Pharmacological Research 172 (2021) 105795

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Proneurogenic and neuroprotective effect of a multi strain probiotic mixture in a mouse model of acute inflammation: Involvement of the gut-brain axis

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ARTICLE INFO

Keywords:
Probiotic
Neurogenesis
Behavior
Gut-brain axis
Inflammation
LPS

ABSTRACT

Neuroinflammation can severely affect brain homeostasis and adult hippocampal neurogenesis with detrimental effects on cognitive processes. Brain and gut are intimately connected via the "gut-brain axis", a bidirectional communication system, and the administration of live bacteria (probiotics) has been shown to represent an intriguing approach for the prevention or even the cure of several diseases. In the present study we evaluated the putative neuroprotective effect of 15-days consumption of a multi-strain probiotic formulation based on food-associated strains and human gut bacteria at the dose of 10^9 CFU/mouse/day in a mouse model of acute inflammation, induced by an intraperitoneal single injection of LPS (0.1 mg/kg) at the end of probiotic administration. The results indicate that the prolonged administration of the multi-strain probiotic formulation not only prevents the LPS-dependent increase of pro-inflammatory cytokines in specific regions of the brain (hippocampus and cortex) and in the gastrointestinal district but also triggers a potent proneurogenic response capable of enhancing hippocampal neurogenesis. This effect is accompanied by a potentiation of intestinal barrier, as documented by the increased epithelial junction expression in the colon. Our hypothesis is that pre-treatment with the multi-strain probiotic formulation helps to create a systemic protection able to counteract or alleviate the effects of LPS-dependent acute pro-inflammatory responses.

1. Introduction

Neuroinflammation is a physiological response of brain cells to damage-associated neurodegenerative diseases, injury or infection aimed at preserving homeostasis and repairing the tissue [1–3]. Following acute inflammation, circulating pro-inflammatory cytokines promote a constellation of neurochemical and hormonal changes, resulting in profound effects on motivational states, mood (depression and anxiety disorders) and cognitive functions (impairments in learning and memory) [4,5]. Among the brain processes, hippocampal adult neurogenesis is particularly affected by neuroinflammation [6,7]. The hippocampal structure represents the main brain region responsible for the formation and consolidation of new memories, with the pivotal contribution of adult hippocampal neurogenesis, which provides a continuous supply of new neurons and neuroplasticity throughout life [8–10]. Adult neurogenesis is a cell-based form of neuronal plasticity consisting in a life-long production of new neurons in specific brain region of adult mammals, the dentate gyrus of hippocampus and the subventricular zone [8,11,12]. Adult hippocampal neurogenesis has been identified in rodents [13–17], non-human primates [15,18] and

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https://doi.org/10.1016/j.phrs.2021.105795
Received 27 May 2021; Received in revised form 20 July 2021; Accepted 27 July 2021
Available online 31 July 2021

humans [19,20].

Inflammatory responses strongly reduce adult neurogenesis and hippocampal homeostasis, with detrimental effects on learning and memory processes [3].

Mounting evidence suggests a direct link between the brain and gastrointestinal function. Brain and gut are intimately connected via the "gut-microbiota-brain axis", which is a bidirectional communication system involved in neuronal and humoral mechanisms [21]. The modulation of microbiota through administration of live bacteria (probiotics) could be an intriguing approach for the prevention or even cure of some diseases. The emerging evidence that some bacteria may have positive mental health benefit (psychobiotic) is attracting the attention of many researchers.

According to Sarkar et al. [22], psychobiotics are beneficial bacteria (probiotics) or support for such bacteria (prebiotics) that influence bacteria–brain relationships. Among bacterial strains showing psychobiotic properties, several belong to the species *B. animalis* subsp. *lactis*, *B. breve*, *Lactobacillus acidophilus*, L. *helveticus*, *L. paracasei,* L. *plantarum*, *Streptococcus thermophilus* [22–24]. During the last two decades, probiotic consumption has been linked with positive changes in neural activity in specific brain areas involved in mood, cognition and emotional processing [25,26]. Moreover, specific probiotics have been shown to reduce the levels of pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6 or tumor necrosis alpha (TNF-α) [27,28], often associated with certain psychiatric disorders [29,30]. Thus, these evidences strongly suggest that the immune-modulatory role of probiotics may be crucial to alleviate and/or counteract inflammation-related brain deficits. Notably, mice treated with a single, peripheral dose of Lipopolysaccharide (LPS) from Gram-negative bacteria, display evident signs of neuro-inflammation and "sickness behaviour" [31,32]. A recent study demonstrated that a probiotic mixture is able to beneficially affect brain function in the context of LPS-dependent inflammation occurring within the body [33].

Based on these evidences, here we tested the potential of a new multi-strain formulation (OttaBac®) to prevent or attenuate the acute, low-grade systemic inflammation induced by injection of the bacterial toxin LPS. OttaBac® is a multi-strain formulation which is a blend of 8 strains belonging to the above species [34,35].

In this study, mice were gavaged with OttaBac® at the dose of 10^9 CFU/mouse/day for a period of 15 days [36–38] before a single intraperitoneal (i.p.) injection of low dose of LPS (0.1 mg/kg). We sacrificed the animal after 2 and 24 hours from LPS treatment and analyzed a putative neuroprotective effect of OttaBac® administration by evaluating physiological and behavioral changes, proliferation and differentiation of the new neurons within the hippocampal dentate gyrus, the neuroinflammatory response, the modifications of intestinal permeability and of the inflammatory state in OttaBac® versus vehicle-administered mice.

2. Material and methods

2.1. Animals

Mice (C57BL/6 J, males, 8 weeks old) were used for all studies (Charles River Laboratory). All efforts were made to minimize animal suffering and to reduce the number of mice used, in accordance with the European Union Directive of September 22, 2010 (2010/63/EU). All experiments were approved by the Italian Ministry of Health (Legislative Decree Nr. 549/2020-PR).

2.2. The multi strain probiotic product OttaBac®

OttaBac® is a probiotic mixture containing eight live, freeze-dried bacterial strains: *Bifidobacterium animalis* subsp. *lactis* BL03*, B. animalis* subsp. *lactis* BI04*, B. breve* BB02, L. *acidophilus* BA05, L. *helveticus* BD08, *Lcb. paracasei* BP07, *Lpb. plantarum* BP06*,* and *Streptococcus thermophilus* BT01. According to the recent reclassification of the genus *Lactobacillus*, *Lactobacillus paracasei* has been reclassified as *Lacticaseibacillus (Lcb.) paracasei* and *Lactobacillus plantarum* has been reclassified as *Lactiplantibacillus (Lpb.) plantarum* [39]. The new taxonomic nomenclature will be therefore applied in this study.

2.3. Evaluation of OttaBac® microbiological quality

The detailed description of the methods analyzing the microbiological quality of OttaBac® (total cells quantification and microbial composition) is reported in Supplementary Material.

2.4. Experimental protocol

The animals were group-housed (3 mice/cage) with temperature (22–23 °C) and humidity (60 ± 5%) controlled, under a 12:12 h light/dark cycle, with food and water freshly available throughout the study. Male mice were used to have consistency with previous behavioral studies using probiotics [40–42].

Animals were randomly assigned to the following groups:

- Saline-injected mice pre-treated with placebo (Control Group, n = 12)
- Saline-injected mice pre-treated with OttaBac® (OB Group, n = 12)
- LPS-injected mice pre-treated with placebo (LPS Group, n = 12)
- LPS-injected mice pre-treated with OttaBac® (OB-LPS Group, n = 12)

Mice were orally gavaged with OttaBac® (10^9 CFU/mouse), or placebo in vehicle (100 µl) for 15 days. On day 16, mice were randomly separated into two groups (n = 24 mice each) and intraperitoneally (i.p.) injected respectively with LPS (0.1 mg/kg dissolved in saline) or saline.

All mice were subjected to behavioral test 90 minutes after LPS or saline injection, at the end of which (at 2 hours post LPS) four groups of mice (n = 6 per group) were euthanized, while four other groups of mice (n = 6 per group) were re-exposed to behavioral tests 24 hours later and then euthanized. We analyzed the inflammatory response in mice 2 and 24 hours post LPS in order to study the effect of the probiotic mixture at an early time point (2 hours) in which the whole inflammatory response is rising to the peak (approximately 4 hours post LPS) and at a later time (24 hours) when specific inflammatory phenomena are still present, but characterized by a partial and slow rescue. Experimental protocol is illustrated in Fig. 1.

2.5. Sickness behaviour

Sickness behavior induced by LPS injection was assessed by measuring body temperature, weight loss and locomotor activity. Body temperature was measured 2 and 24 hours after LPS injection using a digital thermometer (BAT-12, Physitemp Instruments, Clifton, New Jersey, USA) equipped with a rectal probe for mice. Body weight was measured before LPS treatment and 24 hours later. The percentage of weight change is calculated as the ratio between the final and the initial body weight. Locomotor activity was assessed 90 minutes after LPS injection by introducing mice in an open field apparatus consisting of a squared arena (40 ×40 cm). Mice behavior was recorded and locomotor activity was evaluated by measuring the time spent in immobility.

2.6. Explorative and anxiety behaviour

24 h after LPS injection, mice were subjected to open field test and elevated plus maze test with 15-minutes inter-test-interval. The floor of open field apparatus (described above) was subdivided into in 16 squared sectors (10-cm side each) delimitating the peripheral and the central sub-areas of the arena. Each mouse was placed in the center of the arena and left free to explore the environment for 5 min. The number

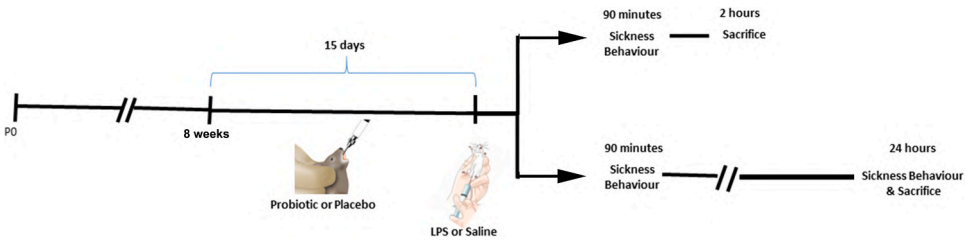

Fig. 1. Schematic timeline of the experimental protocol. Mice were administered with OttaBac® or Placebo for 15 days. At the end of treatment (day 16) mice were intraperitoneally injected with a single dose of LPS (0.1 mg/kg) or Saline. We analyzed 4 different experimental groups: Placebo + Saline mice (Control), Placebo + LPS mice (LPS), OttaBac® + Saline mice (OB) and OttaBac® + LPS mice (OB-LPS). 90 minutes after LPS/Saline injection, 6 mice/group were tested for sickness behaviour. At the end of the tests, 6 mice/groups were sacrificed for biochemical and histological analysis, while other 6 mice/groups were re-tested for sickness behaviour 24 hour following LPS/Saline treatment and then sacrificed.

of sectors crossed and the time spent in periphery vs center were measured as parameters for exploratory behavior and anxiety, respectively.

The elevated plus maze apparatus consisted of a four arms cross maze with two arms open and the other two embedded in 10-cm high walls. Each mouse was placed at the crossing of the four arms and left free to explore for 5 min. The time spent in closed vs open arms and the number of entries in closed arms were measured to assess anxious behavior.

2.7. Immunohistochemistry

Left hemispheres of brains were collected and kept overnight at + 4 °C in PFA. They were subsequently equilibrated in sucrose diluted at 30% in PBS and finally cryopreserved at −80 °C. Dissection was carried out by embedding the brain in *Tissue-Tek* OCT (Sakura, Torrence, CA, USA) and then cut using a cryostat at −25 °C throughout the whole rostro-caudal extent. The coronal sections were processed in a one-in-six series protocol at a 40 μm thickness. Sections were then stained for multiple labelling using different fluorescence techniques. Sections were initially washed with glycine 0.1 M for 10 minutes followed by permeabilization using 0.3% Triton X-100 in PBS for further 10 minutes. The sections were then incubated for 30 minutes in a blocking solution containing 3% normal donkey serum (NDS) in 0.3% Triton X-100 in PBS to saturate the aspecific sites, followed by incubation with the same blocking solution containing primary antibodies for 16–18 hours at 4 °C. The primary and secondary antibodies used are listed in Table 1. Nuclei were observed incubating sections with Hoechst (1:500).

2.8. Quantification of cell number

Slices were collected using systematic random sampling. Approximately 40 coronal sections of 40 μm were obtained from each brain; about 1-in-6 series of sections (each slice thus spaced 240 μm apart from the next) were analyzed by Olympus (FV 1200) confocal microscopy and used to count the number of cells expressing the indicated markers throughout the rostro-caudal extent of the whole hippocampus. The total estimated number of cells positive for Ki67 (specific marker of cell proliferation), SOX2 and GFAP (both expressed in the Neural Stem Cells) and finally DCX (marker of neural precursors), within the Dentate Gyrus (DG) was obtained by multiplying the average number of positive cells per section by the total number of 40 μm sections comprising the entire DG (spaced 240 μm). Cell number for $Iba1^+$ cells for each DG and cerebral cortex section was divided by the corresponding area of the individual section to calculate the average number of cells per 100 μm^2 of DG and cerebral cortex area. Region of interest was calculated by tracing the outline of the desired structure, identified by the presence of nuclei stained with Hoechst 33258, on a digital picture captured using ImageJ system, which was also used to count the labeled cells.

2.9. NeuronJ measurement of DCX^+ cells arborization

Cell arborization determination was performed on DCX^+ cells by the NeuronJ plugin of ImageJ software as described previously [43]. Olympus confocal microscopy was utilized to collect full thickness 63x z-stack images of DG hippocampus from 40-μm-thick brain sections that were immunostained for DCX and DAPI. Z-stacks were collected at 512 × 512 resolution with 3 frame averages for each color channel and 1 μm z-step size. Olympus Software was used to prepare a maximum intensity projection image of the DCX channel, which was thresholded before the analysis with Fiji Software. For each image, surrounding processes were manually removed in Fiji, thereby isolating a total of 10 DCX^+ cells per mouse. The tracing of ramification was performed automatically by following the path of neurite by the Fiji Plugin named NeuronJ, which allows also to determine the total length of the tracings

Table 1
List of antibodies used in this study.

Antibody	Company	Cat #	Dilution	Test
Primary antibodies:				
GFAP	Sigma-Aldrich	G3893	1:400	Immunohistochemistry
DCX	Santa Cruz Biotechnology	sc-8066	1:300	Immunohistochemistry
SOX2	Santa Cruz Biotechnology	sc-17320	1:300	Immunohistochemistry
Iba-1	Abcam	ab5076	1:500	Immunohistochemistry
Ki67	Lab Vision	RM-9106-S	1:150	Immunohistochemistry
TNFα	GeneTex	GTX110520	1:1000	Western Blot
IL10	GeneTex	GTX130513	1:1000	Western Blot
β-actin	Thermofisher	AM4302	1:5000	Western Blot
Occludin	GeneTex	GTX114949	1:1000	Western Blot
E-Cadherin	GeneTex	GTX100443	1:1000	Western Blot
Secondary antibodies:				
Donkey anti-goat Cy2	Jackson Immunoresearch	705–225–147	1:200	Immunohistochemistry
Donkey anti-rabbit Cy3	Jackson Immunoresearch	711–165–152	1:300	Immunohistochemistry
Donkey anti-mouse Alexa-647	Thermofisher	A-21235	1:300	Immunohistochemistry
goat anti-rabbit IgG-HRP	Thermofisher	G-21234	1:5000	Western Blot
goat anti-mouse IgG-HRP	Thermofisher	G-21040	1:5000	Western Blot

of a single cell (Arborization Length). The counting of number of neurites and branching point was carried out manually with Fiji Software.

2.10. Sholl analysis of microglial morphology

Sholl analysis of microglial morphology was carried out as described previously [44]. Briefly, Olympus confocal microscopy was utilized to collect full thickness 63x z-stack images of DG hippocampus and cerebral cortex from 40 μm-thick brain sections that were immunostained for Iba-1 and DAPI (as outlined above; n = 6 mice/ group, 10 image stacks per animal). Z-stacks were collected at 512 × 512 resolution with 3 frame averages for each color channel and 1 m z-step size. Olympus Software was utilized to prepare a maximum intensity projection image of the Iba-1 channel, which was thresholded before the analysis. For each image, surrounding processes were manually removed in Fiji, thereby isolating a total of 10 microglia cells per mouse. The line segment tool was adopted to draw a line from the center of each soma to the tip of its longest process, which provided the maximum process length (Ending Radius). The Sholl analysis plugin with the first shell set at 10-μm step size, was utilized to determine intersections at each Sholl radius. This also provided Maximum intersection (the maximum value of intersection between arborization and Sholl radii), Sum intersection (sum of the intersection between microglia process and Sholl radii), Intersecting radii (number of Sholl radii intersecting the arborization at least once). The soma size of the cell was manually measured in Fiji.

2.11. Expression analysis by qRT-PCR

Total RNA from Cerebral Cortex and Hippocampus was obtained by homogenizing the tissues with a tissue homogenizer (OMNI GLH INTERNATIONAL; power 1; 5 seconds) in TriReagent (Sigma-Aldrich Cat#: T9424). The RNA extraction was performed following TriReagent manufacturer's protocol. RNA was quantified with NanoDrop (Thermo Scientific NanoDrop 2000C). For mRNA analysis, total RNA (500 ng) was retro-transcribed with a retrotranscription kit (Thermo Fisher Scientific Cat#: 8080234) by using random examers. Quantitative Real Time PCR (qRT-PCR) analysis was performed with SYBR Green Master Mix (PowerUp - Applied Biosystems) and primer pairs designed with Primer3 Input software (primer3.ut.ee). All the murine expression primers used in this study span an exon-exon junction. The sequences of murine expression oligonucleotides for qRT-PCR are listed in Table 2. The reactions were run on 7900HT ABI prism PCR machine (Applied Biosystems). All Ct values were obtained in duplicate or triplicate and the analysis of output values was made using standard ΔΔCt method.

2.12. Western blot analysis of colonic mucosa samples

Immediately after sacrifice, colon samples were removed and rinsed with saline. Colonic mucosa samples were stored frozen at −80 °C. Tissue proteins were extracted with RIPA buffer (1% Igepal,0.5% deoxycholic acid, and 0.1% sodium dodecyl sulphate in Tris buffered saline 1 ×; pH 7.4) with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Clear lysates were prepared by centrifugation at 10,000g for 10 min, and protein concentrations were assessed using the BC Assay Uptima kit (Interchim). Equivalent amounts of proteins (25 μg) were electrophoresed on 12% or 4–12% Bis-Tris Bolt Gels (Thermofisher, Italy) and transferred to 0.45μn PVDF membrane. After blocking with 5% non fat dry milk for 1 h, membranes were incubated overnight with the primary and secondary antibodies listed in Table 1. Immunoreactivity was determined using the enhanced chemiluminescence reaction and captured by iBright CL1500 Imaging System. Densitometric analysis was performed using Image J software. Data were expressed, after normalization respect to β-actin housekeeping, as fold of change respect to Control groups.

Table 2
List and sequence of primers used in this study.

Gene Symbol	Forward	Reverse
GAPDH	CACCATCTTCCAGGAGCGAG	CCTTCTCCATGGTGGTGAAGAC
IL-6	TCCTCTCTGCAAGAGACTTCC	TTGTGAAGTAGGGAAGGCCG
IL-1β	GACCTTCCAGGATGAGGACA	TCCATTGAGGTGGAGAGCTT
TNF-α	TCTTCTCATTCCTGCTTGTGG	CACTTGGTGGTTTGCTACGA

2.13. Statistical analysis

For comparison of four groups, a two-way (OttaBac®, LPS) ANOVA was used and when the interaction was significant, a Bonferroni post hoc pairwise multiple-comparison procedure was used. For behavioral studies, data measured at 2 time points, were compared using two-way (OttaBac®, LPS) ANOVA for repeated measures. For all analyses, $p < 0.05$ was considered significant. Post-hoc comparisons within logical sets of means were performed using the Tukey's test, the use of which is permissible or even recommended also in the absence of significant main or interaction effects in the ANOVA, in order to minimize frequency errors of both type I and II following the indications given by Wilcox (1987, pp. 187–189).

3. Results

3.1. Evaluation of OttaBac® microbiological quality

Evaluation of probiotics quality is fundamental to provide a better comparison between different studies and contribute to further assessing the role of probiotics in human health [35]. To this aim the multi-strain OttaBac® was subjected to the quantification of: i) cell viability by single cell analysis, and ii) microbial and strains composition by shotgun metagenomic sequencing. Results indicate high level of cells viability and confirmed that OttaBac® is composed of a blend of 7 species and 8 different strains (see Supplementary results).

3.2. LPS induces sickness behavior in both OttaBac® and Placebo treated mice

We first evaluated the impact of LPS treatment in both OttaBac® (OB) and Placebo treated mice. At the end of the 15-days treatment (OB or placebo), mice were administered with 0.1 mg/kg LPS or saline and sickness behavior was assessed 1.5 and 24 hours later. In both OB and placebo treated mice, LPS treatment induced hypothermia (1.5 hours post LPS, LPS effect: $F_{(1, 20)} = 37{,}46$; $p < 0.0001$, Table 3), and weight loss (24 hours post LPS, LPS effect: $F_{(1, 20)} = 59{,}14$; $p < 0.0001$, Table 4), two well characterized symptoms of sickness behavior [45–47].

As shown in Table S5, compared to relative saline-injected control groups, locomotor activity was significantly reduced 90 minutes after LPS injection, as evidenced by the high amount of time spent in immobility by both OB and placebo treated mice (effect of LPS: $F_{(1,20)} = 62{,}51$, $p < 0.0001$) during the 5-minutes exposition in the open field.

Table 3
Effect of LPS on body temperature.

Treatment	Body Temperature (°C) 2 Hours Post LPS	Body Temperature (°C) 24 Hours Post LPS
CONTROL	36,2 ± 0,14	36,7 ± 0,16
OttaBac®	36,5 ± 0,21	37,3 ± 0,19
LPS	34,6 ± 0,44***	36,7 ± 0,17
OttaBac®-LPS	34,7 ± 0,22§§§	36,9 ± 0,06

Values are given as mean ± SEM (n = 6). The intergroup variation was measured by two-way ANOVA followed by Bonferroni post hoc pairwise multiple-comparison.
***p < 0.001 vs CONTROL; §§§ p < 0.001 vs OttaBac®

Table 4
Effect of LPS on body weight in LPS-treated mice.

Treatment	Body Weight (grams) PRE LPS	Body Weight (grams) 24 HOURS POST LPS	% Weight Loss
CONTROL	28,17 ± 0,87	27,7 ± 1,20	2% ± 1,60
OttaBac®	26,00 ± 0,85	25,8 ± 0,75	0,6% ± 0,57
LPS	27,81 ± 0,65	24,7 ± 0,55	11,3% ± 0,97***
OttaBac®-LPS	26,7 ± 1,02	23,5 ± 0,67	11,6% ± 1,78§§§

The percentage of weight loss is calculated from the ratio between the weight at 24 hours post LPS and the initial weight, multiplied by 100.Values are given as mean ± SEM (n = 6). The intergroup variation was measured by two-way ANOVA followed by Bonferroni post hoc pairwise multiple-comparison.
***p < 0.001 vs CONTROL; §§§ p < 0.001 vs OttaBac®

These data indicate that LPS injection causes sickness behavior in both OttaBac® and Placebo treated mice. We next probed whether the effect of LPS was transient by measuring the same parameters 24 hours later. With the exception of weight loss which needs some days to recover ($F_{(1,20)} = 59,14$, $p < 0.0001$, Table 4), values for body temperature ($F_{(1,20)} = 6,77$, $p = 0,21$, Table 3), and time spent in immobility ($F_{(1,20)} = 0074$, $p = 0,78$, Table S5) were rescued 24 hours after LPS administration. This data indicates that LPS effects on sickness behavior are transient and recovered 24 hours after LPS administration.

3.3. OttaBac® enhances exploratory behavior in mice

We next investigated the long-term effects of OttaBac® administration on exploration, a key behavior in rodents. As parameter for exploration, we refer to the number of sectors crossed during the 2nd exposition to the open field arena (24 hours-time point), when the effect of LPS is over. OttaBac® treated mice crossed more sectors than their placebo controls (Effect of OB: $F_{(1,20)} = 9958$, $p < 0.01$) in the open field (Fig. 2A) with no differences due to LPS (effect of LPS: $F_{(1,20)} = 0,7114$, $p = 0.408$).

To better investigate the exploratory behavior in these mice, we compared their performances between the first and the second exploration of the same environment. To avoid any bias due to LPS effect on locomotor activity during the 1st exposition (see Table S5), we analyzed LPS and saline-injected mice separately. Since exploration normally decreases during the re-exposition to the same environment (a process known as habituation), we performed an ANOVAs for repeated measures in order to assess habituation effect in both groups of mice. As shown in Fig. 2B, in the group of saline-injected mice, the number of crossed sectors was reduced between the first and the second exposition (time effect: $F_{(1,10)} = 12,91$, $p < 0,01$), and multiple comparisons indicated that this effect was more pronounced in placebo control mice ($p < 0,01$) than in OB treated mice ($p > 0,05$) meaning that the latter group maintains elevated exploration during the 2nd exposition to the open field. On the other side, exploration was low soon after LPS injection in both OB and placebo mice but when these mice were re-exposed to the same environment 24 hours later, exploration was increased (time effect: $F_{(1,10)} = 13,62$, $p < 0,01$, Fig. 2C) in OB ($p < 0,01$) but not placebo mice ($p > 0,05$). Together, these data indicate that mice treated with OttaBac® display elevated exploratory behavior during the second exposition to the same environment, and once the effect of LPS is over.

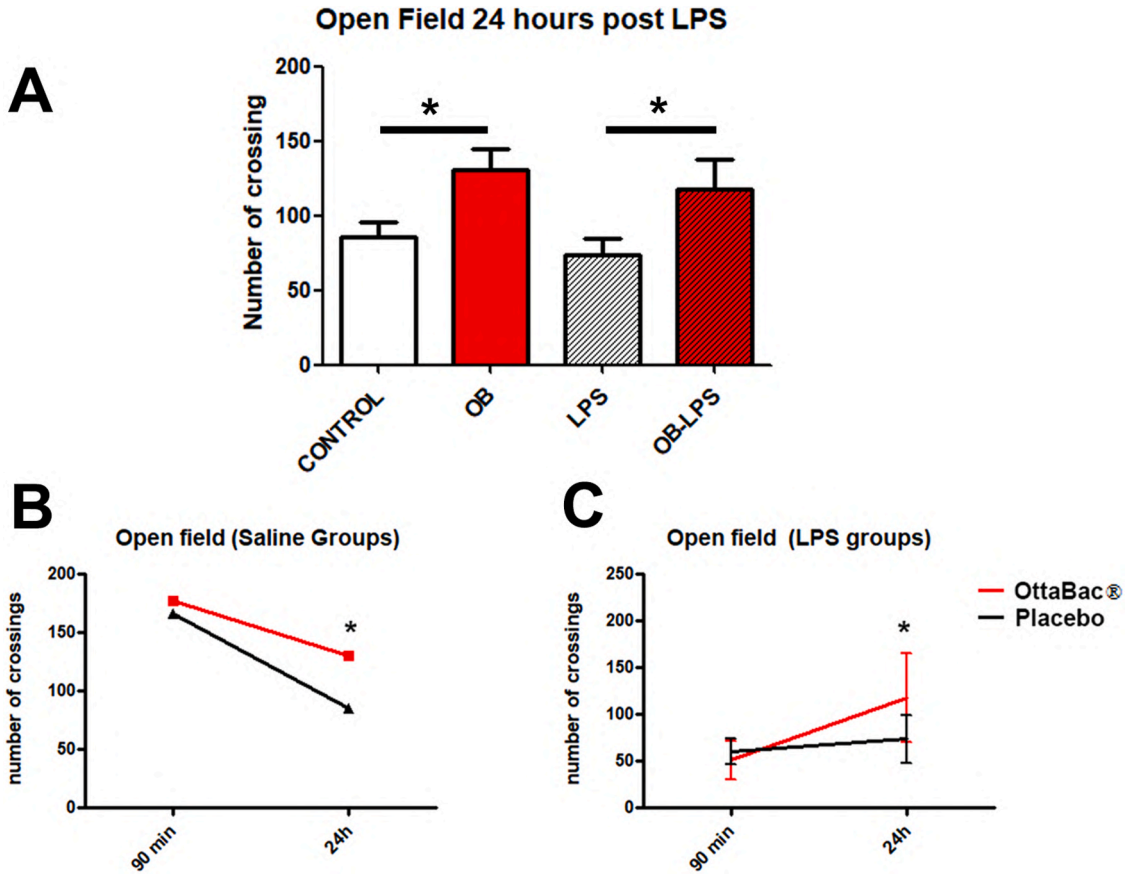

Fig. 2. Effects of OttaBac® administration on Exploratory Behaviour. A) Graphs showing the enhanced exploratory behaviour in the Open Field arena of OB mice, as compared to relative placebo- and LPS-treated groups after 24 hours from saline administration. Data were collected after saline/LPS injection. B) Graph shows the reduction of exploration in saline-injected mice re-exposed to the same environment 24 hours after the first exposition. C) Graph indicates that in LPS-treated mice, re-exposition to the same environment leads to increased exploration only in mice treated with OttaBac®. Statistical significance: * $p < 0.05$. All data reported are mean ± SEM. Two-way ANOVA analysis (Fig. 2 A) and ANOVA for repeated measures analysis were performed (in Fig. 2 B and C).

As enhanced exploration may be indicative of anxiety, we assessed whether OttaBac® administration was associated with the tendency to avoid open spaces, which is a typical anxiety behavior. In mice exposed to open field, we did not detect any group difference for time spent in the central area (Supplementary Table S6), although the number of crossings in central sectors of the open field was higher in OB vs placebo groups (main effect of OB: $F_{(1,20)} = 7589$, $p < 0,05$), consistent with the overall enhanced exploration. Furthermore, when mice were tested in the Elevated Plus Maze, we did not detect group differences for time spent or number of entries in the open arms (Supplementary Table S6). Overall, these data indicate that OttaBac® has no effect on anxiety and confirm that its administration rises exploratory behavior in rodents.

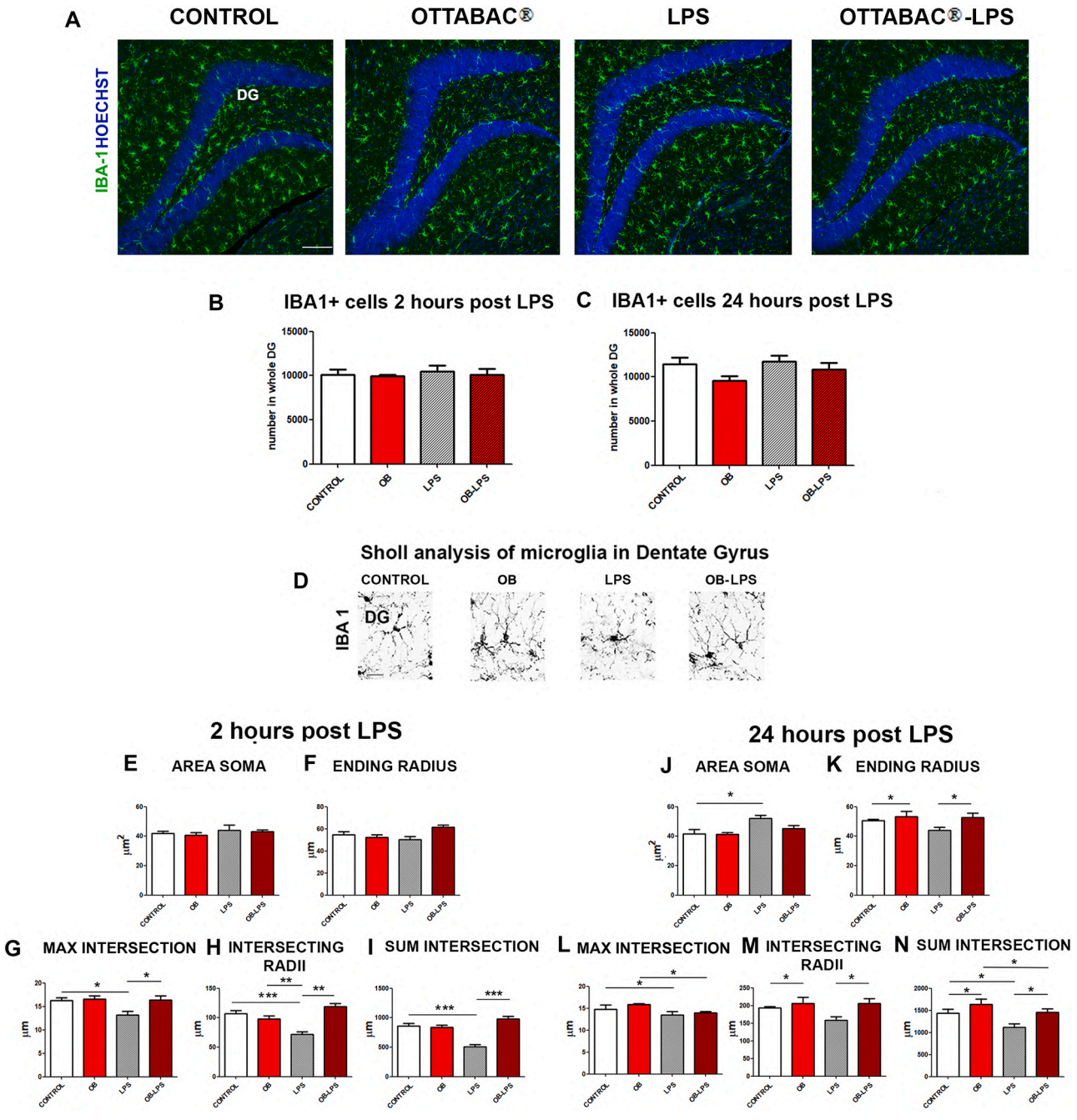

Fig. 3. Effect of OttaBac® on microglia resident in the Dentate Gyrus. A) Representative images showing the distribution of Iba1$^+$ cells (in green) in the Dentate Gyrus (DG) of mice from the 4 experimental groups. Scale bar: 100 µm. B and C) Histograms indicating the comparable total number of Iba1$^+$ cells in the DG among the groups at 2 and 24 hours after LPS injection. D) Representative images displaying the retraction of microglial processes and the decreased arborization detected in DG microglia of the LPS mice group. Scale bar: 100 µm E-I) Diagrams showing the decreased arborization in LPS group and the neuroprotective effect exerted by OB at 2-hour post LPS injection. The graphs indicate a diminution of three different parameters of Sholl Analysis, Max Intersection (G), Intersecting radii (H) and Sum Intersection (I), in the LPS group, with respect to the Control and the OB-LPS groups. J-N) Graphs displaying the evident activation of microglial cells in the DG of LPS group after 24 hours from endotoxin injection, as assessed by the enlarged area soma with respect to the Control group (J) and the decreased arborization and processes complexity in comparison to the Control (L, Max Intersection and N, Sum Intersection) and OB-LPS mice (K, Ending Radius, M, Intersecting radii and N Sum Intersection). Data are obtained from the analysis of six animals *per* condition. Statistical significance: * $p < 0.05$, * * $p < 0.01$ and * ** $p < 0.001$. Two-way ANOVA analysis. DG = Dentate Gyrus.

3.4. OttaBac® exerts an anti-inflammatory effect on hippocampus and cerebral cortex

LPS injection stimulates microglia cells to promptly switch from a resting/surveillance form to an activated state, which is characterized by an increase of microglial density and modification of their morphological features toward an amoeboid phenotype [48–50]. In order to analyze the modulation of microglia after OttaBac® administration and LPS injection, we quantified the density of Iba1^{+} cells and evaluated the microglia morphology by Sholl Analysis in dentate gyrus (DG) and cerebral cortex of the different experimental groups. In DG, we observed that microglial density was not modulated within the four experimental groups at the two different time points upon LPS injection (Fig. 3 A, B). However, the Sholl Analysis revealed that 2 hours after treatment with LPS, the microglial population of LPS group showed a significant retraction of the processes, as assessed by the decrease of Max intersection (LPS effect: $F_{(1,\ 20)} = 5{,}19$; $p = 0.03$ Fig. 3 G) and Sum intersection (LPS x OB interaction: $F_{(1,\ 20)} = 34.12$, $p < 0.001$; followed by Bonferroni post-test Control vs LPS, $p > 0.001$, Fig. 3 I). OttaBac® administration prevented microglia activation in the OB-LPS group, as assessed by overall increased of Sum intersection (LPS x OB interaction: $F_{(1,\ 20)} = 34.12$, $p < 0.001$; followed by Bonferroni post-test LPS vs

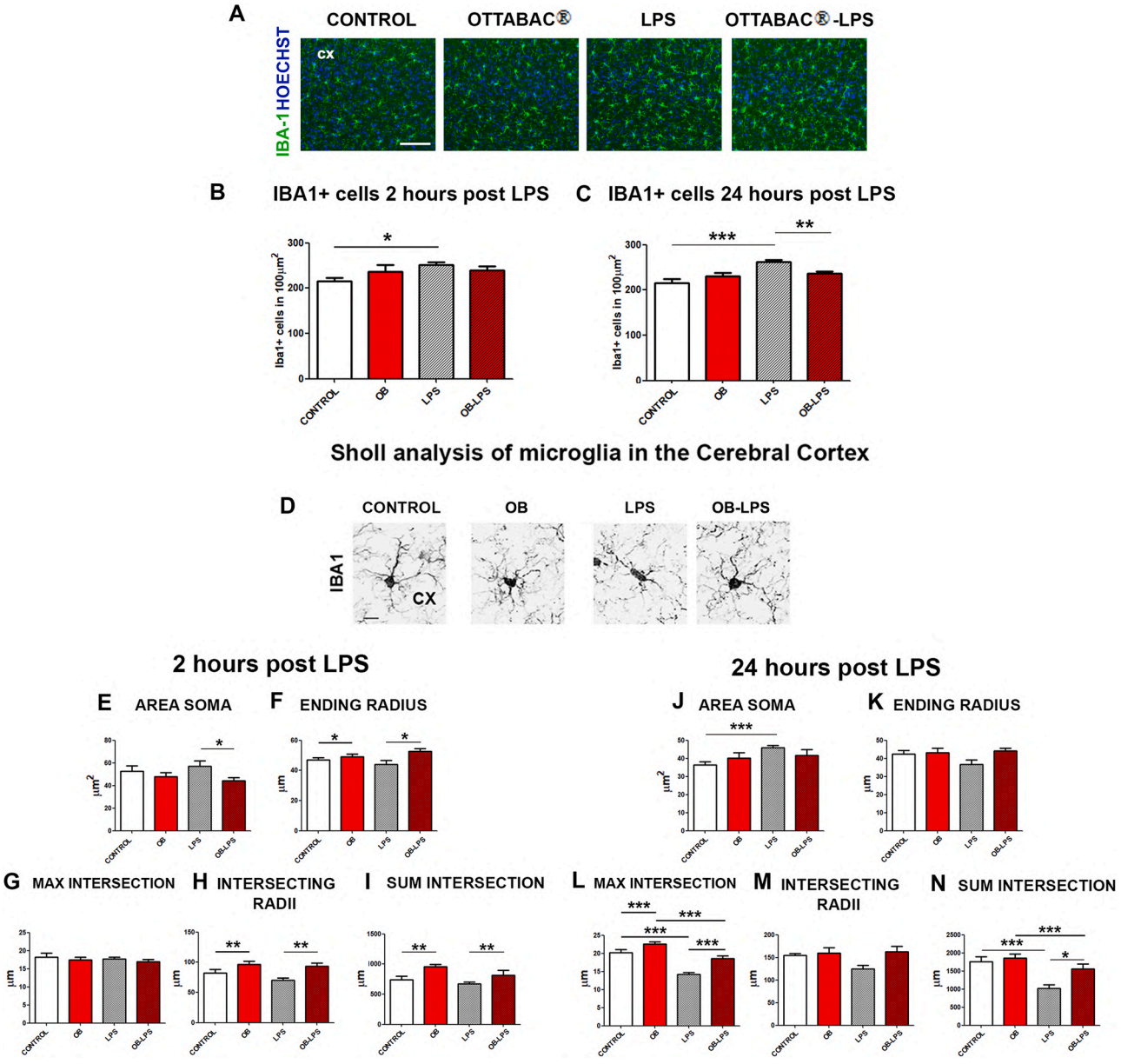

Fig. 4. Effect of OttaBac® on cortical microglia A) Representative images showing the increased cortical density of Iba^{+} cells (in green) in the LPS group. B) Histogram showing the enhanced microglial density in the cerebral cortex of LPS group, when compared to the Control mice at 2-hour after LPS injection. C) Graph indicating the increase of the cortical microglial number in the LPS mice, if compared with Control and OB-LPS groups after 24 hours from LPS injection. D) Representative images displaying the morphological changes (retraction of the processes and enlargement of cell body) observed in cortical microglia of the LPS mice groups. Scale bar: 100 µm. E) Diagrams showing the increased area soma of the LPS group cortical microglia when compared to the OB-LPS mice, 2 hours after LPS injection. F-I) Graphs illustrating the increase of microglial complexity and arborization in term of Ending Radius (F), Intersecting Radii (H) and Sum Intersection (I) observed in the cerebral cortex the OB-treated groups when compared to their respective counterpart 2 hours after LPS injection. J-N). In mice examined 24 hours after LPS treatment we observed in the LPS group an increase of Area Soma respect to the Control mice (J). At this time point Sholl Analysis revealed in the LPS mice a reduction of Max Intersection (L) and Sum Intersection (N) in comparison with Control and OB-LPS groups. Data are obtained from the analysis of six animals *per* condition. Statistical significance: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Two-way ANOVA analysis. DG = Dentate Gyrus.

OB-LPS, $p > 0.001$, Fig. 3 I), Max intersection (OB effect: $F_{(1, 20)} = 5,61$; $p = 0.028$ Fig. 2 G) and Intersecting radii (LPS x OB interaction: $F_{(1, 20)} = 29,77$, $p < 0.0001$; followed by Bonferroni post-test LPS vs OB-LPS, $p = 0.0036$, LPS vs OB, $p = 0.0015$ Fig. 3 H), with respect the LPS group.

In the LPS mice sacrificed 24 hours after LPS injection, the microglia activation was even more pronounced and characterized not only by the decrease in the extension of the microglial processes, but also by the enlarged soma area (LPS effect: $F_{(1, 20)} = 11,11$; $p = 0.003$, Fig. 3 J). At this time point, 15 days of OttaBac® administration was able to maintain the microglia in the resting state at values comparable to the physiological level (Fig. 3 M, N). Finally, under physiological conditions, OttaBac® induces a significant increase, compared to the control group, in the length of the microglia branches assessed by the ending radius (OB effect: $F_{(1, 20)} = 5.08$; $p = 0.035$, Fig. 3 K) as well as of their complexity of arborization (Intersecting radii: OB effect: $F_{(1, 20)} = 6.34$; $p = 0.02$; Sum intersection: OB effect: $F_{(1, 20)} = 7.94$; $p = 0.010$, Fig. 3 M, N).

In cerebral cortex, two-way ANOVA analysis of microglia revealed a main effect of LPS in increasing the microglia density both after 2 hours (LPS effect: $F_{(1, 20)} = 5,03$; $p = 0.036$, Fig. 4B) and 24 hours (LPS x OB interaction: $F_{(1, 22)} = 8,76$; $p = 0.0072$, followed by Bonferroni post-test Control vs LPS, $p < 0.001$, Fig. 4 A, C) after LPS injection. The administration of OttaBac® did not exert any significant effect in the mice sacrificed after 2 hours from LPS injection (OB effect: $F_{(1, 20)} = 0,60$; $p = 0.44$, Fig. 4 B), while after 24 hours we observed that the density of microglia in the OB-LPS group was comparable to the Control group (LPS x OB interaction: $F_{(1, 22)} = 8,76$; $p = 0.0072$, followed by Bonferroni post-test LPS vs OB-LPS, $p = 0.006$, Fig. 4 A,C).

The Sholl analysis did not reveal any significant modification in microglial morphology after 2 hours from LPS injection (Fig. 4 E-I). The OB administration resulted in a significant increase of Ending Radius = OB effect: $F_{(1, 20)} = 6,76$; $p = 0.017$, Fig. 4 F), Intersecting radii (OB effect: $F_{(1, 20)} = 14,44$; $p = 0.001$, Fig. 4 H), and sum intersection (OB effect: $F_{(1, 20)} = 10,29$; $p = 0.0044$ Fig. 4 I) in OB and OB-LPS groups compared to their respective counterpart.

In the mice sacrificed 24 hours following the toxin injection we observed a LPS-dependent modification of microglia toward an activated phenotype characterized by an enlarged area of the soma (LPS effect: $F_{(1, 20)} = 5,67$; $p = 0.027$, Fig. 4J) and by a retraction of the arborization complexity with a significant decrease of Sum intersection (LPS effect: $F_{(1, 20)} = 19,53$; $p = 0.0003$, Fig. 4N) and Max intersection (LPS effect: $F_{(1, 20)} = 50,49$; $p > 0.0001$, Fig. 4G). Administration of OttaBac® was able to induce an improvement of the resting/surveillance morphology of microglia in the OB group and to protect the OB-LPS group by the LPS-dependent activation of these cells.

Taken together, the data obtained in the study of microglia reveal that LPS induces a shift towards a pro-inflammatory phenotype of microglia both in DG and in cortex through an increase in cell density and morphological changes. Treatment with OttaBac® a) improves the surveillance microglial phenotype by increasing the ramification complexity in the OB mice group, b) prevents the transition to an activated neurotoxic phenotype in the DG and cortex of OB-LPS mice.

The anti-inflammatory effect of OttaBac® was also tested by analyzing the expression of pro-inflammatory markers both in DG and cerebral cortex.

The expression level of the main pro-inflammatory factors was analyzed by qRT-PCR. As expected, we found both in cortex and in hippocampus a significant induction of inflammatory genes such as Il-6 (interleukin-6), Il-1β (interleukin-1β) and Tnf-α (Tumor Necrosis Factor α) 2 hours after LPS injection, with or without OB administration (Fig. 5 A and C), according with the activation of an inflammatory response. Il-1β and Tnf-α are still robustly expressed 24 hours after LPS injection in cortex and hippocampus and significantly induced in both LPS and OB-LPS groups (Fig. 5 B and D); Il-6 expression is not consistently detectable 24 hours upon LPS injection (data not shown). Notably, OttaBac® per se does not induce the expression of inflammatory markers. Interestingly, the two-way ANOVA analysis revealed that the expression of some inflammatory markers is significantly reduced upon LPS injection in the mice treated with OttaBac®. Specifically, we found, at 2 hours after LPS injection, an OttaBac®-dependent reduction of the pro-inflammatory

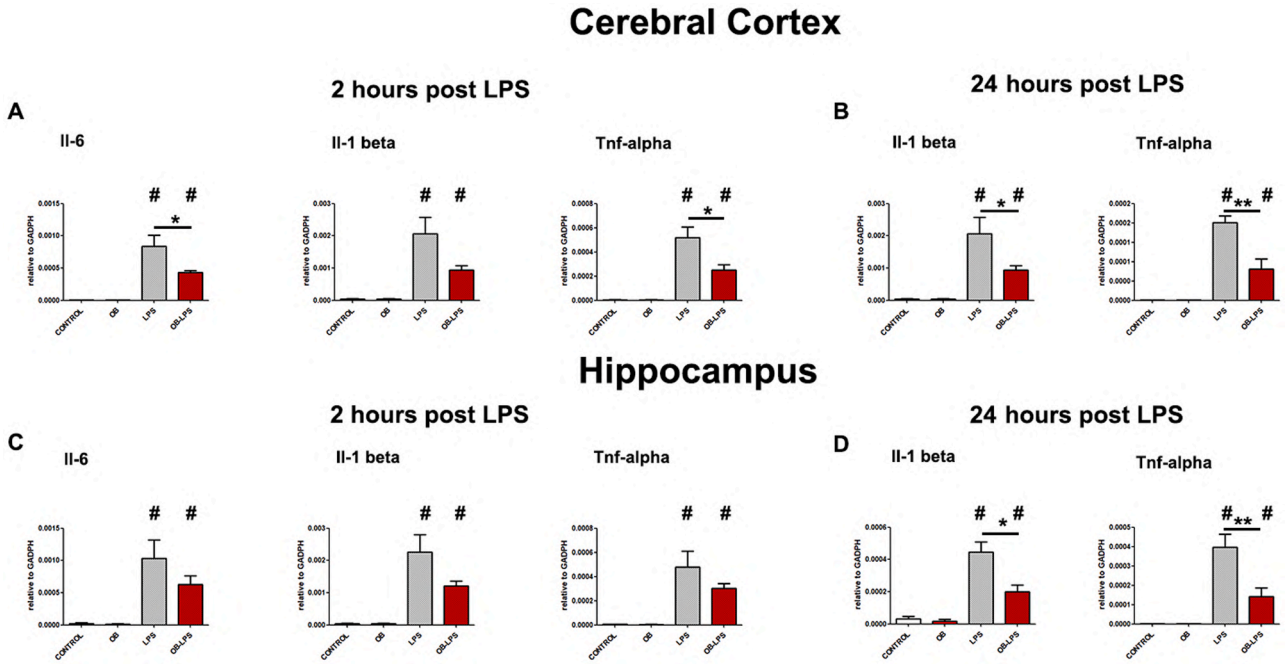

Fig. 5. Anti-inflammatory role of OttaBac® on the expression of pro-inflammatory markers in cortex and hippocampus. Expression analysis by qRT-PCR of inflammatory markers in Cerebral cortex (A and B) and Hippocampus (C and D) isolated from mice treated with OB or placebo for 15 days and injected with LPS or saline 2 hours (A and C) or 24 hours (B and D) before sacrifice. The data are reported as relative to housekeeping gene GAPDH, and represented as mean ± SD (n = 4 biological replicates for each experimental group). The induction of inflammatory markers upon LPS stimulation is always significant related to control animals (#), both in LPS and OB-LPS groups. A two-way (OB, LPS) ANOVA was used for comparison related to LPS group (*, $p < 0.05$; **, $p < 0.01$).

markers, even not always significant, especially in the hippocampus (Fig. 5 C), while the neuroprotection exerted by OttaBac® is quite evident in the cerebral cortex (Cortex: OB effect on Il-6: LPS vs OB-LPS: $F_{(1,12)} = 5,4$; $p < 0,05$. OB effect on Tnf-α: LPS vs OB-LPS: $F_{(1,12)} = 7,15$; $p < 0,05$; Fig. 5 A). The data obtained at 24 hours after LPS injection demonstrate a more evident and significant effect of OttaBac® both in cortex and hippocampus (Cortex: OB effect on Il-1β: LPS vs OB-LPS: $F_{(1,12)} = 8,54$; $p < 0,05$; OB effect on Tnf-α: LPS vs OB-LPS: $F_{(1,12)} = 13,73$; $p < 0,01$; Hippocampus: OB effect on Il-1β: LPS vs OB-LPS: $F_{(1,12)} = 8,56$; $p < 0,05$; OB effect on Tnf-α: LPS vs OB-LPS: $F_{(1,12)} = 9,45$; $p < 0,01$; Fig. 5 B and D). Collectively, these results suggested that the treatment with OttaBac® could attenuate the inflammatory response under acute inflammatory condition and prevent the pro-inflammatory over-activation leading to neuronal damage.

3.5. *OttaBac® induces an enhancement of hippocampal neurogenesis both in saline and LPS-injected mice*

In this section we analyzed the effect of OttaBac® treatment and LPS injection in the early phase of DG adult neurogenesis, by dissecting the different stages of differentiation of the newly generated neurons.

As a first, we evaluated the proliferation rate of newborn neurons by using the specific marker of proliferation named Ki67. The two-way ANOVA did not reveal any significant effect of LPS injection both at 2 hours (LPS effect: $F_{(1, 20)} = 2,39$; Control and OB vs LPS and OB-LPS, $p = 0.137$, Fig. 6B) and 24 hours (LPS effect: $F_{(1, 20)} = 1,02$; $p = 0.32$, Fig. 6D) after injection. Conversely, two-way ANOVA analysis indicated a main effect of the prolonged administration of OttaBac® in increasing the number of proliferating cells in the DG of OB and OB-LPS mice in comparison to Control and LPS mice respectively, at 2 hours and 24 hours after LPS injection (2 hours: OB effect: $F_{(1, 20)} = 66,8$; Control and LPS vs OB and OB-LPS $p > 0.0001$, Fig. 6A and B, 24 hours: OB effect: $F_{(1, 20)} = 27,6$; $p > 0.0001$, Fig. 6 A and C).

We next defined which differentiation stage of adult neurogenesis was modulated by the administration of OttaBac® and the following injection of LPS. For this purpose, we analyzed the differentiation dynamics of Type-1 neural stem cells (NSCs), characterized by the expression of stemness markers GFAP and SOX2, and of the Type-2b, Type-3 neural progenitors, expressing the marker DCX.

The statistical analysis of NSCs in the mice sacrificed after 2 hours from LPS injection did not revealed any significant effect exerted by the toxin in the LPS and OB-LPS groups, compared to their respective saline-injected counterpart, Control and OB mice (Fig. 7 A–C).

Conversely, we observed in the OB and OB-LPS groups a main effect of OttaBac® treatment in the percentage of NSCs that exited the quiescent state and were recruited in the cell cycle (expressed as the ratio of $Sox2^+GFAP^+Ki67^+$ cells to total $Sox2^+GFAP^+$ cells) (OB Effect: $F_{(1, 20)} = 32.89$; $p < 0.0001$, Fig. 7 A), as well as in the number of proliferating Type-1 NSCs ($Sox2^+GFAP^+Ki67^+$ cells, OB Effect: $F_{(1, 20)} = 14.17$; $p = 0.0012$, Fig. 7 B), while the NSCs pool size (assessed by the counting of Sox2 + GFAP + cells) was not affected (OB effect: $F_{(1, 20)} = 0,10$; $p = 0.75$, Fig. 7 C).

In mice from LPS and OB-LPS groups sacrificed after 24 hours from LPS injection, we observed a significant effect of toxin treatment in decreasing the total number of Type-1 NSCs (LPS effect: $F_{(1, 20)} = 5,10$; $p = 0.035$, Fig. 7 F). OttaBac® treatment induced a striking increase of NSCs recruitment (OB effect: $F_{(1, 20)} = 18.05$; $p = 0.0004$, Fig. 7D) and proliferation (OB effect: $F_{(1, 17)} = 13.15$; $p = 0.0021$, Fig. 7 E) in the OB and OB-LPS mice respect to their respective counterpart, Control and LPS mice. However, probiotic mixture treatment did not fully prevent the reduced Type-1 pool size in the OB-LPS mice group (OB Effect: $F_{(1, 20)} = 1,58$; $p = 0.22$, Fig. 7F).

The two-way ANOVA analysis of DCX^+ progenitor cells highlighted a main effect of LPS in the significant reduction of the pool size of neural progenitors in the LPS and OB-LPS mice sacrificed 24 hours after the bacterial toxin injection (LPS Effect: $F_{(1, 20)} = 4,65$; $p = 0.043$, Fig. 8 A, E) when compared to Control and OB mice, respectively.

OttaBac® administration induced in the DG a striking increase of the number of proliferating progenitor cells ($Ki67^+DCX^+$ cells) after 2 and 24 hours from LPS injection (2 hours: OB effect: $F_{(1, 20)} = 10,77$; $p = 0.0037$, Fig. 8 B; 24 hours: OB effect: $F_{(1, 24)} = 36,45$; $p > 0.0001$, Fig. 8 D). This event induced a significant enhancement in the neural progenitor pool size, assessed by the counting of the DCX^+, regardless the administration of LPS 2 hour and 24 hours before the sacrifice

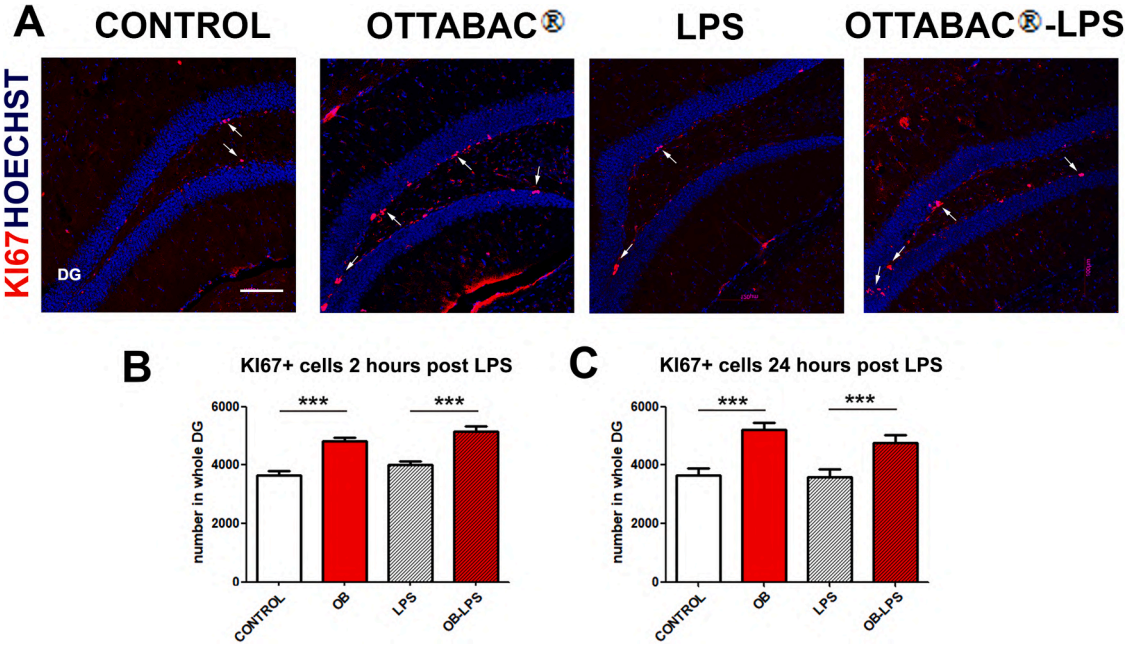

Fig. 6. OttaBac® increases proliferation in the Dentate Gyrus of Saline- and LPS-injected mice. A) Representative pictures describing the enhanced number of $Ki67^+$ cells (in red) detected in the OB and OB-LPS groups, in comparison to their respective counterpart Control and LPS groups. Arrow indicate $Ki67^+$ cells. Scale bar: 100 μm. B and C) Graphs showing the OB-dependent increased proliferation after 2 (B) and 24 (C) hours after LPS treatment. Data are obtained from the analysis of six animals *per* condition. Statistical significance: *** $p < 0.001$. Two-way ANOVA analysis.

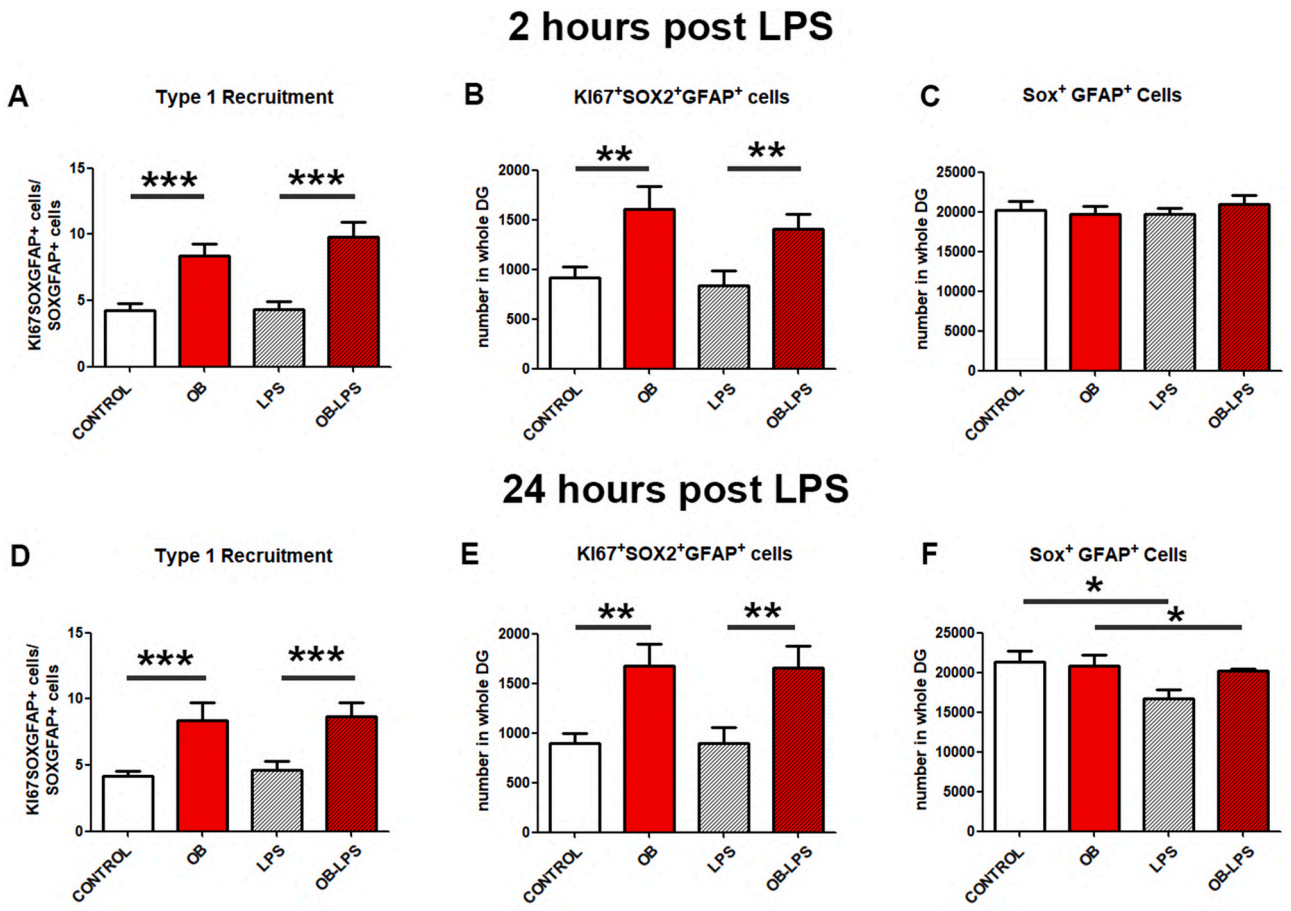

Fig. 7. OttaBac® induces Neural Stem Cells recruitment and expansion in the Dentate Gyrus of Control and LPS-injected mice. A, B) Histograms showing the increase of Neural Stem Cells (NSCs) recruitment (A) and proliferation (B) observed in the OB and OB-LPS mice, when compared to their respective counterpart Control and LPS groups, 2 hours after LPS injection. C) NSCs pool size remains unvaried among the experimental groups analyzed. D, E) The data obtained 24 hours after LPS injection show that the toxin induces a significant drop of NSCs pool, which is counteracted by pre-treatment with OB. F) LPS induces a significant decrease of NSCs pool after 24 hours from injection. Data are obtained from the analysis of six animals *per* condition. Statistical significance: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Two-way ANOVA analysis.

(2 hours: OB effect: $F_{(1, 20)} = 14{,}64$; $p = 0.0011$, Fig. 8 A, C, 24 hours: OB effect: $F_{(1, 20)} = 15{,}06$; $p = 0.0006$, Fig. 8 A, E).

We next evaluated different parameters describing the dendritic arborization complexity of DCX^+ neural progenitors, such as the arborization length, the number of dendrites and branching points of the ramification.

Our data revealed a main effect of LPS treatment in the LPS and OB-LPS groups sacrificed 2 hours later in decreasing the arborization length (LPS Effect: $F_{(1, 20)} = 10{,}26$; $p = 0.0045$, Fig. 9 A, B), and number of neurites (LPS effect: $F_{(1, 20)} = 7{,}56$; $p = 0.012$, Fig. 9 A, C), when compared to their relative counterpart, Control and OB mice groups. The administration of OttaBac® is able to increase the dendritic complexity of DCX^+ cells in the Control group and to counteract the detrimental effect of the bacterial toxin in the OB-LPS group (arborization length: OB effect: $F_{(1, 20)} = 24{,}4$; $p < 0.0001$; branching points: OB effect: $F_{(1, 20)} = 7{,}19$; $p = 0.014$; dendrites number: OB effect: $F_{(1, 20)} = 4{,}51$; $p = 0.04$ Fig. 9 A-D).

In the mice sacrificed at 24 hours from injection, two-way ANOVA analysis revealed a main effect of LPS in the general impairment of the dendritic complexity of the DCX^+ cells (arborization length: LPS effect: $F_{(1, 20)} = 21{,}26$; $p = 0.0002$; branching points: LPS effect: $F_{(1, 20)} = 4{,}74$; $p = 0.041$; dendrites number: LPS effect: $F_{(1, 20)} = 7{,}88$; $p = 0.011$, Fig. 9 E-G), while also at this time point OttaBac® treatment had a main effect in improving the differentiation parameters in DCX^+ progenitors cells both in the Control and OB-LPS experimental groups.

Collectively, our data clearly indicate that a single injection of LPS induced a decrease in the NSCs and neural progenitors 24 hours later; surprisingly, the 15-days administration of OttaBac® provided a powerful proneurogenic effect, by increasing the proliferation and differentiation of the neural progenitor in the Control mice, and by neutralizing the anti-neurogenic processes triggered by LPS injection in OB-LPS mice.

3.6. *OttaBac® increases e-cadherin and occludin colonic expression*

The integrity of the intestinal barrier is an essential prerogative for the maintenance of intestinal homeostasis and also influences the stability of the whole organism.

We performed an expression analysis at protein level of the tight junctions, occludin, and adherens junctions, e-cadherin, under the different experimental conditions. The statistical analysis revealed that LPS injection did not alter epithelial junction expression in mice sacrificed 2 and 24 hours after toxin administration. (Fig. 10 A, B). However, multiple comparison analyses showed that prolonged consumption of OttaBac® induced a significant increase in both e-cadherin (OB effect: $F_{(1,20)} = 53{,}05$; $p < 0{,}0001$, Fig. 10 A, B) and occludin colonic expression (OB effect: $F_{(1,20)} = 19.53$; $p = 0{,}0003$, Fig. 10 A, B), in comparison with Control group. Moreover, in LPS groups, OttaBac® maintained increased expression of occludin 2 hours after toxin injection (OB effect $p = 0.0262$, Fig. 10 A), and of e-cadherin 24 hours after LPS (OB effect $p = 0.0059$, Fig. 10 B).

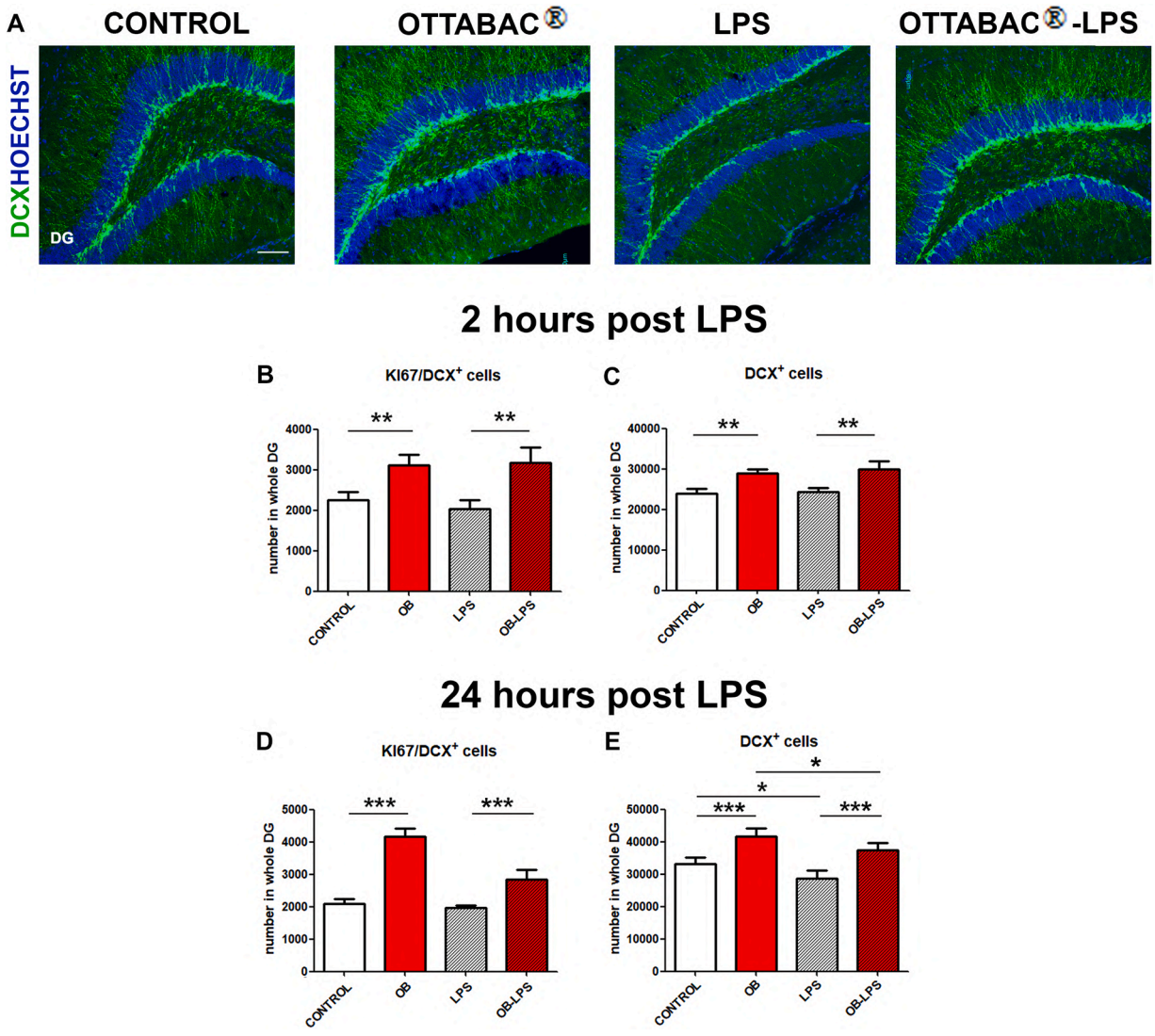

Fig. 8. OttaBac® enhances cell proliferation in the Dentate Gyrus of Control and LPS mice. A) Representative pictures describing the enhanced number of DCX^+ neural progenitors (in green) observed in the OB and OB-LPS groups, in comparison to their counterpart Control and LPS groups, respectively. Scale bar: 100 μm. B, D) Histograms showing the increased DCX^+ cell proliferation in OB and OB-LPS groups in comparison with Control and LPS mice, respectively, after 2 (B) and 24 (D) hours from LPS injection. C, E) The graphs show that at 2 (C) and 24 (E) hours post LPS injection the total number of DCX^+ cells in the DG of OB and LPS OB mice significantly exceeds the value observed in the relative counterparts. At 24 hours post LPS injection it is possible to observe a significant decrease of the DCX^+ progenitors in the DG of LPS group in comparison to the other experimental groups (E). Data are obtained from the analysis of six animals *per* condition. Statistical significance: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Two-way ANOVA analysis. DG = Dentate Gyrus.

3.7. OttaBac® prevents LPS-induced colonic TNF-α expression and increases IL-10 levels in LPS treated mice

We have analyzed the expression of a pro-inflammatory cytokine, TNF-α, and of the anti-inflammatory IL-10, in the different experimental conditions.

A single injection of LPS caused a significant increase of colonic TNF-α expression both 2 and 24 hours after toxin administration (LPS effect 2 h: $F_{(1,20)} = 54.82$, $p < 0{,}0001$; LPS effect a 24 h: $F_{(1,\ 20)} = 18{,}22$, $p = 0{,}0004$, Fig. 11 A, B). OttaBac® consumption, that was not able to modify TNF-α expression in basal condition, significantly counteracted cytokine increase, at both time points analyzed (OB effect 2 h: $F_{(1,\ 20)} = 25{,}02$, $p < 0{,}0001$; OB effect 24 h: $F_{(1,\ 20)} = 4.625$, $p = 0.0439$, Fig. 11 A, B).

24 hours after LPS injection, prolonged ingestion of OttaBac® caused an increased expression of colonic IL-10, revealed after endotoxin treatment ($F_{(1,\ 20)} = 4766$, $p = 0{,}0411$ Fig. 11 B).

4. Discussion

In the present paper we explored the role of the probiotic mixture OttaBac® in counteracting the acute inflammatory effects induced by LPS. Using a LPS-based mouse model of systemic inflammation, we showed that the prolonged administration of OttaBac® (15 days) before the LPS injection induces a robust pro-neurogenic and neuroprotective response capable of enhancing hippocampal neurogenesis.

Moreover, the pre-treatment with OttaBac® counteracts both the LPS-dependent activation of microglia and the increase of pro-inflammatory cytokines in brain and in the gastrointestinal district. We have also demonstrated a specific effect of OttaBac® in enhancing the stability of the colonic barrier, as documented by the increased expression of epithelial junction proteins. While the pre-treatment with OttaBac® could not prevent the typical sickness behavior associated with LPS injection, the positive effects of OttaBac® administration were accompanied by enhanced exploratory behavior in mice after that the acute LPS effect is over. This enhanced exploration manifests with

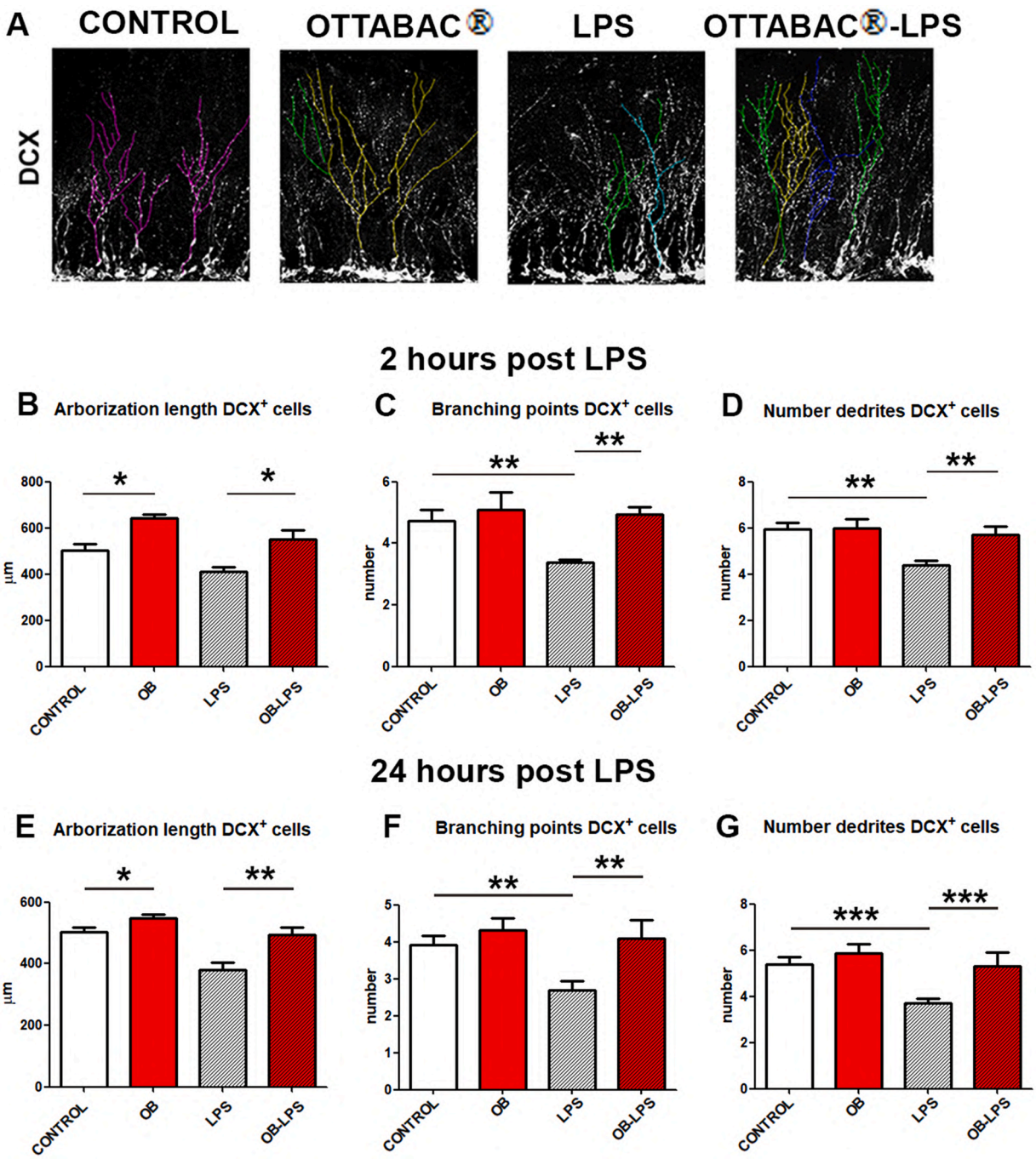

Fig. 9. OttaBac® increases arborization complexity of DCX^+ neural progenitors. A) Representative images illustrating the decreased arborization complexity detected in the LPS group and the improved differentiative rate induce by the OB consumption in the saline and LPS-injected mice. Scale bar: 100 µm. B, E) Histograms showing the pro-differentiative effect exerted by OttaBac® in the saline and LPS-injected mice at 2 and 24 hours after LPS injection, assessed by the significant increase of arborization length in OB and OB-LPS groups. C, D, F, G) At the two time-points analyzed, LPS causes a reduction of branching point (C, F) and dendrites number (D, G) in the DCX^+ neural progenitors, which is totally counteracted by OB pre-administration. Data are obtained from the analysis of six animals *per* condition. Statistical significance: * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Two-way ANOVA analysis.

reduced habituation to surrounding environment, which may be associated with enhanced anxiety and/or reduced memory. We excluded any effect of OttaBac® on anxiety, but future studies are needed to investigate the impact of OttaBac® on memory, with particular relevance to the correlation with enhanced hippocampal neurogenesis.

The most unexpected finding of this study is that the prolonged consumption of OttaBac® exerted an evident pro-neurogenic effect even in physiological condition. Previous studies have never highlighted the ability of probiotics to improve neurogenic processes [51,52]. Our data show for the first time that in physiological state, 15 days of OttaBac® administration induces a significant increase of adult hippocampal neurogenesis, as assessed by enhanced NSCs recruitment from quiescence, proliferation and progenitor differentiation.

Our data suggest that OttaBac® could exert its pro-neurogenic effect by mediating the induction in length and complexity of microglia branches in the hippocampal dentate gyrus and therefore increasing the surveillance activity of microglia. In this regard, it has been widely demonstrated that microglia is an essential component of the neurogenic

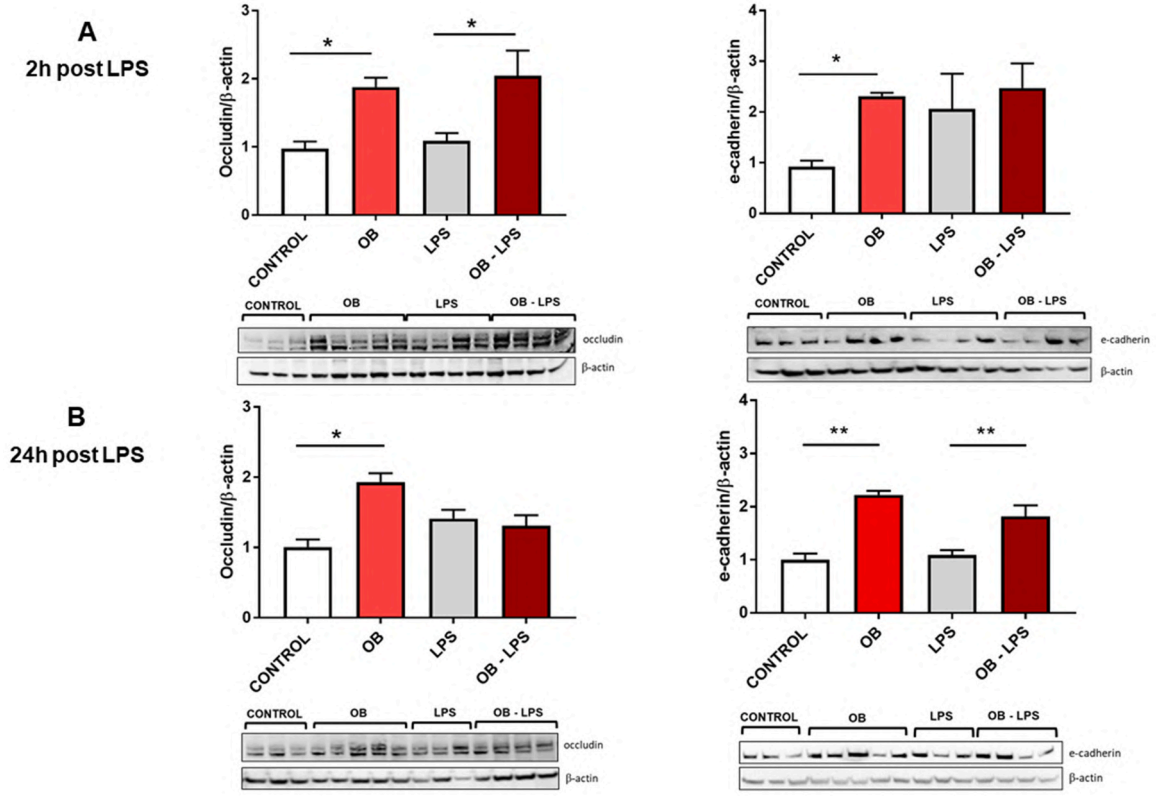

Fig. 10. OttaBac® increases e-cadherin and occludin colonic expression. Histograms and representative bands of colonic epithelial junction expression under different experimental conditions. 15 days-treatment with OB caused an enhanced expression of occludin and e-cadherin (A and B), respect to the Control group. LPS didn't show any significant differences in epithelial junction expression, neither 2 hours (A) or 24 hours (B) after endotoxin treatment, respect to Control group. OB consumption maintains enhanced expression of occludin (2 hours post LPS, A) and of e-cadherin (24 hours post LPS, **B**). Statistical significance: * $p < 0.05$, * * $p < 0.01$. Two-way ANOVA analysis.

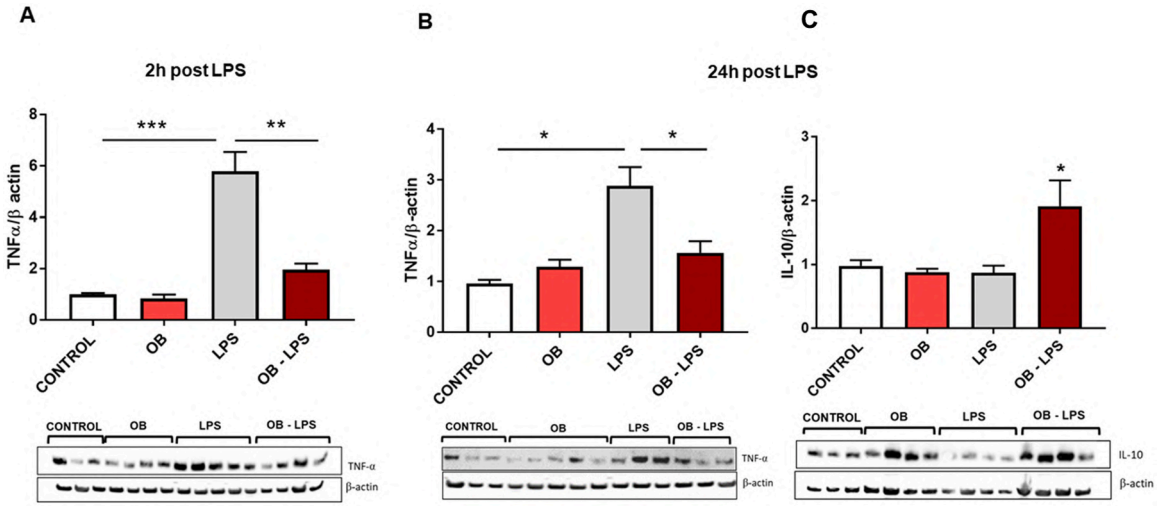

Fig. 11. OttaBac® prevents LPS-induced colonic TNF-α expression and increases IL-10 levels in LPS treated mice Histograms and representative bands of colonic inflammatory state. LPS group showed an increased expression of TNF-α, both at 2 hours (A) and 24 hours (B) after treatment. OB pre-treatment prevented colonic inflammation at both time points (A and B). 24 hours after LPS injection, an increased level of colonic IL-10 was observed only in OB-LPS group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Two-way ANOVA analysis.

dentate gyrus due to its pivotal role in phagocytosing excess of newborn cells undergoing apoptosis [53], in remodeling the volume and geometry of extracellular space, thus allowing the higher concentration of signaling molecules in the niche environment [54], and finally in producing pro-neurogenic factors such as IGF-1 [54–56], fractalkine [57, 58] and VEGF [59]. An alternative and not mutually exclusive hypothesis is that OttaBac® may induce the increase in the expression of specific pro-neurogenic factors such as BDNF, a pleiotropic neurotrophin that plays a fundamental role in neurogenic processes, and which can be regulated by probiotic administration [60,61].

Another relevant aspect that arises from our data is that OttaBac® not only maintains its pro-neurogenic effect, but also exerts a

neuroprotective action in conditions of LPS-dependent neuroinflammation. Several studies have demonstrated that systemic inflammation induces a profound impairment of hippocampal adult neurogenesis, which can be at the origin of cognitive decline during aging as well as neuropsychiatric and neurodegenerative pathologies [3,62–65]. The impact of systemic or intracerebral LPS-dependent inflammatory response on hippocampal neurogenic niche has been widely investigated [7,66] and the effects include decrease in DCX^+ cell population, impairment of the survival of new neurons [67] and deficiency in the integration of newborn neurons into behaviorally relevant pre-existing neural networks [68].

Systemic inflammatory diseases are usually associated with enhanced cytokine levels, which reach the brain through the systemic circulation [69,70] or the vagal nerve [71], increasing in local blood flow, adhesion and extravasation of circulating monocytes, neutrophils and lymphocytes [72]. In line with other studies [7,69,73–75], we found that the peripheral injection of LPS triggers in brain tissue the expression of pro-inflammatory cytokines, such as Il-6, Il-1β, and Tnf-α, which are the primary negative modulators of the neurogenic processes in adult hippocampus. The astrocytic overexpression of Il-6 can reduce proliferation and progenitor differentiation in the hippocampus of transgenic mice [76], while in vitro studies have evidenced that treatment of mouse NSCs with Tnf-α was able to inhibit neuronal and glial differentiation [77]. Furthermore, the anti-neurogenic role of Tnf-α has been also highlighted by the discovery that circulating Tnf-α can trigger the activation of microglia [78,79] which, in turn, produces and releases pro-inflammatory cytokines into the brain tissue, with profound detrimental effects on proliferation, differentiation and survival of newborn neurons [80]. Our data suggest that the decrease in hippocampal neurogenesis observed in LPS-treated mice may have been caused by the increase in pro-inflammatory cytokines Il6, Il-1β, and Tnf-α, detectable at the hippocampal level both at 2 and 24 hours after LPS treatment. In our study, we observed that a single injection of LPS was sufficient to induce a decrease in NSCs pool size as well as in the number and differentiation rate of DCX^+ progenitors within 24 hours, in line with previous studies reporting a temporal shift between the LPS-dependent inflammatory peak, and the alteration of neurogenic parameters [81].

We also assumed that the LPS-dependent activation of microglia within the Dentate Gyrus may play an important anti-neurogenic role. To this regard, several studies have demonstrated that activated microglia plays a pivotal role in the production and release of pro-inflammatory cytokines, capable of exacerbating the inhibitory processes of neurogenesis in the hippocampus [33,82,83]. In our hands, Sholl Analysis revealed numerous morphological changes (process retraction and soma increase) characteristic of the transition of microglia to a state of pro-inflammatory activation, which may have played an important role in the decrease of neurogenesis observed in LPS-treated mice.

Our data clearly indicate that pretreatment with OttaBac® supports a powerful systemic defense able to counteract or partially alleviate the anti-neurogenic processes and the pro-inflammatory response triggered by the administration of LPS. In particular, we demonstrate how the levels of proinflammatory cytokines Il-6, Il-1β, and Tnf-α are significantly lower in the cortex and hippocampus of OttaBac®-treated mice upon LPS injection, compared to those measured in the LPS group. In this regard, it has been shown that an eight strains probiotic mixture was able to significantly reduce the circulating levels of Tnf-α, in mouse models of systemic inflammation with a consequent improvement in sickness behavior, attenuation of the microglia activation and reduced recruitment of monocytes into the brain vasculature and brain parenchyma [33,78,79]. Furthermore, clinical studies have found that the intake of the above eight strains probiotic mixture reduces the levels of circulating pro-inflammatory cytokines, including Tnf– α, in patients suffering from various inflammatory diseases [84–86]. Consequently, we believe that the new probiotic formula may induce an anti-inflammatory and neuroprotective effect through the modulation of some still unexplored molecular pathways and cellular processes. Another important aspect that reflects the anti-inflammatory properties of OttaBac® is represented by the lack of activation of the microglia in OttaBac®-LPS animals; indeed, in these mice microglia remains in a state of resting/surveillance comparable to that observed in the control animals. All these observations suggest that this probiotic blend could represent an important therapeutic tool in preventing peripheral inflammation-associated brain dysfunction and in particular altered adult hippocampal neurogenesis.

In our experimental model LPS caused inflammatory condition, not only at central level, but also in the colon, where we detected increased expression of TNF-α, both at 2- and 24 hours after LPS injection. Pretreatment with OttaBac® was able to counteract both colonic and central inflammation, reinforcing the hypothesis that probiotics exert a protective effect both in peripheral and central districts. Interestingly, OttaBac® increased colonic anti-inflammatory Il-10 in OttaBac®-LPS group 24-hours after endotoxin treatment, without affecting basal condition, suggesting that OttaBac® stimulates anti-inflammatory pathways. Many studies have established that neurological disorders may be associated with intestinal inflammatory process, which could trigger or maintain the pathological condition [87]. Generally, impairments in the intestinal compartment do not remain locally confined, and pro-inflammatory and pro-oxidant mediators may reach the brain through systemic circulation, thereby influencing neuronal homeostasis. In particular, some cytokines (i.e. TNF-α and IL-6), have the ability to modify the permeability of the blood-brain barrier allowing the movement of peripheral immune cells to the brain, and the release of inflammatory markers, finally promoting neuronal damage [88,89]. Considering that, OttaBac® consumption, driving anti-inflammatory pathways could represent an interesting new preventive approach in the inhibition of inflammation-related states.

We also evaluated the intestinal barrier integrity, as the preservation of a functional intestinal permeability is of primary importance for the maintenance of the homeostasis in the entire organisms. In our experimental conditions, LPS did not cause an alteration of colonic epithelial junctions, as shown by the unmodified expression of occludin and e-cadherin upon LPS injection. Probably the low dose of LPS triggers an inflammatory state, which is not sufficient to elicit a disruption of intestinal barrier integrity. However, a very interesting finding is that the prolonged consumption of the probiotic mixture caused a significant increase of tight and adherens junctions, under physiological conditions, that persists under inflammatory condition. In particular, OttaBac® consumption maintains high occludin expression 2 hours after LPS injection, whilst the increased e-cadherin level is observed 24 hours after administration of the toxin. This temporal distinction supports the idea that the junction system is a dynamic network that acts in concert to ensure intestinal homeostasis [90]. Several studies showed a protective effect of probiotics in counteracting gut epithelial alterations caused by different pathological conditions; in addition, we found that the new multi-strain formula produced a strong reinforce of the epithelial “glue”, independently from the negative stimuli. We speculate that the different strains included in OttaBac® mix could work in cooperation to elicit an effect difficult to be reached by a single strain. A correlation between modification of gut permeability and some neurodegenerative disorders has been documented. Indeed, in Parkinson’s disease (PD) the gut is a site of crucial events underlying the onset, progression and symptomatology of this pathology [91]. Gut leakiness has often been found increased in PD patience and in mouse models of the pathology [92,93] and it is accompanied by α-synuclein deposit in the enteric nervous system. More in general, the leaky gut may be considered a silent driver that in cooperation with inflammatory mediators self-feeds a progressive inflammation that, finally, could involve the brain compartment.

The main limitation of this work is represented by the lack of a detailed description of the putative molecular mechanisms underlying the pro-neurogenic and anti-inflammatory effect exerted by the probiotic mixture. However, this is the first in vivo study on the novel

probiotic product and we primarily focused on its effectiveness in a mouse model of acute systemic inflammation, leaving out the identification of the underlying molecular pathways for future research.

5. Conclusion

This study highlights for the first time that a multi-strain probiotic formulation based on food-associated strains and human gut bacteria is effective in inducing a significant increase in initial neurogenic processes (increase in NSC pool, cytogenesis and differentiation rate of neural progenitors) both in basal conditions and in an acute neuroinflammatory context induced by LPS. Our research also reveals the powerful anti-inflammatory action of OttaBac® which is expressed with an evident reduction of specific pro-inflammatory parameters in the brain and gut, and with a reinforcement of the intestinal barrier, suggesting an involvement of the gut-brain axis in orchestrating the underlying molecular processes. Certainly, the comprehension of such pathways represents an intriguing aspect of the investigation, to identify putative metabolites and gut-brain communication routes involved in the effects induced by mixture consumption. Thus, our study provides pivotal reference for designing future strategy of probiotic usage in the pre-clinical and medical context as a pro-neurogenic and anti-inflammatory agent.

CRediT authorship contribution statement

Carla Petrella: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing – review & editing, Funding Acquisition. **Georgios Strimpakos:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – review & editing, Funding acquisition. **Alessio Torcinaro:** Methodology, Investigation. **Silvia Middei:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Valentina Ricci:** Formal analysis, Investigation. **Giorgio Gargari:** Formal analysis, Investigation, Methodology. **Diego Mora:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **Francesca De Santa:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – review & editing, Funding acquisition. **Stefano Farioli-Vecchioli:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – review & editing, Funding acquisition, Project administration, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This study was funded by Beingpharma s.r.l (Italy), which also provided both the OttaBac® product and the placebo. Beingpharma s.r.l had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.phrs.2021.105795.

References

[1] P.A. Carpentier, T.D. Palmer, Immune influence on adult neural stem cell regulation and function, Neuron 64 (2009) 79–92, https://doi.org/10.1016/j.neuron.2009.08.038.

[2] D.J. DiSabato, N. Quan, J.P. Godbout, Neuroinflammation: the devil is in the details, J. Neurochem 139 Suppl 2 (2016) 136–153, https://doi.org/10.1111/jnc.13607.

[3] R.A. Kohman, J.S. Rhodes, Neurogenesis, inflammation and behavior, Brain Behav. Immun. 27 (2013) 22–32, https://doi.org/10.1016/j.bbi.2012.09.003.

[4] Y. Zhu, E.A. Klomparens, S. Guo, X. Geng, Neuroinflammation caused by mental stress: the effect of chronic restraint stress and acute repeated social defeat stress in mice, Neurol. Res. 41 (2019) 762–769, https://doi.org/10.1080/01616412.2019.1615670.

[5] E. O'Neill, É.W. Griffin, R. O'Sullivan, C. Murray, L. Ryan, J. Yssel, A. Harkin, C. Cunningham, Acute neuroinflammation, sickness behavior and working memory responses to acute systemic LPS challenge following noradrenergic lesion in mice, Brain. Behav. Immun. 94 (2021) 357–368, https://doi.org/10.1016/j.bbi.2020.12.002.

[6] J. Valero, G. Mastrella, I. Neiva, S. Sánchez, J.O. Malva, Long-term effects of an acute and systemic administration of LPS on adult neurogenesis and spatial memory, Front. Neurosci. 8 (2014) 83, https://doi.org/10.3389/fnins.2014.00083.

[7] S. Zonis, V.A. Ljubimov, M. Mahgerefteh, R.N. Pechnick, K. Wawrowsky, V. Chesnokova, p21Cip restrains hippocampal neurogenesis and protects neuronal progenitors from apoptosis during acute systemic inflammation, Hippocampus 23 (2013) 1383–1394, https://doi.org/10.1002/hipo.22192.

[8] S. Farioli-Vecchioli, F. Tirone, Control of the cell cycle in adult neurogenesis and its relation with physical exercise, Brain Plast. (Amst., Neth.) 1 (2015) 41–54, https://doi.org/10.3233/BPL-150013.

[9] G. Kempermann, Adult neurogenesis: an evolutionary perspective, Cold Spring Harb. Perspect. Biol. 8 (2015), 018986, https://doi.org/10.1101/cshperspect.a018986.

[10] T. Toda, F.H. Gage, Review: adult neurogenesis contributes to hippocampal plasticity, Cell Tissue Res 373 (2018) 693–709, https://doi.org/10.1007/s00441-017-2735-4.

[11] S. Farioli-Vecchioli, D. Saraulli, M. Costanzi, S. Pacioni, I. Cinà, M. Aceti, L. Micheli, A. Bacci, V. Cestari, F. Tirone, The timing of differentiation of adult hippocampal neurons is crucial for spatial memory, PLoS Biol. 6 (2008) 246, https://doi.org/10.1371/journal.pbio.0060246.

[12] G. Kempermann, The neurogenic reserve hypothesis: what is adult hippocampal neurogenesis good for? Trends Neurosci. 31 (2008) 163–169, https://doi.org/10.1016/j.tins.2008.01.002.

[13] J. Altman, G.D. Das, Autoradiographic and histologicalevidence of postnatal hippocampal neurogenesis in rats, J. Comp. Neurol. 124 (1965) 319–335. http://www.ncbi.nlm.nih.gov/pubmed/5861717.

[14] M.S. Kaplan, D.H. Bell, Neuronal proliferation in the 9-month-old rodent-radioautographic study of granule cells in the hippocampus, Exp. Brain Res. 52 (1983) 1–5, https://doi.org/10.1007/BF00237141.

[15] E. Gould, N. Vail, M. Wagers, C.G. Gross, Adult-generated hippocampal and neocortical neurons in macaques have a transient existence, Proc. Natl. Acad. Sci. U.S.A 98 (2001) 10910–10917, https://doi.org/10.1073/pnas.181354698.

[16] H.G. Kuhn, H. Dickinson-Anson, F.H. Gage, Neurogenesis in the dentate gyrus of the adult rat: age-related decrease of neuronal progenitor proliferation, J. Neurosci. 16 (1996) 2027–2033, https://doi.org/10.1523/JNEUROSCI.16-06-02027.1996.

[17] T. Seki, Y. Arai, Distribution and possible roles of the highly polysialylated neural cell adhesion molecule (NCAM-H) in the developing and adult central nervous system, Neurosci. Res. 17 (1993) 265–290, https://doi.org/10.1016/0168-0102(93)90111-3.

[18] D.R. Kornack, P. Rakic, Continuation of neurogenesis in the hippocampus of the adult macaque monkey, Proc. Natl. Acad. Sci. U. S. A 96 (1999) 5768–5773, https://doi.org/10.1073/pnas.96.10.5768.

[19] E.P. Moreno-Jiménez, M. Flor-García, J. Terreros-Roncal, A. Rábano, F. Cafini, N. Pallas-Bazarra, J. Ávila, M. Llorens-Martín, Adult hippocampal neurogenesis is abundant in neurologically healthy subjects and drops sharply in patients with Alzheimer's disease, Nat. Med. 25 (2019) 554–560, https://doi.org/10.1038/s41591-019-0375-9.

[20] M.K. Tobin, K. Musaraca, A. Disouky, A. Shetti, A. Bheri, W.G. Honer, N. Kim, R. J. Dawe, D.A. Bennett, K. Arfanakis, O. Lazarov, Human hippocampal neurogenesis persists in aged adults and Alzheimer's disease patients, Cell Stem Cell 24 (2019) 974–982.e3, https://doi.org/10.1016/j.stem.2019.05.003.

[21] C. Petrella, S. Farioli-Vecchioli, G.Y. Cisale, G. Strimpakos, J.J. Borg, M. Ceccanti, M. Fiore, G. Monteleone, R. Nisticò, A healthy gut for a healthy brain: preclinical, clinical and regulatory aspects, Curr. Neuropharmacol. (2020), https://doi.org/10.2174/1570159X18666200730111528.

[22] A. Sarkar, S.M. Lehto, S. Harty, T.G. Dinan, J.F. Cryan, P.W.J. Burnet, Psychobiotics and the manipulation of bacteria-gut-brain signals, Trends Neurosci. 39 (2016) 763–781, https://doi.org/10.1016/j.tins.2016.09.002.

[23] T.G. Dinan, C. Stanton, J.F. Cryan, Psychobiotics: a novel class of psychotropic, Biol. Psychiatry 74 (2013) 720–726, https://doi.org/10.1016/j.biopsych.2013.05.001.

[24] A. Tremblay, L. Lingrand, M. Maillard, B. Feuz, T.A. Tompkins, The effects of psychobiotics on the microbiota-gut-brain axis in early-life stress and neuropsychiatric disorders, Prog. Neuropsychopharmacol. Biol. Psychiatry 105 (2021), 110142, https://doi.org/10.1016/j.pnpbp.2020.110142.

[25] M. Messaoudi, R. Lalonde, N. Violle, H. Javelot, D. Desor, A. Nejdi, J.-F. Bisson, C. Rougeot, M. Pichelin, M. Cazaubiel, J.-M. Cazaubiel, Assessment of psychotropic-like properties of a probiotic formulation (Lactobacillus helveticus R0052 and bifidobacterium longum R0175) in rats and human subjects, Br. J. Nutr. 105 (2011) 755–764, https://doi.org/10.1017/S0007114510004319.

[26] K. Tillisch, J. Labus, L. Kilpatrick, Z. Jiang, J. Stains, B. Ebrat, D. Guyonnet, S. Legrain-Raspaud, B. Trotin, B. Naliboff, E.A. Mayer, Consumption of fermented milk product with probiotic modulates brain activity, Gastroenterology 144 (2013) 1394–1401, https://doi.org/10.1053/j.gastro.2013.02.043.
[27] H. Hardy, J. Harris, E. Lyon, J. Beal, A.D. Foey, Probiotics, prebiotics and immunomodulation of gut mucosal defences: homeostasis and immunopathology, Nutrients 5 (2013) 1869–1912, https://doi.org/10.3390/nu5061869.
[28] J. Vulevic, A. Juric, G. Tzortzis, G.R. Gibson, A mixture of trans-galactooligosaccharides reduces markers of metabolic syndrome and modulates the fecal microbiota and immune function of overweight adults. J. Nutr. 143 (2013) 324–331, https://doi.org/10.3945/jn.112.166132.
[29] E.S. Wohleb, D.B. McKim, J.F. Sheridan, J.P. Godbout, Monocyte trafficking to the brain with stress and inflammation: a novel axis of immune-to-brain communication that influences mood and behavior, Front. Neurosci. 8 (2014) 447, https://doi.org/10.3389/fnins.2014.00447.
[30] M.J. Stuart, G. Singhal, B.T. Baune, Systematic review of the neurobiological relevance of chemokines to psychiatric disorders, Front. Cell. Neurosci. 9 (2015) 357, https://doi.org/10.3389/fncel.2015.00357.
[31] S. Biesmans, T.F. Meert, J.A. Bouwknecht, P.D. Acton, N. Davoodi, P. De Haes, J. Kuijlaars, X. Langlois, L.J.R. Matthews, L. Ver Donck, N. Hellings, R. Nuydens, Systemic immune activation leads to neuroinflammation and sickness behavior in mice, Mediat. Inflamm. 2013 (2013), 271359, https://doi.org/10.1155/2013/271359.
[32] C. Cunningham, S. Campion, K. Lunnon, C.L. Murray, J.F.C. Woods, R.M.J. Deacon, J.N.P. Rawlins, V.H. Perry, Systemic inflammation induces acute behavioral and cognitive changes and accelerates neurodegenerative disease, Biol. Psychiatry 65 (2009) 304–312, https://doi.org/10.1016/j.biopsych.2008.07.024.
[33] C. D'Mello, N. Ronaghan, R. Zaheer, M. Dicay, T. Le, W.K. MacNaughton, M. G. Surrette, M.G. Swain, Probiotics improve inflammation-associated sickness behavior by altering communication between the peripheral immune system and the brain. J. Neurosci. 35 (2015) 10821–10830, https://doi.org/10.1523/JNEUROSCI.0575-15.2015.
[34] F.P. Douillard, D. Mora, R.T. Eijlander, M. Wels, W.M. de Vos, Comparative genomic analysis of the multispecies probiotic-marketed product VSL#3, PLoS One 13 (2018), 0192452, https://doi.org/10.1371/journal.pone.0192452.
[35] D. Mora, R. Filardi, S. Arioli, S. Boeren, S. Aalvink, W.M. de Vos, Development of omics-based protocols for the microbiological characterization of multi-strain formulations marketed as probiotics: the case of VSL#3, Microb. Biotechnol. 12 (2019) 1371–1386, https://doi.org/10.1111/1751-7915.13476.
[36] A. Ait-Belgnaoui, H. Durand, C. Cartier, G. Chaumaz, H. Eutamene, L. Ferrier, E. Houdeau, J. Fioramonti, L. Bueno, V. Theodorou, Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats, Psychoneuroendocrinology 37 (2012) 1885–1895, https://doi.org/10.1016/j.psyneuen.2012.03.024.
[37] W. Yu, D. Gao, Z. Wang, W. Jin, X. Peng, A. Zhao, C. Li, S. Qi, Probiotics alleviate cognitive dysfunction associated with neuroinflammation in cardiac surgery, Am. J. Transl. Res. 11 (2019) 7614–7626.
[38] J.E. Beilharz, N.O. Kaakoush, J. Maniam, M.J. Morris, Cafeteria diet and probiotic therapy: cross talk among memory, neuroplasticity, serotonin receptors and gut microbiota in the rat, Mol. Psychiatry 23 (2018) 351–361, https://doi.org/10.1038/mp.2017.38.
[39] J. Zheng, S. Wittouck, E. Salvetti, C.M.A.P. Franz, H.M.B. Harris, P. Mattarelli, P. W. O'Toole, B. Pot, P. Vandamme, J. Walter, K. Watanabe, S. Wuyts, G.E. Felis, M. G. Gänzle, S. Lebeer, A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae, Int. J. Syst. Evol. Microbiol. 70 (2020) 2782–2858, https://doi.org/10.1099/ijsem.0.004107.
[40] J.A. Bravo, P. Forsythe, M.V. Chew, E. Escaravage, H.M. Savignac, T.G. Dinan, J. Bienenstock, J.F. Cryan, Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve, Proc. Natl. Acad. Sci. U.S.A 108 (2011) 16050–16055, https://doi.org/10.1073/pnas.1102999108.
[41] H.M. Savignac, M. Tramullas, B. Kiely, T.G. Dinan, J.F. Cryan, Bifidobacteria modulate cognitive processes in an anxious mouse strain, Behav. Brain Res. 287 (2015) 59–72, https://doi.org/10.1016/j.bbr.2015.02.044.
[42] H.M. Savignac, Y. Couch, M. Stratford, D.M. Bannerman, G. Tzortzis, D.C. Anthony, P.W.J. Burnet, Prebiotic administration normalizes lipopolysaccharide (LPS)-induced anxiety and cortical 5-HT2A receptor and IL1-β levels in male mice, Brain Behav. Immun. 52 (2016) 120–131, https://doi.org/10.1016/j.bbi.2015.10.007.
[43] M.L. Woolley, K.A. Waters, J.E. Gartlon, L.P. Lacroix, C. Jennings, F. Shaughnessy, A. Ong, D.J. Pemberton, M.H. Harries, E. Southam, D.N. Jones, L.A. Dawson, Evaluation of the pro-cognitive effects of the AMPA receptor positive modulator, 5-(1-piperidinylcarbonyl)-2,1,3-benzoxadiazole (CX691), in the rat, Psychopharmacol 202 (2009) 343–354.
[44] K.E. Binley, W.S. Ng, J.R. Tribble, B. Song, J.E. Morgan, Sholl analysis: a quantitative comparison of semi-automated methods, J. Neurosci. Methods 225 (2014) 65–70, https://doi.org/10.1016/j.jneumeth.2014.01.017.
[45] A. Farzi, F. Reichmann, A. Meinitzer, R. Mayerhofer, P. Jain, A.M. Hassan, E. E. Fröhlich, K. Wagner, E. Painsipp, B. Rinner, P. Holzer, Synergistic effects of NOD1 or NOD2 and TLR4 activation on mouse sickness behavior in relation to immune and brain activity markers, Brain Behav. Immun. 44 (2015) 106–120, https://doi.org/10.1016/j.bbi.2014.08.011.
[46] R. Abulkassim, R. Brett, S.M. MacKenzie, T.J. Bushell, Proteinase-activated receptor 2 is involved in the behavioural changes associated with sickness behaviour, J. Neuroimmunol. 295–296 (2016) 139–147, https://doi.org/10.1016/j.jneuroim.2016.04.016.
[47] R.P. Toyama, J.C. Xikota, M.L. Schwarzbold, T.S. Frode, Z. da, S. Buss, J.C. Nunes, G.D.G. Funchal, F.C. Nunes, R. Walz, M.M. de, S. Pires, Dose-dependent sickness behavior, abortion and inflammation induced by systemic LPS injection in pregnant mice, J. Matern. Neonatal Med. 28 (2015) 426–430, https://doi.org/10.3109/14767058.2014.918600.
[48] I.C.M. Hoogland, C. Houbolt, D.J. van Westerloo, W.A. van Gool, D. van de Beek, Systemic inflammation and microglial activation: systematic review of animal experiments, J. Neuroinflamm. 12 (2015) 114, https://doi.org/10.1186/s12974-015-0332-6.
[49] S. Nair, K.S. Sobotka, P. Joshi, P. Gressens, B. Fleiss, C. Thornton, C. Mallard, H. Hagberg, Lipopolysaccharide-induced alteration of mitochondrial morphology induces a metabolic shift in microglia modulating the inflammatory response in vitro and in vivo, Glia 67 (2019) 1047–1061, https://doi.org/10.1002/glia.23587.
[50] D.H. Allendorf, E.H. Franssen, G.C. Brown, Lipopolysaccharide activates microglia via neuraminidase 1 desialylation of toll-like receptor 4, J. Neurochem. 155 (2020) 403–416, https://doi.org/10.1111/jnc.15024.
[51] A. Ait-Belgnaoui, A. Colom, V. Braniste, L. Ramalho, A. Marrot, C. Cartier, E. Houdeau, V. Theodorou, T. Tompkins, Probiotic gut effect prevents the chronic psychological stress-induced brain activity abnormality in mice, Neurogastroenterol. Motil. J. Eur. Gastrointest. Motil. Soc. 26 (2014) 510–520, https://doi.org/10.1111/nmo.12295.
[52] L. Möhle, D. Mattei, M.M. Heimesaat, S. Bereswill, A. Fischer, M. Alutis, T. French, D. Hambardzumyan, P. Matzinger, I.R. Dunay, S.A. Wolf, Ly6C(hi) monocytes provide a link between antibiotic-induced changes in gut microbiota and adult hippocampal neurogenesis, Cell Rep. 15 (2016) 1945–1956, https://doi.org/10.1016/j.celrep.2016.04.074.
[53] A. Sierra, J.M. Encinas, J.J.P. Deudero, J.H. Chancey, G. Enikolopov, L. S. Overstreet-Wadiche, S.E. Tsirka, M. Maletic-Savatic, Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis, Cell Stem Cell 7 (2010) 483–495, https://doi.org/10.1016/j.stem.2010.08.014.
[54] M.S. Tremblay, R.C. Colley, T.J. Saunders, G.N. Healy, N. Owen, Physiological and health implications of a sedentary lifestyle, Appl. Physiol. Nutr. Metab. Physiol. 35 (2010) 725–740, https://doi.org/10.1139/H10-079.
[55] Y. Ziv, N. Ron, O. Butovsky, G. Landa, E. Sudai, N. Greenberg, H. Cohen, J. Kipnis, M. Schwartz, Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood, Nat. Neurosci. 9 (2006) 268–275, https://doi.org/10.1038/nn1629.
[56] O. Butovsky, G. Landa, G. Kunis, Y. Ziv, H. Avidan, N. Greenberg, A. Schwartz, I. Smirnov, A. Pollack, S. Jung, M. Schwartz, Induction and blockage of oligodendrogenesis by differently activated microglia in an animal model of multiple sclerosis, J. Clin. Invest. 116 (2006) 905–915, https://doi.org/10.1172/JCI26836.
[57] A.D. Bachstetter, J.M. Morganti, J. Jernberg, A. Schlunk, S.H. Mitchell, K. W. Brewster, C.E. Hudson, M.J. Cole, J.K. Harrison, P.C. Bickford, C. Gemma, Fractalkine and CX 3 CR1 regulate hippocampal neurogenesis in adult and aged rats, Neurobiol. Aging 32 (2011) 2030–2044, https://doi.org/10.1016/j.neurobiolaging.2009.11.022.
[58] Y. Wolf, S. Yona, K.-W. Kim, S. Jung, Microglia, seen from the CX3CR1 angle, Front. Cell. Neurosci. 7 (2013) 26, https://doi.org/10.3389/fncel.2013.00026.
[59] T. Kreisel, B. Wolf, E. Keshet, T. Licht, Unique role for dentate gyrus microglia in neuroblast survival and in VEGF-induced activation, Glia 67 (2019) 594–618, https://doi.org/10.1002/glia.23505.
[60] K. Leung, S. Thuret, Gut microbiota: a modulator of brain plasticity and cognitive function in ageing, Healthcare 3 (2015) 898–916, https://doi.org/10.3390/healthcare3040898.
[61] H.M. Savignac, G. Corona, H. Mills, L. Chen, J.P.E. Spencer, G. Tzortzis, P.W. J. Burnet, Prebiotic feeding elevates central brain derived neurotrophic factor, N-methyl-D-aspartate receptor subunits and D-serine, Neurochem. Int. 63 (2013) 756–764, https://doi.org/10.1016/j.neuint.2013.10.006.
[62] R.M. Barrientos, M.M. Kitt, L.R. Watkins, S.F. Maier, Neuroinflammation in the normal aging hippocampus, Neuroscience 309 (2015) 84–99, https://doi.org/10.1016/j.neuroscience.2015.03.007.
[63] H.F. Green, Y.M. Nolan, Inflammation and the developing brain: consequences for hippocampal neurogenesis and behavior, Neurosci. Biobehav. Rev. 40 (2014) 20–34, https://doi.org/10.1016/j.neubiorev.2014.01.004.
[64] R. Yirmiya, I. Goshen, Immune modulation of learning, memory, neural plasticity and neurogenesis, Brain Behav. Immun. 25 (2011) 181–213, https://doi.org/10.1016/j.bbi.2010.10.015.
[65] E. Molina-Holgado, F. Molina-Holgado, Mending the broken brain: neuroimmune interactions in neurogenesis, J. Neurochem 114 (2010) 1277–1290, https://doi.org/10.1111/j.1471-4159.2010.06849.x.
[66] H. Fujioka, T. Akema, Lipopolysaccharide acutely inhibits proliferation of neural precursor cells in the dentate gyrus in adult rats, Brain Res. 1352 (2010) 35–42, https://doi.org/10.1016/j.brainres.2010.07.032.
[67] C.T. Ekdahl, J.-H. Claasen, S. Bonde, Z. Kokaia, O. Lindvall, Inflammation is detrimental for neurogenesis in adult brain, Proc. Natl. Acad. Sci. U.S.A. 100 (2003) 13632–13637, https://doi.org/10.1073/pnas.2234031100.
[68] K. Belarbi, T. Jopson, C. Arellano, J.R. Fike, S. Rosi, CCR2 deficiency prevents neuronal dysfunction and cognitive impairments induced by cranial irradiation, Cancer Res 73 (2013) 1201–1210, https://doi.org/10.1158/0008-5472.CAN-12-2989.
[69] R. Dantzer, J.C. O'Connor, G.G. Freund, R.W. Johnson, K.W. Kelley, From inflammation to sickness and depression: when the immune system subjugates the brain, Nat. Rev. Neurosci. 9 (2008) 46–56, https://doi.org/10.1038/nrn2297.
[70] M. Neuman, P. Angulo, I. Malkiewicz, R. Jorgensen, N. Shear, E.R. Dickson, J. Haber, G. Katz, K. Lindor, Tumor necrosis factor-alpha and transforming growth

factor-beta reflect severity of liver damage in primary biliary cirrhosis, J. Gastroenterol. Hepatol. 17 (2002) 196–202, https://doi.org/10.1046/j.1440-1746.2002.02672.x.
[71] R.M. Bluthé, V. Walter, P. Parnet, S. Layé, J. Lestage, D. Verrier, S. Poole, B. E. Stenning, K.W. Kelley, R. Dantzer, Lipopolysaccharide induces sickness behaviour in rats by a vagal mediated mechanism, C. R. Acad. Sci. Iii. 317 (1994) 499–503.
[72] K. Newton, V.M. Dixit, Signaling in innate immunity and inflammation, Cold Spring Harb. Perspect. Biol. 4 (2012), https://doi.org/10.1101/cshperspect.a006049.
[73] S. Layé, P. Parnet, E. Goujon, R. Dantzer, Peripheral administration of lipopolysaccharide induces the expression of cytokine transcripts in the brain and pituitary of mice, Brain Res. Mol. Brain Res. 27 (1994) 157–162, https://doi.org/10.1016/0169-328x(94)90197-x.
[74] N. Quan, E.L. Stern, M.B. Whiteside, M. Herkenham, Induction of pro-inflammatory cytokine mRNAs in the brain after peripheral injection of subseptic doses of lipopolysaccharide in the rat. J. Neuroimmunol. 93 (1999) 72–80, https://doi.org/10.1016/s0165-5728(98)00193-3.
[75] A.M. van Dam, M. Brouns, S. Louisse, F. Berkenbosch, Appearance of interleukin-1 in macrophages and in ramified microglia in the brain of endotoxin-treated rats: a pathway for the induction of non-specific symptoms of sickness? Brain Res. 588 (1992) 291–296, https://doi.org/10.1016/0006-8993(92)91588-6.
[76] L. Vallières, I.L. Campbell, F.H. Gage, P.E. Sawchenko, Reduced hippocampal neurogenesis in adult transgenic mice with chronic astrocytic production of interleukin-6, J. Neurosci. 22 (2002) 486–492, https://doi.org/10.1523/JNEUROSCI.22-02-00486.2002.
[77] S. Johansson, J. Price, M. Modo, Effect of inflammatory cytokines on major histocompatibility complex expression and differentiation of human neural stem/progenitor cells, Stem Cells 26 (2008) 2444–2454, https://doi.org/10.1634/stemcells.2008-0116.
[78] C. D'Mello, K. Riazi, T. Le, K.M. Stevens, A. Wang, D.M. McKay, Q.J. Pittman, M. G. Swain, P-selectin-mediated monocyte-cerebral endothelium adhesive interactions link peripheral organ inflammation to sickness behaviors, J. Neurosci. 33 (2013) 14878–14888, https://doi.org/10.1523/JNEUROSCI.1329-13.2013.
[79] C. D'Mello, T. Le, M.G. Swain, Cerebral microglia recruit monocytes into the brain in response to tumor necrosis factoralpha signaling during peripheral organ inflammation, J. Neurosci. 29 (2009) 2089–2102, https://doi.org/10.1523/JNEUROSCI.3567-08.2009.
[80] A. Sierra, M.-È. Tremblay, H. Wake, Never-resting microglia: physiological roles in the healthy brain and pathological implications, Front. Cell. Neurosci. 8 (2014) 240, https://doi.org/10.3389/fncel.2014.00240.
[81] M. Perez-Dominguez, E. Ávila-Muñoz, E. Domínguez-Rivas, A. Zepeda, The detrimental effects of lipopolysaccharide-induced neuroinflammation on adult hippocampal neurogenesis depend on the duration of the pro-inflammatory response, Neural Regen. Res. 14 (2019) 817–825, https://doi.org/10.4103/1673-5374.249229.
[82] J. Knight, C. Hackett, J. Breton, Y. Mao-Draayer, Cross-talk between CD4+ T-cells and neural stem/progenitor cells, J. Neurol. Sci. 306 (2011) 121–128, https://doi.org/10.1016/j.jns.2011.03.030.
[83] A. Sierra, O. Abiega, A. Shahraz, H. Neumann, Janus-faced microglia: beneficial and detrimental consequences of microglial phagocytosis, Front. Cell. Neurosci. 7 (2013) 6, https://doi.org/10.3389/fncel.2013.00006.
[84] R.K. Dhiman, B. Rana, S. Agrawal, A. Garg, M. Chopra, K.K. Thumburu, A. Khattri, S. Malhotra, A. Duseja, Y.K. Chawla, Probiotic VSL#3 reduces liver disease severity and hospitalization in patients with cirrhosis: a randomized, controlled trial, Gastroenterology 147 (2014) 1327–1337.e3, https://doi.org/10.1053/j.gastro.2014.08.031.
[85] C. Loguercio, A. Federico, C. Tuccillo, F. Terracciano, M.V. D'Auria, C. De Simone, C. Del Vecchio Blanco, Beneficial effects of a probiotic VSL#3 on parameters of liver dysfunction in chronic liver diseases, J. Clin. Gastroenterol. 39 (2005) 540–543, https://doi.org/10.1097/01.mcg.0000165671.25272.0f.
[86] E. Vaghef-Mehrabany, B. Alipour, A. Homayouni-Rad, S.-K. Sharif, M. Asghari-Jafarabadi, S. Zavvari, Probiotic supplementation improves inflammatory status in patients with rheumatoid arthritis, Nutrition 30 (2014) 430–435, https://doi.org/10.1016/j.nut.2013.09.007.
[87] D. Serra, L.M. Almeida, T.C.P. Dinis, The impact of chronic intestinal inflammation on brain disorders: the microbiota-gut-brain axis, Mol. Neurobiol. 56 (2019) 6941–6951, https://doi.org/10.1007/s12035-019-1572-8.
[88] N. Powell, M.M. Walker, N.J. Talley, The mucosal immune system: master regulator of bidirectional gut-brain communications, Nat. Rev. Gastroenterol. Hepatol. 14 (2017) 143–159, https://doi.org/10.1038/nrgastro.2016.191.
[89] M. Sochocka, B.S. Diniz, J. Leszek, Inflammatory response in the CNS: friend or foe? Mol. Neurobiol. 54 (2017) 8071–8089, https://doi.org/10.1007/s12035-016-0297-1.
[90] M.A. Garcia, W.J. Nelson, Cell-cell junctions organize structural and signaling networks, Cold Spring Harb. Perspect. Biol. 10 (2018), a029181, https://doi.org/10.1101/cshperspect.a029181.
[91] D. Yang, D. Zhao, S.Z. Ali Shah, W. Wu, M. Lai, X. Zhang, J. Li, Z. Guan, H. Zhao, W. Li, H. Gao, X. Zhou, L. Yang, The role of the gut microbiota in the pathogenesis of parkinson's disease, Front. Neurol. 10 (2019) 1155, https://doi.org/10.3389/fneur.2019.01155.
[92] M.C. Houser, M.G. Tansey, The gut-brain axis: is intestinal inflammation a silent driver of Parkinson's disease pathogenesis? NPJ Park. Dis. 3 (2017) 3, https://doi.org/10.1038/s41531-016-0002-0.
[93] L.P. Kelly, P.M. Carvey, A. Keshavarzian, K.M. Shannon, M. Shaikh, R.A.E. Bakay, J.H. Kordower, Progression of intestinal permeability changes and alpha-synuclein expression in a mouse model of Parkinson's disease, Mov. Disord. 29 (2014) 999–1009, https://doi.org/10.1002/mds.25736.