Inhaled molecular hydrogen reduces hippocampal neuroinflammation, glial reactivity and ameliorates memory impairment during systemic inflammation

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A B S T R A C T

Sepsis is associated with numerous physiological and biochemical abnormalities that result in a life-threatening condition. The involvement of the Central Nervous System (CNS) during sepsis has received considerable attention, especially the hippocampus which plays a key role in the learning and memory processes. The increased interest in this limbic region during systemic inflammation (SI) is related to the number of sepsis survivor patients who have cognitive impairments. A single injection of lipopolysaccharide (LPS)-induced systemic inflammation is the most commonly used murine endotoxemia model because it replicates several pathophysiological changes observed in severe sepsis. Molecular hydrogen (H_2) has been used as an anti-inflammatory therapeutic strategy to prevent neuroinflammation. However, the mechanisms by which inhaled H_2 mitigate memory loss during SI remains unknown. To understand how H_2 acts in the hippocampus, the current study focused on specific mechanisms that may be involved in reducing neuroinflammation in rats during SI. We hypothesized that inhaled H_2 decreases LPS-induced hippocampal pro-inflammatory cytokines surges and this effect is associated with reduced memory loss. Using different and integrative approaches, *i.e.*, from hippocampal cells electrophysiology to animal behavior, we report that inhaled H_2 decreased LPS-induced peripheral and hippocampal inflammation, decreased microglial and astrocytic activation, lessen memory loss without affecting long-term potentiation (LTP). To our knowledge, this is the first evidence showing that inhaled H_2 reduces hippocampal microglial and glial cells inflammation, which may be associated with a reduced memory impairment induced by SI.

1. Introduction

Lipopolysaccharide (LPS)-induced systemic inflammation (SI) mimics the inflammatory component of sepsis and can trigger neuro-inflammation in different regions of the brain involved with learning and memory (Annane and Sharshar, 2015). These remarkable cognitive impairments are also associated with significant changes in the control of cardiovascular system and the mechanisms involved with thermoregulation (Amorim et al., 2019; Saramago et al., 2019), which ultimately leads to multiple organs dysfunctions (Fernandez et al., 2014; Parrillo et al., 1990; Singer et al., 2016). In humans, despite the high mortality rates caused by sepsis (5.3 million annually - Fleischmann et al., 2016), there is an increment in the number of sepsis survivor patients but with an impairment of cognitive functions, which ultimately has a negative impact on the quality of life and an eventual delay in resuming normal daily activities (Iwashyna et al., 2010).

One acceptable explanation for cognitive decline after sepsis is the neurodegenerative hypothesis, in which the activation of microglia and axonopathy bring persistent damage to the blood-brain barrier causing a chronic decline in the neurotransmission, particularly in the hippocampus (Annane and Sharshar, 2015). Similarly, in the hippocampus of animals that received LPS, some changes can be observed such as increased pro-inflammatory cytokines surges (Donzis and Tronson, 2014), microglial activation (Anderson et al., 2015; Zhang et al., 2017), worst performance in memory tests (Morris Water Maze- MWM and Object Recognition- OR) (Da Ré et al., 2020; Shaw et al., 2001) and changes in the synaptic plasticity (Abareshi et al., 2016). In this scenario, microglia and astrocytes are activated by SI producing proinflammatory cytokines surges, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 that can modulate long-term potentiation (LTP) and long-term depression (LTD) (Donzis and Tronson, 2014), which are related with memory and learning process.

Worldwide there is an effort from researchers to find therapeutic strategies to treat the complex neuroinflammatory consequences of SI. The role of molecular hydrogen (H_2) was first documented in 1975 after regression of skin tumors in mice that received H_2 (Dole et al., 1975), and also by several reports that have shown therapeutic effects of H_2 in a broad range of diseases (For review see Ichihara et al., 2015). Furthermore, its anti-inflammatory effects during LPS-induced SI have been demonstrated (for review see Ohta, 2014). Our group has reported that inhaled H_2 causes a reduction in plasma pro-inflammatory cytokine surges during LPS-induced SI (Saramago et al., 2019), reduces acute exercise-induced hippocampal inflammation (Nogueira et al., 2018) and prevents memory loss in polymicrobial sepsis (Jesus et al., 2020). Previously, Spulber et al. (2012) proposed that chronic consumption of water enriched with H_2 before LPS administration improves neuroinflammation in the hippocampus and sickness behavior.

Consistent with the idea that: a) the hippocampus plays a pivotal role in cognitive functions (learning and memory), b) the impact of LPS-induced SI in limbic areas and c) the anti-inflammatory properties of H_2, in the present study we tested the hypothesis that this gas could protect the hippocampus against inflammation during LPS-induced SI. For this, we investigated microglial and astrocytic activation, measured hippocampal and peripheral cytokines, and tested memory and synaptic plasticity, in rats receiving LPS combined or not with H_2 inhalation. Our results provide evidence that the inhaled H_2 decreased neuroinflammation, decreased microglial or astrocytic reactivity in the hippocampus, and improved short and long-term memory without recovering the LPS-induced inhibition of LTP after the LPS challenge.

2. Methods

The experimental protocols used in this study were carried out following the rules of research in the National Council for Control of Animal Experimentation and approved by The Ethics Committee on Animal Research of the Dental School of Ribeirao Preto - University of Sao Paulo, Ribeirao Preto, Brazil, (protocol # 068/2019).

2.1. Animals

The experiments were performed on 60-70 days-old male Wistar rats obtained from the Central Animal Facility of the Ribeirao Preto campus of the University of Sao Paulo, Brazil. Animals were kept in Plexiglas cages (3–4 per cage) in the animal facility of the Dental School under daily 12 h light/12 h dark cycle (06:00–18:00), controlled room temperature (23–24 °C) and access to water and chow *ad libitum*. After venous catheterization, the animals were placed in a sealed plexiglas chamber and divided into four groups: 1) Saline + Air (Sal + Air; n = 25), that received intravenous (i.v.) saline and were exposed to room air in the chamber at the flow rate of 2 L/min; 2) Saline + H_2 (Sal + H_2; n = 23) that received saline i.v. and gas mixture of H_2 (2%), O_2 (21%), and N_2 (77%) through the same chamber supplied through a gas flowmeter

(Cole-Parmer Instrument Company, IL, USA); 3) LPS + Air (n = 23) group that received i.v. -Escherichia coli LPS [(LPS, 1.5 mg/kg^{-1}, 0111: B4 dissolved in pyrogenic-free saline- Sigma Aldrich (Amorim et al., 2020)] and 4) LPS + H_2 group (n = 22).

2.2. Surgery: venous catheterization

Initially, rats were deeply anesthetized with an intraperitoneal injection of a mixture of ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}) and after the absence of withdrawal reflex to paw and tail pinching, they were implanted with polyethylene catheters (PE-10 connected to PE-50 tubing; Clay Adams, Parsippany, NJ, USA, Intramedic, Becton Dickinson, Sparks, MD, USA), into the vein for saline or LPS administration. This surgical procedure was carried out in aseptic conditions and additional doses of analgesic were given if any sign of pain was observed. Animals were kept in individual cages and allowed to recover for 48 h at 24 °C before all experimental procedures. After this period, all experiments were done 3 h following saline or LPS (at the dose of LPS, 1.5 mg/kg^{-1}) administration. During this time period, rats inhaled H_2 (2% H_2, 21% O_2, N_2 balance) or room air as the control group that was exposed to a normoxic gas mixture (21% O_2, N_2 balance).

2.3. Plasma cytokines measurements

Three hours after LPS or saline administration, rats that inhaled H_2 or room air were killed by decapitation using a guillotine, trunk blood samples were collected in EDTA-coated tubes and centrifuged (20 min at 3.500 rpm, 4 °C), for plasma extraction and were kept at −80 °C until being assayed. Plasma samples were assayed for measurement of the pro-inflammatory cytokines within detection limits, tumor necrosis factor (TNF)-α (232–56,400 pg/mL), interleukin (IL)-6 (170–41,300 pg/ml), interleukin 1β (18.5–4500 pg/mL) and interferon (IFN)-γ (509–123,600 pg/mL) using multiplex assay kits according to standard instructions (LXSARM - 05, R&D System, Minnesota, USA) with Luminex Magpix Technology (Austin, TX, USA).

2.4. Proinflammatory cytokines measurement in the CA1 from dorsal hippocampus

Wistar rats were decapitated and the brains were carefully collected, frozen in dry-ice cold isopentane, and stored at −80 °C. Dorsal hippocampus was sampled using a cryostat by a punch needle (1.5 mm inner diameter) from a 1200-μm thick slice and stored in plastic tubes at −80 °C until being assayed (de Deus et al., 2021). Samples were lysate in 220 μL of PBS supplemented with protease inhibitor cocktail (Cell Signaling, Massachusetts, USA) and then centrifuged at 13.000 rpm for 20 min at 4 °C. Tissue supernatant samples were used to measure TNF-α, IL-1β, IL-6, and IFN-γ by a Multiplex Assay (LXSARM - 05, R&D System, Minnesota, USA) with Luminex® Magpix™ technology (Austin, TX, USA). Results from interleukins in CA1 homogenates were normalized by protein concentrations, which were assessed by the Bio-Rad protein assay based on the Bradford assay (#5000205, Bio-Rad Laboratories, USA).

2.5. Immunofluorescence and imaging of the hippocampal CA1 region

To study LPS-induced glial (microglial or astrocytic) reactivity, we used antibodies against ionized calcium binding adaptor protein (Iba1) to label microglia, and anti-Glial Fibrillary Acidic Protein (GFAP) to label astrocytes. Animals were anesthetized and perfused transcardially with PBS (0.01M Phosphate-buffered saline, pH 7.4, 250 ml), followed by 4% paraformaldehyde (pH 7.4, 350 ml), at room temperature. The brains were removed immediately after perfusion and post-fixed in 4% paraformaldehyde for 60 min, washed in PBS, and placed in 30% sucrose for 48−72 h at 4 °C. Afterward, brains were frozen at −80 °C in O.C.T. Compound (Sakura Finetek, Torrance, CA). Serial transverse sections

(30 μm) through the dorsal hippocampus were cut according to the rat brain atlas (Paxinos and Watson, 2017) on a cryostat at $-20\,°C$ and were placed on histological laminae and stored at $-20\,°C$ until immunofluorescence procedures. Sections were washed (three times) in PBS, permeabilized with Triton X-100 0.3% v/v (20 min), and placed into 0.1 M glycine (15 min). After washing in PBS, the sections were incubated in block buffer (BSA 3% w/v and 0.05% Triton X-100 v/v) for 60 min at room temperature. Primary antibodies were applied overnight at 4 °C. A rabbit polyclonal antibody for Iba1 was used to label microglia (1:500, Cat. 019–19741, Lot. WEE4506, Wako Pure Chemical Industries, Richmond, VA). A rabbit polyclonal antibody for GFAP was used to label astrocytes (1:500, Cat. 123895, Lot. 5, Cell Signaling Technology, Danvers, MA). On the following day, tissue sections were washed and then visualized with the appropriate secondary fluorescent antibody, goat anti-rabbit IgG Alexa Fluor®-546 (1:400, Cat. A11035, Lot. 1387724, Life Technologies, Eugene, OR) 60 min at room temperature. Finally, cell nuclei were stained with DAPI (4',6-dia-midino-2-phenylindole dihydrochloride hydrate: 300 nM, Cat. D1306, Sigma-Aldrich, St Louis, MO), tissue sections were washed and mounted with Fluoromount G (Cat. 17984-25, Electron Microscopy Sciences, Hatfield, PA). The specificity of each assay was tested by omitting the primary antibody. Images of hippocampal CA1 were acquired in a confocal laser scanning microscope (LSM710, Carl Zeiss) and analyzed with the software ImageJ with Fiji (Schindelin et al., 2012; Schneider et al., 2012). Iba1 or GFAP staining intensity, the area occupied by positive cells (μm^2), and the relative area (% of total area) were examined in a standardized area ($0.1\ mm^2$) of CA1 with 2 images in each slice, 4–6 slices were examined per animal.

2.6. Behavioral assessment: novel object recognition (NOR)

Rats did the NOR test in a wooden box ($50 \times 50 \times 90$ cm), where sessions were filmed by a webcam on the top of the box. The NOR test consisted of 3 phases (two days), in which the animals were allowed to explore the box or objects. During the habituation phase, the rats were allowed to explore the box for 20 min in the absence of objects and stimulus; on training, two equal objects (colored squares: A-A') were present on the opposite side of the box for 5 min (first phase); the second phase occurred 20 min after (short-term memory test), where two objects were presented on the opposite side of the box for 5 min, except that one of the squares has replaced one colored square by a blue triangle (colored square- A and blue triangle- B). The last phase occurred 24 h after the short-term memory test and object B was exchanged for another object (C- white cylinder) where two objects were present such as the first phase to investigate long-term memory. After each session, the box and objects were cleaned up with alcohol 70%. We consider the time exploring the new object as a recognition memory index. For this, we used as a criterion of exploration, animals sniffing or touching objects with their nose and paws. The time (T) spent exploring each object (A or B) was recorded and the recognition index (RI) was measured as the percentage of the time spent at the novel object (TN) divided by the time exploring both objects (TE) as defined by RI = TN/TE*100 (Diano et al., 2006).

2.7. Preparation of hippocampal slices

Rats were decapitated under isoflurane-induced anesthesia. Then, brains were rapidly removed and placed in an ice-cold solution containing (mM): 87 NaCl, 2.5 KCl, 25 $NaHCO_3$, 1.25 NaH_2PO_4, 75 Sucrose, 25 Glucose, 0.2 $CaCl_2$, 7 $MgCl_2$, bubbled with 95% O_2 and 5% CO_2. The brain was glued with cyanoacrylate glue to a support, placed inside the cutting chamber of a vibratome, and the dorsal hippocampus was cut in 400 μm transversal slices in the same solution. Hippocampal sections were dissected out from the slices using ophthalmic scissors and microtweezers and placed in artificial cerebrospinal fluid (aCSF) solution containing (mM): 125 NaCl, 2.8 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10
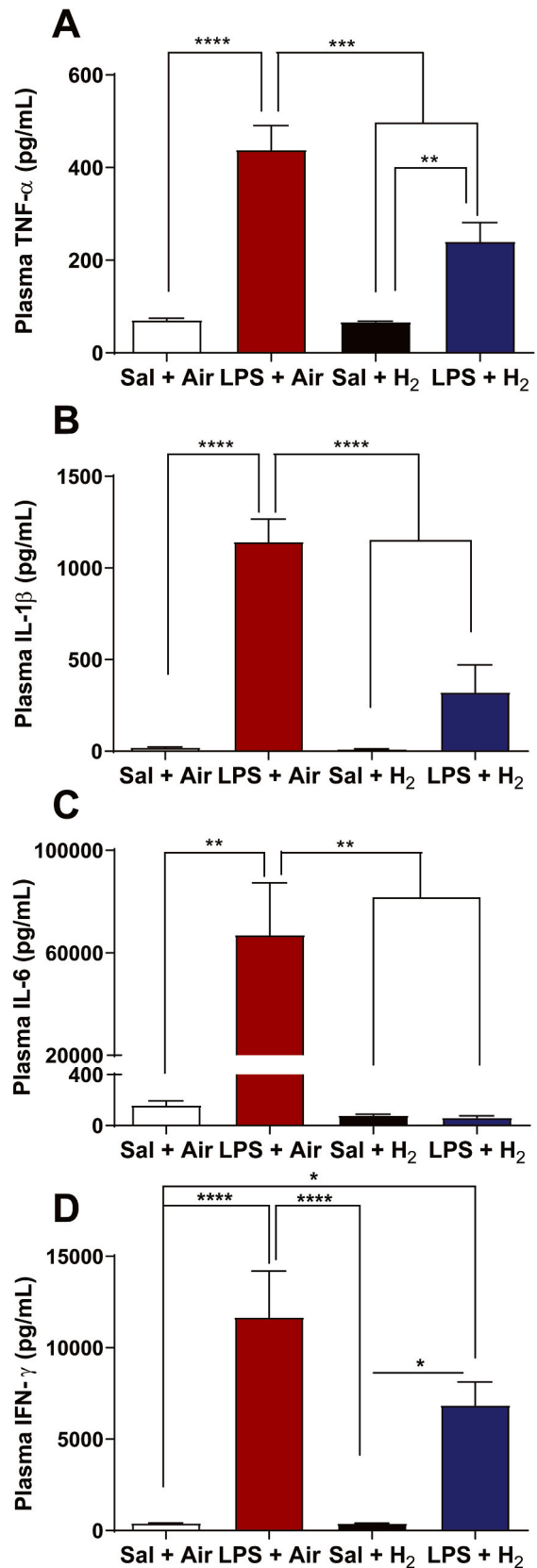

Fig. 1. Plasma levels measurements of proinflammatory cytokines collected after 3 h: TNF-α (A); IL-1β (B); IL-6 (C) and IFN-γ (D) of rats injected with saline or lipopolysaccharide (LPS, 1.5 mg/kg^{-1}) that inhaled H_2 or room air. Data are shown as mean ± SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 (ANOVA and Tukey post-test). N = 8–11 animals per group.
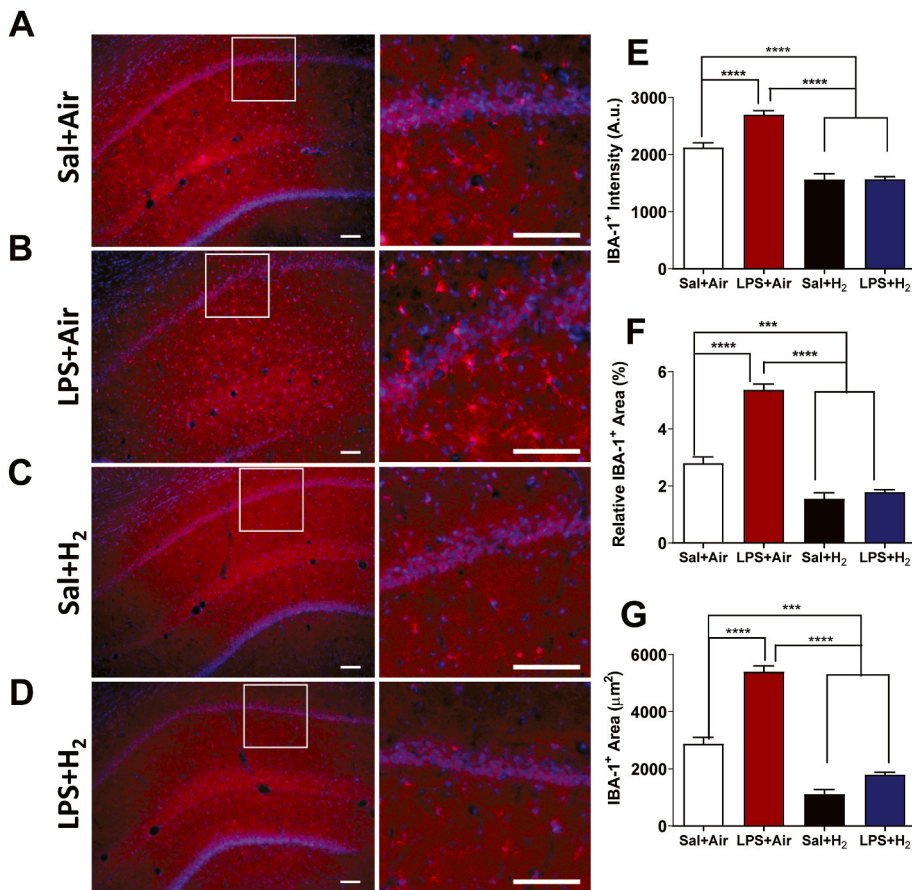
Fig. 2. Photomicrographs showing Iba-1$^+$ microglia marker immunostaining (red) and cell nuclei stained with DAPI (blue) in sections of CA1 from the dorsal hippocampus. Left panel shows representative images from Sal + Air (A), LPS + Air (B), Sal + H$_2$ (C) and LPS + H$_2$ (D) groups. Right panel shows intensity (E), relative area (%-F) and area (μm^2-G) of Iba-1 positive cells. Data are shown as mean ± SEM. ***P $\leq$ 0.001, ****P $\leq$ 0.0001 (ANOVA and Tukey post-test). N = 4 animals per group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Glucose, 2 CaCl$_2$, 1 MgCl$_2$. Slices were left to rest for at least 2 h before use (1 h at 34–35 °C and at least 1 h at room temperature) and continuously bubbled with a carbogenic mixture (95% O2 and 5% CO2) until recordings.

2.8. LTP induction

Field potential recordings were done with a Multiclamp 700B amplifier (Molecular Devices, USA) connected to a Digidata 1440 A AC/DC interface (Molecular Devices, USA). Hippocampal slices were placed in a recording chamber with continuous superfusion of aCSF (1 mL/min) bubbled with carbogenic mixture, and kept in place with a nylon thread in a platinum frame. A stainless steel bipolar concentric microelectrode (FHC - Bowdoin, Maine, USA), connected to a Master-9 voltage stimulator (A.M.P.I., Israel), was placed on the Schaffer-collaterals. Field excitatory postsynaptic potentials (fEPSPs) were recorded at CA1 *stratum radiatum* with borosilicate glass microelectrodes (G85150T, Warner Instruments, USA) filled with aCSF, with tip resistances of 1–2 MΩ and connected to the amplifier probe through a silver wire covered with AgCl. Initially, we constructed input-output curves, where voltage was gradually increased by 10 V increments, until population spikes were observed in the fEPSP. For baseline response, we stimulated the Schaffer fibers (set at 50% of the maximum response) at 0.03 Hz for 25 min. After a stable baseline, LTP was induced on the Schaffer-collateral fibers with 3 trains of high frequency stimulation (HFS) at 100 Hz of 1 s duration (3 s of inter-train interval). HFS trains induced a short post-tetanic potentiation (PTP) followed by LTP of the fEPSPs that lasted at least 80 min. For analysis of LTP, the last 10 min of the fEPSPs. Signals were acquired at 100 kHz and low pass filtered at 3 kHz (Bessel, 8-pole). All data were acquired with pClamp 10.2 software (Molecular Devices, USA).

2.9. Statistical analyses

Data were analyzed by descriptive statistics and are presented as means ± SEM. The comparison between variables was done using one-way ANOVA and Tukey's post-test, and the paired and unpaired T-test with a significance level of 5% (P $\leq$ 0.05). Data analysis was done with Prism version 6.0 (GraphPad Software, USA) and Origin Pro 8.0 (Origin Lab Corporation, USA).

3. Results

3.1. Inhaled H$_2$ reduces plasma pro-inflammatory cytokines surges during SI

Previous results from our group showed that inhaled H$_2$ can reduce plasma pro-inflammatory cytokines surges in rats during LPS-induced SI (Saramago et al., 2019). We observed no changes in plasma pro-inflammatory cytokines surges in animals that received Sal + H$_2$ in comparison with animals of the group Sal + Air (P $\geq$ 0.05, Fig. 1A–D), whereas, during the LPS-induced SI, we observed that the plasma pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, and IFN-γ) surges (Fig. 1A–D; LPS + Air) were significantly reduced in animals that inhaled H$_2$ (Fig. 1A–D; LPS + H$_2$; P $\leq$ 0.05).

3.2. Inhaled H$_2$ decreases microglial or astrocytic reactivity in the hippocampus

Afterward, we examined whether or not LPS-induced SI causes neuroinflammation in the hippocampus, and if the H$_2$ treatment can prevent this process. To do this, we analyzed the microglial and astrocytic immunostaining (Figs. 2 and 3). Our results show that during LPS-
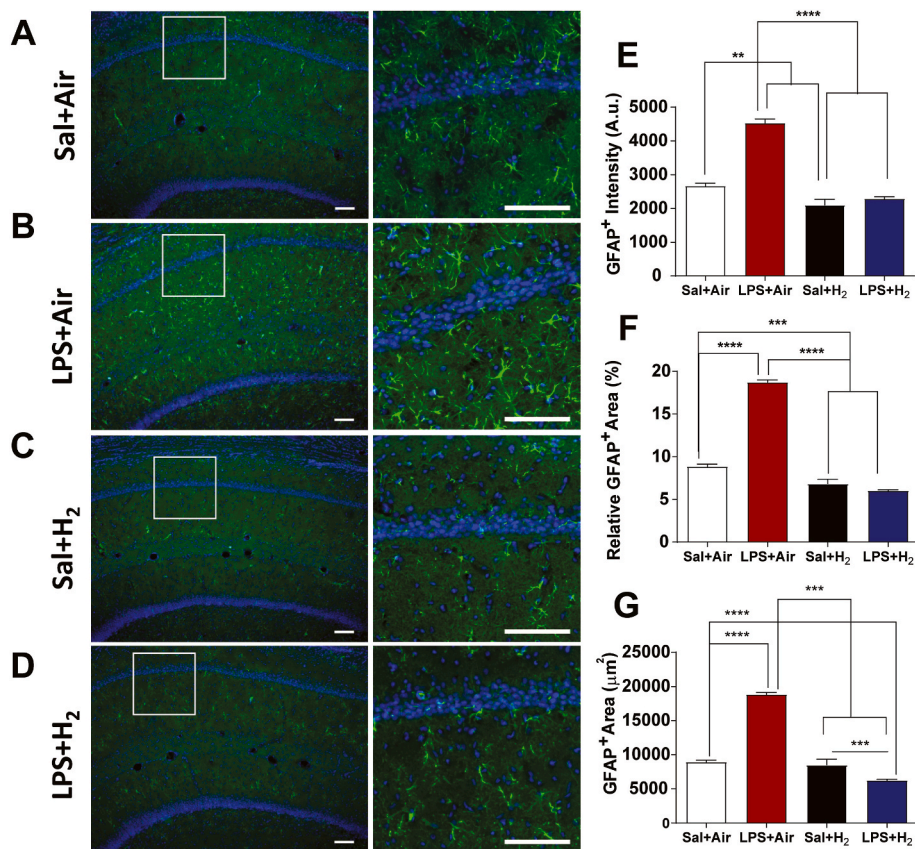
Fig. 3. Photomicrographs showing immunoreactivity of GFAP (green) and cell nuclei stained with DAPI (blue) in sections of CA1 from the dorsal hippocampus. Left panel shows representative images from Sal + Air (A), LPS + Air (B), Sal + H_2 (C) and LPS + H_2 (D) groups. Right panel shows intensity (E), relative area (%-F) and area (μm^2-G) of GFAP positive cells. Data are shown as mean ± SEM. **$P \leq 0.01$, ***$P \leq 0.001$, ****$P \leq 0.0001$ (ANOVA and Tukey post-test). Bars mean 100 μm. N = 4 animals per group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

induced SI (LPS + Air: Figs. 2B and 3B) rats present increased intensity (Figs. 2E and 3E), relative (Figs. 2F and 3F) and total area (Figs. 2G and 3G) demonstrating a high microglial (Iba-1$^+$- Fig. 2B) and astrocytic (GFAP$^+$- Fig. 3B) reactivity in sections of CA1 from the dorsal hippocampus ($P \leq 0.05$). On the other hand, H_2 inhalation significantly blunted LPS-induced increase in the intensity (LPS + H_2: Figs. 2E and 3E), relative area (Figs. 2F and 3F), and total area (Figs. 2G and 3G) demonstrating a lower microglial (Iba-1$^+$; Fig. 2D) and astrocytic reactivity (GFAP; Fig. 3D); ($P \leq 0.05$). Curiously, rats from the Sal + H_2 group showed smaller microglial or astrocytic reactivity than animals from the Sal + Air group ($P \leq 0.05$).

3.3. H_2 inhalation decreases hippocampal pro-inflammatory cytokines surges during LPS-induced SI

Additionally, we decided to confirm whether H_2 treatment can attenuate hippocampal neuroinflammation during SI. To this end, we performed measurements of cytokines from dorsal hippocampus punches, obtained from carefully collected brains, frozen in dry-ice cold isopentane, and stored at −80 °C before the dorsal hippocampus was sampled using a cryostat and a punch needle. Samples were analyzed in order to measure TNF-α, IL-1β, IL-6, and IFN-γ content, normalized by protein concentrations. We found similar levels of TNF-α (Fig. 4A) and IFN-γ (Fig. 4D) between the four experimental groups ($P \geq 0.05$). The levels of IL-6 and IL-1β were similar between Sal + Air and Sal + H_2 (Fig. 4B–C $P \geq 0.05$). However, we observed a significant increase of IL-1β during LPS-induced SI which was blunted in animals that inhaled H_2 (Fig. 4B; $P \leq 0.05$). In addition, we detected high levels of IL-6 in the hippocampus from animals during LPS-induced SI which was blunted in animals that inhaled H_2 (Fig. 4C; $P \leq 0.05$).

3.4. Inhaled H_2 ameliorates short and long-term memory during LPS-induced SI

We sought to investigate whether inhaled H_2 can improve memory compromised during LPS-induced SI. We used the Novel Object Recognition test (NOR), a rapid and simple test for assessing memory impairment in animals (Bertaina-Anglade et al., 2006). Our results demonstrated that during the training phase (A-A; Fig. 5A) rats from all four experimental groups presented a similar percentage of object exploration ($P \geq 0.05$). When evaluating both short (Fig. 5B) and long-term memory (Fig. 5C), we found that H_2 treatment improved the performance of animals treated with LPS ($P \leq 0.05$). Animals that inhaled H_2 spent more time exploring the novel object than non-treated controls (LPS + Air). Interestingly, when we evaluated the total time spent in the exploration of objects (old and new objects), we found that rats that received LPS spent less time in short-term memory when compared to the other groups ($P \leq 0.05$; Fig. 5D) while we observed that all the rats spend the same time in exploration of objects in the long-term memory test ($P \geq 0.05$; Fig. 5E).

3.5. H2 treatment does not revert LTP deficit induced by LPS administration

Because H_2 treatment can improve memory in the NOR after LPS administration, we decided to investigate these effects in the hippocampal long-term synaptic plasticity considering that there is a well-known relationship between LTP and hippocampal learning and memory (Abraham et al., 2019). We induced LTP in the Schaffer-CA1 pathway and observed that while post-tetanic potentiation was similar in slices from all groups tested, ($P \geq 0.05$; Fig. 6E), LTP was significantly reduced in slices from animals that received LPS and also in slices from animals that received LPS and inhaled H_2 in comparison slices from Sal + Air rats ($P \leq 0.05$; Fig. 6B, D and 6F). LTP has developed normally in
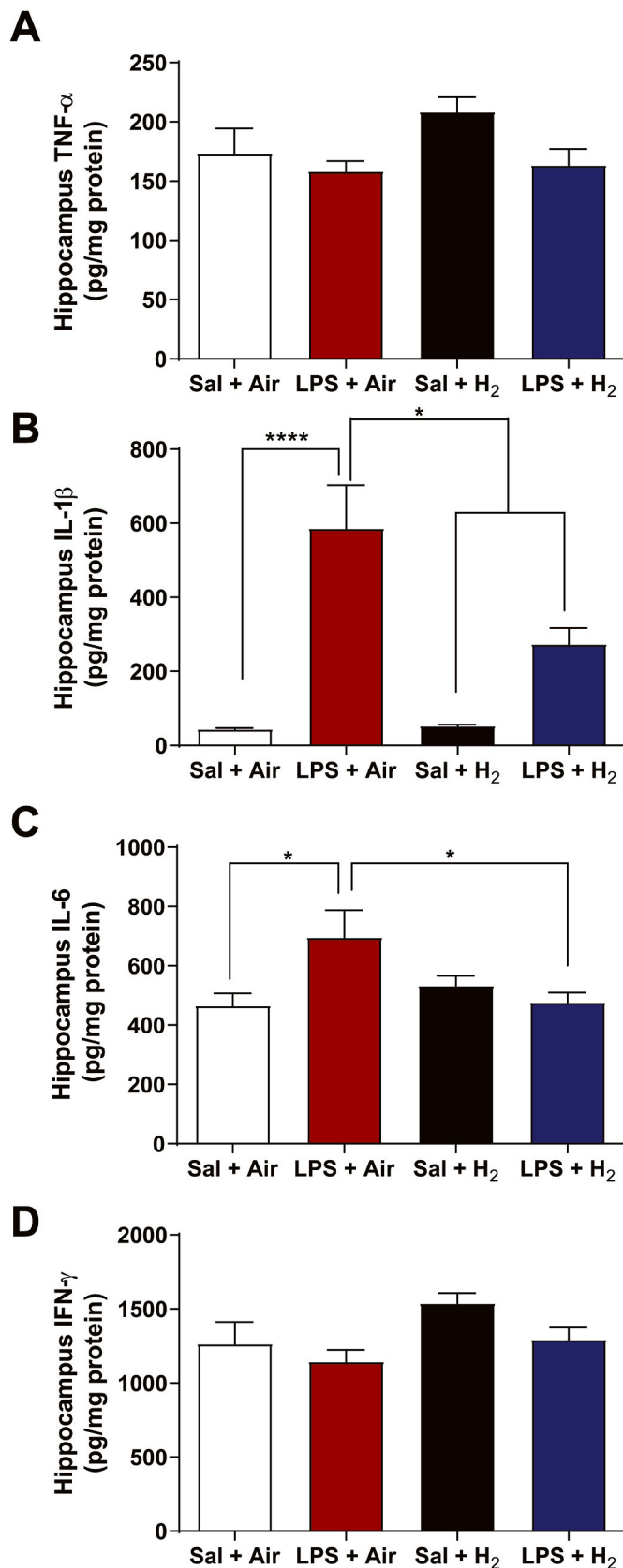
Fig. 4. Dorsal hippocampus levels measurements of proinflammatory cytokines collected after 3 h: TNF-α (A); IL-1β (B); IL-6 (C) and IFN-γ (D) of rats injected with saline or lipopolysaccharide (LPS, 1.5 mg/kg^{-1}) that inhaled H$_2$ or room air. Data are shown as mean ± SEM. *P ≤ 0.05, ****P ≤ 0.0001 (ANOVA and Tukey post-test). N = 8–12 animals per group.

the slices from Sal + Air and Sal + H$_2$ rats (P ≥ 0.05; Fig. 6A, C, and 6F).

4. Discussion

In the present study, we investigated the effects of H$_2$ treatment in the hippocampus during neuroinflammation induced by LPS (1.5 mg/kg^{-1}). Our findings show that our experimental rats showed typical LPS-induced SI characterized by increased plasma levels of TNF-α, IL-1β, IL-6, and IFN-γ (Fig. 1). Besides these systemic parameters, we also observed activation of microglia and astrocytes (Figs. 2 and Fig.3), as well as an increase of IL-1β and IL-6, in the hippocampus (Fig. 4). Eventually, we demonstrated that animals that were exposed to H$_2$ inhalation presented reduced peripheral and hippocampal cytokines surges (Figs. 1 and Fig.4), besides lower microglia and astrocytes reactivity (Figs. 2 and Fig.3). The results from memory and plasticity showed that LPS caused significant memory impairment and affected LTP and that H$_2$ treatment reduced the memory impairment (Fig. 5), without improving hippocampal LTP (Fig. 6). H$_2$ has been shown as an interesting new therapeutic compound, based on the fact that it ameliorates clinical aspects of carcinogenic, cardiovascular, neuromuscular and neurodegenerative, and inflammatory diseases, among other conditions (Nogueira and Branco, 2021). Originally, H$_2$ was thought to be an inert gas until more recent evidence indicated that H$_2$ is able to neutralize the hydroxyl radicals (●OH) and peroxynitrite (ONOO_) within the cells, promoting cytoprotective effects against oxidative stress (Nogueira et al., 2018). Despite this intense production of knowledge related to H$_2$ actions, there are no reports in the literature about the possible effect of H$_2$ inhalation on LPS-induced hippocampal neuroinflammation, glial reactivity, and memory impairment.

We have previously demonstrated that H$_2$ inhalation reduces LPS-induced pro-inflammatory cytokines surges not only in the central nervous system but also in the systemic circulation (Jesus et al., 2020; Nogueira et al., 2018; Saramago et al., 2019). The reduction of neuroinflammation by H$_2$ treatment was observed when rats were subjected to cecal ligation and puncture (CLP) and in the intense acute exercise-induced hippocampal inflammation, demonstrating that H$_2$ is a potent anti-inflammatory in the hippocampus independently of the model used to induce inflammation. Moreover, a previous report demonstrated that chronic *ad libitum* consumption of electrochemically molecular H$_2$-enriched water before the LPS challenge prevents neuroinflammation in the hippocampus (Spulber et al., 2012). Although the inhalation of H$_2$ induces slightly higher H$_2$ concentrations in the brain in comparison to the other routes of administration (Liu et al., 2014), we can consider that H$_2$ administered either orally or inhaled is highly effective in reducing thippocampal neuroinflammation. More recently, Nakamo et al. showed the powerful effect of the H$_2$ against neuroinflammation in intrauterine life in the pups brain that presented a reduction of inflammation when mothers were treated with H$_2$ before the LPS administration (Nakano et al., 2015).

Neuroinflammation is characterized by glial activation associated with the production of a number of pro-inflammatory mediators in the central nervous system. This condition receives special attention within the neurobiological research because this neuroinflammatory response is characteristic not only of most neurological and neurodegenerative diseases (Leng and Edison, 2021), but also takes place during systemic infection related conditions (Muthuraju et al., 2020). Similarly to peripheral inflammation, neuroinflammation has a dual role, *i.e.*, a beneficial protective function that limits the damage and contributes to defeating pathogens, and a deleterious role when it results in neuronal death and neurodegeneration. In this scenario, activated microglia can secrete both pro- and anti-inflammatory cytokines, leading to further damage or promoting regeneration, respectively (Caraffa et al., 2018). For instance, the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 may induce neuronal death (Conroy et al., 2004). We found an LPS-induced increase of IL-1β and IL-6 levels in the hippocampus. These findings are consistent with our previous study that documented the up-regulation of
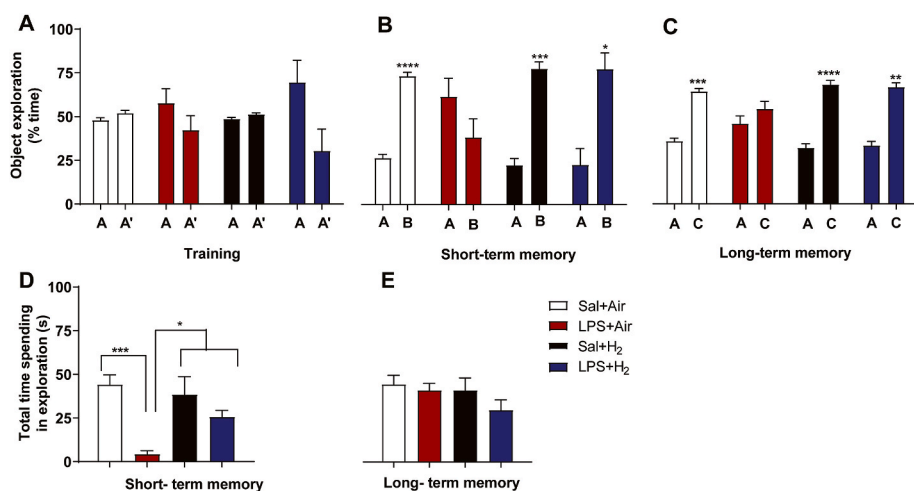
Fig. 5. Novel Object Recognition test. A) Training phase is represented by animals exploring similar squares (A-A') for 5 min. B) 20 min after the training phase, the short-term memory was evaluated by the replacement of the one square with a blue triangle (A–B). On the following day (C), the long-term memory was measured by exchange of the blue triangle with a white cylinder (A–C). Data are shown as mean ± SEM. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 (Test T paired; ANOVA and Tukey post-test). The axis X means the three objects (colored square -A, blue triangle -B and white cylinder-C) that were used to evaluate the animals in three phases of the Novel Object Recognition (NOR). A, A′ means two equal objects (colored squares) that we used during the training phase. B, means the blue triangle that we used during the short-term memory and C, means white cylinder that was used during the long-term memory phase. N = 5–7 animals per group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
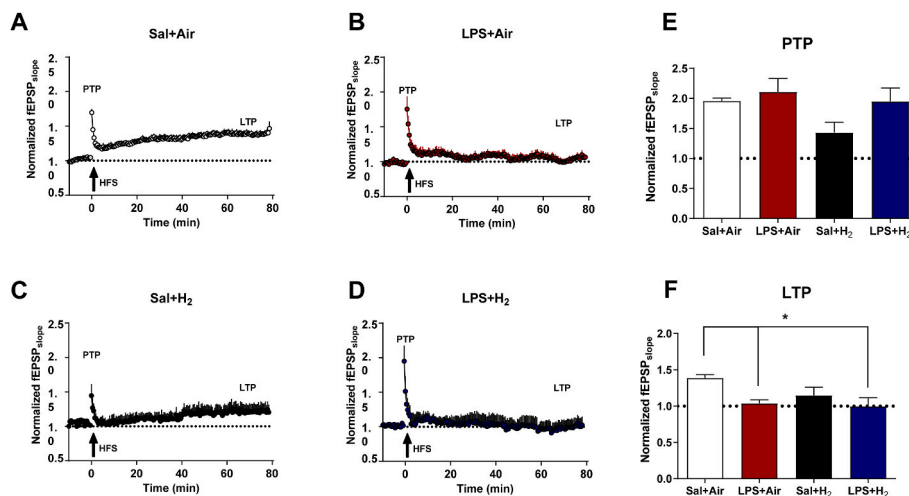

Fig. 6. LTP from the Schaffer-CA1 synapse of Sal + Air (A), LPS + Air (B), Sal + H$_2$(C) and LPS + H$_2$ (D) rats. Normalized fEPSP slopes before and after HFS (arrow) from the Schaffer-CA1 synapse of slices from the dorsal hippocampus to all groups. Bar graphs showing the summary of PTP (E) and LTP (F). Representative recordings are shown above each graph. *P ≤ 0.05 (ANOVA and Tukey post-test). N = 5–7 slices from 4 to 5 animals.

these cytokines in the nucleus tractus solitarius using the same model of neuroinflammation (Amorim et al., 2019). On the other hand, we found no changes in TNF-α and IFN-γ levels in the hippocampus. The lack of LPS-induced increase in these cytokines could be explained by the different time courses of production and release of each cytokine, the dose of LPS administrated and the brain region evaluated. Moreover, IL-1β and IL-6 are known to cause increased production of glutamate in neurons, increasing neuronal excitotoxicity following the activation of NMDA receptors (Ye et al., 2013). In agreement with this notion, mice deficient in the anti-inflammatory cytokine IL-10 have a larger cognitive deficit after LPS-induced neuroinflammation (Richwine et al., 2009).

The activity of anti-inflammatory factors, such as IL-10, limits the severity and duration of the inflammatory response, providing resolution of neuroinflammation. In case this resolution is not achieved, neuroinflammation becomes chronic, maintained by a positive feedback and a pathological condition takes place (Zusso et al., 2020). In this scenario, favoring the resolution process, for instance, with H$_2$, neuroinflammation is reduced (Fig. 4) and thus it may be an effective strategy to lessen the cognitive deficit (Fig. 5) caused by the inflammatory processes. The administration of LPS can activate microglia and astrocyte *in vivo* and *in vitro* (Cerbai et al., 2012; Goshi et al., 2020; Karababa et al.,

2017; Lana et al., 2017; Norden et al., 2016). Here we observed an increase in microglial and astrocytic reactivity after rats received LPS in the dorsal hippocampus and that this increased reactivity was blunted after H$_2$ exposure. To our knowledge, this is the first time that H$_2$ inhalation is reported as a modulator of glial cells in the hippocampus after an immune challenge in adult rats. We speculate that by reducing the pro-inflammatory cytokines surges, H$_2$ indirectly reduces the microglial and astrocytic reactivity, which is likely to be related to the reduced memory impairment induced by SI. It is interesting to note that in a recent study (Nascimento et al., 2023), H$_2$ has been shown to reduce (L-DOPA)-induced dyskinesia (as observed in L-DOPA-treated Parkinsonian patients), and that this effect was associated with decreased striatal and SI.

It is well known that the LPS toxicity model induces memory impairments in rodents, which resemble symptoms of patients that underwent septic events (Iwashyna et al., 2010). Moreover, in many models of sepsis both neuronal and glial alterations leading, in many cases, to cognitive decline (Sonneville et al., 2013) are observed. In order to quantify such cognitive decline, the novel object recognition test is widely used not only to assess short and long-term memory alterations (Ennaceur and Delacour, 1988), but also to assess working
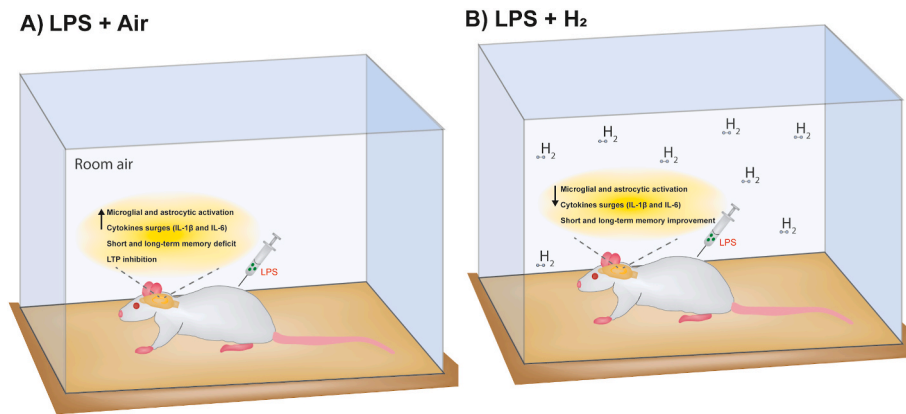
A) LPS + Air   B) LPS + H₂

Fig. 7. Effects of LPS-induced- systemic inflammation in the hippocampus. A) Animals from LPS + Air present an increase of microglial and astrocytic activation, surges of IL-1β and IL-6, a deficit in short and long-term memory, and LTP inhibition. B) Animals from LPS + H2 present a decrease of microglial and astrocytic activation, blunted IL-1β and IL-6, and improvement of short and long-term memory.

memory, attention, anxiety, and preference for novelty in rodents (Goulart et al., 2010). Our data indicate that H$_2$ treatment lessened both short- and long-term LPS-induced memory impairments. Treated animals spent more time exploring the novel object than non-treated controls. Similar results were found by Moraes et al. (2015) where mice submitted to CLP presented impairment in short-term memory during object recognition test, microglial and astrocytic activation and decreased synaptophysin, and PSD-95 in the hippocampus. Furthermore, as demonstrated by Abareshi et al. (2016), rats that received LPS presented a worse performance in the Morris Water Maze test and LTP inhibition when compared to control animals. LTP is considered the model of synaptic plasticity to represent the phenomena related to memory in the hippocampus. We found a robust inhibition of hippocampal LTP in the Schaffer-CA1 synapse in the hippocampus of rats treated with LPS. However, we found no effect of H$_2$ in reverting the LTP despite its beneficial effects on object recognition memory. Although this lack of effect of H$_2$ in reverting the LTP deficit in LPS-treated animals seems counterintuitive as these animals had an improved object recognition ability, deficits in LTP not always reflects in deficits in hippocampal function (Bannerman et al., 2012; Cunha et al., 2015; de Deus et al., 2017). Additionally, hippocampal lesions affect spatial learning and memory rather than object novelty memory which is more dependent on the perirhinal cortex (Mumby et al., 2002). Nevertheless, our data show that although H$_2$ treatment improved new object recognition in LPS-treated rats, it did not revert hippocampal LTP deficits caused by LPS, suggesting that other hippocampal-dependent tasks, like spatial navigation, might still be compromised in these animals.

It is rather appealing the substantial impact of neuroinflammatory mediators in the onset and development of several neurodegenerative disorders (Fan et al., 2020). Furthermore, recent reports address the role of the peripheral immune system in influencing these neurodegenerative disorders and indicating potential and promising new target therapies (Fan et al., 2020). After all, it seems clear now that the peripheral immune system affects the microglia that are renowned for causing neuroinflammation, as observed in classical models of inflammation using LPS (Fan et al., 2020).

5. Conclusions

In the present study, we have shown that H$_2$ suppresses the neuroinflammatory response to LPS by reducing glial activation and proinflammatory cytokines surges, both locally and systemically. This anti-inflammatory effect attenuates LPS-induced memory impairment. In addition, LPS-induced plasma cytokines surge, concurrently associated with hippocampal inflammatory status, is reduced by the holistic H$_2$ inhalation effect (see Fig. 7). This effective anti-inflammatory effect,

combined with the fact that no adverse effects of H$_2$ have been reported, and the gas is relatively easy to use, inexpensive, and effective in daily medical practice indicates H$_2$ as a very interesting compound capable of preventing/treating neurodegenerative disorders.

Declaration of competing interest

We declare no conflict of interest.

Data availability

Data will be made available on request.

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