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Effects of Voluntary Wheel Running on LPS-induced Sickness Behavior in Aged Mice

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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 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.

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

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through various communication pathways to induce a 'mirror image' of cytokine expression within the brain (Dantzer, 2001; Dantzer and Kelley, 2007; Dantzer et al., 2008). 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 (Besedovsky et al., 1986). 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 (Dantzer and Kelley, 2007; Dantzer et al., 2008). 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* (Murray and Murray, 1979).

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 (Gavazzi and Krause, 2002) and animals (Goldmann et al., 2010). Older subjects exhibit exaggerated behavioral and cognitive deficits following immune activation (Abraham and Johnson, 2009; Chen et al., 2008; Godbout et al., 2005; Kohman et al., 2007). 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 (Godbout et al., 2005). 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 (Beutler 2003). 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 (Chen et al., 2008; Dilger and Johnson, 2008; Godbout et al., 2005). 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 (Dilger and Johnson, 2008; Wu et al., 2009; Wynne et al., 2010).

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 (Keylock et al., 2008; Lowder et al., 2006; Vieira et al., 2009a; Vieira et al., 2009b). Studies examining the effects of exercise training on various responses to LPS in younger rodents have produced mixed results (Chen et al., 2007; Conn et al., 1995; Criswell et al., 2004; Oliveira et al., 2011; Rowsey et al., 2006; Wu et al., 2007; Wu et al., 2011). For example, Criswell et al (2004) found 12 weeks of treadmill running led to an exaggerated serum TNF-α response when compared to sedentary rats. In contrast, Chen et al (2007) found the exact opposite; 4 weeks of treadmill

training attenuated the serum TNF-α 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 (Godbout et al., 2005; Park et al., 2011), 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 (Abraham and Johnson, 2009; Godbout et al., 2005) 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.

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 (Godbout et al., 2005), 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 (Dantzer, 2001). 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 CO_2 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 (Godbout et al., 2005). 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 (Godbout et al., 2005; Starr et al., 2009; Wu et al., 2009). 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 $\Delta\Delta C_t$ 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-α, IL-1β, IL-6, and IL-10 because they are critical mediators of inflammation-induced sickness behavior and are affected by aging (Dantzer, 2001; Dilger and Johnson, 2008; Godbout et al., 2005). 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) × 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) × 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.

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 (χ^2 = 3.80; p = 0.15, χ^2 = 0.18; p = 0.94, χ^2 = 2.21; p = 0.33, and χ^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 asses 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 (χ^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 1a 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 postinjection (Figure 1b), but there were no statistically significant differences between groups (time × intervention × 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 × intervention × 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 1c–e).

Fluid intake—For clarity, we present all saline treated groups in Figure 2a 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 × intervention × treatment interactions: $F_{8, 396}$ =0.78, p=0.62; $F_{8, 384}$ =1.84, p=0.068; $F_{8, 408}$ =0.29, p=0.97 for the 0.02, 0.08 and 0.33 mg/kg doses, respectively) (Figure 2b–e). We did find a 3-way interaction at the 0.16 mg/kg dose ($F_{8, 404}$ =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 3a 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 3 a). 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 3b–e). There were no significant differences between groups at the 0.02 and 0.33 mg/kg doses (time × intervention × treatment interactions: $F_{16, 792}$ =0.56, p=0.91; $F_{16, 800}$ =1.38, p=0.15 for 0.02, 0.33 mg/kg, respectively) (Figure 3b,e). We did find significant 3-way interactions at the 0.08 and 0.16 mg/kg doses ($F_{16, 768}$ =2.14; p=0.006 and $F_{16, 808}$ =2.12; p=0.006 for 0.08 and 0.16 mg/kg, respectively)(Figure 3c,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 3a). 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 4a 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 × intervention × 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 4b–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 5). VWR, as applied in this study, could not attenuate the reduction in brain BDNF mRNA expression (intervention × treatment $F_{2,56} = 0.23$; $p = 0.79$) (Figure 5). 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 6a), 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 6b), 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.

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 (Dantzer, 2001). 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 (Kluger, 1979). 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 (Murray and Murray, 1979). 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 (Pawelec et al., 2010; Shanley et al., 2009), 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 (Starkie et al., 2003; Tanaka et al., 2010), 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 (Lenczowski et al., 1999). While we cannot speculate if IL-6 would demonstrate a VWR × 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 (Funk et al., 2011).

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. (2007) 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 (Hung et al., 2008). In contrast, Rowsey et al (2006) 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 (2004) 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 (Conn et al., 1995). In regard to brain inflammation, Wu et al (2007) 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 (Wu et al., 2011). 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 (Nickerson et al., 2005). 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 (Jeneson et al., 2007; Leasure and Jones, 2008).

Much of the recent work on exercise's neuroprotective effects have focused on BDNF as a primary mediator (Zoladz and Pilc, 2010). 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 (Cortese et al., 2011). 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 (Wu et al., 2007; Wu et al., 2011). 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 (Barrientos et al., 2011). *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 upregulation 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 (Dantzer, 2001). 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 (Godbout et al., 2005). 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), we chose to measure cytokine gene expression at only one critical time point. 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 measure IL-1β protein expression. 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.

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Research Highlight

Our data indicate no effect of 10 weeks of voluntary wheel running on lipopolysaccharide-induced exaggerated sickness behavior and inflammation in aged mice.

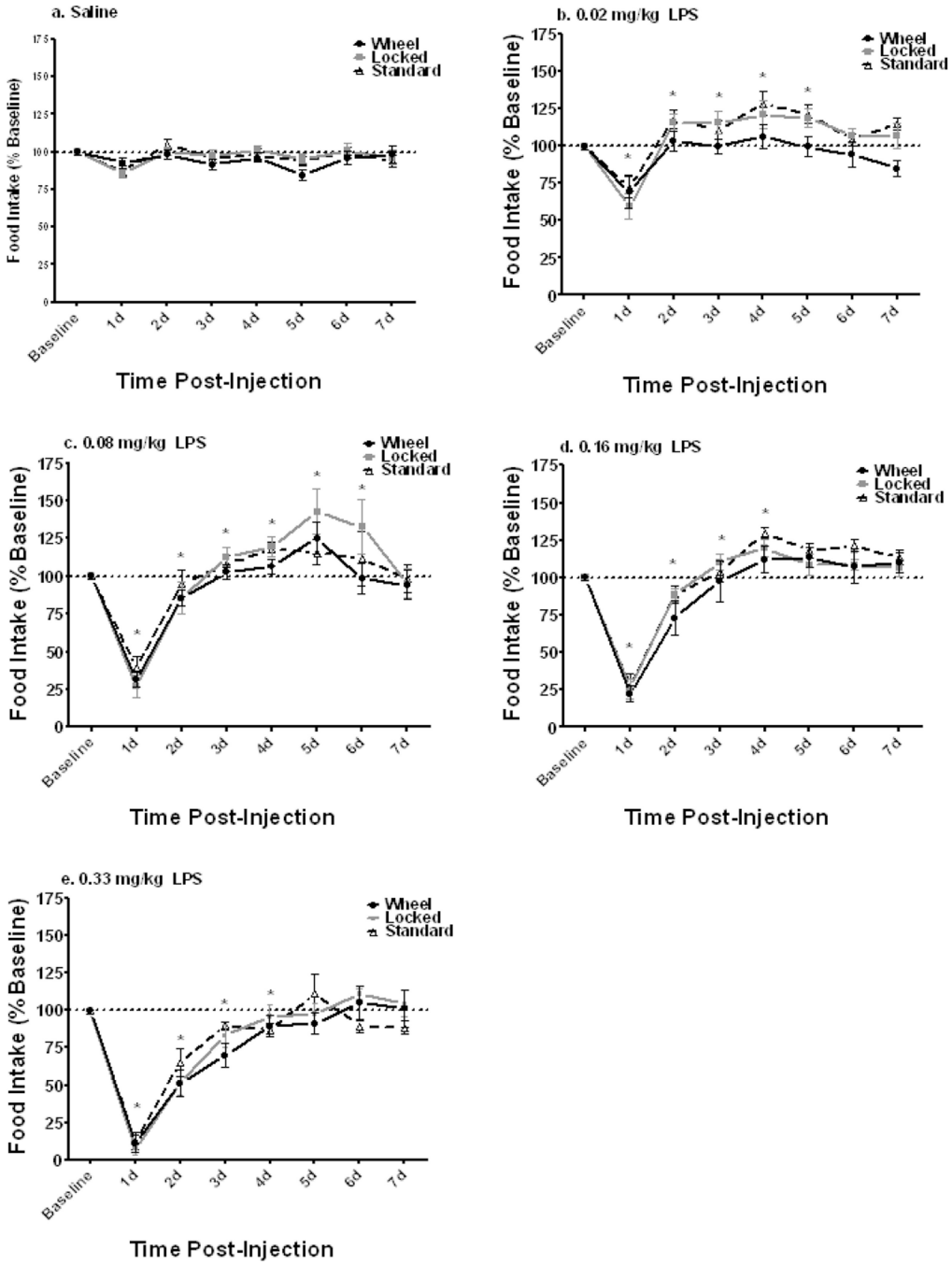

Figure 1. Effects of VWR on LPS-induced changes in food intake in aged mice

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.

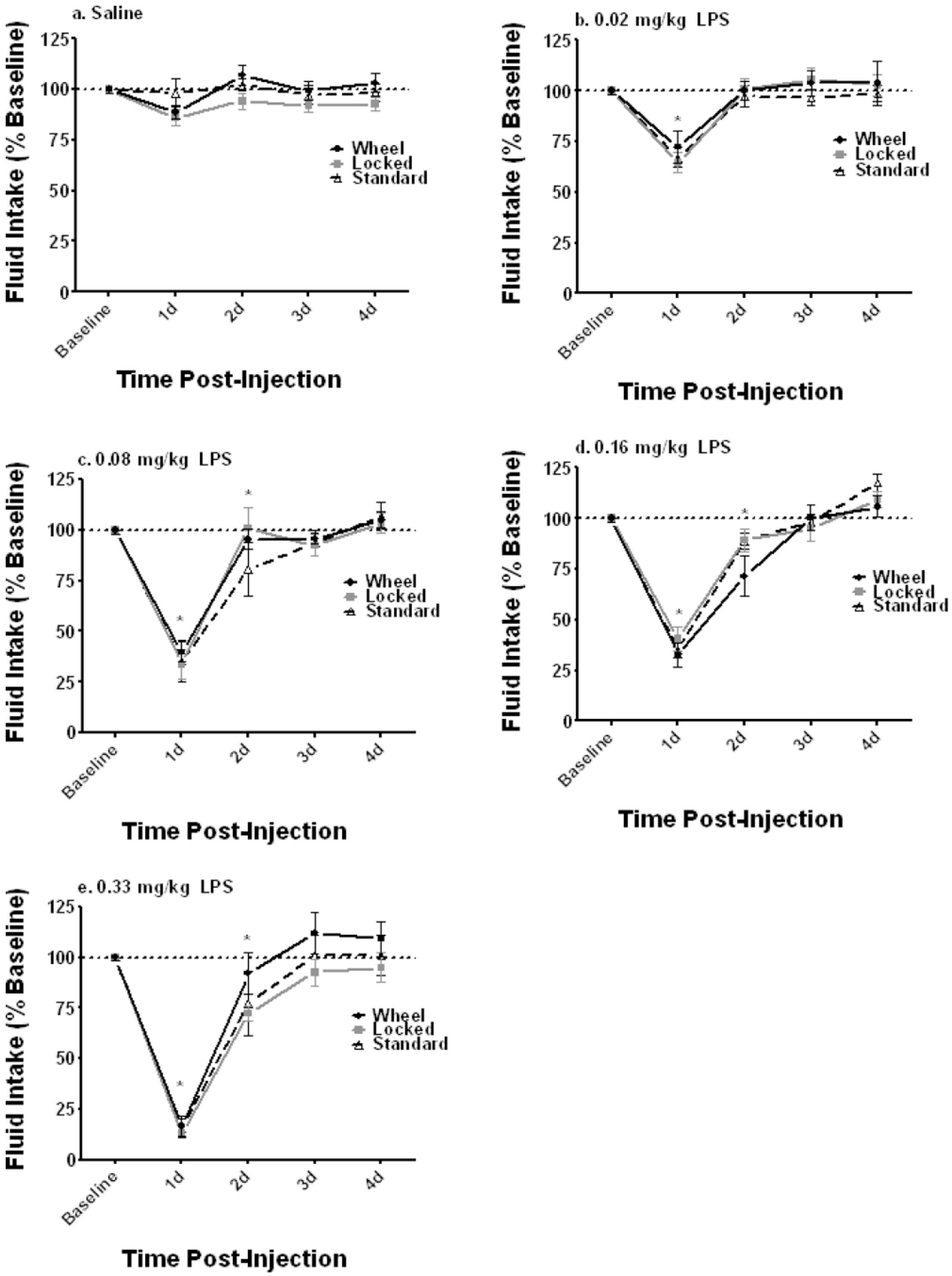

Figure 2. Effects of VWR on LPS-induced changes in fluid intake in aged mice

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.

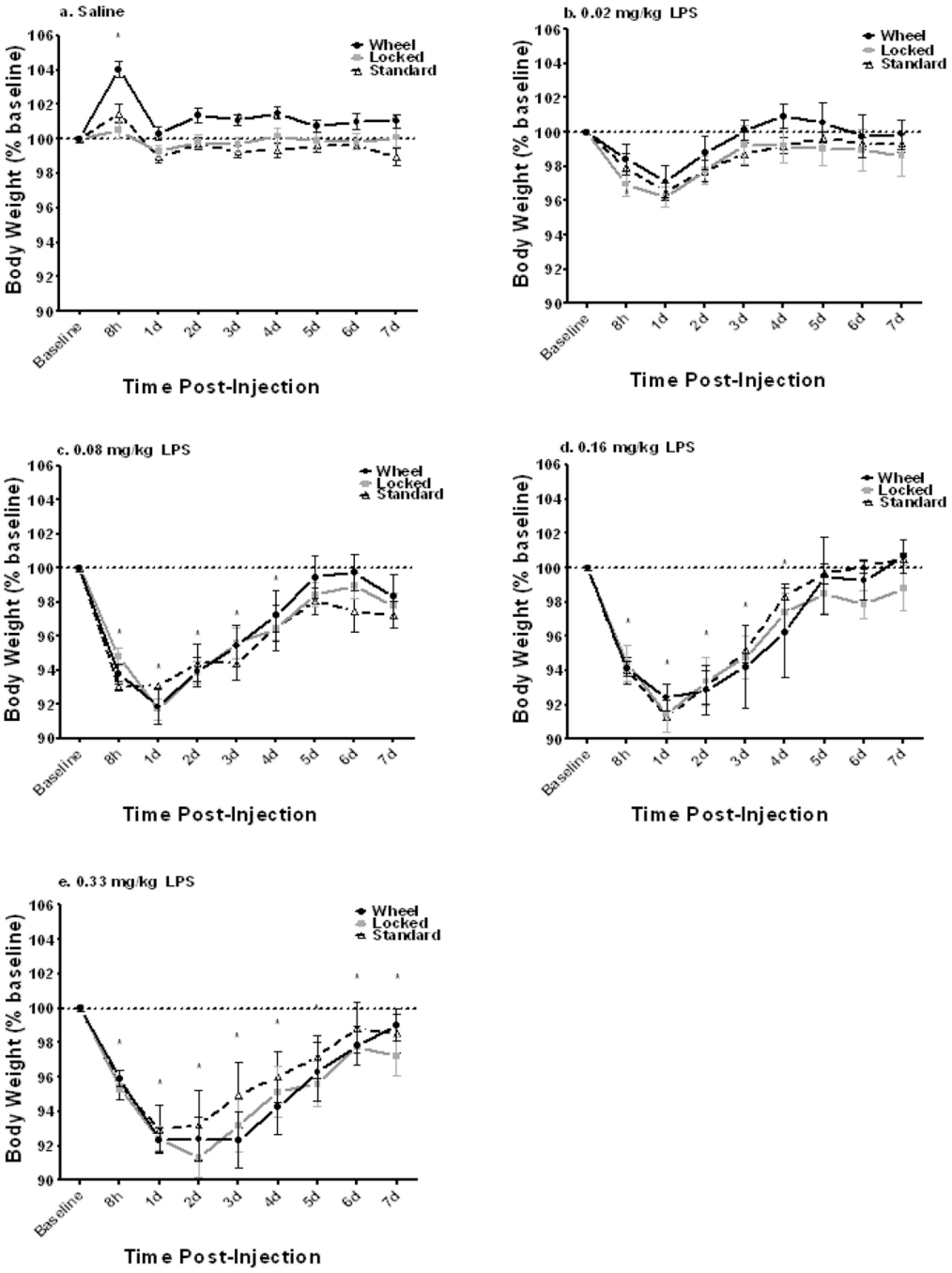

Figure 3. Effects of VWR on LPS-induced changes in body weight in aged mice

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.

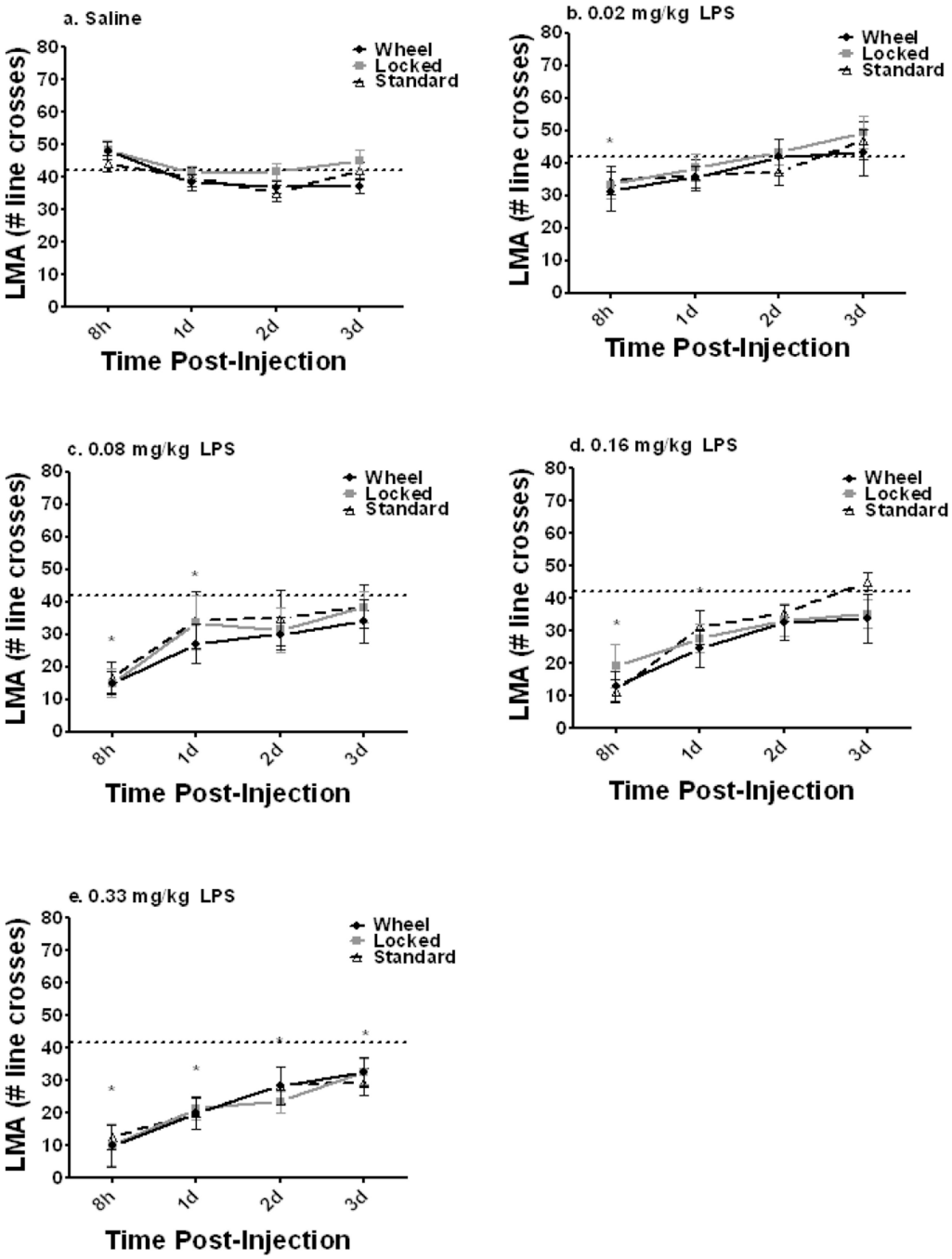

Figure 4. Effects of VWR on LPS-induced reductions in locomotor activity (line crosses) in aged mice

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.

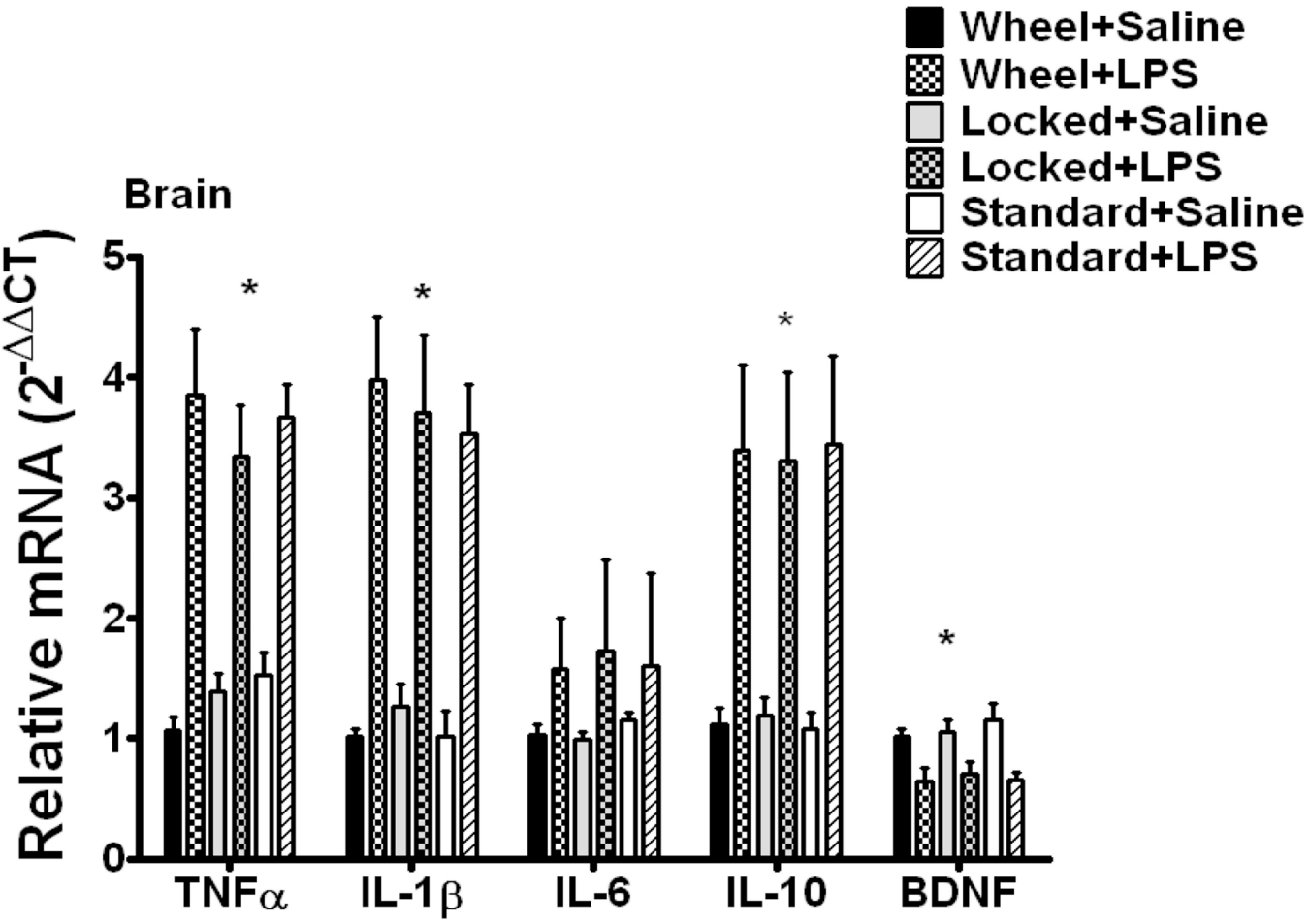

Figure 5. Effect of VWR on LPS-induced brain cytokine and BDNF mRNA expression 24h post-injection

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.

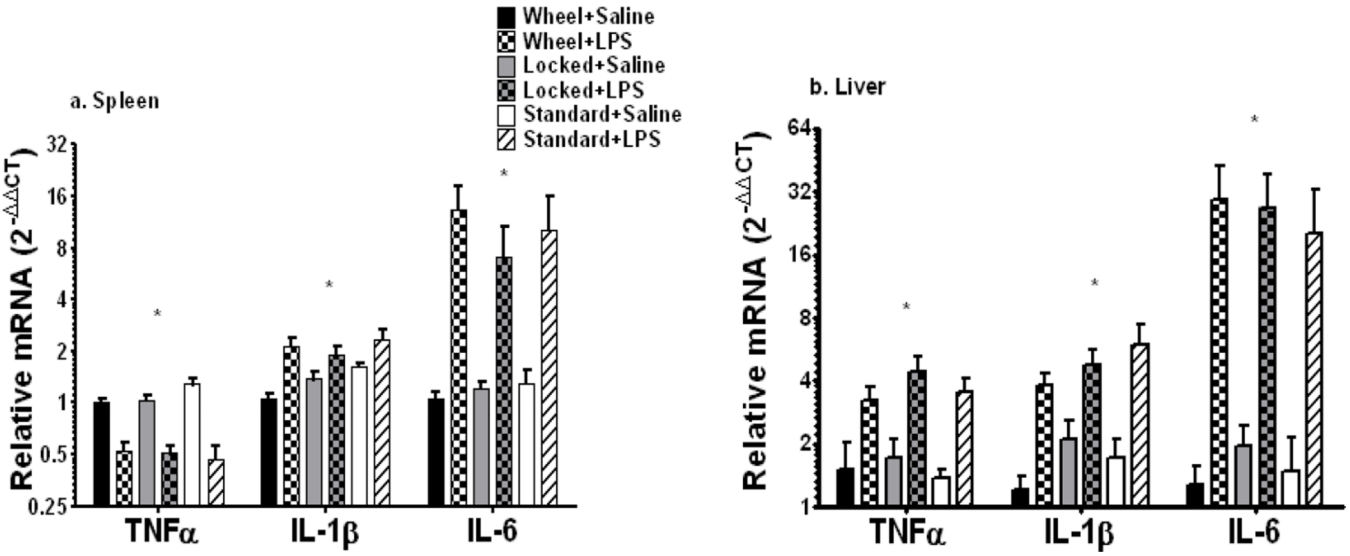

Figure 6. Effect of VWR on LPS-induced peripheral tissue inflammatory gene expression in (a) spleen, and (b) liver 24h post-injection

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.

	Distance Run (km/d)	Pre-Intervention Body Weight (g)	Post-Intervention Body Weight (g)	Body Weight Change (%)	Forced Exercise Time-to-Fatigue (minutes)	Epididymal Adipose Weight (% Body Weight)
Voluntary Wheel	3.54 ± 0.22	34.78 ± 0.38	32.35 ± 0.29	−6.88 ± 0.61 [a]	82.89 ± 5.89 [a]	1.46 ± 0.16 [a]
Locked Wheel	----	34.53 ± 0.35	33.38 ± 0.28	−3.42 ± 0.47 [b]	38.27 ± 3.00	2.10 ± 0.28
Standard	----	33.75 ± 0.45	33.15 ± 0.34	−.332 ± 0.57	26.90 ± 3.36	2.16 ± 0.28

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 2

Mortality in aged mice across intervention and LPS dose.

	LPS Dose				
	Saline	**0.02 mg/kg**	**0.08 mg/kg**	**0.16 mg/kg**	**0.33 mg/kg**
Voluntary Wheel	0/28	2/10 (24h, 48h)	2/10 (72h, 96h)	2/10 (120h, 120h)	1/11 (72h)
Locked Wheel	0/28	0/9	2/11 (24h, 96h)	0/10	3/13 (72h, 96h, 120h)
Standard	0/27	0/9	2/8 (24h, 120h)	1/11 (120h)	4/11 (8h, 8h, 24h, 96h)

Numbers represent: # deaths/ # total mice in group; time of each death.