recovery from infection as evidenced by prolonged body weight loss, reduced voluntary wheel running, and a tendency for reduced sucrose preference.

Thus, while exercise has been shown to be neuroprotective and anti-inflammatory, we did not observe that in our model. Possible explanations for the deviance include: inflammatory model, timing of exercise in relation to immune challenge, and brain regions responsible for behavioral response. Future research is necessary to understand the effects of exercise on microglia priming and how this affects behavioral outcomes. Finally, translational research should investigate the effects of regular exercise training prior to, and after infection, on perceived sickness behaviors and depression progression in aged adults.
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CHAPTER 1
INTRODUCTION

1.1 Significance

Aging of the population and the increased prevalence of chronic diseases among the elderly are major challenges facing our society and medical community. Indeed, by the year 2030, twenty percent of the United States population will be at least 65 years old, and the financial burden of age-related chronic diseases will approach 70% of national healthcare expenditures [1]. Many of these chronic diseases (e.g. cancer, cardiovascular disease, dementia, type 2 diabetes), are associated with inflammation as a key component of their pathophysiology.

Of particular importance is the role of age-related neuroinflammation in the etiology of behavioral abnormalities following physiological (e.g. immune stimulus) or psychological (e.g. stress) challenge. Aged animals, compared to young animals, display protracted sickness behavior, depressive-like behavior, and more severe cognitive impairment after stimulation of the innate immune system, and this is related to an exaggerated and prolonged induction of brain cytokine signaling pathways [1-3]. These observations have been corroborated in elderly humans, who following bacterial or viral infection, exhibit delayed recovery, elevated rates of depression, and prolonged cognitive impairment compared to younger subjects [4-6].

Studies within the past few years have implicated microglia cells and the enzyme indoleamine 2,3 dioxygenase (IDO) as the primary cellular and molecular mediators of inflammation-induced sickness and depressive-like behavior in aged mice [7, 8]. Aged microglial cells exhibit increased sensitivity to stimulation, and upon activation demonstrate elevated production of proinflammatory cytokines (TNFα, IL-1β, IL-6) [8]. In addition to inducing sickness behavior, these proinflammatory cytokines activate IDO leading to the
synthesis of several neurotoxic metabolites implicated in the pathophysiology of inflammation-induced depressive-like behavior [7].

From a clinical perspective, prolonged sickness behavior and increased depressive symptoms result in a reduction in self care, loss of independence, and are often the first steps towards dependent living in elderly individuals. Additionally, depression in elderly subjects is associated with an elevated risk for all cause morbidity and mortality [9]. The current protocol for treating age-related depression involves antidepressant therapy, which is moderately effective, but does not address the underlying inflammatory pathophysiology. Thus, there continues to be a major need to identify safe, alternative approaches for treating depression and reducing inappropriate neuroinflammation in the elderly.

Regular cardiovascular exercise may be one approach to alleviate age-related inflammation-based behavioral disturbances, as numerous studies have reported exercise-induced reductions in systemic and tissue specific inflammation [10]. In regard to age-related neuroinflammation, Barrientos et al. demonstrated voluntary wheel running (VWR) reduced microglia priming, proinflammatory cytokine induction, and cognitive impairment following *E. coli* infection in aged rats [11]. No studies, however, have examined the effects of exercise training on inflammation induced sickness behavior and depressive-like behavior in aged mice.

Therefore, the goal of the following specific aims is to determine if/how exercise training prior to or after immune stimulus can attenuate sickness behavior and depressive-like behavior in mice. Results from these studies may be clinically useful to prescribe prevention and treatment regimens to improve behavioral outcomes following infection in aged adults.
1.2 Specific Aims

This study, the first to examine the role of exercise training on sickness behavior and depressive-like behavior, has three specific aims. These are presented below. Methods, hypotheses, expected results, and alternative hypotheses are presented briefly. A full description of the study methodology, including experimental design and statistical analysis, can be found in Chapter 3.

1.2.1 Specific Aim #1

Does voluntary wheel running attenuate the exaggerated and prolonged sickness behavior observed in aged mice in response to acute LPS-induced inflammation? And, if so, is this effect associated with reduced peripheral and brain proinflammatory cytokine gene expression?

Twenty-two month old male C57bl/6 mice from the NIA Aging Colony were randomized to three housing conditions (Wheel, Locked Wheel, and Shoebox) for a duration of ten weeks. The purpose of the locked wheel and shoebox cages is to control for environmental enrichment created by the running wheel. Following ten weeks of housing intervention, all mice were placed in shoebox cages 24h prior to intraperitoneal (i.p.) injection of lipopolysaccharide (LPS) or equivolume sterile saline. The purpose of placing all mice in shoebox cages is to control for any acute exercise prior to or following LPS treatment; the specific goal of this study is to determine the effects of voluntary exercise training on LPS induced sickness behavior and inflammation. LPS will be injected at four different doses (0.33 mg/kg, 0.16 mg/kg, 0.08 mg/kg, and 0.02 mg/kg). The purpose of the different LPS doses is to ascertain whether potential exercise-induced effects occurred in a dose-dependent manner. This range of LPS doses was selected
based upon previous studies demonstrating that 0.33mg/kg LPS produced prolonged sickness behavior in aged, compared to young mice [2], and 0.02mg/kg being the lowest dose capable of inducing statistically significant changes in sickness behaviors when compared to saline treated mice.

Body weight, food intake, fluid intake, and locomotor activity will be collected at 8h, 24, 48h, and 72h post-injection.

Another set of mice will undergo identical intervention conditions and injections, but will be sacrificed at 24h post-injection for tissue collection. This time-point was determined based on previous data from our lab. Whole brain, liver, and spleen, will be dissected and rapidly frozen for quantification of proinflammatory gene expression.

With the lower doses LPS (0.02 mg/kg, 0.08 mg/kg, and 0.16mg/kg), it is hypothesized there will be a housing intervention dose-response in regard to sickness behavior attenuation and proinflammatory cytokine reduction. That is, VWR will exhibit the greatest attenuation, followed by locked wheel, and shoebox. In contrast, however, we expect to see no beneficial effects of VWR or LW at higher dose LPS (0.33 mg/kg), as this magnitude of inflammation most likely abolishes any exercise or environmental enrichment-induced anti-inflammatory adaptations. These hypotheses are based off Barrientos et. al’s data demonstrating wheel training reduces microglial priming and inflammation induced pro-inflammatory cytokine production in aged rats.

Alternatively, should exercise exert no beneficial effects on sickness behavior or brain inflammation in aged mice, it suggests that an appropriate sickness response may be critical for organism survival and cannot be altered by exercise training.
1.2.2 Specific Aim #2

Does voluntary wheel running attenuate sickness behavior and depressive-like behavior observed in young adult compared to aged mice in response to acute LPS-induced inflammation? And, if so, is this effect associated with reduced brain proinflammatory cytokine and IDO gene expression?

Experiment 1: Sixteen week-old male C57bl/6 mice from the Jackson Labs will be randomized to two housing conditions (Wheel or Shoebox) for a duration of thirty days. Following the housing intervention, mice will be intraperitoneally inoculated with 0.83 mg/kg LPS or equivolume saline, and placed back in their respective cage conditions. This dose of LPS was chosen because it produces transient sickness behavior (~12h) and depressive-like behavior (24h) in young adult C57bl/6J mice. Sickness behavior (body weight, food/fluid intake, locomotor activity) and depressive like behavior (sucrose preference and tail suspension test) will be assessed at 24h. Another group of mice will undergo identical treatment and be used for tissue collection at 4h post-injection. This time-point was chosen on peak IDO expression following LPS injection in young mice [3]. Tissue analysis will include brain proinflammatory cytokine and IDO gene expression.

Experiment 2: Nineteen month old male C57BL/6 mice from the NIA Aging Colony will be randomized to two housing conditions (Wheel or Shoebox) for a duration of seventy days. Following the housing intervention, mice will be intraperitoneally inoculated with 0.33 mg/kg LPS or equivolume saline, and placed back in their respective cage conditions. This dose of LPS was chosen because it is the minimum effective dose to reliably produce depressive-like behavior in aged C57BL/6J mice. Sickness behavior (body weight, food/fluid intake, locomotor activity) and depressive-like behavior (sucrose preference and tail suspension test) will be
assessed at 24h (sucrose preference) and 48h (tail suspension). Another group of mice will undergo identical treatment and be used for tissue collection at 24h post-injection. This time-point was chosen based on data by Godbout et al. indicating it as the critical time-point of prolonged pro-inflammatory cytokine and IDO gene expression, differentiating the aged and young adult neuroinflammatory response. Tissue analysis will include brain proinflammatory cytokine and IDO gene expression.

It is hypothesized there will be a housing intervention dose-response in regard to depressive-like behavior attenuation, and this will be related to a reduction in brain IDO gene expression in both young adult and aged mice. Furthermore, this reduction in IDO expression will be associated with decreased brain TNFα and IFNγ, two primary activators of IDO. These hypotheses are supported by Barrientos’s data demonstrating ex vivo stimulation of microglia from VWR trained rats exhibited significantly blunted TNFα gene expression compared to sedentary rats.

Should VWR yield no beneficial behavioral or anti-inflammatory effects, it could be hypothesized that the sensitivity of IDO to cytokine activation is too low to be affected by exercise training, which has been shown to reduce, but not abolish, inflammation-induced brain proinflammatory cytokines.
1.2.3 Specific Aim #3

**Does exercise intervention after infection prevent the progression of sickness behavior to depressive-like disturbances in aged mice in response to chronic BCG-induced inflammation?**

In contrast to the prior two aims, this aim will examine whether exercise training following immune challenge can attenuate sickness behavior and depressive-like behavior in mice. Twelve week old Balb/c mice will be intraperitoneally inoculated with either 10 micrograms BCG or equivolume saline, and placed back into cages containing running wheels. This dose of BCG was chosen because it adequately allows for the dissociation of sickness behavior (1-5 d) and depressive-like behavior (6-8 d) in young mice. Beginning at 4 h post-inoculation, mice will be treadmill run once per day for thirty minutes at an intensity of 8-12m/m (60-70% VO2max) until the end of the study; a control group of mice will have food removed and be exposed to the noise of the treadmill for the same duration as the runner. Sickness behavior (body weight, food/fluid intake, wheel running activity) will be monitored from inoculation to day 5. On day 6, mice will be subjected to a sucrose preference test.

It is hypothesized that treadmill training post-inoculation will attenuate depressive-like behavior as manifested by increased sucrose preference. There is little evidence to support this hypothesis, but the translational yield is extremely high. Imagine a physician prescribing exercise training post-infection to reduce the risk of developing depression.

Alternatively, post-inoculation exercise training could have no beneficial effects or could even exacerbate inflammation-induced depressive-like behavior. In this case, it would be concluded that aged subjects should completely recover from infection prior to commencing exercise training.
1.3 Summary

The overall aim of these studies is to determine the effects of exercise training, prior to and proceeding immune challenge, on inflammation-induced sickness behavior and depressive-like behavior in young and aged mice. Specifically, these experiments will address whether exercise training can attenuate inflammation induced behavioral abnormalities, and whether this is associated with reduced neuroinflammation. Results from these studies may be clinically useful to prescribe prevention and treatment regimens to improve behavioral outcomes following infection in aged adults.
CHAPTER 2
LITERATURE REVIEW

2.1 Inflammation

While inflammation has only recently garnered greater attention for its relationship to a variety of disease processes, the effects of inflammation have been recognized for centuries. The Roman physician Celsus first characterized inflammation circa 30 BC-38 AD by five cardinal signs: pain (dolor), heat (calor), redness (rubor), swelling (tumor), and loss of function (functio laesa) [12]. Since that time, scientists have come to recognize inflammation as a highly coordinated nonspecific innate immune response to harmful stimuli, with the principal goal of inflammation being the elimination of the stimuli and initiation of tissue healing. Common stimuli include pathogens, damaged cells, and aggregated cellular/metabolic by-products.

The initial step of the inflammatory response is recognition of the inflammatory stimulus. Sentinel cells located throughout the body (e.g., circulating monocytes, tissue macrophages, and tissue dendritic cells) constantly monitor circulating fluids and tissues for the presence of pathogens [12]. Pathogen recognition by these cells is mediated by several classes of receptors referred to as pattern-recognition receptors (PRRs). Unlike most cellular receptors that recognize a unique ligand, PRRs recognize classes of molecules termed pathogen-associated molecular patterns (PAMPS). The most widely studied PRRs are Toll-like receptors (TLRs), which have evolved to recognize proteins, lipids, and unmodified nucleic acid molecules found on infectious pathogens. TLRs are located either within the cell membrane or in endosomes/lysosomes, and the cellular location depends on whether or not the specific TLR

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1 This chapter contains three previously published figures that were not created by the author. The author has received reprint permission from the copyright owners.
recognizes extracellular pathogens (e.g., bacterial components) or intracellular pathogens (e.g., viral components). Ligand binding of most TLRs induces signal transduction through myeloid differentiation primary response gene 88 (MyD88), which acts as an adaptor protein and initiates a mitogen-activated protein kinase. This process ultimately terminates with the transcriptional factor nuclear factor kappa beta (NF-κB) binding DNA and initiating transcription of pro-inflammatory cytokines, such as TNFα, interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and interferon gamma (IFNγ) [13]. The expression of cytokines by macrophages and dendritic cells then recruits other cells of the immune system to the site of infection/damage to clear the pathogen and begin tissue repair.

2.2 Communication between Immune System and Brain

For years, the brain was considered an “immune-privileged” organ in which the blood-brain barrier prevented the influx of large proteins (e.g., cytokines) and cells (e.g., macrophages). However, within the past thirty years, studies have shown that proinflammatory cytokines act not only locally at the site of infection but also centrally in the brain to induce behavioral changes that promote organism recovery and survival from infection. This discovery was based on the observation that intraperitoneal (i.p.) administration of lipopolysaccharide (LPS) induced upregulation of brain proinflammatory cytokine proteins (e.g., I-1β and TNFα) and concurrent sickness behavior [14, 15]. Central administration of IL-1 receptor antagonist (IL-1Ra) and TNFα soluble receptor 1 (TNFαSR1) was shown to abolish the peripherally induced upregulation of brain IL-1β, TNFα, and sickness behavior, experimentally proving the existence of immune-to-brain communication [16-18]. It is now well understood that peripheral infection stimulates the innate immune system to produce pro-inflammatory cytokines, such as IL-1β, TNFα, and IL-
6, which signal through various communication pathways to induce a ‘mirror image’ of cytokine expression within the brain. Centrally, these pro-inflammatory cytokines act directly or indirectly on neurons and supporting cells (e.g., microglia and astrocytes) to alter autonomic nervous and endocrine system output to regulate the body’s response to infection. The brain monitors peripheral inflammatory responses via four primary communication mechanisms, which are briefly explained below and demonstrated in Figure 1.

The initial and fastest route of communication is through the afferent nerves. Locally produced cytokines, in particular IL-1β, bind IL-1 Receptor (IL-1R) on afferent vagal nerves during visceral infections and trigeminal nerves during oro-lingual infections [15, 19]. Upon IL-1β binding, the afferent nerves transduce a signal through the nucleus tractus solaris in the brain stem to secondary projections in the parabrachial nucleus and from there to the hypothalamus and hippocampus [15, 19]. Activation of this neural pathway sensitizes the target brain structures for the action of cytokines propagated via other communication routes and initiates the hypothalamic-pituitary-adrenal (HPA) axis response to inflammation [20]. Bluthe et al. experimentally proved the vagal pathway by demonstrating that a subdiaphragmatic vagotomy attenuated the brain proinflammatory cytokine response and sickness behavior induced by peripheral inflammation [21-23]. These changes were correlated with reduced c-fos activity in the limbic structures and the ventromedial preoptic area, structures known to mediate certain aspects of sickness behavior. c-Fos belongs to the immediate early gene family of transcription factors; expression of c-fos is an indirect marker of neuronal activity, as c-fos is upregulated when neurons initiate action potentials [24]. Interestingly, vagotomized mice still develop i.p. LPS-induced fever, HPA axis activation, and intravenous (i.v.)-induced sickness behavior, indicating neural communication is not required for all components of sickness behavior, and
different pathways mediate sickness behavior based on the infection location/route of delivery [21-23].

In contrast to the rapid activation of the neural pathway, three humoral pathways allow communication between the immune system and the brain during peripheral infection: diffusion across circumventricular organs (CVOs), saturable transporters, and secondary messenger signaling. The majority of the blood brain barrier (BBB) is composed of cellular tight junctions that prevent the passage of large proteins, such as cytokines. However, specific brain regions, such as the circumventricular organs and the subfornical organ, contain ‘leaky’ fenestrated capillaries, which allow diffusion of molecules across the BBB [25]. Blatteis et al. first demonstrated this phenomena by lesioning a CVO located adjacent to the hypothalamic thermoregulatory mechanism and observing a cytokine induced fever [26]. Further studies have confirmed this finding, and it is now well recognized that upon peripheral infection, circulating cytokines and cytokines synthesized by ‘macrophage like cells’ located within the CVOs cross the BBB, bind their respective receptors, and propagate signals to brain regions that regulate the sickness response (e.g., the hypothalamus) [25].

Following the discovery that cytokines cross the BBB, a second humoral route of peripheral-to-brain communication was elucidated. Banks et al. elegantly demonstrated the existence of saturable transporters that actively transport IL-1β, TNFα, and IL-6 across the BBB. The subsequent antagonism of these transporters following i.v. IL-1β administration reduced the central expression of proinflammatory cytokines and protected against inflammation induced behavioral impairments [25, 27, 28]. Recent work indicates these transporters are not static but, instead tightly regulate cytokine transport based on the age of the organism, circadian rhythm, central nervous system (CNS) region, and disease state [25].
The final humoral communication pathway occurs through a second messenger system in the endothelial cells lining the BBB, which express receptors for IL-1β, TNFα, and IL-6. Ligand binding activates the enzyme cyclooxygenase, resulting in prostaglandin E2 (PGE2) and nitric oxide synthesis and subsequent diffusion across the BBB [15, 25, 29, 30]. Upon crossing the brain parenchyma, PGE2 acts on receptors (EP3 and EP4) in the brainstem and hypothalamus to regulate the HPA axis and pyrogenic response to inflammatory stimuli. The synthesis of PGE2 is dependent on cyclooxygenase 2 (COX2) and prostaglandin synthesizing enzymes, both of which are highly expressed in the endothelial cells of cerebral blood vessels; pretreatment with specific COX2 inhibitors abrogates the LPS induced fever and HPA axis response [30].

Regardless of the immune-to-brain communication route, once an inflammatory signal is propagated, brain microglia cells become activated and commence de novo proinflammatory cytokine production. The molecular and cellular mechanisms responsible for de novo microglia cytokine production are not well characterized, but as mentioned earlier, this central response creates a mirror image of the periphery to promote an appropriate behavioral response.
Figure 1. Parallel communication routes between the immune system and brain. The neural pathway involves rapid communication of immune signals via primary afferent nerves. For instance, peripherally produced cytokines directly stimulate vagal afferents from the liver to elicit central responses. In the diffusion pathway, blood-derived cytokines passively traverse the blood-brain barrier at “leaky” brain regions, including the circumventricular organs. Transportation of peripheral cytokines occurs by an energy-dependent, saturable process involving transporters integral in the blood-brain barrier. A fourth route of communication currently being investigated involves endothelial cells composing the blood-brain barrier. It is believed that circulating cytokines bind to endothelial receptors to elicit the release of immune molecules (e.g., cytokines, NOS, etc.) directly into brain regions. Regardless of the pathway, inflammatory mediators released into the CNS activate brain microglial cells to release inflammatory cytokines, which subsequently bind neuronal receptors in specific brain regions (e.g., hippocampus) to initiate the sickness behavior syndrome [31].

2.3 Sickness Behavior and Depressive-like Behavior

Peripherally and centrally produced cytokines act directly or indirectly on neurons and supporting cells (e.g., microglia and astrocytes) to alter autonomic nervous and endocrine system output to regulate the body’s response to infection. IL-1β, for example, induces activation of the hypothalamic-pituitary-adrenal axis and the production of corticosteroids, which attenuate the
pro-inflammatory response in a negative feedback loop [32]. Moreover, these cytokines propagate signals to various brain structures, including the hypothalamus, hippocampus, amygdala, and prefrontal cortex, to invoke a constellation of motivated behavioral adaptations (i.e., ‘sickness behavior’). These adaptations allocate energy and resources towards the immune response and support recovery from infection [15]. Sickness behaviors include loss of appetite and body weight, fatigue, withdrawal from normal social activities, altered cognition, hyperalgesia, and fever [19, 33]. These non-specific symptoms were long believed to be a general weakness and malaise associated with infection. The importance of such behaviors, however, was bolstered by the powerful observation that forced tube feeding of anorexic sick mice to normal *ad libitum* levels resulted in greater mortality to *Listeria monocytogenes* [34]. It is now understood these evolutionarily conserved behaviors are essential for energy conservation, heat regulation, and ultimately organism survival.

IL-1β and TNFα are the primary drivers of inflammation induced sickness behavior. Peripheral or central administration of IL-1β or TNFα induces the entire spectrum of sickness behavior [13, 29]. The behavioral effects of IL-1β and TNFα are mediated through IL-1R1 and TNF-R1 expressed on neurons within the CNS [13, 15]. IL-1R1 knockout (KO) mice do not exhibit a sickness response to IL-1β administration, but do respond to i.p. LPS or TNFα injections [35, 36]. Interestingly, TNFα antibody administration to these mice abrogated LPS induced sickness behavior, experimentally proving the redundant pathways for IL-1β and TNFα for initiating inflammation induced sickness behavior [35, 36]. Exactly how IL-1β and TNFα alter neuron function is not entirely understood, but the following hypotheses have been postulated to suggest these cytokines: 1) signal through p38 and JNK within the hippocampus to
impair long term potentiation via inhibition of calcium signaling or 2) upregulate neuronal ceramide synthesis and subsequent calcium influx, resulting in neuron excitotoxicity [13].

In contrast to IL-1β and TNFα, IL-6 and IFNγ synthesized during infections do not elicit sickness behavior, despite activating the fever and HPA response. Neither IL-6 nor IFNγ administration alone was shown to induce a sickness response [13, 36]. O’Connor et al. demonstrated IFNgR1 KO mice develop a normal sickness response to Bacillus Calmette–Guérin (BCG) infection, suggesting IFNγ is not required for a sickness response [37]. Similarly, Bluthe et al. showed IL-6 knockout mice exhibited an attenuated, but not abrogated, sickness response to LPS, and exogenous administration of IL-6 restored the sickness response to wild-type levels [38]. The lack of a complete abolishment of LPS induced sickness in IL6KO mice indicates rather than inducing sickness behavior, IL-6 amplifies the actions of IL-1β and TNFα and is necessary for the ‘full’ sickness response.

In addition to inducing sickness behavior, peripheral inflammation can also induce major depressive disorders in humans and depressive-like behavior in animal models [15, 39]. Chronic inflammatory conditions, such as aging, atherosclerosis, and rheumatoid arthritis, are associated with an increased prevalence of depression (i.e., co-morbid depression), which is evidenced by a strong correlation between depressive behavior and circulating c-reactive protein (CRP) levels. Furthermore, patients undergoing cytokine immunotherapy for hepatitis and cancers exhibited an initial sickness response, followed by depressive episodes characterized by a depressed mood, guilt, and even suicidal thoughts [40, 41]. These associative observations yielded the “cytokine theory of depression,” which postulates that proinflammatory cytokines produced during immune activation alter brain neurochemistry and mediate inflammation-associated depression. Indeed, preclinical animal models support this theory, as activation of the peripheral innate
immune system with lipopolysaccharide or BCG induced an episode of sickness behavior. The sickness behavior was followed by depressive-like behavior, as measured by increased immobility in the tail suspension test and forced swim test and decreased consumption of a sweetened solution, all of which can be attenuated by chronic pretreatment with anti-depressants [42, 43].

Clinical evidence suggests that altered tryptophan metabolism potentially mediates inflammation-associated depression. In most clinical cases of inflammation-associated depression, a strong correlation between depressive behavior and an increased plasma ratio of kynurenine to tryptophan exists, suggesting a pivotal role for the tryptophan-metabolizing enzyme indoleamine 2,3 dioxygenase (IDO) [41]. IDO activation shunts tryptophan metabolism towards the kynurenine pathway, ultimately resulting in the production of the metabolites kynurenic acid, quinolinic acid (QA), and 3-hydroxykynurenine (3-HK) [41]. Under basal conditions, IDO is expressed at low levels in microglial cells and astrocytes, with the majority of kynurenine activity occurring in astrocytes, and the primary end product being kynurenic acid, a neuroprotective N-methyl-d-aspartate (NMDA) antagonist [41]. However, during immune activation, the proinflammatory cytokines TNFα and IFNγ induce IDO activation in the periphery (spleen, lung, mononuclear cells) and microglial cells, which shifts kynurenine metabolism towards the production of neurotoxic metabolites 3-HK and QA, which have been implicated in inflammation-induced depressive-like behavior [41] (Figure 2). Indeed, O’Connor et al. elegantly demonstrated IDO blockade using IDO KO mice and the nonspecific IDO inhibitor 1-methyltryptophan prior to LPS administration protected against LPS-induced depressive-like behavior [43, 44]. Furthermore, i.p. administration of L-kynurenine induced depressive-like behavior in a dose-dependent manner [43, 44].
Figure 2. Peripheral administration of lipopolysaccharide (LPS) induces sickness behaviour that peaks 2 to 6 hours later and gradually wanes (a). Depression-like behaviour, as measured by increased immobility in the forced-swim test or the tail-suspension test and decreased preference for a sweet solution, emerges on this background. The development of sickness behaviour requires activation of pro-inflammatory cytokine signaling in the brain in response to peripheral LPS (b). Some of the pro-inflammatory cytokines that induce sickness behaviour also enhance activity of the ubiquitous indoleamine 2,3 dioxygenase (IDO) that peaks at 24 hours post-LPS. Activation of IDO results in decreased tryptophan (TRP) levels and increased production of kynurenine (KYN) and other tryptophan-derived metabolites. Pre-treatment with the second-generation tetracycline minocycline, which has potent anti-inflammatory effects both at the periphery and in the brain, blocks both LPS-induced sickness behaviour and depression-like behaviour. By contrast, administration of 1-methyl tryptophan (1-MT), a competitive inhibitor of IDO, blocks LPS-induced depression-like behaviour without altering LPS-induced sickness behaviour [39].

In conclusion, the expression of proinflammatory cytokines induces sickness behavior, and through IDO activation, depressive-like behavior. Understanding the mechanisms of these
behavioral sequelae will allow for therapy targeted toward inflammation induced behavioral disturbances, and in particular, the comorbid depression observed in pathological conditions, such as aging, cardiovascular disease, and diabetes.

2.4 Aging, Microglia Priming, and Behavioral Consequences

The depressive symptoms manifested by the cytokine responses described above have clinical and psychosocial implications. Depressive disorders are estimated to affect up to 50% of elderly patients residing in long-term care facilities, and nearly 70% of these patients reported feelings of depression or sadness to the extent that their quality of life was impaired [45-47]. While the precipitating factors involved in age-related depression are not entirely clear, significant clinical and experimental evidence demonstrates that neuroinflammation, as characterized by increased basal levels of pro-inflammatory cytokines and oxidative stress and reduced anti-inflammatory cytokines and growth, occurs within the brain during normal aging [31]. As a large majority of the population is reaching older ages, it is of interest to determine the causes of age-induced neuroinflammation. In fact, resident microglia cells appear to be the primary mediator of age-induced neuroinflammation [31]. Microglia are innate immune cells of the brain that play critical roles in development, plasticity, and immune surveillance, with essential functions, such as clearing apoptotic bodies, synaptic pruning, and responding to inflammatory stimuli [31, 48, 49]. Young, healthy microglia maintain a ramified morphology with long and thin processes that constantly survey the tissue and respond rapidly to any local homeostatic disturbance (Figure 3). Microglia comprise approximately 15% of human CNS cells and approximately 10% of rodent CNS cells; these cells are derived from the primitive yolk sac myeloid cells and migrate to the CNS during embryonic development, after which replication
and turnover is limited [50, 51]. As mentioned in the previous section, microglia are key cellular players of the coordinated response to infection by the peripheral immune system and the central nervous system; peripheral inflammatory signals are responded to and propagated by microglia, which then produce central pro-inflammatory cytokines, such as TNFα, IL-1β, and IL-6.

In young healthy brains, the activation of microglia and the production of cytokines are transient processes, and microglia return to a quiescent state as the inflammatory stimulus is resolved. However, evidence in the past ten years indicates that aged microglia cells exist in a “primed” state with a heightened sensitivity to activation. Though controversial, the majority of data indicates no age-induced changes in microglia numbers [52]. However, the primed microglia commonly seen with aging are morphologically distinguishable by their shorter deramified processes and the upregulation of the inflammatory cell surface markers MHC II and CD11b (Figure 3) [31, 48, 53]. Additionally, primed microglia express increased steady-state levels of TNFα, IL-1β, and IL-6 mRNA and decreased anti-inflammatory IL-10 [54-56]. Upon immune activation, aged microglia respond with an exaggerated and prolonged pro-inflammatory response. For example, isolated microglia from aged rats produced elevated levels of TNFα and IL-1β following LPS stimulation, and this effect occurred in an LPS dose-response manner [57]. Along those lines, aged mice injected intraperitoneally or centrally with LPS exhibited an exaggerated and prolonged peripheral and central pro-inflammatory cytokine response. More importantly, this exaggerated inflammatory response results in behavioral disturbances compared to young mice. Godbout et al. demonstrated aged mice exhibited protracted sickness behavior (reduced locomotor activity [LMA]) and depressive like behavior (increased Forced Swim Test [FST] and Tail Suspension Test [TST] immobility) compared to young mice following injection with LPS, and this effect was associated with elevated brain IL-
1β and IDO gene expression and increased IDO activity [58, 59]. Kelley et al. corroborated these results by demonstrating an identical effect using BCG as an inducer of the chronic inflammatory response in aged mice [60]. In addition to sickness behavior and depressive like behavior, several labs have reported cognitive impairments following immune activation in aged mice. Using live bacteria, Barrientos et al. demonstrated that *E. coli* infection in aged mice induced central inflammation and hippocampal IL-1β upregulation, which inhibited the production of hippocampal brain derived neurotrophic factor (BDNF), a growth factor that drives neurogenesis and neuron survival and is vital for learning and memory [61-64]. This inhibition of BDNF results in cognitive impairment as measured by the Morris water maze, elevated plus maze, and contextual fear conditioning test [63, 65]. Numerous studies have experimentally implicated microglia as being responsible for age-induced neuroinflammation. Treatment of mice with minocycline, a strong anti-inflammatory agent and microglia inhibitor, attenuated LPS and BCG induced IL-1β, TNFα, and IL-6 in the brains of aged mice and reduced inflammation induced sickness behavior and depressive-like behavior [43, 44].
Figure 3. Microglial cells in the aged brain elicit a discordant inflammatory response upon activation by the peripheral innate immune system. (A) Similar to the priming paradigm proposed for peripheral macrophages, microglial cells in the aged brain may be primed as characterized by phenotypic alterations (e.g., increased expression of cell-surface markers). Upon receiving a triggering stimulus, these primed microglia release excessive concentrations of inflammatory cytokines in the CNS. (B) Evidence suggests the aged mouse brain responds to peripheral infection with a more exaggerated cytokine response compared with the adult mouse brain. Subsequent to peripheral immune stimulation with lipopolysaccharide (LPS), the release of IL-1β is higher in the aged vs. adult mouse brain [10]. (C) Both priming and triggering stimuli appear necessary to cause a discordant response by microglial cells in the aged brain. Cytokines released by microglia normally initiate an adaptive sickness behavior syndrome that includes anorexia, fever, and decreased social exploration. However, excessive cytokine release by primed and activated microglia elicits a discordant, maladaptive sickness behavior syndrome in the aged animal [31].

The mechanisms responsible for the aberrant priming of microglial cells in the aged brain are not entirely understood, but several studies point to the dysregulation of neuron/microglia cross-talk. In the non-diseased young brain, neuronal derived proteins, including fractalkine and
cluster of differentiation 200 (CD200), act as ligands for their respective microglial receptors and maintain the microglia in a quiescent/surveying state [48]. For example, fractalkine, which can either be neuronal membrane bound or free, binds to CX3CR on microglia and maintains the cell in a quiescent state. Following immune activation, fractalkine signaling is critical for attenuating the microglia inflammatory response and restoring brain inflammation to baseline levels. Several studies indicate aging reduces basal levels of fractalkine, which may partially explain microglial priming [48]. Furthermore, following a LPS challenge, microglia from aged mice exhibited a prolonged downregulation of the fractalkine receptor that may contribute to the prolonged proinflammatory cytokine response and protracted sickness behavior observed in aged mice [66]. Microglia from adult mice had restored fractalkine receptor expression 24 h post-LPS injection, while expression was not restored at this time-point in aged mice. Similar to fractalkine, CD200 signaling assists in maintaining microglia in a resting state, and evidence indicates this signaling is impaired during aging. CD200 is a membrane glycoprotein expressed on neurons that binds its corresponding microglia receptor, CD200R. Evidence for the importance of CD200 in modulating the neuroinflammatory response comes from CD200 knockout mice, which exhibited a heightened neuroinflammatory response following inflammatory stimuli [67, 68]. During aging, CD200R is downregulated on microglia compared to adult mice, and CD200 is reduced on neurons [48]. Intrahippocampal injection of CD200 fusion protein or fibroblast growth loop (FGL, a mimetic) reduced the neuroinflammatory response in the aged mouse brain following LPS injection and improved inflammation associated behavioral outcomes [69].

Aging induces morphological and phenotypical changes to microglia cells, and during an inflammatory challenge, the aged organism responds with exacerbated sickness behavior and depressive-like behavior. Therapies targeted at attenuating age-induced neuroinflammation and
microglia priming are important for reducing the prevalence of behavioral disturbances in the rapidly aging population.

2.5 Exercise, Neuroinflammation, and Behavior

The mood enhancing benefits of cardiovascular exercise have been well documented in the literature and through the anecdotal testimony of thousands of exercisers. In fact, exercise may represent a promising approach to attenuate the negative behavior alterations induced by the inflammatory response. Cross-sectional studies have shown an association between a high amount of physical activity and a low amount of depressive symptoms in both middle-aged and aged populations [70, 71]. Physical exercise might be especially efficient in reducing depressive symptoms among patients with mild to moderate depression [71]. This finding is important because a large fraction of older adults (10-20%) have clinically significant depressive symptoms but do not meet all of the criteria for major depression [45]; exercise may be used with these individuals in the absence of pharmacological therapy to mitigate the depressive symptoms. Sjosten and Kivela (2006) reviewed published randomized clinical trials using a rigorous study selection scheme to determine the strength of an effect of regular exercise on depression in older adults [72]. Based on their review, they concluded while more well-controlled studies are needed, exercise was effective in treating depression among those suffering from minor or major depression and in reducing depressive symptoms in those with significant depressive symptoms at baseline. The biological mechanisms responsible for the anti-depressant effects of exercise are not elucidated, but evidence indicates the effects may be associated with an exercise-induced attenuation of systemic and neuroinflammation [73-75]. Depression is often highly correlated with circulating CRP levels in humans, and numerous studies have demonstrated that regular
exercise training reduces CRP in a variety of populations, including aged, obese, and cardiovascular disease patients [74, 76, 77]. Animal models have shown reduced basal brain mRNA expression of IL-1β, TNFα, and IL-6 in several brain regions in response to chronic exercise training [78-80]. However, despite the strong clinical evidence, few studies have examined the effects of exercise training on inflammation-induced behavioral disturbances. Furthermore, when exercise, inflammation, and behavior are studied in conjunction in an animal model, the majority of literature has used unpredictable chronic mild stress (uCMS) as the stimuli for behavioral disturbances rather than a defined inflammatory challenge [81]. uCMS utilizes daily unpredictable stress that typically lasts for 3-6 weeks. For example, one day the mice may be confronted with a flooded cage for an hour, while the next day the mice must tolerate a strobe light for an hour. This experimental paradigm has been shown to increase brain proinflammatory cytokine gene expression and induce depressive-like behavior and cognitive impairments in mice and rats [82], but the relationship between the induction of the proinflammatory response and changes in behaviors has not been well established in this model. However, the data indicating the ability of exercise training to reverse stress-associated depressive-like behavior are quite strong. Solberg et al. found 6 weeks of voluntary wheel running of mice in conjunction with six weeks of uCMS increased sucrose preference and decreased FST immobility compared to mice that received uCMS but no prior wheel training [83]. These results were similar to Zheng et al., who demonstrated four weeks of wheel training in conjunction with uCMS recovered sucrose preference, which was associated with decreased hippocampal corticosterone (CORT) expression and increased hippocampal BDNF expression [84]. Exactly how BDNF may be mediating depressive-like behavior is unclear, but BDNF may be improving the survival of neurons affected by uCMS. Duman et al. proposed an alternative
molecular mechanism; these authors discovered four weeks of wheel training increased prefrontal cortex insulin-like growth factor 1 (IGF-1), and this increase was associated with a protected sucrose preference and FST [85]. While this response appears to be inherently different than the BDNF hypothesis, data by Park [86-88] et al. indicate IGF-1 protects brain BDNF signaling during an inflammatory challenge [86-88]. Therefore, it is plausible that exercise-induced IGF-1 could be mediating its anti-depressant effects via BDNF regulation. More relevant to our specific aims, a recent study by Liu et al. examined the effects of swim training on uCMS induced depressive-like behavior in mice [89]. Following 4 weeks of swim training in combination with uCMS, swim trained mice exhibited increased sucrose preference and decreased FST immobility compared to uCMS mice that did not swim train. Moreover, these behavioral results were associated with reduced prefrontal cortex IFNγ, TNFα, and IDO gene expression. To my knowledge, this is the first study to relate exercise-induced attenuation of depressive-like behavior with decreased IDO expression/activity.

To date, only one study has used a defined inflammatory stimulus to examine the effects of exercise training on inflammation induced behavioral abnormalities. However, this study examined cognitive impairment rather than sickness and depressive-like behavior. Using an E.coli infection model, in which the authors had previously shown elevated IL-1β disrupts BDNF signaling and cognition, Barrientos et al. elegantly demonstrated six weeks of wheel running prevented hippocampal dependent cognitive decline in aged rats [90]. This exercise-induced protection was related to reduced hippocampal IL-1β protein and elevated BDNF protein expression. To further elucidate the mechanisms responsible for the attenuated neuroinflammation, isolated primary microglia from the wheel trained and sedentary rats were stimulated with a range of LPS doses. As expected, the microglia from the voluntary wheel
running (VWR) mice exhibited a significantly reduced sensitivity to LPS, indicating that exercise training may ‘reverse’ age-induced microglia priming and the subsequent neuroinflammation.

Together, these data suggest exercise training may attenuate stress/pathogen induced neuroinflammation and the accompanying behavioral disturbances. The most logical mechanism that has been hypothesized to date is an exercise-mediated protection of BDNF signaling, which may protect synaptic plasticity and neuron survival. A caveat to the BDNF hypothesis is that the majority of exercise studies only show BDNF protection/upregulation in the hippocampus and have not demonstrated a more widespread effect in the brain. Given that depression is a multi-region brain disorder, employing a global approach to examine the effects of exercise on inflammation-induced behavioral disturbances may be more appropriate and realistic. Additionally, understanding the differential mechanisms of exercise modality (e.g., voluntary vs. forced), duration, intensity, and timing (e.g., prior to vs. after an inflammatory challenge) will allow researchers to tailor exercise programs to combat inflammation induced behavioral disturbances in humans [91, 92].

### 2.6 Conclusion

In conclusion, inflammation is a coordinated nonspecific innate immune response to harmful stimuli with the principal goal being elimination of the stimuli and initiation of tissue healing. Proinflammatory cytokines produced during inflammation act not only locally at the site of infection but also centrally in the brain to induce behavioral changes that promote organism recovery and survival from infection. Certain conditions (e.g., aging) sensitize the brain to the behavioral effects of cytokines, resulting in prolonged sickness behavior and increased risk of
depression. Therapeutic interventions, such as exercise training, may attenuate systemic and neural inflammation and may protect against inflammation-induced behavioral abnormalities in older individuals.
CHAPTER 3
METHODOLOGY

The following section is an overview of the methodology used in these studies. The information herein was derived from an excellent review by York et al., regarding *mouse testing methods in Psychoneuroimmunology* [93]. For a more detailed description of experimental design and methodology, please see the respective manuscripts in Chapters 4, 5, and 6.

3.1 Animals

**Aim 1 Animals:** Nineteen month-old male C57BL/6 mice were obtained from the National Institute of Aging (Bethesda, MD), and singly housed in cages with corn-cob bedding in a temperature (23°C) and humidity (45-55%) controlled environment with a 12h dark/light cycle (lights off 0900-2100). Mice were allowed *ad libitum* access to food and water for the entire duration of the study and were given 2 weeks to acclimate to the housing conditions prior to study commencement. Mice that appeared moribund or lost significant body weight (>20%) during the experiments were excluded. All experiments were conducted under the guidelines of the University of Illinois, Urbana-Champaign Animal Care and Use Committee.

**Aim 2 Animals:**

**Experiment 1, Young Adult Mice:** Four month-old male C57BL/6 mice were obtained from Jackson Laboratory (Bar Harbor, ME), and singly housed in cages with corn-cob bedding in a temperature (23°C) and humidity (45-55%) controlled environment with a 12h dark/light cycle (lights off 0900-2100). Mice were allowed *ad libitum* access to food and water for the entire duration of the study and were given 2 weeks to acclimate to the housing conditions prior to
study commencement. All experiments were conducted under the guidelines of the University of Illinois, Urbana-Champaign Animal Care and Use Committee.

**Experiment 2, Aged Mice:** Nineteen month-old male C57BL/6 mice were obtained from the National Institute of Aging (Bethesda, MD), and singly housed in cages with corn-cob bedding in a temperature (23°C) and humidity (45-55%) controlled environment with a 12h dark/light cycle (lights off 0900-2100). Mice were allowed *ad libitum* access to food and water for the entire duration of the study and were given 2 weeks to acclimate to the housing conditions prior to study commencement. Mice that appeared moribund or lost significant body weight (>20%) during the experiments were excluded. All experiments were conducted under the guidelines of the University of Illinois, Urbana-Champaign Animal Care and Use Committee.

**Aim 3 Animals:** Three month-old male balb/c mice were obtained from the Jackson Laboratory (Bar Harbor, ME), and singly housed in cages with corn-cob bedding in a temperature (23°C) and humidity (45-55%) controlled environment with a 12h dark/light cycle (lights off 0900-2100). Mice were allowed *ad libitum* access to food and water for the entire duration of the study and were given 2 weeks to acclimate to the housing conditions prior to study commencement. All experiments were conducted under the guidelines of the University of Illinois, Urbana-Champaign Animal Care and Use Committee.

**3.2 Exercise Training Methodology**

**Voluntary Wheel Running (VWR) Aim 1:** Due to the voluntary nature of wheel running, VWR exerts stronger neuroprotective effects compared to forced treadmill running. For this reason, it was chosen as our primary exercise training modality. Mice were randomized to a voluntary wheel running (VWR), locked wheel (Locked), or ‘normal’ (Standard) housing
condition for a duration of ten weeks. This duration of training is sufficient to induce metabolic adaptations in aged mice. The purpose of using a Locked group is to discern between cage enrichment and wheel running effects. VWR mice were individually housed in a plexiglas cage (48 L × 26 W × 15 H cm) that contained a wireless low-profile running wheel (circumference 37.82 cm)(Med Associates, St. Albans, Vermont). Wheel revolutions were wirelessly relayed via telemetry to a computer in the facility. Locked mice were housed in cages identical to VWR mice, except their wheels were locked in place; this provided cage enrichment (i.e. novel object), but did not allow for exercise training. Standard mice were housed in smaller cages (30 L × 19 W × 12 H cm) without any type of environmental enrichment.

**Voluntary Wheel Running (VWR) Aim 2:** Due to the voluntary nature of wheel running, VWR exerts stronger neuroprotective effects compared to forced treadmill running. For this reason, it was chosen as our primary exercise training modality. Because we found no differences between Locked and Standard mice in Aim 1, we only utilized VWR and Standard housing conditions in Aim 2. Mice were randomized to a voluntary wheel running (VWR) or ‘normal’ (Standard) housing condition for a duration of 30d (Young Adult Mice) and 70d (Aged Mice). This duration of training is sufficient to induce metabolic adaptations for the respective age cohorts (unpublished data and [94]). VWR mice were individually housed in a plexiglass cage (48 L × 26 W × 15 H cm) that contained a wireless low-profile running wheel (circumference 37.82 cm)(Med Associates, St. Albans, Vermont). Wheel revolutions were wirelessly relayed via telemetry to a computer in the facility. Standard mice were housed in smaller cages (30 L × 19 W × 12 H cm) without any type of environmental enrichment.

**Voluntary Wheel Running (VWR) Aim 3:** For Aim 3, we decided to use VWR as a dependent variable rather than an independent variable. That is, VWR was used as a measure of sickness
instead of a training modality. All mice in Aim 3 were individually housed in a plexiglass cage (48 L × 26 W × 15 H cm) that contained a wireless low-profile running wheel (circumference 37.82 cm)(Med Associates, St. Albans, Vermont). Wheel revolutions were wirelessly relayed via telemetry to a computer in the facility. Standard mice were housed in smaller cages (30 L × 19 W × 12 H cm) without any type of environmental enrichment.

**Treadmill Exercise Training Aim 3:** In Aim three we wanted to examine the effects of forced treadmill exercise after BCG inoculation on sickness and depressive-like behavior. Beginning at 4h post-inoculation, mice were treadmill run once per day for thirty minutes at an intensity of 8-12m/m (60-70% VO2max) until the end of the study; a control group of mice had their food removed and was exposed to the noise of the treadmill for the same duration as the treadmill runners.

**Treadmill Test of Fatigability Aims 1 and 2:** Forced treadmill running measures mouse training adaptations compared to wheel running. To assess VWR induced training adaptations in Aims 1 and 2, we measured forced exercise fatigability at the conclusion of the training period, 72h prior to LPS injection. Mice ran until exhaustion on a motor-driven treadmill at gradually increasing speeds from 6-21m/min. Exhaustion was defined as the point at which the mouse refused to run despite prompting by mild prodding with the hand for a period of 10 s; electric shock was not used in this test.

### 3.3 Lipopolysaccharide (LPS) and Bacillus Calmette–Guérin (BCG) Administration

**LPS:** LPS is a component of the Gram negative bacterial cell wall, and is commonly used to stimulate the innate immune system in a similar manner to bacterial infection. However, unlike bacteria which continue replicating and stimulating the immune system, LPS is short lived and
non-replicating, and thus an excellent and widely used model to assess cytokine-induced sickness behavior and depressive-like behavior. LPS interacts in a complex fashion with toll-like receptor (TLR)-4 on host immune cells including macrophages where it signals through nuclear factor kappa beta (NF-κB) to induce pro-inflammatory gene expression (Beutler 2003).

**LPS Administration Aim 1:** After training intervention, all mice were removed from their respective housing conditions cage and singly housed in clean cages (30 L x 19 W x 12 H cm) for a 24h period prior to treatment in order to washout any acute effects of the last wheel training session as acute exercise has been shown to affect LPS responses (Starkie et al., 2003; Tanaka et al., 2010). This was necessary to be able to separate exercise training effects versus influences due to the last exercise session. Following this 24h period, mice were randomized and injected intraperitoneally (i.p.) with saline or *Escherichia coli* LPS (lot 3129, serotype 0127:B8, Sigma) at one of four different doses (0.02 mg/kg, 0.08 mg/kg, 0.16 mg/kg, 0.33 mg/kg). Injection occurred at the onset of the dark cycle. The purpose of the different LPS doses was to ascertain whether potential exercise-induced effects occurred in a dose-dependent manner. This range of LPS doses was selected based upon previous studies demonstrating that 0.33mg/kg LPS produced prolonged sickness behavior in aged, compared to young mice [2], and 0.02mg/kg being the lowest dose capable of inducing statistically significant changes in sickness behaviors when compared to saline treated mice.

**LPS Administration Aim 2.** Given that aged mice exhibit a greater sensitivity to LPS-induced neuroinflammation and behavioral disturbances, we used different LPS doses for Experiment 1 and Experiment 2. Both doses, however, represent the minimum effective dose capable of inducing depressive-like behavior in the respective age cohorts of C57bl/6 mice, as determined by thorough titration experiments (unpublished data).
**Experiment 1, Young Adult Mice:** After 30d of VWR training, mice were randomized and injected intraperitoneally (i.p.) with saline or *Escherichia coli* LPS (lot 3129, serotype 0127:B8, Sigma) at a dose of 0.83 mg/kg.

**Experiment 2, Aged Mice:** Following 70d of VWR training, aged mice were randomized and injected intraperitoneally (i.p.) with saline or *Escherichia coli* LPS (lot 3129, serotype 0127:B8, Sigma) at a dose of 0.33 mg/kg.

**BCG:** Infection of mice with BCG is a well validated model of chronic immune activation. Mice chronically infected with BCG display an acute episode of sickness behavior (0-6d), followed by depressive-like behaviors (7-14d). These behavioral abnormalities are similar to symptoms typically observed in patients undergoing immunotherapy [37, 42]. For this reason, infection of mice with BCG represents a suitable model to study the depressive-like behavioral implications of chronic inflammation. BCG also chronically induces both IDO and proinflammatory cytokines such as IFNγ and TNFα [37, 42]. The advantages of BCG compared LPS are that BCG allows for the temporal dissociation between sickness behavior and depressive-like behavior.

**BCG Administration Aim 3:** In Aim 3, mice were inoculated with either saline or $10^8$ CFU BCG (Organon-Tice; Lot 4511103) via i.p. injection. Injection occurred at the onset of the dark cycle. This dose was based on previous studies from our lab and O’Connor et al. demonstrating it to be the minimum effective dose to induce prolonged (>6d) depressive like behavior in young mice [37].
3.4 Assessment of Sickness Behavior

Proinflammatory cytokines propagate signals to various brain structures, including the hypothalamus, hippocampus, amygdala, and prefrontal cortex, to invoke a constellation of motivated behavioral adaptations (i.e., ‘sickness behavior’). These adaptations allocate energy and resources towards the immune response and support recovery from infection [15]. Sickness behaviors include loss of appetite and body weight, fatigue, withdrawal from normal social activities, altered cognition, hyperalgesia, and fever [19, 33].

All assessments of sickness behavior occurred during the dark cycle at the same time each day. For a more detailed description of behavioral assessments (e.g. time-points), please see the respective manuscripts in Chapters 4, 5, and 6.

**Body Mass:** While body mass loss is not a behavior, per se, it is an extremely sensitive measure of sickness. The loss of body mass observed during sickness is attributed to decreased food and fluid intake, resulting in dehydration and lean body mass loss. To assess body mass changes, mice were weighed at the same time everyday to reduce variability based on circadian feeding patterns. A baseline collection period of >4d should was used to determine a baseline body weight; this is necessary in case post hoc normalization of body weight is required.

**Food Intake:** Food consumption, or rather, food disappearance, is a useful indicator of sickness, as sick animals typically exhibit anorexia. The term food disappearance is used rather than food intake, because some food inadvertently falls in the cage bedding. To measure food disappearance, food should was weighed at the desired intervals (e.g. 24h), and the difference between the measured values was calculated as food disappearance. As with body mass, a
baseline collection period of >4d should was used to determine a baseline food intake; this is necessary in case post hoc normalization of food intake is required.

**Fluid Intake:** Fluid intake, like food intake, is a useful indicator of sickness, as sick animals typically exhibit adipsia. Usually food and fluid intake go “hand in hand” in regard to decrease then recovery. To measure fluid consumption, fluid should was weighed at the desired intervals (e.g. 24h), and the difference between the measured values was calculated as fluid disappearance. A baseline collection period of >4d should was used to determine a baseline fluid intake; this is necessary in case post hoc normalization of fluid intake is required.

**Locomotor activity (LMA):** As lethargy is a primary symptom of sickness behavior, LMA can be used as an easy and high-throughput measure of sickness behavior. LMA is ideally conducted in a novel environment, as the motivation to explore is stronger in this situation in healthy animals compared to sick animals. For this test, mice were individually placed into a clean, novel cage (30 L x 19 W x 12 H cm) devoid of bedding or litter, and LMA was video-recorded for a 5-minute period. Videos were analyzed by dividing the cage into four virtual quadrants and counting the number of quadrant entrances over the 5-minute period; counting was done by a trained observer who was blind to experimental treatments.

### 3.5 Assessment of Depressive-Like Behavior

The underlying cause of depression is unknown, making it difficult to recreate the disease in an animal model [95]. Current models attempt to produce quantifiable measures of human symptoms (e.g. Anhedonia) in animals [95]. Furthermore, a majority of human depression symptoms are subjective, which cannot be assessed in animals[95]. Animal models of depression are evaluated based on their reliability, their ability to accurately predict human
outcomes (predictive validity), their ability to reproduce in animals aspects of illness in humans (face validity), and the extent to which they model depression etiology in humans (construct validity) [95]. The experimental design utilized in our experiments adequately satisfies these criteria. Inflammation-induced depression is a real occurrence in humans, and we use LPS to model this in mice (LPS = construct validity). Anhedonia is a real symptom of human depression, and we use sucrose preference testing to model this in mice (sucrose preference = face validity). Lastly, the tail suspension test is a classic model of predicting the efficacy of antidepressant drugs in humans (tail suspension = predictive validity). When using depressive-like behavior that involves movement (e.g. TST), any sickness-induced reduced locomotion confounds the assessment of depressive-like behavior. Therefore, depressive-like behavior testing should be performed after overt sickness behaviors have subsided and locomotion has returned to normal. These tests are validated as measures of ‘depressive-like behavior’ because pretreatment with anti-depressants reduces the respective depressive-like behavior outcome (e.g. immobility). For a more detailed description of behavioral assessments (e.g. time-points), please see the respective manuscripts.

**Tail Suspension Test (TST):** The tail suspension test (TST), a standardized test of depressive-like behavior in which depression is inferred from increased duration of immobility, was conducted as previously described by O’Connor et al [43]. Briefly, mice were taken from their home cage and hung from their tail on a hook connected to a strain gauge for a period of 6 minutes. A computerized system for processing the force exerted on the gauge (Mouse Tail Suspension Package, MED-TSS-MS; Med Associates, St Albans, VT, USA) collected and analyzed the movements of each individual mouse. An immobility threshold was determined by
establishing an activity level that would exclude all movements and only encompass immobility. Time below this threshold indicated the time of immobility.

**Sucrose Preference Test (SPT).** Anhedonia is a key component of depression, and can be measured in mice by their preference to consume a sweetened solution. A sucrose preference test was performed following a three-day training program, during the 24 h period following LPS administration. Mice were presented with two identical volumetric drinking tubes containing either water or a 1% sucrose solution. Bottles were weighed to before and after the 24h period to measure fluid consumption of each respective solution. Bottle position (right v. left) was alternated each training period to reduce potential bias from place preference. Sucrose preference was calculated using the following formula: \([\text{Sucrose intake} / (\text{water intake} + \text{sucrose intake})] \times 100\).

**3.6 Tissue Cytokine Quantification**

**Quantitative Reverse Transcriptions Polymerase Chain Reaction (qRT-PCR):** qRT-PCR is a commonly used technique in molecular biology to detect RNA expression levels. Briefly, a RNA template is first converted into a complementary DNA (cDNA) using a reverse transcriptase. Following that, the cDNA is then used as a template for exponential amplification using PCR. For our study, quantitative real-time reverse transcription PCR was performed on an Applied Biosystems Prism 7900 using TaqMan gene expression assays for TNF-\(\alpha\) (Mm0043258_m1), IL-1\(\beta\) (Mm00434228_m1), IL-6 (Mm00446190_m1), IL-10 (Mm00439616_m1), BDNF (Mm01334042_m1), IDO (Mm00492586_m) and glyceraldehyde 3-phosphate dehydrogenase (Mm999999_g1) purchased from Applied Biosystems (Foster City,
CA). Reactions were performed in duplicate according to the manufacturer’s instructions.

Relative quantitative measurement of target gene expression was conducted using the $\Delta \Delta C_t$ method with glyceraldehyde 3-phosphate as the endogenous house-keeping gene and VWR saline treated mice were used as the referent group. We chose to analyze TNF-$\alpha$, IL-1$\beta$, IL-6, IL-10, and IDO because they are critical mediators of inflammation-induced sickness and depressive-like behavior and are affected by aging [2, 96, 97]. BDNF is a critical neurogenic growth factor that is highly influenced by exercise (Zoladz et al. 2010). Our group has shown that inflammatory stimuli such as LPS can reduce brain BDNF (Park et al 2011).
CHAPTER 4
THE EFFECTS OF VOLUNTARY WHEEL RUNNING ON LPS-INDUCED SICKNESS BEHAVIOR IN AGED MICE²

4.1 Abstract
Peripheral stimulation of the innate immune system with LPS causes exaggerated neuroinflammation and prolonged sickness behavior in aged mice. Regular moderate intensity exercise has been shown to exert anti-inflammatory effects that may protect against inappropriate neuroinflammation and sickness in aged mice. The purpose of this study was to test the hypothesis that voluntary wheel running would attenuate LPS-induced sickness behavior and proinflammatory cytokine gene expression in ~22-month-old C57BL/6J mice. Mice were housed with a running wheel (VWR), locked-wheel (Locked), or no wheel (Standard) for 10 weeks, after which they were intraperitoneally injected with LPS across a range of doses (0.02, 0.08, 0.16, 0.33 mg/kg). VWR mice ran on average 3.5 km/day and lost significantly more body weight and body fat, and increased their forced exercise tolerance compared to Locked and Shoebox mice. VWR had no effect on LPS-induced anorexia, adipsia, weight-loss, or reductions in locomotor activity at any LPS dose when compared to Locked and Shoebox groups. LPS induced sickness behavior in a dose-dependent fashion (0.33>0.02 mg/kg). Twenty-four hours post-injection (0.33mg/kg LPS or Saline) we found a LPS-induced upregulation of whole brain TNFα, IL-1β, and IL-10 mRNA, and increased IL-1β and IL-6 in the spleen and liver; these effects were not attenuated by VWR. We conclude that VWR does not reduce LPS-induced sickness.

exaggerated or prolonged sickness behavior in aged animals, or 24h post-injection (0.33mg/kg LPS or Saline) brain and peripheral proinflammatory cytokine gene expression. The necessity of the sickness response is critical for survival and may outweigh the subtle benefits of exercise training in aged animals.

4.2 Introduction

Peripheral infection stimulates the innate immune system to produce pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin(IL)-1β, and IL-6, which signal through various communication pathways to induce a ‘mirror image’ of cytokine expression within the brain [19, 33, 96]. Centrally, these pro-inflammatory cytokines act directly or indirectly on neurons and supporting cells (e.g. microglia, astrocytes) to alter autonomic nervous and endocrine system output to regulate the body’s response to infection. IL-1β, for example, induces activation of the hypothalamic-pituitary-adrenal axis and the production of corticosteroids which attenuate the pro-inflammatory response in a negative feedback loop [32]. Moreover, these cytokines invoke a constellation of motivated behavioral adaptations (e.g. ‘sickness behavior’), which allocate energy and resources towards the immune response, and support recovery from infection. Sickness behaviors include: loss of appetite and body weight, fatigue, withdrawal from normal social activities, altered cognition, hyperalgesia, and fever [19, 33]. The importance of such behaviors is bolstered by the powerful observation that forced tube feeding of anorexic sick mice to normal ad libitum levels results in greater mortality to Listeria monocytogenes [34].

Anybody who has been ill has experienced sickness behavior, which, depending on the pathogen, infectious load, and host factors, can either be mild or severely debilitating.
Fortunately for most, these symptoms are transient. One host factor affecting sickness behavior is aging. Normal aging is accompanied by changes in immune function that can result in greater infectious disease susceptibility in humans [98] and animals [99]. Older subjects exhibit exaggerated behavioral and cognitive deficits following immune activation [2, 100-102]. Important for the current study, 24 hr following peripheral lipopolysaccharide (LPS, 0.33 mg/kg) administration, aged mice exhibited significantly decreased social behavior, locomotor activity, food intake, and body weight whereas young mice are fully recovered at that time [2]. LPS is a component of the Gram negative bacterial cell wall, and is commonly used to stimulate the innate immune system in a similar manner to bacterial infection. However, unlike bacteria which continue replicating and stimulating the immune system, LPS is short lived and non-replicating, and thus an excellent and widely used model to assess cytokine-induced sickness behavior. LPS interacts in a complex fashion with toll-like receptor (TLR)-4 on host immune cells including macrophages where it signals through nuclear factor kappa beta (NF-κB) to induce pro-inflammatory gene expression. The protracted behavioral effects associated with aging can be attributed, in part, to exaggerated and less resilient inflammatory responses in the periphery and within the central nervous system, as aged animals show higher pro-inflammatory cytokines levels in the brain in response to peripheral immune activation [2, 97, 101]. The biological basis of this aging phenomenon is not entirely understood, but putative mechanisms indicate an age-related dysregulation of peripheral inflammation, priming of microglia cells, and dysregulated neuron/microglia interaction [66, 97, 103].

Strategies to prevent or attenuate age-associated exaggerated or prolonged inflammation and sickness behavior could assist elderly in recovery from infectious episodes. Our lab has a longstanding interest in exploring the ability of regular exercise to alleviate dysregulated
inflammation in aging, obesity and infectious challenges [104-107]. Studies examining the effects of exercise training on various responses to LPS in younger rodents have produced mixed results [108-114]. For example, Criswell et al [110] found 12 weeks of treadmill running led to an exaggerated serum TNFα response when compared to sedentary rats. In contrast, Chen et al [108] found the exact opposite; 4 weeks of treadmill training attenuated the serum TNFα response to LPS. No studies have examined whether exercise training can affect exaggerated central and peripheral inflammation induced by LPS in aged animals, and whether this translates to attenuated inflammation-induced sickness behavior in aged animals.

Therefore, using a paradigm (LPS-induced sickness behavior) that has previously been shown to be sensitive to endogenous and exogenous factors [2, 115], we sought to examine the influence of 10 weeks of voluntary wheel running (VWR) on LPS-induced sickness behaviors and whether changes in these behaviors were accompanied by changes in central and peripheral proinflammatory cytokine gene expression in aged mice. We purposefully did not include young mice in these experiments because the aging effect has been well documented [2, 100] and our primary interest was whether exercise training impacts age-associated protracted sickness behaviors. We hypothesized voluntary wheel running would induce anti-inflammatory effects and attenuate LPS-induced sickness behaviors in aged mice.

4.3 Materials and Methods

Animals. Nineteen month-old male C57BL/6 mice were obtained from the National Institute of Aging (Bethesda, MD), and singly housed in cages with corn-cob bedding in a temperature (23°C) and humidity (45-55%) controlled environment with a 12h dark/light cycle (lights off 0900-2100). Mice were allowed ad libitum access to food and water for the entire duration of the
study and were given 2 weeks to acclimate to the housing conditions prior to study commencement. Mice that appeared moribund or lost significant body weight (>20%) during the experiments were excluded. All experiments were conducted under the guidelines of the University of Illinois, Urbana-Champaign Animal Care and Use Committee.

**Voluntary Wheel Training.** Following acclimation, mice were randomized to a voluntary wheel running (VWR), locked wheel (Locked), or ‘normal’ (Standard) housing condition for a duration of 10 weeks. The VWR mice were individually housed in a plexiglass cage (48 L × 26 W × 15 H cm) that contained a wireless low-profile running wheel (circumference 37.82 cm)(Med Associates, St. Albans, Vermont). Wheel revolutions were wirelessly relayed via telemetry to a computer in the facility. To discern between cage enrichment and wheel running effects, we used two different control groups. Locked mice were housed in cages identical to VWR mice, except their wheels were locked in place; this provided cage enrichment (i.e. novel object), but did not allow for exercise training. Standard mice were housed in smaller cages (30 L × 19 W × 12 H cm) without any type of environmental enrichment.

**LPS administration.** After ten weeks of training, all mice were removed from their respective housing conditions cage and singly housed in clean cages (30 L × 19 W × 12 H cm) for a 24h period prior to treatment in order to washout any acute effects of the last wheel training session as acute exercise has been shown to affect LPS responses (Starkie et al., 2003; Tanaka et al., 2010). This was necessary to be able to separate exercise training effects versus influences due to the last exercise session. Following this 24h period, mice were randomized and injected intraperitoneally (i.p.) with saline or *Escherichia coli* LPS (lot 3129, serotype 0127:B8, Sigma)
at one of four different doses (0.02 mg/kg, 0.08 mg/kg, 0.16 mg/kg, 0.33 mg/kg). The purpose of the different LPS doses was to ascertain whether potential exercise-induced effects occurred in a dose-dependent manner. This range of LPS doses was selected based upon previous studies demonstrating that 0.33mg/kg LPS produced prolonged sickness behavior in aged, compared to young mice [2], and 0.02mg/kg being the lowest dose capable of inducing statistically significant changes in sickness behaviors when compared to saline treated mice.

**Treadmill Running Test.** To assess VWR induced training adaptations, we measured forced exercise fatigability in a cohort of saline-injected mice, 96h post-injection. Mice ran until exhaustion on a motor-driven treadmill at gradually increasing speeds from 6-21m/min. Exhaustion was defined as the point at which the mouse refused to run despite prompting by mild prodding with the hand for a period of 10 s; electric shock was not used in this test.

**Measurement of sickness behavior.** Sickness behavior was assessed by changes in body weight, food and fluid intake, and locomotor activity (LMA). Body weight was measured daily for eight days post-injection, while food and fluid intake were measured for seven and four days, respectively. Decreased LMA in a novel environment is a sensitive measure of sickness behavior [96]. For this test, mice were individually placed into a clean, novel cage (30 L × 19 W × 12 H cm) devoid of bedding or litter, and LMA was video-recorded for a 5-minute period. Videos were analyzed by dividing the cage into four virtual quadrants and counting the number of quadrant entrances over the 5-minute period; counting was done by a trained observer who was blind to experimental treatments.
**Study design.** In the first experiment, body weight, food and fluid intake, and LMA were measured at numerous time-points after injection of saline or one of 4 different LPS doses. In a separate but identical experiment, mice were killed by CO₂ exposure at 24 h post-Saline or 0.33 mg/kg LPS injection for tissue collection to coincide with sickness behavior data collected in the first experiment. This time-point and LPS dosage were chosen based upon previous research demonstrating clear age-related differences in the sickness and inflammatory response to i.p. LPS [2]. Separate mice were used for behavioral and tissue experiments because behavioral manipulation may confound sensitive measures of tissue gene expression. Tissues were dissected out after transcardial perfusion with ice-cold PBS saline.

**RNA extraction and reverse transcription.** Total RNA from whole brain, epididymal adipose, spleen, and liver was extracted with Qiagen RNeasy Mini Kits (Valencia, CA). We chose these tissues because they demonstrate age-associated exaggerated inflammation following LPS challenge [2, 103, 116]. Reverse transcription reactions were completed in an Eppendorf Mastercycler Thermocycler (Hamburg, Germany) using an Applied Biosystem (Foster City, CA) High Capacity reverse transcriptase kit with 2,000 ng total RNA and random primers for each reaction.

**Real-Time RT-PCR.** Quantitative real-time reverse transcription PCR was performed on an Applied Biosystems Prism 7900 using TaqMan gene expression assays for TNF-α (Mm0043258_m1), IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), IL-10 (Mm00439616_m1), BDNF (Mm01334042_m1), and glyceraldehyde 3-phosphate dehydrogenase (Mm999999_g1) purchased from Applied Biosystems (Foster City, CA).
Reactions were performed in duplicate according to the manufacturer’s instructions. Relative quantitative measurement of target gene expression was conducted using the ΔΔCt method with glyceraldehyde 3-phosphate as the endogenous house-keeping gene and VWR saline treated mice were used as the referent group. We chose to analyze TNF-α, IL-1β, IL-6, and IL-10 because they are critical mediators of inflammation-induced sickness behavior and are affected by aging [2, 96, 97]. BDNF is a critical neurogenic growth factor that is highly influenced by exercise (Zoladz et al. 2010). Our group has shown that inflammatory stimuli such as LPS can reduce brain BDNF (Park et al 2011).

**Statistical Analysis.** Data were analyzed using SPSS v18 (Chicago, IL). All data were tested for normality using the Shapiro-Wilk test. Data not approximating a normal distribution were logarithmically transformed before parametric statistical analysis. In these instances, we report the non-transformed data in the figures for clarity with F and p values from statistical analysis run on transformed data. Mortality data were analyzed using a Mantel-Cox log-rank test. Intervention induced differences in body weight and fatigability were detected using one-way analysis of variance (ANOVA)(intervention = VWR, Locked, Standard). Intervention induced differences in gene expression were detected using a 3 (VWR, Locked, Standard) x 2 (Saline 0.33 mg/kg LPS) ANOVA. Because our primary objective was to assess exercise induced changes in sickness behavior, we analyzed data at each LPS dosage. We did, however, analyze LPS doses independent of intervention to ensure the selected LPS doses affected sickness behavior in a dose-dependent manner. LPS-induced changes in food and fluid intake, body weight and LMA were analyzed using a similar 3 (housing condition) x 2 (LPS, Saline) ANOVA with repeated measures. When appropriate, differences between treatments at each time point
were determined using the Fisher’s least significant difference post-hoc multiple pairwise comparisons. Data are expressed as mean ± SEM. The alpha level was set at $p \leq 0.05$ and all tests were two-tailed.

4.4 Results

**Effects of VWR on LPS-induced mortality, body weight, and fatigability in aged mice.**

There were no statistically significant pre-intervention differences in body weights between housing conditions ($F_{2,203}=1.42; p=0.239$) (Table 1). VWR mice ran an average of 3.54 km per day (Table 1). There were no statistically significant differences in daily running distance between any of the wheel running groups across LPS dose experiments ($F_{4,61}=1.158; p=0.339$).

VWR mice lost significantly more body weight and epididymal fat compared to Locked and Standard mice (Table 1). Interestingly, mice housed with a Locked wheel lost significantly more body weight (but not epididymal fat) when compared to Standard housed mice ($F_{2,203}=3.58; p=0.00$) (Table 1). To assess VWR-induced improvements in muscle endurance, we subjected cohorts ($n = 8-12$) of mice from each intervention to a forced treadmill exercise test to exhaustion. VWR mice ran the longest before reaching exhaustion, more than doubling the length of time run compared to the Locked and Standard groups ($F_{2,29}=37.18; p=0.00$) (Table 1).

Mortality was observed at all LPS doses in these old mice (Table 2) and there were no differences between VWR, Locked, and Standard groups for any of the LPS doses ($\chi^2 = 3.80; p = 0.15, \chi^2 = 0.18; p = 0.94, \chi^2 = 2.21; p = 0.33, \text{ and } \chi^2 = 2.63; p = 0.27$, for the 0.02, 0.08, 0.16 and 0.33 mg/kg doses, respectively) (Table 2). We did not have enough mice to assess potential intervention-induced differences in mortality, but when comparing mortality across LPS doses,
there was no statistically significant LPS dose-effect, indicating that aged mice are extremely sensitive to LPS independent of LPS dose ($\chi^2 = 4.532; p = 0.21$) (Table 2).

Effects of VWR LPS-induced Sickness Indicators

Food intake. For clarity, we present all saline treated groups in Figure 4a as there were no statistically significant differences between them. As expected, 0.33 mg/kg LPS resulted in a significant reduction in food intake compared to saline injected mice at 24h and 48h post-injection (Figure 4b), but there were no statistically significant differences between groups (time x intervention x treatment interaction: $F_{14, 700} = 1.04; p=0.41$). At the 0.02, 0.08, and 0.16 mg/kg LPS doses, we observed a significant reduction in food intake 24h-post injection, but similar to the 0.33 mg/kg LPS dose, there were no statistically significant differences between groups (time x intervention x treatment interactions: $F_{14, 693} = 0.94, p=0.51$; $F_{14, 672} = 0.74, p=0.74$; $F_{14, 707} = 0.72, p=0.76$ for the 0.02, 0.08 and 0.16 mg/kg doses, respectively) (Figure 1c-e). Comparison across LPS doses revealed statistically significant differences in LPS-induced anorexia, with the 0.02 mg/kg dose reducing food intake to a lesser extent at 24h and 48h compared to all other LPS doses, and the 0.33 mg/kg dose reducing food intake to a greater extent than all other LPS doses at 24h, 48h, and 96h. Food intake returned to baseline levels at 48, 48, 72, 96h for the 0.02, 0.08, 0.16, and 0.33 mg/kg doses, respectively. At the 3 lower doses of LPS, we observed a hyperphagic response once food intake returned to baseline levels (Figures 4c-e).

Fluid intake. For clarity, we present all saline treated groups in Figure 5a as there were no statistically significant differences between them. Like LPS-induced anorexia, all LPS doses significantly reduced fluid intake 24h post-LPS injection, but there were no intervention induced differences between 3 of the doses (time x intervention x treatment interactions: $F_{8, 396} = 0.78$,
p=0.62; F\textsubscript{8,384} =1.84, p=0.068; F\textsubscript{8,408} =0.29, p=0.97 for the 0.02, 0.08 and 0.33 mg/kg doses, respectively) (Figure 5b-e). We did find a 3-way interaction at the 0.16 mg/kg dose (F\textsubscript{8,404} =2.11, p=0.033), but post-hoc analysis revealed that the interaction was not a function of intervention-induced differences in LPS response, but rather fluid intake differences between saline injected groups. More specifically, VWR saline-treated mice demonstrated significantly higher fluid intake at 24h and 48h post-injection, compared to Locked and Standard saline-treated mice. Comparison across LPS doses revealed statistically significant differences in LPS-induced adipsia at 24 and 48h post-injection, with 0.02 mg/kg reducing fluid intake to a lesser extent compared to all other LPS doses at 24h and compared to 0.16 and 0.33 mg/kg doses at 48h. The 0.33 mg/kg dose reduced fluid intake to a greater extent than all other LPS doses at 24h; there were no statistically significant fluid intake differences between the 0.16 and 0.08 mg/kg doses. By 48h, fluid intake had recovered near baseline, and there were no significant differences between LPS doses.

**Body weight loss.** For clarity, we present all saline treated groups in Figure 6a separately from LPS-treated groups. Interestingly, we found that the VWR saline-treated mice had higher body weight when compared to locked and standard housed mice (Figure 6a). We believe this to be because the VWR mice were removed from their wheels thus reducing their daily energy expenditure. All LPS injected mice lost statistically significant amounts of body weight post-LPS (Figure 6b-e). There were no significant differences between groups at the 0.02 and 0.33 mg/kg doses (time x intervention x treatment interactions: F\textsubscript{16,792} =0.56, p=0.91; F\textsubscript{16,800} =1.38, p=0.15 for 0.02, 0.33 mg/kg, respectively) (Figure 6b,e). We did find significant 3-way interactions at the 0.08 and 0.16 mg/kg doses (F\textsubscript{16,768} =2.14; p=0.006 and F\textsubscript{16,808} =2.12; p=0.006 for 0.08 and 0.16 mg/kg, respectively)(Figure 6c,d). Similar to fluid intake, these interactions were not a
function of intervention-induced differences in LPS response, but rather body weight differences between saline injected groups (Figure 6a). Comparison across LPS dose revealed a significant effect, where 0.02 mg/kg LPS reduced body weight to a lesser extent at 8, 24, 48, 72, and 96h compared to all other LPS doses. There were no statistically significant body weight loss differences between all other doses.

**Locomotor activity.** For clarity, we present all saline treated groups in Figure 7a as there were no statistically significant differences between them. We assessed LMA at 8, 24, 48, and 72h post-injection, and as expected we observed a significant LPS-induced decrease in LMA. However, there were no significant effects of housing condition (time x intervention x treatment interactions: $F_{6,294}=0.31, p=0.93; F_{6,285}=0.33, p=0.92; F_{6,300}=0.84, p=0.54; F_{6,297}=0.77, p=0.60$ for 0.02, 0.08, 0.16 and 0.33 mg/kg, respectively) (Figure 7b-e). Comparison across LPS doses indicated that at 8, 24, 48, and 72h post-injection, the 0.02 mg/kg dose induced the smallest reduction in LMA compared to the higher three doses, which were not statistically different from each other at any time-point.

**Effects of VWR on LPS-induced brain gene expression 24 hr post-LPS.**

To corroborate our behavioral data, we investigated whether VWR could mitigate LPS-induced (e.g., 0.33mg/kg) gene expression changes in the brain. LPS administration resulted in a significant increase in brain TNF-α (treatment $F_{1,56}=55.5; p < 0.001$), IL-1β (treatment $F_{1,56}=54.4; p < 0.001$), and IL-10 (treatment $F_{1,56}=23.87; p < 0.001$), but not IL-6 (treatment $F_{1,56}=2.22; p = 0.14$) mRNA in all groups (Figure 8). VWR, as applied in this study, could not attenuate the reduction in brain BDNF mRNA expression (intervention x treatment $F_{2,56}=0.23; p = 0.79$) (Figure 8). There were no intervention main effects or intervention by treatment
interactions (p’s for interactions = 0.43, 0.77, 0.95, 0.98, 0.79 for TNF-α, IL-1β, IL-6, IL-10 and BDNF, respectively) indicating that VWR had no effect on expression of these genes within the brain 24 hr post-LPS. These data support our findings of a lack of effect of VWR on sickness behavior induced by LPS.

**Effects of VWR on LPS-induced peripheral pro-inflammatory cytokine gene expression.**

As peripheral inflammation induces brain inflammation (Dantzer, 2001), we sought to determine if VWR attenuated LPS-induced inflammatory cytokine expression in peripheral tissues. In the spleen (**Figure 9a**), LPS significantly reduced TNFα (treatment F_{1,56} = 109; p < 0.001) and increased IL-1β (treatment F_{1,56} = 17; p < 0.001) and IL-6 (treatment F_{1,56} = 9.5; p < 0.005) mRNA in all groups 24hr post-LPS. There were no intervention main effects or intervention by treatment interactions (p’s for interactions = 0.09, 0.36, and 0.59 for TNF-α, IL-1β, and IL-6 respectively). In the liver (**Figure 9b**), LPS significantly increased TNF-α (treatment F_{1,56} = 18.82; p < 0.001), IL-1β (treatment F_{1,56} = 29.818; p < 0.001), and IL-6 (treatment F_{1,56} = 8.25; p = 0.006) in all intervention groups 24hr post-LPS. There were no intervention main effects or intervention by treatment interactions (p’s for interactions = 0.66, 0.50, and 0.92 for TNF-α, IL-1β, and IL-6 respectively). These data support our brain gene expression and behavior observations.

**4.5 Discussion**

We investigated whether a voluntary wheel running intervention could attenuate exaggerated and prolonged LPS induced sickness behavior in aged mice. Ten weeks of voluntary wheel running induced expected training adaptations including weight and fat loss and increased
forced exercise performance. VWR, locked wheel, and standard housed mice injected with LPS exhibited a significant reduction in food and fluid intake post-injection resulting in weight loss that, depending on LPS dose, did not return to baseline until up to 6 days post-injection. In addition to the anorexic/adipsic-induced weight loss, LPS also induced a significant reduction in locomotor activity. Contrary to our hypothesis, there were no differences in sickness behavior responses to LPS between VWR, Locked, and Standard mice. To further support the lack of an exercise effect on sickness behavior, we calculated correlations between average daily wheel running distance and peak sickness behavior (i.e. 24h body weight, 24h food intake, 24h fluid intake, 8h LMA), and as expected they were not significant. These data indicate that exercise training does not protect aged mice from exaggerated or prolonged sickness behavior in this model.

An understanding of the evolutionary basis of an appropriate sickness response may help reconcile this finding that did not support our a priori hypothesis. Sickness behavior is a critical survival mechanism primitive to all organisms and could be too essential of a response to be affected by exercise training [96]. Several studies have shown inhibiting certain aspects of the sickness response results in decreased survival of infected animals. For example, animals housed in a cold environment or treated with an antipyretic drug display higher mortality rates following infection, suggesting an adequate febrile response is critical for host defense and survival [117]. Additionally, mice gavage-fed to levels of non-infected mice following bacterial infection exhibit reduced survival, indicating the importance of anorexia in the sickness response [34]. The highly coordinated responses of sickness behavior are crucial to organism survival. However, it is unclear whether exaggerated and/or prolonged sickness behavior is detrimental or beneficial for the survival of aged organisms. It could be hypothesized that due to immunosenescence and
the longer time that it takes an aged host to clear infection [118, 119], a prolonged sickness response in the aged may be actually benefit recovery.

To examine if exercise affected brain pro-inflammatory cytokine expression independently of sickness behavior, we analyzed brain TNF-α, IL-1β, IL-6 and IL-10 gene expression 24h post LPS injection. We observed an LPS-induced upregulation of TNF-α, IL-1β and IL-10 gene expression in the brain, but no upregulation of IL-6. Furthermore, VWR intervention did not reduce the LPS-induced expression of TNF-α, IL-1β, or IL-10 in the brain, corroborating our negative sickness behavior findings.

Peripheral cytokines act through various communication pathways to induce a ‘mirror image’ of cytokine expression within the brain (Dantzer & Kelley, 2007; Dantzer et al., 2008). Because acute exercise studies have demonstrated inhibition of LPS-induced TNF-α peripherally [120, 121], we investigated if 10 wks of VWR could reduce peripheral proinflammatory cytokine gene expression in response to LPS. As in the brain, we found no differences in LPS-induced cytokine gene expression in the spleen and liver of VWR, Locked, and Standard housed mice when measured 24h after LPS injection.

While we observed no effects of VWR on pro-inflammatory cytokine expression in the brain or periphery, we did find an interesting disconnect between the brain and the periphery. At 24h post-injection, we failed to see a LPS-induced up-regulation of IL-6 in the brain, whereas IL-6 was the highest expressed cytokine in the periphery. The most logical explanation for this is the temporal course of cytokine expression after immune challenge, where TNF-α and IL-1β rapidly increase, followed later by an increase in IL-6. Our data suggest central cytokine gene expression lags behind peripheral cytokine gene expression following i.p. LPS, and supports previous work demonstrating that brain IL-6 is not necessary for the observed sickness response
While we cannot speculate if IL-6 would demonstrate a VWR x LPS interaction at a later time-point, it is intriguing to propose it could be the case, given the finding by Funk et al who demonstrated VWR-induced IL-6 in the brain is responsible for neuronal protection following inflammatory insult [123].

While several studies have demonstrated that acute exhaustive or prolonged exercise can reduce inflammatory responses to LPS in mice (Tanaka et al., 2010) or humans (Starkie et al., 2003), the effects of exercise training on LPS-induced inflammation and sickness behavior are sparse, controversial, and difficult to interpret due to differences in a number of variables including animal species, LPS dosage and route of administration, and exercise training modality and duration. Chen et al. [108] found 4 weeks of treadmill exercise training attenuated septic responses (including arterial pressure, neutrophil count, creatinine, blood urea nitrogen, blood liver enzymes) and reduced plasma TNF-α and IL-1β in response to LPS (10 mg/kg i.v.). In Type 1 diabetic rats, 3 weeks of treadmill exercise training increased survival time and reduced serum TNF-α in response to LPS (15 mg/kg i.v.) when compared to sedentary controls [124]. In contrast, Rowsey et al [112] found that 8 weeks of exercise training increased the febrile response to LPS (0.05 mg/kg i.p.) in rats while having no effect on locomotor activity, while Criswell et al [110] found that 12 weeks of treadmill training increased serum TNF-α and β-glucuronidase activity (5 mg/kg i.p.). In hamsters administered LPS (0.01 mg/kg i.p.), there were no effects seen on serum IL-6 or febrile responses [109]. In regard to brain inflammation, Wu et al [113] demonstrated that 5 weeks of moderate treadmill running attenuated LPS-induced reductions in BDNF, its receptor TrkB, and alleviated LPS-induced cognitive dysfunction albeit not by reducing TNF-α and IL-1β in the hippocampus. Similar protective exercise effects, independent of inflammation within the brain, were seen with LPS-induced dopaminergic neuron
loss in a model of Parkinson’s Disease [114]. In contrast, Nickerson et al. found VWR increased hypothalamic, pituitary, and dorsal vagal IL-1β protein in response to *E. coli*, while reducing circulating IL-1β, suggesting a training-induced disconnect between peripheral and central inflammatory responses [125]. Caution should be taken directly comparing the above-cited studies, as numerous reports have demonstrated differential peripheral and central training adaptations between treadmill training and voluntary wheel running [126, 127].

Much of the recent work on exercise’s neuroprotective effects have focused on BDNF as a primary mediator [128]. BDNF is a neurotrophin that acts primarily in the hippocampus, cortex, and basal forebrain to promote neurogenesis and synaptic plasticity. Inflammatory stimuli, such as LPS, reduce BDNF via an IL-1β dependent mechanism causing suppressed neurogenesis, neuron survival, and synaptic plasticity [62]. Numerous studies have demonstrated exercise training, in particular VWR, prevents the decrease in hippocampal BDNF and attenuates neuronal damage and cognitive impairment following inflammatory injury [113, 114]. Barrientos et al. elegantly demonstrated VWR protected aged rats from *E. coli*-induced cognitive impairments, and this was mediated by a reduction in hippocampal IL-1β and thus protection of BDNF [11]. *Ex vivo* stimulation of microglial from VWR rats revealed reduced sensitivity to LPS, suggesting a potential mechanism for VWR induced neuroprotection. Unfortunately, Barrientos et al. did not measure sickness behavior, and it is difficult to interpret their results in the context of our study paradigm (i.e. *E. coli* vs. LPS; cognitive vs. somatic). Indeed, little evidence supports a role for BDNF in the sickness response indicating exercise-induced protection of BDNF would unlikely influence sickness behavior. We found an LPS-induced reduction in whole brain BDNF, but no protective effect of wheel running on sickness behavior, demonstrating the exercise-induced up-regulation of BDNF shown in the literature is not a global
event but rather a spatially dependent phenomenon predominately observed in the hippocampus. Although no studies have directly compared sickness behavior and cognition in exercise-trained animals, the data reporting beneficial effects of exercise on cognition have used working memory tasks, which are primarily hippocampal-dependent, whereas locomotor activity and sickness behavior are mediated by multiple brain regions including the hypothalamus, hippocampus, amygdala, and prefrontal cortex [96]. These observations support our hypothesis that appropriate sickness behavior is necessary for survival and is a robust stimulus that can’t be affected by exercise training.

While our study clearly demonstrates no effect of voluntary wheel running on LPS-induced sickness behavior in aged mice, we recognize certain limitations. Our study design did not assess brain and peripheral cytokine gene expression at all LPS doses or across numerous time-points. We chose to assess brain and peripheral tissue cytokine gene expression at 24h post-0.33mg/kg LPS injection, based on observations by Godbout et al. demonstrating a clear age-related difference in brain pro-inflammatory cytokine gene expression and sickness behavior between young and aged mice at that time point and dose [2]. Interestingly, our brain IL-6 gene expression data conflicts Godbout et al., who observed a robust increase 24h post-LPS injection. We speculate this is due to different mouse strains, as Godbout et al. used Balb/c mice, which are more sensitive to endotoxin compared to C57bl/6 mice, and thus, would be expected to exhibit a more robust cytokine upregulation (Silvia et al., 1990). Because we did not conduct a dose and time-course analysis of pro-inflammatory cytokine gene expression, we cannot definitively conclude that VWR had no effects on cytokine gene expression, and is possible subtle inflammatory effects may persist in the brain that are not reflected by behavioral measures. However, given that we performed the behavioral experiments first and there were no observed
VWR-induced changes in sickness behavior (our primary outcome), measuring cytokine gene expression at all LPS doses or across numerous time-points would be neither beneficial nor a worthwhile use of resources. Additionally, whole brain IL-1β mRNA may not be the best measure given potential differences between IL-1β mRNA and protein levels due the role of the inflammasome and pro-IL-1β cleavage. However, as stated above, because we found no differences in LPS-induced sickness behavior, we decided not to pursue IL-1β protein expression as we believe it contributes little to the overall findings of the study. Lastly, removing VWR mice from their wheels prior to LPS administration may be perceived as stressful to these animals. However, in order to correctly interpret our exercise training data without the influence of acute wheel running, this was necessary.

In conclusion, we demonstrate that 10 weeks of voluntary wheel exercise training does not affect the LPS-induced exaggeration and prolongation of sickness behavior in aged mice, nor does it have any effect on LPS-induced pro-inflammatory cytokine gene expression in the brain or periphery 24h post 0.33mg/kg LPS injection. These data indicate that a sickness response (even if prolonged and exaggerated as it is in elderly) is likely important for survival and uninfluenced by prior exercise activity.
4.6 Figure Legends, Tables, and Figures

Figure 4. Effects of VWR on LPS-induced changes in food intake in aged mice. LPS administration (a: Saline, b: 0.02 mg/kg LPS, c: 0.08 mg/kg LPS, d: 0.16 mg/kg LPS, and e: 0.33 mg/kg LPS) resulted in significant (*) reductions in food intake, but there were no intervention main effects or intervention by treatment interactions at any LPS dose. Mean ± sem; n = 23-28 for Saline groups, and n=8-13 for all LPS groups.

Figure 5. Effects of VWR on LPS-induced changes in fluid intake in aged mice. LPS administration (a: Saline, b: 0.02 mg/kg LPS, c: 0.08 mg/kg LPS, d: 0.16 mg/kg LPS, and e: 0.33 mg/kg LPS) resulted in significant (*) reductions in fluid intake, but there were no intervention main effects or intervention by treatment interactions at any LPS dose. Mean ± sem; n = 23-28 for saline groups, and n=8-13 for all LPS groups.

Figure 6. Effects of VWR on LPS-induced changes in body weight in aged mice. LPS administration (a: Saline, b: 0.02 mg/kg LPS, c: 0.08 mg/kg LPS, d: 0.16 mg/kg LPS, and e: 0.33 mg/kg LPS) resulted in significant (*) reductions in body weight, but there were no intervention by treatment interactions at any LPS dose. VWR saline-treated mice exhibited significantly higher post-injection body weights at 8h compared to both Locked (*) and Standard saline-treated mice (^) at 72, 96, and 168h. Mean ± sem; n = 23-28 for saline groups, and n=8-13 for all LPS groups.

Figure 7. Effects of VWR on LPS-induced reductions in locomotor activity (line crosses) in aged mice. LPS administration (a: Saline, b: 0.02 mg/kg LPS, c: 0.08 mg/kg LPS, d: 0.16 mg/kg LPS, and e: 0.33 mg/kg LPS) resulted in significant (*) reductions in locomotor activity in all intervention groups, but there was no intervention main effect or intervention by treatment interaction at any LPS dose. Mean ± sem; n = 23-28 for saline groups, and n=8-13 for all LPS groups.

Figure 8. Effect of VWR on LPS-induced brain cytokine and BDNF mRNA expression 24h post-injection. LPS administration (0.33 mg/kg i.p.) resulted in significant (*) up-regulation of TNFα, IL-1β, and IL-10 and a significant (*) down-regulation of BDNF. There were no significant intervention main effects or intervention by treatment interaction effects for any gene measured. IL-6 mRNA expression was unaffected by LPS or VWR. Mean ± sem; n = 8-14/group.

Figure 9. Effect of VWR on LPS-induced peripheral tissue inflammatory gene expression in (a) spleen, and (b) liver 24h post-injection. LPS administration (0.33 mg/kg i.p.) resulted in significant (*) upregulation of IL-1β and IL-6 in spleen and liver. TNF-α mRNA was significantly (*) elevated in response to LPS in liver and significantly (*) reduced in spleen. Mean ± sem; n = 8-14/group.
### Table 1. Intervention-induced adaptations and body composition changes.
Mean ± SEM. *a* Significantly (p<0.05) reduced compared to Locked-Wheel and Standard housing conditions. *b* Significantly different compared to Standard housing condition.

<table>
<thead>
<tr>
<th></th>
<th>Distance Run (km/d)</th>
<th>Pre-Intervention Body Weight (g)</th>
<th>Post-Intervention Body Weight (g)</th>
<th>Body Weight Change (%)</th>
<th>Forced Exercise Time-to-Fatigue (minutes)</th>
<th>Epididymal Adipose Weight (% Body Weight)</th>
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<tr>
<td>Voluntary Wheel</td>
<td>3.54 ± 0.22</td>
<td>34.78 ± 0.38</td>
<td>32.35 ± 0.29</td>
<td>-6.88 ± 0.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>82.89 ± 5.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.46 ± 0.16&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Locked Wheel</td>
<td>----</td>
<td>34.53 ± 0.35</td>
<td>33.38 ± 0.28</td>
<td>-3.42 ± 0.47&lt;sup&gt;b&lt;/sup&gt;</td>
<td>38.27 ± 3.00</td>
<td>2.10 ± 0.28</td>
</tr>
<tr>
<td>Standard</td>
<td>----</td>
<td>33.75 ± 0.45</td>
<td>33.15 ± 0.34</td>
<td>-332 ± 0.57</td>
<td>26.90 ± 3.36</td>
<td>2.16 ± 0.28</td>
</tr>
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</table>

### LPS Dose

<table>
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<th>Saline</th>
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<th>0.08 mg/kg</th>
<th>0.16 mg/kg</th>
<th>0.33 mg/kg</th>
</tr>
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<tbody>
<tr>
<td>Voluntary Wheel</td>
<td>0/28</td>
<td>2/10 (24h, 48h)</td>
<td>2/10 (72h, 96h)</td>
<td>2/10 (120h, 120h)</td>
<td>1/11 (72h)</td>
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<tr>
<td>Locked Wheel</td>
<td>0/28</td>
<td>0/9</td>
<td>2/11 (24h, 96h)</td>
<td>0/10</td>
<td>3/13 (72h, 96h, 120h)</td>
</tr>
<tr>
<td>Standard</td>
<td>0/27</td>
<td>0/9</td>
<td>2/8 (24h, 120h)</td>
<td>1/11 (120h)</td>
<td>4/11 (8h, 8h, 24h, 96h)</td>
</tr>
</tbody>
</table>

### Table 2. Mortality in aged mice across intervention and LPS dose.
Numbers represent: # deaths/ # total mice in group; time of each death.
Figure 4.
Figure 5.
Figure 6.
Figure 7.
Figure 8.
Figure 9.

(a) Spleen

(b) Liver
CHAPTER 5

VOLUNTARY WHEEL RUNNING DOES NOT AFFECT LIPOPOLYSACCHARIDE-INDUCED DEPRESSIVE-LIKE BEHAVIOR IN YOUNG ADULT AND AGED MICE

5.1 Abstract

Peripheral stimulation of the innate immune system with lipopolysaccharide (LPS) causes prolonged depressive-like behavior in aged mice that is dependent on indoleamine 2,3-dioxygenase (IDO) activation. Regular moderate intensity exercise training has been shown to exert neuroprotective effects that might reduce depressive-like behavior in aged mice. The purpose of this study was to test the hypothesis that voluntary wheel running would attenuate LPS-induced depressive-like behavior and brain IDO gene expression in 4-month-old and 22-month-old C57BL/6J mice. Mice were housed with a running wheel (Voluntary Wheel Running, VWR) or no wheel (Standard) for 30 days (young adult mice) or 70 days (aged mice), after which they were intraperitoneally injected with LPS (young adult mice: 0.83 mg/kg; aged mice: 0.33 mg/kg). Young adult VWR mice ran on average 6.9 km/day, while aged VWR mice ran on average 3.4 km/day. Both young adult and aged VWR mice increased their forced exercise tolerance compared to their respective Standard control groups. VWR had no effect on LPS-induced anorexia, weight-loss, increased immobility in the tail suspension test, and decreased sucrose preference in either young adult or aged mice. Four (young adult mice) and twenty-four (aged mice) hours after injection of LPS transcripts for TNF-α, IL-1β, IL-6, and IDO were upregulated in the whole brain independently of VWR. These results indicate that prolonged...
physical exercise has no effect on the neuroinflammatory response to LPS and its behavioral consequences

5.2 Introduction

Major Depressive Disorders (MDD) are a multi-factorial set of psychiatric disorders with varying symptomatology and presumably, etiologies. Common symptoms include: depressed mood, reduced energy, loss of pleasure (anhedonia), dysregulated sleep and appetite, and feelings of guilt and/or suicide. The worldwide prevalence of depressive disorders is ~15% in adults, and upwards of ~ 20% in adults over the age of 65. [47, 129, 130]. Further compounding the age-associated risk of MDD, older adults exhibit longer durations of depressive symptoms compared to young adults, and often suffer less dramatic depressive symptoms that go undiagnosed as merely a “consequence” of aging, yet significantly reduce social interactions and quality of life [45, 131, 132].

The mechanisms of age-related depression are not fully elucidated, but several lines of evidence indicate inflammation can be a driving factor [39]. Aging is associated with chronic low-level inflammation, a condition known as ‘inflammaging’ (Franceschi, 2007). Indeed, apparently healthy subjects >65 years old exhibit significantly elevated circulating C-reactive protein (CRP) levels compared to healthy young adults [133, 134]. The relationship between chronic inflammation and increased prevalence of depression is highlighted by the strong correlations between symptoms of depression and CRP and IL-6 levels in older adults [133, 134]. In addition to inducing a chronic low-level of systemic inflammation, aging sensitizes the brain to subsequent immune stimuli such as infections and inflammatory stimuli [48]. Microglia cells from aged mice are primed in the sense they exhibit exaggerated and prolonged pro-
inflammatory cytokine production following stimulation with lipopolysaccharide (LPS). This age-associated priming results in numerous behavioral consequences [48]. For example, peripheral injection of LPS lengthens the duration of sickness behavior (i.e. anorexia, weight loss, lethargy) in aged mice compared to young adult mice, and this is associated with an exaggerated and prolonged up-regulation of brain IL-1β, TNF-α, and IL-6 [59, 94].

Aged mice also exhibit greater sensitivity to inflammation-induced depressive-like behavior, which is related to the activity of the tryptophan degrading enzyme indoleamine 2,3 dioxygenase (IDO). IDO is the initial and rate-limiting enzyme of tryptophan catabolism through the kynurenine pathway [39, 41]. Activation of IDO is a required step in the development inflammation-induced depressive-like behavior [37, 43, 44]. Aging is associated with prolonged duration of IDO activation in response to inflammatory stimuli, resulting in protracted depressive-like behavior [58]. The same results are obtained in aged mice inoculated with Bacillus Calmette-Guérin (Kelley et al., 2013).

The increased prevalence and burden of MDD is partially due to suboptimal treatment options [95]. The current protocol for treating age-related depression involves antidepressant therapies (e.g. tricyclic antidepressants and serotonin-selective reuptake inhibitors), which are moderately effective, but do not completely address the underlying inflammatory pathophysiology. Furthermore, a significant percentage of patients are non-responders to currently available drugs [135]. Thus, there continues to be a major need to identify safe, alternative approaches for treating depression and reducing inappropriate neuroinflammation. Regular, moderate-intensity aerobic exercise may be one approach to alleviate depressive symptom burden in older adults. The mood enhancing benefits of cardiovascular exercise have been well documented in the literature and through the anecdotal testimony of thousands of
exercisers. Cross-sectional studies demonstrate an association between a high amount of physical activity and a low amount of depressive symptoms in both middle-aged and aged populations [72]. For example, we found that depressive symptoms were related to low physical activity, low aerobic fitness, high CRP, and adiposity in elderly women [70]. Furthermore, physical exercise might be especially efficient in reducing depressive symptoms among patients with mild to moderate depression, which is important because a large fraction of older adults (10-20%) have clinically significant depressive symptoms that do not meet the criteria for major depression [136]. It is plausible that exercise interventions could be utilized in these individuals, in the absence of pharmacological therapy, to mitigate depressive symptoms. Sjosten and Kivela (2006) conducted a meta-analysis of published randomized clinical trials to determine the strength of an effect of regular exercise on depression in older adults, and concluded exercise was effective in treating depression among those suffering from minor or major depression and reducing depressive symptoms in those with significant depressive symptoms at baseline [72].

There are many unsubstantiated but potential ways in which exercise may improve mood and reduce depressive symptoms, including increased brain derived neurotrophic factor (BDNF), insulin growth factor (IGF), hippocampal neurogenesis, and anti-inflammatory effects [90, 137-140]. Unfortunately, no studies have examined whether exercise training can attenuate inflammation-induced depressive like behavior in young adult and aged mice using a defined inflammatory stimulus.

Therefore, we sought to examine the influence of voluntary wheel running (VWR) on LPS-induced depressive-like behavior and IDO activation in young adult mice and aged mice. We hypothesized that VWR would induce anti-inflammatory effects and attenuate LPS-induced
depressive-like behavior and brain proinflammatory cytokine and IDO gene expression in both young adult and aged mice.

5.3 Materials and Methods

Due to significant phenotypic differences between young adult and aged mice, such as the training duration necessary to induce metabolic adaptations and their endogenous sensitivity to LPS [58-60, 141], and the fact that the young adult and aged mice were obtained from two different sources, we conducted two separate and different experiments in the young and aged mice. Therefore, the data from the young adult mice and aged mice cannot be directly compared. Our purpose for this study design was not to examine the aging effect, which is already well documented [58-60], but rather to determine whether regular exercise can attenuate LPS-induced depressive like behavior under the optimal experimental conditions for each age category. For this paper, we designate these two experiments as Experiment 1 (young [4 months] adult mice) and Experiment 2 (aged [19 months] mice).

Animals

All experiments were conducted under the guidelines of the University of Illinois, Urbana-Champaign Institutional Animal Care and Use Committee.

Experiment 1 (Young Adult Mice): Four month-old male C57BL/6J mice (N=40) were obtained from the Jackson Labs (Bar Harbor, Maine), and singly housed in cages with corn-cob bedding in a temperature (23°C) and humidity (45-55%) controlled environment with a 12h dark/light cycle (lights off 0900-2100). Mice were allowed ad libitum access to food and water
for the entire duration of the study and were given 2 weeks to acclimate to the housing conditions prior to study commencement.

**Experiment 2 (Aged Mice):** Nineteen month-old C57BL/6 mice (N=40) were obtained from the National Institute of Aging (Bethesda, MD) singly housed in cages with corn-cob bedding in a temperature (23°C) and humidity (45-55%) controlled environment with a 12h dark/light cycle (lights off 0900-2100). Mice were allowed *ad libitum* access to food and water for the entire duration of the study and were given 2 weeks to acclimate to the housing conditions prior to study commencement.

**Voluntary Wheel Training**

**Experiment 1 (Young Adult Mice):** Following acclimation, mice were randomized to a voluntary wheel running (VWR) or ‘normal’ (Standard) housing condition for a duration of 30 days, which is sufficient time to induce training associated metabolic adaptations (unpublished data). The VWR mice were individually housed in plexiglass cages (36 L × 20 W × 15 H cm) that contained a stainless steel running wheel (diameter 22.86 cm) (Mini-Mitter Repironics, Bend, OR). Wheel revolutions were relayed via telemetry to a computer in the facility. Standard mice were housed in smaller cages (30 L × 19 W × 13 H cm) without any type of environmental enrichment.

**Experiment 2 (Aged Mice):** Exercise training adaptations occur more slowly in aged mice compared to younger mice due to the lower volume of daily running and metabolic differences (Derbre et al., 2012). Therefore, aged mice were randomized to VWR or Standard housing condition for a duration of 70 days, which is sufficient time to induce training associated
metabolic adaptations in aged mice [94]. All other aspects of the intervention were identical to Experiment 1.

**Treadmill Running Test**

**Experiment 1 (Young Adult Mice):** To assess VWR-induced training adaptations, we measured forced exercise fatigability on day 26 of the 30-day VWR intervention. Mice ran until exhaustion on a motor-driven treadmill at 5% grade at gradually increasing speeds from 10-26 m/min. Exhaustion was defined as the point at which mice refused to run despite prompting by mild prodding with the hand for a period of 10 s; electric shock was not used in this test.

**Experiment 2 (Aged Mice):** To assess VWR-induced training adaptations in aged mice, we measured forced exercise fatigability on day 65 of the 70-day VWR intervention. Mice ran until exhaustion on a motor-driven treadmill at gradually increasing speeds from 6-21m/min. As above, exhaustion was defined as the point at which the mouse refused to run despite prompting by mild prodding with the hand for a period of 10 s; electric shock was not used in this test.

**LPS administration**

Given that aged mice exhibit a greater sensitivity to LPS-induced neuroinflammation and behavioral disturbances [58-60], we used different LPS doses for Experiments 1 and Experiment 2. Based upon our preliminary data (not shown), both doses represent the minimum effective dose capable of inducing depressive-like behavior in the respective age cohorts of C57bl/6 mice. As rationale, we reasoned that if an exercise effect would be found it would be found at the minimum effective dose of LPS.
**Experiment 1 (Young Adult Mice):** After 30 days of VWR training, mice were randomized and injected intraperitoneally (i.p.) with saline or *Escherichia coli* LPS (lot 3129, serotype 0127:B8, Sigma) at a dose of 0.83 mg/kg. To control for any effects of acute wheel exercise on LPS response, we injected mice 11 h after the end of their dark cycle on the final day of training. Thus, injection occurred during the final hour of the proceeding light cycle, a time-period where very little VWR occurs. Following injection, mice were placed back into their respective home cages.

**Experiment 2 (Aged Mice):** Following 70 days of VWR training, aged mice were randomized and injected intraperitoneally (i.p.) with saline or *Escherichia coli* LPS (lot 3129, serotype 0127:B8, Sigma) at a dose of 0.33 mg/kg. As above, acute effects of wheel exercise were controlled for by injecting mice with LPS 11h after the end of their dark cycle on the final day of training.

**Measurement of sickness response.**

To ensure LPS had the intended effect, physiological sickness responses were assessed by changes in body weight and food intake. Decreased food intake and the resulting body mass loss are sensitive measures of sickness in animals, and dependent on pro-inflammatory cytokine expression [14].

**Experiment 1 (Young Adult Mice):** Body weight and food intake were measured at 24 h post-injection and calculated as the change from pre-injection baseline.

**Experiment 2, (Aged Mice):** Body weight and food intake were measured at 24 h and 48 h post-injection and calculated as the change from pre-injection baseline.
Measurement of depressive-like behavior

Tail Suspension Test: Given that aged mice exhibit prolonged depressive-like behavior compared to young mice [58], we measured TST immobility at 24 h post-injection in Experiment 1, and at 48 h post-injection in Experiment 2. The tail suspension test (TST), a standardized test of depressive-like behavior in which depression is inferred from increased duration of immobility, was conducted as previously described [43]. Briefly, mice were taken from their home cage and hung from their tail on a hook connected to a strain gauge for a period of 6 minutes (360 sec). A computerized system for processing the force exerted on the gauge (Mouse Tail Suspension Package, MED-TSS-MS; Med Associates, St Albans, VT, USA) collected and analyzed the movements of each individual mouse. An immobility threshold was determined by establishing an activity level that would exclude all movements and only encompass immobility. Time below this threshold indicated the time of immobility.

Sucrose Preference Test: Anhedonia is a key component of depression, and can be measured in mice by their preference to consume a sweetened solution. A sucrose preference test was performed following a 3 day acclimation program during the 24h period following LPS administration. As LPS-induced decreased sucrose preference resolves by 24h in both young adult and aged mice (unpublished data), this was the only time period measured for both Experiment 1 and Experiment 2. Mice were presented with two identical volumetric drinking tubes containing either water or a 1% sucrose solution. Drinking tubes were weighed before and after the 24h period to measure fluid consumption of each respective solution. Drinking tube position (right v. left) was alternated each training period to reduce potential bias from place
preference. Sucrose preference was calculated using the following formula: [Sucrose intake / (water intake + sucrose intake)] × 100.

**Tissue Collection**

**Experiment 1 (Young Adult Mice):** A separate group of mice underwent an identical training intervention and LPS treatment as Experiment 1, but instead of behavioral testing mice were killed by CO₂ exposure at 4 h post-injection for tissue collection to examine pro-inflammatory cytokine and IDO gene expression. This time-point was chosen based on previous data indicating it as the peak of pro-inflammatory cytokine and IDO gene expression [142]. Tissues were dissected out after transcardial perfusion with ice-cold PBS saline.

**Experiment 2, (Aged Mice):** A separate group of mice underwent an identical training intervention and LPS treatment as Experiment 2, but instead of behavioral testing mice were killed by CO₂ exposure at 24 h post-injection for tissue collection to examine pro-inflammatory cytokine and IDO gene expression. This time-point was chosen based on data by Godbout et al. indicating it as the critical time-point of prolonged pro-inflammatory cytokine and IDO gene expression, differentiating the aged and young adult neuroinflammatory response [58].

**RNA extraction and reverse transcription and Real-Time RT-PCR**

Total RNA from whole brain was extracted with Qiagen RNeasy Mini Kits (Valencia, CA). Reverse transcription reactions were completed in an Eppendorf Mastercycler Thermocycler (Hamburg, Germany) using an Applied Biosystem (Foster City, CA) High Capacity reverse transcriptase kit with 2,000 ng total RNA and random primers for each reaction. Quantitative real-time reverse transcription PCR was performed on an Applied Biosystems Prism 7900 using
TaqMan gene expression assays for TNF-α (Mm0043258_m1), IL-1β (Mm00434228_m1), IL-6 (Mm00446190_m1), IL-10 (Mm00439616_m1), BDNF (Mm01334042_m1), IDO (Mm00492586_m1) and glyceraldehyde 3-phosphate dehydrogenase (Mm999999_g1) purchased from Applied Biosystems (Foster City, CA). Reactions were performed in duplicate according to the manufacturer’s instructions. Relative quantitative measurement of target gene expression was conducted using the ΔΔCt method with glyceraldehyde 3-phosphate (GAPDH) as the endogenous house-keeping gene and Standard+saline treated mice were used as the referent group. We chose to analyze TNF-α, IL-1β, IL-6, IL-10, and IDO because they are critical mediators of inflammation-induced sickness and depressive-like behavior in mice [2, 96, 97]. BDNF is a critical neurogenic and anti-inflammatory growth factor that is highly influenced by exercise [143]. Our group has shown that inflammatory stimuli such as LPS can reduce brain BDNF [87, 88].

Statistical Analysis

Data from Experiment 1 and Experiment 2 were analyzed independently using SPSS v18 (Chicago, IL). All data were normally distributed as determined by the Shapiro-Wilk test. Exercise-induced differences in body weight and fatigability were detected using an independent sample t-test (VWR versus Standard). Exercise-induced differences in behavioral and gene expression responses to LPS were detected using a 2 (VWR vs. Standard) x 2 (Saline vs LPS) ANOVA with repeated measures when necessary. Any reference to “groups” implies the following: (VWR+Saline, VWR+LPS, Standard+Saline, Standard+LPS). Data are expressed as mean ± SEM. The alpha level was set at p ≤ 0.05 and all tests were two-tailed. When
appropriate, between-group differences were determined using Fisher’s least significant difference post-hoc multiple pairwise comparisons.

5.4 Results

Effects of VWR on body weight and fatigability

Experiment 1 (Young Adult Mice): There were no statistically significant differences in baseline body weights between exercise conditions (t_{36}=-0.31, p=0.76) (Table 3). Young adult VWR mice ran an average of 6.91 ± 0.25 km per day (Table 3), and there were no differences in daily running distance between young adult VWR+Saline and young adult VWR+LPS groups (t_{18}=1.4, p=0.18). As a result of increased energy expenditure due to VWR, young adult VWR mice gained less body weight compared to young adult Standard mice (time x exercise: F_{1,36}=15.8, p=0.00) (Table 3). There were no differences in body weight change (t_{18}=-0.06, p=0.96) between VWR+Saline and VWR+LPS mice. To assess VWR-induced improvements in muscle endurance, we subjected all young adult mice to a forced treadmill exercise test to exhaustion. Young adult VWR mice ran the longest before reaching exhaustion, running approximately 50% longer than young adult Standard mice, indicating that the 30-day VWR protocol induced the expected metabolic adaptations responsible for improved endurance performance (intervention: t_{33}=-2.7, p=0.01) (Table 3). There were no differences in forced exercise performance (t_{18}=-0.80, p=0.44) between young adult VWR+Saline and young adult VWR+LPS mice.

Experiment 2 (Aged Mice): There were no statistically significant differences in baseline body weights between exercise conditions (t_{33}=-0.24, p=0.81) (Table 3). Aged VWR mice ran an
average of 3.40 ± 0.40 km per day (Table 3), and there were no differences in daily running distance between aged VWR+Saline and aged VWR+LPS groups (t₁₆=-0.98, p=0.33). Aged VWR mice lost more body weight compared to aged Standard mice (time x exercise: F₁,₃₃=15.1, p=0.00) (Table 3), and ran significantly longer before reaching exhaustion, indicating the efficacy of the 70 d VWR protocol (intervention: t₃₃=-4.7, p=0.00) (Table 3). There were no differences in body weight change (t₁₆=0.64, p=0.53) or forced exercise performance (t₁₆=0.92, p=0.37) between aged VWR+Saline and aged VWR+LPS mice.

**Effects of VWR on LPS-induced brain gene expression**

**Experiment 1 (Young Adult Mice):** We investigated whether VWR could mitigate LPS-induced gene expression changes in the brains of young mice 4 h post-injection (Figure 10a). LPS administration resulted in a significant increase in whole brain TNF-α, IL-1β, IL-6, and IFN-γ mRNA (treatment: F₁,₃₂=100.0, p=0.00; F₁,₃₂=76.7, p=0.00; F₁,₃₂=60.8, p=0.00; F₁,₃₂=32.6, p=0.00 for TNF-α, IL-1β, IL-6, and IFN-γ, respectively), which was not attenuated by VWR (intervention x treatment interaction: F₁,₃₂=0.54, p=0.47; F₁,₃₂=0.69, p=0.41; F₁,₃₂=0.00, p=0.99; F₁,₃₂=0.89, p=0.35 for TNF-α, IL-1β, IL-6, and IFN-γ, respectively) (Figure 1e). Similarly, LPS significantly upregulated IDO gene expression (treatment: F₁,₃₂=27.3, p=0.00), which was also not altered by VWR (intervention x treatment: F₁,₃₂=1.2, p=0.28). VWR, as applied in this study, could not attenuate the LPS-induced reduction in brain BDNF mRNA expression (intervention x treatment F₁,₃₂=1.98; p = 0.17). There were no intervention main effects (p’s for intervention main effects = 0.50, 0.41, 0.99, 0.28, 0.99, and 0.36 for TNF-α, IL-1β, IL-6, IDO, IFN-γ, and BDNF, respectively). These data support our findings of a lack of effect of VWR on sickness and depressive-like behavior induced by LPS in young adult mice.
**Experiment 2 (Aged Mice):** At 24h post-injection, LPS administration resulted in a significant increase in whole brain TNF-α, IL-1β, and IL-6 mRNA, (treatment: F\(_{1,36}=36.2\), p=0.00; F\(_{1,36}=46.3\), p=0.00; F\(_{1,36}=9.2\), p=0.005 for TNF-α, IL-1β, and IL-6, respectively), none of which were attenuated by VWR (intervention x treatment interaction: F\(_{1,36}=0.63\), p=0.43; F\(_{1,36}=0.30\), p=0.59; F\(_{1,32}=0.07\), p=0.80 for TNF-α, IL-1β, and IL-6, respectively) (**Figure 11a**). LPS significantly upregulated brain IDO (treatment: F\(_{1,36}=12.0\), p=0.01), which was not affected by VWR (intervention x treatment: F\(_{1,36}=0.004\), p=0.98). LPS did not significantly increase whole brain IFN-γ at 24h post-injection (treatment: F\(_{1,36}=1.6\), p=0.21). Additionally, VWR, as applied in this study, could not attenuate the LPS-induced reduction in brain BDNF mRNA expression (intervention x treatment F\(_{1,36}=376\); p = 0.54). There were no intervention main effects (p’s for intervention main effects = 0.74, 0.58, 0.68, 0.95, and 0.51 for TNF-α, IL-1β, IL-6, IDO, and BDNF, respectively). Interestingly, however, there was a main effect for IFN-γ, in that VWR training reduced whole brain IFN-γ mRNA expression (intervention: F\(_{1,36}=4.34\), p=0.04).

Collectively, these data support our findings of a lack of effect of VWR on sickness and depressive-like behavior induced by LPS in aged mice.

**Effects of VWR on LPS-induced sickness responses**

**Experiment 1 (Young Adult Mice):** As expected, LPS injection resulted in a significant reduction in food intake compared to young adult saline-injected mice at 24 h post-injection (time x treatment: F\(_{1,32}=43.7\), p=0.00), but there were no statistically significant differences between groups (time x exercise x treatment: F\(_{1,32}=0.08\), p=0.78) (**Figure 10b**). There was no difference food intake between young saline-injected VWR and Standard mice (time x intervention: F\(_{1,32}=0.58\), p=0.45). As a result of LPS-induced anorexia, all young adult LPS-
injected mice lost statistically significant amounts of body weight during the 24 h post-LPS (time x treatment: F_{1,32}=36.8, p=0.00), and there were no significant differences between VWR and Standard housed mice in response to LPS (time x intervention x treatment: F_{1,32}=0.19, p=0.67) (Figure 10c). There was no difference in body weight changes between young adult saline-injected VWR and Standard mice (time x intervention: F_{1,32}=0.44, p=0.51).

**Experiment 2 (Aged Mice):** Like young adult LPS-injected mice, aged LPS-injected mice exhibited a significant reduction in food intake at 24 h and 48 h post-injection compared to aged saline-injected mice (time x treatment: F_{2,60}=36.9, p=0.00), but there was no significant effect of VWR training (time x intervention x treatment (F_{2,60}=0.1, p=0.93) (Figure 11b). There was no difference in food intake between aged saline-injected VWR and Standard mice (time x exercise: F_{2,60}=0.43, p=0.65), nor was there an exercise main effect (exercise: F_{1,30}=0.5, p=0.49). Aged LPS-injected mice lost significant body weight compared to aged saline-injected mice (time x treatment: F_{2,60}=36.1, p=0.00), and there were no significant differences between VWR and Standard housed mice in response to LPS (time x exercise x treatment (F_{2,60}=0.03, p=0.97) (Figure 11c). There was no difference in body weight changes between aged saline-injected VWR and Standard mice (time x exercise: F_{2,60}=0.1, p=0.93), nor was there an exercise main effect (intervention: F_{1,30}=0.04, p=0.83).

**Effects of VWR on LPS-induced depressive-like behavior**

**Experiment 1 (Young Adult Mice):** We assessed depressive-like behavior at 24 h post-LPS injection via the tail suspension test and sucrose preference test. LPS significantly increased duration of immobility in the tail suspension test (treatment: F_{1,32}=6.3, p=0.02), but there was no interaction (exercise x treatment interaction: F_{1,32}=0.10, p=0.75) (Figure 10d). There were no
differences in baseline sucrose preference between groups (F3,32=0.15, p=0.93). LPS significantly decreased sucrose preference compared to baseline (time x treatment interaction: F1,32=5.97, p=0.02), but exercise did not affect this (time x exercise x treatment interaction: F1,32=0.18, p=0.67) (Figure 10e). There were no exercise main effects (p’s for exercise main effects = 0.86 and 0.83, for TST and SPT, respectively). Taken together, these data indicate VWR training had no effects on LPS-induced depressive-like behavior in young adult mice.

**Experiment 2 (Aged Mice)**: We assessed depressive-like behavior via the decreased sucrose preference that is apparent at 24h post-injection and the increased duration of immobility that is measured in the tail suspension test at 48h post-injection. LPS significantly increased the duration of immobility (treatment: F1,32=19.2, p=0.00), but there was no interaction (exercise x treatment interaction: F1,32=0.00, p=0.96) (Figure 11d). There were no differences in baseline sucrose preference between groups (F3,34=0.83, p=0.49). LPS significantly decreased sucrose preference compared to baseline (time x treatment interaction: F1,32=8.9, p=0.006), but exercise did not affect this (time x exercise x treatment interaction: F1,32=0.28, p=0.60) (Figure 11e). There were no exercise main effects (p’s for exercise main effects = 0.97 and 0.22, for tail suspension test and sucrose preference tests, respectively). Taken together, these data indicate VWR training had no effects on LPS-induced depressive-like behavior in aged mice.

**5.5 Discussion**

In Experiment 1 we investigated whether a voluntary wheel running intervention could attenuate LPS-induced depressive-like behavior in young adult mice. Thirty days of voluntary wheel running induced the expected training adaptations including reduced weight gain and increased forced treadmill exercise performance. Regardless of treatment, young adult mice
injected with LPS exhibited a significant reduction in food intake, resulting in weight loss at 24 h post injection. Contrary to our hypothesis, the measures of depressive-like behavior did not differ according to the exercise condition. To further support the lack of an exercise effect on LPS-induced depressive-like behavior, we analyzed brain proinflammatory cytokine and IDO gene expression, and found no significant wheel running effects.

Because aging leads to exaggerated and prolonged inflammation and behavioral effects [58-60], in Experiment 2 we investigated whether a voluntary wheel running intervention could attenuate exaggerated and prolonged LPS-induced depressive-like behavior in aged mice. Similar to the effects observed in the young adult mice, the VWR training program induced body weight loss and increased forced exercise performance in aged mice indicating that it had its intended effects. VWR and Standard housed mice injected with LPS exhibited a similar significant reduction in food intake, resulting in body weight loss at 24h and 48h post-injection. These data confirm our previous research demonstrating no beneficial effects of VWR on LPS-induced sickness behavior across multiple LPS doses [94]. Contrary to our hypothesis, there were no differences in LPS-induced depressive-like behavior responses in the tail suspension test and the sucrose preference test between aged VWR and aged Standard mice, and VWR intervention did not attenuate brain IDO and/or proinflammatory gene expression in response to LPS. Taken together, these data show clearly that prolonged voluntary exercise in mice has no effect on the neuroinflammatory and behavioral response to LPS and this lack of effect is the same in both young adults and aged individuals.

A potential explanation for our lack of significant findings could be an insufficient study sample size to detect between group differences. However, we conducted an a priori power analysis (type I and type II errors set at 5% and 80%, respectively) to determine the minimum
sample size necessary to observe a significant intervention x treatment interaction for our most variable measure, sucrose preference. Based upon a thorough investigation of the literature examining the effects of exercise training on depressive-like behavior in rodent models, we determined that we would need 10 mice per group to detect a ~40-50% (effect size ~0.65) exercise-induced difference in the sucrose preference test [144]. Therefore, we believe our data reflect a lack of VWR effect rather than insufficient statistical power. An alternative interpretation of our findings could be that our respective LPS doses were too high and masked any subtle benefits of VWR. However, as stated in the methods section, we utilized the minimum effective LPS dose necessary to induce depressive-like behavior in the respective mouse models. We previously examined the effects of VWR training on LPS-induced sickness behavior at a very low LPS dose (0.02 mg/kg), and observed no protective effects of VWR [94], which lends further credence to the lack of effect within our current experiments.

To the best of our knowledge, this is the first study to utilize a defined inflammatory challenge (i.e., LPS) to examine the effects of exercise training on inflammation-induced depressive-like behavior. Several groups have investigated the effects of exercise training on unpredictable chronic mild stress-induced depressive-like behavior and neuroinflammation, and found promising results. Solberg et al. found 6 weeks of voluntary wheel running of mice in conjunction with six weeks of chronic stress increased sucrose preference and decreased FST immobility compared to mice that were exposed to chronic stress but without prior wheel training [145]. These results were similar to Zheng et al., who demonstrated four weeks of wheel training in conjunction with chronic mild stress recovered sucrose preference, which was associated with decreased hippocampal corticosterone (CORT) expression and increased hippocampal brain-derived neurotrophic factor (BDNF) expression [146]. Exactly how BDNF
may be mediating depressive-like behavior is unclear, but one possibility is that BDNF may be improving survival of neurons affected by chronic stress. Duman et al. proposed an alternative molecular mechanism; these authors discovered four weeks of wheel training increased prefrontal cortex insulin-like growth factor 1 (IGF-1), and this increase was associated with a protected sucrose preference and immobility in the forced swim test [147]. While this response appears to be inherently different than the BDNF hypothesis, data by Park et al. indicate IGF-1 protects brain BDNF signaling during an inflammatory challenge [87, 88]. Therefore, it is plausible that exercise-induced IGF-1 could be mediating its anti-depressant effects via BDNF regulation. These studies support much of the recent work on exercise’s neuroprotective effects, which have focused on BDNF regulation within the hippocampus. BDNF is a neurotrophin that acts predominantly in the hippocampus, cortex, and basal forebrain to promote neurogenesis, neuron survival, and synaptic plasticity. Like LPS, chronic stress reduced BDNF via an IL-1β dependent mechanism causing suppressed hippocampal neurogenesis and neuron survival [148]. Numerous studies have demonstrated exercise training upregulates hippocampal BDNF gene and protein expression, and this is associated with improved behavioral outcomes during inflammatory challenge. We observed an LPS-induced reduction in whole brain BDNF, but failed to detect a VWR-induced increase in whole brain BDNF gene expression in both our young adult and aged mice. This indicates the exercise-induced upregulation of BDNF as demonstrated in the literature is not a global event, but rather a spatially dependent phenomenon primarily observed in the hippocampus.

More relevant to our specific aims, a recent study by Liu et al. examined the effects of swim training on depressive-like behavior and IDO activity in rats submitted to chronic mild stress [144]. Following 4 weeks of swim training in combination with chronic stress, swim
trained rats exhibited increased sucrose preference and decreased FST immobility compared to chronically stressed rats that did not swim train. Moreover, these behavioral results were associated with reduced prefrontal cortex IFN-γ, TNF-α, and IDO gene expression, indicating the protective effects of exercise training in this chronic stress model may be mediated by inhibiting IDO activation and metabolism. In relation to our results, these data indicate that depressive-like behavior in response to chronic mild stress is amenable to the beneficial effects of VWR, whereas LPS-induced depressive-like behavior is not. Again, it is important to note that we utilized the minimum LPS dosage which induces depressive-like behavior in our models. What is unknown is whether other inflammatory stimuli, perhaps that act more chronically, are equally insensitive to VWR.

A potential explanation for the differential findings between our study and those using chronic mild stress, is that LPS-induced depressive-like behavior is a well-defined acute phenomenon dependent on activation of the IDO pathway [43], while chronic mild stress-induced depression is a chronic paradigm dependent on numerous cellular and molecular mechanisms (e.g., HPA axis dysregulation, aberrant monoamine signaling, and reduced growth factor synthesis) which synergize to induce a depressive-like behavior phenotype [149]. Under basal conditions, IDO is expressed at low levels in microglial cells and astrocytes, with the majority of IDO activity occurring in astrocytes, and the primary end product being kynurenic acid, a neuroprotective NMDA antagonist [39, 41, 150]. However, following immune activation (e.g., LPS), proinflammatory cytokines TNF-α and IFN-γ induce IDO activation in microglial cells, shifting kynurenine metabolism towards the production of 3-hydroxykynurenine (3-HK) and quinolic acid (QA), which are implicated in inflammation-induced depressive-like behavior [151]. An alternative explanation could be due to temporal differences in administration of
exercise and ‘inducer’ of behavioral disturbance. In chronic mild stress studies, exercise was performed chronically *during* the administration of the stressors, whereas in our study exercise was performed *prior* to LPS administration. Mice were not allowed wheel access after LPS as it has been shown that they do not run during this acute inflammatory challenge. This hypothesis concurs with the BDNF literature, which indicates exercise-induced BDNF improves neuron survival during stress, rather than protecting against acute inflammatory injury.

Regarding the effects of exercise training on age-related neuroimmune alterations, several reports have demonstrated exercise may attenuate microglial priming. Vukovic et al. found VWR training reduced hippocampal microglia MHCII expression and increased neuronal fractalkine (CX3CL1) in young mice [152]. Fractalkine binds to its receptor (CX3CR) on microglia and maintains the cell in a quiescent state. Following immune activation, fractalkine signaling is critical for attenuating the microglia inflammatory response and restoring brain inflammation to baseline levels. Several studies indicate aging reduces basal levels of fractalkine, which may partially explain microglial priming [48]. Furthermore, following a LPS challenge, microglia from aged mice exhibited a prolonged down-regulation of the fractalkine receptor compared to young mice, which may contribute to the prolonged proinflammatory cytokine response and protracted behavioral disturbances observed in aged mice. Kohman et al. demonstrated similar anti-priming effects of exercise in hippocampal microglia of aged mice. Eight weeks of VWR in aged Balb/c mice reduced microglia proliferation and microglia IGF-1 gene expression [153]. Barrientos et al. further addressed the effects of exercise on microglia priming by isolating primary hippocampal microglia from the wheel trained and sedentary aged rats [21]. Following stimulation with numerous doses of LPS (0-100ng), the microglia from the voluntary wheel running rats exhibited a significantly reduced TNF-α and IL-1β gene expression.
compared to microglia from rats housed in locked wheels, indicating that exercise training may ‘reverse’ age-induced microglia priming and subsequent neuroinflammation. Interpreting this study in the context of ours is difficult, as ex vivo microglia stimulation occurs under tightly controlled and optimal experimental conditions. The researchers did not report the upper-limit LPS concentration where they observed no differences in microglia priming between wheel running and locked wheel rats. It is quite possible that the minimum effective LPS doses we utilized to induce depressive-like behavior corresponded to a higher ‘stimulus’ than 100ng LPS applied to hippocampal microglia in vitro, thus abolishing any protective effect of exercise.

Additionally, similar to the BDNF literature, these studies only examined the effects of exercise on hippocampal microglia priming, the brain region most affected by exercise training. Our lack of observed exercise induced neuroprotection in whole brain tissue furthers supports our hypothesis that exercise’s protective effects are not a global event, but rather a brain-region dependent phenomenon primarily observed in the hippocampus.

While our study clearly demonstrates no effect of voluntary wheel running on LPS-induced depressive-like behavior in young adult and aged mice, we recognize certain limitations. We did not include a ‘locked’ wheel control group for the voluntary wheel intervention. It is plausible that any observed VWR effects could have been due to environmental enrichment rather than adaptations induced by the exercise component of wheel running. However, we have previously reported no differences between VWR, locked wheel, and standard housing interventions on LPS-induced sickness behavior in aged mice [94]. Furthermore, Kobilo et al. elegantly demonstrated the act of running, rather than environmental enrichment, is responsible for neurotrophic effects of voluntary wheel running [154]. A second limitation is that our study design did not assess brain cytokine and IDO gene expression across numerous time-points,
which would strengthen our conclusion that voluntary wheel running does not affect LPS-induced IDO induction and the subsequent depressive-like behavior. We chose to assess brain gene expression at the selected time-points based on observations that 4h post-injection was the peak of inflammation in young mice, and 24h post-injection was the critical time-point of prolonged pro-inflammatory cytokine and IDO gene expression, differentiating the aged and young adult neuroinflammatory response [58, 59]. Because we did not conduct a time-course analysis of brain cytokine and IDO gene expression, we cannot definitively conclude that VWR had no effects of neuroinflammation at the given doses of LPS, and it is possible that subtle inflammatory effects persist in the brain that are not reflected by behavioral outcomes.

Similarly, we recognize whole brain analysis does not reveal potential exercise-induced neuroprotective regional differences, and thus can only speculate that exercise’s protective effects occur in a region specific manner. Given that depressive-like behavior was our primary outcome and there were no observed VWR-induced changes in depressive-like behavior, measuring brain cytokine and IDO gene expression at numerous time points and across numerous brain regions did not seem worthwhile in the context of our experiments.

In conclusion, we demonstrate that voluntary wheel exercise training does not affect LPS-induced depressive-like behavior, nor does it influence LPS-induced brain cytokine or IDO gene expression at 4h or 24h post-injection, in young adult and aged mice, respectively. These lack of neuroprotective effects occurred despite using the minimum effective doses and optimal behavioral time-points in our respective mouse models. Collectively, these data indicate the anti-depressant and neuroprotective effects of exercise are sensitive to the inflammatory stimulus utilized (i.e., LPS vs. CMS), and further research is necessary to elucidate the complex effects of exercise training on inflammation-induced depressive-like behavior.
Acknowledgements

Supported by NIH RO1 AG-029573-S1 to K.W. Kelley.
5.6 Figure Legends, Tables, and Figures

Figure 10. Effects of VWR on LPS-induced changes in sickness behavior, depressive-like behavior, and brain gene expression in young adult mice. LPS administration resulted in significant (*) up-regulation of TNF-α, IL-1β, IL-6, IDO, and IFN-γ, and a significant (*) down-regulation of BDNF at 4h post-injection (a). There were no significant intervention main effects or intervention by treatment interaction effects for any gene measured. LPS administration resulted in significant (*) reductions in food intake (b) and body weight (c), increased 24h TST immobility (d), and reduced 24h sucrose preference (e), but there were no intervention main effects or intervention by treatment interactions. Mean ± sem; n = 9-10/group.

Figure 11. Effects of VWR on LPS-induced changes in sickness behavior, depressive-like behavior, and brain gene expression in aged mice. LPS administration resulted in significant (*) up-regulation of TNF-α, IL-1β, IL-6, and IDO and a significant (*) down-regulation of BDNF at 4h post-injection. There were no significant intervention main effects or intervention by treatment interaction effects for any gene measured (a). LPS administration resulted in significant (*) reductions in food intake (b) and body weight (c), increased 48h TST immobility (d), and reduced 24h sucrose preference (e), but there were no intervention main effects or intervention by treatment interactions. Mean ± sem; n = 9-10/group.
<table>
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<th>Distance Run (km/d)</th>
<th>Pre-intervention body weight (g)</th>
<th>Post-intervention body weight (g)</th>
<th>Body weight change (%)</th>
<th>Forced exercise time-to-fatigue (minutes)</th>
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<td><strong>Experiment 1:</strong></td>
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<td>Young Adult Mice</td>
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<td>Voluntary Wheel</td>
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<td>24.72 ± 0.56</td>
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<td>0.09 ± 1.9</td>
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<td>26.45 ± 0.24</td>
<td>6.11 ± 0.47</td>
<td>47.29 ± 2.86</td>
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<td><strong>Experiment 2:</strong></td>
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<tr>
<td>Voluntary Wheel</td>
<td>3.4 ± 0.4</td>
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<td>32.22 ± 0.49</td>
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**Table 3.** Intervention-induced adaptations. Mean ± SEM; n=9-10/group

a Significantly (p < 0.05) reduced compared to Young Adult Standard housing conditions.
b Significantly (p < 0.05) reduced compared to Aged Standard housing conditions.
Figure 10.

(a) Relative mRNA levels for TNFα, IL-1β, IL-6, IDO, IFNγ, and BDNF in different treatment groups. The results are presented as mean ± SEM. Significant differences are indicated by asterisks above the bars.

(b) Food intake as a percentage of baseline for different treatment groups. The results are presented as mean ± SEM. Significant differences are indicated by asterisks above the bars.
Sucrose Preference (% Total Fluid Intake)

- Standard+Saline
- Standard+LPS
- VWR+Saline
- VWR+LPS

* indicates significant difference
Figure 11.

(a) Relative mRNA expression levels of TNFα, IL-1β, IL-6, IDO, IFNγ, and BDNF across different conditions: Standard+Saline, Standard+LPS, VWR+Saline, and VWR+LPS.

(b) Food intake (% Baseline) over 24h and 48h in Standard+Saline, Standard+LPS, VWR+Saline, and VWR+LPS conditions.
Sucrose Preference (% Total Fluid Intake)

- Standard+Saline
- Standard+LPS
- VWR+Saline
- VWR+LPS

* denotes significant difference
6.1 Abstract

We tested the effects of daily forced treadmill exercise on BCG-induced sickness behavior in young adult mice balb/c mice. Mice were intraperitoneally injected with BCG; 4 hours later they underwent a 30-minute treadmill running bout or remained sedentary, and treadmill running continued for one session/day for the remainder of the study. As expected, BCG induced significant anorexia, body weight loss, anhedonia via sucrose preference, and reduced voluntary wheel running. BCG-infected mice forced to treadmill exercise post-infection exhibited prolonged weight loss, reduced voluntary wheel running, and a tendency toward decreased sucrose preference compared to BCG-infected mice allowed to remain sedentary. Treadmill exercise may act as a stressor, which reduces the efficacy of the immune response to BCG, decreases BCG clearance, and prolongs BCG-induced sickness behavior.

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4 This chapter is used with permission of the co-authors: J.C. O’Connor, R. Dantzer, K.W. Kelley, J.A. Woods
6.2 Introduction

Peripherally and centrally produced cytokines act directly or indirectly on neurons and supporting cells (e.g., microglia and astrocytes) to alter autonomic nervous and endocrine system output to regulate the body’s response to infection [15]. Interleukin-1 beta (IL-1β), for example, induces activation of the hypothalamic-pituitary-adrenal axis and the production of corticosteroids, which attenuate the pro-inflammatory response in a negative feedback loop. Moreover, these cytokines propagate signals to various brain structures, including the hypothalamus, hippocampus, amygdala, and prefrontal cortex, to invoke a constellation of motivated behavioral adaptations, often termed ‘sickness behavior [15].’ Sickness behaviors include loss of appetite and body weight, fatigue, withdrawal from normal social activities, altered cognition, hyperalgesia, anhedonia, and fever. Long believed to be a general weakness and malaise associated with infection, it is now realized the purpose of these adaptations is to allocate energy and resources towards the immune response and support recovery from infection. The importance of such behaviors was bolstered by powerful observations that inhibiting certain aspects of the sickness response resulted in decreased survival of infected animals [155-157]. A pioneering study by Murray et al. demonstrated forced tube feeding of anorexic sick mice to normal levels resulted in a doubling of mortality from Listeria monocytogenes infection compared to mice allowed to eat ad libitum [156]. In a similar manner, animals housed in a cold environment or treated with an antipyretic drug displayed higher mortality rates following numerous infection types, suggesting an adequate febrile response is critical for host defense and survival [155, 157-160]. While these studies were conducted more than thirty years ago, it is now understood that sickness induced anorexia limits foraging behavior and nutrient available to
invading pathogens, and the pyretic response blunts microorganism replication capacity and enhances immune cell effector mechanisms.

As energy conservation and allocation toward the immune response is the primary goal of sickness behavior, it is fitting that a classical indicator of sickness is reduced locomotor activity. Animals injected with live viruses or bacteria, viral or bacterial mimetics (poly I:C or LPS), or proinflammatory cytokines (TNFα or IL-1B) exhibit a robust reduction in locomotor activity [94]. For example, young mice injected with LPS exhibit decreased home cage locomotor activity, as well as reduced exploration in a novel environment within an hour of inoculation. Similarly, young healthy balb/c mice injected with the attenuated bacterium Bacillus Calmette-Guérin (BCG) reduce their voluntary wheel running activity to virtually nothing for approximately three days post-infection (unpublished data). These observations prompted Skinner et al. to propose that avoidance of physical activity may be the most sensitive indicator of sickness [161]. Measuring fever (febrile response), food intake (anorectic response), and voluntary wheel running (locomotor response) following LPS injection across three doses in rats, the researchers discovered, via statistical regression analysis, that decreased voluntary wheel running was the most sensitive measure of the sickness response to LPS [161]. Given the energetic cost of locomotor activity, it makes logical sense on an evolutionary basis that proinflammatory cytokines produced during infection would act in the brain to limit locomotor and energy expenditure. However, the importance of reduced locomotor activity during sickness has never been empirically demonstrated in a similar manner to the anorectic and febrile response.

Therefore, the purpose of our study was to examine the effect of forced treadmill exercise (i.e. forced locomotor activity) on sickness behavior following BCG infection. We hypothesized
mice forced to treadmill exercise would exhibit prolonged sickness behavior and protracted recovery from infection.

### 6.3 Materials and Methods

**Animals**

Four month-old male balb/c mice (N=40) were obtained from the Jackson Labs (Bar Harbor, Maine), and singly housed in cages with corn-cob bedding in a temperature (23°C) and humidity (45-55%) controlled environment with a 12h dark/light cycle (lights off 0900-2100). Mice were allowed *ad libitum* access to food and water for the entire duration of the study and were given two weeks to acclimate to the housing conditions prior to study commencement. All experiments were conducted under the guidelines of the University of Illinois, Urbana-Champaign Animal Care and Use Committee.

**Voluntary Wheel Running**

In contrast to our previous studies using voluntary wheel running (VWR) as an exercise training modality, in this study we used VWR as a measure of sickness behavior. As previously mentioned, mice infected with BCG significantly reduce their voluntary wheel running for ~3d post-infection. Following facility acclimation, all mice were individually housed in a plexiglass cage (36 L × 20 W × 15 H cm) that contained a stainless steel running wheel (diameter 22.86 cm) (Mini-Mitter Repirons, Bend, Oregon). Wheel revolutions were relayed via telemetry to a computer in the facility. Ten days of baseline wheel running data was collected prior to BCG inoculation to account for individual running variability between mice.
Infection with *Bacillus Calmette-Guérin*

Following ten days of baseline wheel running, mice were randomized and intraperitoneally injected with $10^8$ CFU BCG per mouse or equivolume saline. BCG is a live attenuated bacterium prepared from *mycobacterium bovis*, a strain of bovine tuberculosis bacillus [42]. It is commonly used, with little efficacy, in humans to vaccinate against human tuberculosis. Infection of mice with BCG is a well-validated model of chronic immune activation. Mice chronically infected with this dose of BCG display a robust chronic pro-inflammatory cytokine response and a resulting episode of sickness behavior which lasts a duration of ~6d.

**Forced Treadmill Exercise Protocol**

Beginning at 4h post-inoculation, mice were randomized to two intervention groups: forced treadmill exercise or sedentary. Forced treadmill mice were treadmill exercised once per day for thirty minutes at an intensity of 8-12m/m (60-70% VO2max), for nine days post-infection; sedentary exposed to the noise of the treadmill, and had their food and water removed for the same duration as the runners, but did not undergo forced treadmill running.

**Measurement of Sickness Behaviors**

Sickness behavior was assessed by changes in body weight, food and fluid intake, voluntary wheel running, and anhedonia via sucrose preference. Body weight, food and fluid intake, and voluntary wheel running were measured for nine days post-injection. Anhedonia is a key component of sickness behavior, and can be measured in mice by their preference to consume a
sweetened solution. A sucrose preference test was performed six days after BCG infection. Mice were presented with two identical volumetric drinking tubes containing either water or a 1% sucrose solution. Bottles were weighed before and after the 24h period to measure fluid consumption of each respective solution. Bottle position (right v. left) was alternated each training period to reduce potential bias from place preference. Sucrose preference was calculated using the following formula: \[ \text{Sucrose preference} = \left( \frac{\text{Sucrose intake}}{\text{water intake} + \text{sucrose intake}} \right) \times 100. \]

**Statistics**

Data were analyzed independently using SPSS v18 (Chicago, IL). All data were tested for normality using the Shapiro-Wilk test. Data not approximating a normal distribution were logarithmically transformed before parametric statistical analysis. Intervention (treadmill or sedentary) induced differences in BCG-induced sickness behaviors were detected using a 2 (Treatment = BCG, Saline) x 2 (Intervention = Treadmill, Sedentary) ANOVA, and with repeated measures when necessary. Any reference to “groups” implies the following: (Saline+Sedentary, Saline+Treadmill, BCG+Sedentary, BCG+Treadmill). Data are expressed as mean ± SEM. The alpha level was set at \( p \leq 0.05 \) and all tests were two-tailed.
Study Design

6.4 Results

Baseline Characteristics

There were no statistically significant pre-intervention differences in body weights between the different groups ($F_{3,39}=0.72, p=0.549$). During days 7-10 of the wheel acclimation period, mice ran an average of 3.4 km per day, and there were no differences in daily running distance between groups ($F_{3,39}=0.97, p=0.419$).

Effects of forced treadmill exercise on BCG-induced sickness behaviors:

Food Intake

As expected, BCG infection induced a significant reduction in food intake that peaked during the initial 24h, and did not return to baseline levels until 7d post-infection (time x treatment: $F_{8,280}=73.15, p=0.000$) (Figure 12). Forced treadmill exercise did not alter BCG-induced anorexia (time x treatment x intervention: $F_{8,280}=1.14, p=0.334$). There were no differences in
food intake between saline injected groups (time x intervention: $F_{8,280}=0.88$, $p=0.534$), nor was their an intervention main effect (Intervention: $F_{1,35}=0.00$, $p=0.993$).

**Body weight loss**

As a result of anorexia, all BCG-infected mice lost significant amounts of body weight compared to saline treated mice (time x treatment: $F_{8,280}=62.65$, $p=0.000$) (**Figure 13**). Mice infected with BCG that remained sedentary, other than voluntary wheel running, during their recovery regained their baseline body weight by 8d post-infection. Interestingly, mice that were forced to treadmill exercise daily following BCG infection, exhibited significantly slower body weight recovery (time x treatment x intervention $F_{8,280}=2.015$, $p=0.045$). Post-hoc analysis revealed that BCG+Treadmill mice exhibited reduced body weight compared to BCG+Sedentary mice at every time-point past 24h post-infection. There were no differences in body weight between Saline+Treadmill and Saline+Sedentary mice during the study.

**Voluntary wheel running**

BCG infected mice significantly reduced their wheel running post-infection compared to saline groups, and it never recovered to baseline levels for the duration of the study (time x treatment: $F_{8,280}=42.78$, $p=0.000$) (**Figure 14**). However, BCG-infected mice that were forced to treadmill exercise daily exhibited slower VWR recovery compared to BCG-infected mice that were allowed to remain sedentary (time x treatment x intervention: $F_{8,280}=1.978$, $p=0.049$). Post hoc analysis revealed BCG+Treadmill mice exhibited reduced VWR at days 5-7 post-infection compared to BCG+Sedentary mice. There were no differences in VWR between Saline+Treadmill and Saline+Sedentary mice during the study.
Sucrose preference (anhedonia)

There were no differences in baseline sucrose preference between the four groups prior to BCG-infection (group: $F_{3,38}=0.12$, $p=0.049$) (Figure 15). At 6d post-infection, BCG-infected mice exhibited reduced sucrose preference at compared to Saline treated mice (treatment: $F_{1,35}=5.23$, $p=0.028$). Interestingly, treadmill exercised mice, independent of BCG/Saline exhibited reduced sucrose preference compared to sedentary mice ($F_{1,35}=4.89$, $p=0.034$). There was a trend for BCG+Treadmill mice to exhibit reduced sucrose preference compared to BCG+Sedentary mice, although this did not reach statistical significance ($p=0.100$). There were no differences in total fluid intake between groups during the sucrose preference testing period.

6.5 Discussion

We investigated whether forced exercise following BCG infection affects sickness behaviors in young adult mice. BCG infection induced significant anorexia, body weight loss, anhedonia, and decreased voluntary wheel running compared to saline treated mice. Mice infected with BCG that were forced to treadmill exercise post-infection exhibited protracted body weight and voluntary wheel running recovery, and a tendency for exacerbated anhedonia compared to BCG-infected mice allowed to remain sedentary post-infection.

This is the first study to examine forced treadmill exercise post-infection in the context of sickness behavior. Our results support the previously mentioned studies, highlighting the necessity of a proper sickness response (i.e., reduced locomotor activity) in animals’ recovery from infection [155-157]. However, further insight into forced treadmill running provides an alternative interpretation of our data. Several studies from our lab indicate the ‘forced’ nature of
treadmill exercise may be perceived as a stressor by the mice, and induce immunomodulating effects. Lowder et al., demonstrated prolonged treadmill exercise following influenza infection increased mouse mortality, which was attributed to an elevated HPA response, and a subsequent suppression of immune effector mechanisms [105, 162]. In contrast, Pence et al. found forced exercise prior to subcutaneous wounding increased inflammation and speeded wound healing in obese mice [163]. Additionally, recent data indicate treadmill exercise, prior to DSS ingestion exacerbates, while voluntary wheel running attenuates, DSS induced colitis in mice (unpublished data). The interaction between stress and the immune response is complicated, and partially based on the timing of the stressor in relation to the immune challenge. For example, inescapable tailshock prior to LPS challenge enhances peripheral and central pro-inflammatory cytokine production, indicating stress sensitizes the immune system to subsequent immune challenge [164, 165]. Alternatively, stressors applied following immune challenge reduce pro-inflammatory cytokine expression, virus/bacteria clearance, and increase animal mortality to infection. Goujon et al. demonstrated this phenomenon by subjecting mice to restraint stress (RS) immediately following LPS injection, RS mice exhibited significantly lower brain IL-β, TNFα, and IL-6 gene expression [166]. Along similar lines, but using a live virus rather than bacterial mimetic, Dobbs et al. injected mice with influenza, and then exposed them to six days, sixteen minutes per day, of RS [167]. RS significantly reduced influenza virus specific cell production of IL-2, IFNγ, and IL-6 from regional lymph node and spleen cells; interestingly, pre-treatment of the mice with RU486, a type II glucocorticoid receptor antagonist, completely abolished the immunosuppressive effects of RS, implicating stress-induced glucocorticoids as the primary mediators of this response [167]. Glucocorticoid receptors are capable of transrepressing nuclear factor-kappa B (NF-kB) transcriptional activity, which is vital to
mounting a proper inflammatory response [168]. The dichotomous effect of the timing of stress in relationship to immune stressor could be explained by research from several groups, who proposed that stress hormones exert a bi-directional effect on immune function [169]. Stress-induced glucocorticoids sensitize immune cells to a subsequent immune challenge, which results in a more robust inflammatory response. This theory makes sense from an evolutionary perspective as stress often precedes wounding/pathogen entry. In contrast, stress-induced glucocorticoid production following immune challenge acts in an anti-inflammatory fashion and suppresses the immune response. Under this model, stress-induced glucocorticoids re-prioritize energy allocation, diverting energy away from the ‘energy intensive’ immune response toward the more pressing fight/flight response. While our previous studies have not assessed glucocorticoid response to treadmill running, we found that chronic treadmill training induces adrenal hypertrophy and thymic atrophy, two classical indicators of stress (unpublished data). Furthermore, in the current study, treadmill running reduced sucrose preference in saline treated mice, which is a common observation in stressed animals. Based on these data we speculate treadmill running mice immediately post-infection induced an exaggerated HPA and glucocorticoid response, which inhibited the initial immune response to BCG, and ultimately prolonged BCG clearance and sickness behavior. This hypothesis is supported by a study from Ceddia et al., which found that treadmill exercise following peritoneal thioglycolate inflammation reduces macrophage antigen presentation [170, 171]. Unfortunately we did not assess BCG-specific cellular immune response, or measure lung BCG colonies, so we can only speculate on this theory.

An alternative explanation for the protracted sickness behaviors observed in BCG+Treadmill mice may simply be a matter of energy balance. That is, the energy expended
during the treadmill bout may have induced a caloric deficit in BCG-infected mice, leading to prolonged weight loss. There were no differences in food intake or body weight between the Saline+Treadmill and Saline+Sedentary mice, indicating the daily treadmill exercise bouts did not induce energy imbalance healthy mice. However, as BCG-infected mice significantly reduced their food intake post-infection, it is possible treadmill exercise exacerbated this anorexia-induced caloric imbalance, and caused prolonged weight loss. In a similar manner, the forced exercise, coupled with exacerbated weight loss, may have altered hypothalamic energy sensing in such a way that it induced a reduction in voluntary wheel running in an attempt to restore energy homeostasis. Again, as we did not measure energy expenditure or hypothalamic energy sensing neurocircuitry, these hypotheses are merely speculation.

While our study demonstrates forced exercise prolongs certain aspects of BCG-induced sickness behavior in young adult mice, we recognize the above-mentioned limitations. In hindsight, we should have collected tissues to assess stress-induced changes in peripheral/brain inflammation and immune response to BCG. Additionally, measuring energy expenditure would have allowed us to rule out energy balance as a mediator of the treadmill-induced differences BCG–induced sickness behaviors.

In conclusion, this is the first study to demonstrate forced treadmill exercise induces protracted sickness responses following BCG infection. Further research is necessary to elucidate the cellular and molecular mechanisms responsible for this effect. From a public health perspective, these data support previous research indicating stress (physical and psychological) slow recovery from infection, and should be avoided in the post-infection time-period.
6.6 Figure Legends and Figures

**Figure 12. Effects of treadmill exercise on BCG-induced changes in food intake.** BCG administration resulted in significant (*) reductions in food intake, but there were no intervention main effects or intervention by treatment interactions. Mean ± sem.

**Figure 13. Effects of treadmill exercise on BCG-induced body weight loss.** BCG administration resulted in a significant (*) reduction in body weight. BCG+Treadmill mice exhibited reduced body weight compared to all other groups at all time-points after 24h (#). Mean ± sem.

**Figure 14. Effects of treadmill exercise on BCG-induced reduction in voluntary wheel running.** BCG administration resulted in a significant (*) reduction in voluntary wheel running. BCG+Treadmill mice exhibited reduced voluntary wheel running compared to all other groups at days 6-8 post-infection. (#). Mean ± sem.

**Figure 15. Effects of treadmill exercise on BCG-induced anhedonia.** BCG administration resulted in a significant (*) reduction in sucrose preference at 6d post-infection. Treadmill exercise reduced mouse sucrose preference, independent of BCG/Saline treatment ($). There was a tendency (p=0.10) for BCG+Treadmill mice to exhibit lower sucrose preference compared to BCG+Sedentary mice. Mean ± sem.
Figure 12.
Figure 13.
Figure 14.
Figure 15.
CHAPTER 7
CONCLUSIONS

Aging of the population and the increased prevalence of chronic diseases among the elderly are major challenges facing our society and medical community. Of particular importance is the role of age-related neuroinflammation in the etiology of behavioral abnormalities following physiological (e.g. immune stimulus) or psychological (e.g. stress) challenge. Aged animals, compared to young animals, display protracted sickness behavior, depressive-like behavior, and cognitive impairment after stimulation of the innate immune system, and this is related to an exaggerated and prolonged induction of brain inflammation [1-3]. From a clinical perspective, prolonged sickness behavior and increased depressive symptoms result in a reduction in self care, loss of independence, and are often the first steps towards dependent living in elderly individuals.

The overarching aim of these studies was to investigate the effects of voluntary wheel training and forced treadmill running on inflammation-induced behavioral disturbances in young adult and aged mice. Our data indicate voluntary wheel training did not attenuate sickness behavior or brain proinflammatory cytokine gene expression in old mice. Similarly, voluntary wheel training had no effect on lipopolysaccharide-induced depressive-like behavior of brain indoleamine 2,3 dioxygenase gene expression in young adult or aged mice. In contrast, forced treadmill exercise post-BCG infection significantly slowed sickness behavior recovery in young adult mice.

Collectively, these data indicate that exercise training provides little protection against inflammation-induced behavioral abnormalities, and may actually exacerbate sickness behavior if the exercise is perceived as a stressor. These data contradict a majority of the literature
demonstrating a neuroprotective, anti-inflammatory, and anti-depressant effects of exercise.

Potential explanations for the dichotomous results within the literature include: inflammatory model, behavioral outcome, and brain region responsible for observed effect. Future research should examine the effects of exercise on numerous brain regions, and attempt to elucidate the regional specificity for the protective effects of exercise. Additionally, understanding how exercise affects microglia priming and neuron/microglia communication will provide novel insight into the neuroprotective effects regular exercise training. Finally, the animal data needs to be translated to human models of aging and sickness. Randomized-controlled clinical trials should investigate the effects of regular exercise training prior to, and after infection, on perceived sickness behaviors and depression progression in aged adults.
CHAPTER 8

REFERENCES


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