EXERCISE ATTENUATES SICKNESS BEHAVIOR AND PROTECTS AGAINST DOPAMINERGIC IMPAIRMENT INDUCED BY NEUROINFLAMMATION

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ABSTRACT

Neuroinflammation affects dopamine metabolism and produces a set of symptoms known as sickness behavior, including fever, anhedonia, anorexia, weight loss, decreased sociability and mobility, and cognitive impairment. Motor and cognitive impairments related to sickness behavior are associated with dopamine (DA) metabolism imbalance in the prefrontal cortex. Lipopolysaccharide (LPS) administration induces neuroinflammation and causes sickness behavior in mice, while physical exercise has anti-inflammatory properties and may attenuate sickness behavior and DA impairment. We investigated the effect of exercise on DA levels and sickness behavior induced by LPS in mice. Adult Swiss male mice (8–10 weeks, 47.1 ± 0.7 g, n=495) performed six weeks of voluntary exercise in free-running wheels (RW group) or had the blocked wheel in their cages (sedentary, SED group). After six weeks of exercise, both groups received an intraperitoneal injection (i.p.) of either saline (SAL) or LPS (0.33 mg/kg, i.p.). All animals were submitted to behavioral tests for sickness behavior assessment (fatigue, locomotion, anhedonia, and social interaction). Neuroinflammation markers and DA metabolism were assessed in the prefrontal cortex. LPS administration provoked anorexia, body weight loss, impaired motor function, social withdrawal, and anhedonia. This sickness behavior was accompanied by reduced cortical DA metabolism and its metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC). Neuroinflammation was confirmed through increased levels of the proinflammatory cytokines IL-1β and IL-6. Inflammation was also confirmed in the blood by an increased content of IL-1β. Physical exercise intervention prevented animals from neurochemical, biochemical, and behavioral alterations. These findings provide new evidence of physical exercise's potential as an environmental approach to treating neuroinflammatory conditions.

Keywords Physical Exercise; Neuroinflammation; Lipopolysaccharide; Sickness Behavior; Dopamine.

INTRODUCTION

Normal levels of inflammatory cytokines are essential for brain neurocircuitry maintenance [1]. However, exacerbated and chronic inflammation and excess of inflammatory cytokines affect neuronal integrity and the monoamine neurotransmitter system in the brain, producing a range of behavioral disturbances consistent with dopamine (DA) function changes [2]. Sickness behavior is a set of symptoms induced by increased inflammatory cytokines due to viral infections, neuroinflammation, or even immune trauma [3–8]. Sickness behavior's primary symptomatology is fever, anhedonia, anorexia, weight loss, decreased sociability, mobility, and cognitive impairment [9, 10]. Although sickness behavior has been described as a conservation-withdrawal behavioral state due to metabolic constraints [11], its symptoms impair general activity, quality of life, daily and professional activities [10]. Motor and cognitive disturbances of sickness behavior are associated with DA metabolism imbalance in the prefrontal cortex [12–16].

Lipopolysaccharide (LPS) is an endotoxin present in the outer membrane of Gram-negative bacteria [17] that increases the concentration of proinflammatory cytokines interleukin (IL)-1β, IL-6, and tumor necrosis factor-α

(TNF-α) in the central nervous system (CNS) [8, 10, 18]. Neuroinflammation is a common mechanism in the pathogenesis of several neurodegenerative and psychiatric diseases, *e.g.*, Alzheimer's disease and Parkinson's disease [16, 19, 20]. Proinflammatory cytokines may affect multiple aspects of DA neurotransmission, including DA synthesis, reuptake, or DA packing and release, reducing DA function [1]. Basal ganglia-mediated fatigue and decreased mobility are resistant to treatment with classical stimulant medications that increase dopamine transporter (DAT) mediated DA release and/or block DA reuptake, indicating cytokine effects on DA function may impair or avoid the mechanism of action of pharmacological treatment [21].

Regular exercise contributes to brain health maintenance [22–26] through reduction of the inflammatory response [27, 28], improvement of dopaminergic neurotransmission [29], neurogenesis, cell survivor, angiogenesis [30], mitochondrial morphology [31], and blood-brain barrier integrity [31]. However, exercise effects on LPS induced-neuroinflammation and sickness behavior are contradictory [32–35]. The literature points out conflicting data on TNF-α response in exercised LPS-treated animals [36, 37]. Because these studies were carried out on a treadmill, we evaluated the effect of a light-intensity voluntary exercise protocol using running wheels (RW). Exploring the effects of physical exercise on sickness behavior will provide insights about complementary strategies to pharmacological interventions in the treatment of neuroinflammatory diseases [10, 38]. Further understanding of how neuroinflammation affects DA metabolism will enhance our knowledge of neuroinflammatory conditions' behavioral consequences.

MATERIALS AND METHODS

Animals and running wheel (RW)

Adult Swiss male mice (8–10 weeks, 47.1 ± 0.7 g, n=495) from the Central Facility of Federal University of Santa Catarina (UFSC) were used. The UFSC Animal Care and Use Committee (IACUC) approved the research (protocols IACUC-PP10616 and PP10519). The animals remained in the mouse vivarium of the Biochemistry Department of UFSC. They were housed in polypropylene collective cages (38 · 32 · 17 cm) lined with wood shavings, inside ventilated cabinets (Alesco Ind, Monte Mor, SP, Brazil) under a controlled environment (12h light-dark cycle, lights on at 7:00 h, room temperature 21±1°C). The access to commercial food (3.9 kcal/g, Nuvilab CR1, Nuvital Nutrientes S/A, Colombo, PR, Brazil) and tap water was *ad libitum*.

First, the animals were divided into sedentary (SED) and exercise groups (RW). Running mice were housed in individual polypropylene cages (27 × 18 × 13 cm) equipped with RW (4½", Super Pet, USA) to stimulate voluntary running [28, 39, 40]. Control SED animals were also isolated and had access to a "locked" RW (not spinning). Mice that performed 2 km/day in the RW for two weeks were selected to the exercise group (RW) and performed four more weeks of exercise. Running distance was measured every 24 hours at the same time (19:00 h). The free and locked wheels remained in the same location in the cage [28, 39, 40]. Wheels blockade reduces the environmental enrichment bias of running wheels, and, therefore, the effects observed derive from physical exercise only. Animals that did not meet RW or SED group criteria were allocated to other experiments (about 70%). The allocation for experimental groups was random. For each test, the experimental unit was an individual animal.

LPS administration and sickness behavior

After six weeks of exercise, RW and SED groups received an intraperitoneal injection (volume 10 mL/kg) of either LPS 0.33 mg/kg (serotype 0127: B8, Sigma) to induce neuroinflammation [7, 41–43] or vehicle (NaCl 0,9%). We monitored running performance for another week to indicate fatigue, body mass, and food intake. Treatments were administered at 9:00-10:00h to conduct behavioral tests (open-field, splash test, and social interaction) after 4 hours (13:00-14:00h). No animals died during the experimental protocol until euthanasia. Animals were immediately euthanized (cervical dislocation) after behavioral tests. Blood and prefrontal cortex were collected for ELISA (enzyme-linked immunosorbent assay) and HPLC (high-performance liquid chromatography) analysis. Blood samples were centrifuged at 5000 rpm for 5 min for serum collection. The serum and prefrontal cortex were stored at -80°C until biochemical analysis.

The locomotor activity of animals was assessed on the open-field test. Each animal could freely explore a circular apparatus (60 cm diameter) for 5 minutes in a 10 lux lighting test room [44]. The total distance (m), rearings (number and duration), and speed (maximal and average speed, m/min) were measured using the ANY-maze™ software. The splash test evaluates the self-care and anhedonic-like behaviors of animals. Sucrose 10% was sprinkled in the back of animals [45], and grooming (frequency and duration) was manually measured for 5 minutes. The social interaction test analyzes socialization. A female mouse (male matched-age) was placed in the center of the open field with the SAL- or LPS-treated male. The interaction was manually timed for 5 minutes [46].

Enzyme-linked immunosorbent *assay* (*ELISA*)

The prefrontal cortex was homogenized into five volumes of tris 10 mM solution containing 1 mM ethylenediamine tetra-acetic acid, triton 1%, protease inhibitors aprotinin 1μ/mL, chemostatin 1 μ/mL, leupeptin 1 μ/mL, and 1 μ/mL of phenylmethylsulfonyl fluoride. After, the sample was centrifuged (14.000 RPM, 10 minutes, 4ºC), and the supernatant was used for the quantification of IL-1β e IL-6 cytokines using commercial kits (R&D System®).

High-performance liquid chromatography (HPLC)

The prefrontal cortex was sonicated (5 minutes) in 10 volumes of 0.1 N perchloric acid/0.02% sodium metabisulfite. DA and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) levels were determined by analyzing 20 μL of the supernatant in a system composed of a mobile phase containing sodium phosphate (90 mM), citric acid (50 mM), sodium heptane sulfonate (1.7 mM), EDTA (48 μM), 10% acetonitrile and pH 3.0 with a flow of 0.3 mL/minute in a column 120x2.0 mm C18 (Synergi Hydro). Under these conditions, monoamines' retention time and their metabolites were approximately 2.7 minutes for DA and 4.2 minutes for DOPAC. The concentrations of DA and DOPAC were determined by an electrochemical detector (Waters 2465) on the HPLC (Alliance e2695, Waters, Milford, USA) and calculated as ng/mg protein [47].

Statistics

Ten independent experiments were carried out. Data are presented as mean±SEM in graphs built using the GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA (www.graphpad.com).

Statistical analyzes were performed according to an intention-to-treat principle using StatSoft, Inc. (2007). STATISTICA (data analysis software system), version 8.0. www.statsoft.com. Unicaudal two-way analysis of variance ANOVA was used to evaluate open field, splash test, social interaction, IL-6, IL-1β, DA and DA/DOPAC ratio, followed by Newman-Keuls post hoc test. ANOVA with repeated measures evaluated the evolution of running distance, body weight, and food intake, followed by the Bonferroni post hoc test. The differences were considered significant when $p < 0.05$.

Effect sizes (Cohen's partial eta-square) were calculated for between-group changes, where a Cohen's was used for ANOVA, defined as 0.01 small, 0.09 medium, and 0.25 large.

Data availability

Datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

RESULTS

Physical exercise attenuates LPS-induced sickness behavior in mice

Swiss mice had a 22.6 % ± 5.6 adherence to the RW, and the runners performed 2.4 ± 0.9 km/day. There was a significant increase in the running distance from day 1, reaching a plateau on day 10 [F (41,8) = 3.8, $p < 0.05$, Figure 1A]. LPS decreased running distance within six days after LPS treatment [F (5,1) = 4.4, $p < 0.05$, Figure 1A].

There was a significant increase in the body mass of animals throughout the six weeks before treatment [F (5,245) =118.7, $p < 0.05$, Figure 1B], with no difference between exercised and sedentary animals. However, LPS treatment decreased the body weight [F (1,47) = 11.7, $p < 0.05$, Figure 1B] and food intake [F (1,43) = 7.3, $p < 0.05$, Figure 1C] in both SED and RW mice. However, the decrease in food intake returned to control levels (SAL) on the third day after LPS treatment, with no difference between SED and RW.

LPS decreased locomotor activity [F (1,28) = 11.08, $p < 0.05$, $\eta^2 = 0.30$, Figure 2A] as well as number [F (1,28) = 26.47, $p < 0.05$, Figure 2B] and time [F (1,28) = 23.48, $p < 0.05$, Figure 2C] of rearings in the open field with large Cohen's effect sizes ($\eta^2 = 0.48$). The exercise did not protect against LPS-induced motor impairments (Figure 1A), but partially recovered the rearings (Figure 2B-C). LPS also decreased number [F (1,28) = 15.13, $p < 0.05$, Figure 2D] and time [F (1,28) = 7.73, $p < 0.05$, Figure 2E] of grooming in the splash test with moderate Cohen's effect size ($\eta^2 = 0.23$). Exercise was effective in recovering self-care of animals (Figure 2D-E). In addition, the large ($\eta^2 = 0.52$) social isolation in SED animals treated with LPS was partially reversed by exercise [F (1,28) = 27.27, $p < 0.05$, Figure 2F].

Physical exercise attenuates LPS-induced cortical neuroinflammation and DA depletion

LPS increased serum [F (1,19) = 7.86, $p < 0.05$, $\eta^2 = 0.67$, Figure 3A] and cortical levels of IL-1β [F (1,19) = 3.93, $p < 0.05$, $\eta^2 = 0.19$, Figure 3B], with no effects of exercise. LPS also increased cortical IL-6 levels in the prefrontal cortex, which was attenuated by exercise [F (1,19) = 4.80, $p < 0.05$, $\eta^2 = 0.23$, Figure 3C].

LPS decreased DA [F (1,24) = 5.4, $p < 0.05$, $\eta^2 = 0.27$, Figure 3D]; and DOPAC levels [F (1,27) = 11.2, $p < 0.05$, $\eta^2 = 0.29$, Figure 3D] in the prefrontal cortex of animals, effects mitigated by exercise. Thus, LPS increased cortical dopaminergic turnover, decreased by exercise [F (1,25) = 5.9, $p<0.05$, $\eta^2 = 0.32$, Figure 3E].

DISCUSSION

This study demonstrated that LPS induced neuroinflammation and disruption of DA neurotransmission in the prefrontal cortex, as indicated by increased IL-1β and decreased DA levels, respectively, neuroinflammation-induced sickness behavior, and decreasing locomotor activity, food intake, self-care, and social interaction. Physical exercise decreased cortical DA turnover, maintaining DA levels in the prefrontal cortex, and attenuated neuroinflammation and sickness behavior in mice.

Decreased exploration of new environments (*e.g.*, open field) is a sensitive measure of sickness behavior [6]. Martin et al. [33, 48] did not observe the effects of exercise on LPS-induced mobility impairments in his experimental design: a rectangular arena of 0.57 m^2. Here, exercise benefits to motor behavior were observed in a larger, circular arena, with 113 m^2. The animal strains were different, C57BL6 for Martins et al. [33, 48], and Swiss in this study. These strains have different sensitivity to LPS, being higher in C57BL6 [49]. In common, a decrease in food intake and RW fatigue were observed in this work, and Martins et al. studies [33, 48], with no benefits from the previous exercise.

LPS also induces social withdrawal [42, 50]. We observed exercise-induced protection to self-care, anhedonia, social withdrawal in LPS-treated mice, and emotional components of behavior. Although there is no evidence of these latter effects, the literature is robust on the anxiolytic and antidepressant effects of exercise [51–53] in various animal models of depression and other neurological diseases [54, 55]. Sickness behavior is an adaptive reorganization of the host's priorities during an infectious period, necessary for survival [33]. The benefits of exercise in attenuating, rather than disappearing, corroborate sickness behavior as a phenotype necessary for recovery.

The immune system is responsive to exercise in an intensity-dependent manner [56, 57]. High-intensity physical training increases the risk of upper respiratory tract infections, while mild-moderate intensity training protects against these infections [56, 58]. In this study, LPS increased serum levels of IL-1β and cortical levels of

IL-1β and IL-6. Harden et al. (2008) [35] reported that both interleukin IL-1β and IL-6 act synergistically in the brain to induce sickness behavior. Godbout et al. (2005) also demonstrated that LPS increases IL-6 levels in the mouse brain [43]. However, the anti-inflammatory effects of exercise for LPS on the CNS are controversial. Martins et al. [33, 48] observed no effects of exercise (RW, 4-10 weeks) on increasing brain levels of IL-1β and IL-6 in LPS-treated C57BL6 mice. We used Swiss mice; light exercise partially reversed the cortical increase in IL-6 in LPS-treated animals. The greater sensitivity of the C57BL6 strain to LPS may explain this difference [49], making it impossible to detect differences. Evidence is robust in demonstrating anti-inflammatory effects of exercise on the brain of aged rodents [28] or models of Alzheimer's [59] and Parkinson's disease [60]. One mechanism is the negative regulation of TLR signaling [61]. TLR4 is the receptor for LPS that signals the cascade of proinflammatory cytokines. In humans, exercise decreases the expression of TLR4 in circulating monocytes [62, 63]. However, we did not see changes in serum and cortical levels of IL-1β.

Exercise protected from LPS-induced anhedonia, which is associated with low DA metabolism in the prefrontal cortex [64–66]. Here, LPS decreased DA concentration and its metabolite DOPAC in animals' prefrontal cortex and induced anhedonia. Moreover, exercise reversed LPS-induced DA losses and DOPAC/DA ratio in the prefrontal cortex. DOPAC/DA ratio is an index of DA turnover and reflects synaptic DA terminals [29]. The exercise-induced decreased DOPAC/DA ratio suggests a decreased extracellular DA degradation at dopaminergic synapses, resulting in a higher probability of successful neurotransmission. Previous studies have also demonstrated protection from exercise against LPS-induced DA loss [60] and decreased cortical DOPAC/DA ratio in exercised animals [28, 52], associated with antidepressant and cognitive effects.

CONCLUSION

Our study demonstrated that six weeks of light-intensity exercise attenuates sickness behavior and protects from dopaminergic impairment induced by neuroinflammation in mice. Previous studies also observed the effects of inflammatory cytokines on DA function, but the precise mechanism is still unknown. Our findings support physical exercise as a complementary strategy to recovery from behavioral impairments and exacerbated inflammation underlying neuroinflammatory conditions.

DECLARATIONS

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ETHICAL PUBLICATION STATEMENT

We confirm that we have read the Journal's position involving ethical publication and confirm that this report is accurate to the guidelines.

DISCLOSURE

All authors declared no conflict of interest.

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AUTHOR CONTRIBUTIONS

Study conception and design: Alves, A.C.B.; Martins, R.P., Prediger, R.D.; Latini, A.; Aguiar Jr, AS.

Acquisition of data: Alves, A.C.B.; Pires, A.C.S.; Speck, A.E.; Scheffer, D.L.

Analysis and interpretation of data: Alves, A.C.B.; Pires, A.C.S.; Melo H.M.; Lopes, S.C.; Martins, R.P.

Drafting of the manuscript: Alves, A.C.B.; Aguiar Jr, AS.

Critical revision: Aguiar Jr, A.S.; Martins, R.P.; Alves, A.C.B. Latini, A.

AVAILABILITY OF DATA AND MATERIALS

Any data used in this report that is not found in the reading can be available when requested to the corresponding author Aguiar Jr, A.S.

CONSENT TO PARTICIPATE

All authors agreed to participate in this study and approved the final version.

CONSENT FOR PUBLICATION

All authors have read and agreed to publish this manuscript.

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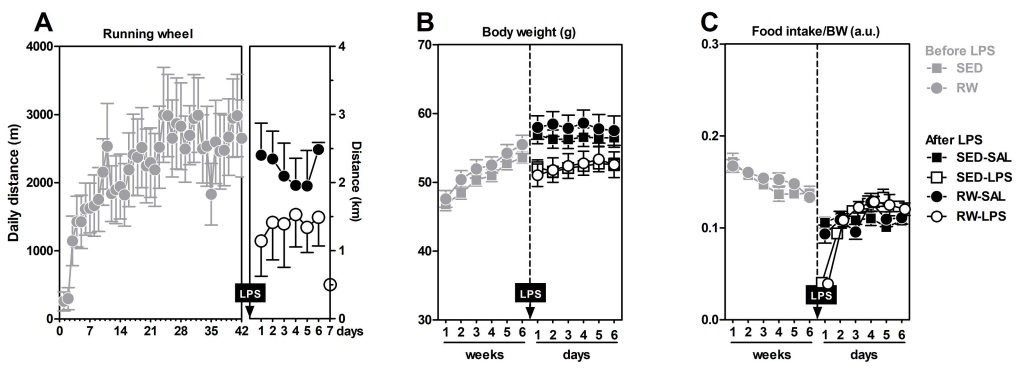
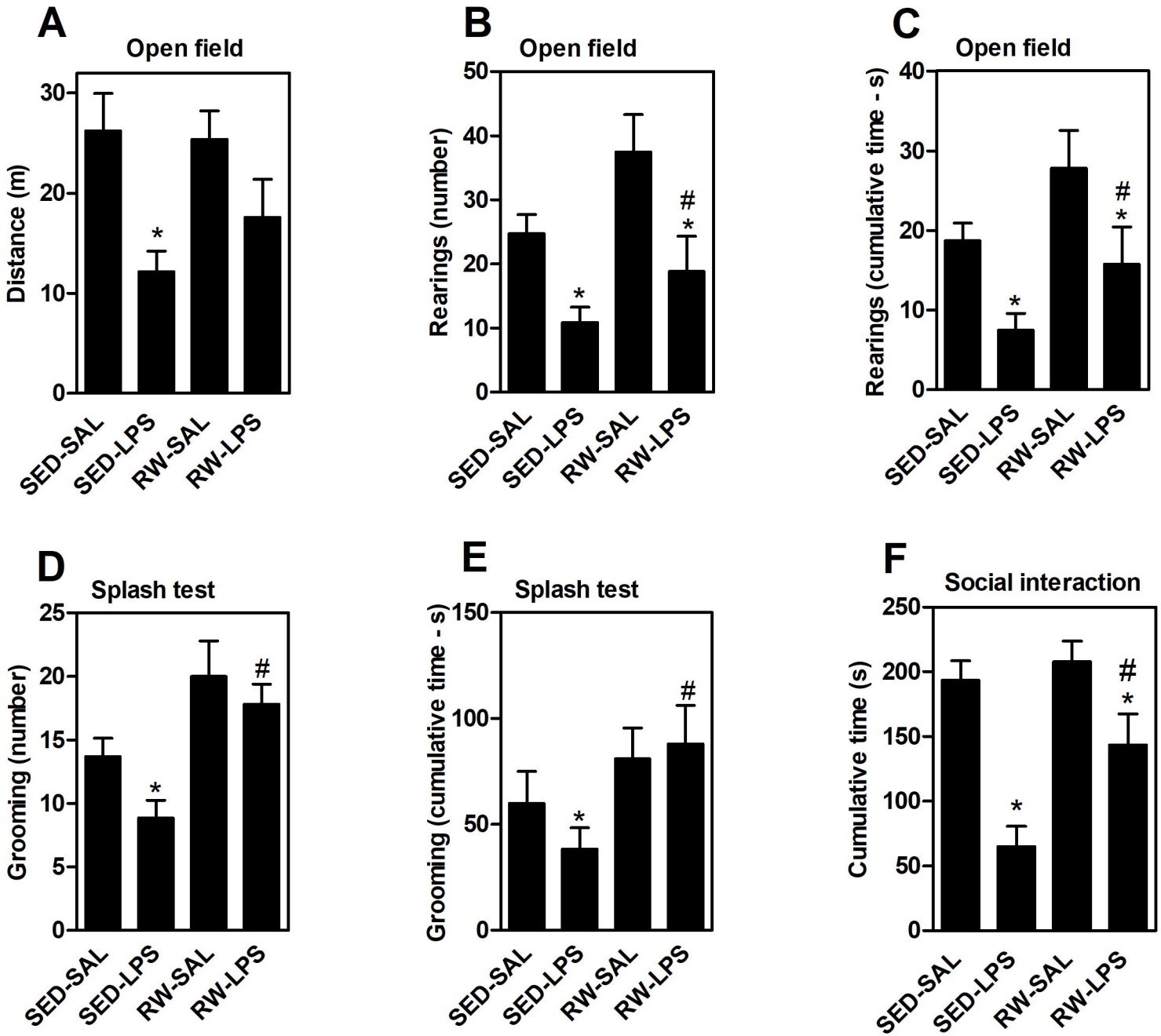
Figure legends

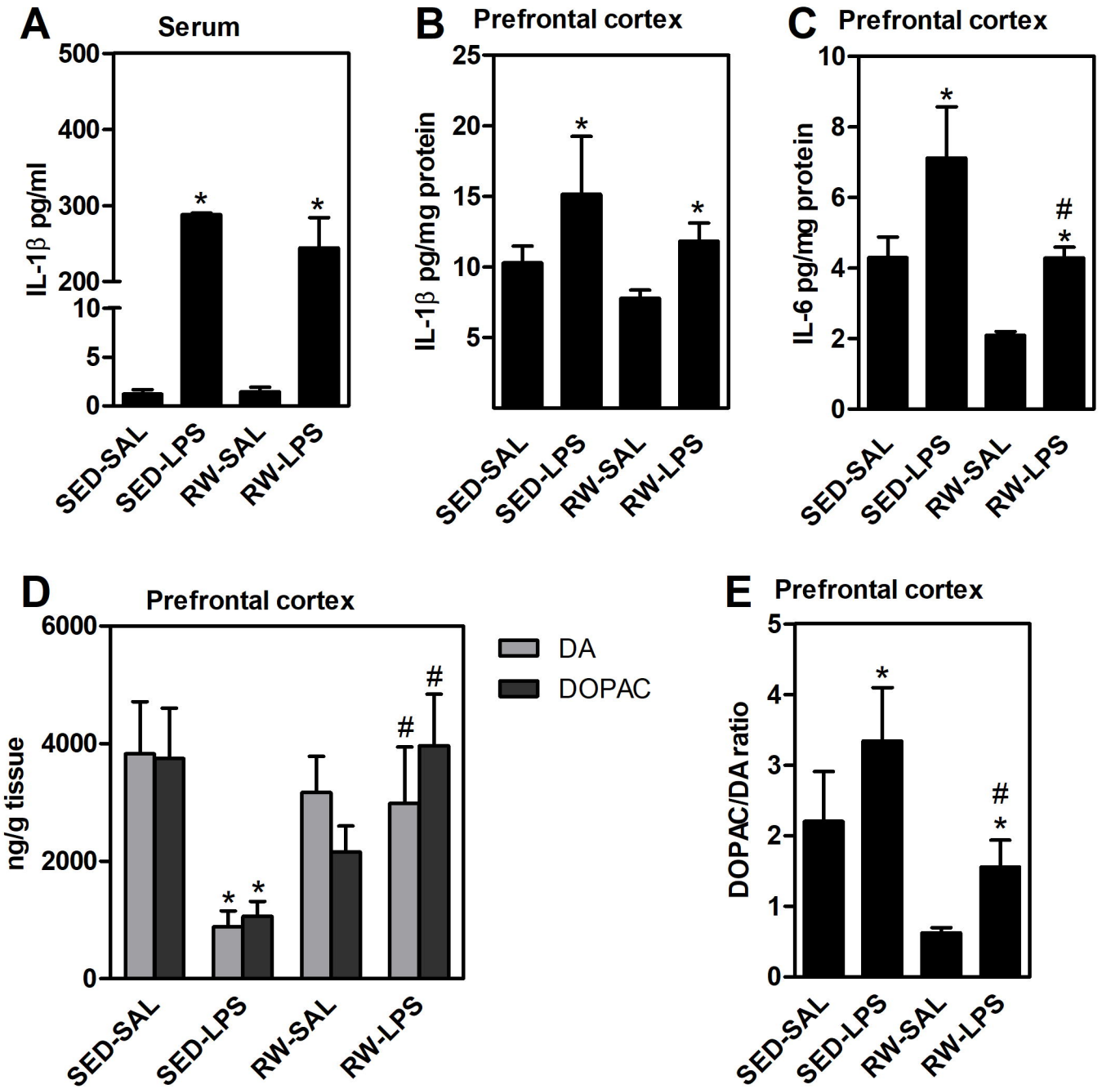
Figure 1. LPS (0.33 mg/kg, i.p.) induced fatigue in the running wheels (A) and loss of weight (B), and food intake (C) within one week after intoxication. Data expressed as mean ± SEM for 9-10 independent experiments. LPS – Lipopolysaccharide. RW – running wheel. SAL – saline. SED – sedentary.

Figure 2. LPS (0.33 mg/kg, i.p.) induced sickness behavior in mice: motor impairment (A-C), anhedonia (D-E), and social isolation (F). Long-term running partially reversed these losses. Data expressed as mean ± SEM for 9-10 independent experiments. * $P<0.05$ from SAL (ANOVA two-way). # $P<0.05$ from SED (ANOVA two-way). LPS – Lipopolysaccharide. RW – running wheel. SAL – saline. SED – sedentary.

Figure 3. LPS (0.33 mg/kg, i.p.) induced systemic inflammation (A) and neuroinflammation (B-C). LPS reduced DA and DOPAC (D) and increased DOPAC/DA turnover (E) in the animals' prefrontal cortex. The exercise partially reversed the increase in IL-6 (C) and DOPAC/DA turnover (E) in the prefrontal cortex. The recovery of DA and DOPAC levels was total (D). Data expressed as mean ± SEM for 9-10 independent experiments. * $P<0.05$ from SAL (ANOVA two-way). # $P<0.05$ from SED (ANOVA two-way). DA – dopamine. DOPAC – 3,4-dihydroxyphenylacetic acid. LPS – Lipopolysaccharide. RW – running wheel. SAL – saline. SED – sedentary.


A
Running wheel
Daily distance (m)
Distance (km)
LPS
0 7 14 21 28 35 42 1 2 3 4 5 6 7 days
B
Body weight (g)
LPS
1 2 3 4 5 6 1 2 3 4 5 6
weeks
days
C
Food intake/BW (a.u.)
LPS
1 2 3 4 5 6 1 2 3 4 5 6
weeks
days
Before LPS
SED
RW
After LPS
SED-SAL
SED-LPS
RW-SAL
RW-LPS


A
Open field
Distance (m)
0
10
20
30
*
SED-SAL
SED-LPS
RW-SAL
RW-LPS
B
Open field
Rearings (number)
0
10
20
30
40
50
*
#
*
SED-SAL
SED-LPS
RW-SAL
RW-LPS
C
Open field
Rearings (cumulative time - s)
0
10
20
30
40
*
#
*
SED-SAL
SED-LPS
RW-SAL
RW-LPS
D
Splash test
Grooming (number)
0
5
10
15
20
25
*
#
SED-SAL
SED-LPS
RW-SAL
RW-LPS
E
Splash test
Grooming (cumulative time - s)
0
50
100
150
*
#
SED-SAL
SED-LPS
RW-SAL
RW-LPS
F
Social interaction
Cumulative time (s)
0
50
100
150
200
250
*
#
*
SED-SAL
SED-LPS
RW-SAL
RW-LPS


A
Serum
IL-1β pg/ml
500
400
300
200
10
5
0
SED-SAL
SED-LPS
RW-SAL
RW-LPS
B
Prefrontal cortex
IL-1β pg/mg protein
25
20
15
10
5
C
Prefrontal cortex
IL-6 pg/mg protein
10
8
6
4
2
0
#
D
Prefrontal cortex
ng/g tissue
6000
4000
2000
0
DA
DOPAC
E
Prefrontal cortex
DOPAC/DA ratio
5
4
3
2
1