● RESEARCH ARTICLE

The detrimental effects of lipopolysaccharide-induced neuroinflammation on adult hippocampal neurogenesis depend on the duration of the pro-inflammatory response

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Abstract

Adult hippocampal neurogenesis is a finely tuned process regulated by extrinsic factors. Neuroinflammation is a hallmark of several pathological conditions underlying dysregulation of neurogenesis. In animal models, lipopolysaccharide (LPS)-induced neuroinflammation leads to a neurogenic decrease mainly associated to the early inflammatory response. However, it is not well understood how the neuroinflammatory response progresses over time and if neurogenesis continues to be diminished during the late neuroinflammatory response. Moreover, it is unknown if repeated intermittent administration of LPS along time induces a greater reduction in neurogenesis. We administered one single intraperitoneal injection of LPS or saline or four repeated injections (one per week) of LPS or saline to young-adult mice. A cohort of new cells was labeled with three 5-bromo-2-deoxyuridine injections (one per day) 4 days after the last LPS injection. We evaluated systemic and neuroinflammation-associated parameters and compared the effects of the late neuroinflammatory response on neurogenesis induced by each protocol. Our results show that 1) a single LPS injection leads to a late pro-inflammatory response characterized by microglial activation, moderate astrocytic reaction and increased interleukin-6 levels. This response correlates in time with decreased neurogenesis and 2) a repeated intermittent injection of LPS does not elicit a late pro-inflammatory response although activated microglia persists. The latter profile is not accompanied by a continued long-term hippocampal neurogenic decrease. Hereby, we provide evidence that the neuroinflammatory response is a dynamic process that progresses in a milieu-dependent manner and does not necessarily lead to a neurogenic decrease, highlighting the complex interaction between the immune system and neurogenesis.

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Introduction

Adult hippocampal neurogenesis is a finely tuned process regulated by multiple extrinsic factors which in turn remodel cellular and molecular niche components modulating different stages of the neurogenic process (Gonçalves et al., 2016). One condition that has been described as an important anti-neurogenic factor is neuroinflammation which is a physiological response of brain cells to damage-associated neurodegenerative diseases, injury or infection in an attempt to maintain homeostasis and repair the tissue (Carpentier and Palmer, 2009; Whitney et al., 2009; Kohman and Rhodes, 2013; Fan and Pang, 2017).

Microglia and astrocytes are involved in maintaining the physiological brain function and in orchestrating the neuroinflammatory response. These cells respond to inflammatory signals undergoing cellular, molecular and functional changes that occur in a context-dependent manner. In addition, microglia and astrocytes are components of the cellular niche regulating directly the neurogenic microenvironment (Hanisch and Kettenmann, 2007; Sofroniew, 2015;

Gonçalves et al., 2016; de Miranda et al., 2017).

In animal models, administration of lipopolysaccharide (LPS), an endotoxin present in the outer membrane of Gram-negative bacteria and a potent activator of the innate immune system, has been widely used to induce a neuroinflammatory state (Buttini et al., 1996; Turrin et al., 2001; Qin et al., 2007). It has been demonstrated that LPS-induced neuroinflammation consistently leads to a neurogenic decrease independently of the administration model (*i.e.* intracerebroventricular or systemic) and regardless of the frequency of the injections (single, consecutive and even intermittent repetitive intraperitoneal LPS injection) (Ekdahl et al., 2003; Monje et al., 2003; Wu et al., 2007; Fujioka and Akema, 2010; Zonis et al., 2013). Moreover, in almost all of these studies the neurogenic rate was evaluated shortly (3 to 24 hours) after LPS administration (Monje et al., 2003; Wu et al., 2007; Fujioka and Akema, 2010; Zonis et al., 2013), which correlates in time with an early neuroinflammatory response (in the first 2 to 4 hours post-injection) when a peak in the pro-inflammatory mediators has been

reported to occur (Skelly et al., 2013; Pardon, 2015; Lopes, 2016). However, it is unknown how the neuroinflammatory response progresses and if a diminished neurogenesis continues during the late neuroinflammatory response (days post-injection). Even when it has been proposed that intermittent repeated LPS administration induces chronic neuroinflammation and induce a neurogenic decrease soon after the last LPS exposure (Wu et al., 2007), it is unknown if this model induces a greater reduction in hippocampal neurogenesis over time.

The aim of this study was therefore to further characterize the effects of neuroinflammation on adult hippocampal neurogenesis evaluating the effects of the late neuroinflammatory response and to analyze if repeated LPS exposure induces a long-term neurogenic decrease.

Materials and Methods

Animals

All animals were handled in accordance with local government rules (Official Mexican Standard NOM-062-ZOO-1999) and the Animal Care Committee of the Animal Facility approved the methods under the protocol named Central nervous system plasticity: neurogenesis and signaling pathways (approval number 077) on September 5, 2014. Two-month-old C57Bl/6J male mice ($n = 44$, body weight 25.1 ± 0.3 g) were used throughout the experiments and were randomly assigned to the following groups: Saline-SI (saline, single injection; $n = 8$), LPS-SI (LPS, single injection; $n = 10$), Naive-CSI (uninjected control for a single injection; $n = 6$), Saline-RI (saline, repeated injections; $n = 8$), LPS-RI (LPS, repeated injections; $n = 10$) and Naive-CRI (uninjected control for repeated injections; $n = 6$). During the entire procedure, mice were housed in laboratory environment conditions with an inverted 12-hour artificial light/dark cycle and water and food *ad libitum*. Animals were sacrificed 1 week after treatment and brains were extracted for further analyses (**Figure 1**).

Treatment

A group of animals received a single i.p. injection of LPS (1 mg/kg, *Escherichia coli* serotype O127:B8, Cat# L3129; Sigma-Aldrich, St. Louis, MO, USA) or repeated LPS injections (4 in total, one per week for 4 weeks); freshly dissolved in 0.9 % sterile saline solution. Another group was injected with the vehicle solution (0.9% sterile saline; 1 mL/kg) at the same time points and two groups of naive mice were used as controls (see below). All treatments were administered in the early active phase of the mice (first half of the dark phase).

Experimental groups

Saline or LPS single injection (SI): Animals received one single injection of the treatment and were sacrificed 7 days afterwards.

Saline or LPS repeated injections (RI): Animals received one injection of the treatment per week for four consecutive weeks and were sacrificed 7 days after the last injection.

Control for a single injection (naive-CSI): Naive animals

were subjected to behavioral and weight testing as the SI group, but they did not receive any injection (saline, LPS or 5-bromo-2-deoxyuridine (BrdU)) and followed the same timeline as the SI groups.

Control for repeated injections (naive-CRI): Naive animals were subjected to behavioral and weight testing as the RI group, but they did not receive any injection (saline, LPS or BrdU) and followed the same timeline as the RI groups.

Open field test

An open field square arena ($40 \times 40 \times 25$ cm^3) was used to evaluate spontaneous locomotor activity. The arena consisted of 16 squares (10×10 cm^2) of which 4 were "central" and 12 were "peripheral"; the arena was lit from one side with a red light. To promote habituation to the experimenter and decrease the levels of anxiety all mice were handled 5 minutes for 3 consecutive days before the behavioral evaluation. Before every trial, the arena was wiped with cleaning solution (10% extran, 10% ethanol, 80% water). Each mouse was placed in a corner of the arena and was allowed to explore freely; behavior was videotaped for 5 minutes. The evaluation was carried out at 2 hours (day 0 for the SI groups; day 21 for the RI groups) and 24 hours (day 1 for the SI groups; day 22 for the RI groups) after the administration of saline or LPS (for the naive groups, CSI and CRI, the same analysis was performed according to the matching experimental groups). General locomotion was evaluated by counting the total crossings performed by each mouse. Mice from all groups were pre-exposed for 5 minutes to the arena 1 day before the evaluation to avoid an initial burst of exploration due to the innate exploratory behavior of these animals. All tests were performed in the dark phase of the light/dark cycle (**Figure 1**).

BrdU injection

Three days before sacrifice all animals received one daily i.p. injection of BrdU (Cat# 19-160; Sigma-Aldrich) for 3 consecutive days (**Figure 1**). BrdU was administered at a dose of 50 mg/kg of body weight in a 0.9% sterile saline solution previously vortexed and heated at 60°C.

Immunoblot analysis

Mice ($n = 22$) were deeply anesthetized with an overdose of sodium pentobarbital (210 mg/kg) and brains were removed and washed with ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 2 mM KH$_2$PO$_4$). The hippocampus was immediately dissected on top of a petri dish lying on ice. The tissue was homogenized in 600 μL of lysis buffer (MilliQ water, 50 mM Tris base, 60 mM NaCl, 1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% tergitol-type NP-40, complete inhibitor cocktail). Samples were sonicated (10-second pulse of 40 A), incubated (30 minutes on ice with intermittent vortexing every 10 minutes) and centrifuged ($17,949 \times g$, 10 minutes at 4°C). Finally, the supernatants were transferred into pre-chilled tubes and protein levels were measured using the Bio-Rad DC™ protein assay kit (Cat# 5000111; Bio-Rad, Hercules, CA, USA).
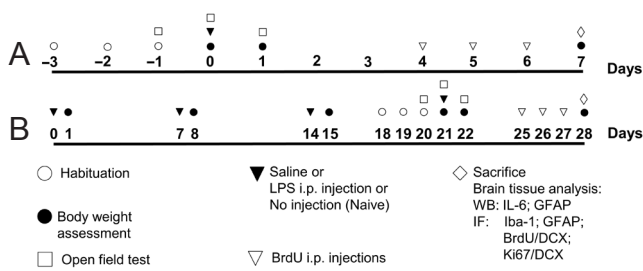
Figure 1 Experimental timeline.
The experimental timeline shows the days in which different experimental procedures were performed. All animals were two-month-old at the beginning of the experiment. (A) The "SI" protocol and (B) the "RI" protocol of saline or LPS. Locomotion and body weight were recorded as indicators of sickness behavior at the indicated time points (open circles, open squares and black circles, see "Materials and methods" for details). Locomotor activity was analyzed using the open field test. All animals, except for the ones in the naive groups, received intraperitoneal injection of BrdU; proliferation in the naive groups was assessed through Ki67 immunofluorescence. Naive groups served as handling-associated anxiety control for each experimental group. Saline-SI: $n = 8$; LPS-SI: $n = 10$; naive-CSI: $n = 6$; saline-RI: $n = 8$; LPS-RI: $n = 10$; naive-CRI: $n = 6$. LPS: lipopolysaccharide; SI: single injection; CSI: control for a single injection; RI: repeated injections; CRI: control for a repeated injection; BrdU: 5-bromo-2-deoxyuridine; WB: western blotting; IF: immunofluorescence; Ki67: endogenous proliferative marker; IL-6: interleukin-6; GFAP: glial fibrillary acidic protein; Iba-1: ionized calcium binding adaptor molecule-1; DCX: doublecortin.

Equal amounts, 60 μg for IL-6 and 30 μg for glial fibrillary acidic protein (GFAP), of protein were loaded into 15% or 10% SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) Bis-Tris acrylamide gels respectively and then transferred to a polyvinylidene difluoride (PVDF) membrane (Immuno-Blot LF PVDF Membrane Roll, Bio-Rad, Cat# 162-026). The membranes were blocked with 5% non-fat-milk in TBS-T (Tris-buffered saline with 0.1% Tween 20; pH 8.0) for 1 hour at room temperature (RT) and then incubated with one of the following primary antibodies: anti-IL-6 (Cat# ab7737; rabbit polyclonal; dilution 1:200; incubated for 48 hours at 4°C; Abcam, Cambridge, MA, USA), anti-GFAP (Cat# Z0334; rabbit polyclonal; dilution 1:1000; incubated overnight at 4°C; Dako, Santa Clara, CA, USA) or anti-β-actin (Cat# A5316; mouse monoclonal; dilution 1:1000; incubated overnight at 4°C; Sigma-Aldrich). After washing three times in TBS-T, membranes were incubated with either, horseradish peroxidase-conjugated goat anti-rabbit (Cat# sc-2030; dilution 1:5000; Santa Cruz Biotechnology, Dallas, TX, USA) or rabbit anti-mouse (Cat# 616520; dilution 1:10,000; Invitrogen, Carlsbad, CA, USA) for 2 hours at RT and the signal was detected using Immobilon™ Western Chemiluminescent horseradish peroxidase substrate reagents (Cat# WBKLS0500; Millipore, Burlington, MA, USA) and BioMax Ligth Films (Cat# 8689358; Kodak, Rochester, NY, USA). For relative quantification of immunosignals, band intensities were analyzed using ImageJ software (National institutes of Health, Bethesda, MD, USA).

Histological procedures
Animals ($n = 22$) were deeply anesthetized with an overdose of sodium pentobarbital (210 mg/kg) and were transcardially perfused with ice-cold 0.9% saline solution followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed, post-fixed in paraformaldehyde and then transferred to 15% and 30% sucrose in 0.1 M phosphate buffer. 40-μm thick coronal brain sections were obtained using a cryostat (Microm HM550, Thermo Fisher Scientific, Waltham, MA, USA); sections were then maintained in cryoprotection solution (25% ethylene glycol, 25% glycerol, 50% 0.2 M phosphate buffer, pH 7.4) and stored at 4°C until processing for immunofluorescence.

Immunofluorescence
Free-floating sections were washed 3 times in PBS (137 mM NaCl, 2.7 mM KCl; 10 mM Na_2HPO_4; 2 mM KH_2PO_4) and then permeabilized in 0.3% PBS-Triton X-100 for 30 minutes. For BrdU detection, sections were transferred to 2 N HCl at 37°C for 30 minutes and washed three times in PBS. Then, sections were blocked with 5% of normal horse serum (Cat# S-2000; Vector Laboratories, Burlingame, CA, USA) diluted in 0.3% PBS-Triton X-100 for 1 hour at RT. After blocking, sections were incubated with primary antibodies for 48 hours at 4°C: anti-BrdU (Cat# 11170376001; mouse monoclonal; dilution 1:500; Roche, Basel, Switzerland), anti-doublecortin (DCX; Cat# sc-8066; goat polyclonal; dilution 1:250; Santa Cruz Biotechnology), anti-Ki67 (Cat# GTX16667; rabbit monoclonal; dilution 1:200; GeneTex, Irvine, CA, USA), anti-ionized calcium binding adaptor molecule-1 (Iba-1; Cat# 019-19741; rabbit monoclonal; dilution 1:250; Wako, Neuss, Germany) and anti-GFAP (Cat# Z0334; rabbit polyclonal; dilution 1:1000; Dako) in blocking solution. After washing three times in 0.3% PBS-Triton X-100, sections were incubated with the appropriate secondary antibodies for 2 hours at RT: Alexa Fluor 488 donkey anti-goat (Cat# A11055; dilution 1:250; Invitrogen), Alexa Fluor 555 donkey anti-mouse (Cat# A31570; dilution 1:500; Invitrogen) or Alexa Fluor 647 donkey anti-rabbit (Cat# A31573; dilution 1:500; Invitrogen) in blocking solution. After washing three times in PBS, sections were mounted on slides coated with poly-L-lysine (Cat# P8920; Sigma-Aldrich); Dako fluorescent mounting medium (Cat# S3023; Dako) was applied and sections were covered with a coverslip.

Confocal microscopy and stereological-based cell estimation
Analysis of Iba-1⁺ and GFAP⁺ cells in the dentate gyrus was performed using a Nikon A1R+ confocal microscope (Nikon Instruments Inc, Tokyo, Japan) with a 40× objective lens and digital images were taken with NIS-Elements C imaging software (Nikon). Two fields corresponding to the suprapyramidal blade of the dentate gyrus from one section were obtained. For qualitative assessment of cell morphology, "z" stacks containing information from 63 and 52 optical slices for Iba-1 and GFAP immunodetection respectively, separated by 0.4 μm interval, were acquired. "z" projections containing the sum of pixels for each image per stack were obtained using ImageJ software. Quantification of Ki67⁺,

BrdU$^+$ and DCX$^+$ cells in the SGZ was performed using a Zeiss LSM5 confocal microscope (Zeiss, Oberkochen, Germany) with a 40× oil objective lens and digital images were obtained using a Zeiss LSM Image Browser software. "z" stacks containing information from 10 optical slices separated by a 2.0 μm interval were acquired. Stereological-based cell estimates of one of every six serial sections comprising the dorsal hippocampus were performed; analysis was performed in five fields per section: one in the crest, two in the suprapyramidal blade and two in the infrapyramidal blade of the dentate gyrus. Cells were counted as double-labeled if analyzed markers appeared simultaneously in at least two adjacent focal planes of orthogonal projections. Cell estimates were obtained after multiplying the total number of cells in each category by 6.

Statistical analysis

All analyses were performed using Statistica (Version 12.5, Dell Software, Round Rock, TX, USA) and all graphs were built using GraphPad Prisma for Windows (Version 7, GraphPad Software, Inc., La Jolla, CA, USA). All values are expressed as the mean ± SEM. Normally distributed data were analyzed by independent samples Student's *t*-test for the Ki67$^+$ counts, mixed analysis of variance (ANOVA) for weight, two-way ANOVA for open field test and one-way ANOVA for the remaining data sets. All ANOVAs were followed by Tukey's *post hoc* test when necessary. All tests were considered significant when *P* < 0.05.

Results

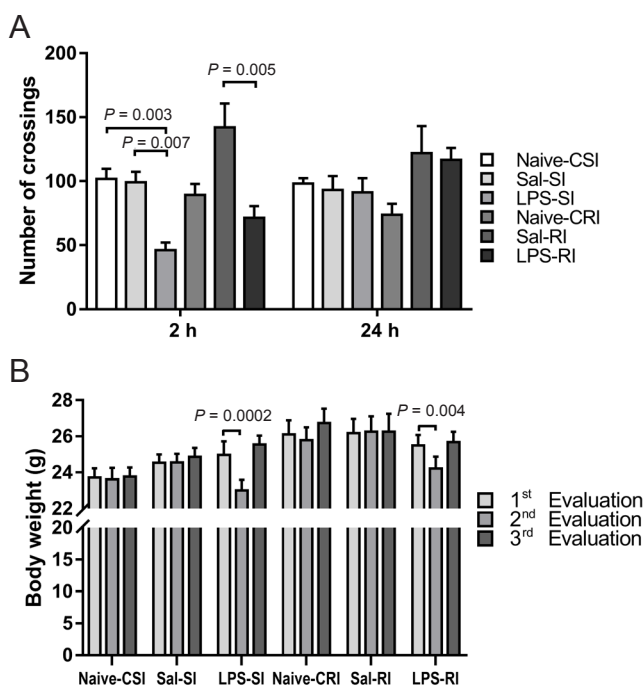### Single and repeated LPS injection protocols induce sickness behavior and body weight loss

Open field test

To evaluate sickness-related behavioral changes, we assessed spontaneous locomotor activity in the open field test at 2 and 24 hours post treatment (after the last injection for the RI groups). Two-way ANOVA showed a significant effect for group ($F_{(5,48)} = 6.7214$, *P* = 0.000081), which indicates that the two LPS-treated groups display a lower number of total crossings on the 5-minute period recorded. Also, we observed a significant interaction between group and time of evaluation ($F_{(5,48)} = 3.3871$, *P* = 0.01). At 2 hours after the administration of LPS, both the SI and RI groups show a decreased number of total crossings when compared to their respective saline group (*P* = 0.007 and *P* = 0.003, respectively, Tukey's *post hoc* test). In addition, the number of total crossings was significantly lower in the LPS-SI group when compared to the naive-CSI group (*P* = 0.005, Tukey's *post hoc* test) (**Figure 2A**). During the second evaluation (24 hours after treatment) there were no significant differences in the number of total crossings between any groups. These results show that the inflammatory-associated sickness behavior takes place soon after LPS challenge but gets resolved within 24 hours. In addition, we evaluated the percentage of central crossings as control for differences in handling-associated anxiety. Results did not show any significant effect or interaction on the percentage of central crossings, which

indicates that neither of the used handling protocols induced changes in the levels of anxiety in any group (data not shown).

Body weight

Body weight assessment was used as another correlate for the systemic sickness-response associated to the LPS challenge. Mixed ANOVA showed a significant effect for time of evaluation ($F_{(2,100)} = 23.295$, *P* = 0.000000005), indicating a decrease in body weight after one day of treatment. Also, a significant interaction between group and time of evaluation was observed ($F_{(10,100)} = 6.32$, *P* = 0.0000002). Both LPS-SI and LPS-RI showed a significant decrease in body weight one day after LPS administration (*P* =0.0002 and *P* = 0.004, respectively, Tukey's *post hoc* test). After 7 days of treatment, the body weight of these groups had returned to baseline (*P* = 1.0 for both groups, Tukey's *post hoc* test). None of the remaining groups showed significant differences on body weight at one or seven days of evaluation (**Figure 2B**). In agreement with the behavioral data, these results show that after a single or repeated administration of LPS, a systemic

Figure 2 Sickness and anxiety-related signs after the single and repeated LPS injection protocols.
(A) Total crossings (central and peripheral squares) recorded on a 5-minute interval at 2 and 24 hours after the administration of saline or LPS (Days 0 and 1 of the experimental timeline for the SI and naive-CSI groups; days 21 and 22 for the RI and naive-CRI groups; *n* = 3–8, two-way ANOVA). (B) Body weight assessment on days 0 (1st evaluation; on the injection day), 1, and 7 (2nd and 3rd evaluations; after the injection day). This assessment was carried out on days 0, 1 and 7 of the experimental timeline for the SI and naive-CSI groups; and days 21, 22 and 28 in agreement with the last LPS or saline injection for the RI and naive-CRI groups; *n* = 6–13, mixed ANOVA. For all graphs bars represent mean ± SEM. Sal: Saline; LPS: lipopolysaccharide; SI: single injection; CSI: control for a single injection; RI: repeated injections; CRI: control for a repeated injection; ANOVA: analysis of variance.

inflammatory-associated response takes place and induces a transitory decrease in body weight.

LPS-SI leads to a late pro-inflammatory response whereas LPS-RI does not induce a sustained pro-inflammatory effect

Microglial response

To evaluate LPS-associated neuroinflammation at a cellular level, we performed a qualitative analysis of microglial cell activation based on morphological changes. We used Iba-1, a constitutively expressed protein, as a specific immunohistochemical marker for microglial cells (Ito et al., 1998). In the naive and saline groups, hippocampal microglia exhibited small cell bodies with fine and highly ramified processes corresponding to a resting state (**Figure 3**). One week after LPS-SI, microglia exhibited a reactive profile with elongated cell bodies and long thick processes as well as increased Iba-1 immunoreactivity. All these changes are consistent with a stage II of microglial activation (Mathieu et al., 2010) (**Figure 3**). One week after LPS-RI, microglia also showed an activated morphology but corresponding to stage III with small and thin processes in addition to a round-body shape with increased Iba-1 levels suggesting a sustained microglial activation (Mathieu et al., 2010) (**Figure 3**).
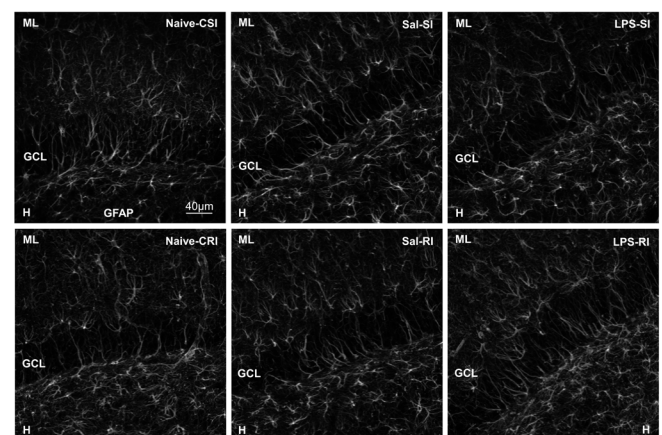
Astrocytic response

To evaluate a second neuroinflammation-associated cellular parameter, we performed a qualitative analysis of astrocytic morphological response using GFAP as a specific marker to identify astrocytes (Bignami et al., 1972). In both naive-CSI and -CRI groups, hippocampal astrocytes displayed a bushy morphology with fine processes proper of non-activated as-
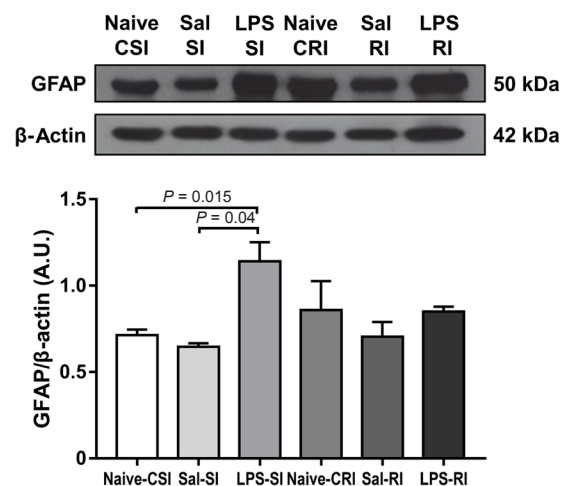
trocytes. We did not observe differences between saline and naive groups (**Figure 4A**). Seven days after the LPS-SI challenge, astrocytes showed a mild cell body hypertrophy, extended cell processes and an increased GFAP immunoreactivity that suggested a moderate reactive astrocytic response in the hippocampus (**Figure 4A**). A repeated LPS exposure did not elicit an evident astrocytic reaction suggesting a lack of persistent astrocytic response after a repeated inflammatory challenge (**Figure 4A**).

To corroborate the astrocytic response induced by both LPS-SI and LPS-RI protocols, we performed a western blot assay to quantify GFAP protein levels. One-way ANOVA showed a significant main effect for group ($F_{(5,14)}$ = 4.2849,

Figure 3 A single LPS injection promotes microglial activation that is enhanced by repeated LPS injections.
Confocal microscopy images show Iba-1 immunodetection in gray scale. Left column: Naive; middle column: Saline; right column: LPS. Top row: SI groups; bottom row: RI groups. Naive groups did not receive injections but were sacrificed together with the SI groups (naive-CSI) and together with the RI groups (naive-CRI) respectively. LPS: Lipopolysaccharide; ML: molecular layer; GCL: granular cell layer; H: hilus; Iba-1: ionized calcium binding adaptor molecule-1; SI: single injection; CSI: control for a single injection; RI: repeated injections; CRI: control for a repeated injection. Arrowheads indicate characteristic cell morphology for each condition. Inserted images show a zoom of the corresponding cells.
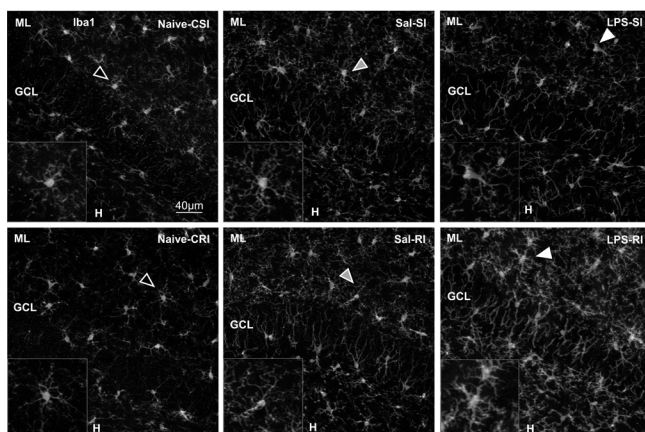

Figure 4 Increased hippocampal levels of GFAP after a single LPS injection but not after repeated LPS injections.
(A) Confocal microscopy images showing GFAP immunofluorescence in gray scale. Left column: Naive; middle column: Saline; right column: LPS. Top row: SI groups; bottom row: RI groups. Naive groups did not receive injections but were sacrificed together with the SI groups (naive-CSI) and together with the RI groups (naive-CRI) respectively. ML: molecular layer; GCL: granular cell layer; H: hilus; GFAP: glial fibrillary acidic protein. (B) Representative western blots and quantitative analysis results of GFAP protein levels obtained from hippocampal homogenates. Protein content was normalized to β-actin. Values are expressed as the mean ± SEM (*n* = 3–4). One-way analysis of variance followed by Tukey's *post hoc* test. GFAP: glial fibrillary acidic protein; LPS: lipopolysaccharide; SI: single injection; CSI: control for a single injection; RI: repeated injections; CRI: control for a repeated injection.

$P = 0.014$) and only the LPS-SI injection protocol induced an increase in GFAP levels compared with its respective saline and naive groups ($P = 0.015$ and $P = 0.04$, respectively, Tukey's *post hoc* test). In agreement with qualitative morphological analysis, GFAP levels in the LPS-RI group were not different from its respective saline and naive groups (**Figure 4B**). This also shows that astrocytic activation is only induced by a single LPS injection, but a repetitive inflammatory stimulus does not lead to a sustained response.

IL-6 protein levels

The molecular neuroinflammatory response was analyzed through the levels of IL-6 cytokine, one of the main molecules involved in the pro-inflammatory response mediated by microglia and astrocytes. One-way ANOVA showed a significant main effect for group ($F_{(5,17)} = 4.1999$, $P = 0.011$) and only the LPS-SI group showed an increase in IL-6 levels when compared to the saline-SI group ($P = 0.013$, Tukey's *post hoc* test; **Figure 5**). We did not observe any differences between any naive and saline groups. Neither the contents of IL-6 were different between the saline-RI and LPS-RI groups, showing that repeated injections of LPS do not induce a sustained pro-inflammatory cytokine-associated response.

LPS-SI leads to a hippocampal neurogenic decrease whereas LPS-RI does not trigger a long-term reduction

To assess the neurogenic rate under neuroinflammatory conditions after single and repeated injections of LPS, we analyzed two markers: BrdU, an exogenous proliferative marker of a specific cohort of cells and DCX which is a microtubule-associated protein related to late neuronal commitment progenitors as well as immature neurons. Cell proliferation in the naive-CSI and -CRI groups was assessed through Ki67, an endogenous proliferative marker, along with DCX.

We observed a 48% decrease ($P = 0.008$) in the total number of Ki67$^+$ cells in the naive-CRI group compared with the naive-CSI group (**Figure 6A** and **B**). We also observed that both Ki67$^+$/DCX$^-$ and Ki67$^+$/DCX$^+$ cell number was significantly decreased by 51% and 45% respectively ($P = 0.009$ and $P = 0.048$, respectively, Student's *t*-test) in the naive-CRI group, suggesting an age-dependent effect in the neurogenic rate (**Figure 6A** and **C**). When we analyzed the phenotype of Ki67$^+$ cells, most were DCX$^+$ suggesting that these cells undergo the highest proliferative rate during neurogenesis.

One-way ANOVA revealed a significant effect of LPS treatment ($F_{(3,12)} = 26.59$, $P = 0.00001$) on total BrdU$^+$ cells, with a 19% decrease of BrdU$^+$ cells in the LPS-SI group compared with the saline-SI group ($P = 0.025$, Tukey's *post hoc* test). In addition, there was a 43% decrease in the number of BrdU$^+$ cells in the saline-RI group compared with the saline-SI group ($P = 0.0003$, Tukey's *post hoc* test) again suggesting an age-dependent effect. Interestingly, there was no significant difference in the number of BrdU$^+$ cells between the saline-RI and LPS-RI groups (**Figure 6A** and **D**). When we analyzed the phenotype of BrdU$^+$ cells, almost all were DCX$^+$ in each experimental condition (**Figure 6A** and **E**), also suggesting that these cells exhibit the highest

proliferative rate during the neurogenic process. One-way ANOVA revealed a significant effect of LPS treatment ($F_{(3,12)} = 25.66$, $P = 0.00002$) on BrdU$^+$/DCX$^+$ cells. We found a 17% decrease in BrdU$^+$/DCX$^+$ cells in LPS-SI treated mice compared with the Saline-SI group ($P = 0.046$, Tukey's *post hoc* test). In addition, we observed a 42% decrease in the number of BrdU$^+$/DCX$^+$ cells in the saline-RI group compared with the saline-SI group ($P = 0.0003$, Tukey's *post hoc* test). In contrast, no significant difference was found in the number of BrdU$^+$/DCX$^+$ between the saline-RI and LPS-RI groups (**Figure 6A** and **E**). Interestingly, we did not find any effect on BrdU$^+$/DCX$^-$ cells. These data indicate that LPS-SI induces a decrease in the neurogenic rate, whereas LPS-RI does not promote a long-term neurogenic reduction.

Discussion

Our results show that a single LPS injection leads to a late pro-inflammatory response whereas a repeated LPS administration does not induce a pro-inflammatory sustained effect.

Regarding systemic inflammation, our data show that either single or repeated LPS exposure, promotes transitory sickness-related symptoms such as reduced locomotion and body weight-loss. These responses are resolved within one week after LPS challenge, as previously described (Dantzer et al., 2008; Biesmans et al., 2013).

In terms of the neuroinflammatory responses, our results indicate that a single LPS administration elicits a pro-inflammatory profile characterized by microglial activation, moderate astrocytic reaction and increased IL-6 levels. In contrast, repeated LPS exposure only induces microglial activation, which could be due to the fact that microglial activation is not only associated with a pro-inflammatory profile but also with an anti-inflammatory phenotype as a consequence of the dynamics and reversibility of the inflammatory process (Perry et al., 2007; Mathieu et al., 2010). This could also underlie our observation showing microglial activation in the absence of other pro-inflammatory parameters such as reactive astrocytes. The moderate response that we observe in astrocytes after a single LPS injection is in agreement with a diffuse innate immune activation as previously reported (Wilhelmsson et al., 2006; Sofroniew, 2009, 2015). The absence of an evident astrocytic activation after repeated LPS injection suggests that a pro-inflammatory state is not sustained, and this is also supported by the lack of a long-term increase in IL-6 levels. However, we cannot rule out the possibility that other neuroinflammatory markers may be increased and this remains to be tested in further experiments. In addition, it is possible that repeated injections of LPS induce an increase in the release of neurotrophic factors (Kuno et al., 2006; Tanaka et al., 2008; Buffo et al., 2010; Heneka et al., 2010; Jurgens and Johnson, 2012; Bian et al., 2013) rather than pro-inflammatory associated molecules by an autocrine loop as previously reported (Kuno et al., 2006; Tanaka et al., 2008) thus modulating the effects of LPS. Moreover, it has been shown that after a LPS challenge, activated astrocytes can modulate microglial functions indicating that both cells can influence each other (*i.e.*, astrocytes

providing negative feedback to activated microglia) (Min et al., 2006; Lynch, 2009). Overall, our data indicate that the brain inflammatory response gets resolved to some extent after repeated LPS stimuli emphasizing that brain inflammation is not a simple all-or-none response, but rather is a finely controlled phenomenon. The intensity and duration of the response to immune stimuli relies on the balance and combination of immune soluble mediators secreted into the local microenvironment.

Our results show that a late pro-inflammatory response, induced by a single LPS injection, correlates in time with a decrease in adult hippocampal neurogenesis whereas after repeated LPS administration there is no sustained neurogenic decrease beyond control levels. The latter response could be explained by the absence of a persistent pro-inflammatory state. Although LPS-SI diminishes the neurogenic rate, these levels continue to be higher than those observed for the saline or LPS repeated injection protocols suggesting that the neurogenic decrease in the latter groups is age-dependent as previously documented (Wu et al., 2008; Ben Abdallah et al., 2010; Encinas et al., 2011). The aforementioned results show that the LPS-RI protocol fails to decrease the neurogenic rate beyond its respective control. Interestingly, our findings indicate that DCX$^+$ progenitor cells undergo the highest proliferative rate during neurogenesis.

Several neuroinflammation protocols, such as a single, consecutive and even intermittent repetitive intraperitoneal LPS injections lead to a consistent reduction in the number of new DCX$^+$ cells in the DG indicating a neurogenic decrease (Monje et al., 2003; Wu et al., 2008; Fujioka and Akema, 2010; Zonis et al., 2013; Valero et al., 2014). However, this effect has been evaluated mainly during the early inflammatory response when there is a peak in the pro-inflammatory mediators (Skelly et al., 2013; Pardon, 2015; Lopes, 2016). In this work, we provide further support to the idea that brain inflammation has a detrimental effect on adult hippocampal neurogenesis. We demonstrate that a diminished number of BrdU$^+$/DCX$^+$ cells continues along time during the late inflammatory response induced by a single LPS injection. This effect could either reflect a downregulation in the proliferative capacity or in the survival rate of neural precursor cells. In this regard, it has been recently reported that the LPS-associated neurogenic decline is a consequence of a proliferative restrain of type 2 progenitor cells (Melo-Salas et al., 2018). If the decrease in DCX$^+$ new cells also depends on an exacerbated apoptotic death remains to be evaluated.

Concerning the possibility that intermittent repeated LPS injections represent a model for chronic neuroinflammation inducing a greater reduction in hippocampal neurogenesis over time, our results indicate that this protocol does not lead to a long-term reduction in the number of BrdU$^+$/DCX$^+$ cells beyond control levels. This result correlates in time with the absence of a persistent pro-inflammatory profile. Considering the impact of neurogenesis in cognitive functions, it would be of interest to analyze if LPS-RI impairs other neurogenic stages as maturation and functional integration of

the adult-born neurons during this period, which could in turn impair functional outcomes such as learning, memory and behavior.

Although we observed activated microglial cells after repeated LPS injections, it is possible that their phenotype is different from the one elicited by one injection, which is in agreement with previous reports indicating that activated microglia is not always detrimental for neurogenesis and can be even beneficial under certain conditions producing growth factors such as brain derived neurotrophic factor (Wu et al., 2007; Littlefield et al., 2015) and transforming growth factor-β (Battista et al., 2006).

In addition, the absence of increased IL-6 levels is in agreement with the lack of a neurogenic decrease. It has been reported that LPS-induced pro-inflammatory cytokines are key mediators of the neurogenic decrease through promoting a deficit in proliferation and by impairing neuronal differentiation of neural precursor cells (reviewed in Pérez-Domínguez et al., 2017). In particular, IL-6 has been shown to reduce neurogenesis (Vallières et al., 2002; Monje et al., 2003; Nakanishi et al., 2007; Zonis et al., 2013) and IL-6-blocking antibodies prevent this effect (Monje et al., 2003; Nakanishi et al., 2007). We also propose that there are no long-term effects of the proinflammatory-induced neurogenic decrease elicited by the first LPS exposure in the model of repeated LPS injection.

Taken together, our data show that the LPS-induced neurogenic decrease correlates in time with a pro-inflammatory profile. The absence of the neuroinflammatory markers evaluated in the repeated LPS protocol may underlie the lack of LPS sustained effects on neurogenesis. Analyzing the mechanisms mediating the absence of a decreased neurogenesis after repeated exposure to LPS opens new venues in the study of biology of neural precursor cells and brain immune response.

Figure 5 IL-6 protein levels in the hippocampus increase with single LPS injection but not with repeated LPS injections.

Representative western blots and quantitative analysis results of IL-6 protein from hippocampal homogenates. Protein contents were normalized to β-actin. Values are expressed as the mean ± SEM. One-way analysis of variance followed by Tukey's *post hoc* test. n = 3–5. Sal: Saline; LPS: lipopolysaccharide; IL-6: interleukin-6; SI: single injection; CSI: control for a single injection; RI: repeated injections; CRI: control for a repeated injection.

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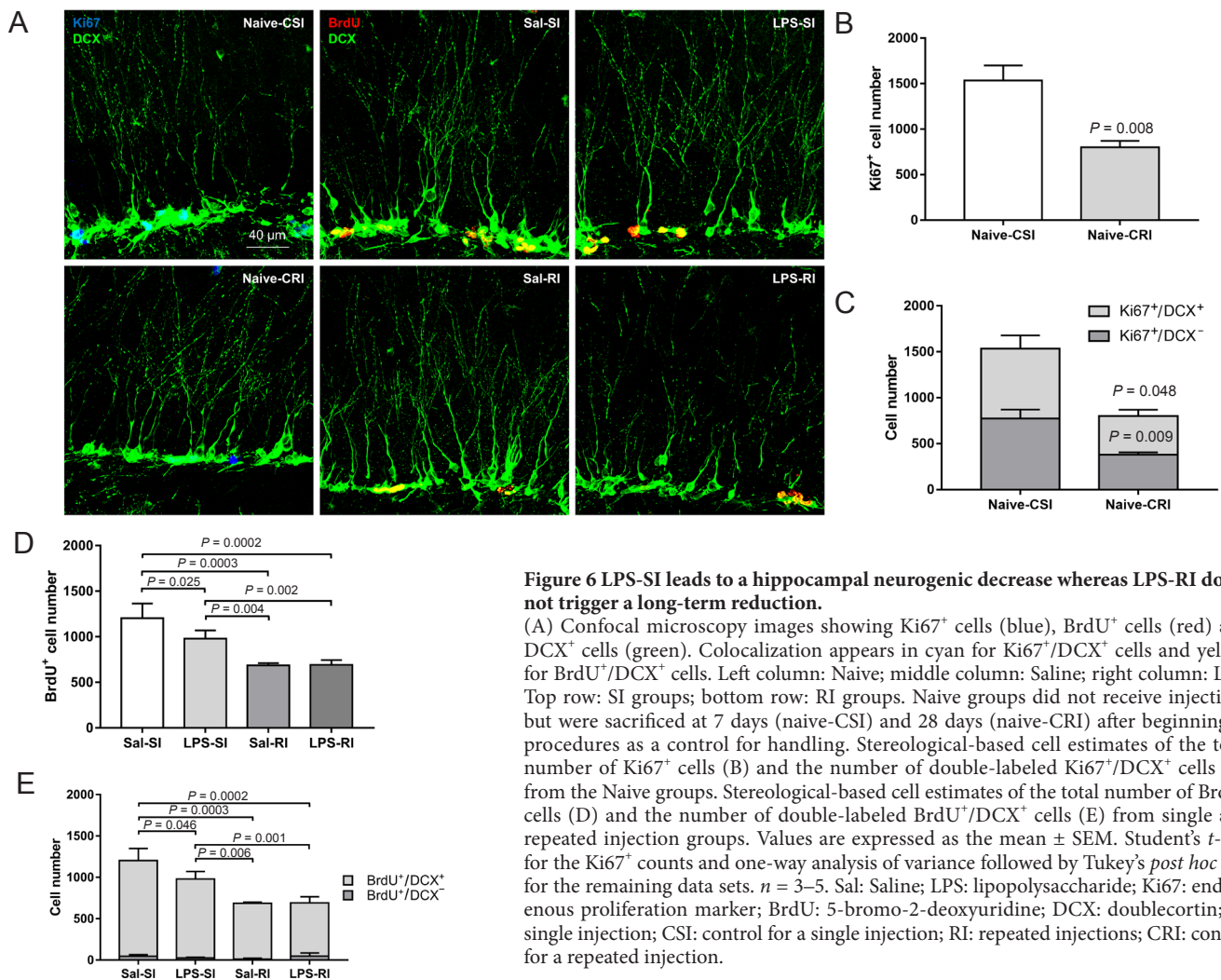
Figure 6 LPS-SI leads to a hippocampal neurogenic decrease whereas LPS-RI does not trigger a long-term reduction.

(A) Confocal microscopy images showing Ki67$^+$ cells (blue), BrdU$^+$ cells (red) and DCX$^+$ cells (green). Colocalization appears in cyan for Ki67$^+$/DCX$^+$ cells and yellow for BrdU$^+$/DCX$^+$ cells. Left column: Naive; middle column: Saline; right column: LPS. Top row: SI groups; bottom row: RI groups. Naive groups did not receive injections but were sacrificed at 7 days (naive-CSI) and 28 days (naive-CRI) after beginning of procedures as a control for handling. Stereological-based cell estimates of the total number of Ki67$^+$ cells (B) and the number of double-labeled Ki67$^+$/DCX$^+$ cells (C) from the Naive groups. Stereological-based cell estimates of the total number of BrdU$^+$ cells (D) and the number of double-labeled BrdU$^+$/DCX$^+$ cells (E) from single and repeated injection groups. Values are expressed as the mean ± SEM. Student's *t*-test for the Ki67$^+$ counts and one-way analysis of variance followed by Tukey's *post hoc* test for the remaining data sets. n = 3–5. Sal: Saline; LPS: lipopolysaccharide; Ki67: endogenous proliferation marker; BrdU: 5-bromo-2-deoxyuridine; DCX: doublecortin; SI: single injection; CSI: control for a single injection; RI: repeated injections; CRI: control for a repeated injection.

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