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Systemic LPS-induced neuroinflammation increases the susceptibility for proteasome inhibition-induced degeneration of the nigrostriatal pathway

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ABSTRACT:

Introduction: Besides proteasome dysfunction, neuroinflammation is a common feature in the pathogenesis of Parkinson's disease (PD). Accordingly, peripheral inflammation has been shown to increase the susceptibility of the brain for nigrostriatal degeneration by inducing activation of glial cells and release of pro-inflammatory cytokines in the brain. Given that current animal models of PD fail to recapitulate the pathophysiology occurring in idiopathic PD, the aim of this study was to combine two pathogenic mechanisms (i.e. neuroinflammation and proteasome inhibition) to create a dual-hit mouse model of PD.

Methods: We repeatedly injected mice with a low dose of LPS (250 µg/kg/day i.p. for four days) to induce neuroinflammation, followed by a unilateral intranigral injection of lactacystin (LAC; 3 µg). Seven days later, mice were evaluated behaviorally to assess locomotion, anxiety- and depressive-like behavior. Nigrostriatal degeneration was analyzed by measuring striatal dopamine loss as well as loss of nigral dopaminergic neurons. Neuroinflammation was confirmed by quantifying microglial cells in the substantia nigra (SN) and cytokine expression in the striatum.

Results: Repeated systemic LPS injections increase the number of microglial cells in the SN and induce a mixed profile of pro- and anti-inflammatory cytokines in the striatum without affecting the integrity of the nigrostriatal pathway. Systemic LPS-induced neuroinflammation, however, increases the susceptibility of the nigrostriatal pathway for LAC-induced degeneration.

Conclusion: Recapitulating two relevant etiopathogenic mechanisms of PD - neuroinflammation and proteasome inhibition-, we propose this dual-hit model as a relevant mouse model for PD that could be used to investigate potential therapeutic targets.

INTRODUCTION

Parkinson's disease (PD) is an age-related debilitating neurodegenerative disorder characterized by a selective and gradual loss of dopaminergic innervations from the Substantia Nigra (SN) pars compacta (SNpc) to the striatum. Over the years, findings from epidemiological studies, *post-mortem* PD brains and animal PD models have provided evidence to support the role for central and peripheral inflammation in the pathogenesis of PD [1]. Microglia participate to central nervous system homeostasis by removing debris and responding to injury, while synthetizing a variety of cytokines and neurotrophic factors. However, in the context of neurodegenerative disorders, it is hypothesized that high levels of pro-inflammatory mediators released by chronically activated microglia, damage neurons and further activate microglia, resulting in a feed-forward inflammatory cycle [2]. Preclinical studies demonstrated that neuroinflammation induced by single or repeated systemic lipopolysaccharide (LPS) exposure replicates some characteristics of PD: a prolonged and widespread microglial activation results in progressive loss of dopaminergic neurons in the nigrostriatal system in days, weeks or months depending on the paradigm used [3]. Moreover, microglia react upon LPS exposure and the subsequent increased cytokine levels by adopting an atypical or primed state; a phenomenon also observed in the aged central nervous system. These primed microglia could exert an exaggerated pro-inflammatory response, thereby enhancing the neurodegenerative effects of later exposure to a second stimulus [4].

Multiple-hit animal PD models might represent a valid alternative to well-established models of PD. For instance, novel models of idiopathic PD are generated by exposure to a combination of the classical toxins/agents such as LPS, MPTP, 6-OHDA, rotenone, etc [5]. In the early 2000s, an intriguing new factor has been associated with the pathogenesis of PD. *Post-mortem* data from sporadic PD patients indicated that proteasome function is impaired in the SN, including loss of 20S core alpha subunits, decreased expression of 19S and general loss of all

three peptidase activities. The underlying cause of proteasome dysfunction in PD has not been elucidated [6,7]. A recent approach to model proteasome dysfunction *in vivo* has been the use of the toxin lactacystin (LAC), a selective proteasome inhibitor that inhibits all three peptidase activities of the 20S proteasome. LAC has been successfully used in rats and mice as its nigral administration produces a fast-onset PD-like phenotype, including α-synuclein accumulation, dopaminergic cell loss and behavioral deficits [6,8,9]. Despite neuroinflammation and proteasome dysfunction being two significant hallmarks of many neurodegenerative diseases, the relationship between both factors is poorly understood. In this study, we evaluated the effect of prior exposure to an inflammogen (i.e. LPS) on proteasome inhibition-induced parkinsonism in mice.

MATERIAL AND METHODS

Animals

Male C57BL/6J mice (11-12 weeks of age; Charles River Laboratories) were group-housed (2-6 mice/cage) in a 14/10 h light/dark cycle with free access to food and tap water. Temperature (21-25 ºC) and relative humidity (30-60 %) were maintained constant during the experiments, which were carried out according to the Belgian animal welfare legislation (Royal Decree of 29 May 2013) and the regulations covering animal experimentation in the EU (European Communities Council Directive 2010/63/EU). The Ethical Committee for Animal Experiments (Vrije Universiteit Brussel) approved the experiments.

Induction of peripheral inflammation

Peripheral inflammation was induced by repeated intraperitoneal (i.p.) LPS (*Escherichia coli*, O55:B5, Sigma-Aldrich) injections of 250 µg/kg over four consecutive days while control mice received i.p. injections of vehicle (physiological saline, 0.9% w/v of NaCl). Solutions were made freshly before administration. Prior to each injection, mice were weighed, and body temperature was measured to evaluate LPS-induced sickness behavior.

Stereotaxic surgery

Mice were anesthetized (i.p. injection of a mixture of ketamine (100 mg/kg; Ketamine 1000 Ceva, Ceva Sante Animale) and xylazine (10 mg/kg; Rompun 2%, Bayer N.V)) and positioned in an Ultra Precise Small Animal Stereotaxic Frame (David Kopf Instruments). A small hole was made through the skull above the left SNpc (AP -3.0, LM -1.0, DV -4.5 from Bregma). A volume of 1.5 µl freshly-dissolved LAC (2 µg/µl in NaCl 0.9%; Cayman Chemical) was injected at a flow rate of 0.5 µl/min into the left SNpc. Sham-operated mice received the same volume of saline. After injection, the syringe was left in place for five additional min, and then slowly removed. The skin was sutured, and mice received 4 mg/kg ketoprofen subcutaneously (Ketofen, Merial) for post-operative analgesia.

Behavioral assessment

Seven days after LAC lesion, mice underwent behavioral assessment to test motor dysfunction using the rotarod and open field test, and anxiety- and depressive-like behavior using the open field, light-dark and tail suspension test [6,10] as described in Supplementary material.

Neurochemical analysis of striatal dopamine content

Striatal content of dopamine and the selected metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured using HPLC (see Supplementary material for more details).

Immunohistochemistry

The caudal part of the brain was post-fixed for three days in 4% paraformaldehyde and sliced into 40 µm vibratome sections to immunohistochemically detect tyrosine hydroxylase (TH) and ionized calcium binding adapter molecule 1 (Iba1), using rabbit anti-TH (1/2000; AB152; Millipore) and rabbit anti-Iba1 antibody (1/1000; 019-19741, RRID: AB_839504), and employing the Vectastain ABC kit (Vector Laboratories). The mean number of TH+ profiles per mice was counted blindly in six serial sections throughout the rostro-caudal extent of the

SNpc (-2.92 mm to -3.60 mm relative to Bregma [11]). Similarly, the number of Iba1+ profiles/mm^2 was evaluated in three serial sections covering the SN. Immunoreactivity was visualized using 3,3'-diaminobenzidine as chromogen. Microscopic analysis and cell count of the sections were performed using ImageJ software (U.S. National Institutes of Health, Bethesda).

Western Blot analysis

In a separate cohort of mice, striatal TH expression was quantified using semi-quantitative Western blotting, using primary rabbit anti-TH antibody (1/2000, AB152) and enhanced chemiluminescence (ECL prime, GE Healthcare) as described in Supplementary material. Optical densities of TH-immunoreactive bands were normalized to those of the total amount of proteins loaded, visualized on the same membrane (SERVA Purple, Serva Electrophoresis GmbH).

Real-time PCR

IL-1β, TNF-α, nitric oxide synthase (NOS2) and arginase 1 (Arg1) mRNA expression was quantified using real-time polymerase chain reaction (qPCR). Total RNA was extracted from the striatum (RNeasy® Lipid Tissue Mini Kit; Qiagen) and the RNA concentration and purity were determined using the Nanodrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher). After cDNA synthesis (iScript™ cDNA Synthesis Kit, Bio-Rad Laboratories), real-time PCR was performed using the StepOnePlus™ qPCR system (Applied Biosystems; Foster City) in combination with TaqMan® reagents (Applied Biosystems) and TaqMan® Gene Expression Assays (more details in Supplementary material). The results were processed according to the 2-ΔΔCT method and mRNA expression levels were expressed as fold changes relative to vehicle-treated mice and normalized against the geometric means of Bcl2113 mRNA expression levels.

Statistical analysis

Data were expressed as mean ± Standard Error of the Mean (SEM). Statistical analysis was performed using GraphPad Prism 6.01 software. To study one variable within one group of animals, we employed Mann Whitney U test. Two paired groups were analyzed using the two-sided Wilcoxon matched-pairs signed-rank test. For analysis of multiple variables within multiple groups of animals, we applied two-way ANOVA followed by Tukey's *post hoc* test on the significant main effects. The α-value was set at 0.05.

RESULTS

Four daily LPS injections induce changes in body weight

Mice were injected for four consecutive days with either vehicle or LPS and were sacrificed on the fifth day (96 hours after the first injection). Body weight and temperature were measured 24 hours after each injection to assess sickness behavior. At the end of the four-day treatment period, LPS-treated mice had a significant decrease in body weight compared to baselines (i.e. before the first injection) [$p < 0.0001$], contrary to vehicle-injected mice [$p > 0.05$, Figure 1A]. However, whereas the first [$p < 0.0001$] and second [$p < 0.01$] LPS injection resulted in a significant decrease in body weight after 24 hours, the third and fourth LPS injection induced a significant increase in body weight compared to vehicle injection [$p < 0.05$, Figure 1B]. Repeated LPS treatment did not induce changes in body temperature as no significant differences could be observed at the end or during the four-day treatment between LPS- and vehicle-treated mice [$p > 0.05$, Figure 1C, D].

Repeated LPS injections activate microglial cells, without affecting the integrity of the nigrostriatal pathway

We observed a significant increase in the number of Iba1+ cells per mm^2 in the SN after the four-day LPS treatment, compared to vehicle treatment [VEH: 339.9 ± 8.774 cells/mm^2, LPS: 402.3 ± 22.80 cells/mm^2, $U = 5.000$, $p = 0.0221$; Figure 2A, B]. The mRNA expression levels of IL-1β [6.6-fold, $p = 0.0006$] and Arg1 [3.5-fold, $p = 0.0401$] were significantly higher in the

striatum of the LPS group compared to the control group, whereas no significant differences were observed for TNF-α and NOS2 [$p > 0.05$, Figure 2C]. Four repeated LPS injections do neither affect the striatal TH protein expression [$p > 0.05$; Figure 2D] nor the number of TH+ profiles in the SNpc [$p > 0.05$; Figure 2E], as compared to vehicle-treated mice.

LPS-induced neuroinflammation potentiates nigrostriatal degeneration induced by proteasome inhibition

The total number of Iba1+ cells was evaluated in the SN to study the consequences of LPS pre-treatment on LAC-induced neuroinflammation. A significant increase in the number of Iba1+ cells could be detected in the ipsilateral SN after intranigral LAC injection, compared to sham-treatment [lesion factor: $F(1,18) = 4.993$, $p = 0.0384$], independent of LPS pre-treatment (Figure 3A). We next investigated whether prior systemic LPS-induced neuroinflammation increases susceptibility of dopaminergic neurons to proteasome inhibition-induced degeneration. Immunohistochemical analyses revealed that intranigral LAC administration led to a significant loss of TH-expressing cells in the ipsilateral SNpc [lesion factor: $F(1,37) = 8.273$, $p = 0.0066$; Figure 3B, C]. In addition, neurodegeneration induced by LAC was more pronounced in mice pre-treated with LPS compared to vehicle, as *post hoc* analysis revealed a significant decrease in TH+ cells only in the LPS group. Interestingly, LPS pre-treatment significantly enhanced the LAC-induced loss of dopamine in the ipsilateral striatum [lesion factor: $F(1, 37) = 35.05$, $p < 0.0001$; treatment factor: $F(1, 37) = 4.527$, $p = 0.041$; Figure 3D]. Whereas LAC lesion induced a significant loss of striatal dopamine content in both vehicle- and LPS-treated mice, LPS pre-treatment resulted in a significantly lower dopamine content after LAC lesion, compared to vehicle pre-treatment. As an alternative measure for neuronal function, we assessed dopamine turnover (DOPAC+HVA/dopamine) in the ipsilateral striatum. There was a significant interaction between lesion and treatment [interaction factor: $F(1,37) = 4.274$, $p = 0.0458$; Figure

3E], with the dopamine turnover being significantly increased in the LPS LAC group compared to all other experimental groups.

Systemic LPS injection has only minor effects on proteasome inhibition-induced changes in motor function, without affecting depressive-like behavior

LAC-injected mice displayed a global impairment in motor coordination and balance compared to sham-treated mice on the rotarod test [lesion factor: $F(1,49) = 19.86$, $p < 0.0001$; Figure 4A], which was less pronounced after LPS treatment. In contrast, a significant increase in distance traveled, was seen in the open field test after LAC treatment [lesion factor: $F(1,49) = 15.11$, $p = 0.0003$; Figure 4B], which was more evident in the LPS pre-treated group. For all behavioral tests described, we failed to reveal any effect related to anxiety-like behavior as seen in the latency to exit and time spent outside of the shelter in the light dark test (Figure 4C, D) as well as time spent in the center of the open field test (Figure 4E). Finally, we investigated depressive-like symptoms using the tail suspension test. Interestingly, we could observe a paradoxical decrease in immobility time after LAC [lesion factor: $F(1,35) = 10.52$, $p = 0.0026$; Figure 4F], unrelated to LPS or vehicle pre-treatment.

DISCUSSION

Exposure to LPS - an inflammogen known to activate microglia and to induce widespread (neuro)inflammation depending on the dose and paradigm used - can selectively induce dopaminergic neuron loss in animals [1]. Microglia produce pro-inflammatory mediators and reactive species, which, when present in excess or over a prolonged period of time, could lead to neuronal damage and in turn contribute to sustained inflammation in PD. Microglia are considered pro-inflammatory if they produce higher levels of pro-inflammatory factors such as IL-1β, TNF-α or iNOS, while increased levels of Arg1, Ym1 and IL-10 indicate a more anti-inflammatory phenotype. Badshah *et al.* (2016) described increased Iba1 expression in the hippocampus and cortex after seven days of 250 μg/kg LPS treatment together with increased

protein expression of IL-1β, TNF-α and NOS2 [12]. Our data show that daily administration of LPS 250 μg/kg for four days resulted in a significantly increased number of Iba1+ cells in the SN. In addition, increased striatal mRNA levels of the pro-inflammatory IL-1β and anti-inflammatory Arg1 were detected 24 hours after the last LPS challenge. This mixed inflammatory profile was reported before by Beier et al. (2017), who showed increases in the pro-inflammatory markers (iNOS, IL-1β, TNF-α, IL-6) on day five after four daily LPS injections of 1 mg/kg followed by a mixed expression profile of pro- and anti-inflammatory cytokines on day 19, ending with a predominantly anti-inflammatory profile on day 36 together with a total cessation of neuronal loss [13].

The mixed inflammatory profile that we observe in the striatum after four consecutive LPS injections possibly reflects a mixed systemic inflammatory profile and could as such explain the transient weight loss in these mice: only the first and second LPS challenge induced a robust change in body weight, while the third and fourth challenge did not lead to further decreases and mice even seemed to start recovering, according to the observation made by Püntener et al. (2012) [14]. Besides sickness behavior, systemic LPS injections were shown to trigger progressive neurodegeneration in the SN especially at higher doses. Whereas a single systemic LPS (5mg/kg, i.p.) injection was reported to induce a strong inflammatory response and a slow progressive loss of TH+ neurons in the SN after 10 months of treatment [3], repeated 1 mg/kg LPS injections significantly reduced the amount of TH+ cells after 19 days [15], with no neuronal loss present on day 5 [13]. We therefore examined the possible deleterious effects of four systemic low-dose LPS injections on the nigrostriatal pathway and found no loss of dopaminergic neurons in the SNpc nor decreased striatal TH expression levels, indicating that this paradigm of LPS treatment does not affect the nigrostriatal pathway, despite the significant nigral microglial activation. The indication of a mixed pro- and anti-inflammatory profile in the

striatum could provide an explanation for this intact nigrostriatal pathway, as anti-inflammatory cytokines might gain the upper hand and protect against LPS-induced degeneration.

Prolonged systemic inflammation can give rise to hypo- (tolerance) as well as hyper- (priming) innate immune responses in the brain in response to a subsequent stimulus. Indeed, studies with double-hit animal models have shown that local or systemic application of bacterial LPS in both single or repeated challenges can aggravate toxin-induced neurodegeneration in models of PD [16–21]. In this study we administered the proteasome inhibitor LAC as a second stimulus after four consecutive injections of LPS. According to previous reports, we showed that local administration of LAC to the SN leads to degeneration of the nigrostriatal pathway [6,8,9]. Moreover, we report that seven days after intranigral LAC injection a significant increase in number of microglial cells is present in the SN compared to sham surgery, suggesting that proteasome inhibition and concomitant dopaminergic neurodegeneration triggers microglial activation. When mice were pre-treated with LPS prior to LAC exposure, we observed an enhancement of nigrostriatal degeneration, suggesting a sensitizing effect of LPS, in line with previous work in the MPTP [16], rotenone [22], paraquat [20] and 6-OHDA models [17,21]. In addition to striatal dopamine loss, a significant increase in dopamine turnover in the ipsilateral striatum was detected exclusively in the LPS pre-treated LAC-lesioned mice. Past research postulated that an increased dopamine turnover is one of the functional compensatory changes, conserving the normal motor function in PD, arising when dopamine depletion reached a certain threshold [23,24]. Local administration of LAC to the nigrostriatal pathway induces PD-related motor and non-motor symptoms [6]. Consistent with previous studies investigating the effect of nigrostriatal proteasome inhibition in rats [25–27] and mice [6,9], we observed strong impairment in rotarod performance in LAC-injected mice. This motor deficit was not affected by LPS pre-treatment, possibly as a result of the elevated dopamine turnover which could compensate for the dopamine depletion and related motor

impairment. In contrast, the open field test revealed an increase in spontaneous motor activity following LAC lesion, suggesting hyperkinetic behavior. Multiple studies discovered that unilateral intracerebral administration of a neurotoxin (i.e. MPTP, rotenone, epoxomicin and LAC) may induce hyperactivity of surviving dopaminergic neurons in the ipsilateral SNpc, which eventually results in selective increased firing frequency and consequent hyperkinetic behavior [6,28]. Besides motor symptoms, PD is also characterized by non-motor symptoms such as anxiety and depression, which are important determinants for the patient's quality of life. No differences could be noticed in the light-dark test and in the open field test between both treatment and lesion groups. These results should be interpreted with caution since both the open field and the light-dark test can be biased by the hyperactive behavior that was observed in LAC-treated animals. Consistent with the results of the open field test, we also noted an increased restlessness of both LPS- and vehicle-treated LAC-lesioned mice in the tail suspension test, suggesting hyperactivity or an increased perseverance to engage in escape-oriented behavior [6]. Our current findings strengthen the hypothesis that nigrostriatal neuroinflammation induced by peripheral inflammation increases the susceptibility of the nigrostriatal dopaminergic pathway for proteasome inhibition-induced degeneration, thereby identifying a novel and relevant mouse model for studying PD.

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FIGURE LEGENDS

Figure 1: Four daily LPS injections induce sickness behavior. (A-D) Sickness behavior was measured by determining changes in body weight and temperature 24 hours after each LPS or vehicle injection (Inj I-IV). (A) At the end of the four-day treatment period, LPS-treated mice had a significant decrease in body weight compared to baseline (i.e. before the first injection, time 0), contrary to vehicle-injected mice. (B) The first and second injection of LPS led to a significant decrease in body weight 24 hours after each injection, while the third and fourth injection induced an increase in body weight compared to vehicle-treatment. (C, D) Repeated LPS treatment did not induce persistent changes in body temperature as no significant differences could be observed between LPS- and vehicle-treated mice at the end or during the four-day treatment. Statistical analysis was performed using the (A, C) Wilcoxon matched paired test on the absolute values of the 96h timepoint; $^{\#\#\#\#}p<0.0001$ vs baseline, (B, D) Mann Whitney test; $^{*}p<0.05$, $^{**}p<0.01$, $^{****}p<0.0001$ vs. vehicle. Arrows (Inj I-IV): i.p. injection with LPS or vehicle. Sample size is indicated in the figure. LPS: lipopolysaccharide, VEH: vehicle.

Figure 2: Four daily LPS injections promote microglial activation. (A) Representative images of Iba1+ stainings in the SN of vehicle and LPS-treated mice. (B) Immunohistochemical analysis of microglia in the SN revealed an increase in the number of Iba1+ cells one day after the last LPS challenge. (C) At the same timepoint, the striatal mRNA expression profile of the pro-inflammatory cytokines IL-1β, TNF-α, NOS2 and anti-inflammatory cytokine Arg1 was evaluated by real-time PCR. Repeated doses of LPS induced significant increases in IL-1β and Arg1. (D) Our LPS treatment paradigm did not affect striatal TH protein expression levels or (E) the number of TH+ profiles in the SNpc. Data are presented as mean ±SEM. Statistical analysis were performed using Mann-Whitney test (B-E); $^{*}p<0.05$, $^{***}p<0.01$ vs. vehicle. Sample size is indicated in the figure. LPS: lipopolysaccharide, Iba1: ionized calcium binding adapter molecule 1, IL-1β: interleukin-1 beta, TNF-α: tumor necrosis factor alpha, NOS2: nitric oxide synthase 2, Arg1: arginase-1. TH: tyrosine hydroxylase, VEH: vehicle. Scalebar=50µm.

Figure 3: **LPS-induced neuroinflammation enhances proteasome inhibition-induced degeneration of the nigrostriatal pathway.** (A) Intranigral LAC injection increases the amount of Iba1+ cells in the SN seven days after lesioning, independent of pretreatment with LPS. (B) Unilateral nigral administration of LAC significantly decreased the mean number of TH+ cells in the ipsilateral SNpc compared to sham treatment. LPS treatment prior to LAC administration resulted in a more pronounced TH+ cell loss compared to vehicle treatment. (C) Representative microphotographs of TH-staining in the SNpc in the four experimental groups. (D) LPS pre-treatment significantly increased striatal dopamine depletion and (E) dopamine turnover induced by LAC, seven days after lesioning. Data are presented as mean ±SEM. Two-way ANOVA followed by a Tukey's *post hoc* test; $^{*}p<0.05$, $^{****}p<0.0001$ (LAC vs sham); $^{\#}p<0.05$ (LPS vs vehicle), $^{\$}p<0.05$ (vs all treatment groups). Sample size is indicated in the figure. LAC: lactacystin, LPS: lipopolysaccharide, TH: tyrosine hydroxylase, Iba1: ionized calcium binding adapter molecule 1, DA: dopamine, DOPAC: 3,4-dihydroxyphenylacetic acid, HVA: homovanillic acid, VEH: vehicle. Scalebar=250µm.

Figure 4: LAC-treated mice develop motor and non-motor symptoms. (A) The accelerating rotarod test showed decreased time spent on the rod after LAC treatment suggesting an impaired motor coordination and balance, which was less pronounced after LPS treatment. (B) Spontaneous horizontal activity was globally increased in the open field test after LAC lesion; an effect that was enhanced by LPS pre-treatment. (C, D) Anxiety- like behavior was unaffected by LPS or LAC treatment as shown in the light-dark test and (E) the open field test. (F) LAC-induced a decrease in immobility time in the tail suspension test. Two-way ANOVA followed by a Tukey's *post hoc* test; $^{*}p<0.05$, $^{**}p<0.01$ (LAC vs sham). Data are presented as mean ±SEM. Sample size is indicated in the figure. LAC: lactacystin, LPS: lipopolysaccharide, VEH: vehicle.

SUPPLEMENTARY MATERIAL

Rotarod test

Prior to surgery, mice were trained on a rotarod (TSE RotaRod Advanced, TSE Systems) and baseline performance was evaluated. Mice were allowed to acclimate to the testing room for 1h prior to testing. During the initial training phase, each mouse was placed on the rod at a constant speed of five rpm. When mice dropped off the rod, they were immediately placed back on the rotarod system for five consecutive min. In the second training phase, mice underwent three consecutive trials of one min at a constant speed of five rpm, with three min of rest between each trial. For evaluating rotarod performance at baseline and seven days after surgery, mice underwent five consecutive trials, each starting at five rpm for 30 seconds, followed by a five to 25 rpm accelerating protocol for 200 seconds, eventually resulting in a maximum total rod time of 230 seconds. During the test, mice were allowed to pause three min in between each trial. The mean time spent on the rod of the five trials was used for statistical analysis.

Open field test

Spontaneous locomotion and anxiety-like behavior were evaluated using the open field test. Mice were placed in a corner of a square box (60 cm × 60 cm; height 60 cm) with black opaque Plexiglas walls and automatically recorded for 10 min by a video tracking system (Ethovision software, Noldus). Endpoints for analysis are the spontaneous horizontal activity which is expressed as distance travelled (as a measure for spontaneous locomotion) and time spent in the center of the arena defined as the central 40 x 40 cm zone (as a measure for anxiety-like behavior).

Tail suspension test

The tail suspension test was used to examine depressive-like behavior. At the level of the tail suspension set-up we created an aversive illumination of 400 lux. Mice were suspended by the tip of their tail for five min to induce an inescapable situation. The time of immobility was

scored off-line by a blinded researcher and used as a parameter to assess depressive-like behavior. Mice that climbed their tail during the trial were excluded from the experiment.

Light-dark test

The light-dark test investigates anxious behavior by comparing the spontaneous exploratory activity of mice with their innate aversion to well-illuminated areas. At the beginning of the experiment, each mouse was placed in a dark shelter (30 x 30 x 8.5 cm; ±0 lux), positioned in the corner of a brightly-illuminated open field area (60 x 60 x 60 cm; 700 lux), in order to induce a conflict situation. During this experiment, two anxiety-related parameters (time spent outside the shelter and latency to exit the shelter) were manually evaluated for five min by a blinded researcher.

Neurochemical analysis of striatal dopamine content

Striata were dissected out on an ice-cold petri dish, weighed and homogenized in 400 µl antioxidant solution [0.05M HCl, 0.5% $Na_2S_2O_5$, and 0.05% Na_2EDTA], containing 10 ng/100µl 3,4-dihydroxybenzylamine as internal standard. After centrifugation (12.000 rpm, 20 min, 4 °C), the supernatant was diluted (1:5 in 0.5 M acetic acid) and stored at -80°C until use. Twenty µl of the diluted sample was analyzed for dopamine and the selected metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), using a narrow-bore (C18 column, 5 µm, 150 mm x 2.1 mm; Altima Grace) HPLC system with an electrochemical detector (Antec).

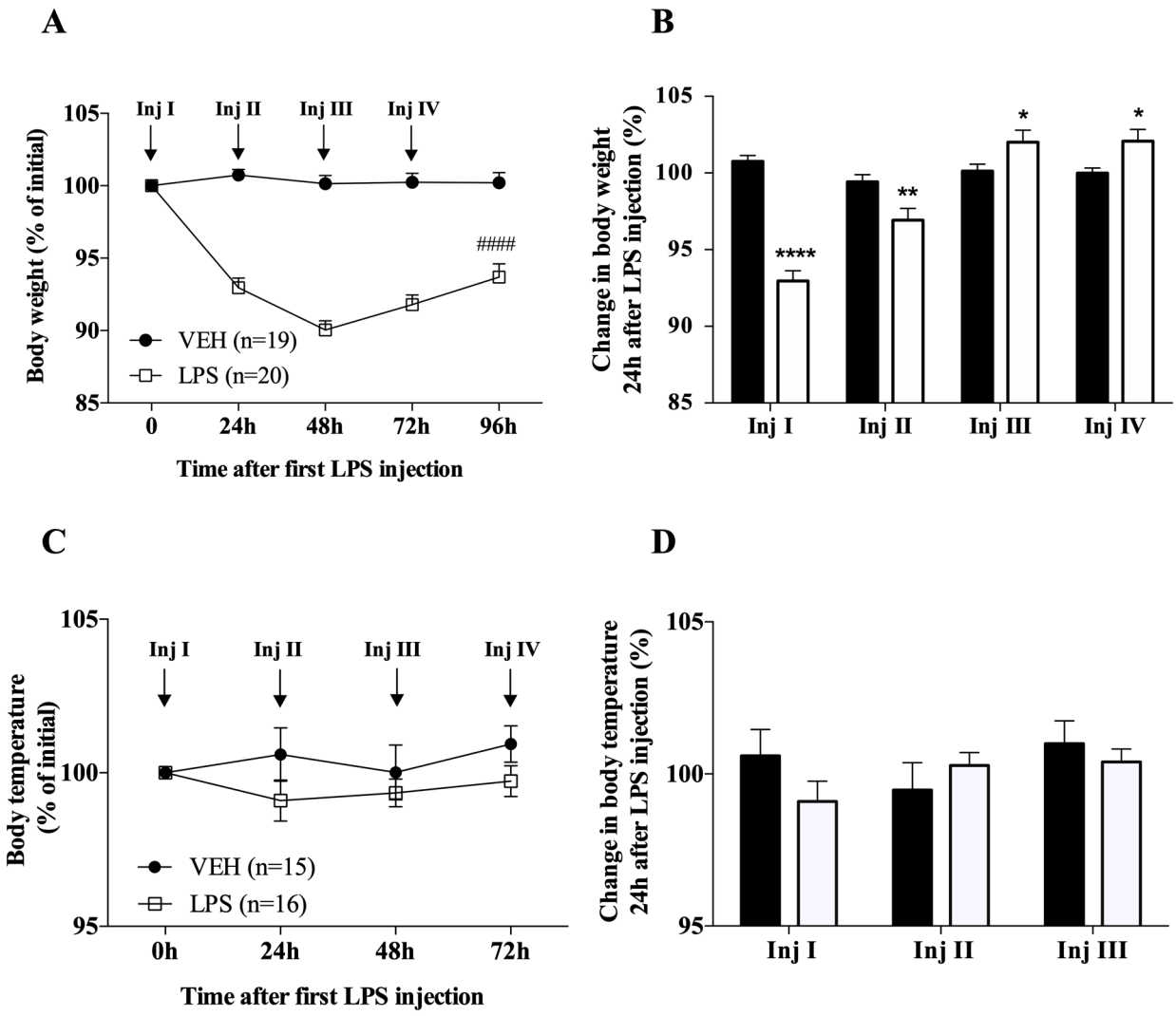
Western Blot analysis

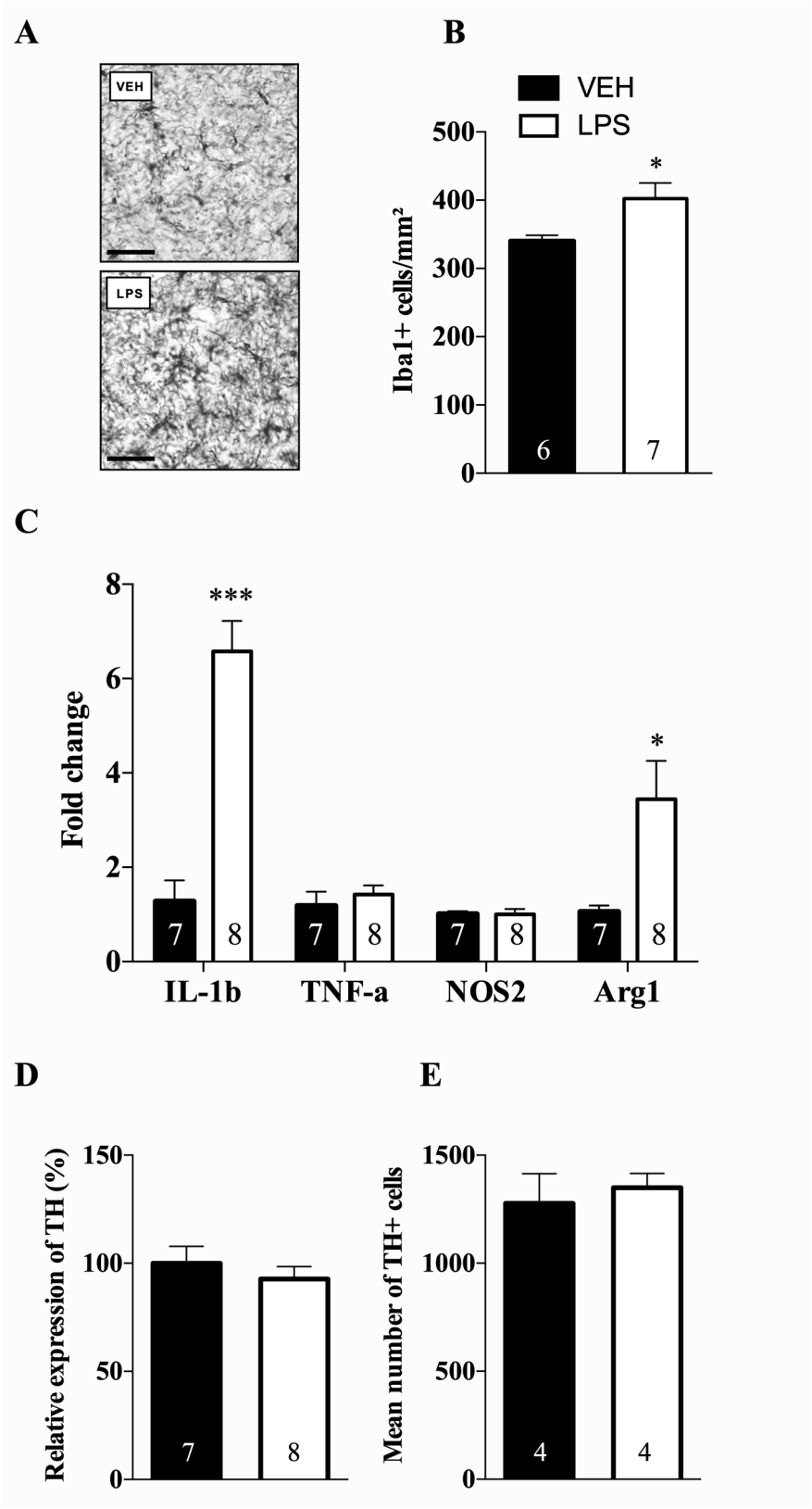
Striatal tissue was collected and homogenized in 300 µl of extraction buffer (2% sodium dodecyl sulphate (SDS), 60 mM Tris base, 100 mM dithiothreitol and phosphatase & protease inhibitor cocktail (pH 6.8, Sigma-Aldrich)) and samples were incubated for 30 min at 37 °C. After centrifugation (10.000 rpm, 10 min, 4 °C), the supernatant was stored at -20 °C until use. Total protein concentration was determined using a fluorometric method (Qubit, Invitrogen,

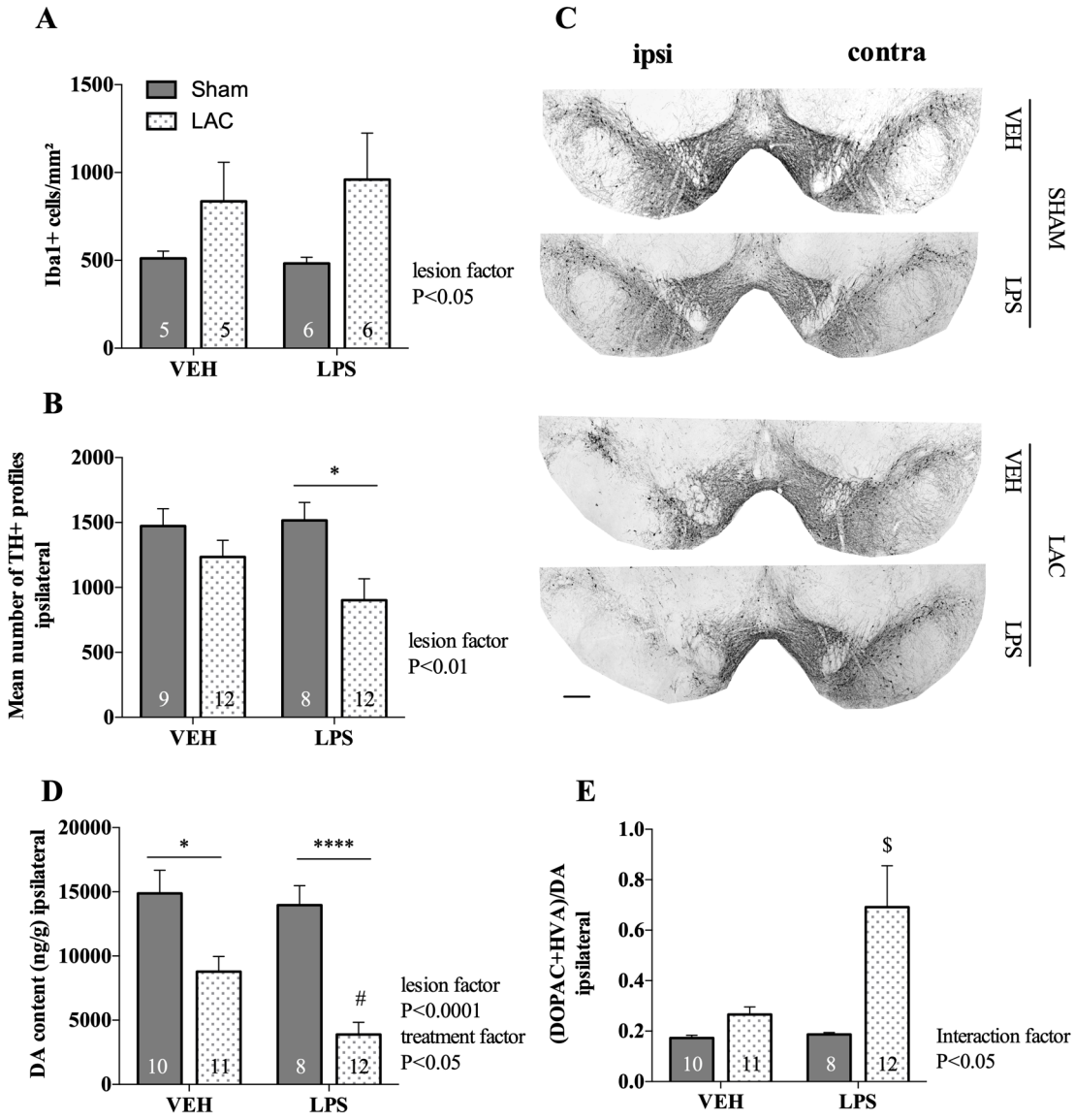
Life Technologies). Equal concentrations of protein were loaded on an SDS-polyacrylamide gel (4-12% gel, Bio-Rad Laboratories) in order to separate the proteins on molecular weight. Subsequently, immunoblotting was performed by transferring the proteins to a polyvinylidene fluoride membrane by means of a semi-dry system (Trans-Blot Turbo Transfer system; Bio-Rad Laboratories). Non-specific binding of the antibody was prevented by incubating the membrane in blocking agent (5% ECL Prime Membrane Blocking Agent; GE Healthcare) for at least one hour at room temperature. Afterwards, the membrane was overnight incubated at room temperature with the primary rabbit antibody. The next day, membranes were incubated with horseradish-peroxidase-conjugated anti-rabbit immunoglobulin G antiserum (1/4000, Dako) for 30 min and protein bands were visualized by enhanced chemiluminescence (ECL prime; GE Healthcare) using the LAS4000 detector (GE Healthcare). Densitometric analysis of immunopositive protein bands was done using ImageJ software (National Institute of Health) and normalized to the density of total amount of proteins loaded, visualized on the same membrane (SERVA Purple, SERVA Electrophoresis GmbH, Heidelberg, Germany).

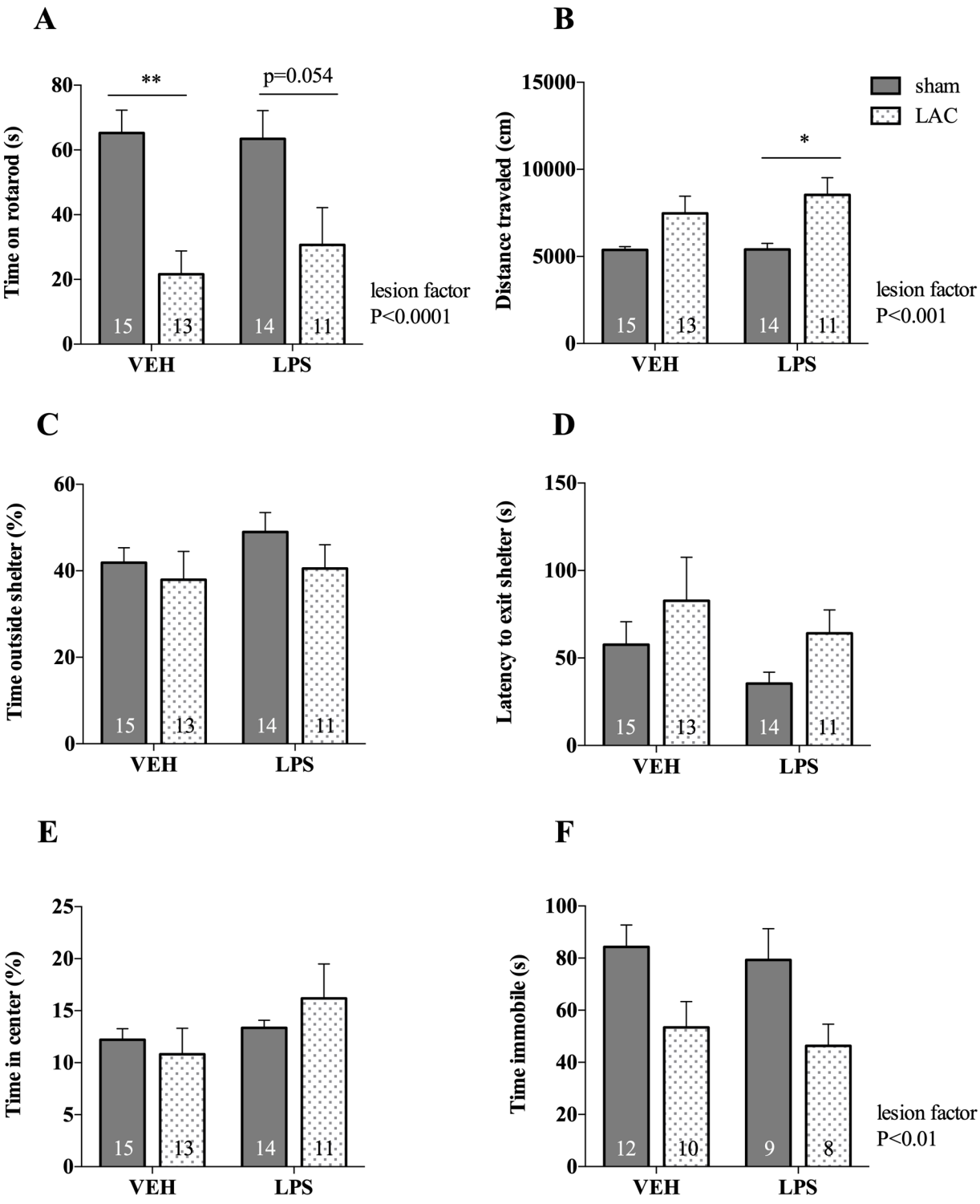
Real-time PCR

The qPCR reaction mix consisted of 10 µl TaqMan® Universal Master Mix, 1 µl of TaqMan® Gene Expression Assay and 2 µl of cDNA in a 20 µl volume adjusted with DNase-/RNase-free water. The TaqMan® Gene Expression Assays used were the following: IL-1β (assay ID: Mm00434228_m1), TNF-α (assay ID: Mm00443258_m1), Nos2 (assay ID: Mm00440502_m1), Arg1 (assay ID: Mm00475988_m1), Ywhaz (assay ID: Mm03950126_s1), Brap (assay ID: Mm00518493_m1) and Bcl2l13 (assay ID: Mm00463355_m1). Bcl2l13 was identified as the most stable housekeeping gene of the three housekeeping genes tested (Ywhaz, Brap and Bcl2l13) using Normfinder.


A
Inj I
Inj II
Inj III
Inj IV
105
100
95
90
85
Body weight (% of initial)
####
VEH (n=19)
LPS (n=20)
0
24h
48h
72h
96h
Time after first LPS injection
B
Change in body weight 24h after LPS injection (%)
****
**
*
*
Inj I
Inj II
Inj III
Inj IV
C
Body temperature (% of initial)
VEH (n=15)
LPS (n=16)
0h
24h
48h
72h
Time after first LPS injection
D
Change in body temperature 24h after LPS injection (%)
Inj I
Inj II
Inj III


A
VEH
LPS
B
VEH
LPS
Iba1+ cells/mm²
500
400
300
200
100
0
*
6
7
C
Fold change
8
6
4
2
0
***
*
7
8
7
8
7
8
7
8
IL-1b
TNF-a
NOS2
Arg1
D
Relative expression of TH (%)
150
100
50
0
7
8
E
Mean number of TH+ cells
1500
1000
500
0
4
4


A
Sham
LAC
Iba1+ cells/mm²
1500
1000
500
0
5
5
6
6
VEH
LPS
lesion factor
P<0.05
B
Mean number of TH+ profiles
ipsilateral
2000
1500
1000
500
0
*
9
12
8
12
VEH
LPS
lesion factor
P<0.01
C
ipsi
contra
VEH
LPS
SHAM
VEH
LPS
LAC
D
DA content (ng/g) ipsilateral
20000
15000
10000
5000
0
*
****
#
10
11
8
12
VEH
LPS
lesion factor
P<0.0001
treatment factor
P<0.05
E
(DOPAC+HVA)/DA
ipsilateral
1.0
0.8
0.6
0.4
0.2
0.0
$
10
11
8
12
VEH
LPS
Interaction factor
P<0.05


A
Time on rotarod (s)
80
60
40
20
0
**
p=0.054
15
13
14
11
VEH
LPS
lesion factor
P<0.0001
B
Distance traveled (cm)
15000
10000
5000
0
sham
LAC
*
15
13
14
11
VEH
LPS
lesion factor
P<0.001
C
Time outside shelter (%)
60
40
20
0
15
13
14
11
VEH
LPS
D
Latency to exit shelter (s)
150
100
50
0
15
13
14
11
VEH
LPS
E
Time in center (%)
25
20
15
10
5
0
15
13
14
11
VEH
LPS
F
Time immobile (s)
100
80
60
40
20
0
12
10
9
8
VEH
LPS
lesion factor
P<0.01